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Fully Automated Enzymatic Synthesis of Glycans

Jiabin Zhang

FULLY AUTOMATED ENZYMATIC SYNTHESIS OF GLYCANS

by

JIABIN ZHANG

Under the Direction of Jun Yin, PhD

ABSTRACT

Oligosaccharides, together with oligonucleotides and oligopeptides, comprise the three major classes of natural biopolymers. Automated systems for oligonucleotide and oligopeptide synthesis have significantly advanced developments in biological science by allowing non-specialists to rapidly and easily access these biopolymers. Researchers have endeavored for decades to develop a comparable general automated system to synthesize oligosaccharides. Such a system would have a revolutionary impact on the understanding of the roles of glycans in biological systems. The main challenge to achieving automated synthesis is the lack of general synthetic methods for routine synthesis of glycans. Currently, the two main methods to access homogeneous glycans and glycoconjugates are chemical synthesis and enzymatic synthesis. Enzymatic glycosylation can proceed stereo- and regiospecifically without protecting group manipulations. Moreover, the

reaction conditions of enzyme-catalyzed glycosylations are extremely mild when compared to chemical glycosylations. Over the past few years, methodology towards the automated chemical synthesis of oligosaccharides has been developed. Conversely, while automated enzymatic synthesis is conceptually possible, it is not as well developed.

Inspired by the success of automated oligosaccharide synthesis through chemical glycosylation, a fully machine-driven automated system is built up here for oligosaccharides synthesis through enzymatic glycosylation in aqueous solution. The designed automation system is based on the use of a thermosensitive polymer and a commercially available peptide synthesizer to fully achieve automation process.

An automated platform for chemo-enzymatic glycopeptide synthesis is built up which easily assembles glycopeptides in an organic phase solvent system before extending oligosaccharide residues by enzymatic glycosylation. Our system is based on the use of an amine-functionalized silica resin to facilitate the linkage of the primers needed to begin the chemical or enzymatic synthesis of the target compounds to a solid support. Using our platform, a peptide from mucin 1 with different important glycan epitopes was successfully prepared with a resin transfer step by hand.

INDEX WORDS: automated synthesis, peptide synthesizer, PNIPAM, silica gel

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DEDICATION

I want to thank for my families for their love, support and understanding. I will thank every friend for helping me in science or in life.

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LIST OF ABBREVIATIONS

Poly(N-isopropylacrylamide) (PNIPAM)

Uridine-5'-triphosphate (UTP)

Guanosine-5'-triphosphate (GTP)

Cytidine-5'-triphosphate (CTP)

Glucose (Glc)

Galactose (Gal)

Xylose (Xyl)

N-acetylglucosamine (GlcNAc)

N-acetylgalactosamine GalNAc

Fucose (Fuc)

Mannose (Man)

N-Acetylneuraminic acid (Neu5Ac)

N-Acetylmannosamine (ManNAc)

Uridine 5'-diphospho-galactose (UDP-Gal)

Uridine 5'-diphospho-N-acetylglucosamine (UDP-GlcNAc)

Uridine 5'-diphospho-N-acetylgalactosamine (UDP-GalNAc)

Guanosine 5'-diphospho-L-fucose (GDP-Fuc)

Escherichia coli (E. coli)

Helicobacter pylori β1,3-N-acetylglucosaminyltransferase (HpLgtA)

Neisseria meningitides β1,4-galactosyltransferase (NmLgtB)

Neisseria meningitidis CMP-sialic acid synthetase (NmCSS)

Pasteurella multocida multifunctional α2,3-sialyltransferase 1 M144D mutant (PmST1 M144D)

Campylobacter jejuni β1,4-N-acetyl-galactosaminyltransferase (CgtA)

Campylobacter jejuni β1,3-galactosyltransferase mutant (CgtB)

Helicobacter mustelae α1,2 -fucosyltransferase (1,2-FucT)

Helicobacter mustelae α1,3-N-acetyl-galactosaminyltransferase (BgtA)

Human blood group B glycosyltransferase (GTB)

Glycine (Gly, G)

Alanine (Ala, A)

Serine (Ser, S)

Threonine (Thr, T)

Cysteine (Cys, C)

Valine (Val, V)

Leucine (Leu, L)

Isoleucine (Ile, I)

Methionine (Met, M)

Proline (Pro, P)

Phenylalanine (Phe, F)

Tyrosine (Tyr, Y)

Tryptophan (Trp, W)

Aspartic Acid (Asp, D)

Glutamic Acid (Glu, E)

Asparagine (Asn, N)

Glutamine (Gln, Q)

Histidine (His, H)

Arginine (Arg, R)

Lysine (Lys, K)

matrix-assisted laser desorption/ionization (MALDI) mass spectrometry

high-performance liquid chromatography (HPLC)

ultraviolet (UV)

electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS)

Nuclear magnetic resonance (NMR)

1 INTRODUCTION

1.1 Background

1.1.1 General Introduction to Glycans

Glycans are biopolymers which contain a large number of monosaccharides linked glycosidically. Glycans also called glycoconjugates, include glycoproteins, glycolipids, proteoglycans, or polysaccharides.[1-3] Monosaccharides are the simple sugars with multiple OH groups, and can be a triose, tetrose, pentose or hexose based on number of carbons. Hundreds of monosaccharides have been found right now. However, only a few of them are commonly found and well-studied in living organism. A universal symbol for the representation of glycans has been well developed in glycoscience. Monosaccharide was presented by simple shapes, such as round, square, triangle, diamond, fivepointed star and so on, with different standard colors including yellow, green, blue, red, orange and purple (Figure 1.1).[4]

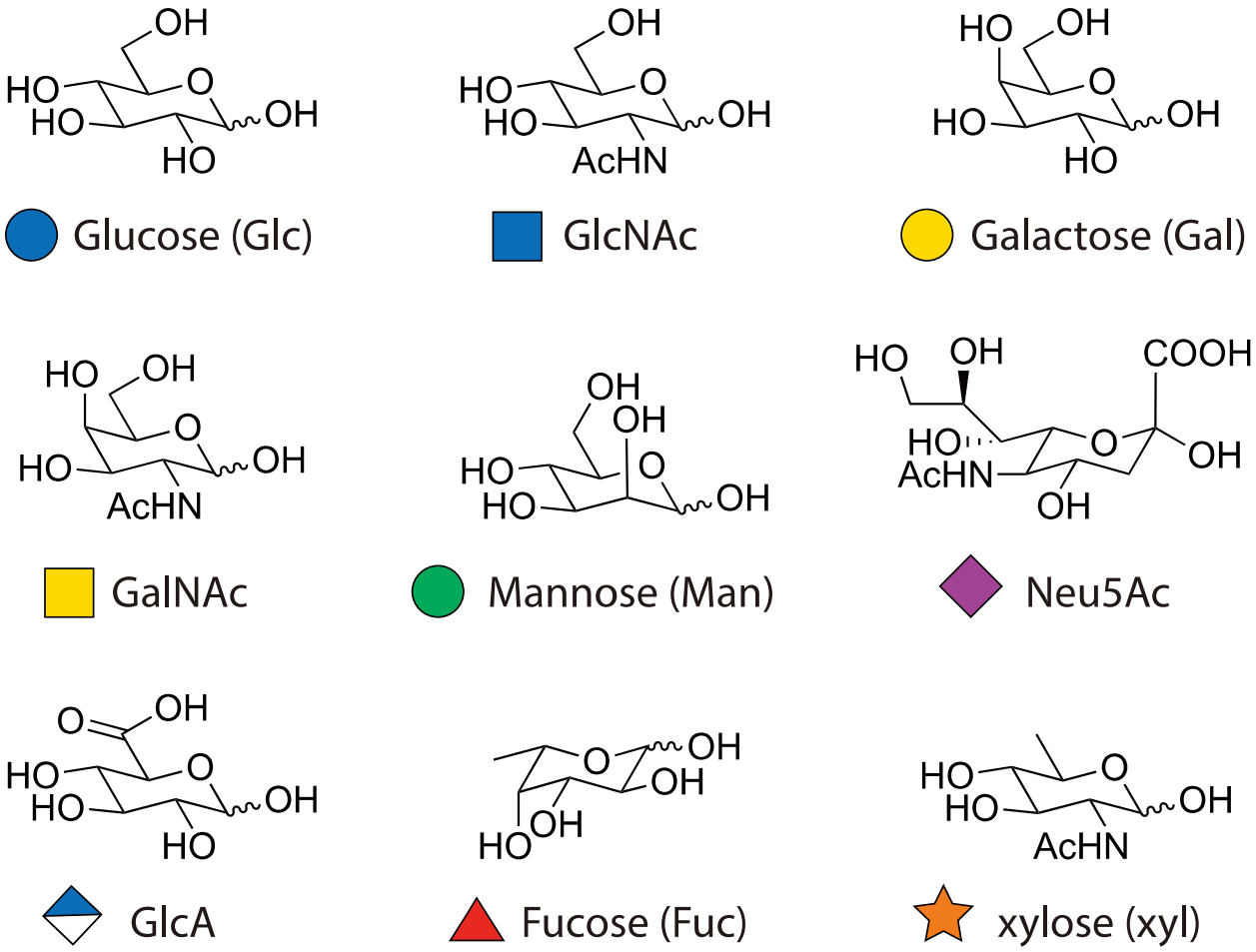


Figure 1.1 Structures and symbols of common monosaccharides

1.1.2 Automated Platform for Biopolymer Synthesis

Reliable and rapid access to oligonucleotides and oligopeptides by commercial automated synthesizers has fundamentally altered biological research. A major breakthrough in the synthesis of oligonucleotides and oligopeptides was the use of solid-phase synthetic methodologies.[5] This development in synthetic chemistry enabled the subsequent use of automated synthesizers based on the methodology.[6] A number of automated platforms have been developed for automated assembly of the natural biopolymers (oligosaccharides, oligonucleotides and oligopeptides), since solid phase peptide synthesis was first reported by R. B. Merrifield in 1963.[7-10] In 1982, the First commercially available oligonucleotide synthesizer was developed by Biosearch of Novato.[11] This

platform was built up based on solid phase synthesis with a peptide synthesizer. In 1984, R. B.Merrifield won Nobel Prize in Chemistry because of his special contribution for solid phase synthesis. Over the past few decades, many advanced automated synthesizers have been put into market to produce almost every possible oligopeptides and oligonucleotides which greatly advance the fields of biology and medicine.

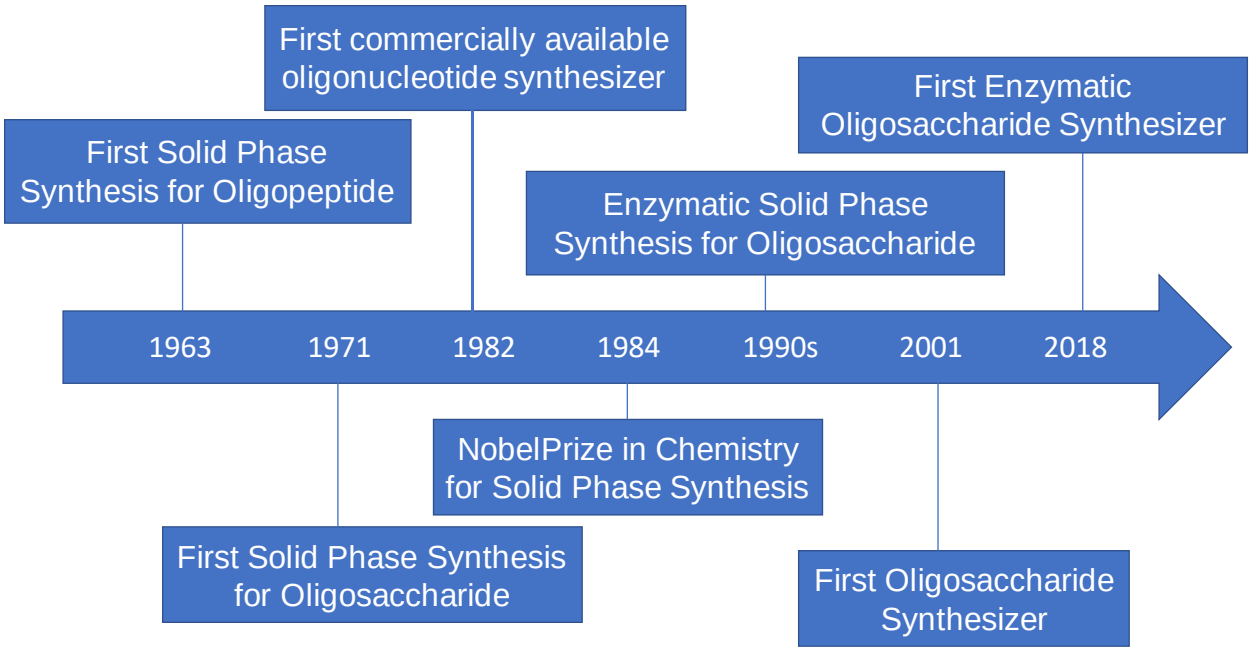


Figure 1.2 Timeline of automated synthesis for bioplolymers

However, a general commercial system for the synthesis of oligosaccharides has yet to be implemented, mainly due to the lack of general synthetic methods. With a goal of achieving automated synthesis of complex oligosaccharides, many methodologies are currently being developed by synthetic chemists to address the challenges in carbohydrate chemistry that have prohibited automation. The use of solid-phase synthesis for the construction of oligosaccharides was initiated with Frechet and co-workers in 1971.[12] However, it was not until 2001 that Seeberger and co-workers reported the first automated synthesis of oligosaccharides based on a solid-phase synthetic method.[13] The first automated glycan synthesizer was constructed by modifying a peptide synthesizer, and the synthetic process closely resembles protocols established for the generation

of peptides. To achieve automated synthesis, the first monosaccharide residue is attached through its reducing end to a solid support via a linker. The linker must be cleavable to release the final product upon the completion of the synthesis. Once the hydroxyl group to be modified in the coupling reaction is exposed by selective deprotection, addition of a glycosyl donor and activator will initiate the glycosylation reaction. Typically, the activator is a reagent that can generate a leaving group at the anomeric position. Upon the completion of one reaction cycle, subsequent sugar residues can be added by repeating the same overall process. The acquisition of final product requires cleavage of the fully protected material from the solid support and removal of the protecting group. One of the main advantages of the use of a solid-phase support in oligosaccharide synthesis is that considerable excess of the glycosyl donor and other regents can be used to drive the reaction to completion without the need to separate out the final product from these reagents. Side products and other unwanted materials can be removed by a single wash while the desired glycan remains covalently attached to the solid support.

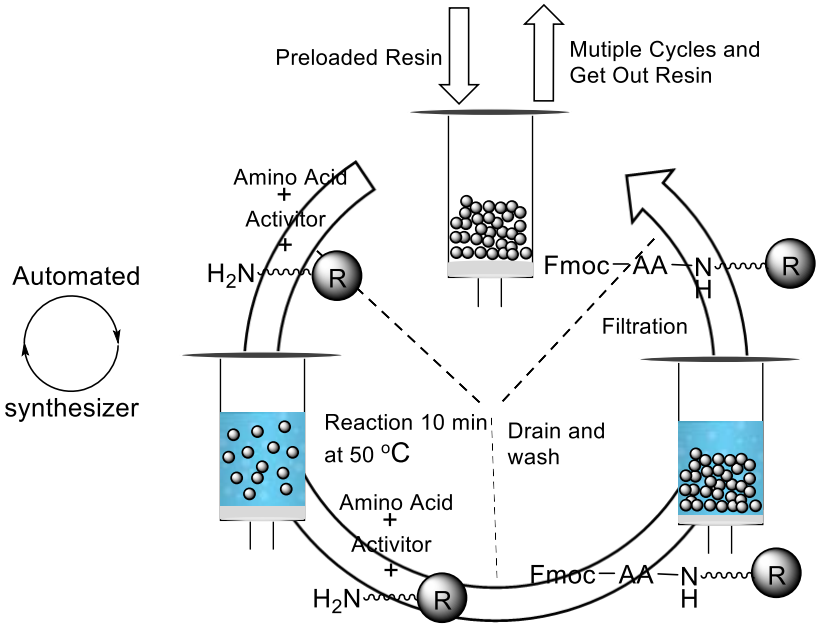


Figure 1.3 The Principle of Solid Phase Synthesis

1.1.3 Enzymatic Glycosylation

Enzymatic glycosylations are reactions which are catalyzed by glycotransferase to extend sugars on the oligosaccharide chains.[14] Sugar nucleotides, which are shown below (Figure 1.4), are used as donors in these reactions.[1] Enzymatic glycosylation is able to proceed stereo- and regioselectively without the need to undergo tedious protecting group manipulations. Even for very sterically demanding couplings such as those involving sialic acid, enzymatic glycosylation can be performed selectively. Enzymatic reactions occur in aqueous solutions and only require limited control over reaction conditions such as temperature, system pH, and the presence or absence of metal ions. Also, there are no toxic byproducts of the glycosylation process, making the enzymatic synthesis of oligosaccharides an environmental friendly approach.

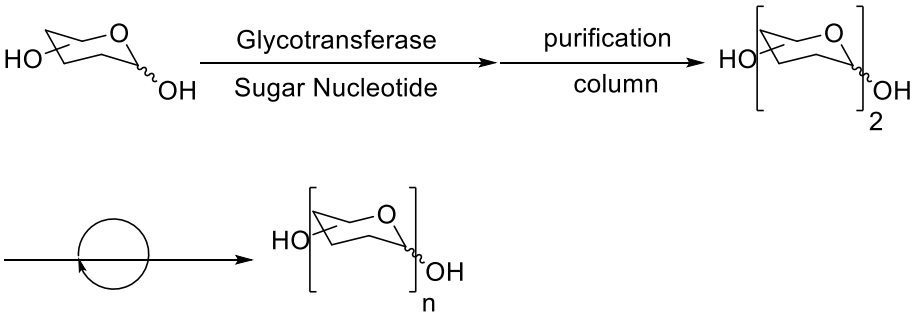


Figure 1.4 Enzymatic synthesis of oligosaccharide

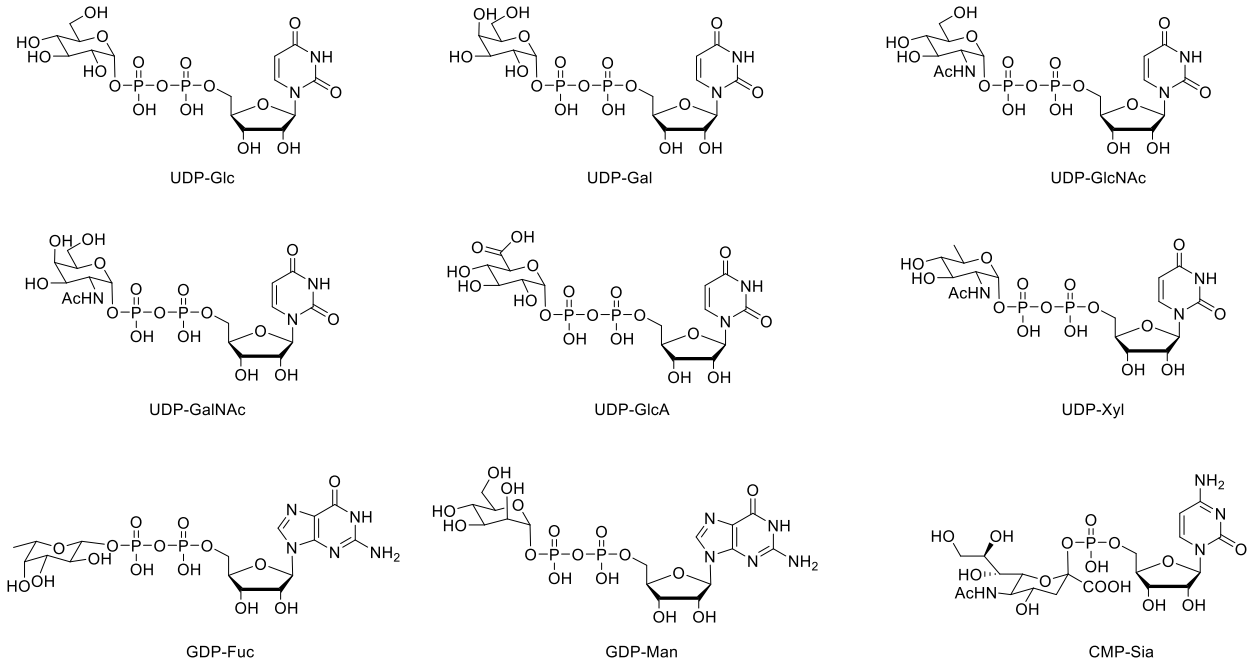


Figure 1.5 Structures of Common Sugar Nucleotides

1.2 Significance

Automated synthesis of biopolymers could save researchers lots of time and energy by using machine. The researchers just need to set up a well-defined program before making these biopolymers and click start button to get the targeting molecules. In this case, non-experts could synthesize complex structural long chain biopolymer. The peptide synthesizer, which has a long history, shows a highly automated production process and was commercialized for a long time. This work have built up an automated platform for glycan synthesis and will greatly promote the area of chemical biology and pharmacy.

2 FIRST GENERATION AUTOMATED PLATFORM

Oligosaccharides, oligonucleotides, and oligopeptides comprise the three major classes of natural biopolymers.[15] Structurally defined glycans have many important biological and medical applications. They find use in glycan microarray studies,[16] clinical diagnostics,[17] enzymatic activity tests,[18] and the development of carbohydrate-based vaccines.[3, 19] However, it is difficult to obtain suitable quantities of homogeneous oligosaccharides from nature by extraction for biological studies. Synthetic preparation by either chemical or enzymatic glycosylation is the only way to obtain amounts of homogeneous oligosaccharides sufficient for research applications. Chemical glycosylation can produce structurally defined glycans with diverse natural and unnatural structures,[20] while enzymatic glycosylation can produce both simple and complicated glycans with high regio- and stereo-specificity under mild reaction conditions.[21] Regardless, both chemical and enzymatic methods require skilled researchers to perform the operation, which is a time-consuming and labor-intensive process.

The advent of automated systems for the synthesis of oligonucleotides and oligopeptides allows non-specialists to rapidly produce these biopolymers and is responsible for greatly advancing the fields of biology and medicine.[22] Over the past few decades, researchers have put considerable efforts into developing a comparable automated synthesizer to produce oligosaccharides.[12, 23-25] In 1999, Wong and co-workers developed a programmable one-pot solution-phase strategy for the synthesis of oligosaccharides.[26] In 2001, a landmark breakthrough from Seeberger and co-workers opened the machine-driven synthesis of oligosaccharides based on solid-phase synthesis and chemical glycosylation.[13] This work adopted the same concept of solid-phase synthesis, which was demonstrated by Merrifield for peptide synthesis.[27] Subsequently, a large number of oligosaccharides have been successfully prepared by applying this platform.[28-43] In addition, many

other automated approaches towards chemical glycosylation were also developed to produce oligosaccharides,[44-46] such as an HPLC-based automated system, Yoshida's electrolysis cell-based automated system, and a Fluorous-tag solid phase system. Nevertheless, chemical glycosylations suffer from tedious protection/deprotection manipulations. By comparison, enzymatic reactions are advantageous in both efficiency and specificity. Although automated enzymatic synthesis is conceptually possible, it is not as well developed. Herein, we report an automated system for the preparation oligosaccharides (Figure 2.1). This system is based on enzymatic glycosylation, a temperature-dependent polymer, and a commercially available peptide synthesizer to fully realize the automation process.

Figure 2.1 Automated enzymatic synthesis of oligosaccharide.

The key steps for sugar elongation in the automation process are the reaction and separation steps. Initially, we attempted to use Merrifield's concept on enzymatic glycosylation. Enzymatic synthesis using solid-phase methodology was reported many years ago. However, we and many other researchers found that, in general, enzymatic glycosylation on a solid-phase either does not work well or gives inadequate yields. This is due to the incompatibility between enzymes and solid resin. This problem could not be solved to a satisfactory degree even after we optimized several

parameters such as altering the length of linkers, utilizing different resins, and varying the particle size of the resins. At the same time, many works found that enzymatic glycosylations can be performed on solution-phase resins/polymers. However, the use of solution-phase supports or polymers increases the difficulty of product purification. In 2010, Nishimura and co-workers used a globular protein-like PAMAM dendrimer for automated synthesis,[47] which allows the product to be separated by ultrafiltration. However, ultrafiltration is a difficult manipulation to be controlled by a machine. In addition, significant product loss was also observed during the purification process owing to the physical properties of hollow fiber-based material.

2.1 Resin

In 1963, the first solid phase synthesis was reported by by R. B. Merrifield. Subsequently, a number of automated synthesizers based on the solid phase synthesis were reported by many other research groups and companies. These synthesizers have been used to prepare all types natural biopolymers (peptide, nucleotides and saccharides). All the traditional solid phase resins which have been used for automated synthesis have many holes.

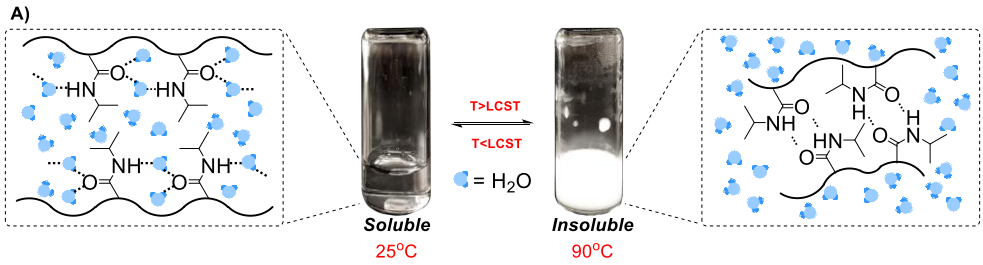


Figure 2.2 Thermosensitive Polymer (PNIPAM). LCST, Lower Critical Solution Temperature.

In our design, Poly(N-isopropylacrylamide) (PNIPAM),[48] a temperature-dependent polymer, is used as a support. When the environmental temperature decreases below that of the lower critical solution temperature (LCST), PNIPAM can form intermolecular hydrogen bonds with water, which allows it to be solubilized in aqueous solutions in which it can be accessed by

glycosyltransferases. When the temperature rises, hydrogen bonds are formed intramolecularly, which causes the aggregation and precipitation of PNIPAM (Figure 2.2). Consequently, a simple filtration manipulation can separate the product from other reagents such as sugar nucleotides and metal ions. The recovery yield of this polymer can reach more than 80% over ten cycles. More importantly, temperature can easily be controlled on an instrument such as a peptide synthesizer.

2.2 Linker

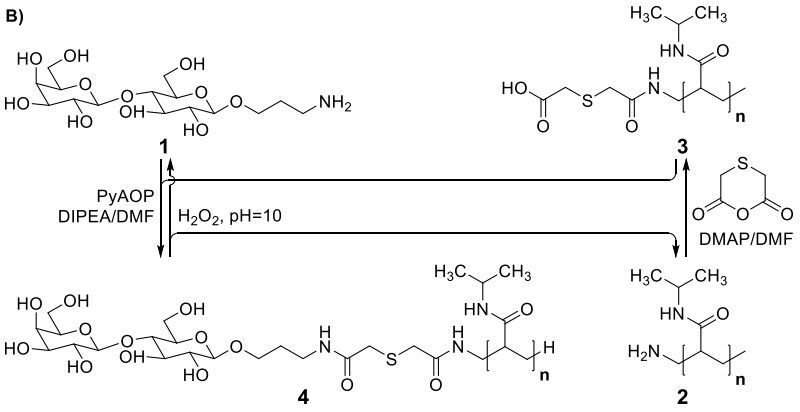


Figure 2.3 Conjugation of Oligosaccharide Primer (lactose) with PNIPAM through a Cleavable Linker.

A cleavable linker is required to conjugate an oligosaccharide primer with the PNIPAM polymer. Most known cleavable linkers require strong acidic or basic conditions during cleavage. However, glycans are not stable under such conditions.[46, 49] To avoid possible damage to the product, we used a thioether linker in this work (Figure 2.3). The thioether bond is oxidized to a sulfone by hydrogen peroxide. After hydrolysis, the oligosaccharide conjugate contains a terminal NH2 in an aqueous solution. Compound 1 (lactose with aminopropyl linker) was conjugated with PNIPAM polymer in the presence of thiodiglycolic anhydride, giving product 4 in 72% yield as confirmed by NMR (Appendix A). Unreacted lactose primer can be removed through dialysis (MWCO: 1000). To test the cleavage efficiency of this linker, a standard peptide linked with lactose was

treated with hydrogen peroxide (1M, pH 10). The results show that more than 80% of lactose can be released in less than 2 hours (Figure 2.4). In addition, we also tested the enzymatic activity of many glycotransferases with both 1 and 4. We found no significant difference between 1 and 4, indicating that enzymatic glycosylation holds almost comparable activity on the acceptor whether in solution or on soluble polymer.

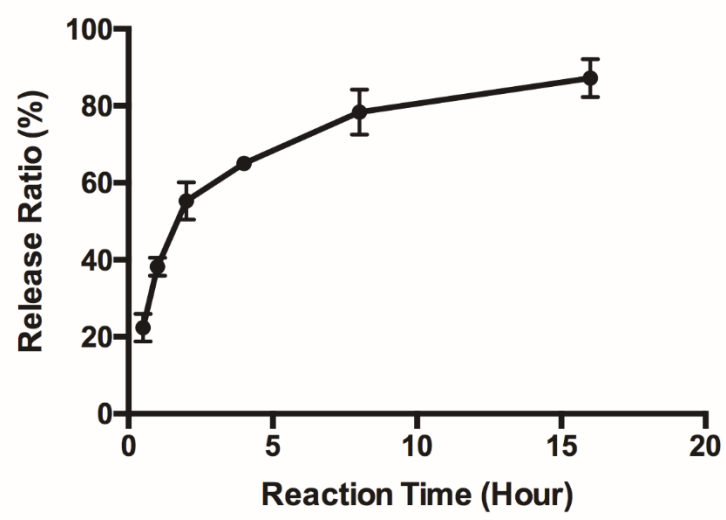


Figure 2.4 Lactose Release from Thioether Linker Bound Peptide

2.3 Instrument

Finally, a CEM Liberty Blue peptide synthesizer was chosen to perform the designed automation process as its functional units fit our strategy well. It has a reaction vessel connected with a filter and a temperature-control system, which is controlled by a computer. The enzymes, nucleotide sugars, and reaction buffers can be stored in the preexisting tubes in which amino acids would normally be stored during peptide synthesis. Reagents are automatically injected into the reaction vessel through a tubing system. (Figure 2.5).

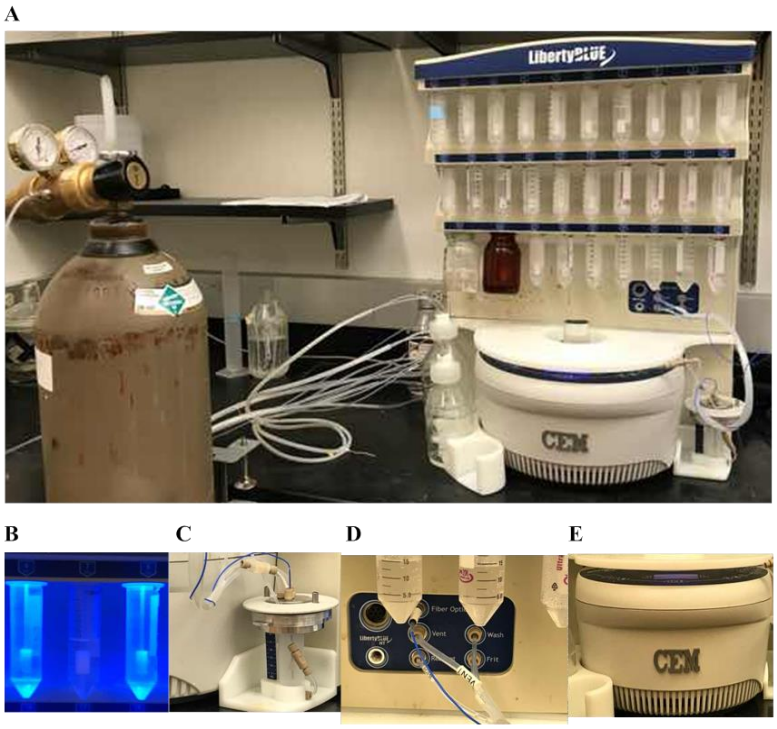


Figure 2.5 Peptide Synthesizer (CEM Liberty Blue) Used for Automation Synthesis in This Work.

2.4 Automated Process

We chose compound 6 (oligosaccharides GM1) as a target to test our system (Figure 2.6). Ganglioside GM1 is a glycan epitope located on the cell surface and plays important roles in many biological processes such as virus-cell interactions.[50] The enzymatic synthesis of GM1 from lactose involves three glycosylation steps.[50] The enzymes used are Pasteurella multocida α2,3-sialyltransferase (PmsT1), Campylobacter jejuni β1,4-N-acetylgalactosaminyltransferase (CgtA) and Campylobacter jejuni β1,3-galactosyltransferase CgtB.[50] The standardized procedure contains three processes (Figure 4B): automated synthesis, product release, and product purification. In order to adapt the CEM peptide synthesizer for this purpose, several optimization procedures were required. The program protocol was first optimized for enzymatic reactions (Figure 2.7). The synthetic glycosylation materials including the NmCSS (CMP-sialic acid synthetase from Neisseria meningitis), PmsT1, CgtA, CgtB, CTP, Neu5Ac, UDP-GalNAc, UDP-Gal, and reaction

buffer were stored in the preexisting tubes. Compound 4, prepared as described above, was used as the glycosylation primer. To the reaction vessel of the CEM Liberty Blue, 330 mg (50 umol) of conjugated polymer (4) was loaded. The automated synthetic process was then started by automatically injecting NmCSS, PmST1, CTP (3 equiv), Neu5Ac (3 equiv), and reaction buffer (10 mL, pH 8.0). The reaction time and the amount of enzyme usage were calculated based on enzyme kinetic parameters. Once the first reaction finished (3 h), the reaction solution temperature was increased to 90 ℃ to precipitate the glycosylation product. Unreacted materials and by-products were removed by filtration. The precipitate was washed and then CgtA, UDP-GalNAc (2 equiv), and reaction buffer (10 mL, pH 8.0) were injected into the reaction vessel to initiate the second glycosylation step. Once this reaction finished (6 h), the glycosylation product was precipitated out and washed as described above. CgtB, UDP-Gal (2 equiv), and reaction buffer (10 mL, pH 8.0) were injected to initiate the third glycosylation step. Once this step finished (6 h), the product was precipitated and washed. The automation process was stopped at this point, and the precipitate was taken out and dissolved in water. The target oligosaccharide GM1 (Figure 5) was released by the treatment of the polymer with hydrogen peroxide (1 M, pH 10). After HPLC purification, 20 mg of GM1 oligosaccharide was obtained in 38% yield from compound 4. The product was confirmed by NMR and MS analysis (see general methods and Appendix A).

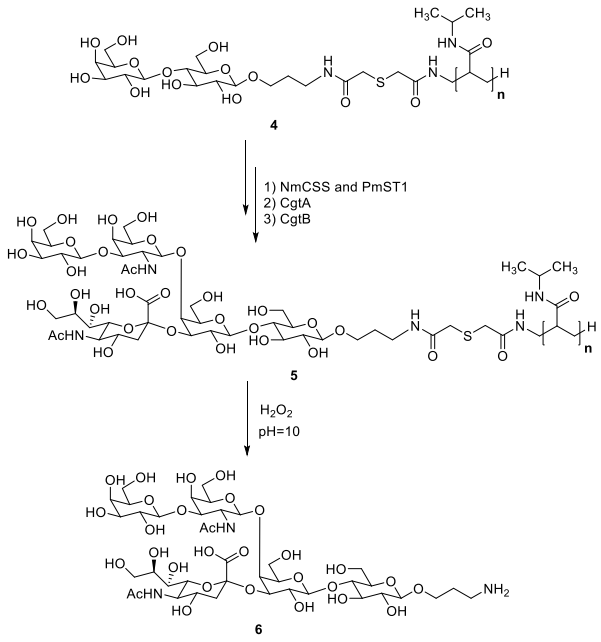


Figure 2.6 The Principle of Automation Synthesis of GM1

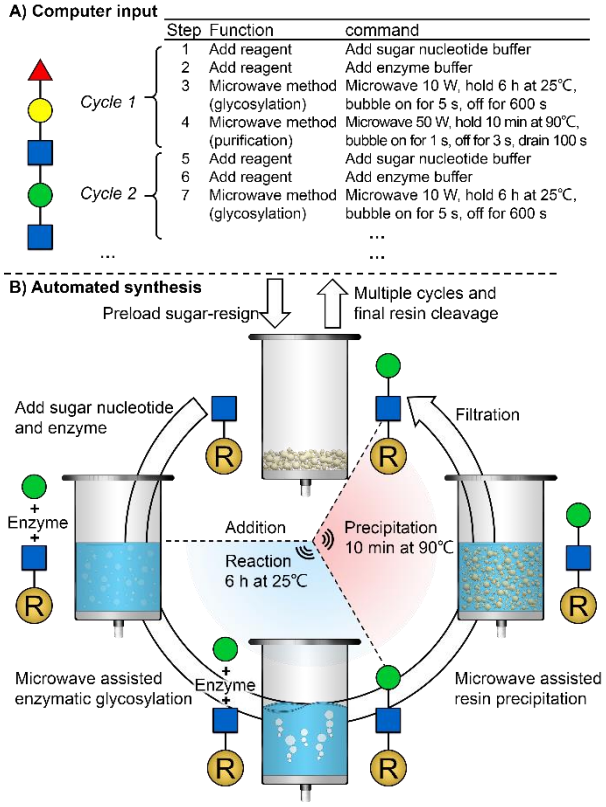


Figure 2.7 Automated Enzymatic Synthesis of Oligosaccharides Synthesis.

2.5 Results

In a similar automated manner, oligosaccharides for Blood group O, A, and B were also successfully prepared (Figure 2.8 and Table 2.1) using enzymes reported previously.[51] Blood group O, A, and B Oligosaccharides were obtained in 27 to 38% yield (more than 10 mg scale, Table 2.1). The product yields obtained by the automated system are only slightly lower than the yields in in vitro synthesis. Nevertheless, the automated synthesis process can save reaction time and labor to a great extent. The products were confirmed by NMR and MS analysis (see general methods and Appendix A). In addition to preparing these important oligosaccharide structures, we also tested our automation system by synthesizing Poly-N-acetyllactosamine (poly-LacNAc). Poly-LacNAc contains Galβ1,4GlcNAc repeating units, and has been identified as an important ligand for galectin-mediated cell adhesion to extra-cellular matrix (ECM) proteins. Recently, it was also found Poly-LacNAc can serve as an age-specific ligand for rotavirus P[11] in neonates and infants.[52] The in vitro enzymatic synthesis of Poly-LacNAc involves two glycosylation reactions catalyzed by LgtA and LgtB.[53] Since LgtA prefers a substrate with a longer chain, trisaccharide GlcNAc 1,3Gal 1,4Glc with aminopropyl linker was conjugated with PNIPAM to serve as a primer. In a similar program and automated manner as mentioned above, 18 reaction cycles (each glycosylation reaction step was performed two times to get high conversion) were performed in seven days to produce Poly-LacNAc with 12 residues in 9% yield (8 mg). Although more glycosylation reaction steps can be performed to produce longer chain, purification is unsuccessful as the product has poor solubility in water when the sugar chain has more than 12 monosaccharide residues.

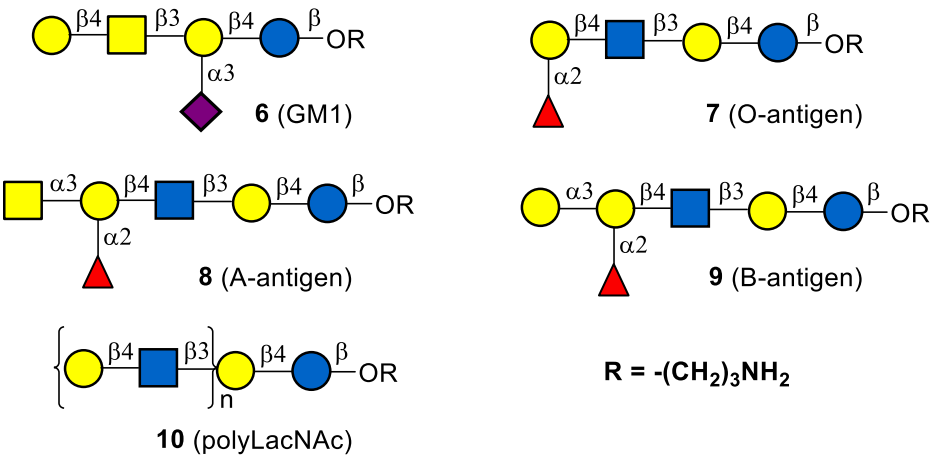


Figure 2.8 Oligosaccharides That Were Used to the Test Automated System in This Work.

Table 2.1 Oligosaccharides That Prepared by Automation System

Product	Enzymes	E/N[a] (mg)			Yield (%)	Time (hours)	Product (mg)
6	PmsT1, CgtA, CgtB	1.6	2.1	1.8	38	27	20
7	LgtB, FucT	1.0	2.5		35	24	13
8	LgtB, FucT, BgtA	1.0	2.5	1.9	29	36	14
9	LgtB, FucT, GTB	1.0	2.5	1.8	27	36	13
10	LgtA, LgtB	1.7	1.0		9	168	8

[a] enzyme usage

2.6 Materials and General Methods

2.6.1 Materials

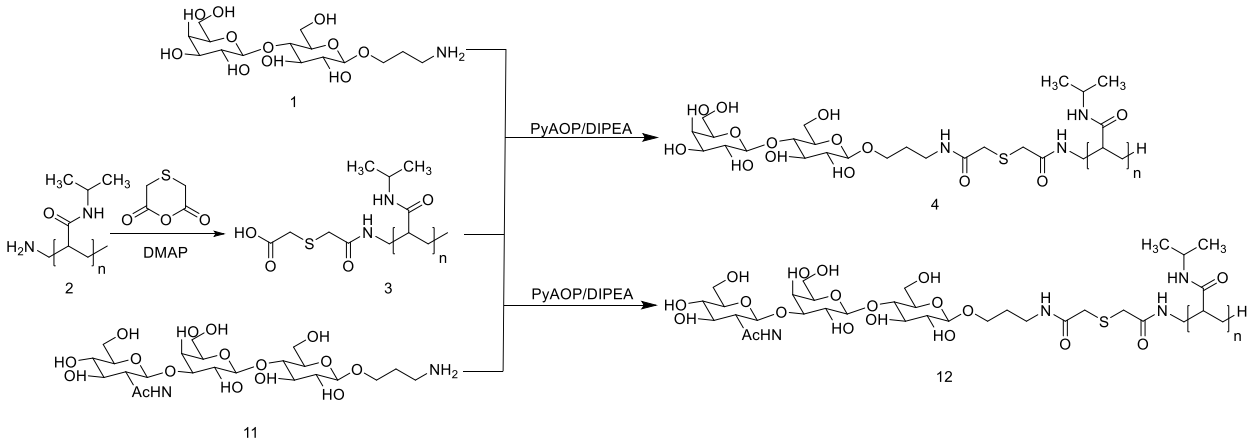
All commercially available solvents and reagents were used without purification. PNIPAM dendrimer (Mn5500) was purchased from Sigma Aldrich Inc (USA). Thiodiglycolic anhydride was purchased from Fisher. N-Acetylneuraminic acid (Neu5Ac) and cytidine 5'-triphosphate (CTP) were purchased from Carbosynth Limited. Sugar nucleotides uridine 5'-diphospho-galactose (UDP-Gal),[54] UDP-N-acetylglucosamine (UDP-GlcNAc),[55] UDP-N-acetylgalactosamine (UDP-GalNAc),[55] and guanosine 5'-diphospho-L-fucose (GDP-Fuc)[55] were prepared as described previously. Helicobacter pylori β1,3-N-acetylglucosaminyltransferase (HpLgtA),[56] Neisseria meningitides β1,4-galactosyltransferase (NmLgtB),[56] Neisseria meningitidis CMP-sialic acid

synthetase (NmCSS),[57] Pasteurella multocida multifunctional α2,3-sialyltransferase 1 M144D mutant (PmST1 M144D),[58] Campylobacter jejuni β1,4-N-acetyl-galactosaminyltransferase (CgtA),[50] Campylobacter jejuni β1,3-galactosyltransferase mutant (CgtB), Helicobacter mustelae α1,2 -fucosyltransferase, Helicobacter mustelae α1,3-N-acetyl-galactosaminyltransferase (BgtA),and human blood group B glycosyltransferase (GTB) were expressed and purified as described previously.

^{1}H-NMR and ^{13}C-NMR spectra were recorded by using a Bruker 400 spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C) or a Bruker 600 spectrometer (600 MHz for ^{1}H, 150 MHz for ^{13}C). All 1H Chemical shifts (in ppm) were assigned in reference to CDCl3 (δ= 7.24 ppm), MeOD (δ= 4.87 ppm), and D2O (δ = 4.79 ppm) and all 13C NMR spectra were calibrated with CDCl3 (δ = 77.00 ppm). HPLC was performed with a Shimadzu Prominence UFLC. Column: Waters XBridge BEH amide column, 130 Å, 5 μm, 4.6 mm × 250 mm. A semi-preparative Waters XBridge BEH amide column (130 Å, 5 μm, 10 mm × 250 mm) was used for purification. Solvent A: 100 mM ammonium formate, pH 3.2. Solvent B: Acetonitrile. Semi-preparative Agilent Eclipse XDB-C18, 5 μm, 9.4 × 250 mm. Aeris PEPTIDE 3.6 μm CB-C18, 4.6 mm x 250 mm. Solvent A: 0.1% TFA Water. Solvent B: 0.1% Acetonitrile. High-resolution electrospray ionization mass spectrometry (ESI-HRMS) experiment was performed on an LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher). Automated synthesis was performed on a Liberty Blue peptide synthesizer (Figure 2.5, CEM) according to the basic manufacture's manual and software for controlling operation with a designated program (Figure 2.7) and sequence for the synthesis of the present target compounds.

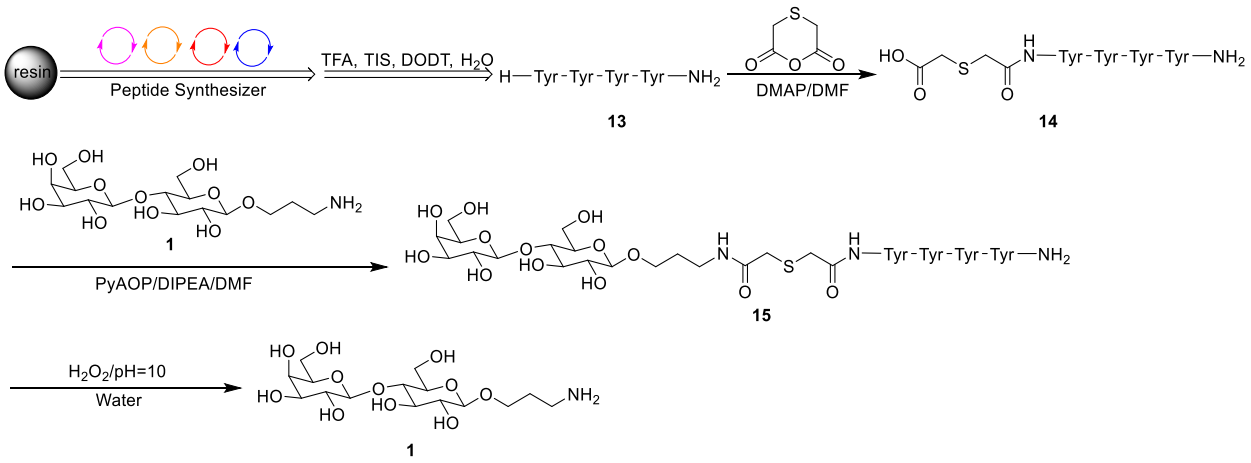
2.6.2 Methods

Scheme 1



Compound 4 was prepared from PNIPAM. In detail, PNIPAM (550 mg, 0.1 mmol) was dissolved in DMF (10 ml). Thiodiglycolic anhydride (132 mg, 1 mmol) and DMAP (122 mg, 1 mmol) were added to the solution, and the reaction mixture was stirred at room temperature overnight. After the reaction completed the solvent was removed by high vacuo and the product purified by dialysis (MWCO 1000) to obtain compound 3. Compound 3 and compound 1 (80 mg, 0.2 mmol) were dissolved in DMF (10 ml). PyAOP (522 mg, 1 mmol) and DIPEA (129 mg, 1 mmol) were added to the solution, and the reaction mixture was stirred at room temperature overnight. The solvent was removed by high vacuo and the product purified by dialysis (MWCO 1000) to obtain compound 4. Compound 12 was prepared from PNIPAM and compound 11 using the protocol as described for the synthesis of compound 4. The sugar loading efficiency is in the range of 72% (Appendix A).

