Supporting Information

for

Efficient chemoenzymatic synthesis of sialyl Tn-antigens and derivatives†

Li Ding,[ab] Hai Yu,[b] Kam Lau,[b] Yanhong Li,[b] Saddam Muthana,[bc] Junru Wang[a] and Xi Chen*[b]

[a]*College of Science, Northwest A&F University, Yangling, Shaanxi, China*
[b]*Department of Chemistry, University of California, One Shields Avenue, Davis, California 95616, USA. E-mail: chen@chem.ucdavis.edu.edu; Fax: +1 530-752-8995; Tel: +1 530-754-6037*
[c]*Current address: National Cancer Institute, Frederick, Maryland, 21702, USA.*

*Correspondence to: Xi Chen, Department of Chemistry, University of California, One Shields Avenue, Davis, California, 95616, USA. Tel: 530-754-6037; Fax: 530-752-8995; E-mail: chen@chem.ucdavis.edu

Table of Contents

Table S1. Apparent kinetic parameters of Psp2,6ST(15–501)-His_6 and His_6-Pd2,6ST(16–497).

Enzymes	**Psp2,6ST(15–501)-His_6**			**His_6-Pd2,6ST(16–497)**		
Substrates	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
CMP-Neu5Ac	0.62±0.04	$(1.4±0.1)\times10^2$	2.2×10^2	0.90±0.20	$(1.6±0.1)\times10^2$	1.7×10^2
LacβMU	0.36±0.07	$(1.0±0.1)\times10^2$	2.9×10^2	[a]0.80±0.10	[a]$(1.4±0.1)\times10^2$	[a]1.8×10^2
CMP-Neu5Ac	3.9±0.4	4.8±0.2	1.2	11±1	0.38±0.01	0.034
GalNAcα2AA	9.5±2.7	31±5	3.3	51±5	4.7±0.2	0.09
GalNAcαOSer	1.4±0.1	34±3	24	1.6±0.2	24±1	15
GalNAcαOThr	5.9±1.6	16±1	2.7	10±1	8.3±0.2	0.8

[a]Data were cited from a paper reported previously.[1]

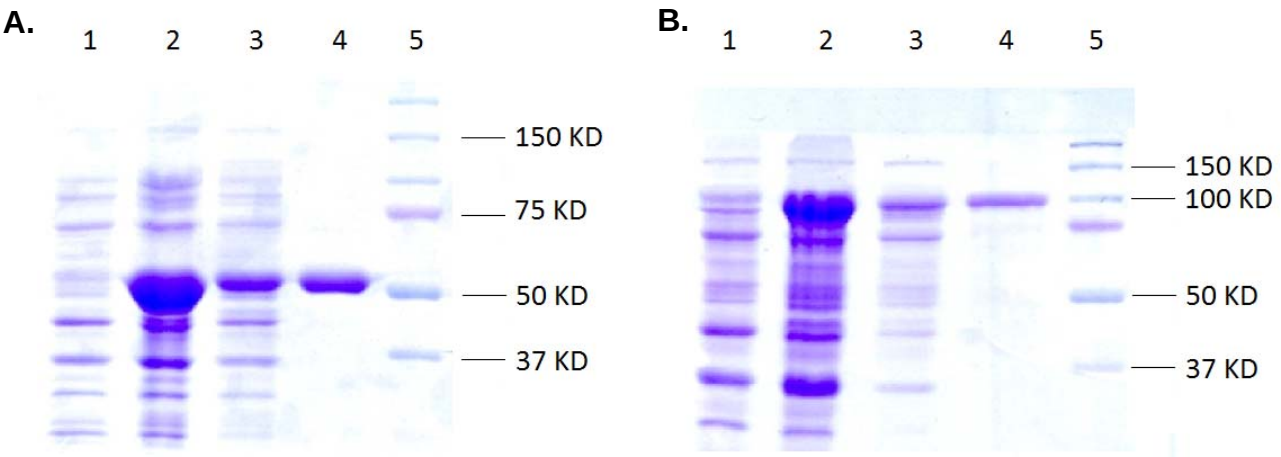


Figure S1. SDS-PAGE of A, Psp2,6ST(15–501)-His_6 and B, MBP-Psp2,6ST(15–501)-His_6. Lanes: 1, whole cell extraction before induction; 2, whole cell extraction after induction; 3, cell lysate after induction; 4, Ni^{2+}-column purified protein; 5, protein standards.