Scheme 2



Compound 13 was prepared with the peptide synthesizer in 4 cycles. In detail, resin (333 mg, 0.25 mmol) was added to the reaction vessel and all the required reagents were loaded into the tubes. The following procedures were performed by the program on the automated synthesizer. The resin was swelled for 300 s with DMF (10 ml). For the first coupling cycle, 5 ml piperidine in the DMF (v/v=20%) was added to the reaction vessel and the mixture was keep at 50 ℃ for 2 min to deprotect the Fmoc group. The operation was repeated to ensure complete deprotection. Then the resin was washed with DMF (3×5 ml). Tyr solution (5 ml), DIPEA (2 ml), HBTU (2 ml) were added to the reaction vessel to form a solution (9 ml) as below: (0.25 mmol resin, 1 mmol Tyr, 1 mmol DIPEA and 1 mmol HBTU). The mixture was kept at 50 ℃ for 10 min. This operation was repeated. After 4 cycles, the peptide bound resin was transferred to a tube and 4 ml cleavage cocktail (TFA:H2O:TIS:DODT=92.5:2.5:2.5:2.5) was added to the tube to give a crude product. The final product was purified by HPLC (acetonitrile, 15%-70%, 40min). ^{1}H-NMR (400 MHz, MeOD), δ 7.051-6.648 (m, 16H), 4.548-4.442 (m, 3H), 3.926 (dd, J1 = 4.8 Hz, J2 = 8.4 Hz 1H), 3.083-2.700 (m, 8H). ^{13}C NMR (101 MHz, MeOD) δ 174.27, 171.60, 171.45, 168.30, 156.86, 155.96, 155.90, 130.24, 130.06, 130.03, 129.89, 127.52, 127.38, 127.32, 124.54, 115.49, 114.90,

114.86, 55.09, 54.56, 54.26, 36.74, 36.65, 36.43. HRMS (ESI): m/z $[M+H]^+$calcd for $C_{36}H_{39}N_5O_8$ 670.2872; found: 670.2829.

Compound 14 was prepared from compound 13. In detail, compound 13 (67 mg, 0.1 mmol) was dissolved in DMF (2 ml). Thiodiglycolic anhydride (66 mg, 0.5 mmol) and DMAP (61 mg, 0.5 mmol) were added to the solution and the reaction mixture was stirred at room temperature overnight. The solvent was removed by high vacuo, and the product was purified by HPLC (acetonitrile, 15%-70%, 30min) to get compound 14 (43 mg) in 53.6% yield regarding compound 13. ^{1}H-NMR (400 MHz, MeOD), 7.04-6.64 (m, 16H), 4.46-4.40 (m, 4H),3.32-2.68 (m, 12H). ^{13}C NMR (101 MHz, MeOD) δ 173.07, 171.11, 170.78, 170.70, 170.26, 169.04, 154.35, 154.27, 128.48, 128.46, 128.40, 126.20, 125.88, 125.87, 125.76, 113.41, 113.36, 54.04, 53.90, 53.68, 53.22, 34.96, 34.86, 34.76, 34.71, 33.43, 32.03. HRMS (ESI): m/z $[M+H]^+$calcd for $C_{40}H_{43}N_5O_{11}S$ 802.2753; found: 802.2744.

Compound 15 was prepared from compound 14. In detail, compound 14 (40 mg, 0.05 mmol) and compound 1 (60 mg, 0.15 mmol) were dissolved in DMF (10 ml). PyAOP (261 mg, 0.5 mmol) and DIPEA (65 mg, 0.5 mmol) were added to the solution, and the reaction mixture was stirred at room temperature overnight. The solvent was removed by high vacuo, and the product was purified by HPLC (acetonitrile, 3%-40%, 30min) to get compound 15 (21 mg) in 35.5% yield regarding compound 14. ^{1}H-NMR (400 MHz, MeOD), δ 7.964-6.835 (m, 8H), 6.610-6.562 (m, 8H), 4.380-4.326 (m, 4H), 4.265-4.246 (d, J = 7.6 Hz, 1H), 4.204-4.185 (d, J = 7.6 Hz, 1H), 3.823-3.317 (m, 12H), 3.208-3.125 (m, 6H), 2.970-2.591 (m, 10H), 1.695-1.665 (m, 2H). HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{55}H_{70}N_6O_{21}S$ 1183.4388; found: 1183.4331.

Sugar release efficiency was determined using compound 15. In detail, 1 mg of compound 15 was dissolved in 1 M H_2O_2 solution at pH=10. The release efficiency was monitored by HPLC

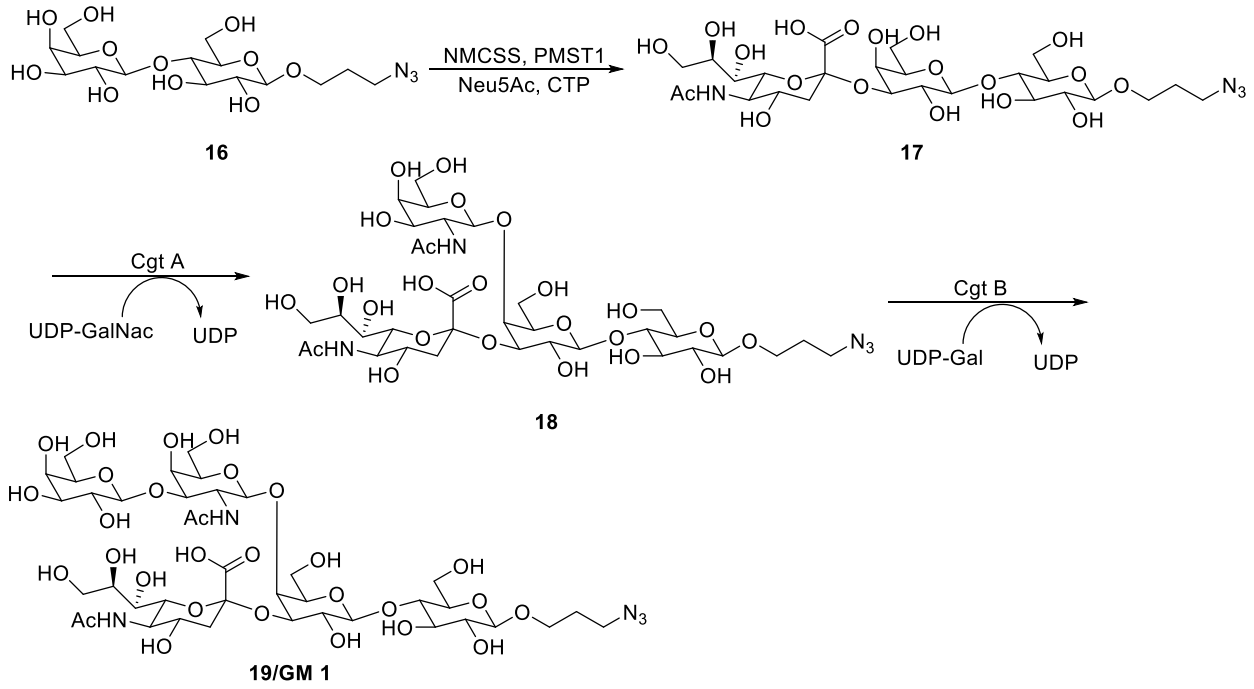
(acetonitrile, 3%-90%, 40min) at time point intervals of 1h, 2h, 4h, and 12h. More than 80% sugar was released from compound 15 (Figure 2.4). All reactions were run in triplicate.

Enzyme activity test. The enzyme activity for the sugar bound PNIPAM was tested with PmST1. In detail, a reaction mixture with a final volume of 100 µl containing 50 mM Tris-HCl, 1mM of Lactose β-pNP, 10 mM of Neu5Ac, 10 mM of CTP, 5 mM of Mg^{2+}, 1×10-3 units of NmCSS, 1×10-4, 2×10-4, 3×10-4, 4×10-4, and 5×10-4 units of PmST1 was incubated at 25°C for 30 min. The reaction was quenched by adding an equal volume of ethanol and freezing at -80 °C for 30 min. The reaction was monitored by an analytical GL Science Inertsil ODS-4 column (100 Å, 5 µm, 4.6 mm × 250 mm) with UV detector (260 nm). The running solvents were solvent A (H2O with 0.1% TFA) and solvent B (acetonitrile with 0.1% TFA). The running condition was a gradient elution with solvent B linear increased from 5% to 10% within 25 min, with a total flow rate of 1 mL/min. All reactions were run in triplicate. The reactivity of sugar bound PNIPAM was tested with Lactose-PNIPAM (compound 4) using the same conditions. The reaction was monitored by Aeris PEPTIDE 3.6 µm CB-C18, 4.6 mm x 250 mm with UV detector (210 nm). The running solvents were solvent A (H2O with 0.1% TFA) and solvent B (acetonitrile with 0.1% TFA). The running condition was gradient elution with solvent B being held at 20% for 5 min, then increasing linearly to 45% over 2 min, then holding at 45% for 5 mins, then increasing linearly to 60% over 2 min, then holding at 60% for 2 mins, then increasing linearly to 80% over 2 min, then holding at 80% for 13 mins with a total flow rate of 1 mL/min.

Time optimization was performed with the same substrate Lactose-pNP and Lactose-PNIPAM (compound 4). In detail, a reaction mixture in a final volume of 100 µl containing 50 mM Tris-HCl, 1mM of Lactose-pNP, 10 mM of Neu5Ac, 10 mM of CTP, 5 mM of Mg^{2+}, 1×10-3 units NmCSS, and 3×10-4 units of PmST1 was incubated at 25°C for 30 min (1 h, 2 h, 3 h and 6 h). The

reaction was quenched by adding an equal volume of ethanol and freezing at -80 °C for 30 min. The reaction was monitored by an analytical GL Science Inertsil ODS-4 column (100 Å, 5 μm, 4.6 mm × 250 mm) with UV detector (260 nm). The running solvents were solvent A (H2O with 0.1% TFA) and solvent B (acetonitrile with 0.1% TFA). The running condition was gradient elution with solvent B linearly increasing from 5% to 10% over 25 mins, with a total flow rate of 1 mL/min. All reactions were run in triplicate. The reactivity of sugar bound PNIPAM was tested by Lactose-PNIPAM (compound 4) with the same conditions. The reaction was monitored by Aeris PEPTIDE 3.6 μm CB-C18, 4.6 mm x 250 mm with UV detector (210 nm). The running solvents were solvent A (H2O with 0.1% TFA) and solvent B (acetonitrile with 0.1% TFA). The running condition was gradient elution with solvent B at 20% for 5 min, then increasing linearly to 45% over 2 min, then holding at B 45% for 5 mins, then increasing linearly to 60% over 2 min, then holding at 60% for 2 mins, then increasing linearly to 80% over 2 min, then holding at 80% for 13 mins with a total flow rate of 1 mL/min.

Scheme 3



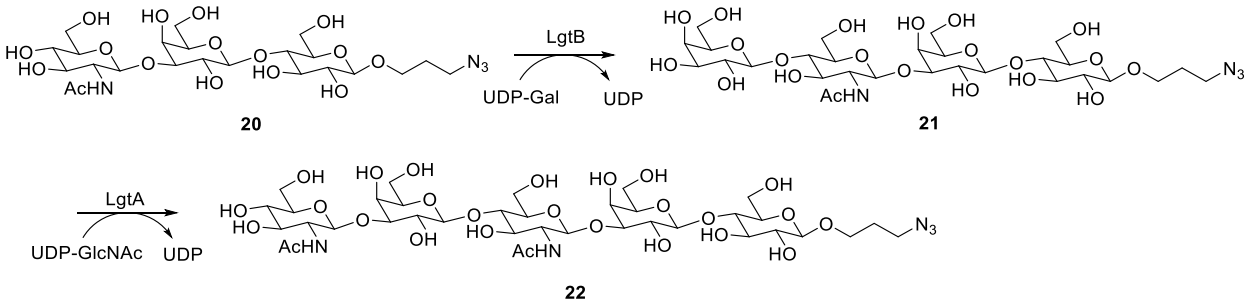
Compound 17 was prepared from compound 16 by using NmCSS and PmST1. In detail, a reaction mixture with a final volume of 10 ml containing 50 mM Tris-HCl, 42.5 mg of 10 (0.1 mmol), 15 mM of Neu5Ac, 15 mM of CTP, 5 mM of Mg^{2+}, 7.4 mg of NmCSS, and 1.6 mg of PmST1 was incubated at 25°C to allow the formation of compound 17. After 3 hours, the reaction was monitored by TLC (EtOAc/MeOH/H2O/HOAc=5:2:1.4:0.4). An equal volume of ethanol was added to remove proteins and the solution was concentrated in vacuo. After purification by Bio-Gel P-2 column 60 mg of compound 17 was obtained in 83.8% yield regarding compound 16. ^{1}H-NMR (400 MHz, D2O), δ 4.44 (d, J = 8.0 Hz, 1H), 4.39 (d, J = 8 Hz, 1H), 4.03 (dd, J1 = 2.8 Hz, J2 = 6 Hz 1H), 3.92-3.45 (m, 19H), 3.37 (t, J = 6.6 Hz, 2H), 3.23 (t, J = 4.4 Hz 1H), 2.68 (dd, J1 = 4.4 Hz, J2 = 12.4 Hz, 1H), 1.93 (s, 3H), 1.84 (m, 2H), 1.73 (t, J = 12.4, 1H). ^{13}C NMR (100 MHz, D2O), δ 174.97, 173.86, 102.60, 102.10, 99.75, 78.18, 75.43, 75.13, 74.73, 74.30, 72.83, 72.76, 71.73, 69.32, 68.32, 68.04, 67.41, 67.32, 62.52, 60.98, 59.99, 51.63, 47.82, 39.58, 28.18, 21.99. HRMS (ESI): m/z $[M+Na]^+$ calcd for C26H44N4O19 739.2492; found: 739.2434.

Compound 18 was prepared from compound 17 by using CgtA. In detail, a reaction mixture with a final volume of 8.4 ml containing 50 mM Tris-HCl, 10 mM trisaccharide (60mg), 15 mM of UDP-GalNAc, 5 mM of Mg^{2+}, and 2.1 mg of CgtA was incubated at 25°C to allow the formation of tetrasaccharide. After 6 hours, the reaction was monitored by TLC (EtOAc/MeOH/H2O/HOAc=5:2:1.4:0.4). An equal volume of ethanol was added to remove proteins and the solution was concentrated in vacuo. After purification by using Bio-Gel P-2 column. 52 mg of compound 18 was obtained in 67.5% yield regarding compound 17. ^{1}H-NMR (400 MHz, D2O), δ 4.74 (d, J = 8.8 Hz, 1H), 4.53 (d, J = 8.0 Hz, 1H), 4.49 (d, J = 8 Hz, 1H), 4.16 (m, 2H), 4.02-3.57 (m, 22H), 3.48-3.27 (m, 5H), 2.68 (dd, J1 = 4.4 Hz, J2 = 12.4 Hz, 1H), 2.02 (m, 6H), 1.95 (m, 3H). ^{13}C NMR (100 MHz, D2O), δ 174.97, 174.79, 174.05, 102.71, 102.54, 102.06, 101.58, 78.53, 77.11, 74.69, 74.32, 74.26, 73.96, 73.01, 72.68, 72.23, 71.21, 69.96, 68.66, 67.95, 67.72, 67.31, 62.78, 61.11, 60.49, 60.04, 52.28, 51.53, 48.06, 47.82, 36.87, 28.18, 22.55, 21.99. HRMS (ESI): m/z $[M+Na]^{+}$ calcd for C34H57N5O24 942.3285; found: 942.3248.

Compound 19 was prepared from compound 18 by using CgtB. In detail, a reaction mixture with a final volume of 5.6 ml containing 50 mM Tris-HCl, 10 mM tetrasaccharide (52mg), 15 mM of UDP-Gal, 5 mM of Mg^{2+}, and 1.8 mg of CgtB was incubated at 25°C to allow the formation of compound 19. After 6 hours, the reaction was monitored by TLC (EtOAc/MeOH/H2O/HOAc=5:2:1.4:0.4). An equal volume of ethanol was added to remove proteins and the solution was concentrated in vacuo. 48 mg of compound 19 was obtained in 79% yield regarding compound 18. ^{1}H-NMR (400 MHz, D2O), δ 4.67 (d, J = 8.8 Hz, 1H), 4.43 (m, 3H), 4.04 (m, 3H), 3.94 (m, 3H), 3.79-3.32 (m, 27H), 3.26-3.16 (m, 2H), 2.56 (m, 1H), 1.91-1.77 (m, 9H). ^{13}C NMR (151 MHz, D2O) δ 175.02, 174.76, 174.11, 104.73, 102.58, 102.49, 102.10, 101.63, 80.31, 78.60, 77.13, 74.87, 74.73, 74.37, 74.34, 74.32, 74.05, 73.07, 72.72, 72.48, 72.25, 70.67,

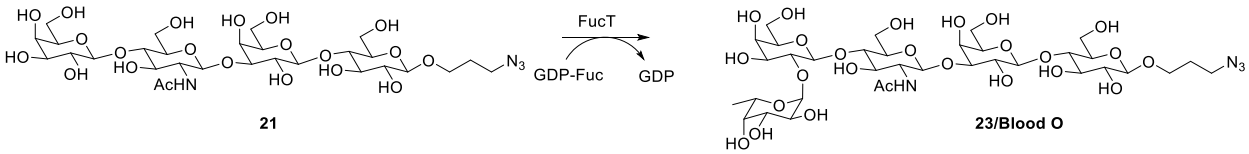
70.00, 68.70, 68.57, 68.00, 67.89, 67.35, 62.81, 61.08, 60.92, 60.59, 60.09, 51.58, 51.17, 48.11, 47.87, 36.92, 28.22, 22.57, 22.03. HRMS (ESI): m/z $[M+Na]^+$ calcd for C40H67N5O29 1104.3814; found: 1104.3766.

Scheme 4



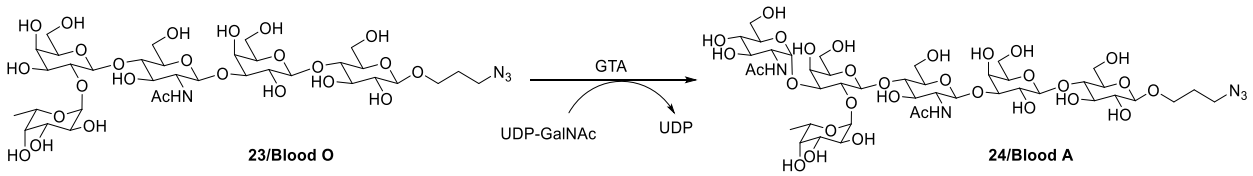
Compound 22 was prepared from compound 21 using LgtA. In detail, a reaction mixture in a final volume of 2 ml mixture containing 50 mM Tris-HCl (pH 8.0), 15.8 mg of compound 21 (10 mM), 5 mM of Mg^{2+}, 15 mM of UDP-GlcNAc, and 0.6 mg of LgtA was incubated at 25°C to allow the formation of compound 22. After 12 hours, the reaction was monitored by TLC (EtOAc/MeOH/H2O/HOAc=5:2:1.4:0.4). An equal volume of ethanol was added to remove proteins and the solution was concentrated in vacuo. The product was purified by using Bio-Gel P-2 column. 13 mg of compound 22 was obtained in 65.5 % isolated yield regarding 21. ^{1}H-NMR (400 MHz, D2O), δ 4.70 (m, 2H), 4.49 (m, 3H), 4.15 (d, J = 2.8 Hz, 1H), 3.99-3.44 (m, 31H), 3.32 (m, 1H), 2.03 (s, 6H), 1.94 (m, 2H). ^{13}C NMR (101 MHz, D2O) δ 174.90, 102.82, 102.71, 102.05, 81.94, 78.23, 78.05, 75.58, 74.81, 74.71, 74.48, 74.28, 73.48, 72.71, 72.10, 69.91, 69.59, 68.26, 67.30, 60.89, 60.38, 59.96, 59.76, 55.58, 55.07, 47.78, 28.16, 22.08. HRMS (ESI): m/z $[M+H]^+$ calcd for C37H63N5O26 994.3834; found: 994.3757.

Scheme 5



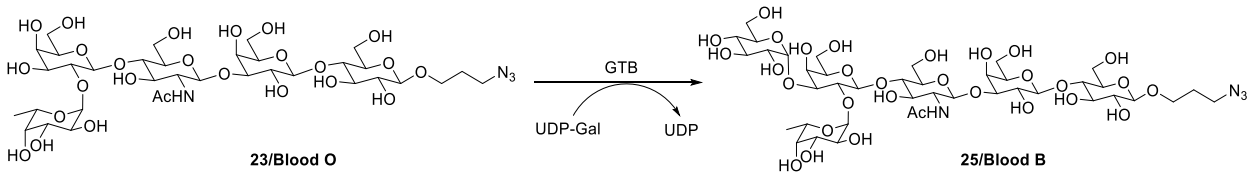
Compound 23 was prepared from compound 21 using FucT from Helicobacter mustelae. In detail, a reaction mixture in a final volume of 4 ml mixture containing 50 mM Tris-HCl (pH 8.0), 31.6 mg of compound 21 (10 mM), 5 mM of Mg^{2+}, 15 mM of GDP-Fucose, and 2.5 mg of 1,2-FucT was incubated at 25°C to allow the formation of compound 23. After 6 hours, the reaction was monitored by TLC (EtOAc/MeOH/H2O/HOAc=5:2:1.4:0.4). An equal volume of ethanol was added to remove proteins and the solution was concentrated in vacuo. The product was purified by using Bio-Gel P-2 column. 32 mg of compound 23 was obtained in 85.5 % isolated yield regarding compound 21. ^{1}H-NMR (400 MHz, D2O), δ 5.29 (d, J = 2.4 Hz, 1H), 4.69 (d, J = 8.4 Hz, 1H), 4.54 (d, J = 7.6 Hz, 1H), 4.48 (d, J = 8 Hz, 1H), 4.43 (d, J = 8 Hz, 1H), 4.22 (dd, J1 = 6.4 Hz, J2 = 13.2 Hz, 1H), 4.13 (d, J = 3.2 Hz, 1H), 4.01-3.54 (m, 26H), 3.46 (t, J = 6.4 Hz, 3H), 3.31 (m, 1H), 2.02 (s, 3H), 1.92 (m, 2H), 1.21 (d, J = 6.4 Hz, 3H). ^{13}C NMR (101 MHz, D2O) δ 174.88, 102.86, 102.73, 102.05, 100.17, 99.35, 81.92, 78.24, 76.35, 75.75, 75.19, 75.03, 74.79, 74.71, 74.28, 73.46, 72.73, 72.00, 71.60, 69.92, 69.54, 69.06, 68.26, 68.12, 67.30, 66.88, 61.07, 60.86, 59.97, 59.92, 55.31, 47.79, 28.17, 22.14, 15.26. HRMS (ESI): m/z $[M+Na]^{+}$ calcd for C35H60N4O25 959.3439; found: 959.3450.

Scheme 6



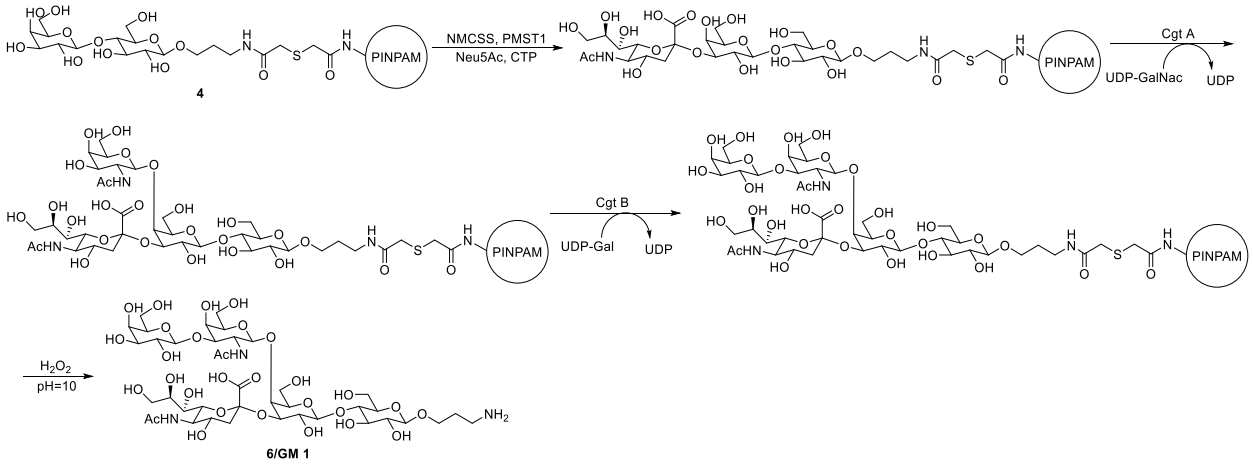
Compound 24 was prepared from compound 23 using BgtA. In detail, a reaction mixture in a final volume of 1.5 ml mixture containing 50 mM Tris-HCl (pH 8.0), 14 mg of compound 23 (10 mM), 5 mM of Mg^{2+}, 15 mM of UDP-GalNAc, and 0.6 mg of BgtA was incubated at 25°C to allow the formation of compound 24. After 6 hours, the reaction was monitored by TLC (EtOAc/MeOH/H2O/HOAc=5:2:1.4:0.4). An equal volume of ethanol was added to remove proteins and the solution was concentrated in vacuo. The product was purified by using HPLC (water/acetonitrile,75-35, 16min/35min). 10.2 mg of compound 24 was obtained in 59.9 % isolated yield regarding compound 23. ^{1}H-NMR (600 MHz, D2O), δ 5.23 (d, J = 2.4 Hz, 1H), 5.06 (d, J = 2.8 Hz, 1H), 4.58 (d, J = 5.6 Hz, 1H), 4.49 (d, J = 5.2 Hz, 1H), 4.37 (d, J = 5.2 Hz, 1H), 4.33 (d, J = 5.6 Hz, 1H), 4.20 (dd, J1 = 4 Hz, J2 = 8.8 Hz, 1H), 4.12 (m, 3H), 4.02 (d, J = 2.4 Hz, 1H), 3.87-3.51 (m, 29H), 3.35(m, 3H), 3.20 (m, 1H), 1.92 (s, 3H), 1.91 (s, 3H), 1.81 (m, 2H), 1.13 (d, J = 4.4 Hz, 3H), ^{13}C NMR (151 MHz, D2O) δ 174.86, 174.73, 102.86, 102.75, 102.06, 99.99, 98.57, 91.25, 81.94, 78.23, 75.86, 75.62, 75.12, 75.08, 74.80, 74.72, 74.29, 72.76, 72.32, 72.08, 71.63, 71.03, 69.95, 69.89, 68.43, 68.26, 67.69, 67.60, 67.30, 66.83, 62.99, 61.26, 61.15, 60.87, 59.97, 59.88, 55.40, 49.45, 47.81, 28.18, 22.19, 21.92, 15.13. HRMS (ESI): m/z $[M+Na]^{+}$ calcd for C43H73N5O30 1162.4232; found: 1162.4176.

Scheme 7



Compound 25 was prepared from compound 23 using GTB from humans. In detail, a reaction mixture in a final volume of 1.5 ml mixture containing 50 mM Tris-HCl (pH 8.0), 14 mg of compound 23 (10 mM), 5 mM of Mg^{2+}, 15 mM of UDP-Gal, and 0.6 mg of GTB was incubated at 25°C to allow the formation of compound 25. After 6 hours, the reaction was monitored by TLC (EtOAc/MeOH/H2O/HOAc=5:2:1.4:0.4). An equal volume of ethanol was added to remove proteins and the solution was concentrated in vacuo. The product was purified by using HPLC (water/acetonitrile,75-35, 18min/35min). 9.6 mg of compound 25 was obtained in 58.4 % isolated yield regarding compound 23. ^{1}H-NMR (600 MHz, D2O), δ 5.29 (d, J = 2.8 Hz, 1H), 5.21 (d, J = 1.6 Hz, 1H), 4.67 (d, J = 5.6 Hz, 1H), 4.59 (d, J = 5.2 Hz, 1H), 4.45 (d, J = 5.2 Hz, 1H), 4.41 (d, J = 5.2 Hz, 1H), 4.27 (m, 2H), 4.17 (t, J = 4 Hz, 1H), 4.11 (d, J = 2.4 Hz, 1H), 3.98-3.53 (m, 31H), 3.43 (m, 3H), 3.29 (m, 1H), 2.00 (s, 3H), 1.88 (m, 2H), 1.20 (d, J = 4.4 Hz, 3H), ^{13}C NMR (151 MHz, D2O) δ 174.88, 102.87, 102.77, 102.07, 100.05, 98.74, 92.96, 81.97, 78.25, 76.11, 75.84, 75.07, 74.91, 74.82, 74.73, 74.31, 72.78, 72.48, 72.10, 71.64, 71.10, 69.97, 69.45, 69.23, 68.27, 68.01, 67.65, 67.32, 66.79, 63.44, 62.44, 61.23, 61.13, 60.89, 59.99, 59.89, 55.39, 47.83, 28.20, 22.21, 15.15. HRMS (ESI): m/z $[M+Na]^{+}$ calcd for C41H70N4O30 1121.3967; found: 1121.3912.

Scheme 8

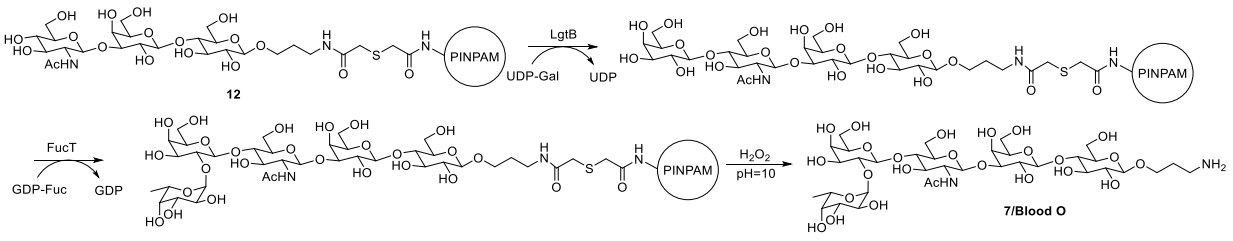


Fully automated enzymatic synthesis of GM 1 on our automated synthesizer. A synthetic program was made for compound 6 that contains three cycles (table 2.1). Compound 4 was loaded into the reaction vessel and all the enzymes and sugar nucleotide solutions added to the corresponding tubes. The following procedures were performed by the program on the automated synthesizer. For the first glycosylation, steps 1 and 2: Neu5Ac, CTP, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0) and NmCSS, PmST1 (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 15 mM Neu5Ac and CTP, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 7.4 mg of NmCSS, 1.6 mg of PmST1, and 5 mM compound 4; step 3: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 3 h (microwave 10 W, hold 3 h at 25°C, bubble on for 5 s, off for 600 s. This is a special case and all the other glycosyl reactions were kept for 6 h); step 4: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s). This glycosylation was only done by once and all the others were done by twice to improve the yield. For the second glycosylation, steps 1 and 2: UDP-GalNAc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and CgtA (5 ml) were

added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-GalNAc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 2.1 mg of CgtA, and 5 mM resin-bound sugar; step 3: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 4: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s); steps 5 and 6: UDP-GalNAc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and CgtA (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-GalNAc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 2.1 mg of CgtA, and 5 mM resin-bound sugar; step 7: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 8: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s). For the third glycosylation, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0) and CgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 1.8 mg of CgtB, and 5 mM resin-bound sugar; step 3: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 4: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only

(microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s); steps 5 and 6: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0) and CgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 1.8 mg of CgtB, and 5 mM resin-bound sugar; step 7: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 8: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s). After three cycles, the resin-bound sugar was transferred to a flask and the sugar released under 1 M H_2O_2 (PH=10). The final compound 6 (20 mg) was purified by HPLC (water/acetonitrile,80-54.2, 35min/45min) in 38% yield from compound 4. ^{1}H-NMR (400 MHz, D2O), δ 4.48 (m, 3H), 4.09 (m, 3H), 3.98 (m, 3H), 3.85 (d, J = 2 Hz, 1H), 3.81-3.52 (m, 22H), 3.46 (m, 2H), 3.30 (m, 3H), 3.10 (t, J = 4.4 Hz, 1H), 2.60 (dd, J1 = 3.2 Hz, J2 = 8.8 Hz, 1H), 1.97-1.78 (m, 9H). HRMS (ESI): m/z $[M+H]^+$ calcd for C40H69N3O29 1056.4090; found: 1056.4045.

Scheme 9

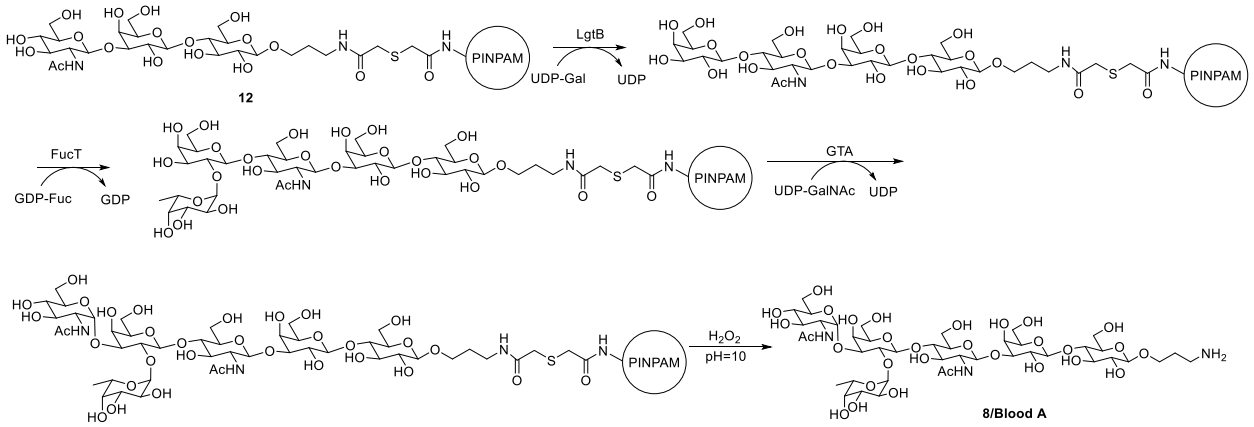


Fully automated enzymatic synthesis of Blood O on our automated synthesizer. A synthetic program was made for synthesizing compound 7 and this program contains two cycles (table 2.1). Compound 12 was loaded into the reaction vessel and all the enzyme and sugar nucleotide

solutions were added to the corresponding tubes. The following procedures were performed by the program on the automated synthesizer. For the first glycosylation, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0) and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 1.0 mg of LgtB, and 4 mM compound 12; step 3: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 4: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s); steps 5 and 6: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0) and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 1.0 mg of LgtB, and 4 mM resin-bound sugar; step 7: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 8: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s). For the second glycosylation, steps 1 and 2: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0) and 1,2-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 2.5 mg of 1,2-FucT, and 4 mM resin-bound sugar; step 3: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble

on for 5 s, off for 600 s); step 4: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s); steps 5 and 6: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0) and 1,2-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 2.5 mg of 1,2-FucT, and 4 mM resin-bound sugar; step 7: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 8: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s). After two cycles, the resin-bound sugar was transferred to a flask and the sugar released under 1 M H_2O_2 (PH=10). The final compound 7 (13 mg) was purified by HPLC (water/acetonitrile, 80-54.2, 34 min/45 min) in 35% yield from compound 12. ^{1}H-NMR (600 MHz, D2O), δ 5.24 (d, J = 2 Hz, 1H), 4.64 (d, J = 5.6 Hz, 1H), 4.48 (d, J = 5.2 Hz, 1H), 4.37 (d, J = 5.2 Hz, 1H), 4.15 (m, 1H), 4.07 (m, 1H), 3.98 (m, 2H), 3.82 (m, 2H), 3.74-3.47 (m, 22H), 3.40-3.07 (m, 4H), 1.97 (s, 3H), 1.81 (m, 2H), 1.16 (d, J = 4.4 Hz, 3H). HRMS (ESI): m/z $[M+Na]^+$ calcd for C35H62N2O25 933.3524; found: 933.3592.

Scheme 10

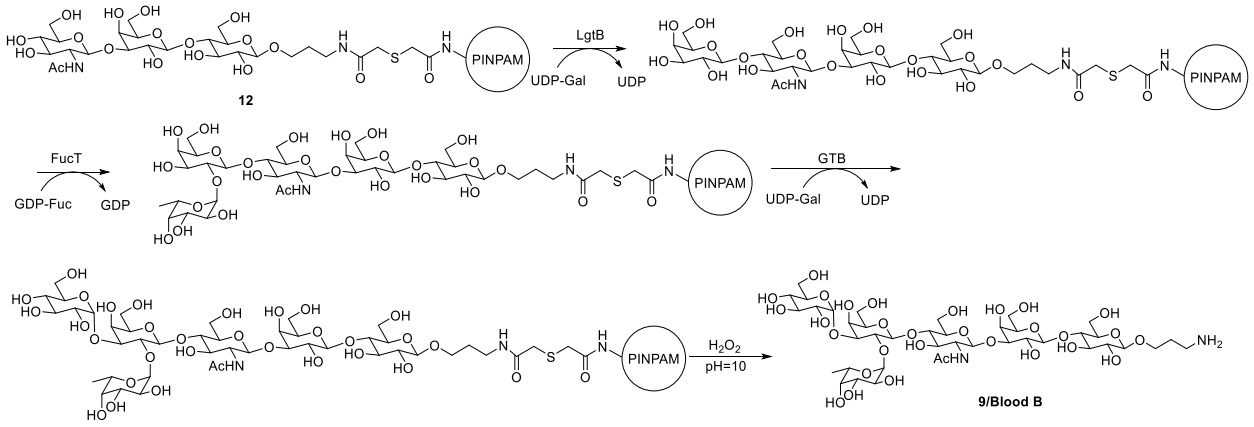


Fully automated enzymatic synthesis of Blood A on our automated synthesizer. A synthetic program was made for compound 8 and this program contains three cycles (table 2.1). Compound 12 was loaded into the reaction vessel and all the enzyme and sugar nucleotide solutions were added to the corresponding tubes. The following procedures were performed by the program on the automated synthesizer. For the first glycosylation, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 1.0 mg of LgtB, and 4 mM compound 12; step 3: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 4: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s); steps 5 and 6: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 1.0 mg of LgtB, and 4 mM resin-bound sugar; step 7: the temperature of the reaction mixture was maintained

at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 8: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s). For second glycosylation, steps 1 and 2: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,2-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 2.5 of 1,2-FucT, and 4 mM resin-bound sugar; step 3: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 4: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s); steps 5 and 6: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,2-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 2.5 of 1,2-FucT, and 4 mM resin-bound sugar; step 7: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 8: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s). For the third glycosylation, steps 1 and 2: UDP-GalNAc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and BgtA (5 ml) were added to the reaction vessel to

form a solution (10 ml) as below: 10 mM UDP-GalNAc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 1.9 mg of GTA, and 4 mM resin-bound sugar; step 3: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 4: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s); steps 5 and 6: UDP-GalNAc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and BgtA (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-GalNAc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 1.9 mg of GTA, and 4 mM resin-bound sugar; step 7: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 8: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s). After three cycles, the resin-bound sugar was transferred to a flask and the sugar released under 1 M H_2O_2 (PH=10). The final compound 8 (14 mg) was purified by HPLC (water/acetonitrile,75-35, 31min/35min) in 29% yield from compound 12. ^{1}H-NMR (600 MHz, D2O), δ 5.29 (d, J = 2.4 Hz, 1H), 5.11 (d, J = 1.6 Hz, 1H), 4.64 (d, J = 5.2 Hz, 1H), 4.54 (d, J = 5.6 Hz, 1H), 4.45 (d, J = 5.6 Hz, 1H), 4.38 (d, J = 4.8 Hz, 1H), 4.25 (m, 1H), 4.18 (m, 3H), 4.07 (m, 1H), 3.97-3.83 (m, 9H), 3.76-3.55 (m, 20H), 3.38-3.08 (m, 4H), 1.99 (m, 8H), 1.18 (d, J = 4.4 Hz, 3H). HRMS (ESI): m/z $[M+H]^+$ calcd for C43H75N3O30 1114.4508; found: 1114.4485.

Scheme 11

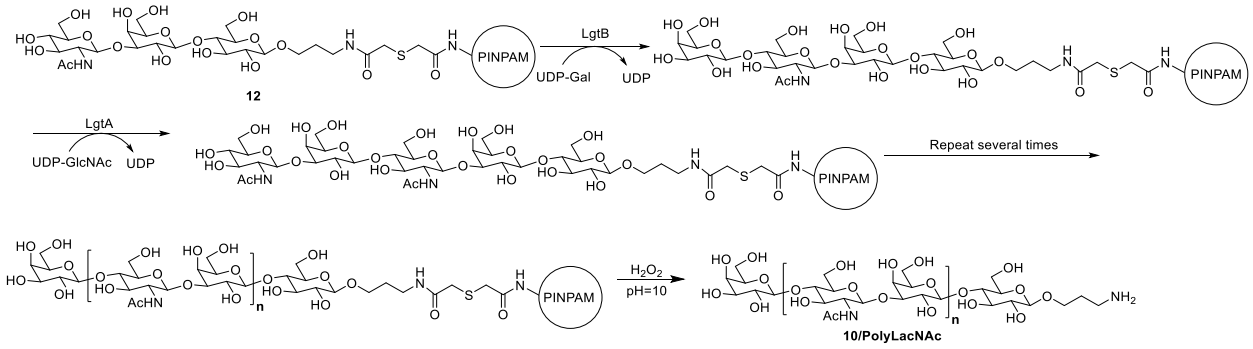


Fully automated enzymatic synthesis of Blood B on our automated synthesizer. A synthetic program was made for compound 9 and this program contains three cycles (table 2.1). Compound 12 was loaded into the reaction vessel and all the enzyme and sugar nucleotide solutions were added to the corresponding tubes. The following procedures were performed by the program on the automated synthesizer. For the first glycosylation, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 1.0 mg of LgtB, and 4 mM compound 12; step 3: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 4: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s); steps 5 and 6: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg2+, 1.0 mg of LgtB, and

4 mM resin-bound sugar; step 7: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 8: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s). For the second glycosylation, steps 1 and 2: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,2-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 2.5 mg of 1,2-FucT, and 4 mM resin-bound sugar; step 3: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 4: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s); steps 5 and 6: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,2-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 2.5 mg of 1,2-FucT, and 4 mM resin-bound sugar; step 7: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 8: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s). For the third glycosylation, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl

(pH 8.0), and GTB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 1.8 mg of GTB, and 4 mM resin-bound sugar; step 3: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 4: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s); steps 5 and 6: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and GTB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 1.8 mg of GTB, and 4 mM resin-bound sugar; step 7: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 8: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s). After three cycles, the resin-bound sugar was transferred to a flask and the sugar released under 1 M H_2O_2 (PH=10). The final compound 9 (13 mg) was purified by HPLC (water/acetonitrile,75-35, 28min/35min) in 27.1% yield from compound 12. ^{1}H-NMR (600 MHz, D2O), δ 5.21 (m, 1H), 5.13 (m, 1H), 4.59 (d, J = 5.2 Hz, 1H), 4.52 (d, J = 4.8 Hz, 1H), 4.40 (d, J = 5.2 Hz, 1H), 4.33 (d, J = 5.2 Hz, 1H), 4.19 (m, 2H), 4.08 (m, 1H), 4.03 (m, 1H), 3.86-3.53 (m, 31H), 3.33-3.04 (m, 4H), 2.00-1.90 (m, 5H), 1.20 (m, 3H). HRMS (ESI): m/z $[M+H]^+$ calcd for C41H72N2O30 1073.4243; found: 1073.4229.

Scheme 12



Fully automated enzymatic synthesis of PolyLacNAc on our automated synthesizer. A synthetic program was made for compound 10 and this program contains nine cycles (table 2.1). Compound 12 was loaded into the reaction vessel and all the enzyme and sugar nucleotide solutions were added to the corresponding tubes. For the first glycosylation, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 1.0 mg of LgtB, and 4 mM compound 12; step 3: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h (microwave 10 W, hold 6 h at 25°C, bubble on for 5 s, off for 600 s); step 4: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s); steps 5 and 6: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 1.0 mg of LgtB, and 4 mM resin-bound sugar; step 7: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 6 h

(microwave 10 W, hold 6 h at 25° C, bubble on for 5 s, off for 600 s); step 8: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s). For the second glycosylation, steps 1 and 2: UDP-GlcNAc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtA (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-GlcNAc, 50 mM Tris-HCl (pH 8.0), 4 mM Mg^{2+}, 1.7 mg of LgtA, and 4 mM resin-bound sugar; step 3: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 12 h (microwave 10 W, hold 12 h at 25°C, bubble on for 5 s, off for 600 s. This is a special case and all the other glycosyl reactions were kept for 6 h); step 4: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s); steps 5 and 6: UDP-GlcNAc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtA (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-GlcNAc, 50 mM Tris-HCl (pH 8.0), 4 mM Mg^{2+}, 1.7 mg of LgtA, and 4 mM resin-bound sugar; step 7: the temperature of the reaction mixture was maintained at 25°C under 10 W of microwave irradiation. Glycosyl reaction was done at 25°C for 12 h (microwave 10 W, hold 12 h at 25°C, bubble on for 5 s, off for 600 s. This is a special one and all the other glycosyl reactions were kept for 6 h); step 8: The temperature of the reaction mixture was quickly brought up to 90°C under 50 W of microwave irradiation. It was kept at 90°C for 10 min and drained to push out all the waste to leave resin-bound sugar only (microwave 50 W, hold 10 min at 90°C, bubble on for 1 s, off for 3 s, drain for 100 s). To obtain a longer polyLacNAc, these two cycles were repeated.

After nine cycles, the resin-bound sugar was transferred to a flask and the sugar released under 1 M H_2O_2 (PH=10). The final compound 10 (8 mg) was purified by HPLC (water/acetonitrile,60-45, 14min/40min) in 9.0% yield from compound 12. ^{1}H-NMR (600 MHz, D2O), δ 4.68 (m, 4H), 4.44 (m, 8H), 4.09 (m, 6H), 4.00 (m, 1H), 3.92 (m, 6H), 3.79-3.64 (m, 39H), 3.61-3.46 (m, 14H), 3.28 (m, 1H), 3.10 (m, 3H), 2.01-1.93 (m, 14H). HRMS (ESI): m/z [M +H+Na]2+ calcd for C85H144N6O61 1124.4158; found: 1124.4159.

2.7 Conclusion

In summary, a fully machine-driven system based on enzymatic glycosylation has been developed for the automated synthesis of oligosaccharides. Several important oligosaccharides (GM1, Blood O, A, and B antigens, and Poly-LacNAc) were selected and successfully prepared in an automated manner. The use of a temperature-dependent polymer, which is soluble in water at room temperature and insoluble at elevated temperatures, overcomes limitations of solid-phase enzymatic synthesis. It allows for both aqueous-phase reactions and solid product separation.

This study represents a proof-of-concept demonstration that enzymatic synthesis of oligosaccharides can be achieved in an automated manner using a commercially available peptide synthesizer. This work will enable the development of an improved instrument for automated oligosaccharide synthesis in the future. Such improvements should include more storage tubes for enzymes and glycosylation donors, a temperature control system for enzyme storage, a detection system for monitoring reaction processes, more reaction vessels to perform multi-oligosaccharide syntheses simultaneously, and a larger reaction vessel to perform large-scale reactions. Moreover, a database on enzymatic reaction kinetics of glycosyltransferase enzymes should also be developed and built into the control program in future studies, which will help to improve the automation process by optimizing reaction time and enzyme usage.

3 SECOND GENERATION AUTOMATED PLATFORM

Glycoproteins are proteins containing oligosaccharide chains linked through covalent bonds. Most of them are important internal membrane proteins (IMPs), where they play a variety of important biological and physiological roles in living organisms.[2, 3, 5, 59-61] IMPs include transporters, linkers, channels, receptors, enzymes, cell attachment recognition site (cell-cell interactions) and so on. Ease of access to targets is vital for research into the relationship between structure and function of glycoproteins. Glycopeptides, fragments of glycoproteins, have been used to study the physiological and biological activity of their original glycoproteins. At the same time, glycopeptides have many important biological and medical applications. They have been used in clinical diagnostics, disease therapy, and biotechnological tools.[62, 63] However, glycopeptides contain both a peptide chain and one or more oligosaccharide chains, the combination of which makes synthesis more complex.[6] Two major approaches have been reported for glycopeptide synthesis. Briefly, the peptide chain could first be synthesized by solid phase peptide synthesis in an organic phase system after which the oligosaccharide portion would be built up through enzymatic reactions in an aqueous phase system.[64] The other strategy is to first prepare an amino acid building block already containing the oligosaccharide and then incorporate the amino acid into a peptide by conventional solid phase peptide synthesis much like any other amino acid.[6] Both ways need skilled researchers to perform synthetic process, which is a time-consuming and labor-intensive process.

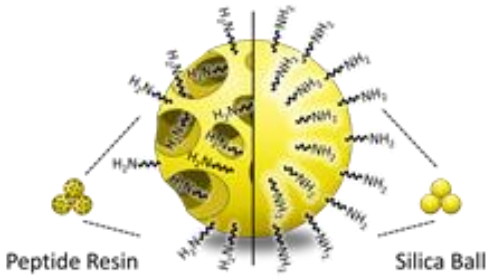


Figure 3.1 Difference of Traditional Peptide Resins and Silica Gel

Automated synthesis allows non-specialists to rapidly prepare compounds which could save considerable time and labor. A number of synthesizers have been developed for automated assembly of the natural biopolymers (oligosaccharides, oligonucleotides and oligopeptides) since solid phase peptide synthesis was first reported by R. B. Merrifield in 1963.[7, 8, 27] Over the past few decades, many advanced automated synthesizers have been put into market to produce almost every possible oligopeptide and oligonucleotide which has greatly advanced the fields of biology and medicine. In 2001, a chemical automated platform was created for oligosaccharide synthesis by Seeberger and co-workers using a solid phase methodology based on a commercially available peptide synthesizer.[13] Subsequently, Seeberger developed his own glycan synthesizer (Glyconeer 2.1) which uses polystyrene as solid support.[43] These approaches are based on Merrifield's concept, where a growing compound is first attached on a solid support through a cleavable linker, allowing purification by simple washing steps and release of the compound after several automated cycles. All the resins which are used as solid supports are highly porous (Figure 3.1) allowing internal attachment of primers to increase loading efficiency. In 2018, G. J. Boons and our group found that these holes block enzymatic glycosylation and published two independent papers on enzymatic automated synthesis based on water soluble resins.[65, 66] Nevertheless, all these reported automated platforms can only be used to synthesize peptides, nucleotides, and saccharides, but not glycopeptides. Herein, we report an automated platform for the preparation of glycopeptides

(Figure 3.2). This platform is based on the use of a silica gel solid support, a peptide synthesizer to prepare a peptide chain in an organic phase system, and another peptide synthesizer to perform enzymatic glycosylation in an aqueous phase system.

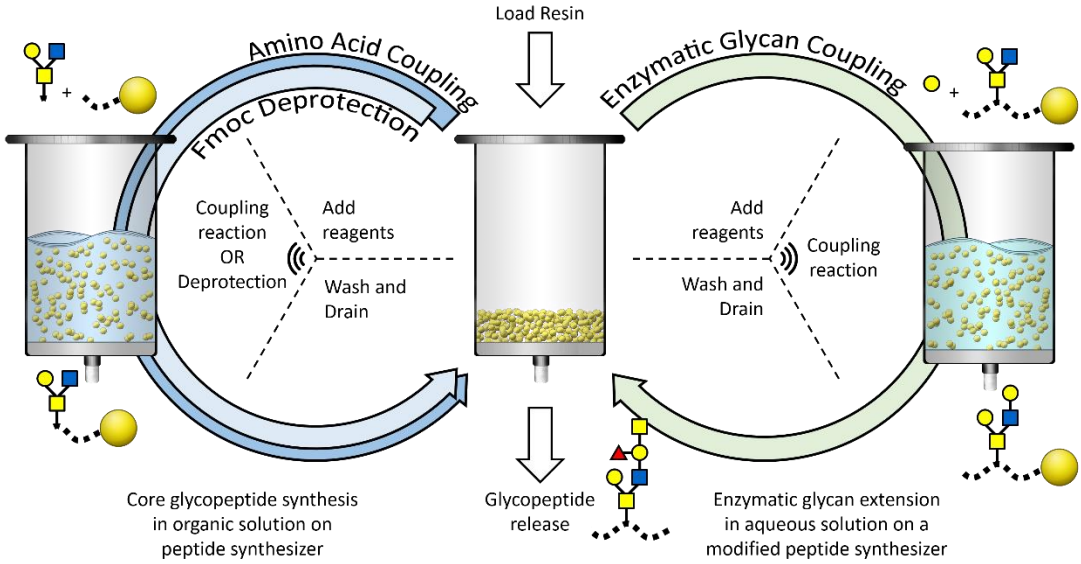


Figure 3.2 Automated Process of Glycopeptides

3.1 Resin

The key point for automated process of glycopeptide synthesis is that the reaction and separation steps are performed in both organic (peptide chain) and water phase (oligosaccharide chain). The traditional solid resins can be used for two solvent system synthesis and have highly loading efficiency, but they are only suitable for small molecule synthesis, as the tiny holes inside would block enzymatic reactions. The water-soluble resins, which are used for enzymatic glycosylation,[23, 67] are hardly used for automated processes in organic phase systems. It is necessary to find an ideal resin which could be used to achieve our goal. In our design, an aminopropyl silica resin is used as a solid support because it is compatible with both water and organic solvents, although it has a much lower loading efficiency that traditional solid resin. In 1994, Chi-Huey Wong's group

reported that this resin can be used for solid phase enzymatic synthesis with a 0.2 mmol/g loading efficiency.[68] A trisaccharide was successfully synthesized by using the solid resin in ideal scale.

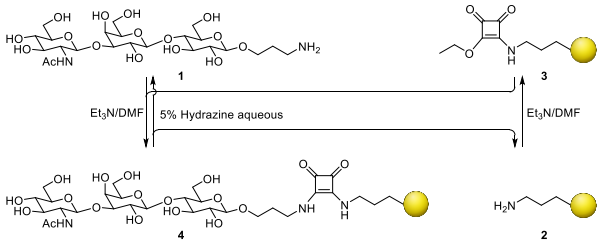


Figure 3.3 Conjugation of Oligosaccharide Primer (Trisaccharide) with Silica Resin through a Cleavable Linker

$$\text{Loading Efficiency} = \frac{\left\{W_{[Resin]} - W_{[Original\ Resin]}\right\} \Big/ Mw_{[Primer+Linker]}}{W_{[Resin]}}$$

$$\text{Release Efficiency} = \frac{W_{[Resin]} - W_{[Released\ Resin]}}{W_{[Resin]} * Loading\ Efficiency} \times 100\%$$

3.2 Linker

In our design, an aminopropyl silica resin is used as a solid support because it is compatible with both water and organic solvents, although it has a much lower loading efficiency that traditional solid resin. In 1994, Chi-Huey Wong's group reported that this resin can be used for solid phase enzymatic synthesis with a 0.2 mmol/g loading efficiency.[68] With a large density of amino group on the surface (Figure 3.1), a cleavable linker is easily attached to the silica resin. Usually, cleavage of a removable linker requires exposure to either harsh acidic or basic condtions. However, glycans are not stable in strong acids and peptides can undergo racemization in strongly basic conditions. Moreover, the side chain of cysteine can be damaged under oxidation condition. In 1991, Glu¨senkamp's group reported 3,4-diethoxy-3-cyclobuten-1,2-dione (squaric acid diethyl ester) as a cleavable linker.[69] This linker can release a compound with a terminal NH2 in a 5% hydrazine aqueous solution (Figure 3.3). Compound 1 was linked to the solid support through the squaric

acid linker, giving a loading efficiency of 0.1 to 0.2 mmol/g of dry silica resin (Figure 2C). Excess amino groups were then capped using acetic anhydride. To test the cleavage efficiency of this linker, compound 4 was treated with 5% hydrazine aqueous solution. The release efficiency is calculated by the given equation. More than 90% of the compound was released in less than 12 h.

3.3 Reaction efficiency

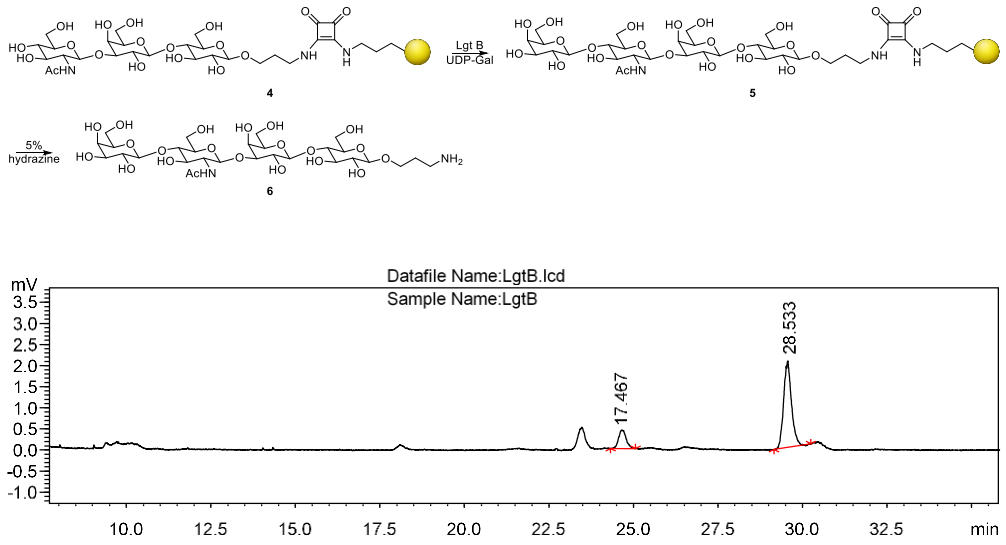


Figure 3.4 Enzymatic Activity Test in the Solid Phase Synthesis

In addition, we also tested the enzymatic activity of many glycotransferases on the silica resin. β-1,4-galactosyltransferase (Lgt B) was chosen as an example to test the enzymatic activity in the solid phase synthesis (Figure 3.4). Compound 4, prepared as described above, was used as the glycosylation primer. To the reaction vessel, 500 mg (50 umol) of conjugated silica resin (compound 4) was loaded. The automated synthetic process was then started by automatically injecting Lgt B (10 mg), UDP-Gal (3 equiv), and reaction buffer (10 mL, pH 8.0). Once the reaction finished (24 h), unreacted materials and by-products were removed by filtration under high pressure. The silica resin was washed and taken out. Compound 5 and 5% aqueous hydrazine (5 ml) were put into a column. After the reaction finished (12 h), the filtration was collected and a HPLC performed. The results show that more than 80% trisaccharide was converted to

tetrasaccharide (Figure 3.4). In a similar manner, the enzymatic activities of 1,2-Fuc T, Gt A and Gt B were obtained (Figure 3.5).[70-72] After 24 h reactions, the activities ranged from 62% to 80%. It turns out that most of the sugars can be put on another monosaccharide after two repetitions of the same enzymatic glycosylation step (double coupling).

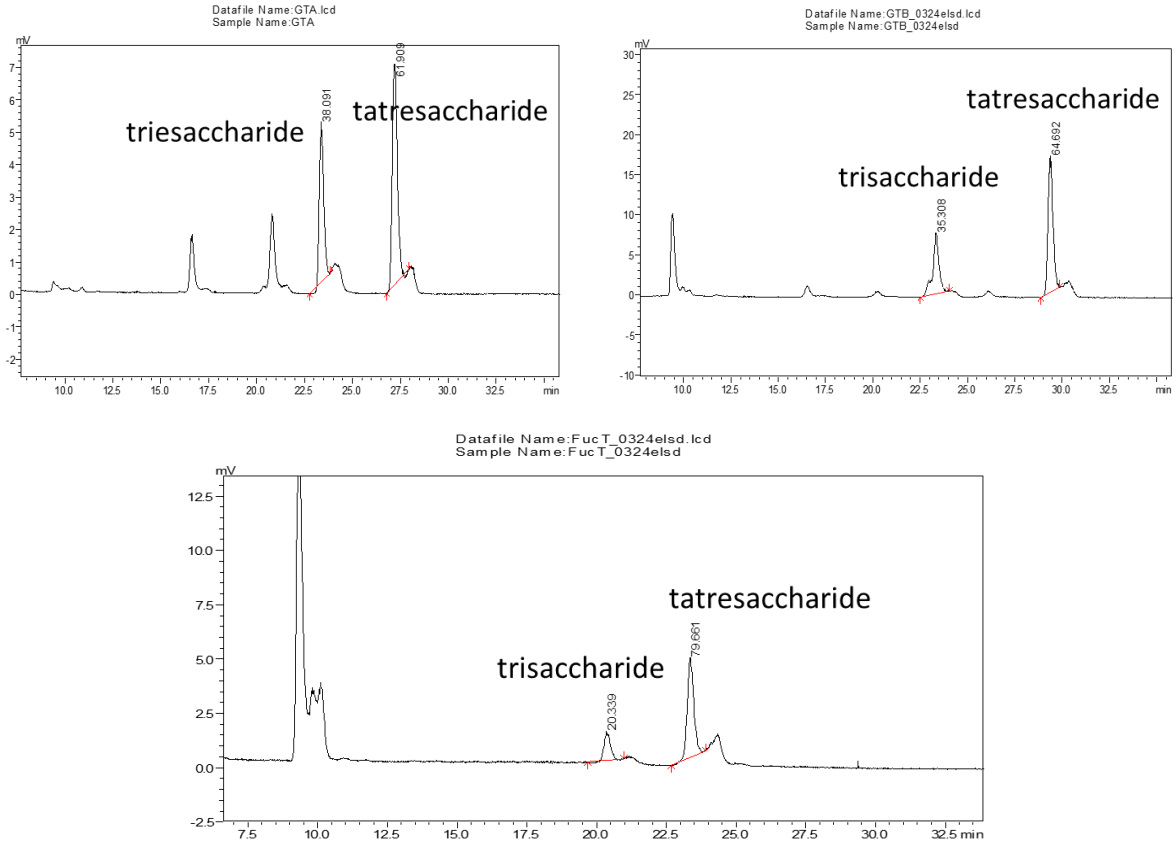


Figure 3.5 Enzyme Activity (GtA, GtB and 1,2-Fuc T)

3.4 Instrument

Finally, a CEM Liberty Blue peptide synthesizer was employed to perform the designed automation process according to previous reports. The synthesizer can perfectly prepare the peptide part without any modification. As it was reported previously, the synthesizer can be used for oligosaccharide chain synthesis with some modifications. The program for automated enzymatic reactions is easily added to the Liberty Blue software (Details are shown in Table 3.1).

Table 3.1 Automated Programm for Enzymatic Glycosylations

Function	Step	Command
Add reagent	1	Add sugar nucleotide buffer
Add reagent	2	Add enzyme buffer
Microwave method	3	Hold 24 h at 37℃, bubble on for 5 s, off for 600 s, drain 100s
Add reagent	4	Add sugar nucleotide buffer
Add reagent	5	Add enzyme buffer
Microwave method	6	Hold 24 h at 37℃, bubble on for 5 s, off for 600 s, drain 100s
Wash	7	

3.5 Automated Process

Compound 10 was chosen as a target to test our platform (Figure 3.7). The first amino acid was attached to the resin by manual operation (Figure 3.6). Mucin 1 is a glycoprotein with extensive O-linked glycosylation, which is overexpressed on the surface of many cancers. The study of Mucin 1 may prove useful in the clinical diagnosis and treatment of cancer. The PAHGVSSAPD peptide is a repeat unit of Mucin 1, which involves nine reactions from the first amino acid (Asp) (Figure 3.7). The automated synthesis of the peptide backbone was controlled by the original program protocols. Compound 8 (1 g) was used a starting material and put into the reaction vessel of peptide synthesizer. The synthesizer was programmed to add deprotection solution (20% piperidine of DMF solution, 6 ml). After 3 min, the solution was removed by filtration. Additional deprotection solution was added and filtration performed again after 3min. The resin was washed three times with DMF (10 ml * 3). Then Fmoc-Pro-OH (5 ml), HATU (2 ml) and DIEA (2 ml)

were injected into the reaction vessel to couple the second amino acid. The solution was removed by filtration after 10 min. The coupling reaction was performed one more time and the resin was washed three times with DMF (10 ml * 3) for the next reaction cycle. The program was set up to perform nine cycles (all the details are provided in methods). The resin was taken out and ready to be used for enzymatic glycosylation. Half of the samples were shaken with 5% hydrazine to release the peptide and the solution collected by filtration. After HPLC purification, 10 mg of peptide was obtained in 12.9% yield from compound 8. The product was confirmed by NMR and MS analysis (see Appendix B).

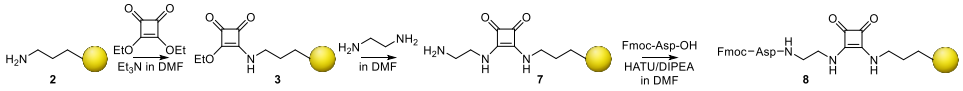
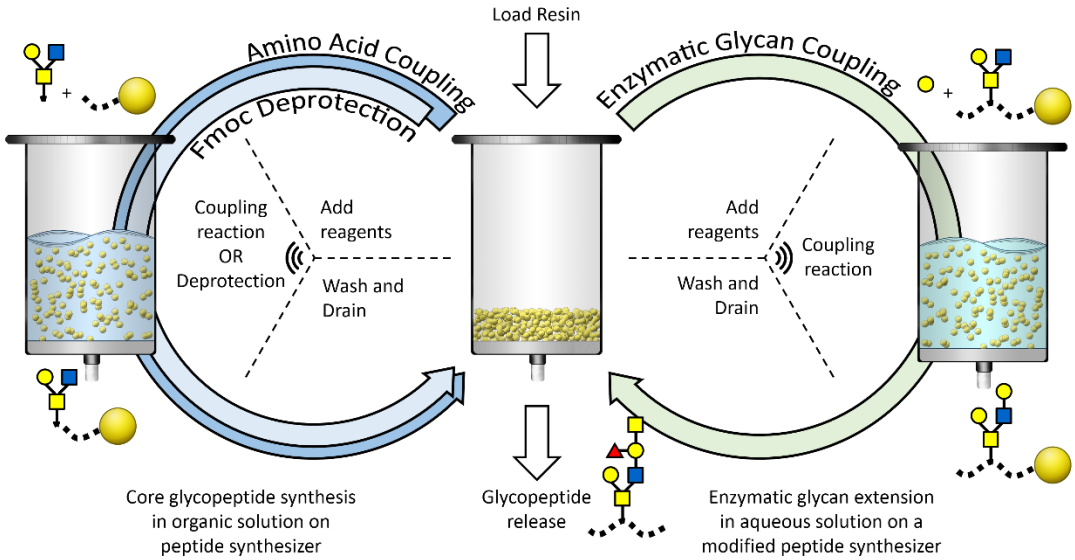


Figure 3.6 Loading the First Amino Acid

	Function	Steps	Command
Fmoc Removal Cycle1	Add reagent	1	Add deprotection solution
	Microwave method	2	Hold 3 min at 50℃, bubble on for 2 s, off for 3 s, drain 100s
	Add reagent	3	Add deprotection solution
	Microwave method	4	Hold 3 min at 50℃, bubble on for 2 s, off for 3 s, drain 100s
	Wash	5	10ml DMF wash three times
Amino Acid Coupling Cycle1	Add reagent	6	Add Amino Acid, HATU and DIPEA solution
	Microwave method	7	Hold 10 min at 50℃, bubble on for 2 s, off for 3 s, drain 100s
	Wash	8	10ml DMF wash three times
Fmoc Removal Cycle2			...

	Function	Steps	Command
Enzymatic coupling Cycle 1	Add reagent	1	Add sugar nucleotide buffer
	Add reagent	2	Add enzyme buffer
	Microwave method	3	Hold 24 h at 37℃, bubble on for 5 s, off for 600 s, drain 100s
	Add reagent	4	Add sugar nucleotide buffer
	Add reagent	5	Add enzyme buffer
	Microwave method	6	Hold 24 h at 37℃, bubble on for 5 s, off for 600 s, drain 100s
Enzymatic coupling Cycle 2	Add reagent	7	Add sugar nucleotide buffer
	Add reagent	8	Add enzyme buffer
	Microwave method	9	Hold 24 h at 37℃, bubble on for 5 s, off for 600 s, drain 100s
			...



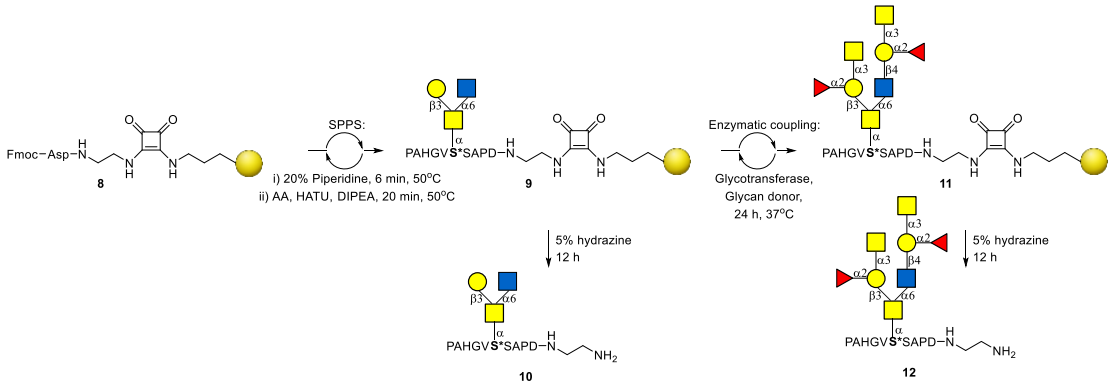


Figure 3.7 Automated Synthesis Process of Glycopeptide

Compound 12 was prepared by extending the oligosaccharide chain with enzymatic glycosylations. Blood A epitope contains three glycosylation reactions from core 2. The enzymes used were Lgt B, 1,2-Fuc T and Gt A (Figure 3.7). All the steps were automated and performed under the control of a new program, which was designed for enzymatic reactions. The resin (compound 9) was transferred to a new reaction vessel. LgtB (5 mg), UDP-Gal (2 equiv), and reaction buffer (10 mL, pH 8.0) were automated injected to the vessel under the control of the program. Once this reaction finished (12 h), unreacted materials and by-products were removed by filtration. The enzymatic glycosylation reaction was performed one more time and the resin was washed three times with water (10 ml * 3). The program was set up to perform three enzymatic reactions catalyzed by Lgt B, 1,2-Fuc T and Gt A to generate Compound 11. Compound 11 was treated with 5% hydrazine to release the peptide and the solution collected by filtration. After HPLC purification, 10 mg of peptide was obtained in 11.8% yield from compound 8. The product was confirmed by NMR and MS analysis (see Appendix B).

3.6 Results

In a similar automated manner, compounds 13-20 were prepared using enzymes reported previously. All the targets were purified by HPLC to give highly pure compounds. All products were confirmed by NMR and MS analysis (see Appendix B).

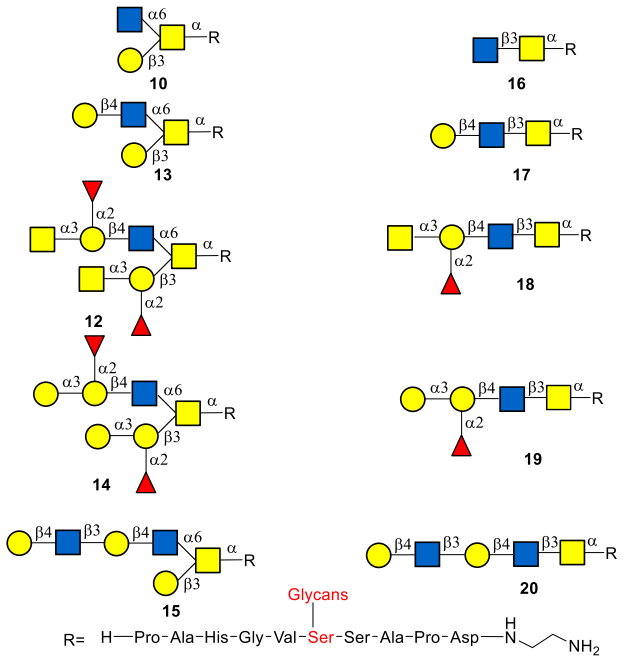


Figure 3.8 Targets That Were Used to the Test Automated System in This Work

3.7 Materials and General Methods

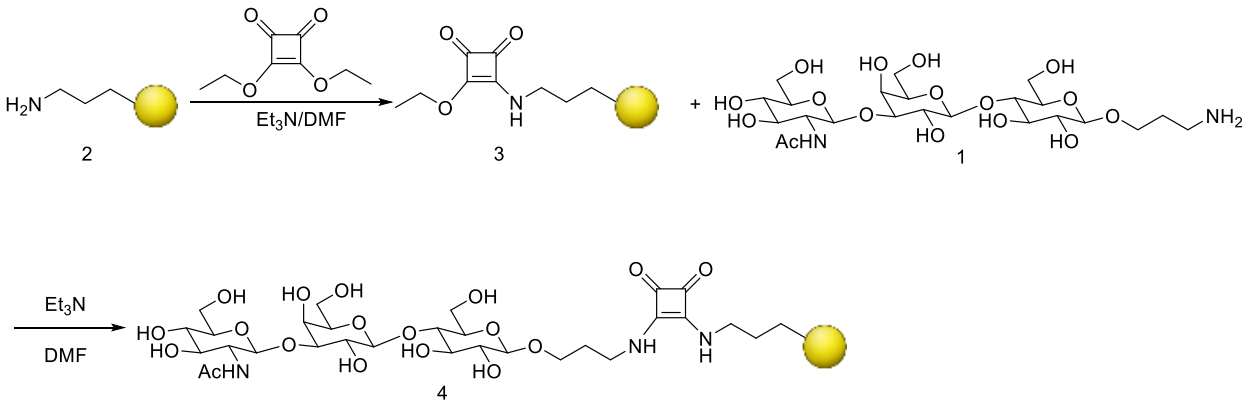
All commercially available solvents and reagents were used without purification. Silica Gel Resin was purchased from Fisher Scientific (USA). 3,4-diethoxy-3-cyclobuten-1,2-dione was purchased from MilliporeSigma. N-Acetylneuraminic acid (Neu5Ac) and cytidine 5'-triphosphate (CTP) were purchased from Carbosynth Limited. Sugar nucleotides uridine 5'-diphospho-galactose (UDP-Gal), UDP-N-acetylglucosamine (UDP-GlcNAc), UDP-N-acetylgalactosamine (UDP-GalNAc), and guanosine 5'-diphospho-L-fucose (GDP-Fuc) were prepared as described previously. Helicobacter pylori β1,3-N-acetylglucosaminyltransferase (HpLgtA), Neisseria meningitides β1,4-galactosyltransferase (NmLgtB), Helicobacter mustelae α1,2 -fucosyltransferase, Helicobacter mustelae α1,3-N-acetyl-galactosaminyltransferase (BgtA), and human blood group B glycosyltransferase (GTB) were expressed and purified as described previously.

^{1}H-NMR and ^{13}C-NMR spectra were recorded by using a Bruker 400 spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C) or a Bruker 600 spectrometer (600 MHz for ^{1}H, 150 MHz for ^{13}C). All 1H Chemical shifts (in ppm) were assigned in reference to $CDCl_3$ (δ= 7.24 ppm), MeOD (δ= 4.87 ppm), and D_2O (δ = 4.79 ppm) and all ^{13}C NMR was calibrated with $CDCl_3$ (δ = 77.00 ppm). HPLC was performed on Shimadzu Prominence UFLC. Column: Waters XBridge BEH amide column, 130 Å, 5 μm, 4.6 mm × 250 mm. Semi-praparative Waters XBridge BEH amide column (130 Å, 5 μm, 10 mm × 250 mm) was applied for purification. Solvent A: 100 mM ammonium formate, pH 3.2. Solvent B: Acetonitrile. Semi-praparative Agilent Eclipse XDB-C18, 5 μm, 9.4 × 250 mm. Aeris PEPTIDE 3.6 μm CB-C18, 4.6 mm x 250 mm. Solvent A: 0.1% TFA Water. Solvent B: 0.1% Acetonitrile. High-resolution electrospray ionization mass spectrometry (ESI-HRMS) experiment was performed on an LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher).

Automated synthesis was performed on a Liberty Blue peptide synthesizer according to the basic manufacture's manual and software for controlling operation with a designated program (Table 3.1) and sequence for the synthesis of the present target compounds.

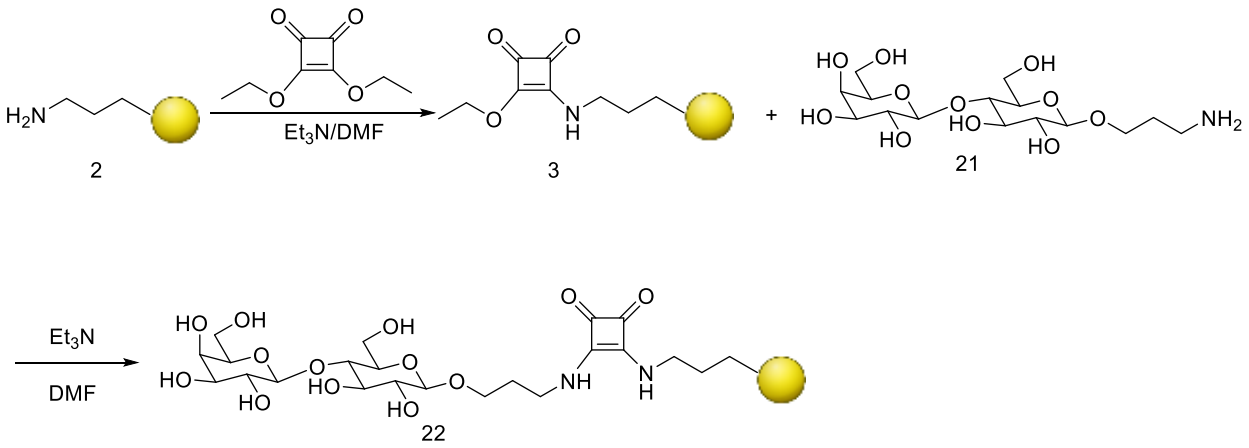
Synthesis.

Scheme 1



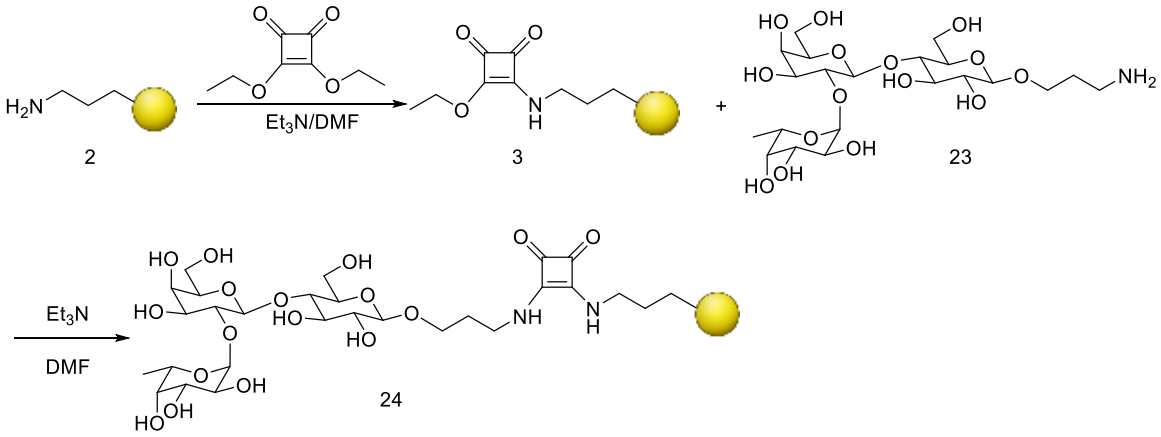
Compound **4** was prepared from amino group functional silica resin. In detail, compound 2 (1 g) was dispersed in the DMF (10 ml). 3,4-diethoxy-3-cyclobuten-1,2-dione (850 mg, 5 mmol) and triethylamine (1 g, 10 mmol) were added to the solution. The reaction mixture was shaken for 8 h at room temperature and filtered to get the resin. The resin was re-dispersed in the DMF (10 ml). 3,4-diethoxy-3-cyclobuten-1,2-dione (850 mg, 5 mmol) and triethylamine (1 g, 10 mmol) were added to the solution. The reaction mixture was shaken for 8 h at room temperature and filtered to get the resin. The resin was re-dispersed in the DMF (10 ml). Compound 1 (625 mg, 1 mmol) and triethylamine (1 g, 10 mmol) were added to the solution. The reaction mixture was shaken for 8 h at room temperature and filtered to get the resin. The resin was re-dispersed in the DMF (10 ml). Compound 1 (625 mg, 1 mmol) and triethylamine (1 g, 10 mmol) were added to the solution. The reaction mixture was shaken for 8 h at room temperature and filtered to get the resin. The loading efficiency was calculated by an equation.

Scheme 2



Compound 22 was prepared from amino group functional silica resin. In detail, compound 2 (1 g) was dispersed in the DMF (10 ml). 3,4-diethoxy-3-cyclobuten-1,2-dione (850 mg, 5 mmol) and triethylamine (1 g, 10 mmol) were added to the solution. The reaction mixture was shaken for 8 h at room temperature and filtered to get the resin. The resin was re-dispersed in the DMF (10 ml). 3,4-diethoxy-3-cyclobuten-1,2-dione (850 mg, 5 mmol) and triethylamine (1 g, 10 mmol) were added to the solution. The reaction mixture was shaken for 8 h at room temperature and filtered to get the resin. The resin was re-dispersed in the DMF (10 ml). Compound 21 (400 mg, 1 mmol) and triethylamine (1 g, 10 mmol) were added to the solution. The reaction mixture was shaken for 8 h at room temperature and filtered to get the resin. The resin was re-dispersed in the DMF (10 ml). Compound 21 (400 mg, 1 mmol) and triethylamine (1 g, 10 mmol) were added to the solution. The reaction mixture was shaken for 8 h at room temperature and filtered to get the resin. The loading efficiency was calculated by an equation.

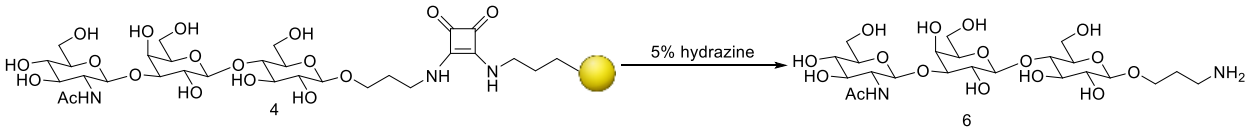
Scheme 3



Compound 22 was prepared from amino group functional silica resin. In detail, compound 2 (1 g) was dispersed in the DMF (10 ml). 3,4-diethoxy-3-cyclobuten-1,2-dione (850 mg, 5 mmol) and triethylamine (1 g, 10 mmol) were added to the solution. The reaction mixture was shaken for 8 h at room temperature and filtered to get the resin. The resin was re-dispersed in the DMF (10 ml). 3,4-diethoxy-3-cyclobuten-1,2-dione (850 mg, 5 mmol) and triethylamine (1 g, 10 mmol) were added to the solution. The reaction mixture was shaken for 8 h at room temperature and filtered to get the resin. The resin was re-dispersed in the DMF (10 ml). Compound 21 (545 mg, 1 mmol) and triethylamine (1 g, 10 mmol) were added to the solution. The reaction mixture was shaken for 8 h at room temperature and filtered to get the resin. The resin was re-dispersed in the DMF (10 ml). Compound 21 (545 mg, 1 mmol) and triethylamine (1 g, 10 mmol) were added to the solution. The reaction mixture was shaken for 8 h at room temperature and filtered to get the resin. The loading efficiency was calculated by an equation.

Compound release experiment

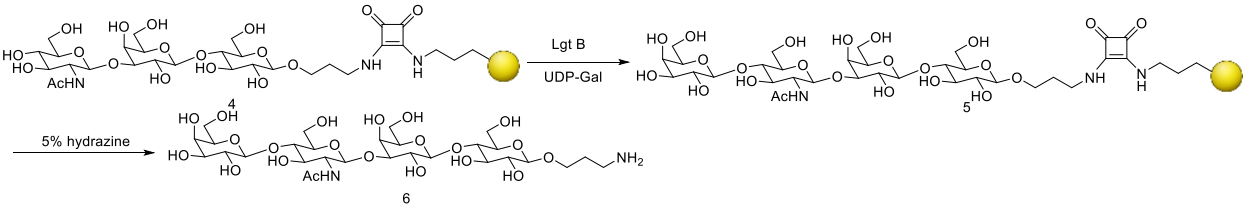
Scheme 4



Compound 4 was used to perform compound release experiment. In detail, compound 4 (200 mg) was dispersed in the 5% hydrazine aqueous solution (5 ml). The reaction mixture was shaken for 12 h at room temperature and compound-released resin was collected by filtration. The release efficiency was calculated by an equation.

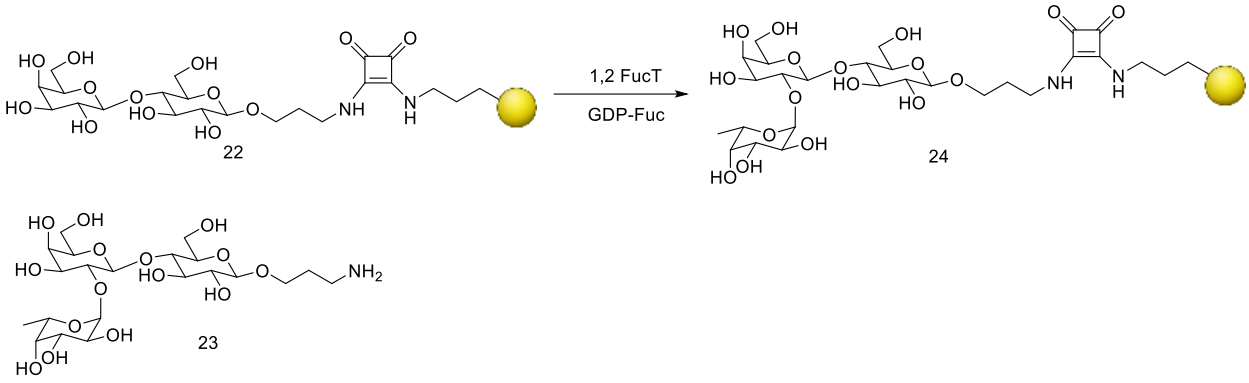
Enzymes activity test

Scheme 5



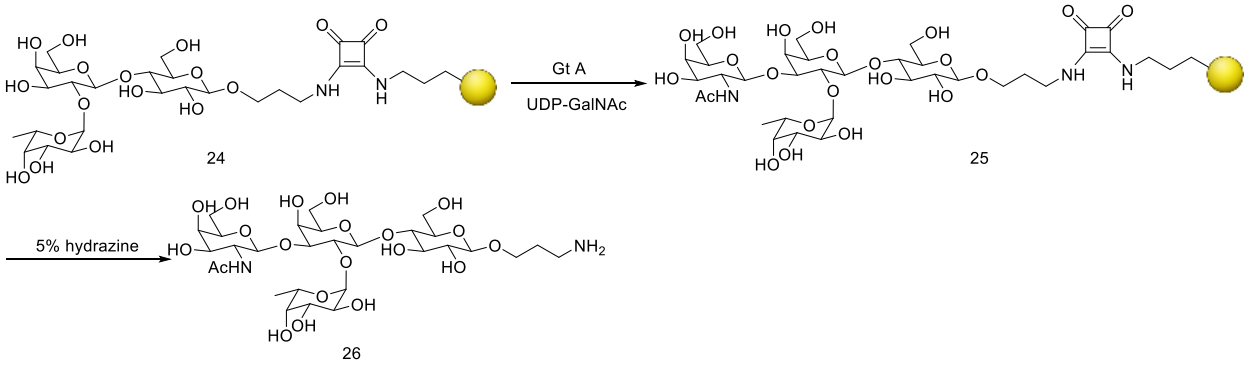
Compound 4 was used to test the activity of Lgt B. In detail, to the reaction vessel, Compound 4 (500 mg, 50 umol) was loaded. The automated synthetic process was then started by automatically injecting Lgt B (5 mg), UDP-Gal (3 eq), and reaction buffer (10 mL, pH 8.0). Once the reaction finished (24 h), unreacted materials and by-products were removed by filtration under high pressure. The silica resin was washed and taken out. Compound 5 and 5% hydrazine aqueous (5 ml) were put into a column. After the reaction finished (12 h), the filtration was collected and injected into HPLC (water/acetonitrile, 80-54.2, 30 min/45 min). The results show that more than 80% trisaccharide was converted to tetrasaccharide (Figure 3.4).

Scheme 6



Compound 22 was used to test the activity of 1,2 Fuc T. In detail, to the reaction vessel, Compound 22 (500 mg, 50umol) was loaded. The automated synthetic process was then started by automatically injecting 1,2 Fuc T (6.5 mg), GDP-Fuc (3 eq), and reaction buffer (10 mL, pH 8.0). Once the reaction finished (24 h), unreacted materials and by-products were removed by filtration under high pressure. The silica resin was washed and taken out. Compound 24 and 5% hydrazine aqueous (5 ml) were put into a column. After the reaction finished (12 h), the filtration was collected and injected into HPLC (water/acetonitrile, 80-54.2, 30 min/45 min). The results show that more than 80% trisaccharide was converted to tetrasaccharide (Figure 3.5).

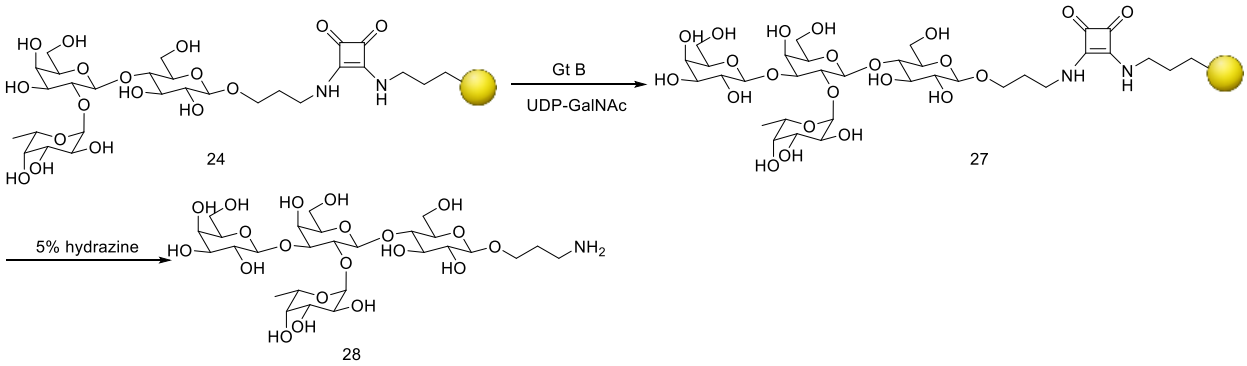
Scheme 7



Compound 24 was used to test the activity of Gt A. In detail, to the reaction vessel, Compound 24 (500 mg) was loaded. The automated synthetic process was then started by automatically injecting

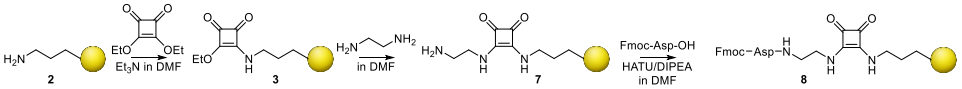
GtA (5 mg), UDP-GalNAc (3 eq), and reaction buffer (10 mL, pH 8.0). Once the reaction finished (24 h), unreacted materials and by-products were removed by filtration under high pressure. The silica resin was washed and taken out. Compound 25 and 5% hydrazine aqueous (5 ml) were put into a column. After the reaction finished (12 h), the filtration was collected and injected into HPLC (water/acetonitrile, 80-54.2, 30 min/45 min). The results show that more than 60% trisaccharide was converted to tetrasaccharide (Figure 3.5).

Scheme 8



Compound 24 was used to test the activity of Gt B. In detail, to the reaction vessel, Compound 24 (500 mg) was loaded. The automated synthetic process was then started by automatically injecting Gt B (10 mg), UDP-Gal (3 eq), and reaction buffer (10 mL, pH 8.0). Once the reaction finished (24 h), unreacted materials and by-products were removed by filtration under high pressure. The silica resin was washed and taken out. Compound 27 and 5% hydrazine aqueous (5 ml) were put into a column. After the reaction finished (12 h), the filtration was collected and injected into HPLC (water/acetonitrile, 80-54.2, 30 min/45 min). The results show that more than 70% trisaccharide was converted to tetrasaccharide (Figure 3.5).

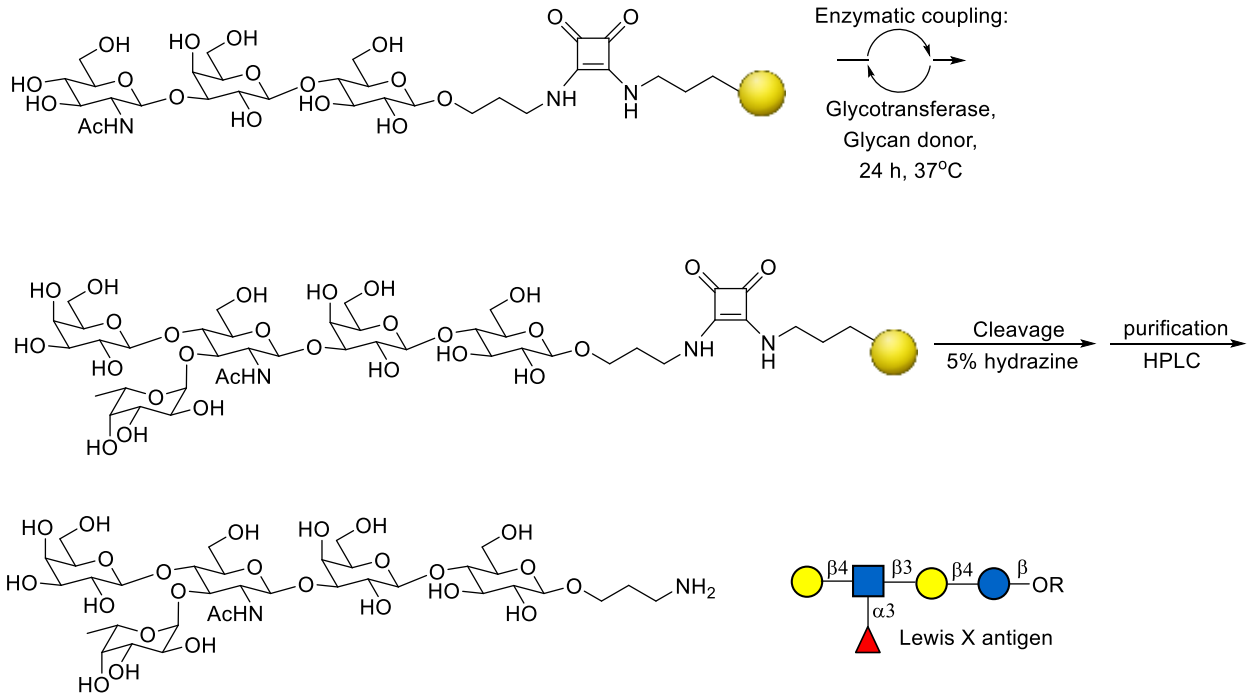
Scheme 9



Compound 8 was prepared from amino group functional silica resin. In detail, compound 2 (10 g) was dispersed in the DMF (100 ml). 3,4-diethoxy-3-cyclobuten-1,2-dione (8.5 g, 50 mmol) and triethylamine (10 g, 100 mmol) were added to the solution. The reaction mixture was shaken for 8 h at room temperature and filtered to get the resin. The resin was re-dispersed in the DMF (100 ml). 3,4-diethoxy-3-cyclobuten-1,2-dione (8.5 g, 50 mmol) and triethylamine (10 g, 100 mmol) were added to the solution. The reaction mixture was shaken for 8 h at room temperature and filtered to get the resin (Compound 3). The resin was re-dispersed in the DMF (100 ml). Ethylenediamine (600 mg, 10 mmol) and triethylamine (10 g, 100 mmol) were added to the solution. The reaction mixture was shaken for 8 h at room temperature and filtered to get the resin. The resin was re-dispersed in the DMF (100 ml). Ethylenediamine (600 mg, 10 mmol) and triethylamine (10 g, 100 mmol) were added to the solution. The reaction mixture was shaken for 8 h at room temperature and filtered to get the resin (Compound 7). The resin was re-dispersed in the DMF (100 ml). Fmoc-Asp-OH (3.55 g,10 mmol), HATU (3.8 g, 10 mmol) and DIPEA (1.29 g, 10 mmol) were added to the solution. The reaction mixture was shaken for 1h at room temperature and filtered to get the resin. The resin was re-dispersed in the DMF (100 ml). Fmoc-Asp-OH (3.55 g,10 mmol), HATU (3.8 g, 10 mmol) and DIPEA (1.29 g, 10 mmol) were added to the solution. The reaction mixture was shaken for 1h at room temperature and filtered to get the resin (Compound 8). The loading efficiency was calculated by an equation.

Automated synthesis of oligosaccharide

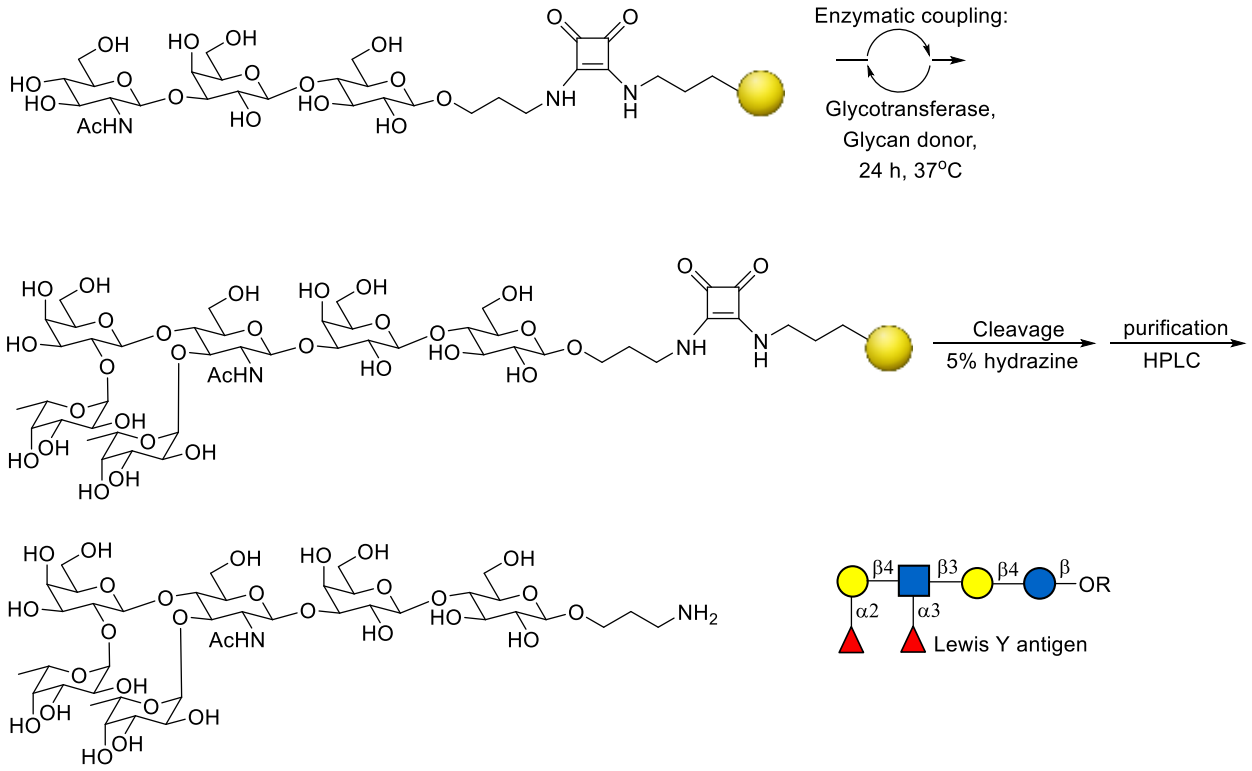
Scheme 10



Fully automated synthesis of lewis X antigen on our automated synthesizer. A synthetic program was made for lewis X antigen and this program contains two cycles (table 3.1). Compound 4 (500 mg) was loaded into the reaction vessel and all the enzyme and sugar nucleotide solutions were added to the corresponding tubes. The following procedures were performed by the program on the automated synthesizer. For the first cycle, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 9; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-Gal, Mg2+ (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 9; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave.

Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the first cycle was finished. For the second cycle, steps 1 and 2: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,3-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 6.5 mg of 1,3-FucT, and 5 mM compound bound resin; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,3-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 6.5 mg of 1,3-FucT, and 5 mM compound bound resin; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the second cycle was finished. Compound X was obtained after three cycles. Compound X was dispersed in the 5% hydrazine aqueous solution and the reaction mixture was shaken at room temperature for 12 h. After the reaction finished (12 h), the filtration was collected and injected to HPLC (water/acetonitrile, 5-20, 30 min/45 min) to get Lewis X antigen (5 mg) in yield 10.6%.

Scheme 11



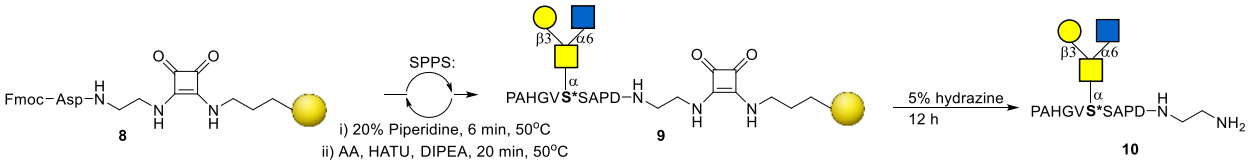
Fully automated synthesis of Lewis Y antigen on our automated synthesizer. A synthetic program was made for Lewis Y antigen and this program contains three cycles (table 3.1). Compound 4 (500 mg) was loaded into the reaction vessel and all the enzyme and sugar nucleotide solutions were added to the corresponding tubes. The following procedures were performed by the program on the automated synthesizer. For the first cycle, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 9; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 9; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave.

Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the first cycle was finished. For the second cycle, steps 1 and 2: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,3-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 6.5 mg of 1,3-FucT, and 5 mM compound bound resin; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,3-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 6.5 mg of 1,3-FucT, and 5 mM compound bound resin; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the second cycle was finished. For the third cycle, steps 1 and 2: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,2-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 6.5 mg of 1,2-FucT, and 5 mM compound bound resin; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,2-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of 1,2-FucT, and 5 mM compound bound resin; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C

for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the third cycle was finished. Compound X was obtained after three cycles. Compound X was dispersed in the 5% hydrazine aqueous solution and the reaction mixture was shaken at room temperature for 12 h. After the reaction finished (12 h), the filtration was collected and injected to HPLC (water/acetonitrile, 5-20, 30 min/45 min) to get Lewis Y antigen (5 mg) in 9.5yeild %.

Automated synthesis of glycopeptide

Scheme 12



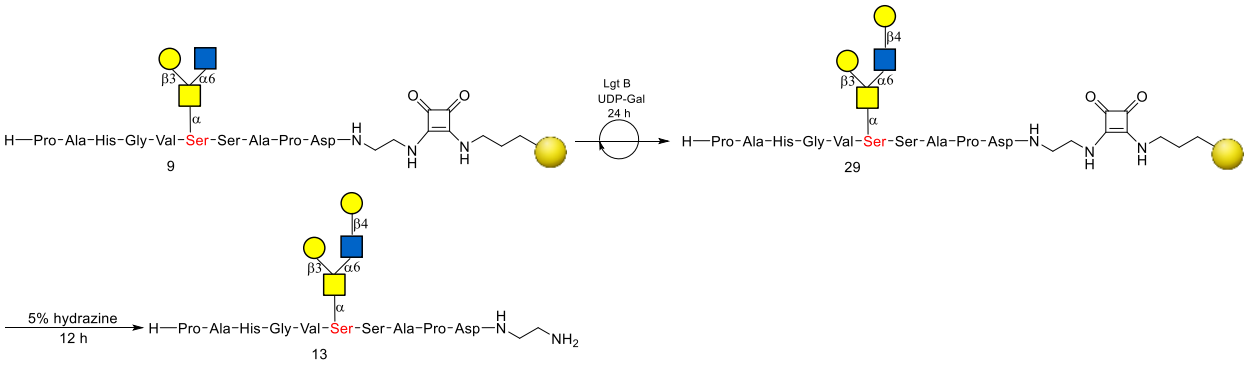
Fully automated synthesis of Mucin 1 peptide (compound 10) on our automated synthesizer. A synthetic program was made for compound 9 and this program contains ten cycles. Compound 8 (500 mg) was loaded into the reaction vessel and all the amino acid, base and activator solutions were added to the corresponding tubes. The following procedures were performed by the program on the automated synthesizer. For the first cycle, step 1: deprotection solution (20% piperidine in DMF solution, 3 ml) was added to the reaction vessel; step 2: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 3 min (microwave 25 W, hold 3 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); Step 3: deprotection solution (20% piperidine in DMF solution, 3 ml) was added to the reaction vessel; step 4: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 3 min (microwave 25 W, hold 3 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); step 5: the resin was washed by DMF (10 ml) three times; step 6: Fmoc-Pro-

OH (2.5 ml, 0.2 M), HATU (1 ml, 0.5 M) and DIEA (1 ml, 0.5 M) were added to the reaction vessel; step 7: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 10 min (microwave 25 W, hold 10 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); step 8: Fmoc-Pro-OH (2.5 ml, 0.2 M), HATU (1 ml, 0.5 M) and DIEA (1 ml, 0.5 M) were added to the reaction vessel; step 9: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 10 min (microwave 25 W, hold 10 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); step 10: the resin was washed by DMF (10 ml) three times; the first cycle was finished. For the second and third cycles, the synthesizer performed the same steps as the first cycle (the corresponding amino acid was injected following the program). For the fourth cycle (sugar amino acid cycle), step 1: deprotection solution (20% piperidine in DMF solution, 3 ml) was added to the reaction vessel; step 2: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 3 min (microwave 25 W, hold 3 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); Step 3: deprotection solution (20% piperidine in DMF solution, 3 ml) was added to the reaction vessel; step 4: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 3 min (microwave 25 W, hold 3 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); step 5: the resin was washed by DMF (10 ml) three times; step 6: Fmoc-Ser(Core 2)-OH (2.5 ml, 0.2 M), HATU (1 ml, 0.5 M) and DIEA (1 ml, 0.5 M) were added to the reaction vessel; step 7: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 1 h (microwave 25 W, hold 10 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); step 8: Fmoc- Ser(Core 2)-OH (2.5 ml, 0.2 M), HATU (1 ml, 0.5 M) and DIEA (1 ml, 0.5 M) were added to the reaction vessel; step 9: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave.

Reaction was done at 50°C for 1 h (microwave 25 W, hold 10 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); step 10: the resin was washed by DMF (10 ml) three times; the fourth cycle was finished. For the 5th to 9th cycles, the synthesizer performed the same steps as the first cycle (the corresponding amino acid was injected following the program). For the 10th cycle, step 1: deprotection solution (20% piperidine in DMF solution, 3 ml) was added to the reaction vessel; step 2: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 3 min (microwave 25 W, hold 3 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); Step 3: deprotection solution (20% piperidine in DMF solution, 3 ml) was added to the reaction vessel; step 4: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 3 min (microwave 25 W, hold 3 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); step 5: the resin was washed by DMF (10 ml) three times; step 6: cocktail (TFA:water:TIS:DODT = 92.5:2.5:2.5:2.5; 10 ml) was added to the reaction vessel; step 7: the temperature of the reaction mixture was maintained at room temperature without microwave. Reaction was done at room temperature for 3 h (microwave 0 W, hold 3 h at room temperature, bubble on for 2 s, off for 3 s, drain 100 s); step 8: the resin was washed by DMF (10 ml) three times; the 10th cycle was finished. Compound 9 was obtained after 10 cycles. Compound 9 was dispersed in the 5% hydrazine aqueous solution and the reaction mixture was shaken at room temperature for 12 h. After the reaction finished (12 h), the filtration was collected and injected to HPLC (water/acetonitrile, 5-20, 30 min/45 min) to get compound 10 (10 mg) in yield 12.9%. ^{1}H-NMR (600 MHz, D2O), δ 8.67 (s, 1H), 7.28 (s, 1H), 4.60-4.56 (m, 2H), 4.49-4.42 (m, 3H), 4.37-4.30 (m, 4H), 4.26-4.20 (m, 3H), 4.11 (m, 1H), 3.97-3.91 (m, 4H), 3.88-3.83 (m, 3H), 3.78-3.61 (m, 13H), 3.48-3.41 (m, 4H), 3.40-3.29 (m, 4H), 3.26-3.21 (m, 1H), 3.16-3.12 (m, 1H), 3.09-3.07 (m, 2H), 2.88-2.78 (m, 2H), 2.41-2.36 (m, 1H), 2.26-2.20 (m, 1H),

2.08-1.84 (m, 14H), 1.31-1.28 (m, 6H), 0.91-0.86 (m, 6H). HRMS (ESI): m/z [M +3H]3+ detected: 516.5758 m/z; calcd for C63H102N16O29 1546.6999 Da.

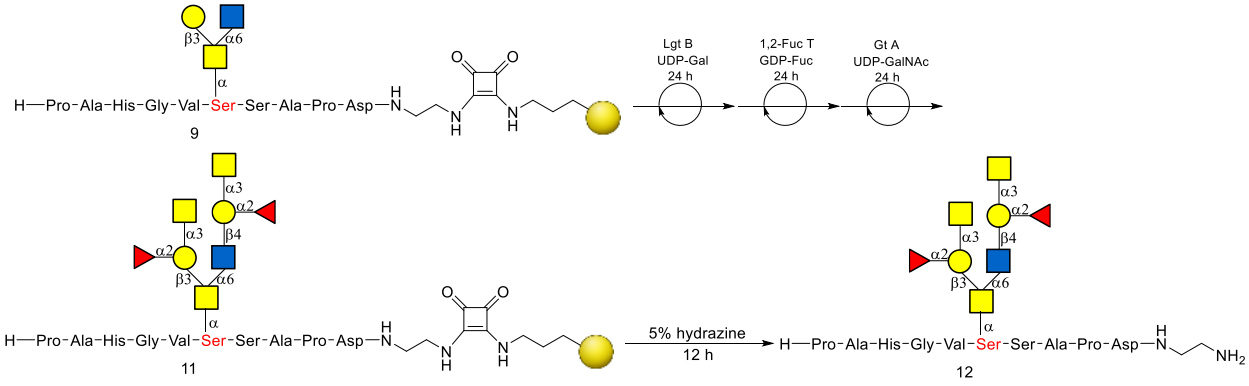
Scheme 13



Fully automated synthesis of compound 13 on our automated synthesizer. A synthetic program was made for compound 29 and this program contains one enzymatic reaction cycle (table 3.1). Compound 9 (500 mg) was loaded into the reaction vessel and all the enzyme and sugar nucleotide solutions were added to the corresponding tubes. The following procedures were performed by the program on the automated synthesizer. For the first cycle, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 9; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 9; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C,

bubble on for 5 s, off for 600 s); step 6: the resin was washed by water (10 ml) three times; the first cycle was finished. Compound 29 was obtained after one cycle. Compound 29 was dispersed in the 5% hydrazine aqueous solution and the reaction mixture was shaken at room temperature for 12 h. After the reaction finished (12 h), the filtration was collected and injected to HPLC (water/acetonitrile, 5-20, 30 min/45 min) to get compound 13 (10 mg) in yield 11.7%. ^{1}H-NMR (600 MHz, D2O), δ 8.67 (s, 1H), 7.28 (s, 1H), 4.60-4.30 (m, 8H), 4.26-4.20 (m, 3H), 4.11 (m, 1H), 3.96-3.83 (m, 9H), 3.79-3.41 (m, 25H), 3.37-3.31 (m, 2H), 3.26-3.21 (m, 1H), 3.16-3.12 (m, 1H), 3.08 (m, 2H), 2.83-2.70 (m, 2H), 2.41-2.36 (m, 1H), 2.26-2.20 (m, 1H), 2.07-1.86 (m, 14H), 1.33-1.28 (m, 6H), 0.92-0.86 (m, 6H). HRMS (ESI): m/z [M +3H]3+ detected: 570.5934 m/z; calcd for C69H112N18O34 1708.7527 Da.

Scheme 14

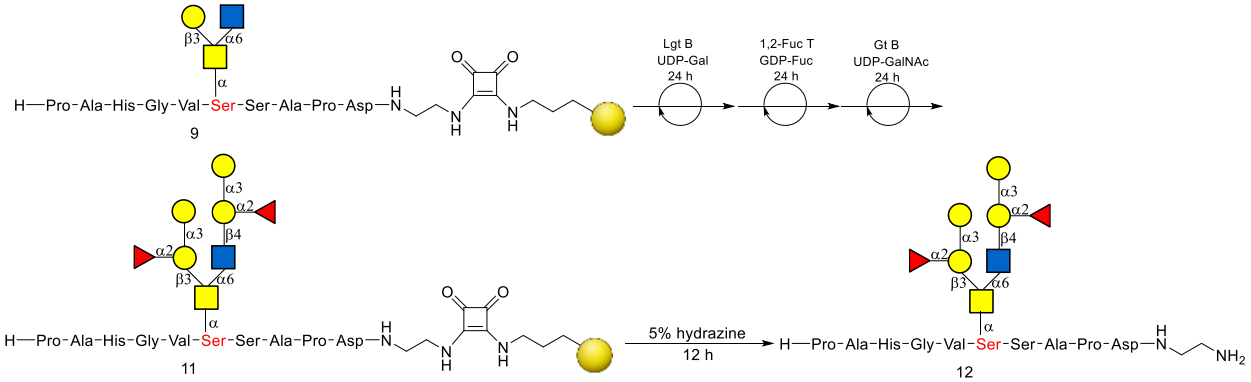


Fully automated synthesis of compound 12 on our automated synthesizer. A synthetic program was made for compound 11 and this program contains three enzymatic cycles (table 3.1). Compound 9 (500 mg) was loaded into the reaction vessel and all the enzyme and sugar nucleotide solutions were added to the corresponding tubes. The following procedures were performed by the program on the automated synthesizer. For the first cycle, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution

(10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 9; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 9; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the first cycle was finished. For the second cycle, steps 1 and 2: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,2-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 6.5 mg of 1,2-FucT, and 5 mM compound bound resin; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,2-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 6.5 mg of 1,2-FucT, and 5 mM compound bound resin; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the second cycle was finished. For the third cycle, steps 1 and 2: UDP-GalNAc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and GtA (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-GalNAc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+},

5.0 mg of GtA, and compound bound resin; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-GalNAc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and GtA (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-GalNAc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of GtA, and compound bound resin; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the third cycle was finished. Compound 11 was obtained after three cycles. Compound 11 was dispersed in the 5% hydrazine aqueous solution and the reaction mixture was shaken at room temperature for 12 h. After the reaction finished (12 h), the filtration was collected and injected to HPLC (water/acetonitrile, 5-20, 30 min/45 min) to get compound 12 (10 mg) in yield 8.3%. ^{1}H-NMR (600 MHz, D2O), δ 8.66 (s, 1H), 7.26 (s, 1H), 5.26 (d, J = 2.4 Hz, 1H), 5.18 (d, J = 2.4 Hz, 1H), 5.08 (m, 2H), 4.56-4.39 (m, 7H), 4.34-4.28 (m, 2H), 4.23-4.02 (m, 12H), 3.94-3.29 (m, 47H), 3.23-3.19 (m, 1H), 3.13-3.10 (m, 1H), 3.06-3.05 (m, 2H), 2.68-2.62 (m, 2H), 2.39-2.33 (m, 1H), 2.24-2.17 (m, 1H), 2.05-1.84 (m, 20H), 1.32-1.26 (m, 6H), 1.16-1.09 (m, 6H), 0.88-0.82 (m, 6H). HRMS (ESI): m/z [M +3H]3+ detected: 803.3528 m/z; calcd for C97H158N18O52 2407.0273 Da.

Scheme 15

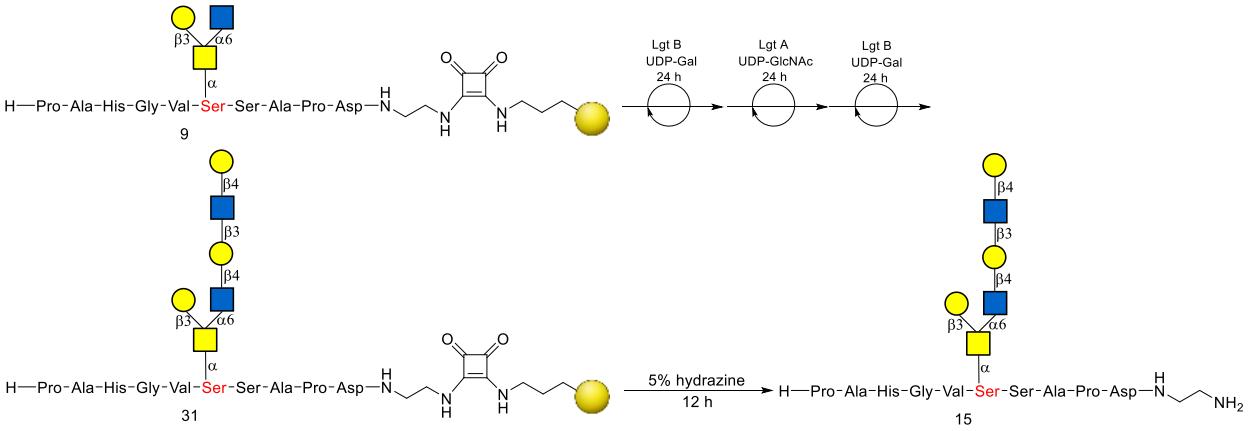


Fully automated synthesis of compound 14 on our automated synthesizer. A synthetic program was made for compound 30 and this program contains three cycles (table 3.1). Compound 9 (500 mg) was loaded into the reaction vessel and all the enzyme and sugar nucleotide solutions were added to the corresponding tubes. The following procedures were performed by the program on the automated synthesizer. For the first cycle, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 9; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 9; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the first cycle was finished. For the second cycle, steps 1 and 2: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,2-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM

GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 6.5 mg of 1,2-FucT, and 5 mM compound bound resin; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,2-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 6.5 mg of 1,2-FucT, and 5 mM compound bound resin; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the second cycle was finished. For the third cycle, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and GtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of GtB, and compound bound resin; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and GtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of GtB, and compound bound resin; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the third cycle was finished. Compound 30 was obtained after three cycles. Compound 30 was dispersed in the 5% hydrazine aqueous solution and the reaction mixture was shaken at room temperature for 12 h. After the reaction finished (12 h), the filtration was collected and injected to

HPLC (water/acetonitrile, 5-20, 30 min/45 min) to get compound 14 (8.9 mg) in yield 7.6%. ^{1}H-NMR (600 MHz, D2O), δ 8.66 (s, 1H), 7.26 (s, 1H), 5.23 (d, J = 2.4 Hz, 1H), 5.16-5.14 (m, 3H), 4.58-4.37 (m, 7H), 4.34-4.28 (m, 2H), 4.24-4.02 (m, 12H), 3.94-3.74 (m, 15H), 3.72-3.54 (m, 26H), 3.47-3.29 (m, 6H), 3.23-3.19 (m, 1H), 3.14-3.10 (m, 1H), 3.07-3.06 (m, 2H), 2.66-2.61 (m, 2H), 2.39-2.33 (m, 1H), 2.23-2.19 (m, 1H), 2.06-1.83 (m, 14H), 1.32-1.26 (m, 6H), 1.14-1.08 (m, 6H), 0.88-0.83 (m, 6H). HRMS (ESI): m/z [M +3H]$^{3+}$ detected: 776.0017 m/z; calcd for C93H152N16O52 2324.9742 Da.

Scheme 16

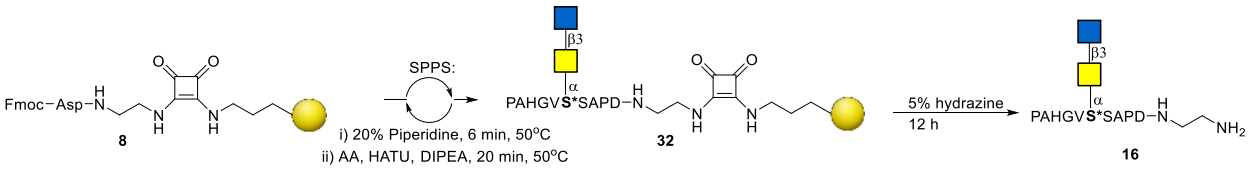


Fully automated synthesis of compound 15 on our automated synthesizer. A synthetic program was made for compound 31 and this program contains three cycles (table 3.1). Compound 9 (500 mg) was loaded into the reaction vessel and all the enzyme and sugar nucleotide solutions were added to the corresponding tubes. The following procedures were performed by the program on the automated synthesizer. For the first cycle, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 9; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W

of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 9; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the first cycle was finished. For the second cycle, steps 1 and 2: UDP-GlcNAc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and Lgt A (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-GlcNAc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of Lgt A, and 5 mM compound bound resin; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-GlcNAc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and Lgt A (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-GlcNAc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of Lgt A, and 5 mM compound bound resin; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the second cycle was finished. For the third cycle, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and compound bound resin; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold

12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and compound bound resin; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the third cycle was finished. Compound 31 was obtained after three cycles. Compound 31 was dispersed in the 5% hydrazine aqueous solution and the reaction mixture was shaken at room temperature for 12 h. After the reaction finished (12 h), the filtration was collected and injected to HPLC (water/acetonitrile, 5-20, 30 min/45 min) to get compound 15 (9.5 mg) in yield 9.2%. ^{1}H-NMR (600 MHz, D2O), δ 8.66 (s, 1H), 7.26 (s, 1H), 4.50-4.28 (m, 10H), 4.23-4.17 (m, 3H), 4.09-4.06 (m, 2H), 3.94-3.80 (m, 10H), 3.76-3.38 (m, 35H), 3.36-3.27 (m, 2H), 3.23-3.19 (m, 1H), 3.14-3.10 (m, 1H), 3.06-3.04 (m, 2H), 2.71-2.63 (m, 2H), 2.39-2.33 (m, 1H), 2.24-2.17 (m, 1H), 2.06-1.81 (m, 17H), 1.31-1.26 (m, 6H), 0.89-0.84 (m, 6H). HRMS (ESI): m/z $[M+3H]^{3+}$ detected: 692.3038 m/z; calcd for C83H135N17O44 2073.8849 Da.

Scheme 17



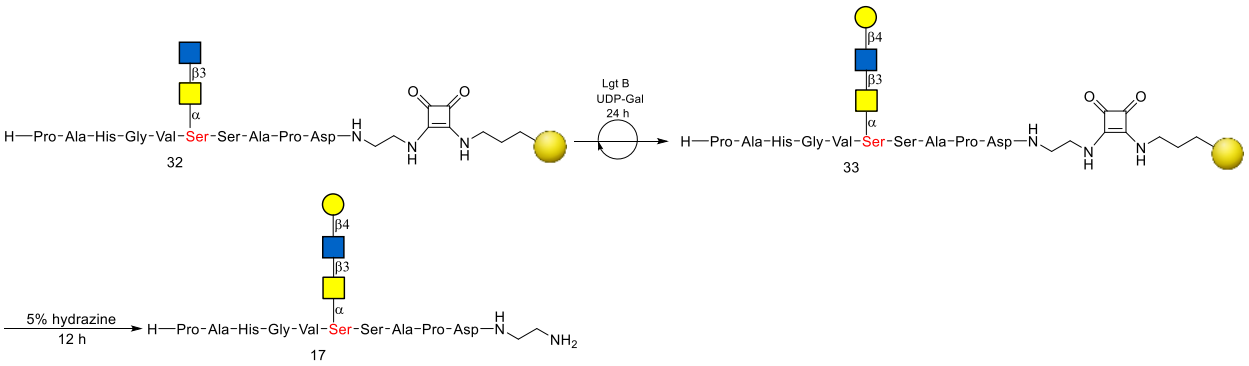
Fully automated synthesis of Mucin 1 peptide (compound 16) on our automated synthesizer. A synthetic program was made for compound 32 and this program contains ten cycles (table S1). Compound 8 (500 mg) was loaded into the reaction vessel and all the amino acid, base and activator solutions were added to the corresponding tubes. The following procedures were

performed by the program on the automated synthesizer. For the first cycle, step 1: deprotection solution (20% piperidine in DMF solution, 3 ml) was added to the reaction vessel; step 2: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 3 min (microwave 25 W, hold 3 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); Step 3: deprotection solution (20% piperidine in DMF solution, 3 ml) was added to the reaction vessel; step 4: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 3 min (microwave 25 W, hold 3 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); step 5: the resin was washed by DMF (10 ml) three times; step 6: Fmoc-Pro-OH (2.5 ml, 0.2 M), HATU (1 ml, 0.5 M) and DIEA (1 ml, 0.5 M) were added to the reaction vessel; step 7: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 10 min (microwave 25 W, hold 10 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); step 8: Fmoc-Pro-OH (2.5 ml, 0.2 M), HATU (1 ml, 0.5 M) and DIEA (1 ml, 0.5 M) were added to the reaction vessel; step 9: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 10 min (microwave 25 W, hold 10 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); step 10: the resin was washed by DMF (10 ml) three times; the first cycle was finished. For the second and third cycles, the synthesizer performed the same steps as the first cycle (the corresponding amino acid was injected following the program). For the fourth cycle (sugar amino acid cycle), step 1: deprotection solution (20% piperidine in DMF solution, 3 ml) was added to the reaction vessel; step 2: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 3 min (microwave 25 W, hold 3 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); Step 3: deprotection solution (20% piperidine in DMF solution, 3 ml) was added to the reaction vessel; step 4: the temperature of the

reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 3 min (microwave 25 W, hold 3 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); step 5: the resin was washed by DMF (10 ml) three times; step 6: Fmoc-Ser(Core 3)-OH (2.5 ml, 0.2 M), HATU (1 ml, 0.5 M) and DIEA (1 ml, 0.5 M) were added to the reaction vessel; step 7: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 1 h (microwave 25 W, hold 10 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); step 8: Fmoc- Ser(Core 3)-OH (2.5 ml, 0.2 M), HATU (1 ml, 0.5 M) and DIEA (1 ml, 0.5 M) were added to the reaction vessel; step 9: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 1 h (microwave 25 W, hold 10 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); step 10: the resin was washed by DMF (10 ml) three times; the fourth cycle was finished. For the 5th to 9th cycles, the synthesizer performed the same steps as the first cycle (the corresponding amino acid was injected following the program). For the 10th cycle, step 1: deprotection solution (20% piperidine in DMF solution, 3 ml) was added to the reaction vessel; step 2: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 3 min (microwave 25 W, hold 3 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); Step 3: deprotection solution (20% piperidine in DMF solution, 3 ml) was added to the reaction vessel; step 4: the temperature of the reaction mixture was maintained at 50°C under 25 W of microwave. Reaction was done at 50°C for 3 min (microwave 25 W, hold 3 min at 50°C, bubble on for 2 s, off for 3 s, drain 100 s); step 5: the resin was washed by DMF (10 ml) three times; step 6: cocktail (TFA:water:TIS:DODT = 92.5:2.5:2.5:2.5; 10 ml) was added to the reaction vessel; step 7: the temperature of the reaction mixture was maintained at room temperature without microwave. Reaction was done at room temperature for 3 h (microwave 0 W, hold 3 h at room temperature, bubble on for 2 s, off for 3 s,

drain 100 s); step 8: the resin was washed by DMF (10 ml) three times; the 10th cycle was finished. Compound 32 was obtained after 10 cycles. Compound 32 was dispersed in the 5% hydrazine aqueous solution and the reaction mixture was shaken at room temperature for 12 h. After the reaction finished (12 h), the filtration was collected and injected to HPLC (water/acetonitrile, 5-20, 30 min/45 min) to get compound 16 (1.1 mg) in yield 18.9%. ^{1}H-NMR (600 MHz, D2O), δ 8.64 (s, 1H), 7.26 (s, 1H), 4.63-4.60 (m, 1H), 4.59-4.58 (m, 1H), 4.56-4.54 (m, 1H), 4.48-4.45 (m, 2H), 4.40-4.38 (m, 1H), 4.32-4.27 (m, 2H), 4.23-4.19 (m, 1H), 4.16-4.09 (m, 3H), 3.91-3.86 (m, 4H), 3.83-3.81 (m, 1H), 3.76-3.54 (m, 10H), 3.46-3.41 (m, 3H), 3.37-3.27 (m, 4H), 3.22-3.19 (m, 1H), 3.13-3.09 (m, 1H), 3.06-3.04 (m, 2H), 2.87-2.77 (m, 2H), 2.38-2.32 (m, 1H), 2.22-2.17 (m, 1H), 2.03-1.81 (m, 14H), 1.28-1.26 (m, 6H), 0.88-0.83 (m, 6H). HRMS (ESI): m/z [M +3H]3+ detected: 462.5574 m/z; calcd for C57H92N16O24 1384.6470 Da.

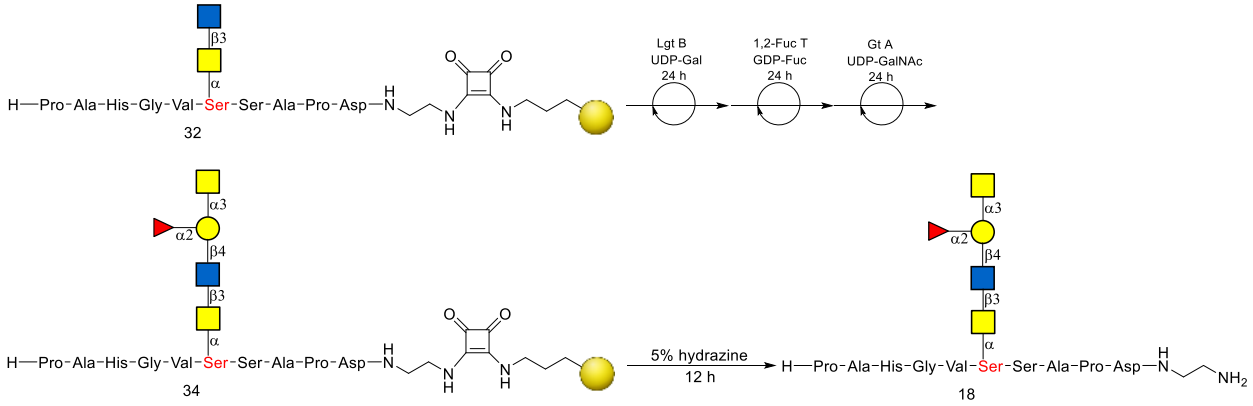
Scheme 18



Fully automated synthesis of compound 17 on our automated synthesizer. A synthetic program was made for compound 33 and this program contains one cycle (table 3.1). Compound 32 (500 mg) was loaded into the reaction vessel and all the enzyme and sugar nucleotide solutions were added to the corresponding tubes. The following procedures were performed by the program on the automated synthesizer. For the first cycle, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM

Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 32; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 32; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 6: the resin was washed by water (10 ml) three times; the first cycle was finished. Compound 33 was obtained after one cycle. Compound 33 was dispersed in the 5% hydrazine aqueous solution and the reaction mixture was shaken at room temperature for 12 h. After the reaction finished (12 h), the filtration was collected and injected to HPLC (water/acetonitrile, 5-20, 30 min/45 min) to get compound 17 (10.2 mg) in yield 13.2%. ^{1}H-NMR (600 MHz, D2O), δ 8.67 (s, 1H), 7.27 (s, 1H), 4.64-4.56 (m, 4H), 4.53-4.47 (m, 2H), 4.43-4.39 (m, 2H), 4.36-4.29 (m, 2H), 4.26-4.12 (m, 1H), 4.18-4.11 (m, 3H), 3.94-3.83 (m, 7H), 3.78-3.58 (m, 15H), 3.47-3.42 (m, 4H), 3.38-3.29 (m, 2H), 3.26-3.21 (m, 1H), 3.16-3.11 (m, 1H), 3.08-3.07 (m, 2H), 2.89-2.77 (m, 2H), 2.41-2.35 (m, 1H), 2.26-2.20 (m, 1H), 2.06-1.82 (m, 14H), 1.32-1.28 (m, 6H), 0.90-0.86 (m, 6H). HRMS (ESI): m/z [M +3H]3+ detected: 516.5756 m/z; calcd for C63H102N16O29 1546.6999 Da.

Scheme 19

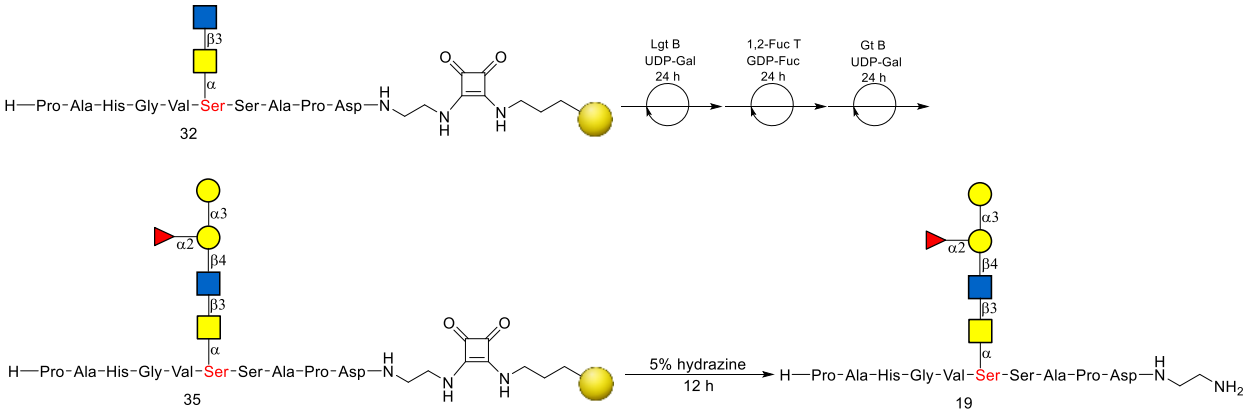


Fully automated synthesis of compound 18 on our automated synthesizer. A synthetic program was made for compound 34 and this program contains three cycles (table S1). Compound 32 (500 mg) was loaded into the reaction vessel and all the enzyme and sugar nucleotide solutions were added to the corresponding tubes. The following procedures were performed by the program on the automated synthesizer. For the first cycle, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 32; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 32; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the first cycle was finished. For the second cycle, steps 1 and 2: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,2-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM

GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 6.5 mg of 1,2-FucT, and 5 mM compound bound resin; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,2-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 6.5 mg of 1,2-FucT, and 5 mM compound bound resin; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the second cycle was finished. For the third cycle, steps 1 and 2: UDP-GalNAc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and GtA (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-GalNAc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of GtA, and compound bound resin; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-GalNAc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and GtA (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-GalNAc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of GtA, and compound bound resin; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the third cycle was finished. Compound 34 was obtained after three cycles. Compound 34 was dispersed in the 5% hydrazine aqueous solution and the reaction mixture was shaken at room temperature for 12 h. After the reaction finished (12 h), the filtration was collected and injected to

HPLC (water/acetonitrile, 5-20, 30 min/45 min) to get compound 18 (9.7 mg) in yield 10.2%. ^{1}H-NMR (600 MHz, D2O), δ 8.66 (s, 1H), 7.26 (s, 1H), 5.26 (d, J = 2.4 Hz, 1H), 5.08 (d, J = 2.8 Hz, 1H), 4.60-4.37 (m, 5H), 4.33-4.27 (m, 2H), 4.23-4.22 (m, 2H), 4.16-4.09 (m, 6H), 3.91-3.56 (m, 29H), 3.47-3.27 (m, 5H), 3.23-3.19 (m, 1H), 3.13-3.10 (m, 1H), 3.06-3.04 (m, 2H), 2.83-2.69 (m, 2H), 2.37-2.33 (m, 1H), 2.22-2.18 (m, 1H), 2.05-1.82 (m, 17H), 1.32-1.26 (m, 6H), 1.16 (d, J = 4.4 Hz, 3H), 0.88-0.84 (m, 6H). HRMS (ESI): m/z [M +3H]$^{3+}$ detected: 632.9555 m/z; calcd for C77H125N17O38 1895.8371 Da.

Scheme 20

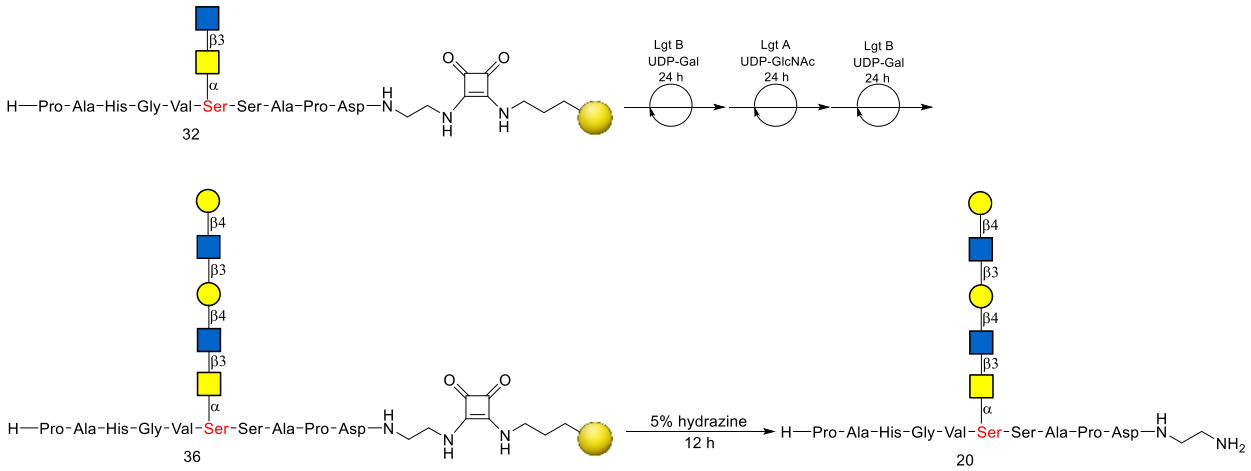


Fully automated synthesis of compound 19 on our automated synthesizer. A synthetic program was made for compound 35 and this program contains three cycles (table 3.1). Compound 32 (500 mg) was loaded into the reaction vessel and all the enzyme and sugar nucleotide solutions were added to the corresponding tubes. The following procedures were performed by the program on the automated synthesizer. For the first cycle, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 32; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W

of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 32; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the first cycle was finished. For the second cycle, steps 1 and 2: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,2-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 6.5 mg of 1,2-FucT, and 5 mM compound bound resin; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: GDP-Fuc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and 1,2-FucT (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM GDP-Fuc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 6.5 mg of 1,2-FucT, and 5 mM compound bound resin; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the second cycle was finished. For the third cycle, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and GtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of GtB, and compound bound resin; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold

12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and GtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of GtB, and compound bound resin; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the third cycle was finished. Compound 35 was obtained after three cycles. Compound 35 was dispersed in the 5% hydrazine aqueous solution and the reaction mixture was shaken at room temperature for 12 h. After the reaction finished (12 h), the filtration was collected and injected to HPLC (water/acetonitrile, 5-20, 30 min/45 min) to get compound 19 (9.3 mg) in yield 10%. ^{1}H-NMR (600 MHz, D2O), δ 8.66 (s, 1H), 7.26 (s, 1H), 5.24 (d, J = 2.4 Hz, 1H), 5.14 (m, 1H), 4.63-4.46 (m, 4H), 4.40-4.38 (m, 2H), 4.34-4.27 (m, 2H), 4.23-4.09 (m, 7H), 3.91-3.56 (m, 30H), 3.46-3.27 (m, 5H), 3.22-3.19 (m, 1H), 3.13-3.09 (m, 1H), 3.06-3.04 (m, 2H), 2.76-2.66 (m, 2H), 2.38-2.33 (m, 1H), 2.22-2.19 (m, 1H), 2.04-1.82 (m, 14H), 1.31-1.26 (m, 6H), 1.16 (d, J = 4.4 Hz, 3H), 0.88-0.84 (m, 6H). HRMS (ESI): m/z $[M+3H]^{3+}$ detected: 619.2802 m/z; calcd for C75H122N16O38 1854.8106 Da.

Scheme 21



Fully automated synthesis of compound 20 on our automated synthesizer. A synthetic program was made for compound 36 and this program contains three cycles (table 3.1). Compound 32 (500 mg) was loaded into the reaction vessel and all the enzyme and sugar nucleotide solutions were added to the corresponding tubes. The following procedures were performed by the program on the automated synthesizer. For the first cycle, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 32; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and 5 mM compound 32; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the first cycle was finished. For the second cycle, steps 1 and 2: UDP-GlcNAc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and Lgt A (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10

mM UDP-GlcNAc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of Lgt A, and 5 mM compound bound resin; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-GlcNAc, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and Lgt A (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-GlcNAc, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of Lgt A, and 5 mM compound bound resin; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the second cycle was finished. For the third cycle, steps 1 and 2: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and compound bound resin; step 3: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); steps 4 and 5: UDP-Gal, Mg^{2+} (5 ml) in 100 mM Tris-HCl (pH 8.0), and LgtB (5 ml) were added to the reaction vessel to form a solution (10 ml) as below: 10 mM UDP-Gal, 50 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, 5.0 mg of LgtB, and compound bound resin; step 6: the temperature of the reaction mixture was maintained at 37°C under 20 W of microwave. Glycosyl reaction was done at 37°C for 12 h (microwave 20 W, hold 12 h at 37°C, bubble on for 5 s, off for 600 s); step 7: the resin was washed by water (10 ml) three times; the third cycle was finished. Compound 36 was obtained after three cycles. Compound 36 was dispersed in the 5% hydrazine aqueous solution and the reaction mixture was shaken at room temperature for 12 h. After the reaction finished (12 h), the filtration was collected and injected to

HPLC (water/acetonitrile, 5-20, 30 min/45 min) to get compound 20 (10.2 mg) in yield 10.7%. ^{1}H-NMR (600 MHz, D2O), δ 8.66 (s, 1H), 7.26 (s, 1H), 4.63-4.59 (m, 5H), 4.56-4.45 (m, 2H), 4.40-4.06 (m, 9H), 3.92-3.56 (m, 30H), 3.49-3.40 (m, 6H), 3.36-3.27 (m, 2H), 3.23-3.19 (m, 1H), 3.13-3.09 (m, 1H), 3.06-3.04 (m, 2H), 2.86-2.73 (m, 2H), 2.39-2.33 (m, 1H), 2.24-2.18 (m, 1H), 2.04-1.80 (m, 17H), 1.30-1.26 (m, 6H), 0.88-0.84 (m, 6H). HRMS (ESI): m/z $[M+3H]^{3+}$ detected: 638.2873 m/z; calcd for C77H125N17O39 1911.8321 Da.

3.8 Conclusion

In summary, a fully machine-driven system based on the use of two peptide synthesizers has been developed for the automated synthesis of glycopeptides. The standardized process has five steps: automated synthesis of peptide backbone, resin transfer, automated synthesis of oligosaccharide portion, compound release and purification. A peptide (piece of Mucin 1) decorated with several important oligosaccharide epitopes was selected and successfully prepared in an automated manner. The use of a functionalized silica gel resin overcomes the limitations of traditional solid supports for enzymatic synthesis. It allows the formation of peptide bonds chemically and glycosidic bonds enzymatically on a single solid phase support.

This study represents a proof-of-concept demonstration that a two-solution system for the synthesis of glycopeptides can be achieved in an automated manner using two commercially available peptide synthesizers. This work will enable an improved, combined instrument for automated glycopeptide synthesis in the future. Such improvements should include more storage tubes for both organic reagents and reagents of enzymatic glycosylation, a temperature control system for enzyme storage, a detection system for monitoring reaction processes, and more wash bottles for organic solvents and water buffers.