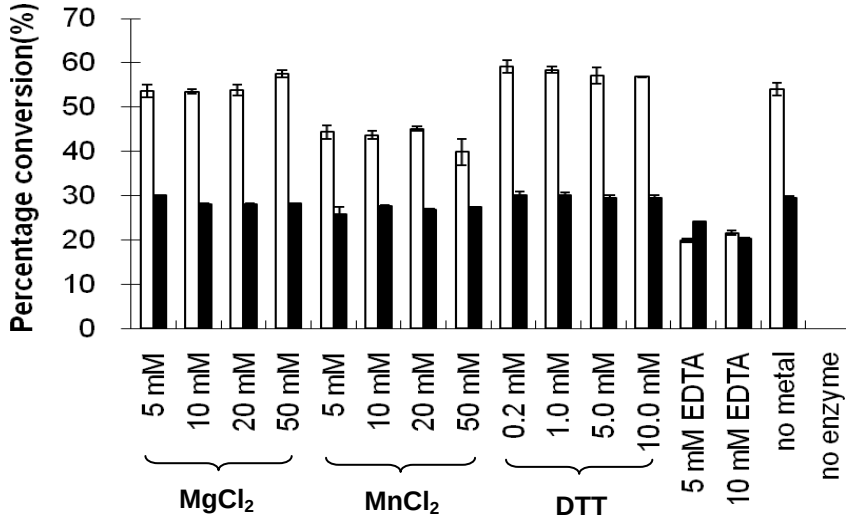


Figure S2. Effects of metal ions (Mg^{2+} and Mn^{2+}), EDTA, and DTT on the sialyltransferase activity of Psp2,6ST(15–501)-His_6 by quantitative HPLC analysis. White columns, CMP-Neu5Ac and LacβMU were used as substrates; black columns, CMP-Neu5Ac and GalNAcα2AA were used as substrates. Reaction conditions were the same as those for pH profiles.

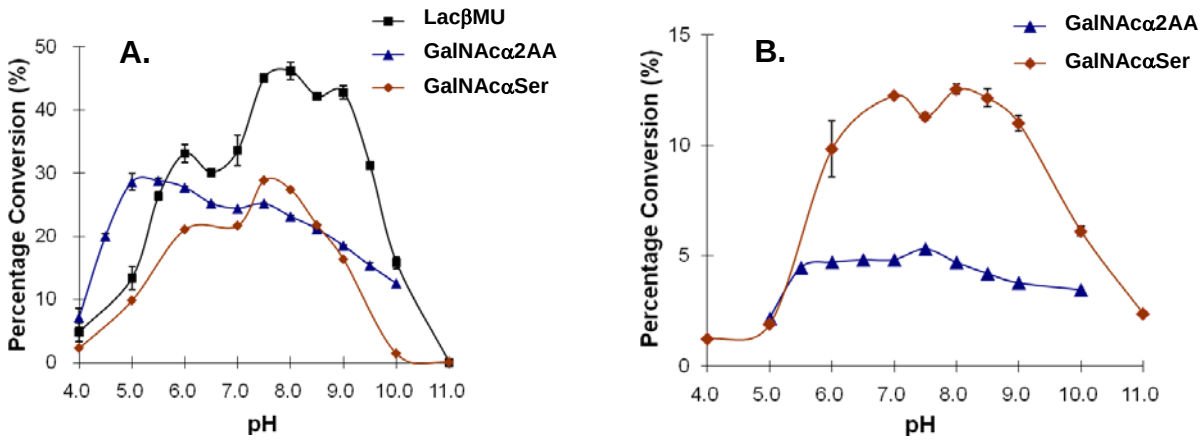


Figure S3. The pH profiles of A, Psp2,6ST(15–501)-His_6 and B, His_6-Pd2,6ST(16–497) when LacβMU (■), GalNAcα2AA (▲), or GalNAcαSer (♦) was used as the sialyltransferase acceptor. 100% conversion was defined as the formation of 1 mM product (Neu5Acα2–6LacβMU or Neu5Acα2–6GalNAcα2AA or Neu5Acα2–6GalNAcαSer). Activities were measured in duplicate at indicated pH at 20 °C for 20 min. Buffers (200 mM) used were: NaOAc-HOAc, pH 4.0–4.5; MES, pH 5.0–6.0; HEPES, pH 7.0; Tris-HCl, pH 7.5–9.0; and CAPS, pH 10.0–11.0. Amounts of enzymes used: 0.3 μM for Psp2,6ST(15–501)-His_6 when LacβMU was used as the acceptor; 6 μM for Psp2,6ST(15–501)-His_6 and 30 μM for His_6-Pd2,6ST(16–497) when GalNAcα2AA was used as the acceptor substrate; 0.6 μM for both Psp2,6ST(15–501)-His_6 and His_6-Pd2,6ST(16–497) when GalNAcαSer was used as the acceptor substrate.

Bacterial strains, plasmids, and materials.

Primers, *Escherichia coli* DH5α electrocompetent cells and BL21 (DE3) chemically competent cells were from Invitrogen (Carlsbad, CA). Full-length synthetic gene of *Photobacterium sp*. JT-ISH-224 α2–6-sialyltransferase with codons optimized for *E. coli* expression system was custom-synthesized by Geneart AG (Regensburg, Germany). Vector plasmids pET15b and pET22b (+) were from Novagen (EMD Biosciences Inc., Madison, WI). Ni^{2+}-NTA agarose (nickel-nitrilotriacetic acid agarose), QIAprep spin miniprep kit, and QIAEX II gel extraction kit were from Qiagen (Valencia, CA). Herculase enhanced DNA polymerase was from Stratagene (La Jolla, CA). T4 DNA ligase, 1 kb DNA ladder, vector plasmid pMAL-c4X, restriction enzymes BamHI, XhoI, NdeI and SalI were from New England Biolabs Inc. (Beverly, MA). Precision Plus Protein Standards were from Bio-Rad (Hercules, CA). Bicinchoninic acid (BCA) protein assay kit was from Pierce Biotechnology Inc. (Rockford, IL). Ni^{2+}-NTA agarose (nickel-nitrilotriacetic acid agarose) was from 5 PRIME (Gaithersburg, MD).