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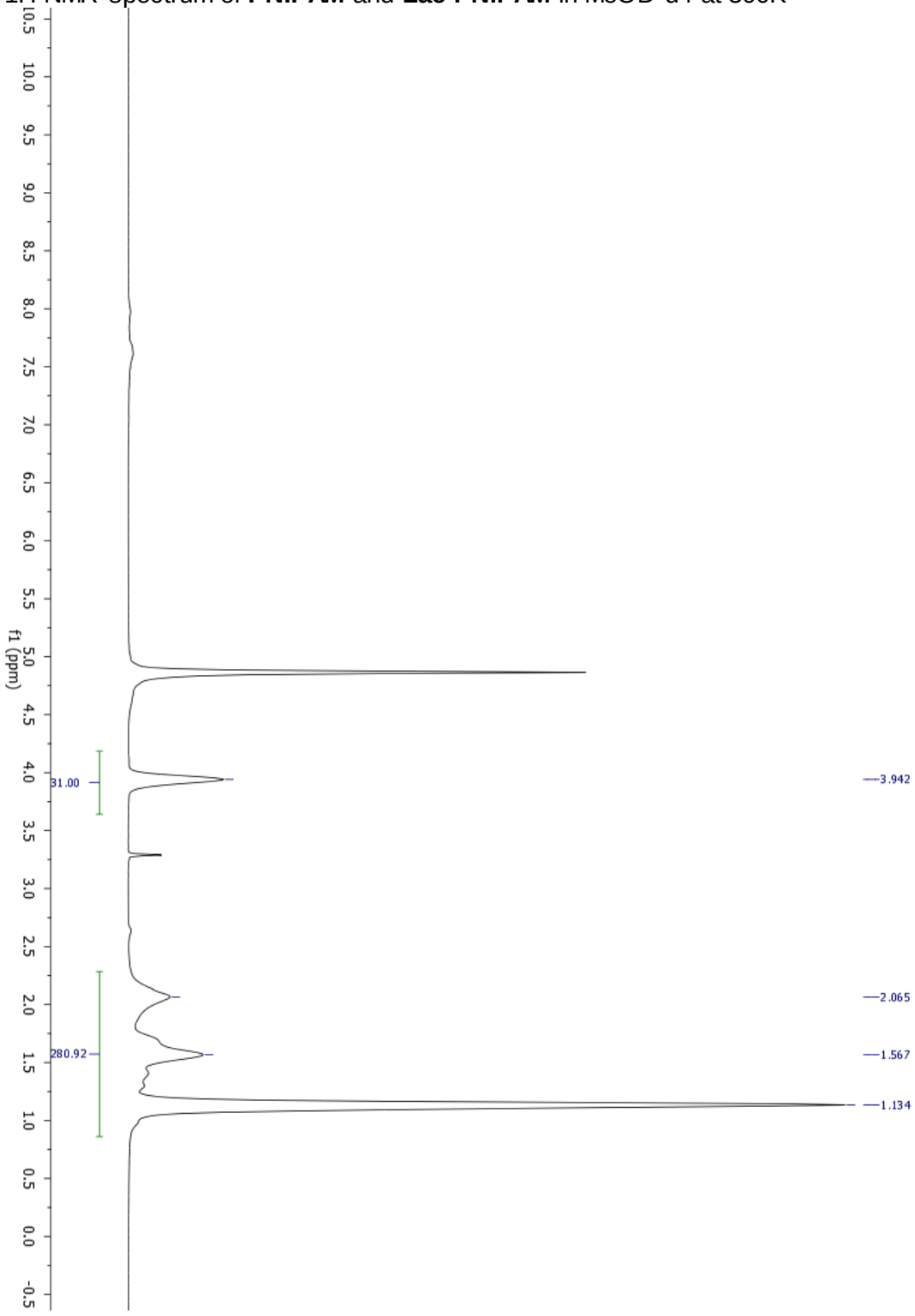
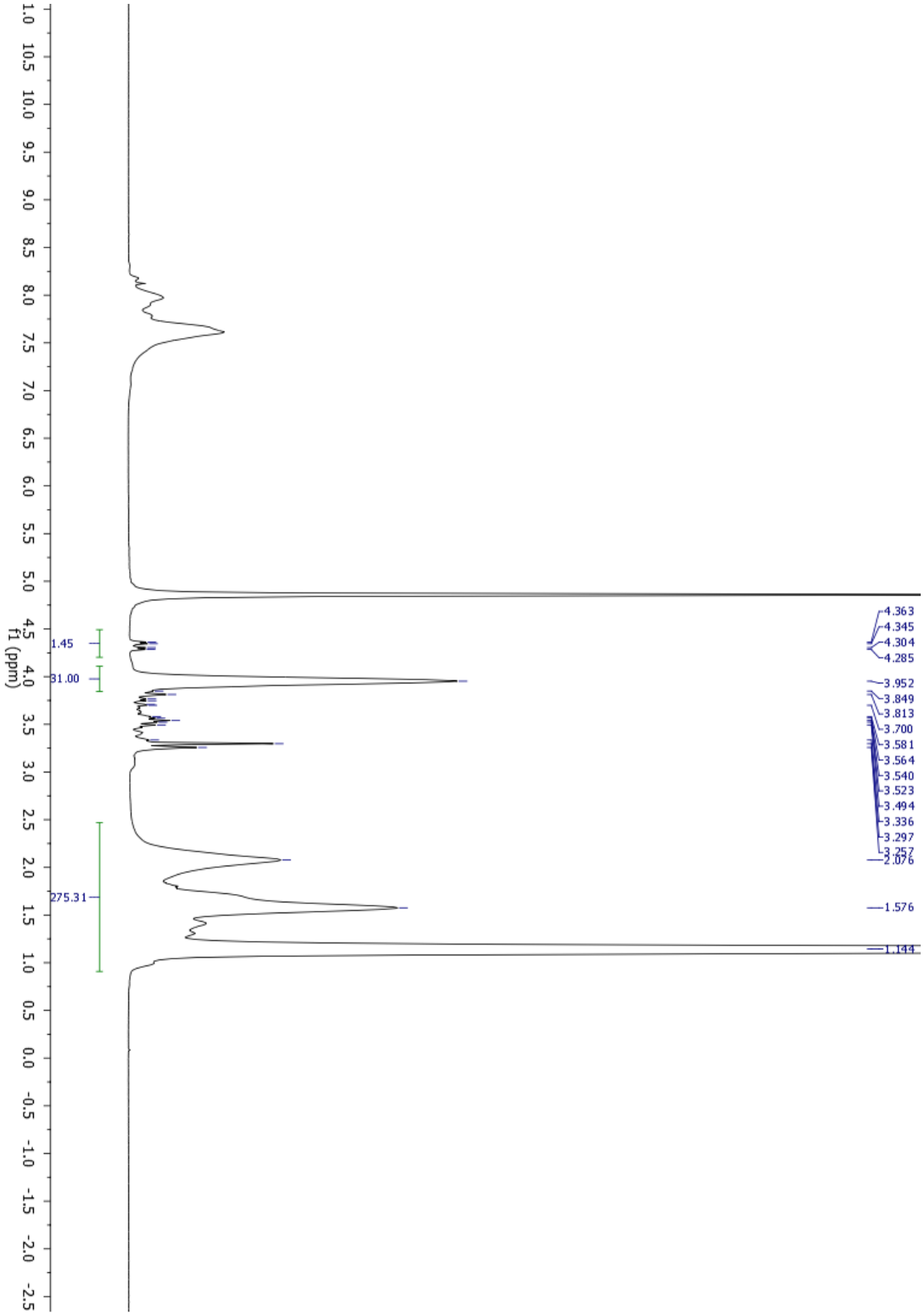
APPENDICES

Appendix A NMR SPECTRUMS OF COMPOUNDS IN PART TWO

1H-NMR spectrum of **PNIPAM** and **Lac-PNIPAM** in MeOD-d4 at 300K

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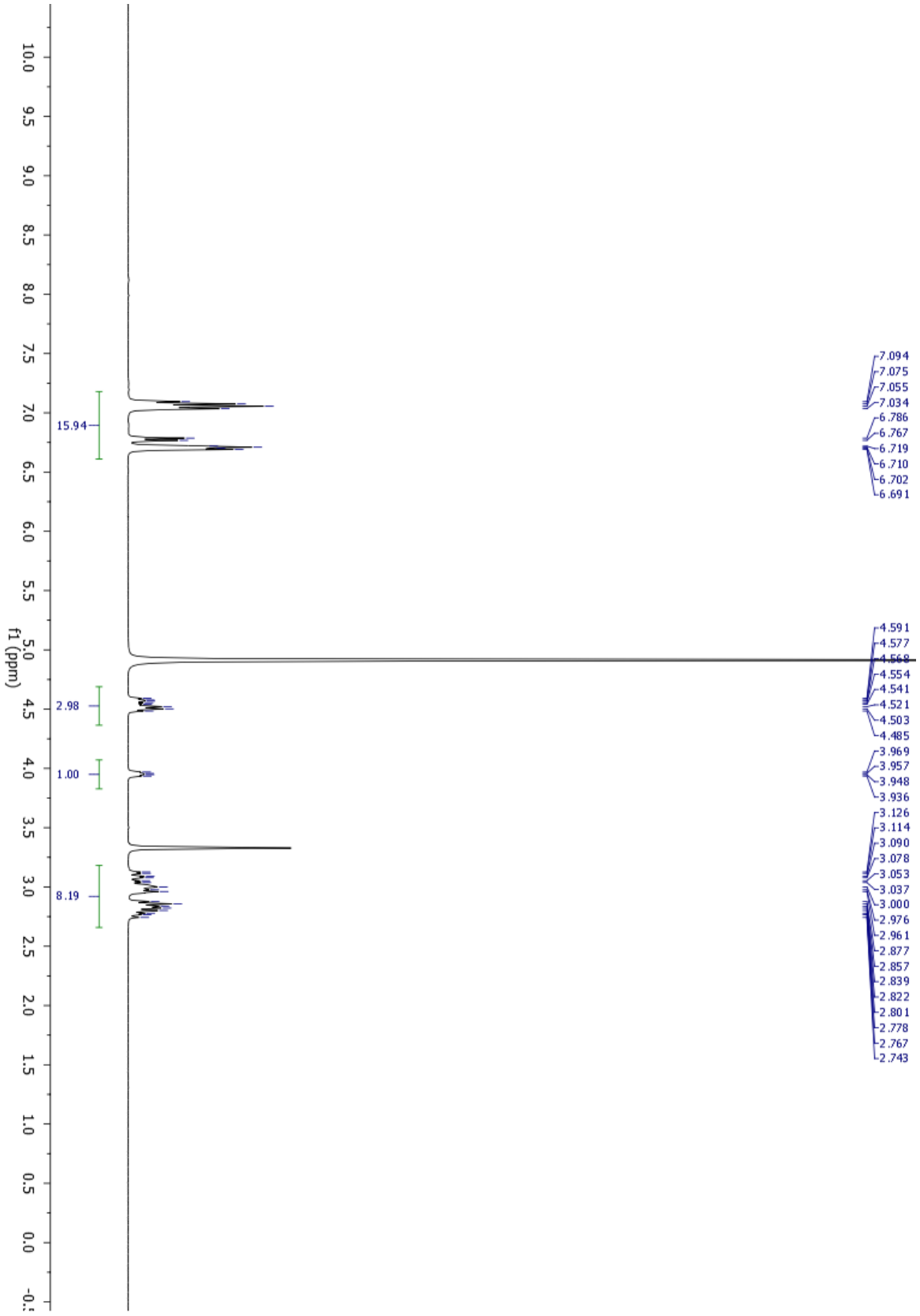
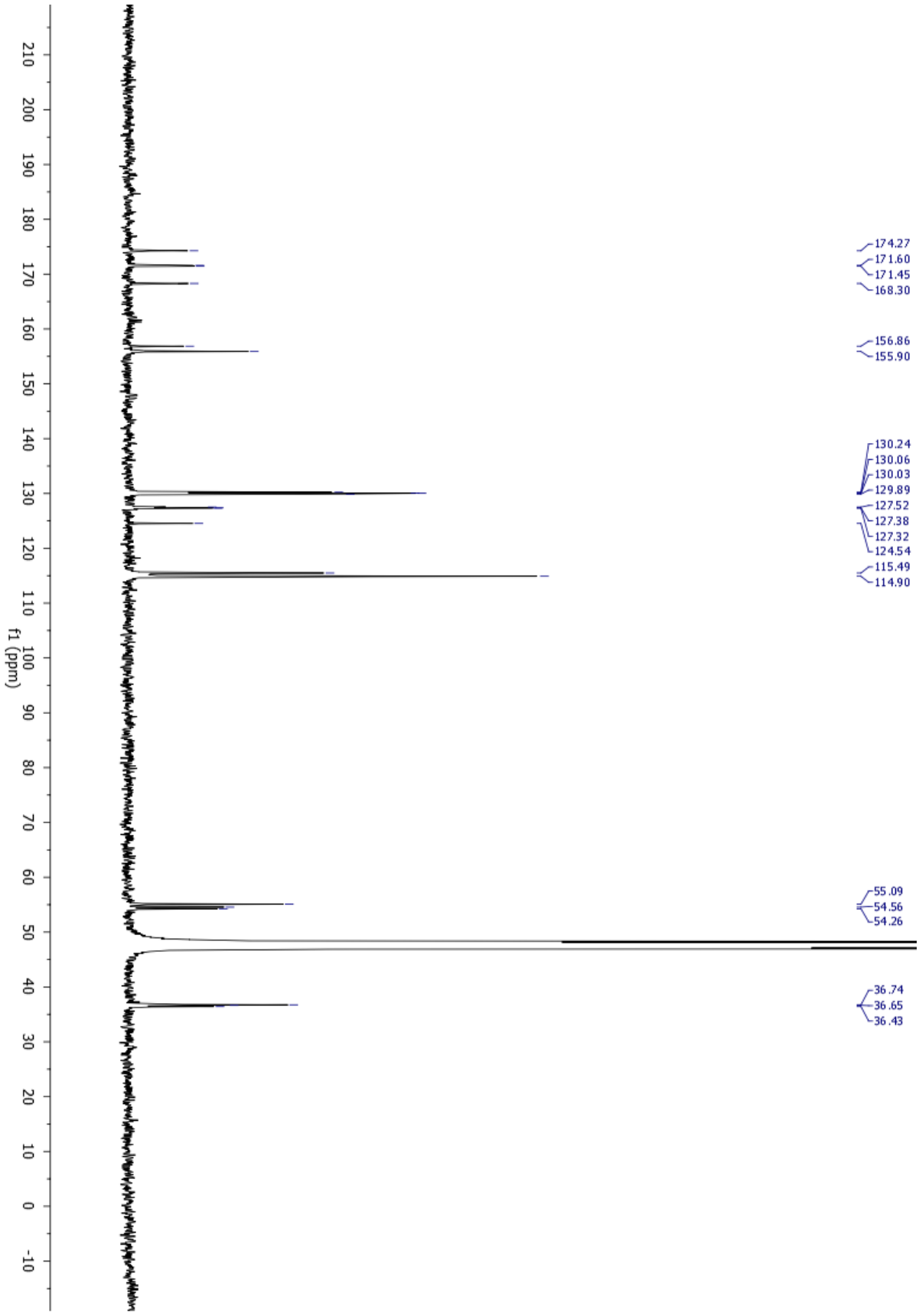
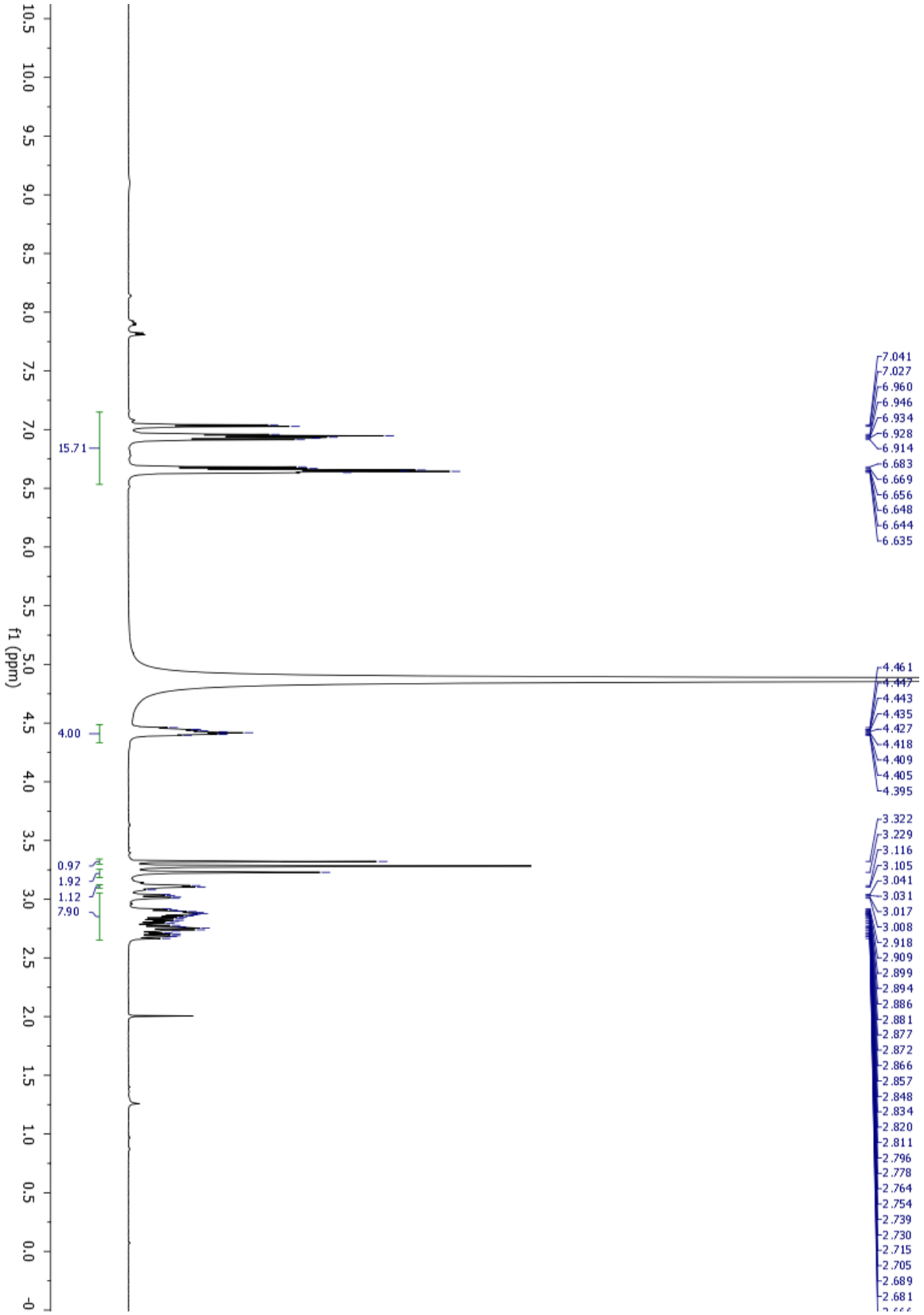
275.31
4.363
4.345
4.304
4.285
3.952
3.849
3.813
3.700
3.581
3.564
3.540
3.523
3.494
3.336
3.297
3.257
2.076
1.576
1.144

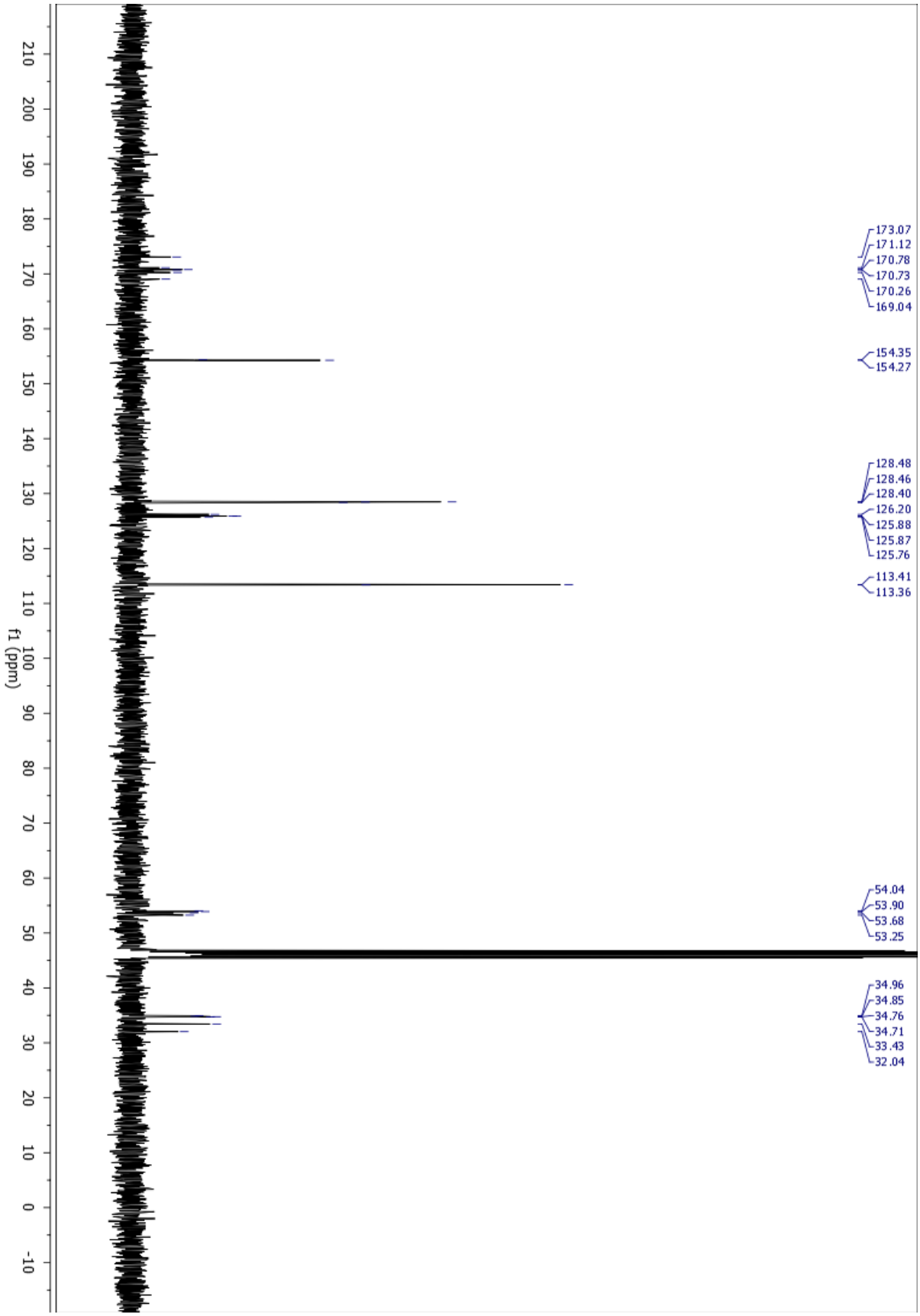
NMR spectrum of compound **13** in MeOD-d4 at 300K



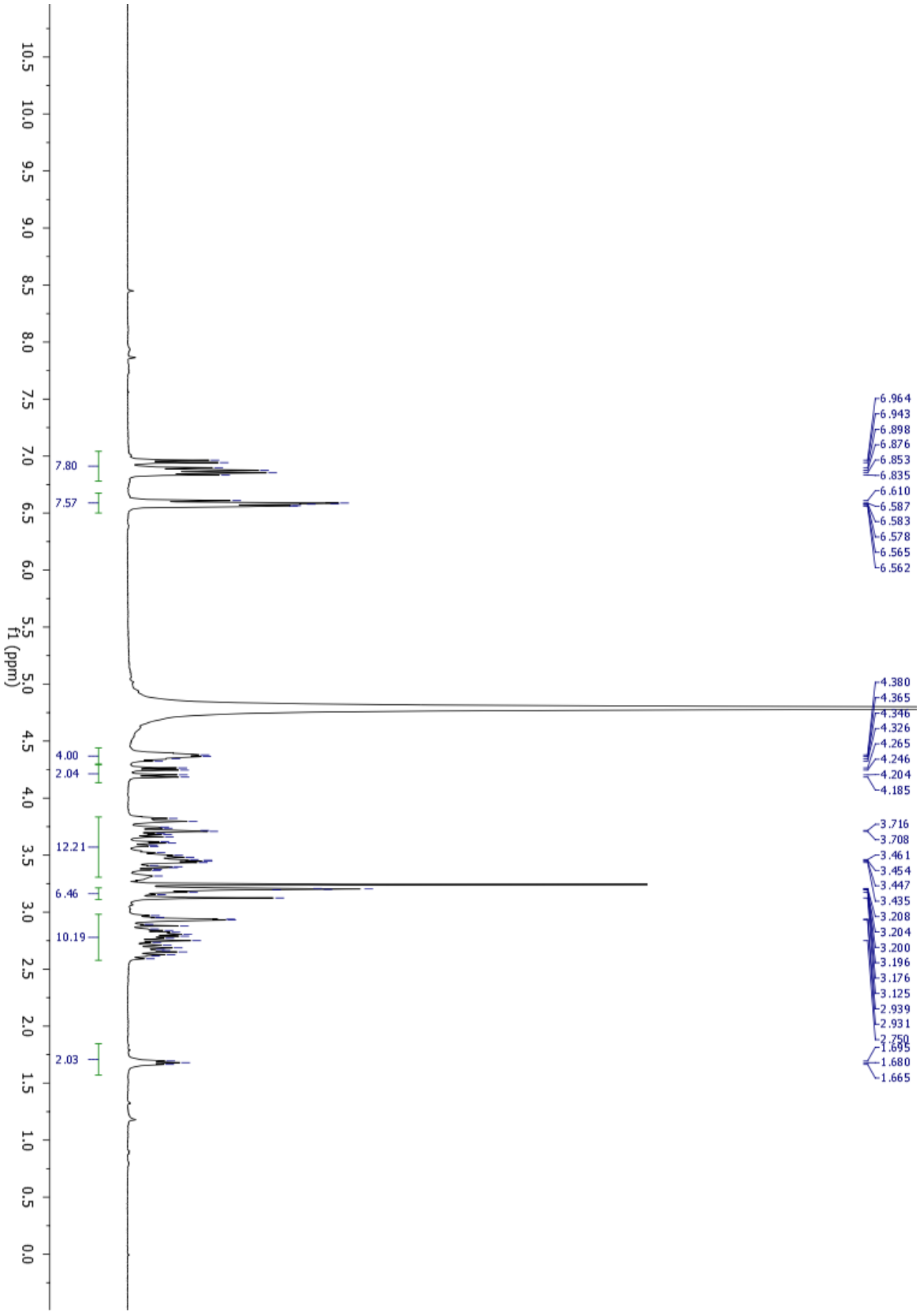

174.27
171.60
171.45
168.30
156.86
155.90
130.24
130.06
130.03
129.89
127.52
127.38
127.32
124.54
115.49
114.90
55.09
54.56
54.26
36.74
36.65
36.43
f1 (ppm)

NMR spectrum of compound **14** in MeOD-d4 at 300K

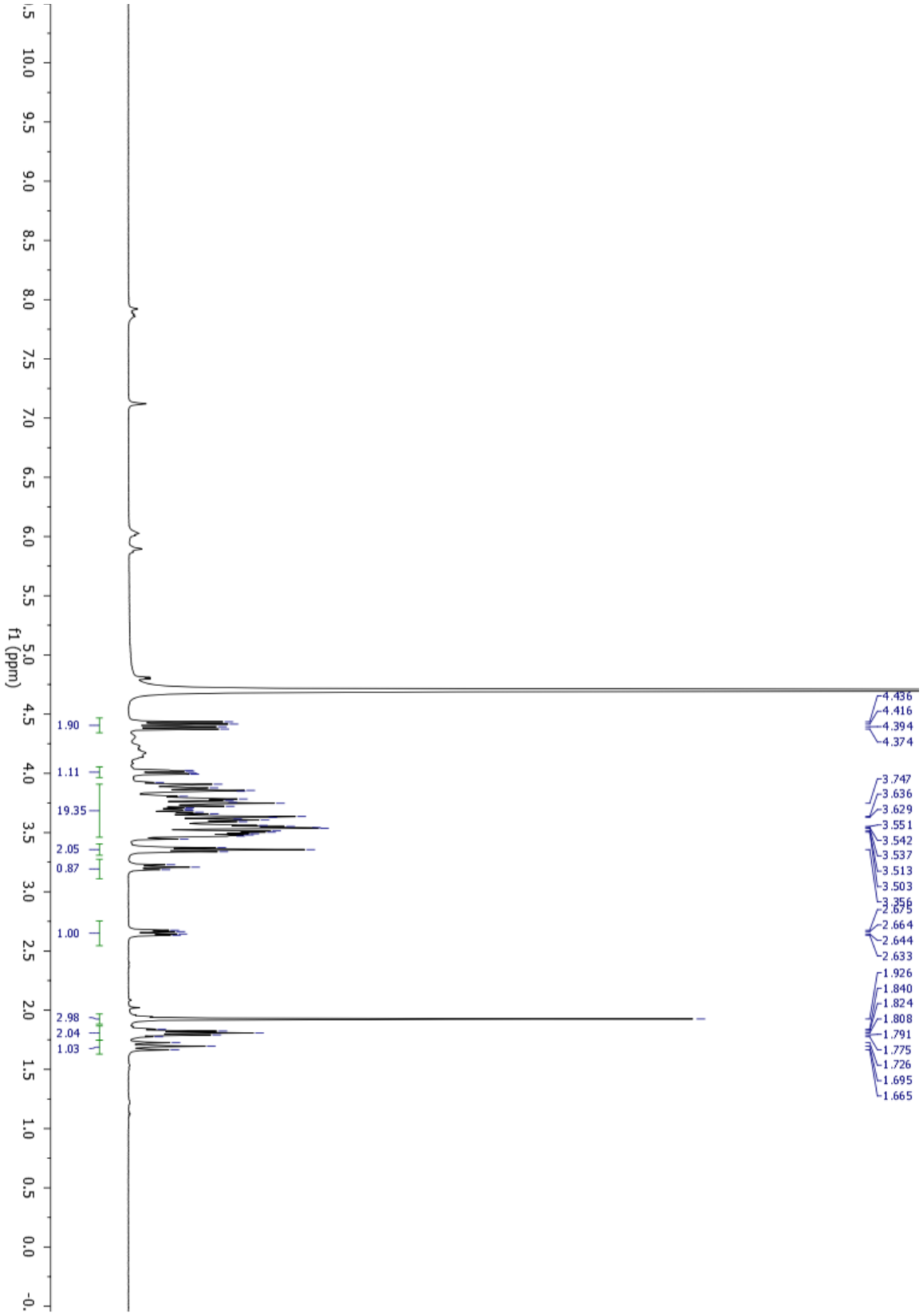


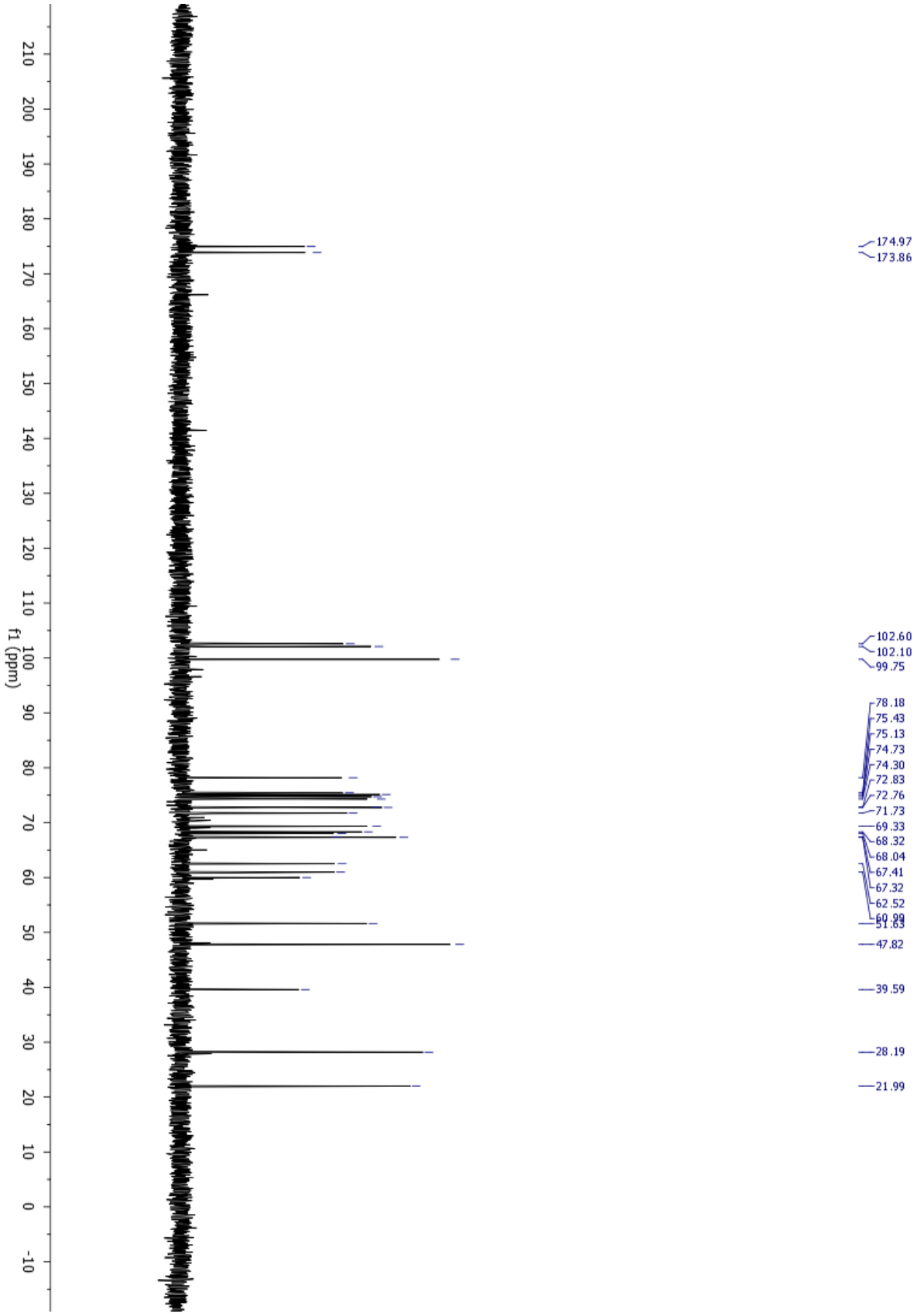

173.07
171.12
170.78
170.73
170.26
169.04
154.35
154.27
128.48
128.46
128.40
126.20
125.88
125.87
125.76
113.41
113.36
54.04
53.90
53.68
53.25
34.96
34.85
34.76
34.71
33.43
32.04
f1 (ppm)

NMR spectrum of compound **15** in MeOD-d4 at 300K



NMR spectrum of compound **17** in D_2O at 300K




f1 (ppm)
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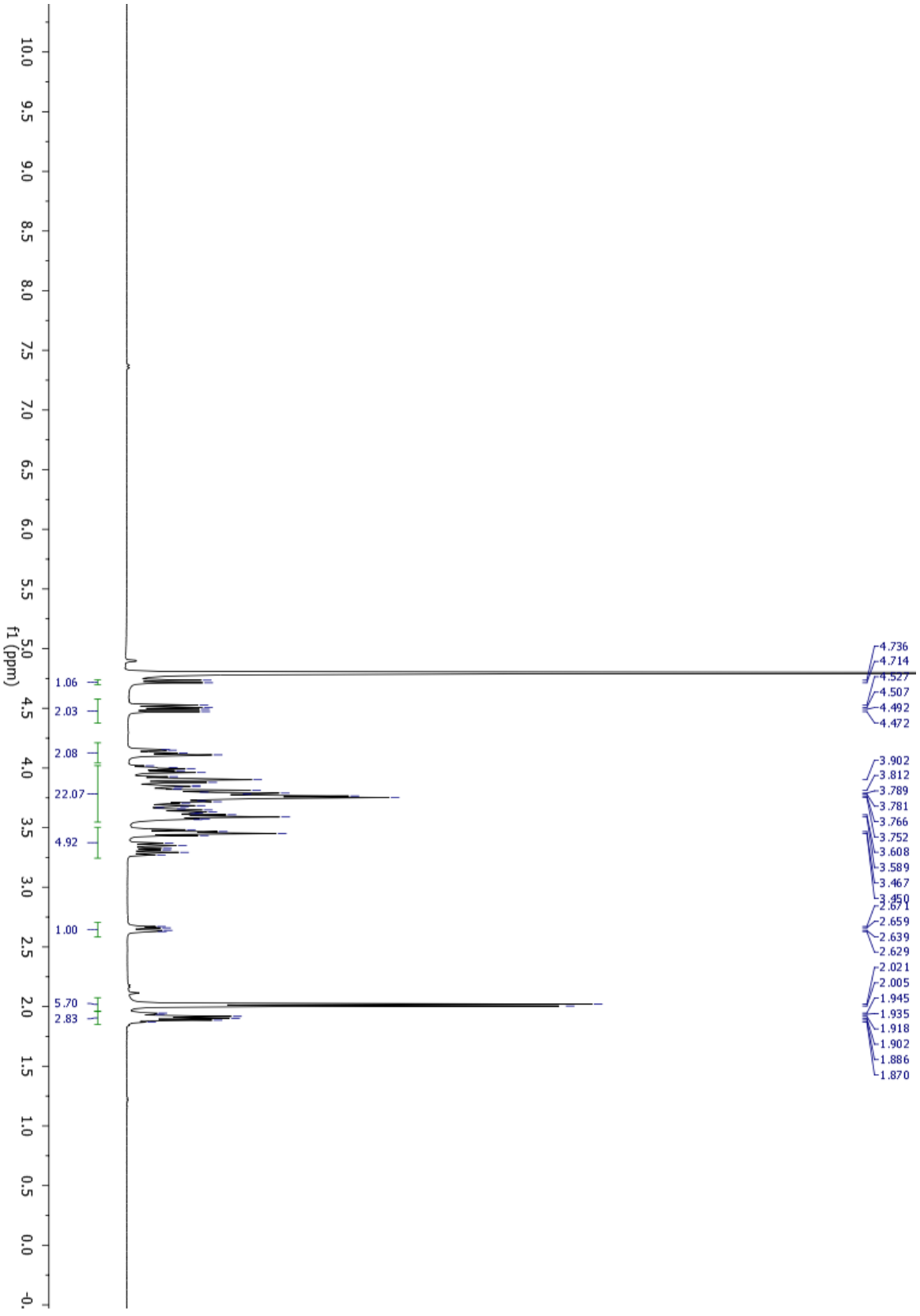
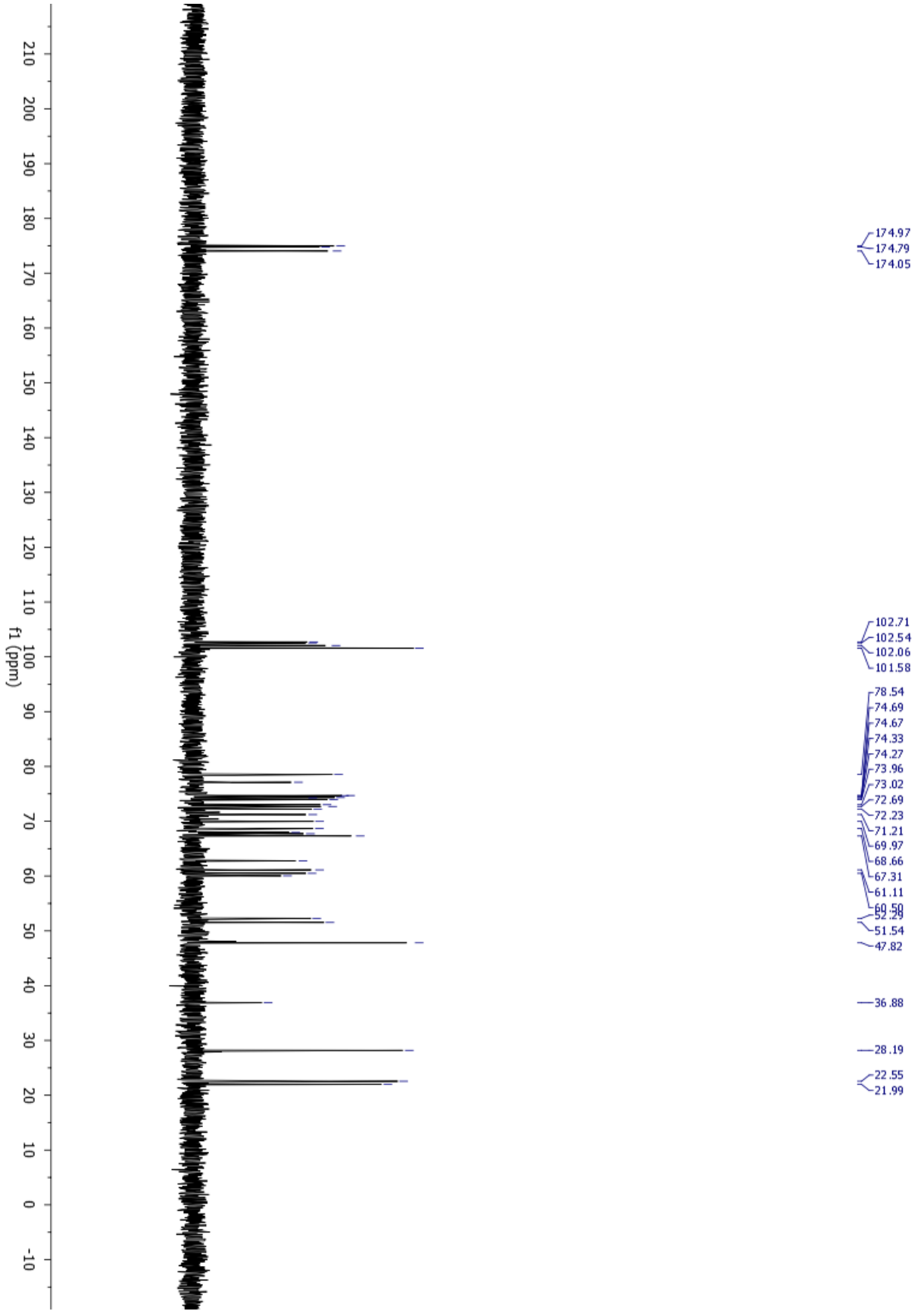
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
174.97
173.86
102.60
102.10
99.75
78.18
75.43
75.13
74.73
74.30
72.83
72.76
71.73
69.33
68.32
68.04
67.41
67.32
62.52
47.82
39.59
28.19
21.99

NMR spectrum of compound **18** in D_2O at 300K




f1 (ppm)
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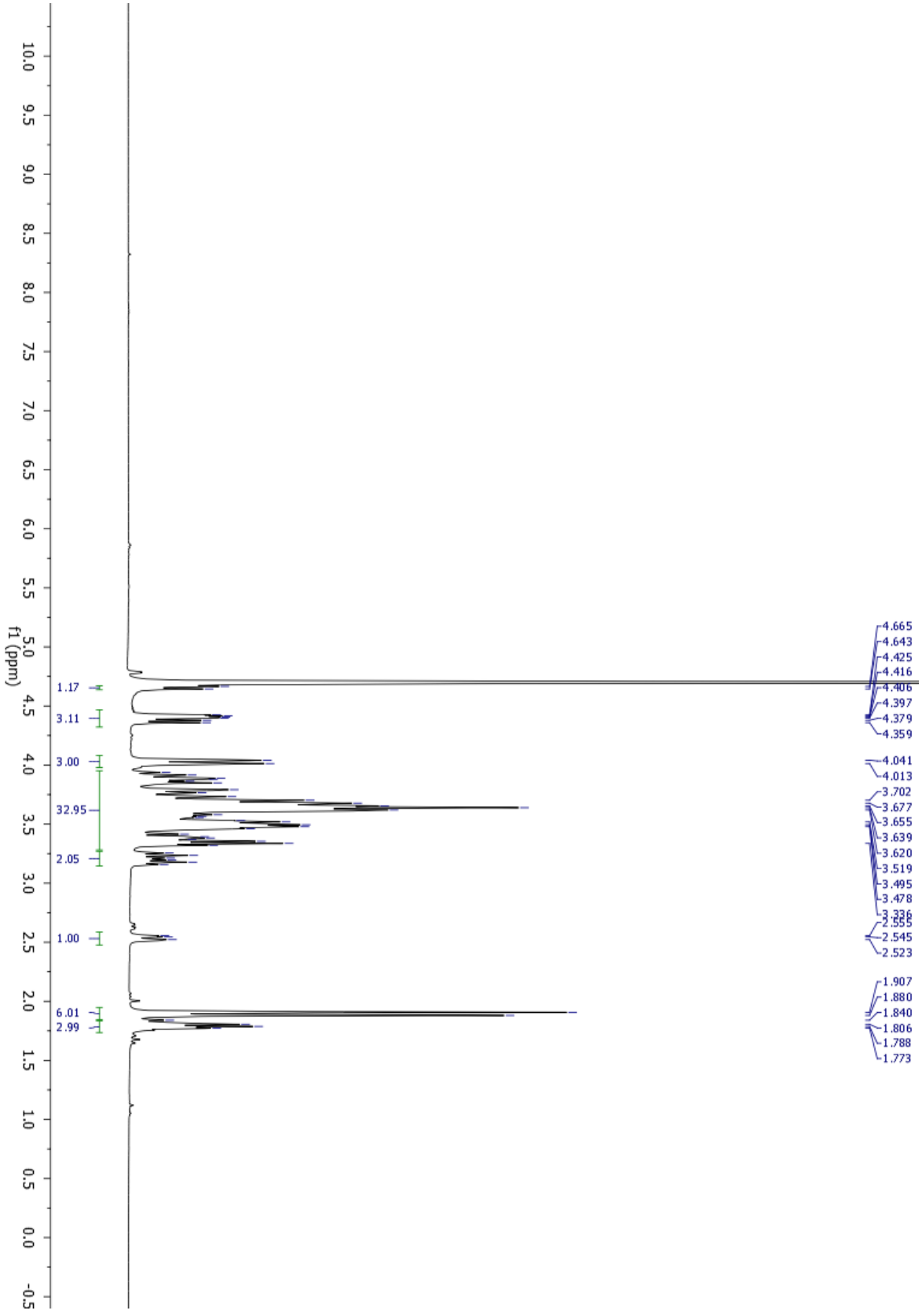
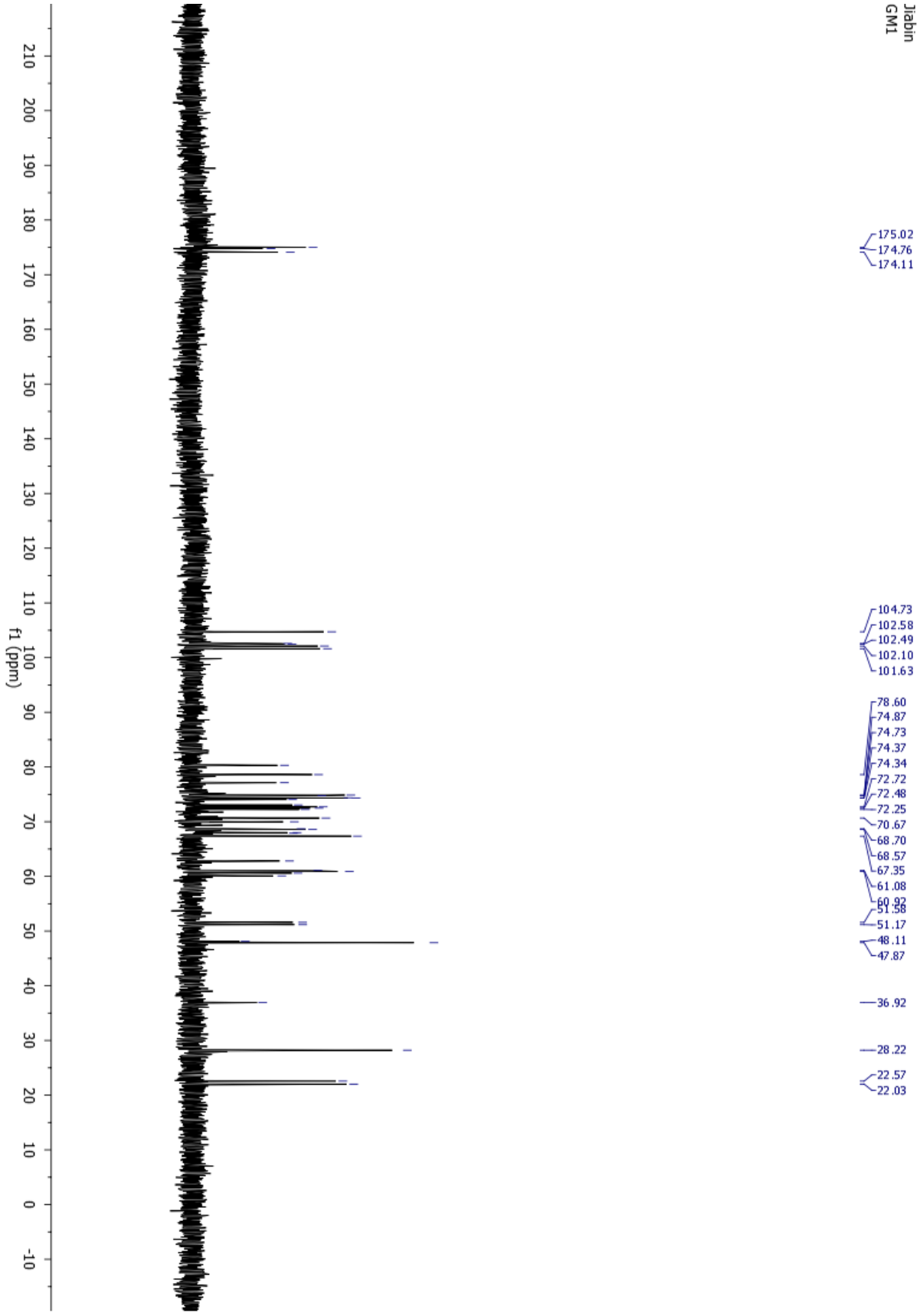
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
174.97
174.79
174.05
102.71
102.54
102.06
101.58
78.54
74.69
74.67
74.33
74.27
73.96
73.02
72.69
72.23
71.21
69.97
68.66
67.31
61.11
60.50
51.54
47.82
36.88
28.19
22.55
21.99

NMR spectrum of compound **19** in D_2O at 300K




Jiabin
GM1
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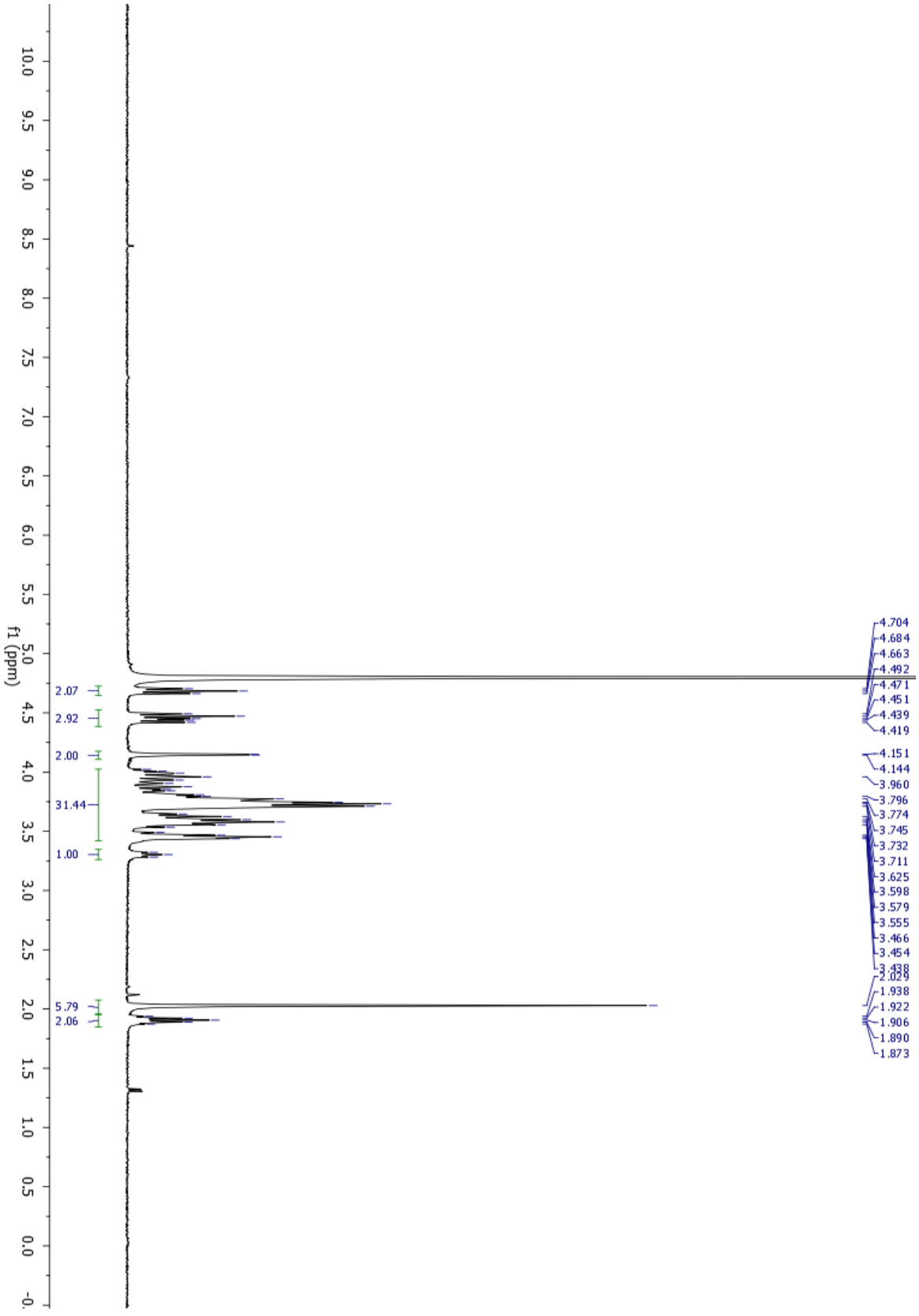
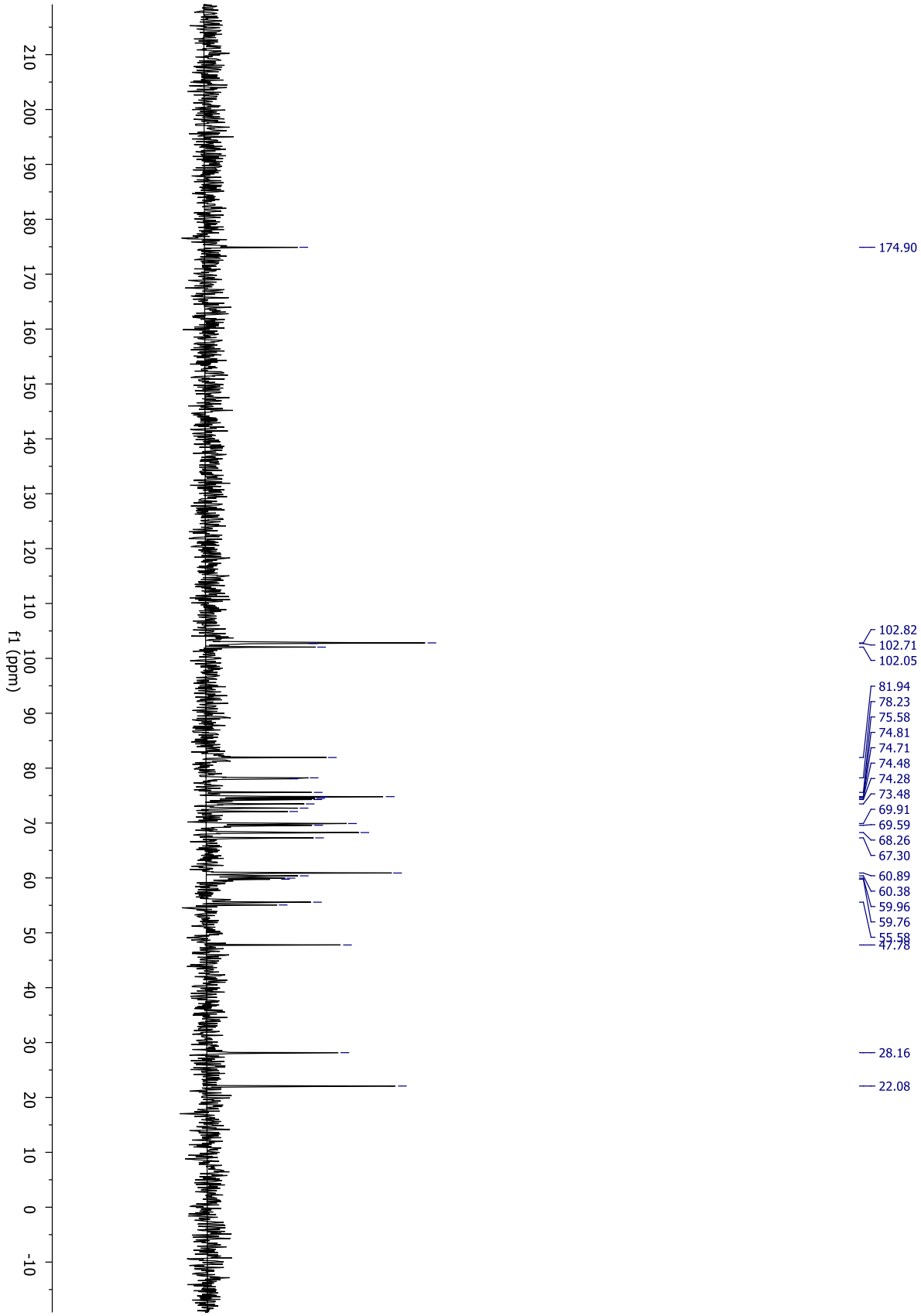
174.76
174.11
104.73
102.58
102.49
102.10
101.63
78.60
74.87
74.73
74.37
74.34
72.72
72.48
72.25
70.67
68.70
68.57
67.35
61.08
60.92
51.58
51.17
48.11
47.87
36.92
28.22
22.57
22.03
f1 (ppm)
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10

NMR spectrum of compound **22** in D_2O at 300K




f1 (ppm)
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180
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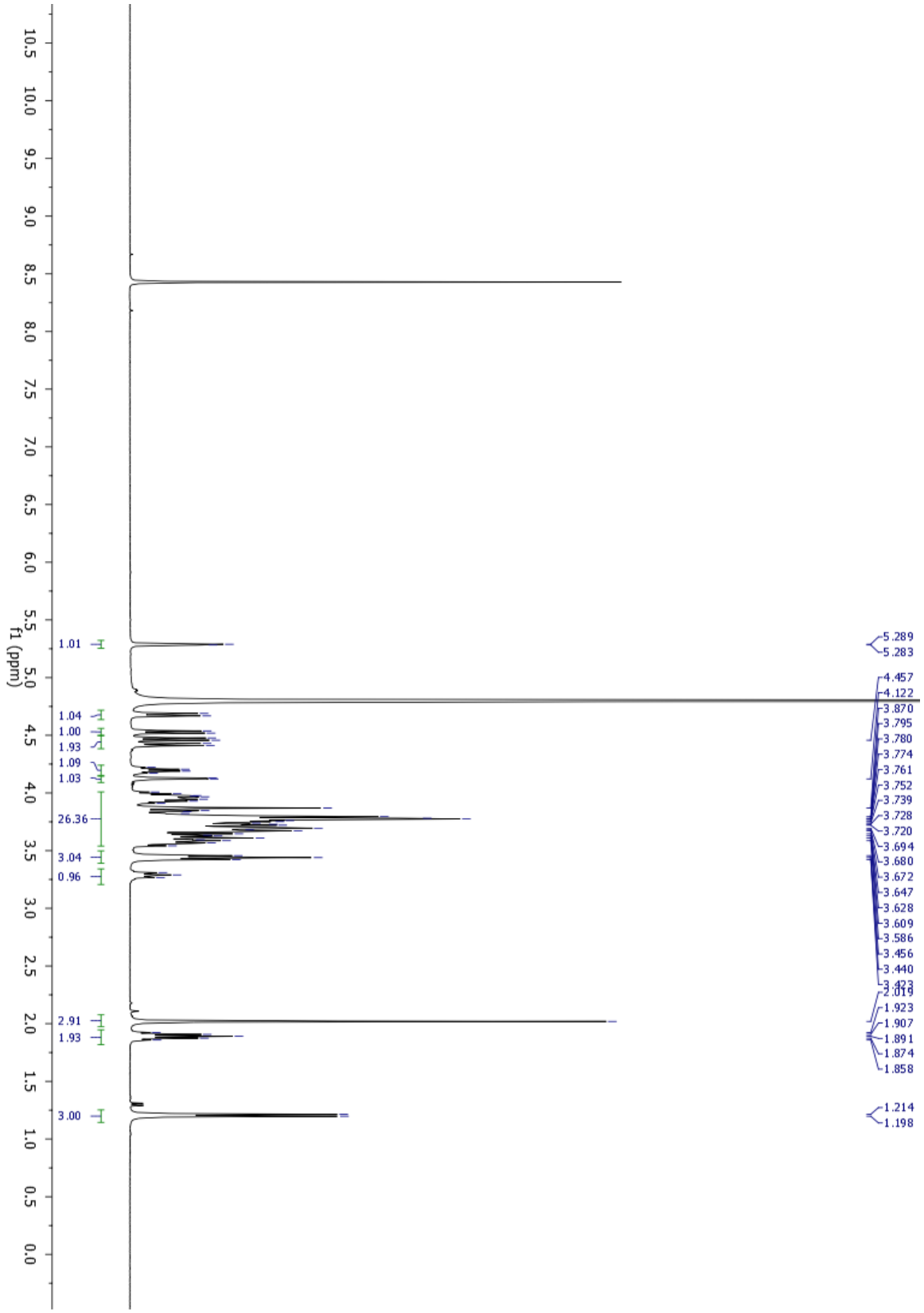
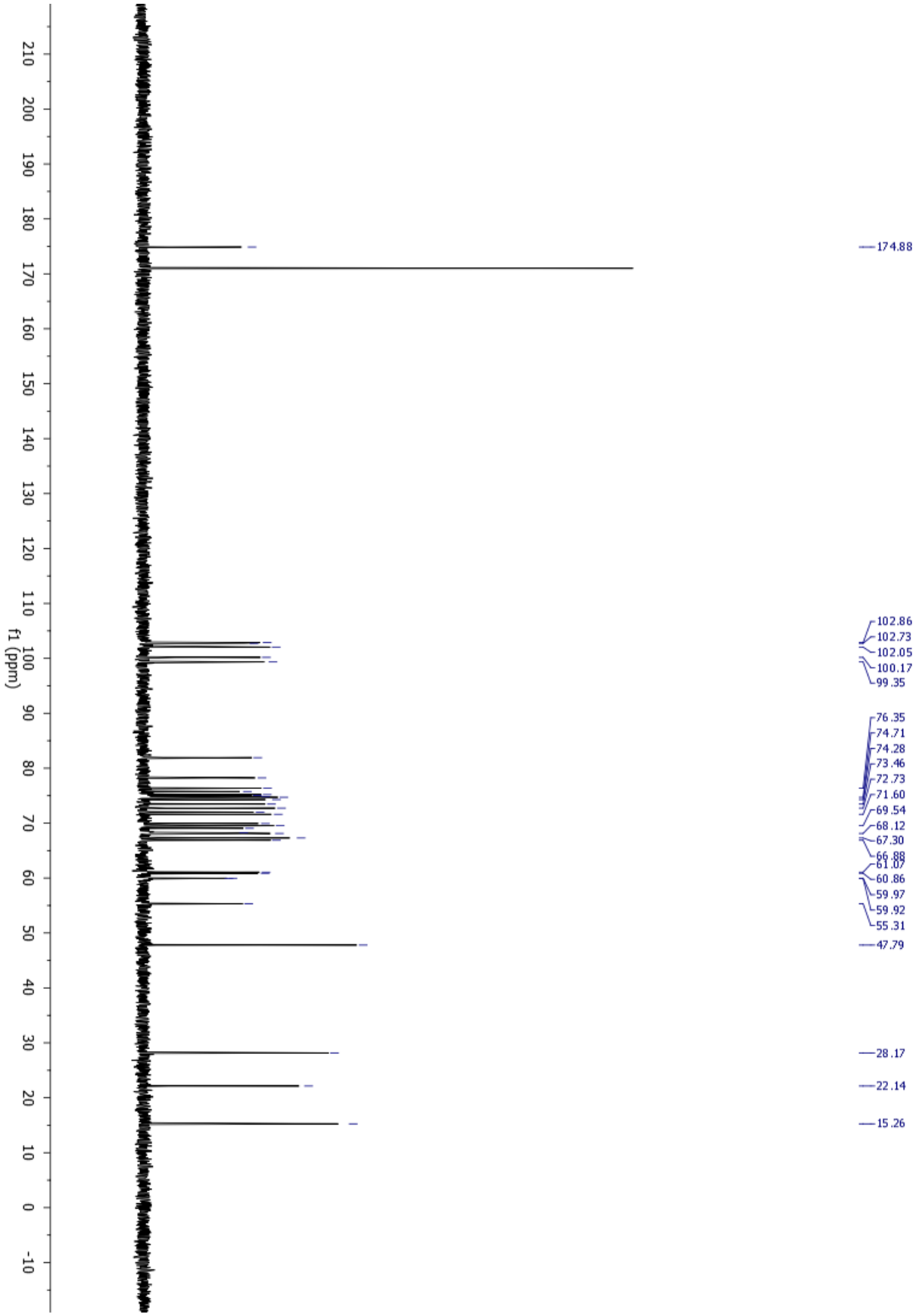
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
174.90
102.82
102.71
102.05
81.94
78.23
75.58
74.81
74.71
74.48
74.28
73.48
69.91
69.59
68.26
67.30
60.89
60.38
59.96
59.76
55.58
47.78
28.16
22.08

NMR spectrum of compound **23** in D_2O at 300K

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180
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140
130
120
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100
90
80
70
60
50
40
30
20
10
0
-10
174.88
102.86
102.73
102.05
100.17
99.35
76.35
74.71
74.28
73.46
72.73
71.60
69.54
68.12
67.30
66.88
61.07
60.86
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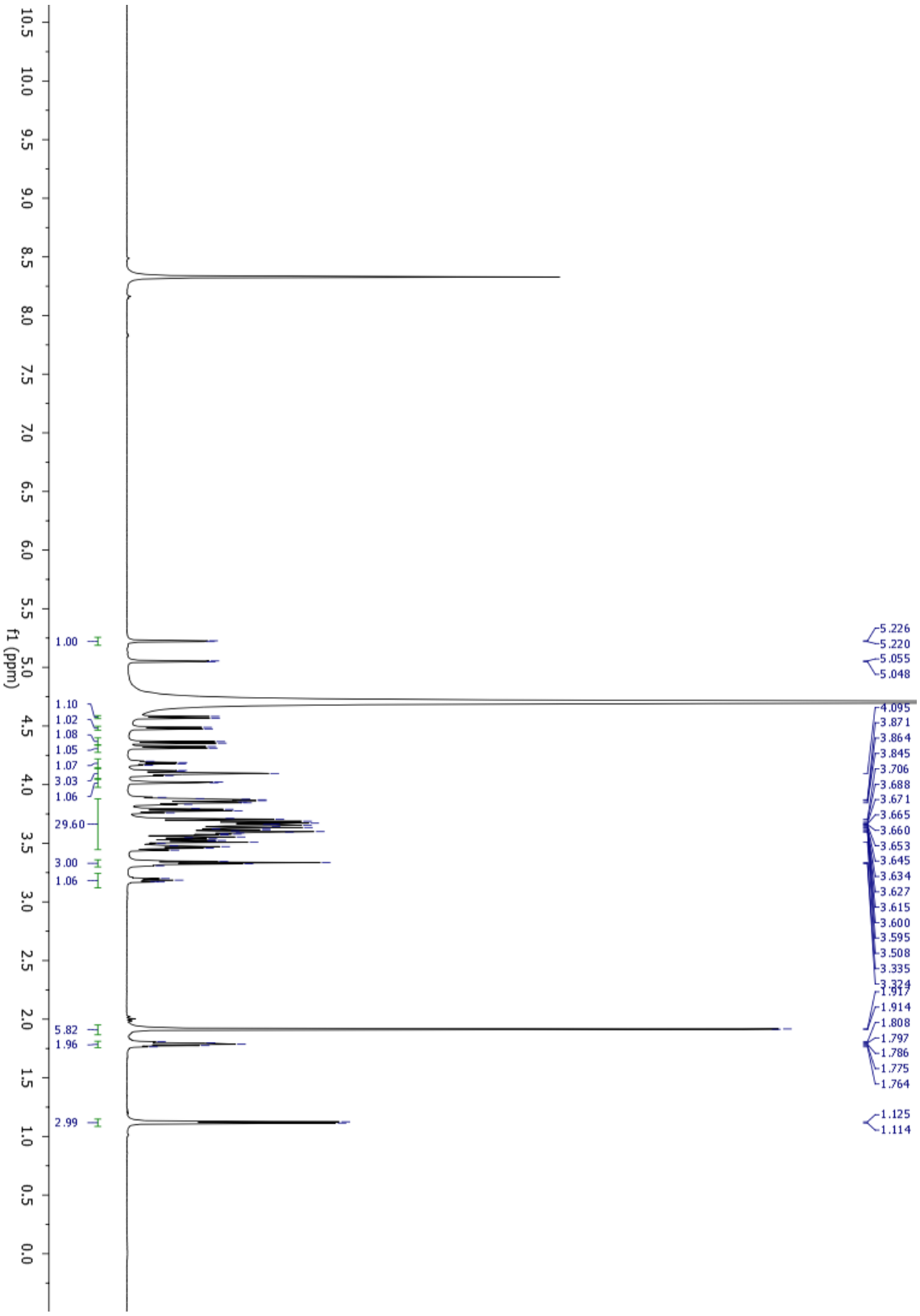
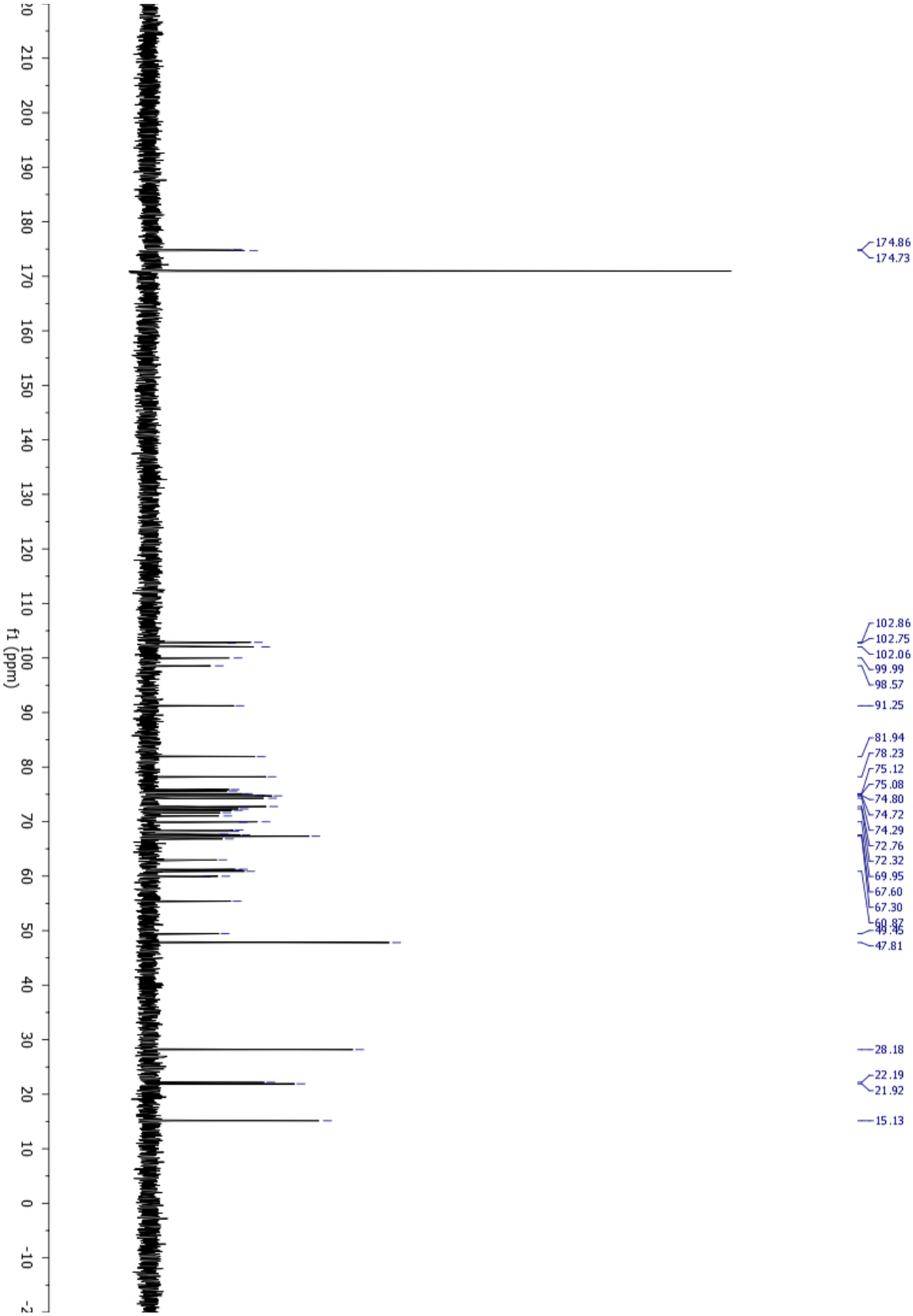
59.92
55.31
47.79
28.17
22.14
15.26

NMR spectrum of compound **24** in D_2O at 300K

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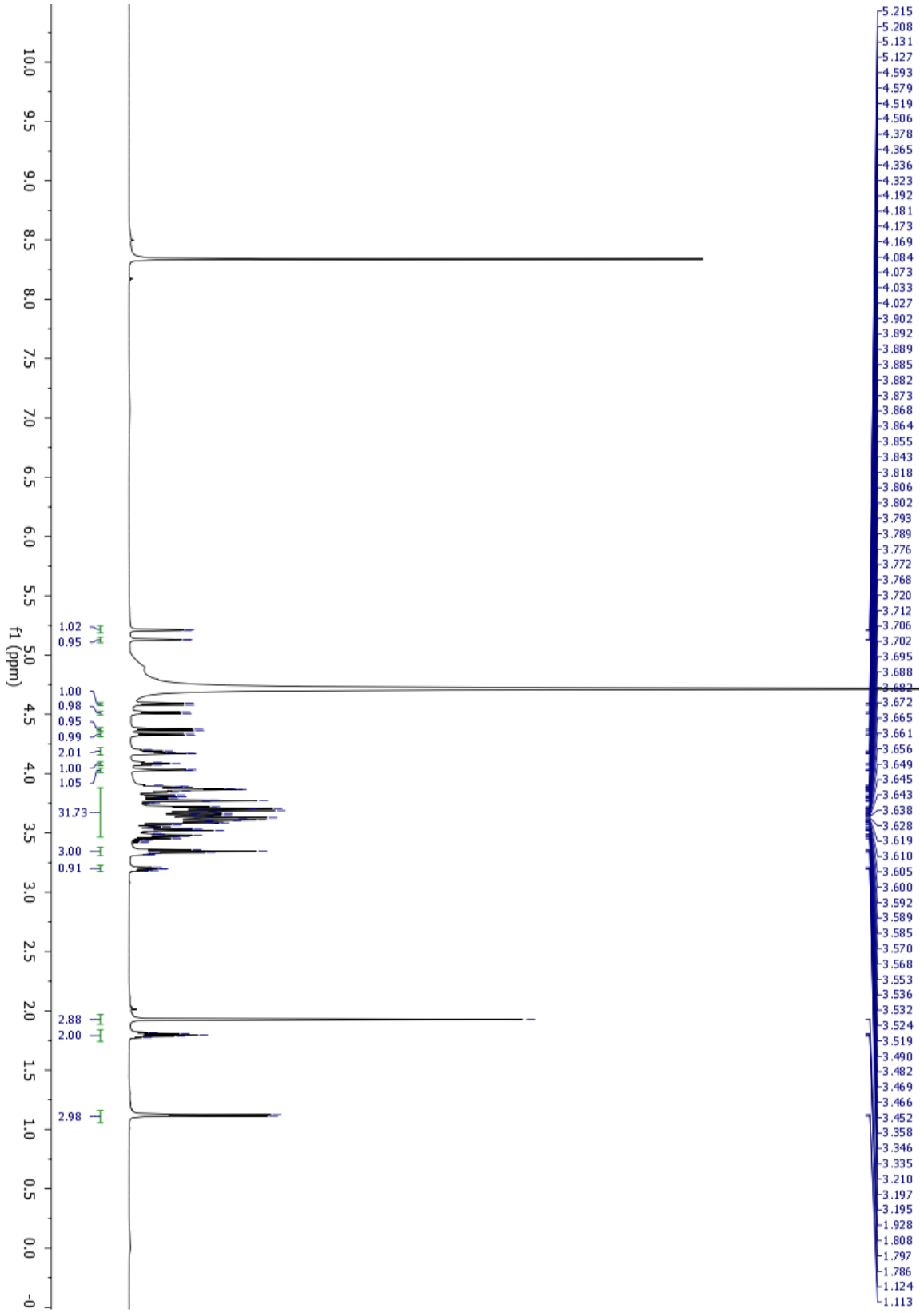
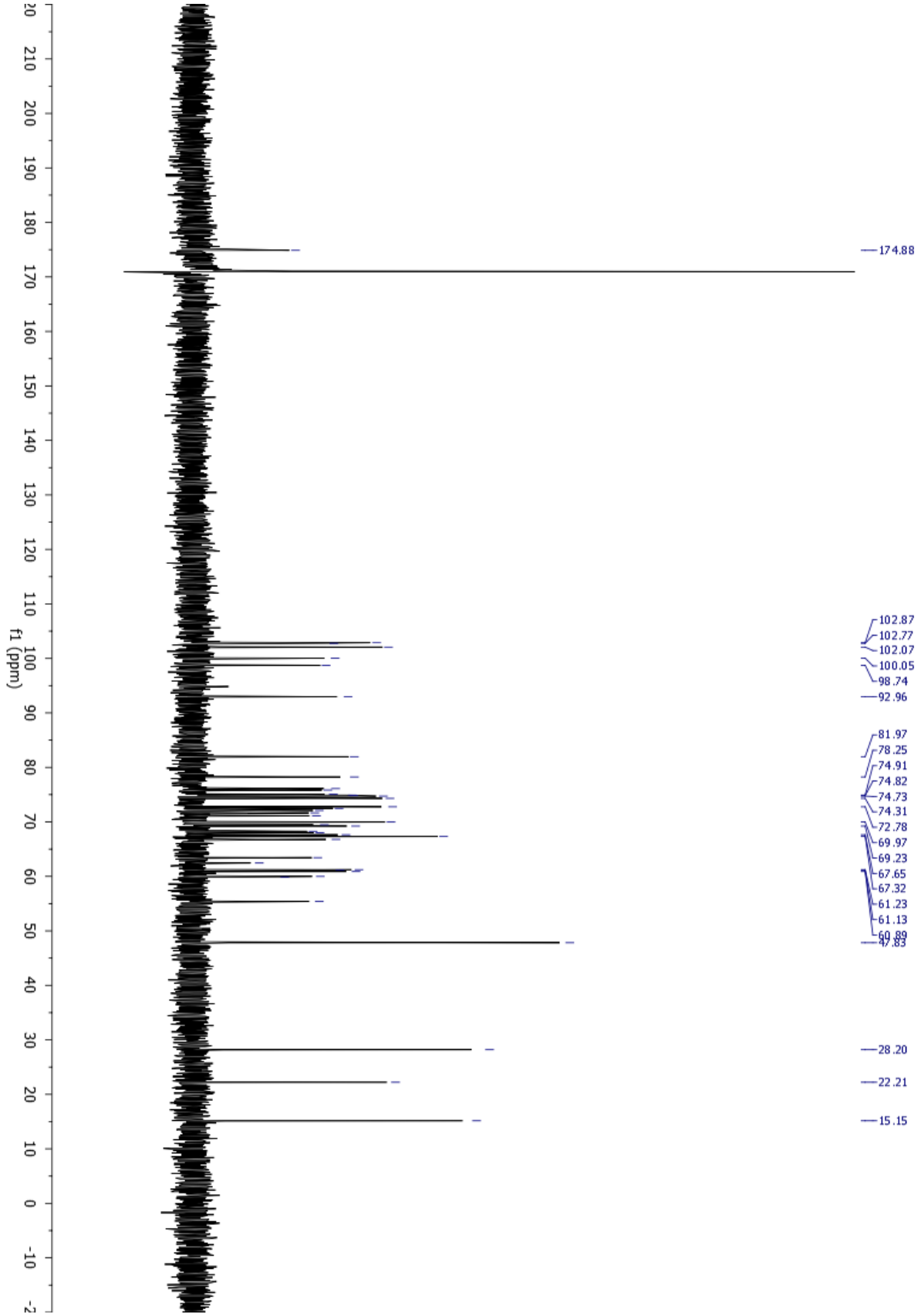
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
174.86
174.73
102.86
102.75
102.06
99.99
98.57
91.25
81.94
78.23
75.12
75.08
74.80
74.72
74.29
72.76
72.32
69.95
67.60
67.30
60.87
49.45
47.81
28.18
22.19
21.92
15.13

NMR spectrum of compound **25** in D_2O at 300K




f1 (ppm)
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210
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180
170
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150
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100
90
80
70
60
50
40
30
20
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0
-10
-2
174.88
102.87
102.77
102.07
100.05
98.74
92.96
81.97
78.25
74.91
74.82
74.73
74.31
72.78
69.97
69.23
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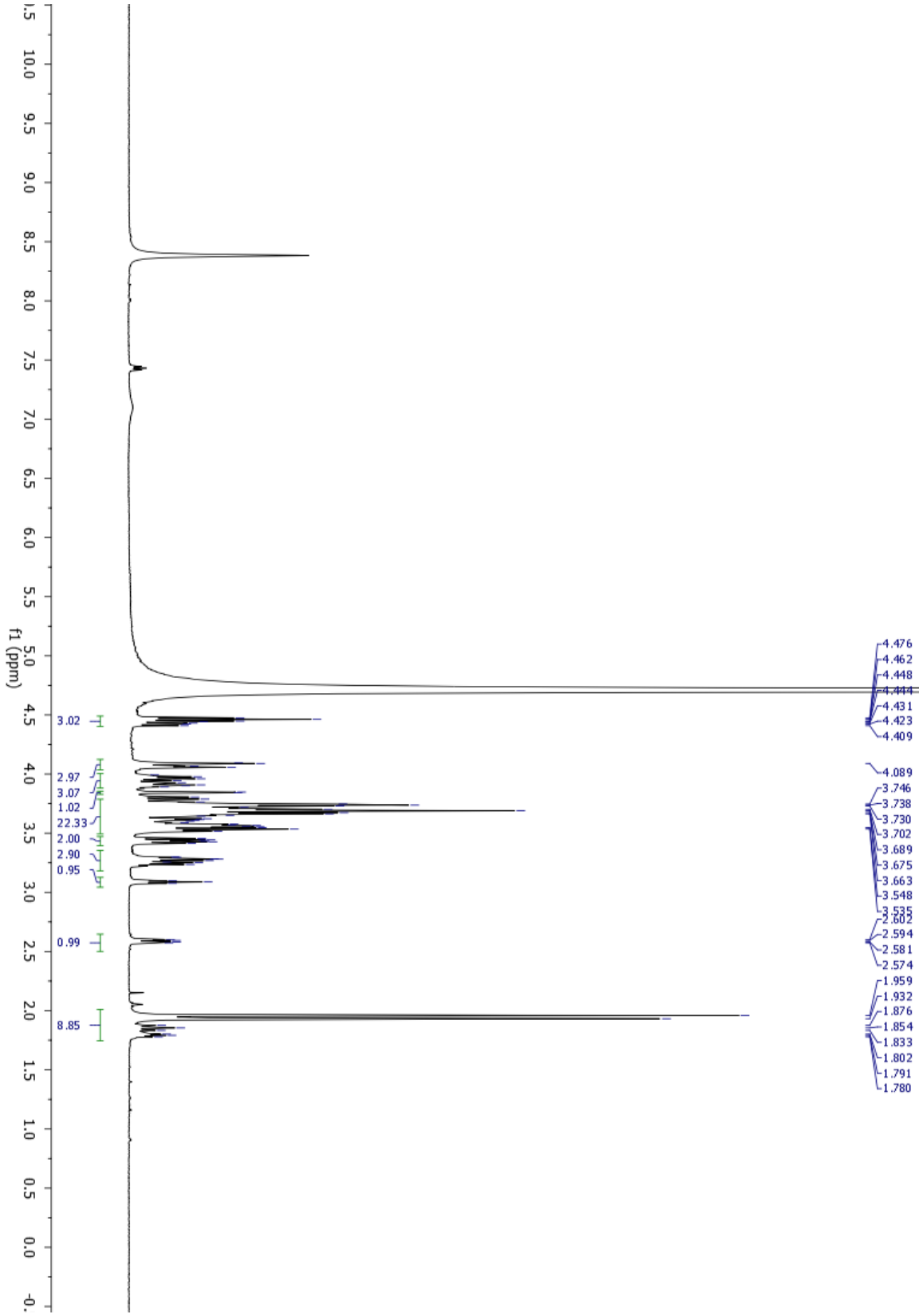
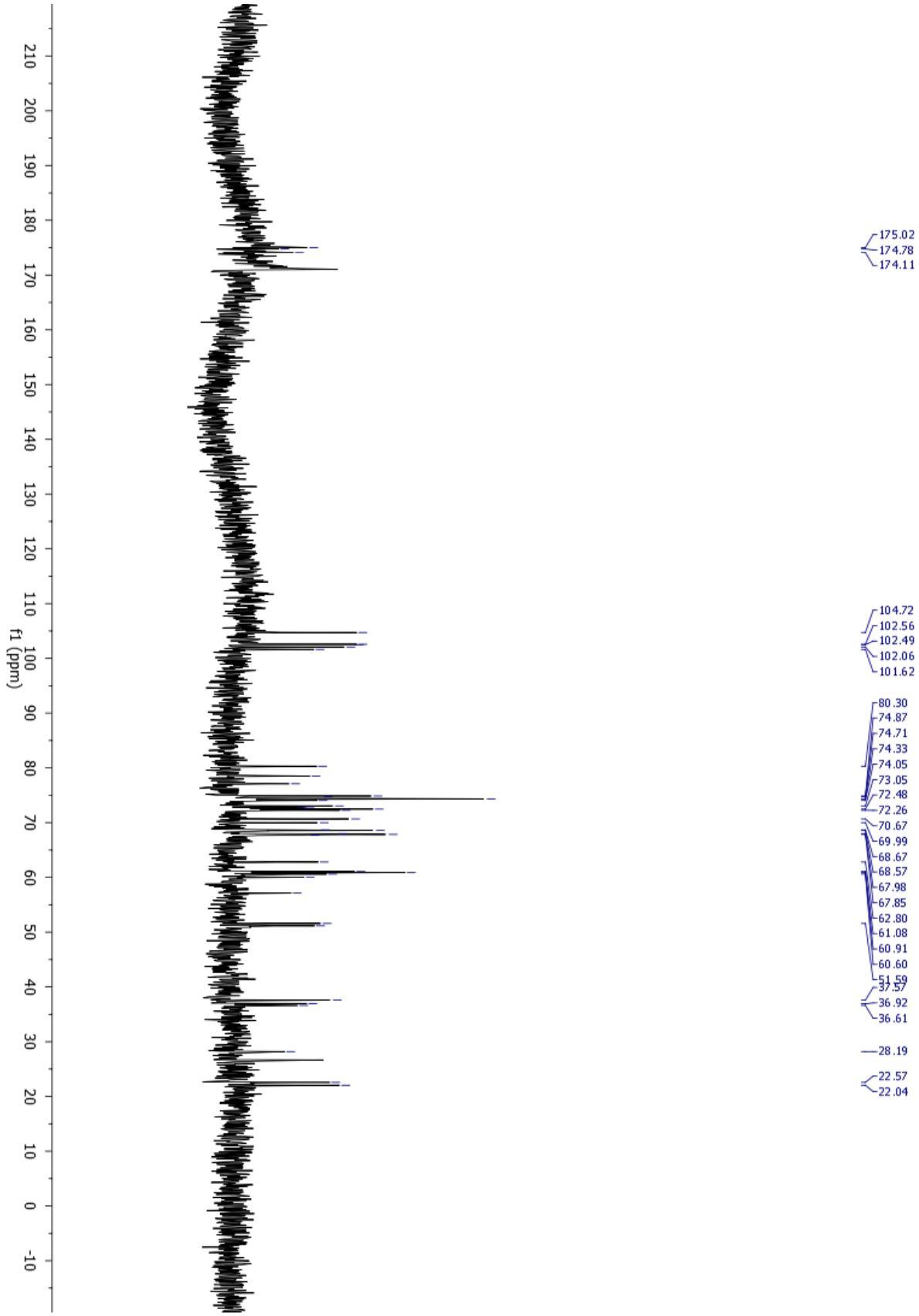
67.32
61.23
61.13
60.89
47.83
28.20
22.21
15.15

NMR spectrum of compound **6** in D_2O at 300K




f1 (ppm)
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170
160
150
140
130
120
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90
80
70
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50
40
30
20
10
0
-10
175.02
174.78
174.11
104.72
102.56
102.49
102.06
101.62
80.30
74.87
74.71
74.33
74.05
73.05
72.48
72.26
70.67
69.99
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68.57
67.98
67.85
62.80
61.08
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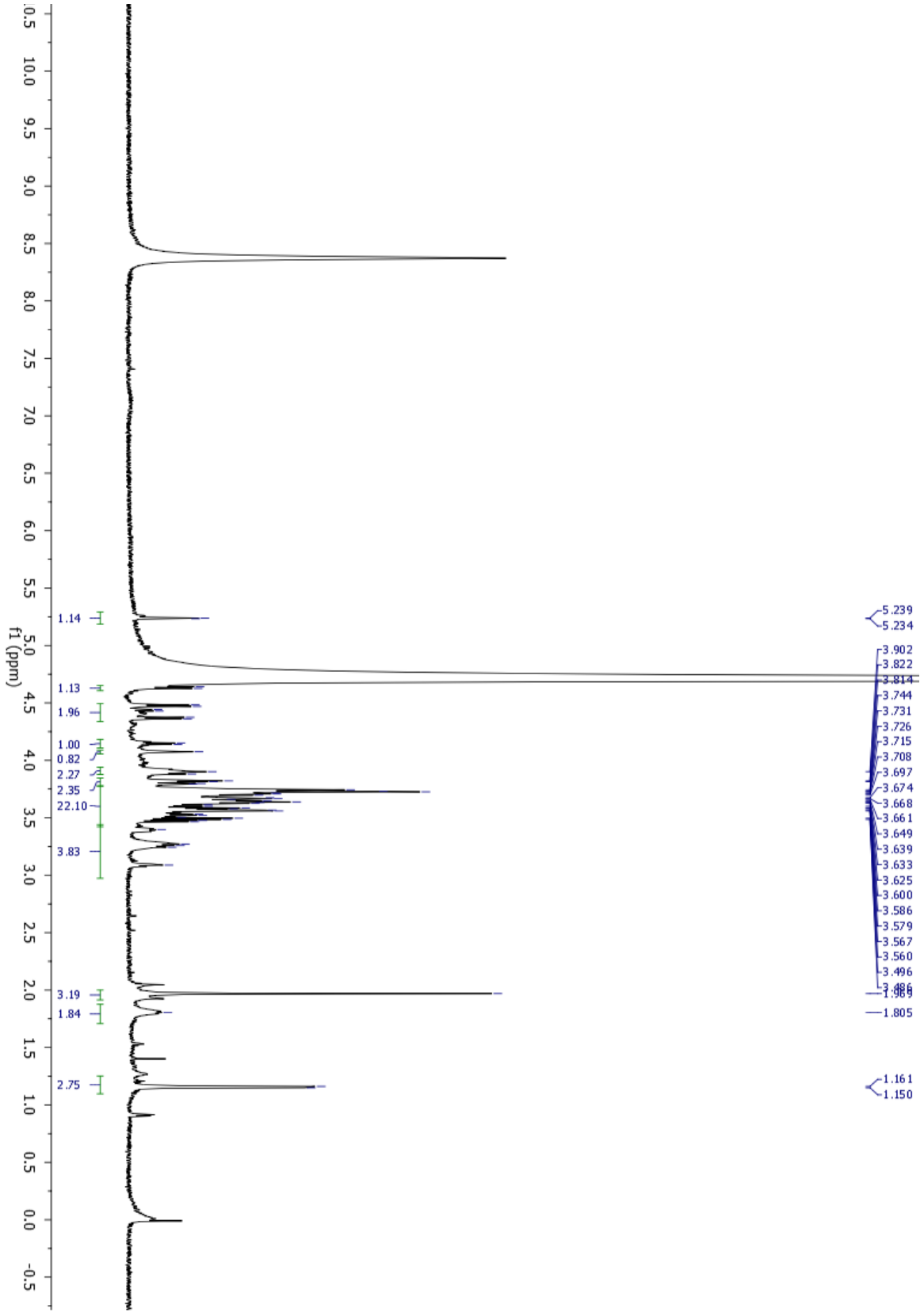
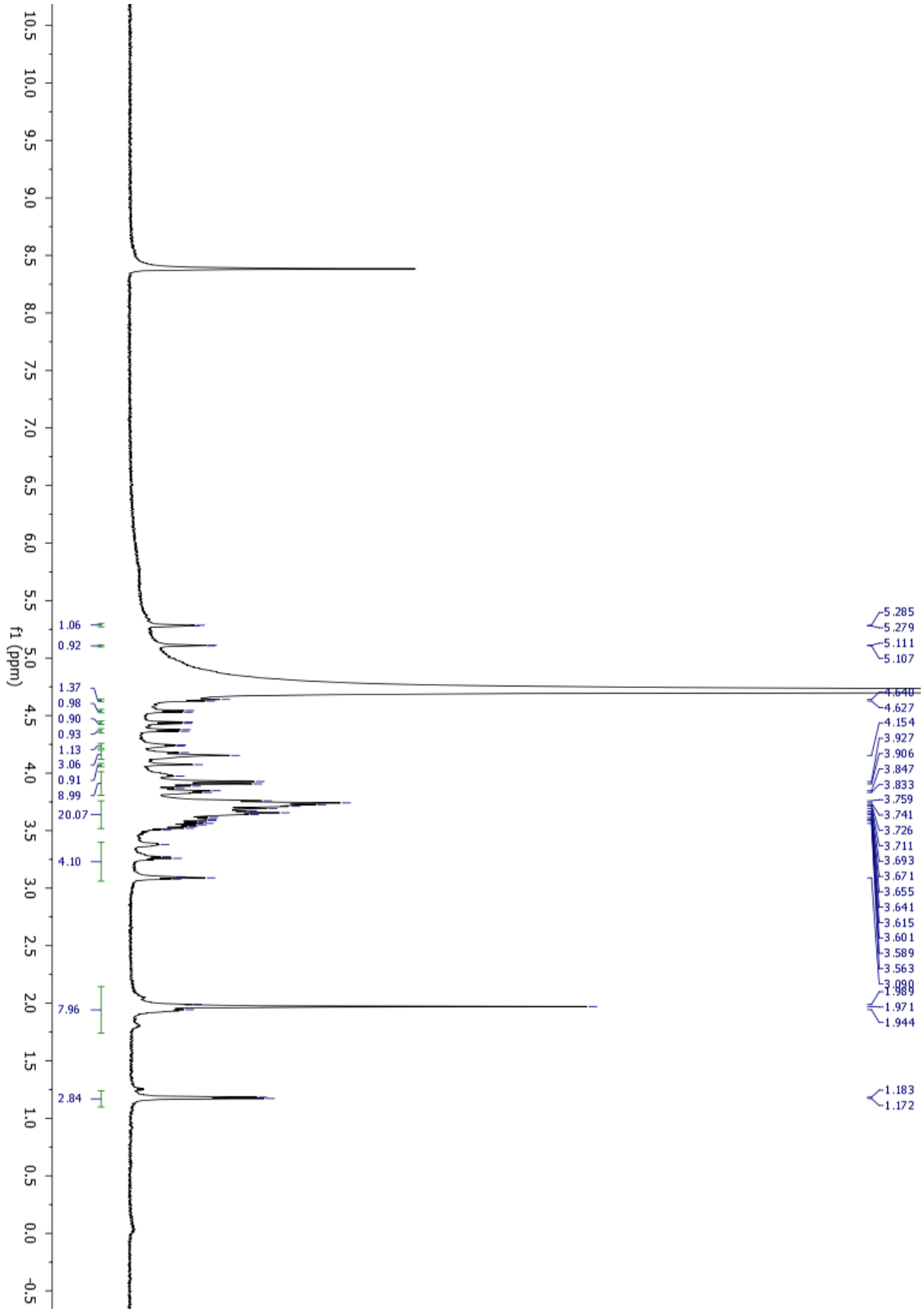
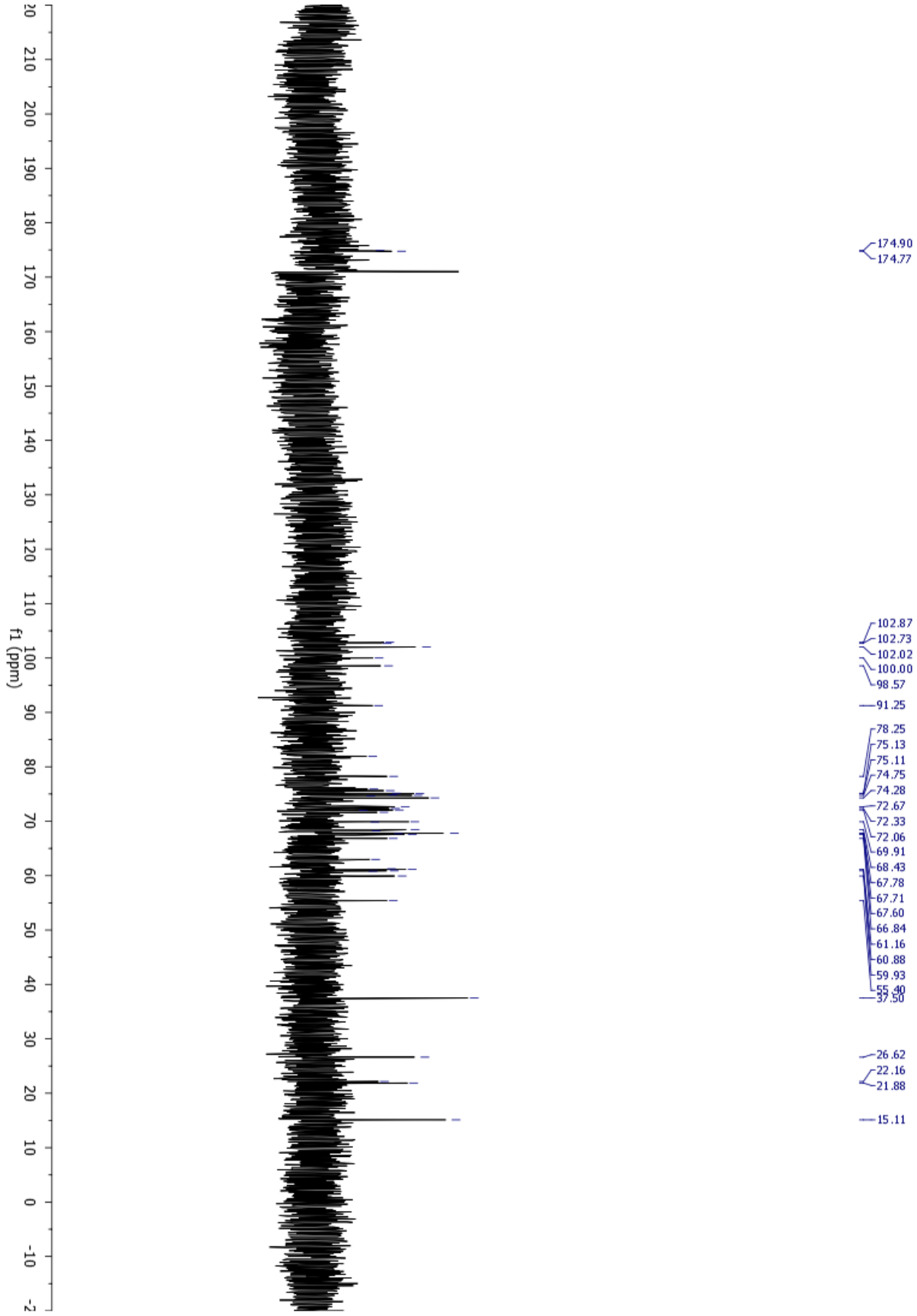
37.57
36.92
36.61
28.19
22.57
22.04