Cloning, expression, and purification of Psp2,6ST(15–501)-His_6

Psp2,6ST was cloned into pET22b(+) and pMal-c4x vectors and expressed in *E. coli* BL21 (DE3) cells as C-His_6-tagged fusion proteins with or without additional N-MBP-tag. The primers used to clone Psp2,6ST(15–501)-His_6 in pET22b(+) vector were: forward primer 5'-GATCCATATGTGTAATAATAGCGAAGAAAATAC-3' (*Nde*I restriction site is underlined), reverse primer 5'-CCGCTCGAGTGCCCAAAACAGGACGTTTT-3' (*Xho*I restriction site is underlined). The primers used to clone MBP-Psp2,6ST(15-501)-His_6 fusion protein were: forward primer is same as the one used in cloning Psp2,6ST(15–501)-His_6 fusion protein, reverse primer 5'-ACGCGTCGACTTA*GTGGTGGTGGTGGTGGTG*TGCCCAA-3' (*Sal*I restriction site is underlined, codons encoding hexa-histidine are in italics). PCRs for amplifying the target gene were performed in a 50 μL reaction mixture containing template DNA (1 μg), forward and reverse primers (1 μM each), 10 × Herculase buffer (5 μL), dNTP mixture (1 mM), and 5 U (1 μL) of Herculase-enhanced DNA polymerase. The reaction mixture was subjected to 29 cycles of amplification with an annealing

temperature of 52 °C. The resulting PCR product was purified and digested with restriction enzymes. The purified and digested PCR product was ligated with predigested vectors and transformed into *E. coli* DH5α electrocompetent cells. Selected clones were grown for minipreps and characterization by restriction mapping and DNA sequencing performed by Davis Sequencing Facility in the University of California-Davis.

Positive plasmid was selected and transformed into BL21 (DE3) chemically competent cells. The plasmid-bearing *E. coli* strain was cultured in LB-rich medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) supplemented with ampicillin (100 μg/mL). Overexpression of the target protein was achieved by inducing the *E. coli* culture with 0.3 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) when the OD_{600nm} of the culture reaches 0.8–1.0 and incubating at 20 °C for 20 h with vigorous shaking at 250 rpm in a C25KC incubator shaker (New Brunswick Scientific, Edison, NJ).

Target proteins were purified from cell lysate. To obtain the cell lysate, cell pellet harvested by centrifugation at 4000 rpm for 2 h was resuspended in lysis buffer (pH 7.5, 100 mM Tris-HCl containing 0.1% Triton X-100) (20 mL/L cell culture). Lysozyme (50 μg/mL) and DNaseI (3 μg/mL) were then added and the mixture was incubated at 37 °C for 1 hour with vigorous shaking. Cell lysate was obtained by centrifugation at 11,000 rpm for 30 min as the supernatant. Purification of His-tagged proteins from the lysate was achieved using an AKTA FPLC system (GE Healthcare) equipped with a HisTrapTM FF 5 mL column. The column was pre-equilibrated with 10 column volumes of binding buffer (5 mM imidazole, 0.5M NaCl, 50 mM Tris-HCl, pH 7.5) before the lysate was loaded. Followed by washing with eight column volumes of binding buffer, 10 column volumes of washing buffer (50 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5), the protein was eluted with a linear gradient of elute buffer containing 50–250 mM imidazole in Tris-HCl buffer (50 mM, pH 7.5, 0.5 M NaCl). The fractions containing the purified enzymes were collected and stored at 4 °C.

Protein concentration was determined in a 96-well plate using bicinchoninic acid with BSA as standard. The absorbance was measured at 562 nm using a plate reader.

SDS-PAGE analysis of Psp2,6ST(15–501)-His_6

SDS-PAGE analysis (Figure S1, A) shows both the recombinant proteins in pET system and pMal-c4x system can be well expressed with molecular weight of about 56 kDa and 99.5 kDa respectively, which are close to 55 kDa calculated for the Psp2,6ST(15–501)-His_6 and 98 kDa calculate for the MBP-Psp2,6ST(15–501)-His_6. The SDS-PAGE analysis of Psp26ST(15–501)-His_6 in pET22b (+) system (Figure S1A) indicates that it can be expressed in a large amount which consists of about 80% of the total protein extracts from *E. coli* host cells. But the soluble portion of the target protein that can be observed in the lysate is not high. So pMal-c4x expression system was tested to improve the protein solubility. However, SDS-PAGE analysis of MBP-Psp2,6ST(15–501)-His_6 expression (Figure S1B) showed that the pMal-c4x expression system did not improve the expression level.