NMR spectrum of compound **7** in D_2O at 300K



NMR spectrum of compound **8** in D_2O at 300K




f1 (ppm)
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200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-2
174.90
174.77
102.87
102.73
102.02
100.00
98.57
91.25
78.25
75.13
75.11
74.75
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72.06
69.91
68.43
67.78
67.71
67.60
66.84
61.16
60.88
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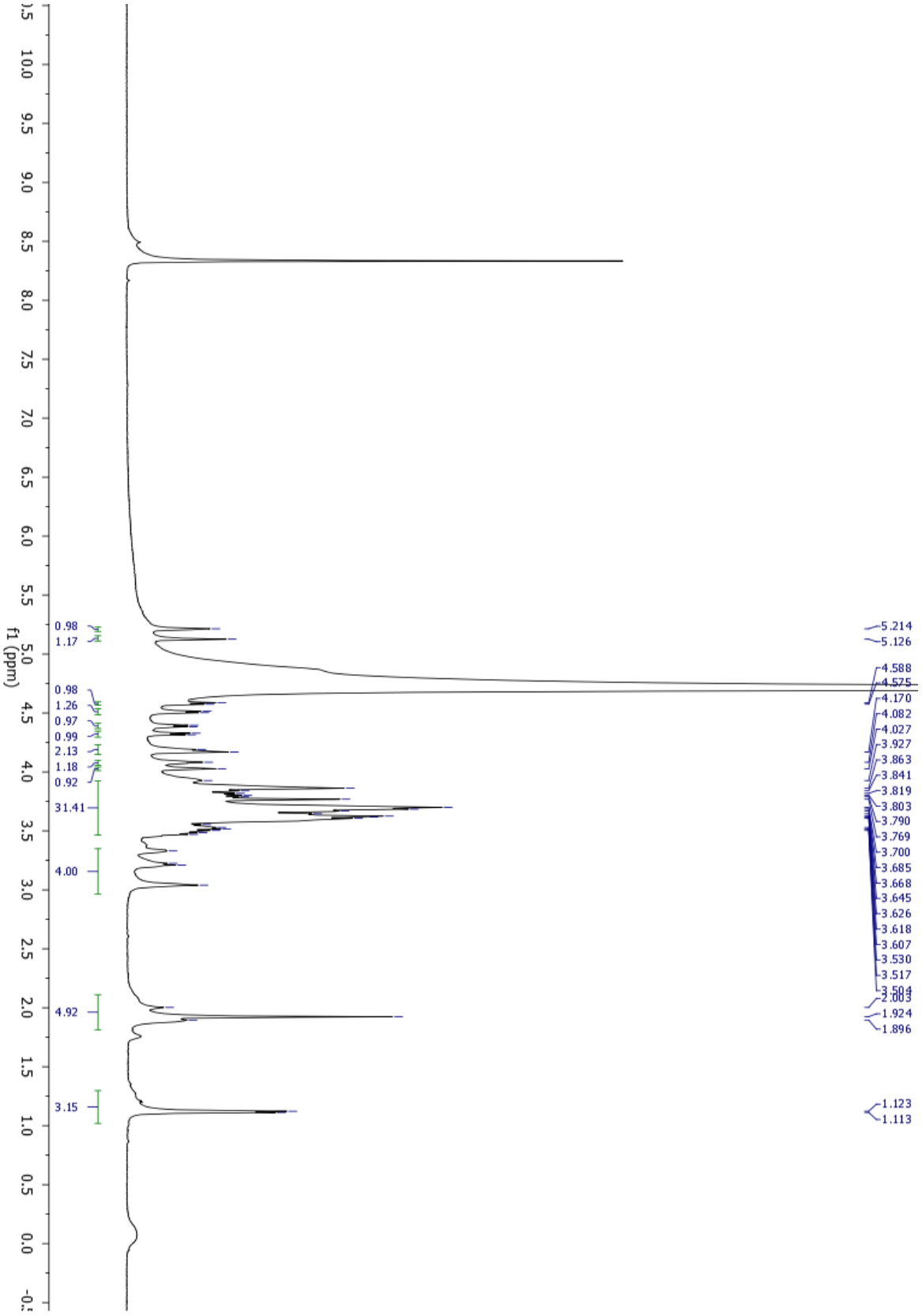
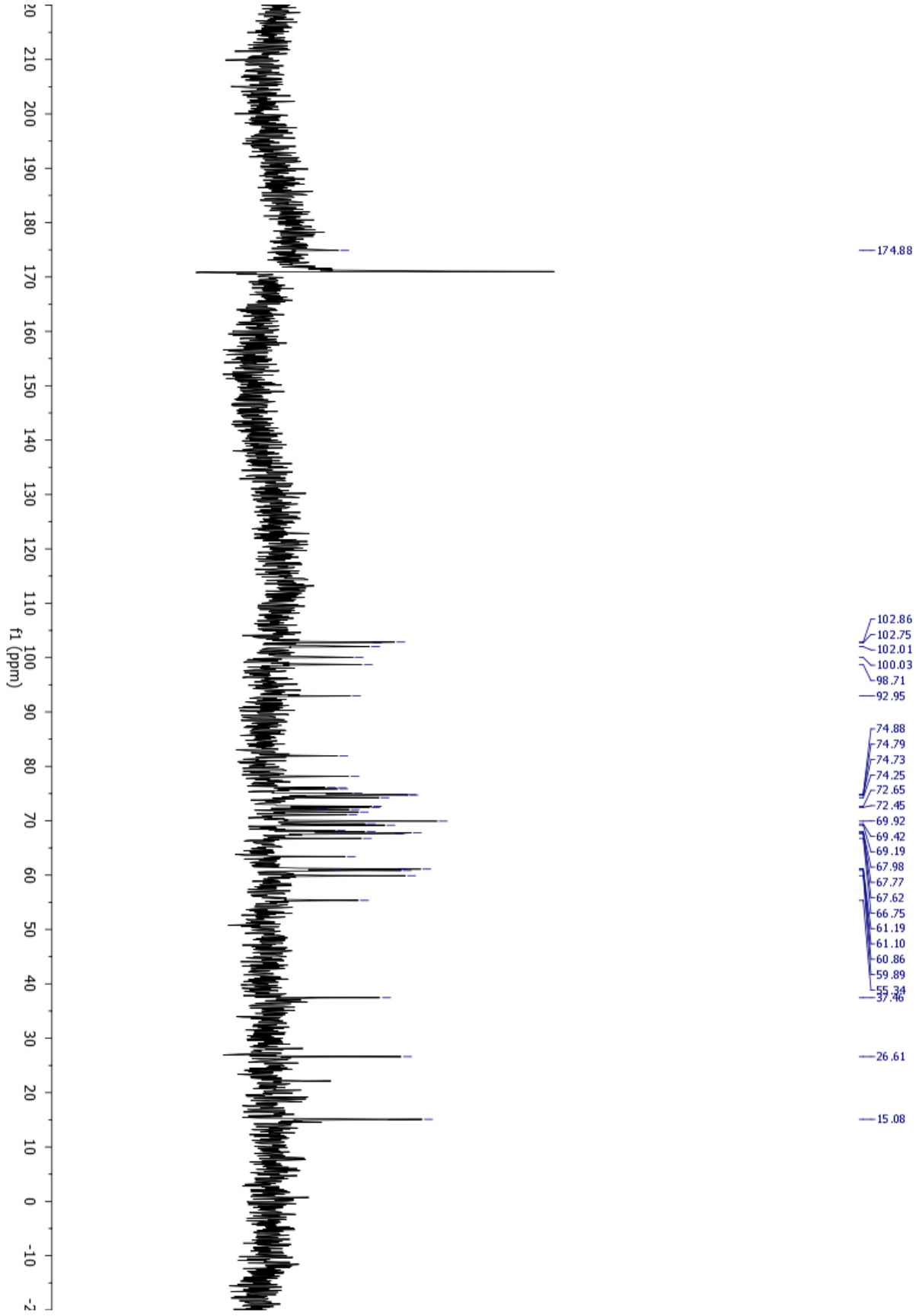
55.40
37.50
26.62
22.16
21.88
15.11

NMR spectrum of compound **9** in D_2O at 300K




f1 (ppm)
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200
190
180
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160
150
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130
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100
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70
60
50
40
30
20
10
0
-10
-2
174.88
102.86
102.75
102.01
100.03
98.71
92.95
74.88
74.79
74.73
74.25
72.65
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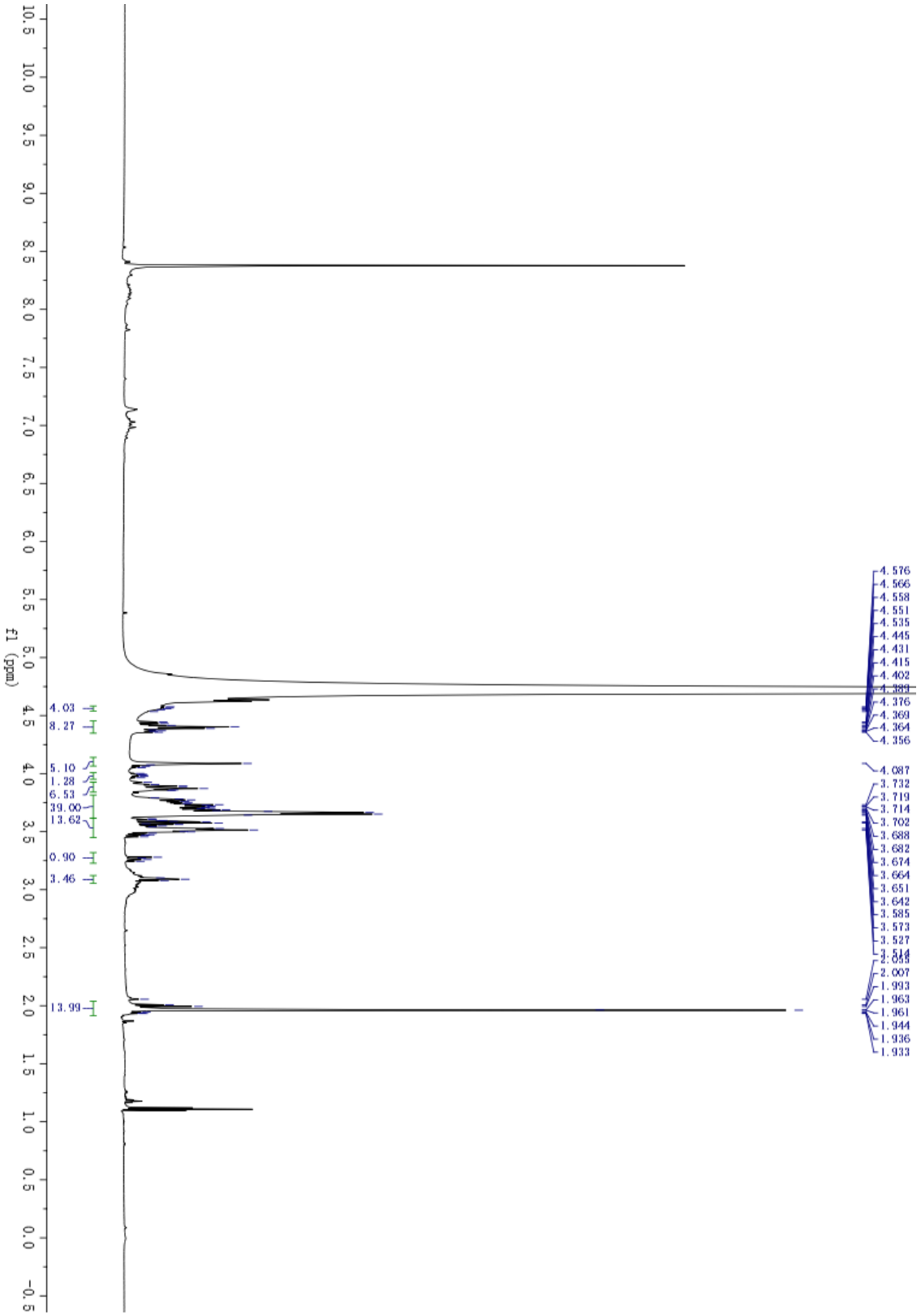
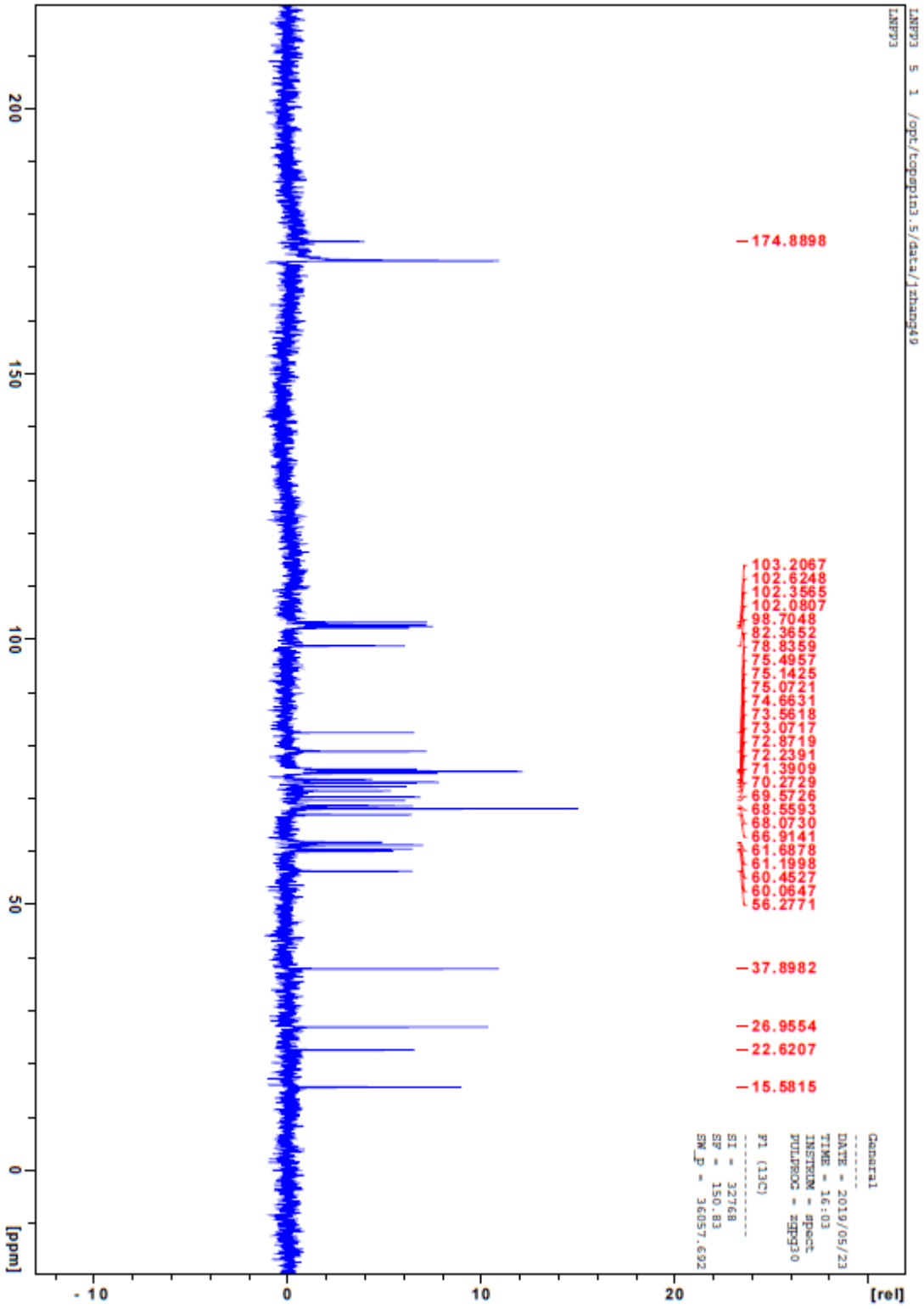
69.42
69.19
67.98
67.77
67.62
66.75
61.19
61.10
60.86
59.89
55.34
37.46
26.61
15.08

NMR spectrum of compound **10** in D_2O at 300K

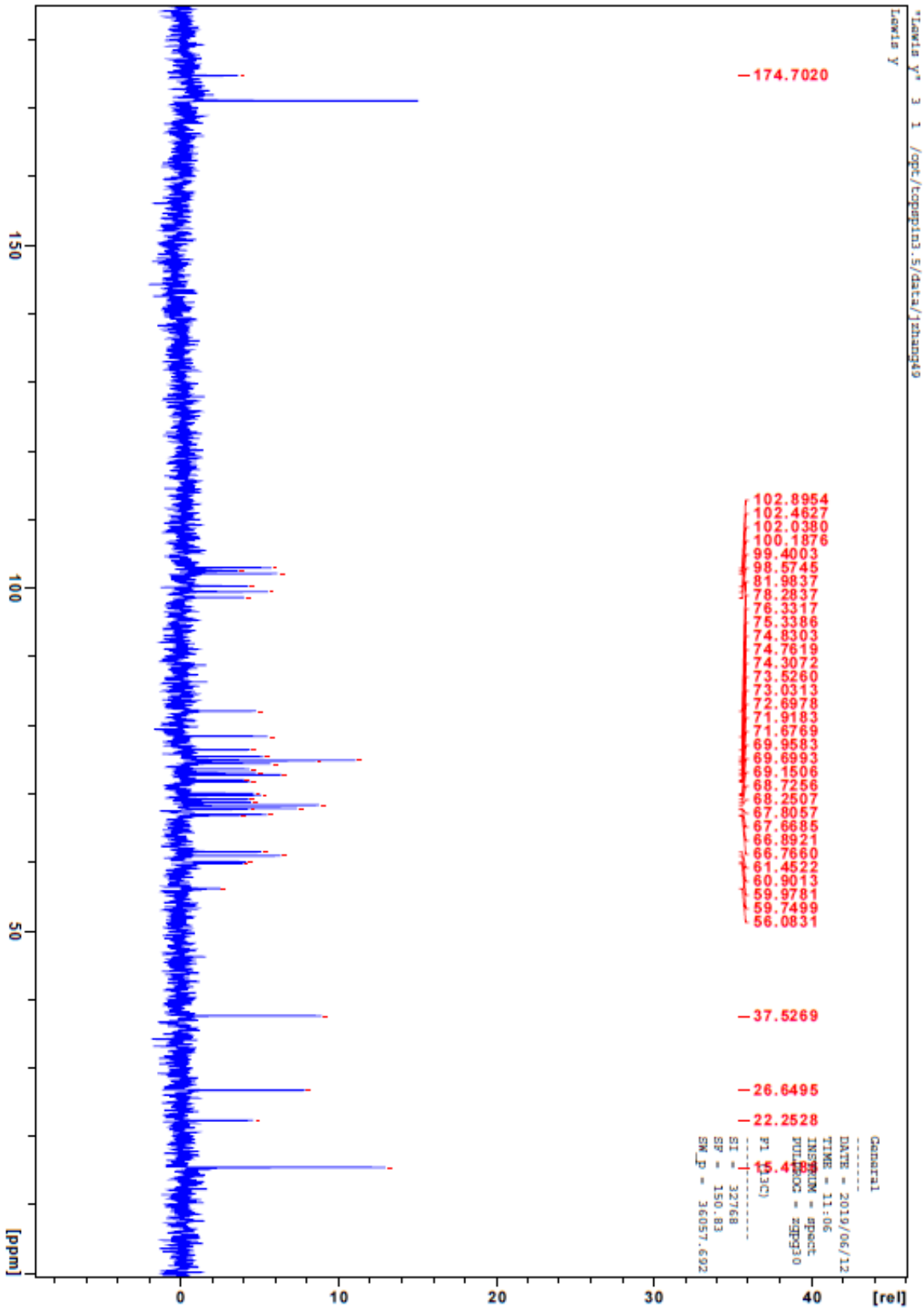


Appendix B NMR SPECTRUMS OF COMPOUNDS IN PART THREE

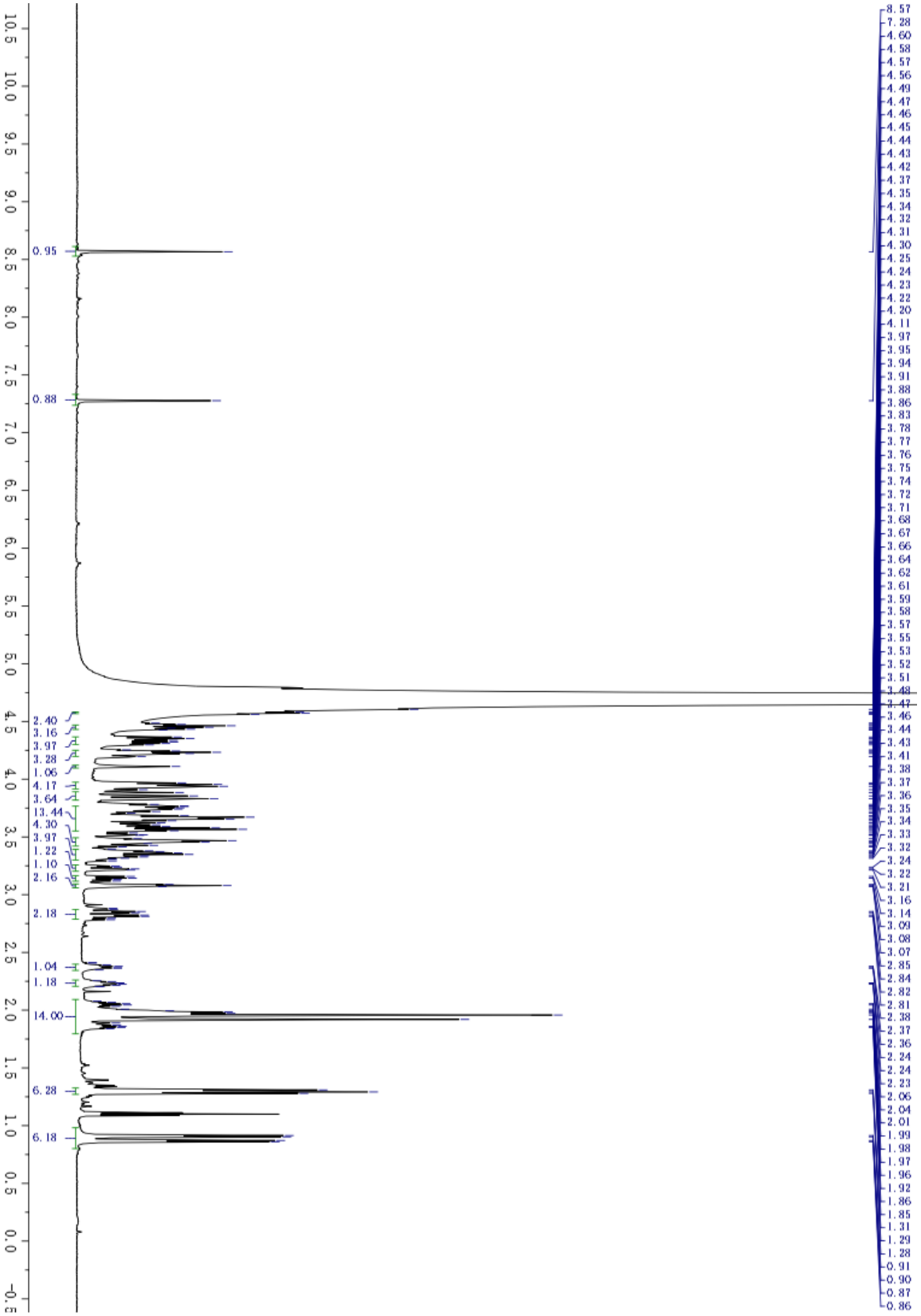
NMR spectrum of lewis X antigen in D_2O at 300K



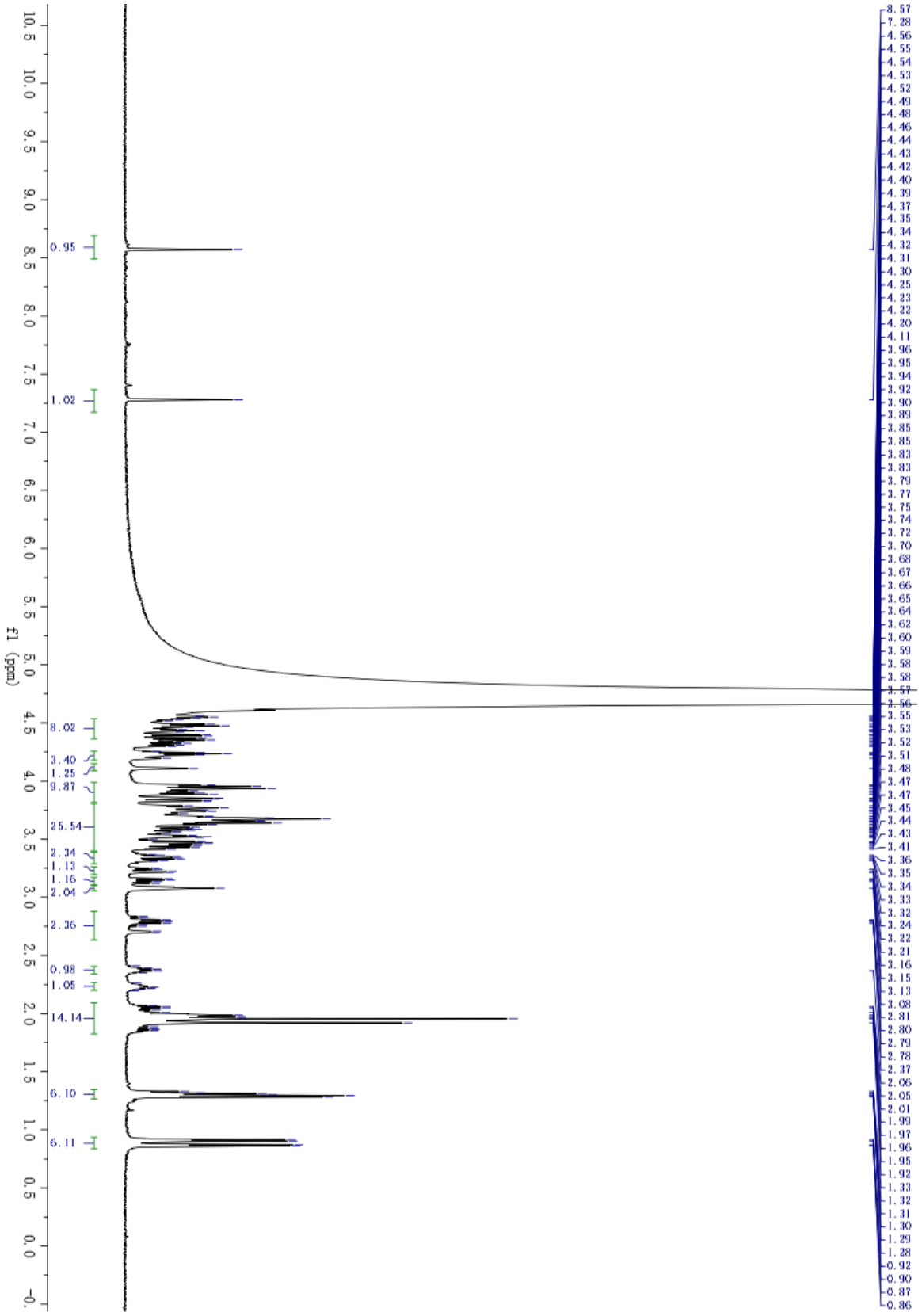
NMR spectrum of lewis X antigen in D_2O at 300K



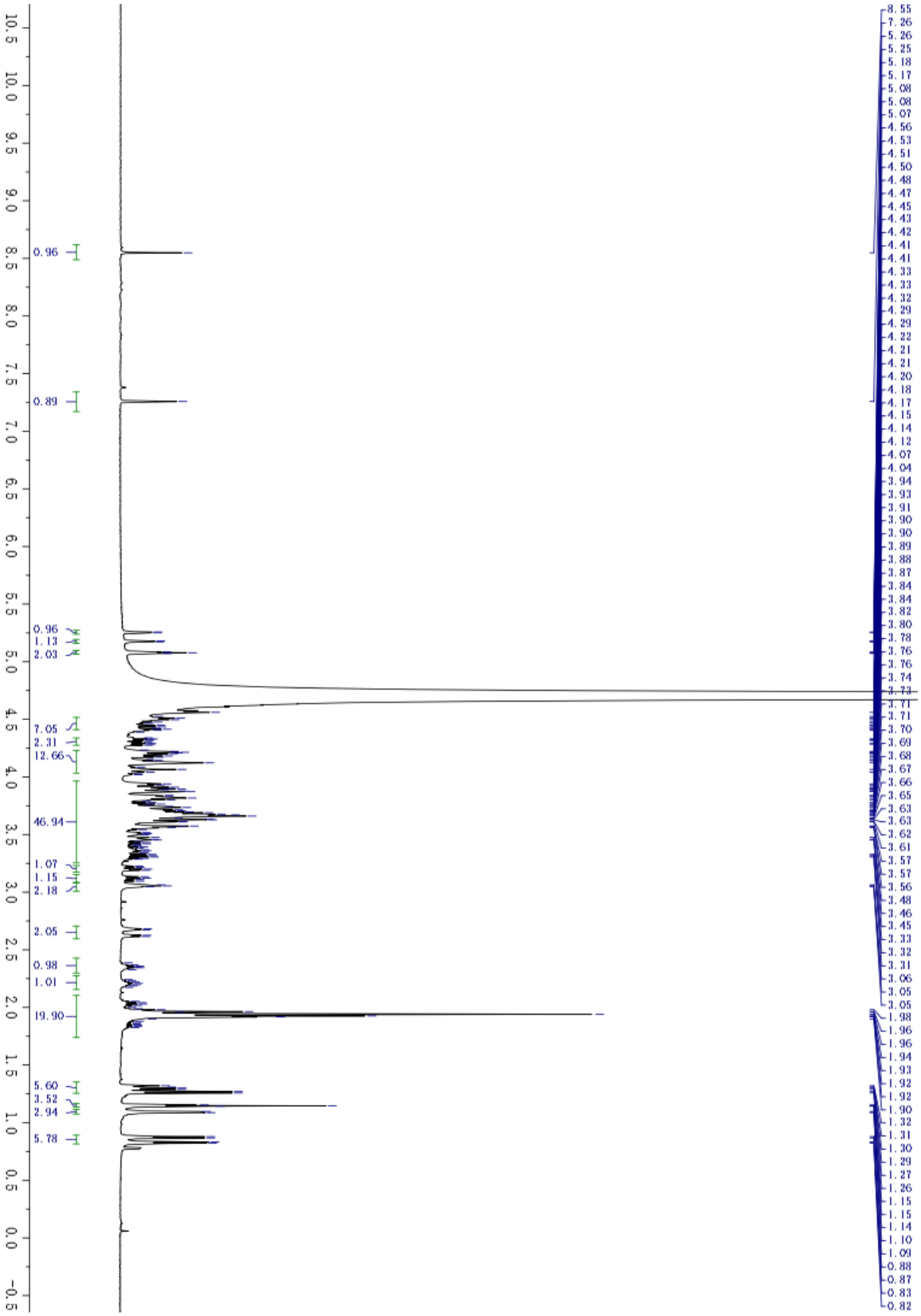
600 MHz 1H-NMR spectrum of **Compound 10** in D_2O at 300K



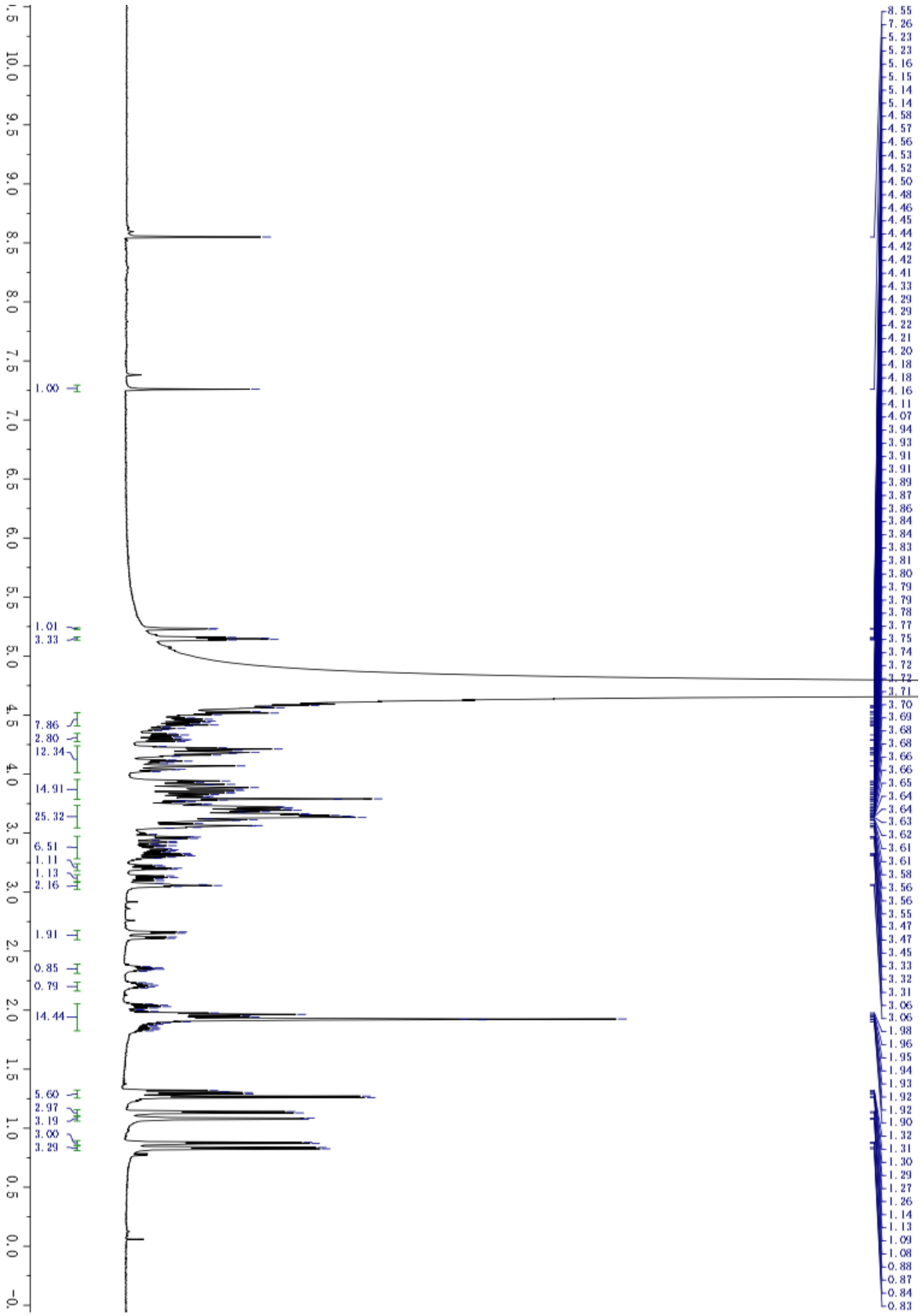
600 MHz 1H-NMR spectrum of **Compound 13** in D_2O at 300K



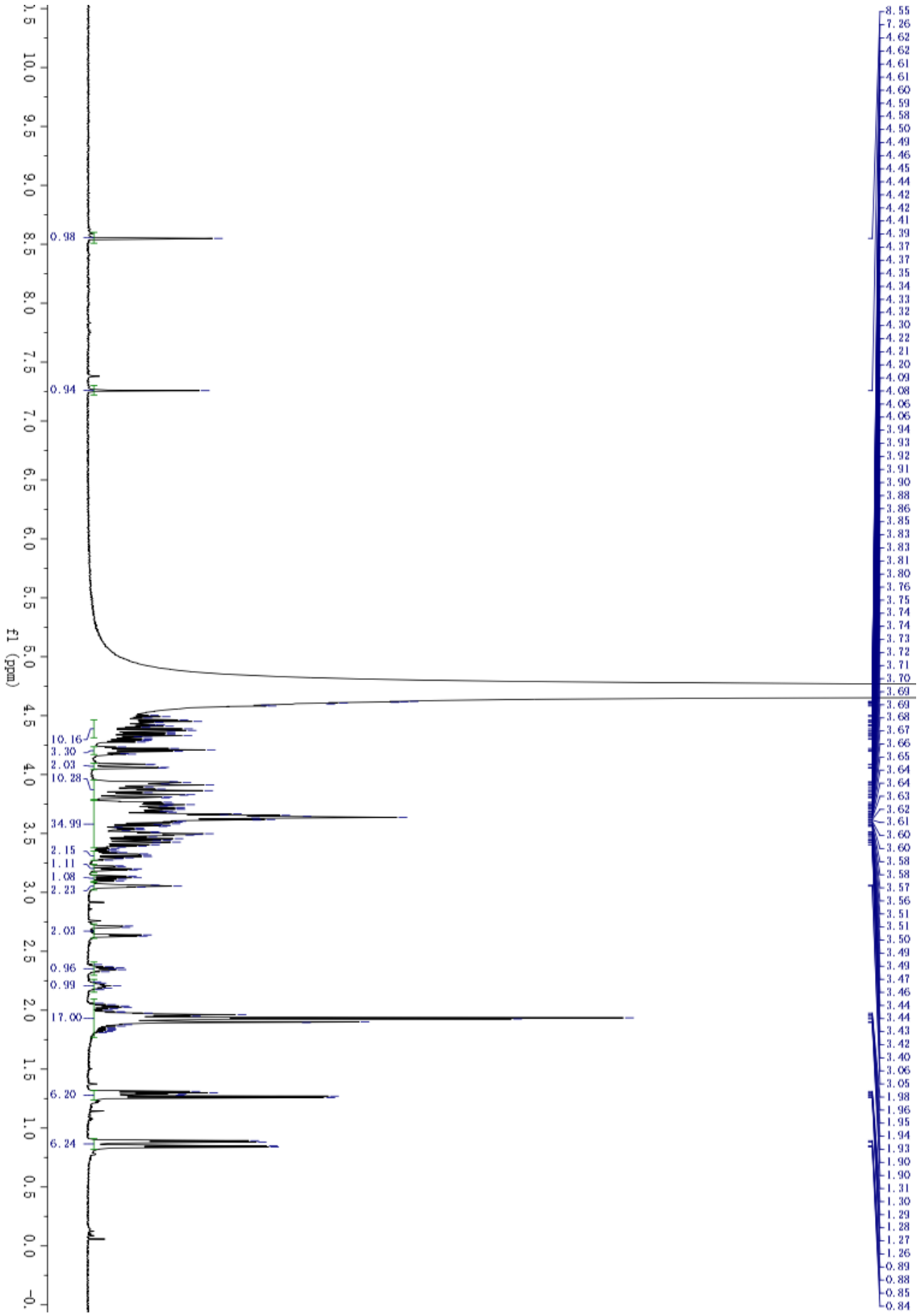
600 MHz 1H-NMR spectrum of **Compound 12** in D_2O at 300K



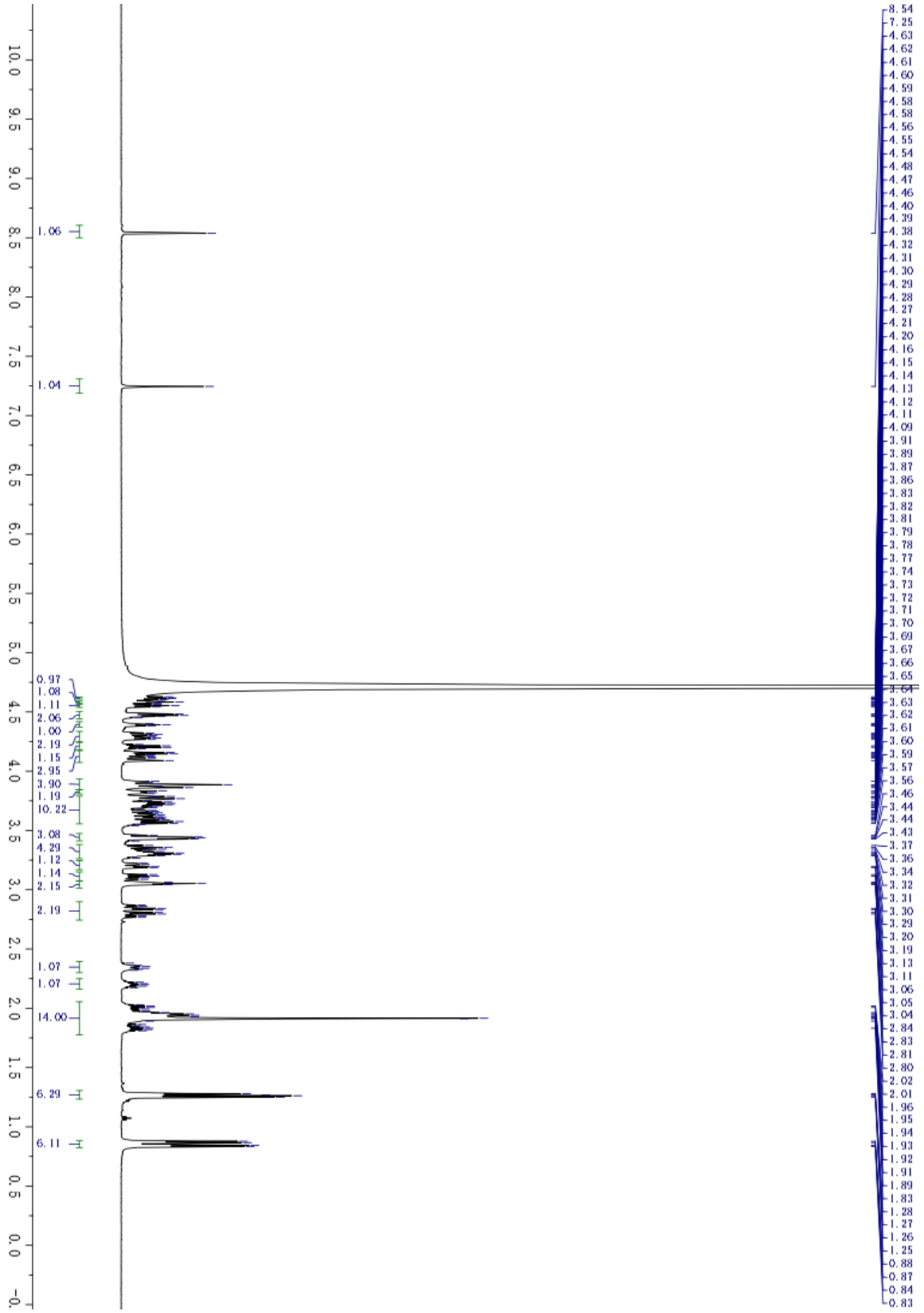
600 MHz 1H-NMR spectrum of **Compound 14** in D_2O at 300K



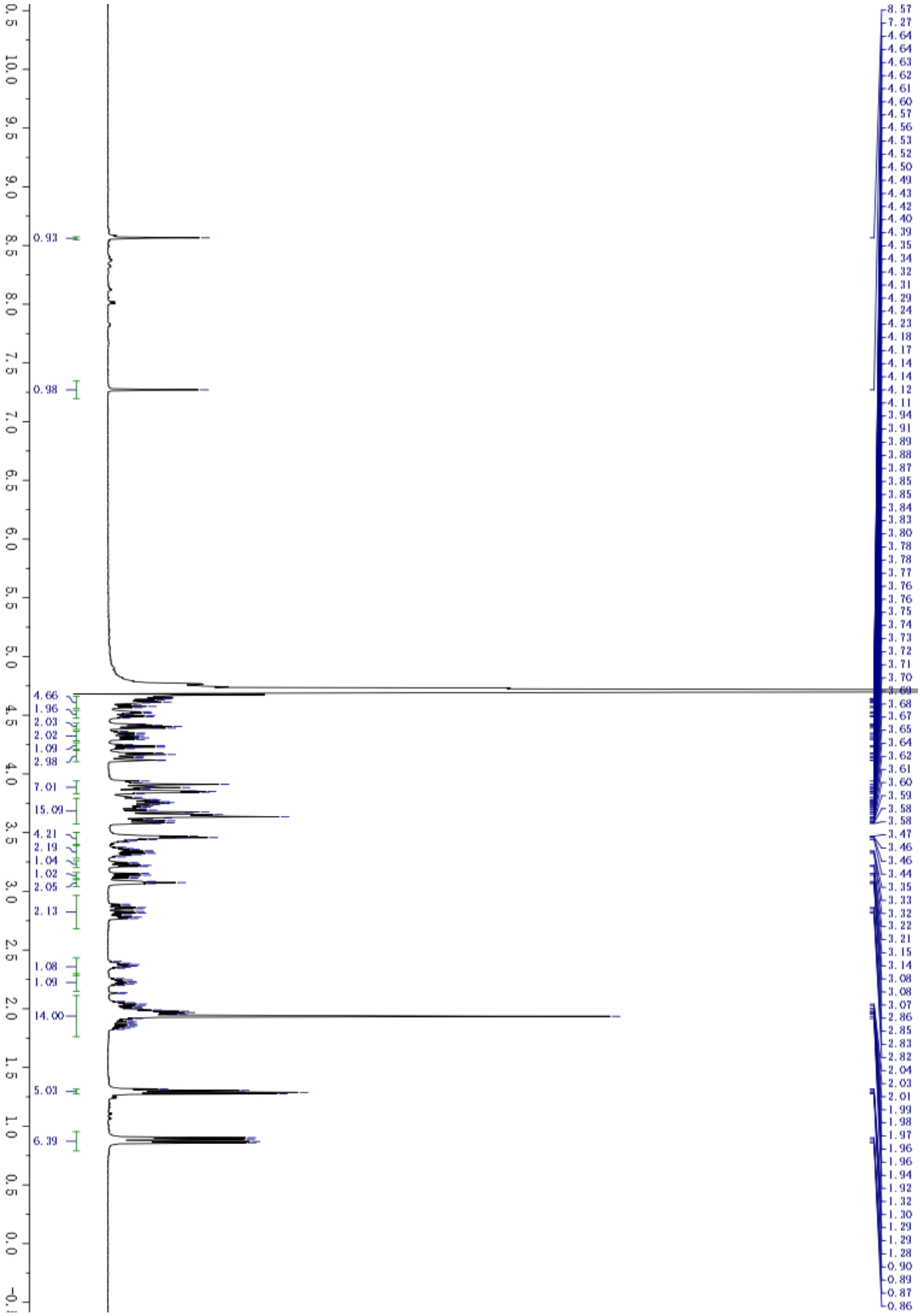
600 MHz 1H-NMR spectrum of **Compound 15** in D_2O at 300K



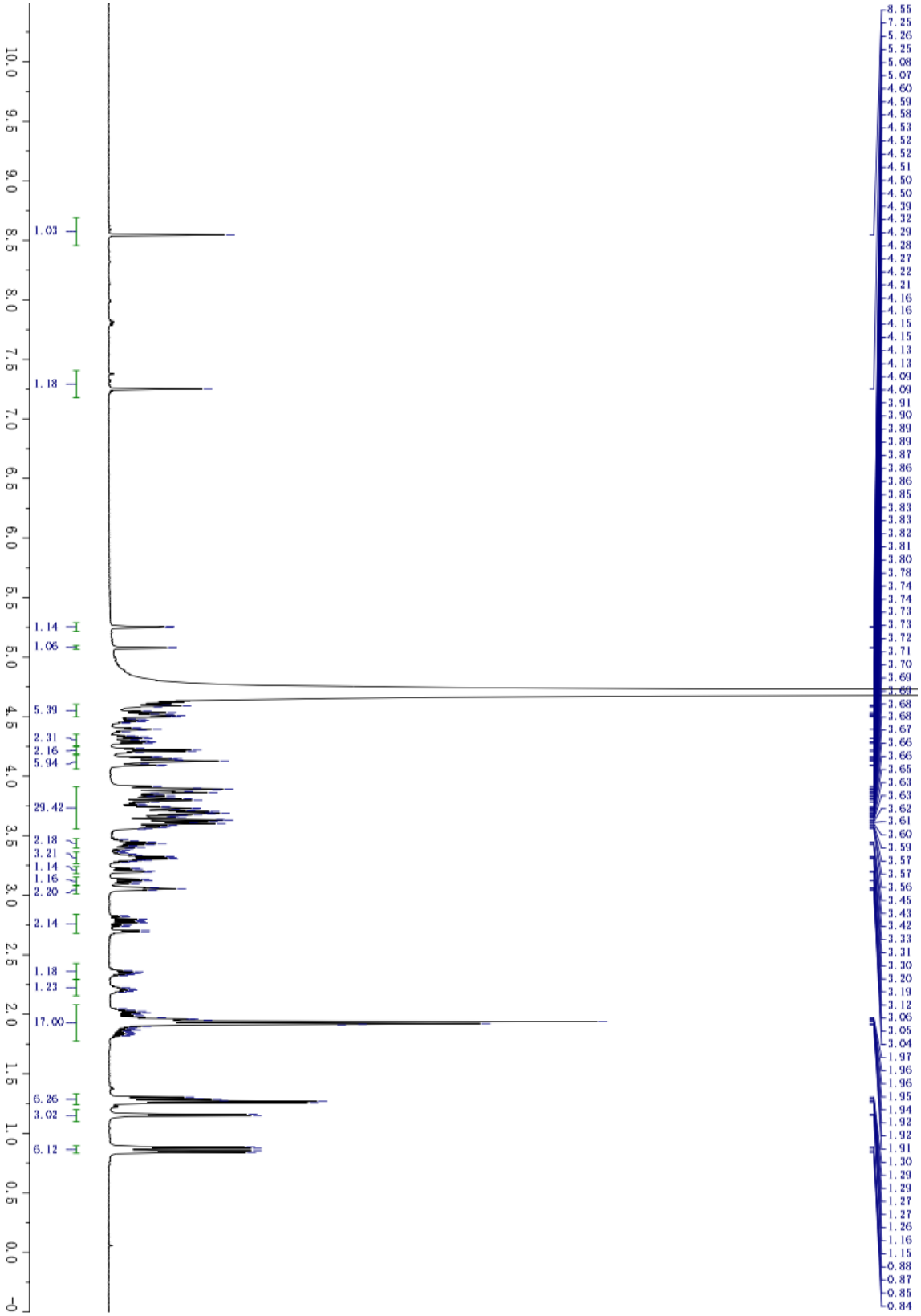
600 MHz 1H-NMR spectrum of **Compound 16** in D_2O at 300K



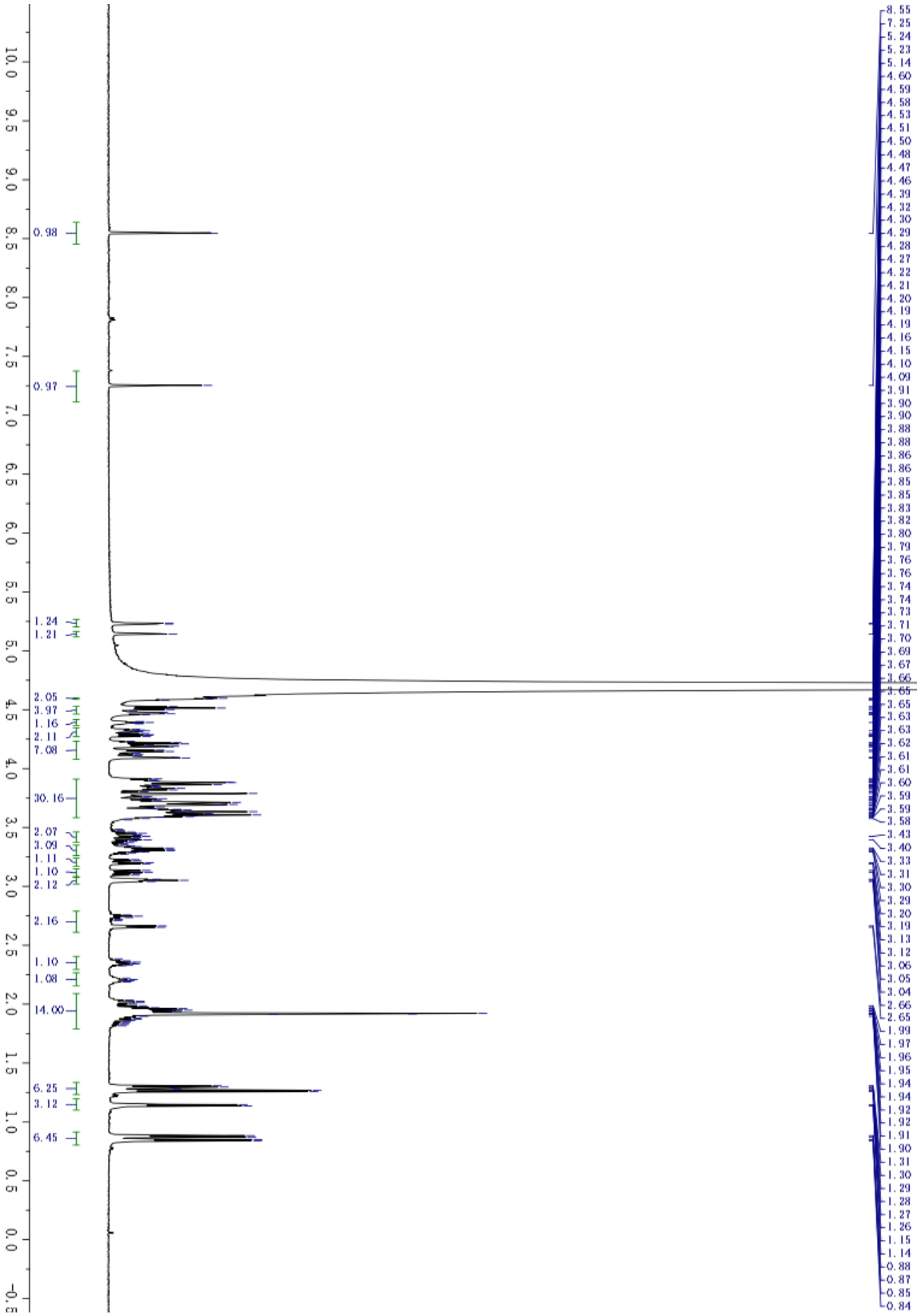
600 MHz 1H-NMR spectrum of **Compound 17** in D_2O at 300K