Effects of metal ions, DTT and EDTA

EDTA (5 mM, 10 mM), different concentrations (5, 10, 20, 50 mM) of $MgCl_2$ or $MnCl_2$, and various concentrations of DTT (0.2, 1, 5, 10 mM) were used in a Tris-HCl buffer (pH 8.0, 200 mM) or MES buffer (pH 5.0, 200 mM) to analyze their effects on the α2–6-sialyltransferase activity of Psp2,6ST(15–501)-His_6 for acceptor LacβMU or GalNAcα2AA. Other components used in the reaction system are the same as used in the pH profile. Reaction without EDTA, DTT, and metal ions was used as a control.

The effects of metal ions Mg^{2+} and Mn^{2+} as well as DTT and the chelating agent EDTA on the α2–6-sialyltransferase activity of Psp2,6ST(15–501)-His_6 toward LacβMU and GalNAcα2AA were examined at pH 5.0 and 8.0 respectively. The results (Figure S2) indicate that a divalent metal ion is required as

the enzyme activity decreased dramatically when EDTA is applied at 5 mM and LacβMU is used as acceptor. However the addition of Mg^{2+} up to 50 mM does not affect the sialyltransferase activity of the enzyme, but the activity drops a little when Mn^{2+} ion is used in the reaction system. DTT up to 10 mM does not significantly increase the activity of the enzyme for both acceptor LacβMU and GalNAcα2AA, indicating disulfide formation is not required for the sialyltransferase activity of Psp2,6ST(15–501)-His_6.

pH profile study for Psp2,6ST(15–501)-His_6 and His_6-Pd2,6ST(16–497)

Typical enzymatic assays were performed in a total volume of 10 μL in a buffer (200 mM) with pH varying from 4.0 to 11.0. The buffers used were: NaOAc-HOAc, pH 4.0–4.5; MES, pH 5.0–6.0; HEPES, pH 7.0; Tris-HCl, pH 7.5–9.0; and CAPS, pH 10.0–11.0. Reaction system for acceptor LacβMU is: CMP-Neu5Ac (1 mM), LacβMU (1 mM), and the recombinant Psp2,6ST(15–501)-His_6 (0.3 μM). Reaction system for acceptor GalNAcα2AA is: CMP-Neu5Ac (4 mM), GalNAcα2AA (1 mM), and the recombinant enzyme Psp2,6ST(15–501)-His_6 (6 μM) or His_6-Pd2,6ST(16–497) (30 μM). Reaction system for acceptor GalNAcαSer is: CMP-Neu5Ac (1 mM), GalNAcαSer (1 mM), and the recombinant enzyme Psp2,6ST(15–501)-His_6 or His_6-Pd2,6ST(16–497) (0.6 μM). All reactions were allowed to proceed for 20 min at 20 °C. The reaction mixture was quenched by adding ice cold 25% (v/v) acetonitrile to make 100-fold dilution for LacβMU, 500-fold dilution for GalNAcαSer, or quenched by adding ice cold acetonitrile 30% (v/v) (for GalNAcα2AA) to make 80-fold dilution. The samples were then kept on ice until aliquots of 5 μL were injected and analyzed by a Shimadzu LC-2010A system equipped with a membrane on-line degasser, a temperature control unit, and a fluorescence detector. A reverse-phase Premier C18 column (250×4.6 mm i.d., 5 μm particle size, Shimadzu) protected with a C18 guard column cartridge was used. The mobile phase was 25% (v/v) acetonitrile (for LacβMU and GalNAcαSer) or 30% (v/v) acetonitrile (for GalNAcα2AA). The fluorescent-labeled LacβMU and Neu5Acα2–6LacβMU were detected by excitation at 325 nm and emission at 372 nm. The fluorescent-labeled GalNAcα2AA and Neu5Acα2–6GalNAcα2AA were detected by excitation at 315 nm and emission at 400 nm. The fluorescent-labeled GalNAcαSer and Neu5Acα2–6GalNAcαSer were detected by excitation at 313 nm and emission at 262 nm. All assays were carried out in duplicate.

Kinetics assay

Enzymatic assays for Psp2,6ST(15–501)-His_6 were carried out at 20 °C for 20 minutes in a total volume of 10 μL in a proper buffer at the optimal pH according to the pH profile. Specific conditions for Psp2,6ST(15–501)-His_6 when LacβMU was used as an acceptor: Tris-HCl buffer (200 mM, pH 8.0), enzyme (0.3 μM), varied concentrations of LacβMU (0.1, 0.25, 0.4, 1, 2, 4, and 6 mM) with a fixed concentration of CMP-Neu5Ac (1 mM) or varied concentrations of CMP-Neu5Ac (0.1, 0.25, 0.4, 1, 2, 4, and 6 mM) with a fixed concentration of LacβMU (1 mM). Specific conditions for Psp2,6ST(15–501)-His_6 when GalNAcα2AA was used as an acceptor: MES buffer (200 mM, pH 5.0), enzyme (6 μM), varied concentrations of GalNAcα2AA (0.5, 0.8, 1, 2, 4, 8, and 10 mM) and a fixed concentration of CMP-Neu5Ac (4 mM) or varied concentrations of CMP-Neu5Ac (0.5, 1, 2, 4, 5, 8, and 10 mM) with a fixed concentration of GalNAcα2AA (1 mM). Specific conditions for His_6-Pd2,6ST(16–497) when GalNAcα2AA was used as an acceptor: Tris-HCl buffer (200 mM, pH 7.5), enzyme (30 μM), varied concentrations of GalNAcα2AA (0.5, 1, 2, 4, 10, 20, 40, 80, and 100 mM) with a fixed concentration of CMP-Neu5Ac (4 mM) or varied concentrations of CMP-Neu5Ac (0.5, 1, 2, 4, 10, 20, 40, 80, and 100 mM) with a fixed concentration of GalNAcα2AA (1 mM). Specific conditions for Psp2,6ST(15–501)-

His_6 and His_6-Pd2,6ST(16–497) when GalNAcαSer was used as an acceptor: Tris-HCl buffer (200 mM, pH 8.0), varied concentrations of GalNAcαSer (0.1, 0.25, 0.5, 1, 2, 4, 8, and 10 mM), a fixed concentration of CMP-Neu5Ac (2 mM), and Psp2,6ST(15–501)-His_6 (0.6 μM) or His_6-Pd2,6ST(16–497) (2.0 μM). Specific conditions for Psp2,6ST(15–501)-His_6 and His_6-Pd2,6ST(16–497) when GalNAcαThr was used as an acceptor: Tris-HCl buffer (200 mM, pH 8.0), varied concentrations of GalNAcαThr (0.5, 1, 2, 4, 10, 20, 40, 60 mM), a fixed concentration of CMP-Neu5Ac (4 mM), and Psp2,6ST(15–501)-His_6 (2.0 μM) or His_6-Pd2,6ST(16–497) (10.0 μM).

General methods

Chemicals were purchased and used without further purification. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were recorded on a Varian VNMRS 600 MHz spectrometer. High resolution electrospray ionization (ESI) mass spectra were obtained at the Mass Spectrometry Facility in the University of California, Davis. Optical rotation was recorded on an Autopol IV Automatic polarimeter at 589 nm wavelength. Infrared spectra were recorded on a PerkinElmer Spectrum 100 ATR-FTIR. Silica gel 60 Å was used for flash column chromatography. Thin-layer chromatography (TLC) was performed on silica gel plates using *p*-anisaldehyde sugar stain or 5% sulfuric acid in ethanol stain for detection. Gel filtration chromatography was performed using a column (100 cm × 2.5 cm) packed with BioGel P-2 Fine resins. Sodium pyruvate, ManNAc (**1**), and mannose (**3**) and were purchased from Sigma. LacβMU, GalNAc derivatives **10**–**13**, ManNAc derivatives **2, 4**–**9** were synthesized as described previously.[2–5]

One-pot three-enzyme preparative-scale synthesis of STn and its derivates

α-GalNAc glycosides (**10**–**13**, 41–55 mg), a sialic acid precursor (**1**–**9**, 1.5 equiv.), sodium pyruvate (5.0 equiv.), and CTP (1.5 equiv.) were dissolved in Tris-HCl buffer (10 mL, 100 mM, pH 8.5) containing $MgCl_2$ (20 mM) and appropriate amounts of Pm aldolase (1.5 mg), NmCSS (1.0 mg), and Psp2,6ST(15–501)-His_6 (2.5–3.0 mg). The pH condition of the mixture was controlled at 8.0 for acceptor **12** or **13** to avoid the loss of Fmoc group, and at 7.5 for donor **5** to avoid the loss of *O*-acetyl. All other reactions were carried out at pH 8.5. All reactions were incubated in an incubator shaker at room temperature for around 2 days with agitation at 140 rpm. The product formation was monitored by TLC developed with EtOAc:MeOH:H_2O:HOAc = 4:2:1:0.1 (by volume) and stained with *p*-anisaldehyde sugar stain. When an optimal yield was achieved, the reaction was stopped by adding the same volume of cold EtOH and kept at 4 °C for 30 min. The mixture was then centrifuged and the precipitates were removed. The supernatant was concentrated, passed through a BioGel P-2 gel filtration column, and eluted with water to obtain partially purified product. A silica gel column was then used to obtain the final pure sialylated products using EtOAc:MeOH:H_2O = 6:2:1 (by volume) as the mobile phase.

3-Azidopropyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-2-acetamido-2-deoxy-α-D-galactopyranoside (14, Neu5Acα2–6GalNAcαProN_3). 69 mg, Yield, 76%; white foam. $[\alpha]_D^{23}$ = +55.0° (*c* 1.0, H_2O); ν_{max}/cm^{-1} 3296 (s, OH), 2932 (s, C–H, alkene), 2100 (s, N_3), 1613 (s, C=O, carboxylic acid), 1560 (m, C=O, amide), 1031 (s, C-N); ^{1}H NMR (600 MHz, D_2O) δ 4.87 (d, 1H, *J* = 3.6 Hz), 4.12 (dd, 1H, *J* = 3.6 and 10.8 Hz), 4.03–3.75 (m, 8H), 3.70–3.39 (m, 8H), 2.72 (dd, 1H, *J* = 4.8 and 12.0 Hz, H-3_{eq}"), 2.03 (s, $2CH_3$), 1.90 (m, 2H), 1.67 (t, 1H, *J* = 12.0 Hz, H-3_{ax}"); ^{13}C NMR (75 MHz, D_2O) δ 175.17, 174.67, 173.55, 100.50, 97.21, 72.71, 71.92, 69.63, 68.68, 68.39, 68.38, 67.63, 65.30, 63.98, 62.77, 52.01, 50.09, 48.31, 40.42, 28.07, 22.20, 22.11. HRMS (ESI) *m/z* calcd for $C_{22}H_{38}N_5O_{14}$ (M+H) 596.2415, found 596.2408.