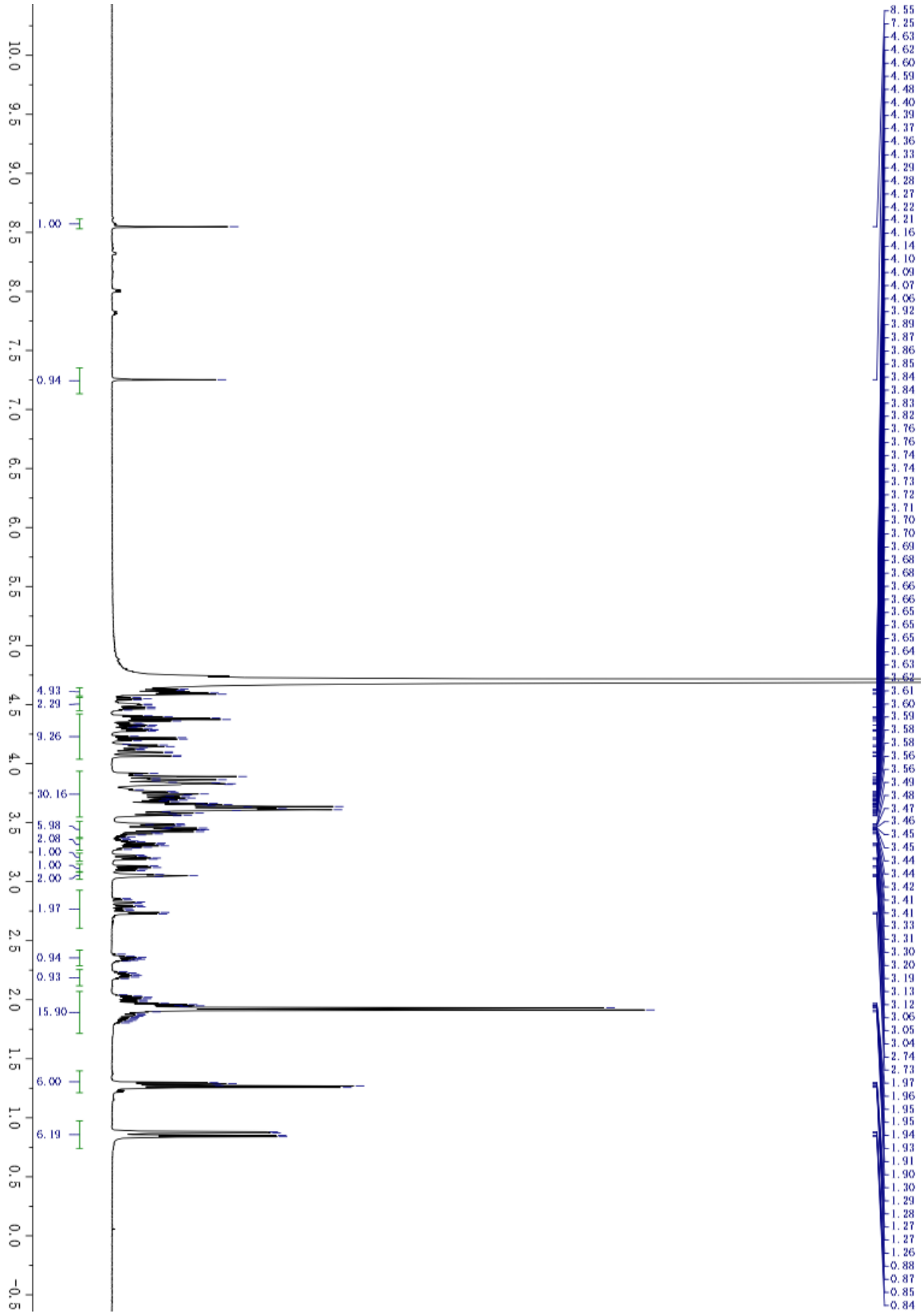
600 MHz 1H-NMR spectrum of **Compound 18** in D_2O at 300K



600 MHz 1H-NMR spectrum of **Compound 19** in D_2O at 300K

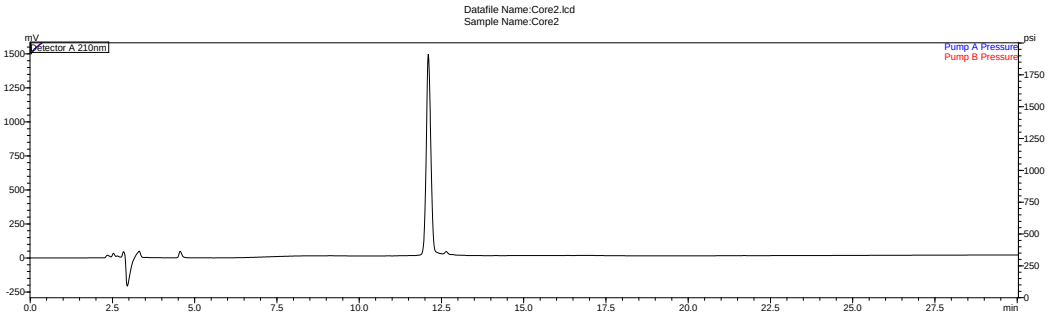


600 MHz 1H-NMR spectrum of **Compound 20** in D_2O at 300K

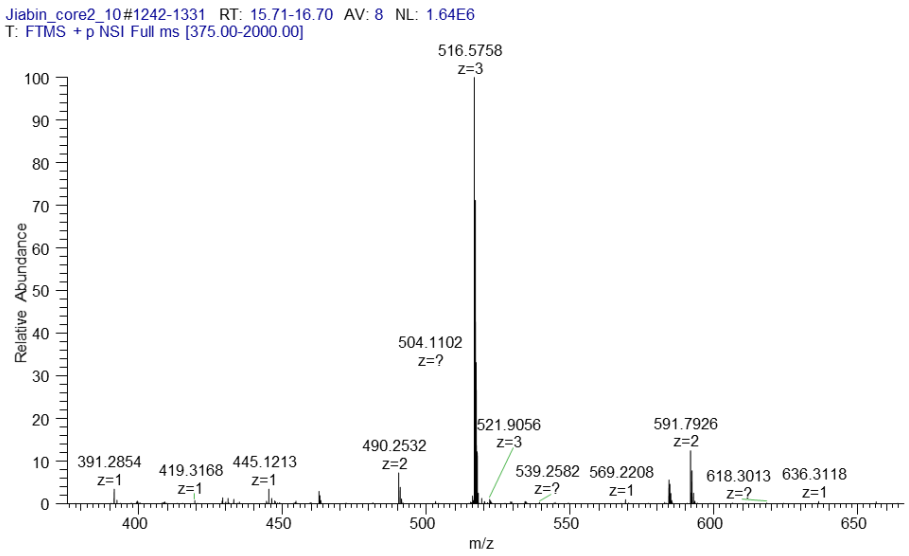


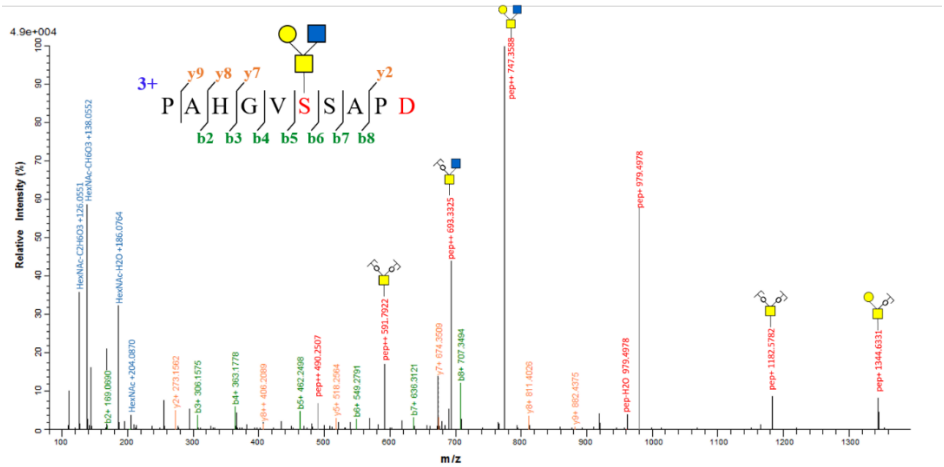
Appendix C MASS SPECTRUMS AND CHROMATOGRAM OF COMPOUNDS IN PART THREE

HPLC and Mass spectrum of **Compound 10**

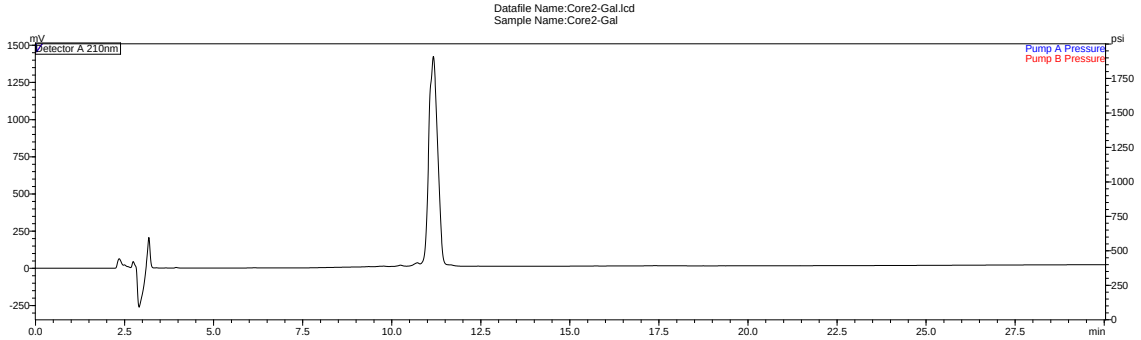


[M+3H]

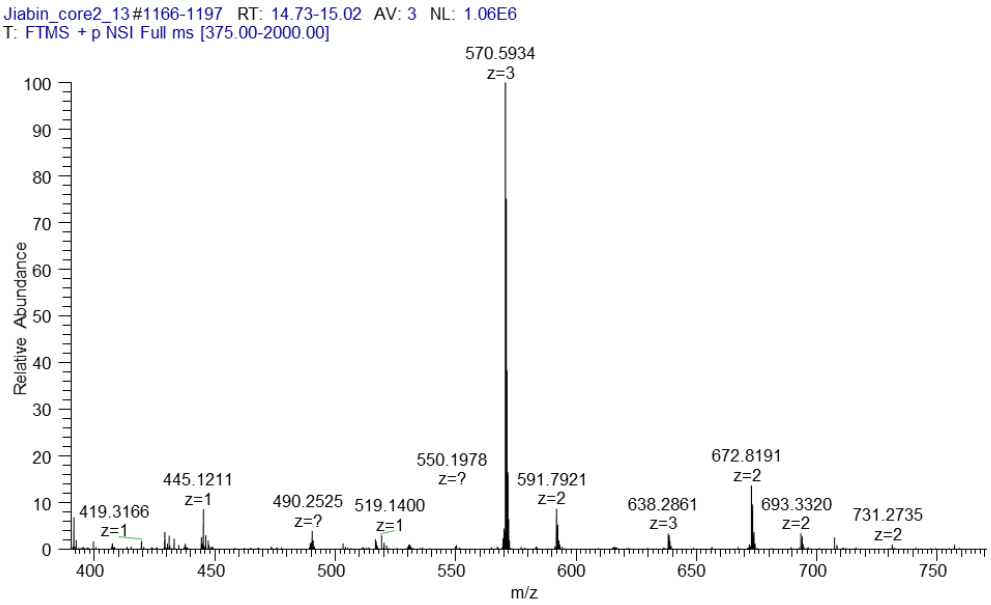


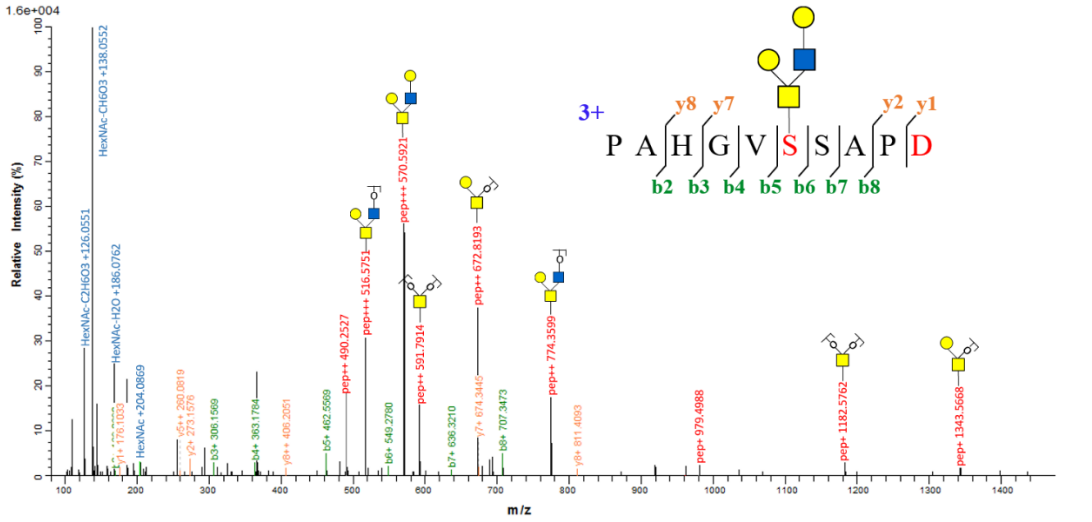


HPLC and Mass spectrum of **Compound 13**

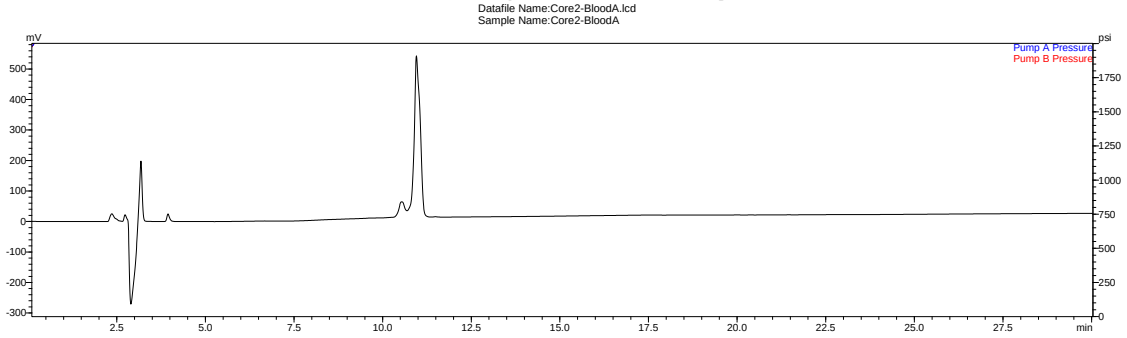


[M+3H]

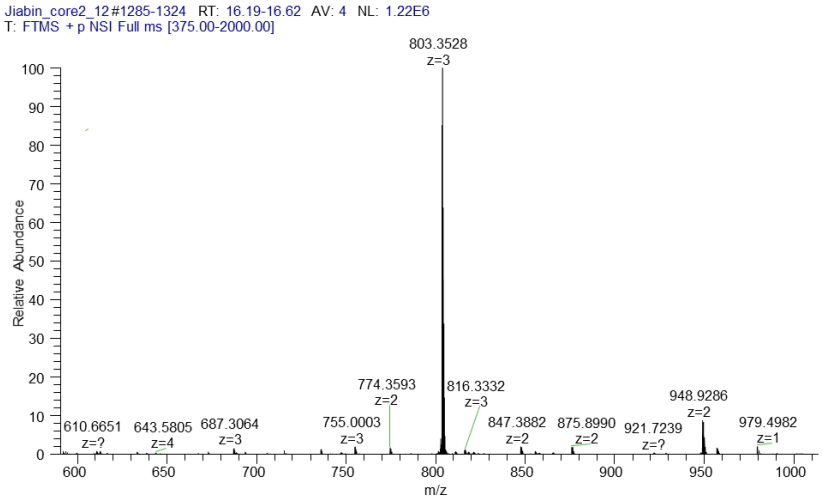


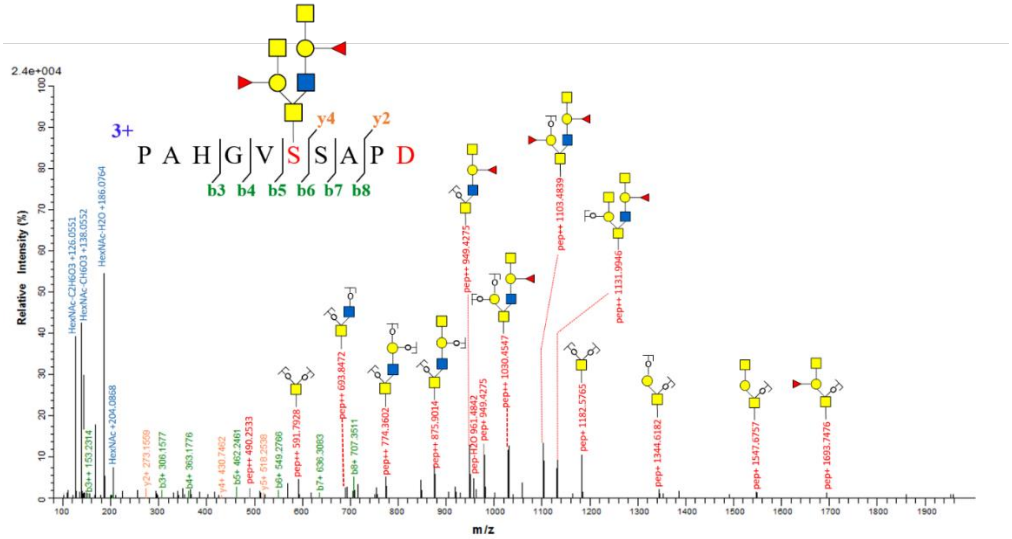


HPLC and Mass spectrum of **Compound 12**

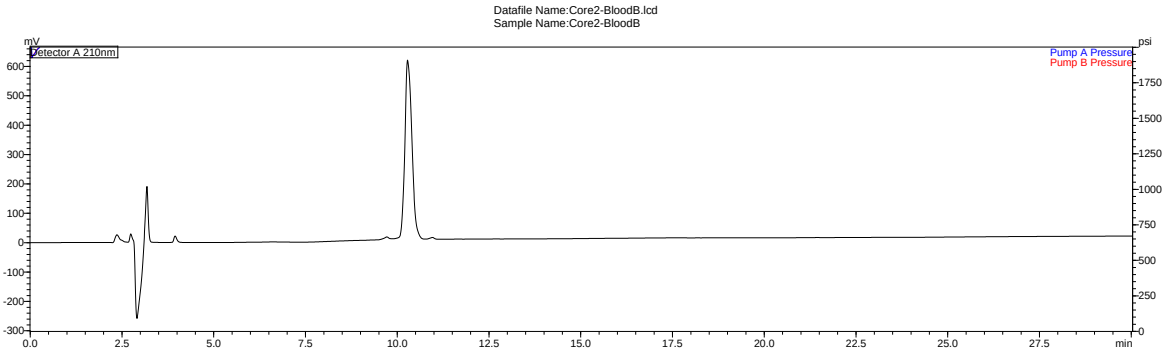


[M+3H]

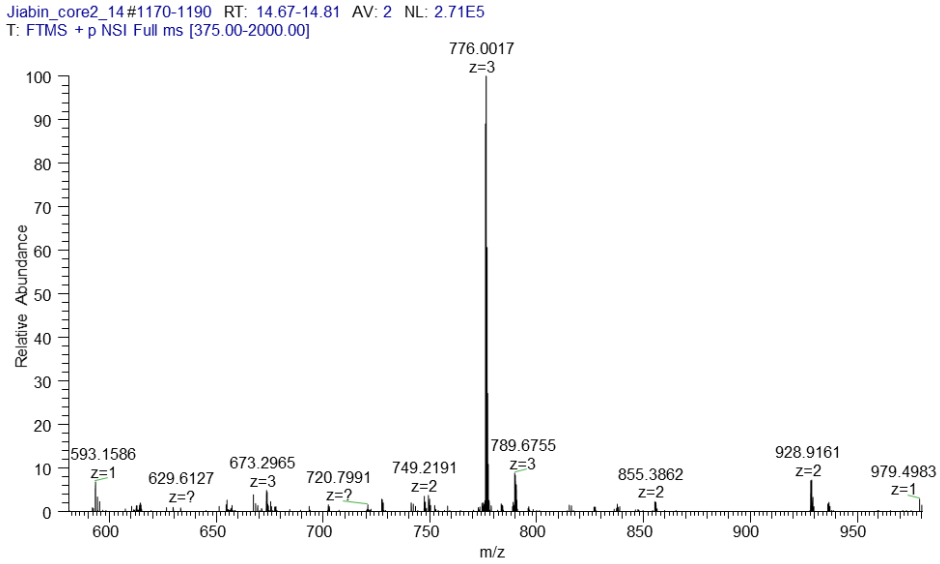


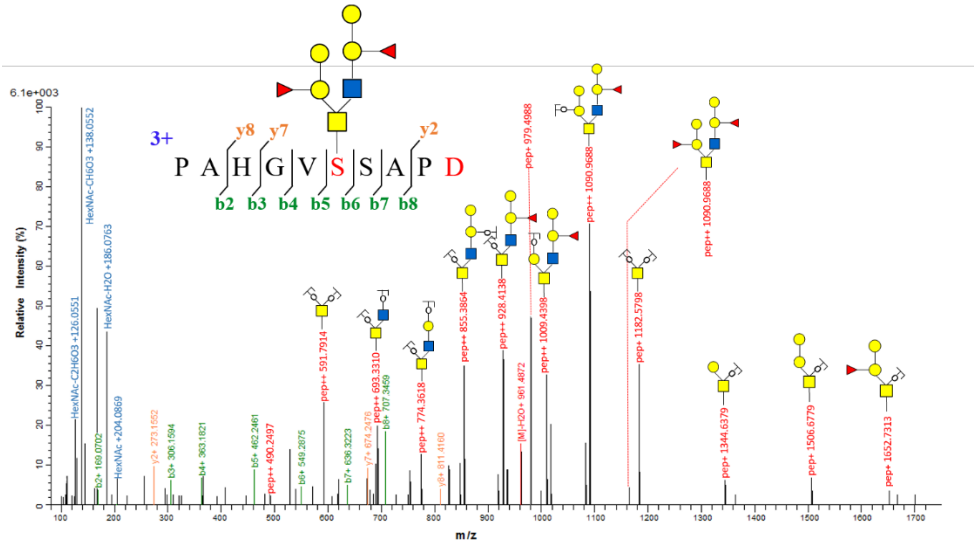


HPLC and Mass spectrum of **Compound 14**

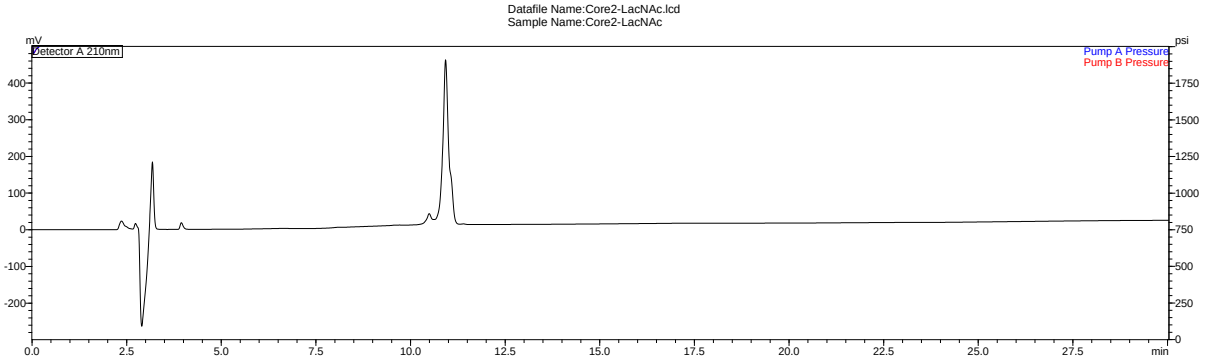


[M+3H]

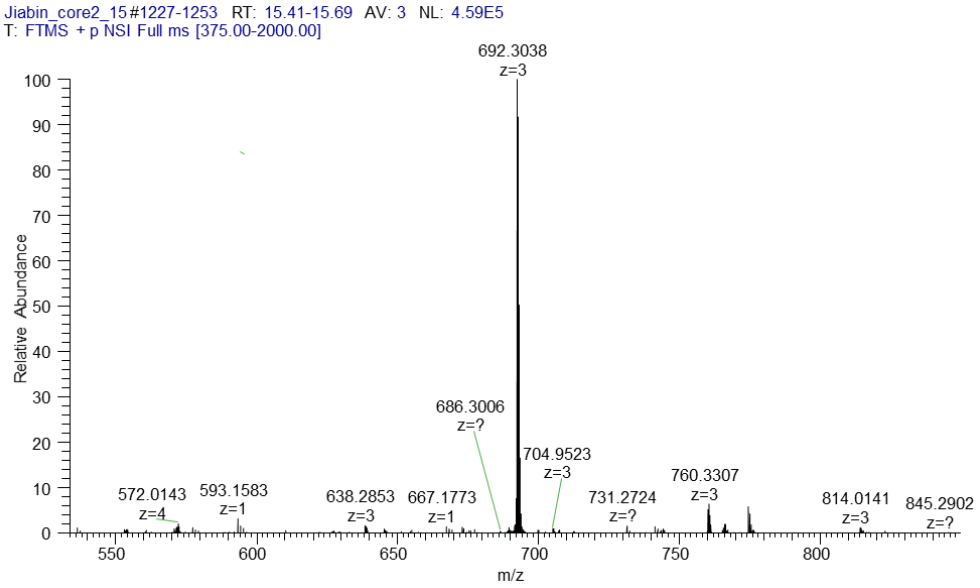


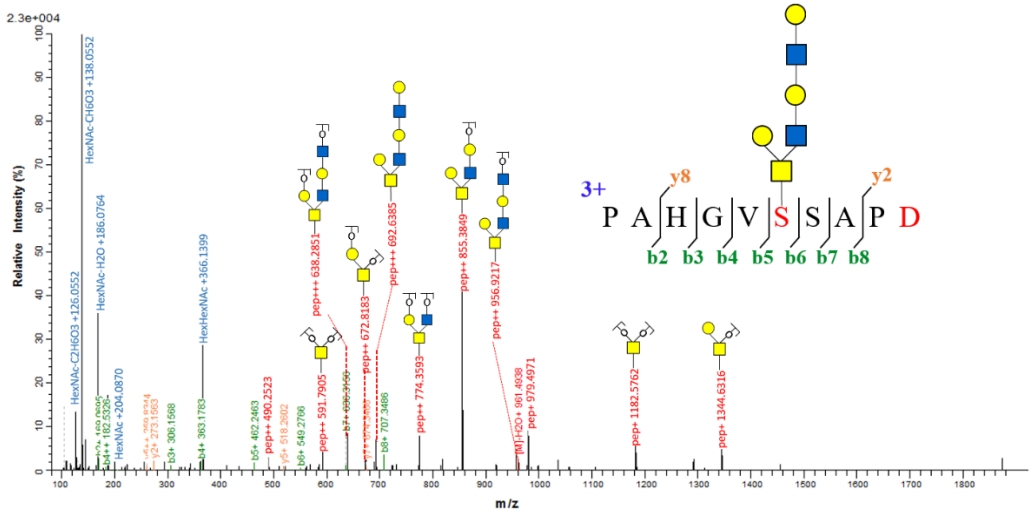


HPLC and Mass spectrum of **Compound 15**

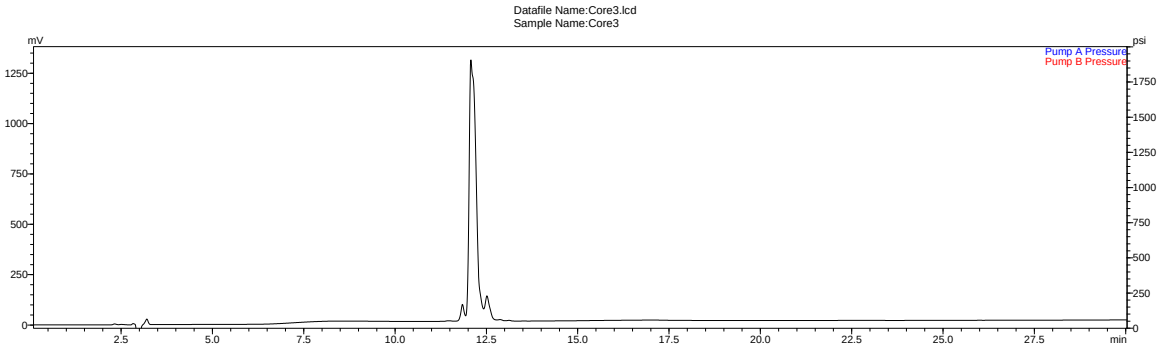


[M+3H]

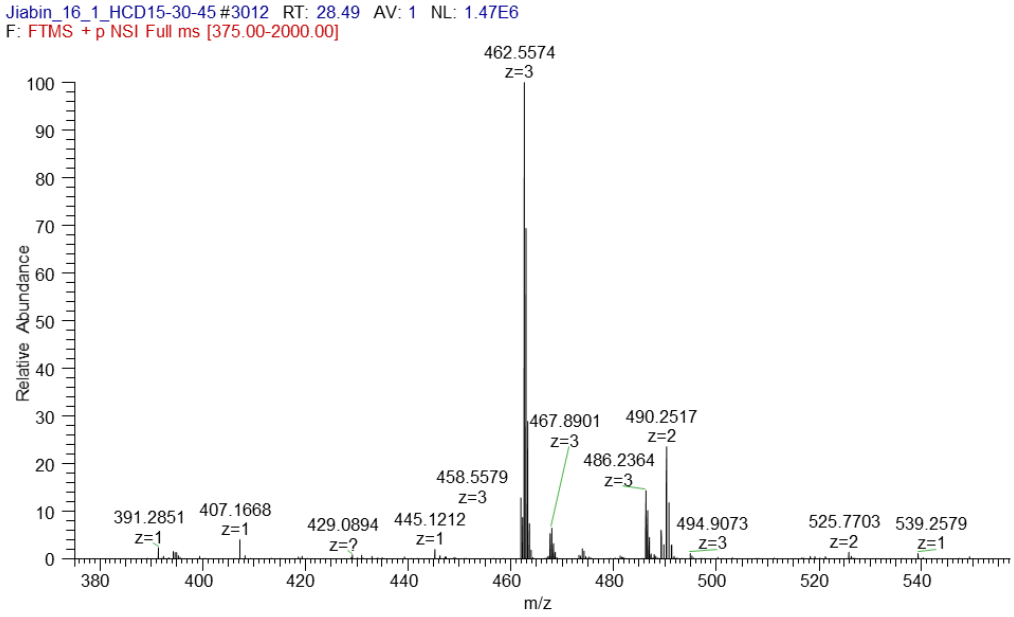


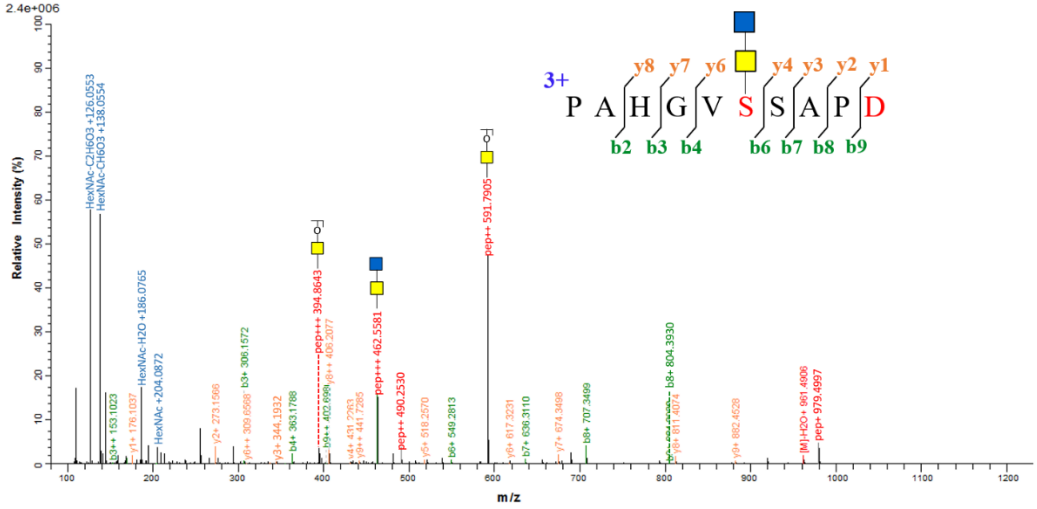


HPLC and Mass spectrum of **Compound 16**

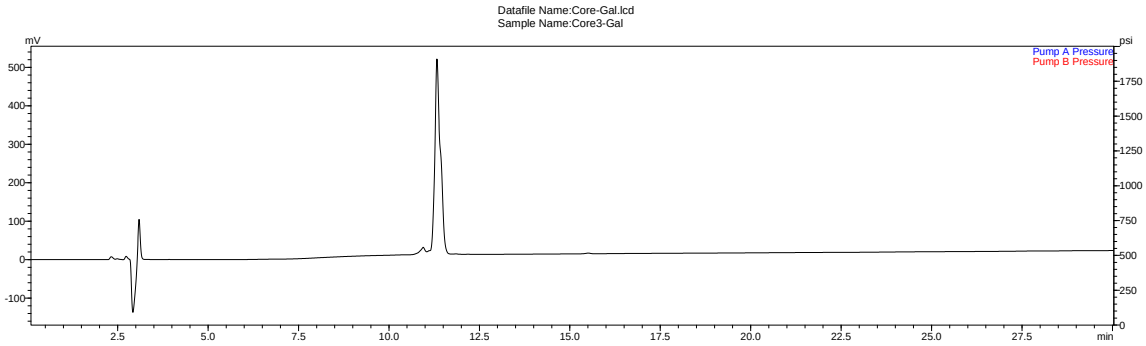


[M+3H]

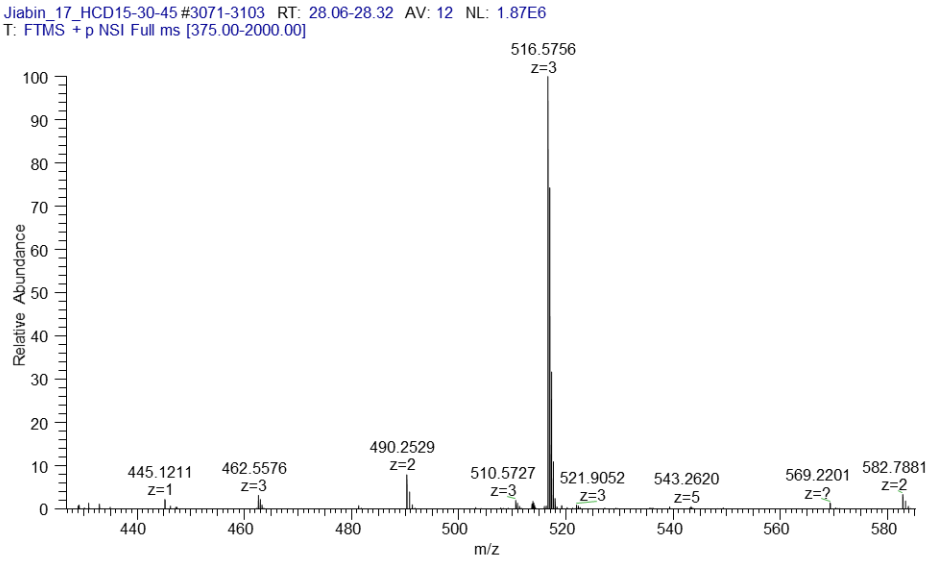


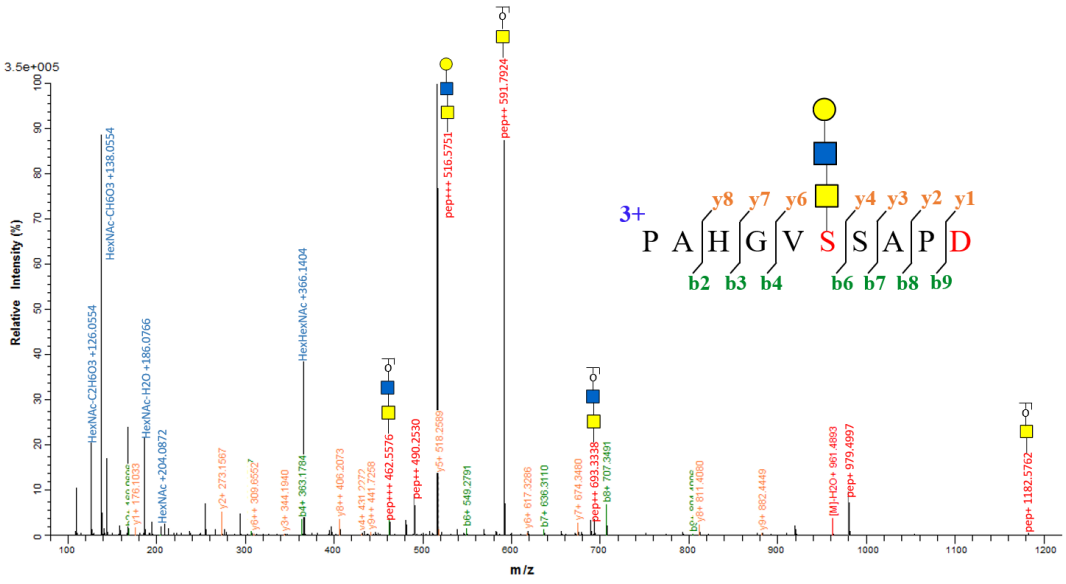


HPLC and Mass spectrum of **Compound 17**

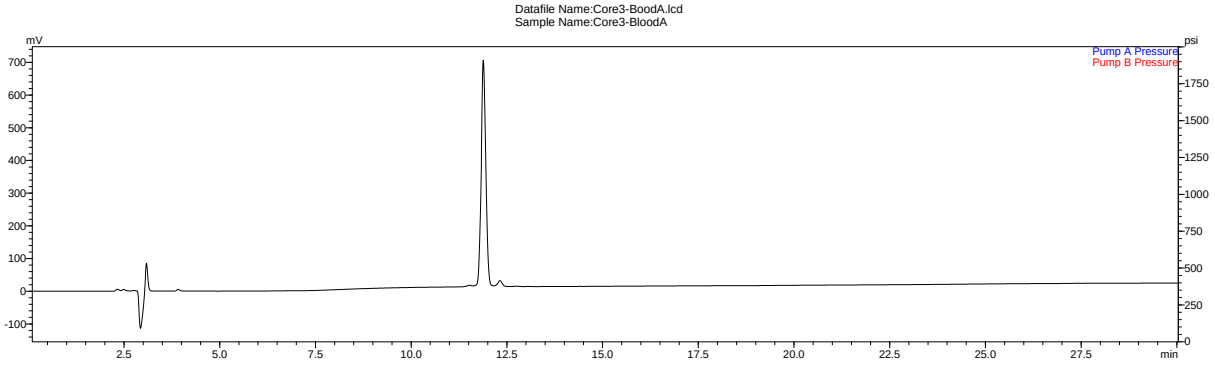


[M+3H]

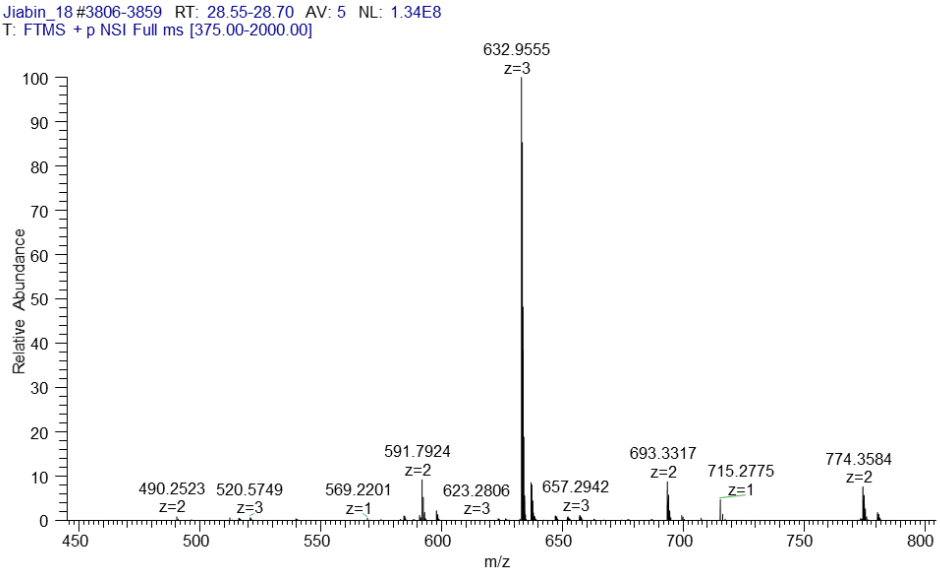


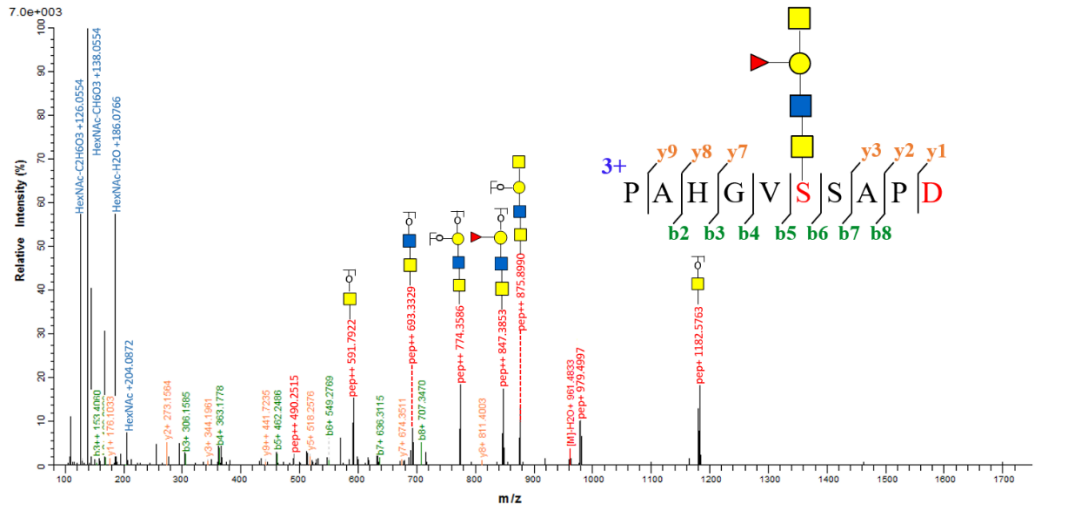


HPLC and Mass spectrum of **Compound 18**

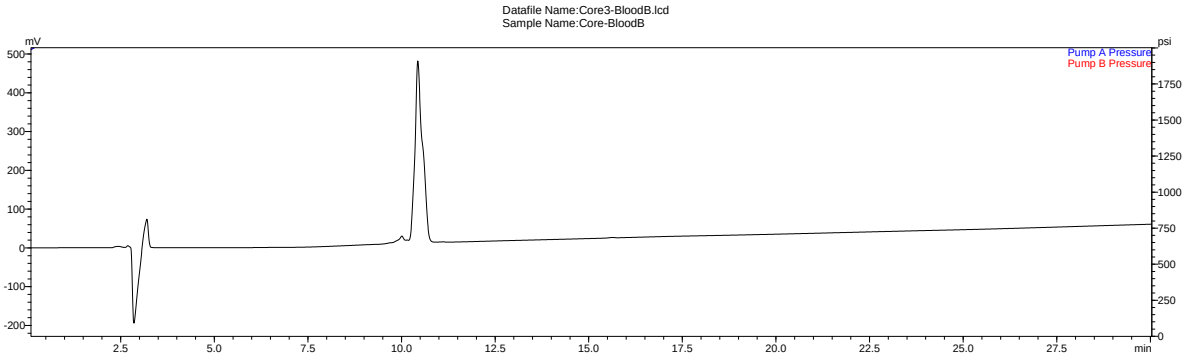


[M+3H]

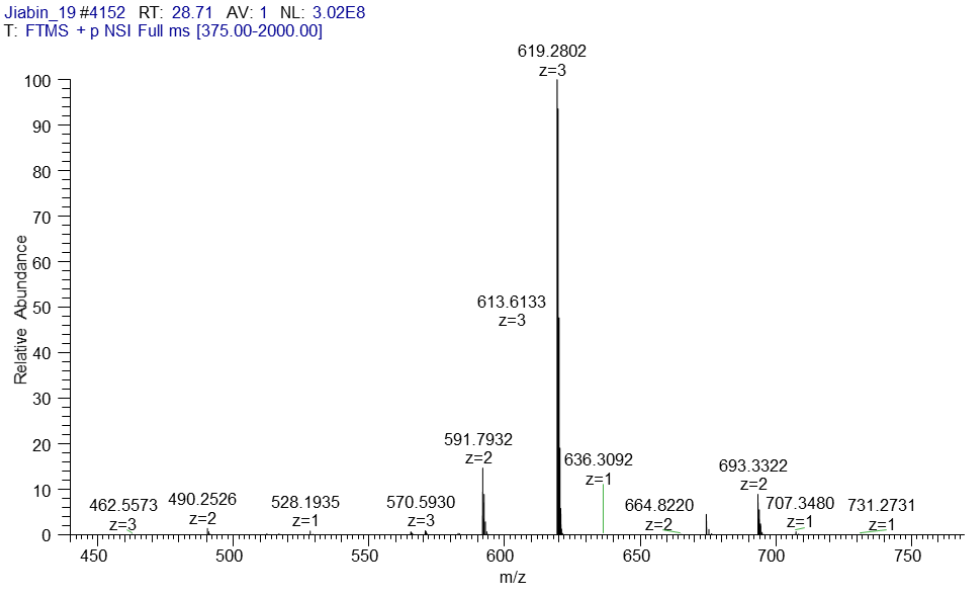


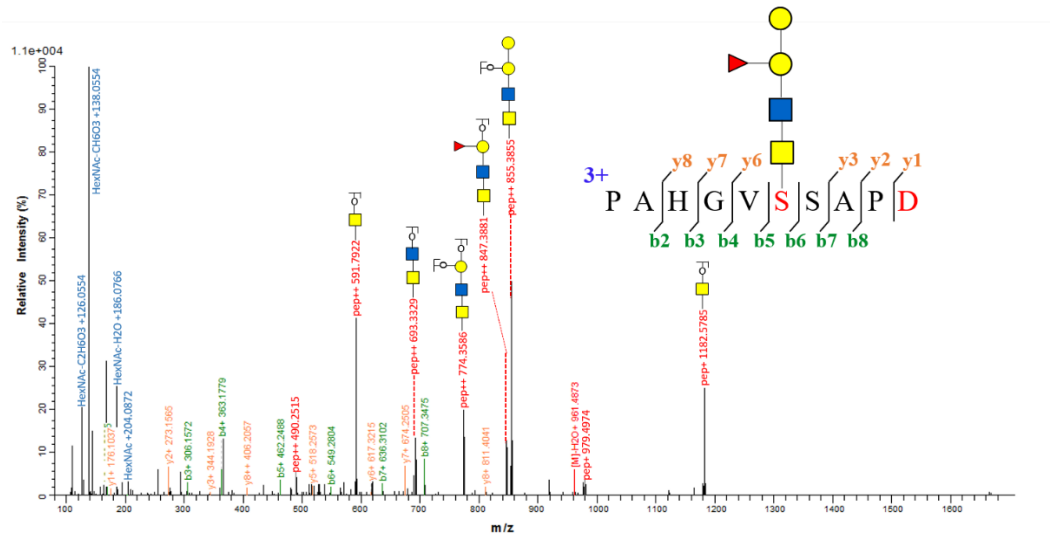


HPLC and Mass spectrum of **Compound 19**

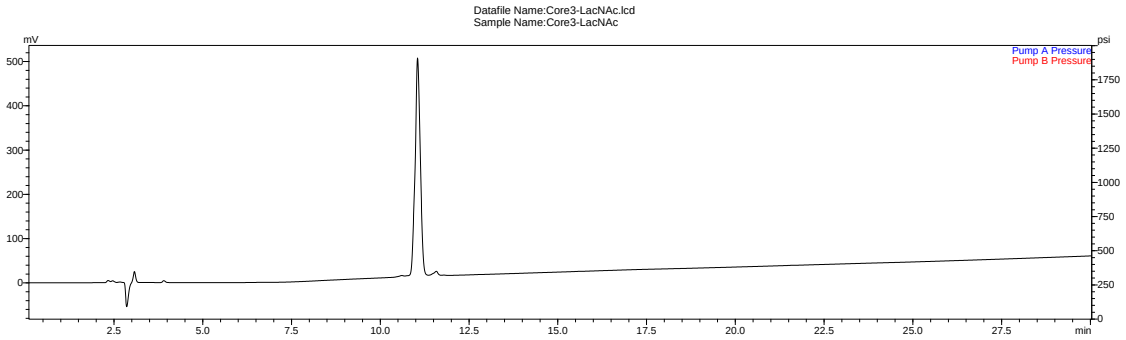


[M+3H]





HPLC and Mass spectrum of **Compound 20**



[M+3H]