3-Azidopropyl *O*-(5-glycolylamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-2-acetamido-2-deoxy-α-D-galactopyranoside (15, Neu5Gcα2–6GalNAcαProN$_3$). 70 mg, Yield, 75%; white foam. $[\alpha]_D^{23}$ = +58.7° (*c* 1.0, H_2O); ν_{max}/cm^{-1} 3290 (s, OH), 2932 (s, C–H, alkene), 2100 (s, N_3), 1611 (s, C=O, carboxylic acid), 1545 (m, C=O, amide), 1032 (s, C-N); ^{1}H NMR (600 MHz, D_2O) δ 4.72 (d, 1H, *J* = 3.6 Hz), 3.98 (dd, 1H, *J* = 3.6 and 11.4 Hz), 3.96 (s, 2H), 3.89 (dd, 1H, *J* = 3.6 and 7.8 Hz), 3.84 (d, 1H, *J* = 3.6 Hz), 3.78–3.59 (m, 8H), 3.50–3.25 (m, 6H), 2.58 (dd, 1H, *J* = 4.8 and 12.0 Hz, H-3$_{eq}$''), 1.88 –s, CH_3), 1.75 (m, 2H), 1.54 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$''); ^{13}C NMR (75 MHz, D_2O) δ 175.92, 174.67, 173.57, 100.51, 97.21, 72.43, 71.96, 69.64, 68.67, 68.32, 68.12, 67.63, 65.29, 64.00, 62.73, 61.11, 51.69, 50.09, 48.30, 40.46, 28.06, 22.09. HRMS (ESI) *m/z* calcd for $C_{22}H_{38}N_5O_{15}$ (M+H) 612.2364, found 612.2356.

3-Azidopropyl *O*-(3-deoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-2-acetamido-2-deoxy-α-D-galactopyranoside (16, Kdnα2–6GalNAcαProN$_3$). 50 mg, Yield, 60%; white foam. $[\alpha]_D^{24}$ = +52.1° (*c* 1.0, H_2O); ν_{max}/cm^{-1} 3271 (s, OH), 2927 (s, C–H, alkene), 2099 (s, N_3), 1603 (s, C=O, carboxylic acid), 1545 (m, C=O, amide), 1026 (s, C-N); ^{1}H NMR (600 MHz, D_2O) δ 4.86 (d, 1H, *J* = 3.6 Hz), 4.12 (dd, 1H, *J* = 3.6 and 10.8 Hz), 4.02 (dd, 1H, *J* = 3.6 and 8.4 Hz), 3.97 (d, 1H, *J* = 2.4 Hz), 3.91–3.40 (m, 14H), 2.66 (dd, 1H, *J* = 4.8 and 12.6 Hz, H-3$_{eq}$''), 2.03 (s, CH_3), 1.89 (m, 2H), 1.62 (t, 1H, *J* = 12.6 Hz, H-3$_{ax}$''); ^{13}C NMR (75 MHz, D_2O) δ 174.67, 173.74, 100.47, 97.20, 73.70, 72.24, 70.30, 70.06, 69.67, 68.70, 68.06, 67.63, 65.29, 64.06, 62.83, 50.09, 48.30, 40.04, 28.06, 22.09. HRMS (ESI) *m/z* calcd for $C_{20}H_{35}N_4O_{14}$ (M+H) 555.2150, found 555.2141.

3-Azidopropyl *O*-(5-methoxyacetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-2-acetamido-2-deoxy-α-D-galactopyranoside (17, Neu5GcMeα2–6GalNAcαProN$_3$). 82 mg, Yield, 86%; white foam. $[\alpha]_D^{24}$ = +58.1° (*c* 1.0, H_2O); ν_{max}/cm^{-1} 3296 (s, OH), 2937 (s, C–H, alkene), 2096 (s, N_3), 1608 (s, C=O, carboxylic acid), 1545 (m, C=O, amide), 1029 (s, C-N); ^{1}H NMR (600 MHz, D_2O) δ 4.88(d, 1H, *J* = 3.6 Hz), 4.13 (dd, 1H, *J* = 3.6 and 10.8 Hz), 3.99 (s, 2H), 3.91–3.42 (m, 16H), 3.42 (s, 3H), 2.73 (dd, 1H, J=4.8, 12.6), 2.04(s, 3H), 1.90 (m, 2H), 1.69 (t, 1H, *J* = 12.6); ^{13}C NMR (75 MHz, D_2O) δ 174.67, 173.60, 171.30, 100.52, 97.21, 72.41, 71.99, 71.01, 69.64, 68.68, 68.40, 68.13, 67.63, 65.30, 64.00, 62.74, 59.13, 51.67, 50.09, 48.31, 40.52, 28.07, 22.09. HRMS (ESI) *m/z* calcd for $C_{23}H_{40}N_5O_{15}$ (M+H) 626.2521, found 626.2514.

3-Azidopropyl *O*-(5-acetoxyacetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-2-acetamido-2-deoxy-α-D-galactopyranoside (18, Neu5GcAcα2–6GalNAcαProN$_3$). 67 mg, Yield, 68%; white foam. $[\alpha]_D^{24}$ = +48.2° (*c* 1.0, H_2O); ν_{max}/cm^{-1} 3292 (s, OH), 2932 (s, C–H, alkene), 2099 (s, N_3), 1613 (s, C=O, carboxylic acid), 1542 (m, C=O, amide), 1028 (s, C-N); ^{1}H NMR (600 MHz, D_2O) δ 4.89 (d, 1H, *J* = 3.6 Hz), 4.14 (s, 2H), 4.17–3.46 (m, 14H), 2.77 (dd, 1H, *J* = 4.8 and 12.6 Hz), 2.06 (s, 3H), 1.93 (m, 2H), 1.72 (t, 1H, *J* = 12.6 Hz); ^{13}C NMR (75 MHz, D_2O) δ 175.92, 174.67, 173.56, 171.00, 100.52, 97.21, 72.43, 71.96, 69.64, 68.68, 68.33, 68.12, 67.64, 65.31, 64.01, 62.74, 61.12, 59.47, 51.70, 50.09, 48.31, 40.46, 28.07, 22.10. HRMS (ESI) *m/z* calcd for $C_{24}H_{39}N_5O_{16}$ (M+H) 653.2392, found 653.2413.

3-Azidopropyl *O*-(5-azidoacetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-2-acetamido-2-deoxy-α-D-galactopyranoside (19, Neu5AcN$_3$α2–6GalNAcαProN$_3$). 73

mg, Yield, 75%; white foam. $[\alpha]_D^{23}$ = +62.3° (*c* 1.0, H_2O); ν_{max}/cm^{-1} 3306 (s, OH), 2927 (s, C–H, alkene), 2106 (s, N_3), 1608 (s, C=O, carboxylic acid), 1547 (m, C=O, amide), 1032 (s, C-N); ^1H NMR (600 MHz, D_2O) δ 4.87 (d, 1H, *J* = 3.6 Hz), 4.13 (dd, 1H, *J* = 3.6 and 10.8 Hz), 4.05 (s, 2H), 3.99–3.42 (m, 16H), 2.73 (dd, 1H, *J* = 4.8 and 12.6 Hz), 2.07 (s, 3H), 1.90 (m, 2H), 1.69 (t, 1H, *J* = 12.6 Hz); ^{13}C NMR (75 MHz, D_2O) δ 174.67, 173.55, 171.31, 100.51, 97.22, 72.37, 71.97, 69.64, 68.66, 68.34, 68.22, 67.63, 65.30, 64.01, 62.75, 52.07, 52.05, 50.09, 48.31, 40.44, 28.07, 21.10. HRMS (ESI) *m/z* calcd for $C_{22}H_{37}N_8O_{14}$ (M+H) 637.2429, found 637.2425.

3-Azidopropyl *O*-(5-fluoroacetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-2-acetamido-2-deoxy-α-D-galactopyranoside (20, Neu5AcFα2–6GalNAcαProN$_3$). 74 mg, Yield, 80%; white foam. $[\alpha]_D^{23}$ = +53.7° (*c* 1.0, H_2O); ν_{max}/cm^{-1} 3292 (s, OH), 2933 (s, C–H, alkene), 2100 (s, N_3), 1606 (s, C=O, carboxylic acid), 1560 (m, C=O, amide), 1028 (s, C-N); ^1H NMR (600 MHz, D_2O) δ 4.87(d, 1H, *J* = 3.6 Hz), 4.13 (dd, 1H, *J* = 3.6 and 11.4 Hz), 4.03 (m, 1H), 3.96–3.42 (m, 15H), 2.74 (dd, 1H, *J* = 4.8 and 12.6 Hz), 2.03 (s, 3H), 1.90 (m, 2H), 1.69 (t, 1H, *J* = 12.6 Hz), 4.95 (s, 1H), 4.88 (s, 1H) ; ^{13}C NMR (75 MHz, D_2O) δ 174.68, 173.54, 171.66, 100.53, 97.22, 79.97, 72.30, 72.02, 69.65, 68.69, 68.25, 68.22, 67.64, 65.31, 64.03, 62.77, 51.61, 50.10, 48.32, 40.43, 28.07, 22.10. HRMS (ESI) *m/z* calcd for $C_{22}H_{37}FN_5O_{14}$ (M+H) 614.2321, found 614.2313.

3-Azidopropyl *O*-(5-phenylmethoxyacetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-2-acetamido-2-deoxy-α-D-galactopyranoside (21, Neu5GcBnα2–6GalNAcαProN$_3$). 77 mg, Yield, 72%; white foam. $[\alpha]_D^{23}$ = +50.8° (*c* 1.0, H_2O); ν_{max}/cm^{-1} 3301 (s, OH), 2932 (s, C–H, alkene), 2096 (s, N_3), 1611 (s, C=O, carboxylic acid), 1542 (m, C=O, amide), 1032 (s, C-N); ^1H NMR (600 MHz, D_2O) δ 7.47–7.42 (m, 5H) 4.87 (d, 1H, *J* = 3.6 Hz), 4.66 (s, 2H), 4.15 (dd, *J* = 3.6, 10.8), 4.11 (s, 2H), 4.05–3.41 (m, 16H), 2.73 (dd, *J* = 4.8 and 12.6 Hz), 2.04 (s, 3H), 1.89 (m, 2H), 1.69 (t, 1H, *J* = 12.0 Hz); ^{13}C NMR (75 MHz, D_2O) δ 174.66, 173.54, 171.67, 136.71, 128.99, 128.80, 128.77, 100.51, 97.22, 73.49, 72.43, 72.00, 69.64, 68.57, 68.43, 68.11, 67.64, 65.30, 64.00, 62.79, 51.74, 50.09, 48.31, 40.53, 28.07, 22.10. HRMS (ESI) *m/z* calcd for $C_{29}H_{44}N_5O_{15}$ (M+H) 702.2834, found 702.2836.

3-Azidopropyl *O*-[5-(N-benzyloxycarboxyamido)glycylamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid]-(2→6)-2-acetamido-2-deoxy-α-D-galactopyranoside (22, Neu5GlyCbzα2–6LacProN$_3$). 75 mg, Yield, 67%; white foam. $[\alpha]_D^{24}$ = +50.7° (*c* 1.0, H_2O); ν_{max}/cm^{-1} 3286 (s, OH), 2942 (s, C–H, alkene), 2103 (s, N_3), 1616 (s, C=O, carboxylic acid), 1542 (m, C=O, amide), 1032 (s, C-N); ^1H NMR (600 MHz, D_2O) δ 7.45–7.39 (m, 5H), 5.12 (m, 2H), 4.85 (d, 1H, *J* = 3.6 Hz), 4.12(dd, *J* = 3.6 and 11.4 Hz), 4.06–3.40 (m, 18H), 2.71 (dd, 1H, 4.8, 12.6), 2.05 (s, 3H), 1.87 (m, 2H), 1.66 (t, 1H, *J* = 12.6 Hz); ^{13}C NMR (75 MHz, D_2O) δ 177.23, 176.10, 175.78, 161.22, 138.93, 131.52, 131.18, 130.49, 103.07, 99.78, 75.06, 74.58, 72.19, 71.23, 70.91, 70.74, 70.20, 70.06, 67.86, 66.53, 65.39, 54.63, 52.65, 50.88, 46.42, 42.96, 30.63, 24.66. HRMS (ESI) *m/z* calcd for $C_{30}H_{45}N_6O_{16}$ (M+H) 745.2892, found 745.2892.

Methyl 2-[[4-[[3-[[*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-2-acetamido-2-deoxy-α-D-galactopyranosyl]oxy]propyl]amino]-1,4-dioxobutyl]amino]-Benzoic acid (23, Neu5Acα2–6GalNAcα2AA). 41 mg, Yield, 64%; white foam. $[\alpha]_D^{24}$ = +50.2° (*c* 1.0, H_2O); ν_{max}/cm^{-1} 3282 (s, OH), 2927 (s, C–H, alkene), 1607 (s, C=O, carboxylic acid), 1529 (m, C=O, amide), 1031 (s, C-N); ^1H NMR (600 MHz, D_2O) δ 7.94–7.27 (m,

4H), 4.79 (d, 1H, *J* = 3.6 Hz), 4.05 (dd, 1H, *J* = 3.6 and 10.8 Hz), 3.84 (s, 3H), 3.91–3.53 (m, H), 3.37 (m, 1H), 3.25 (m, 2H), 2.73 (t, 2H, *J* = 6.6 Hz), 2.69 (dd, 1H, *J* = 4.8 and12.6 Hz), 2.60 (t, 2H, *J* = 6.6 Hz), 1.99 (s, 3H), 1.97 (s, 3H), 1.75 (m, 2H), 1.63 (t, 1H, *J* = 12.0 Hz). ^{13}C NMR (75 MHz, D_2O) δ 175.16, 174.60, 174.56, 173.56, 173.50, 169.26, 137.27, 134.16, 131.02, 125.39, 123.45, 120.90, 100.47, 97.04, 72.70, 71.90, 69.56, 68.66, 68.39, 67.81, 65.15, 63.93, 62.76, 52.93, 52.01, 49.99, 40.44, 36.28, 32.61, 31.18, 28.14, 22.17,. 22.06. HRMS (ESI) *m/z* calcd for $C_{34}H_{51}N_4O_{18}$ (M+H) 803.3198, found 803.3202.

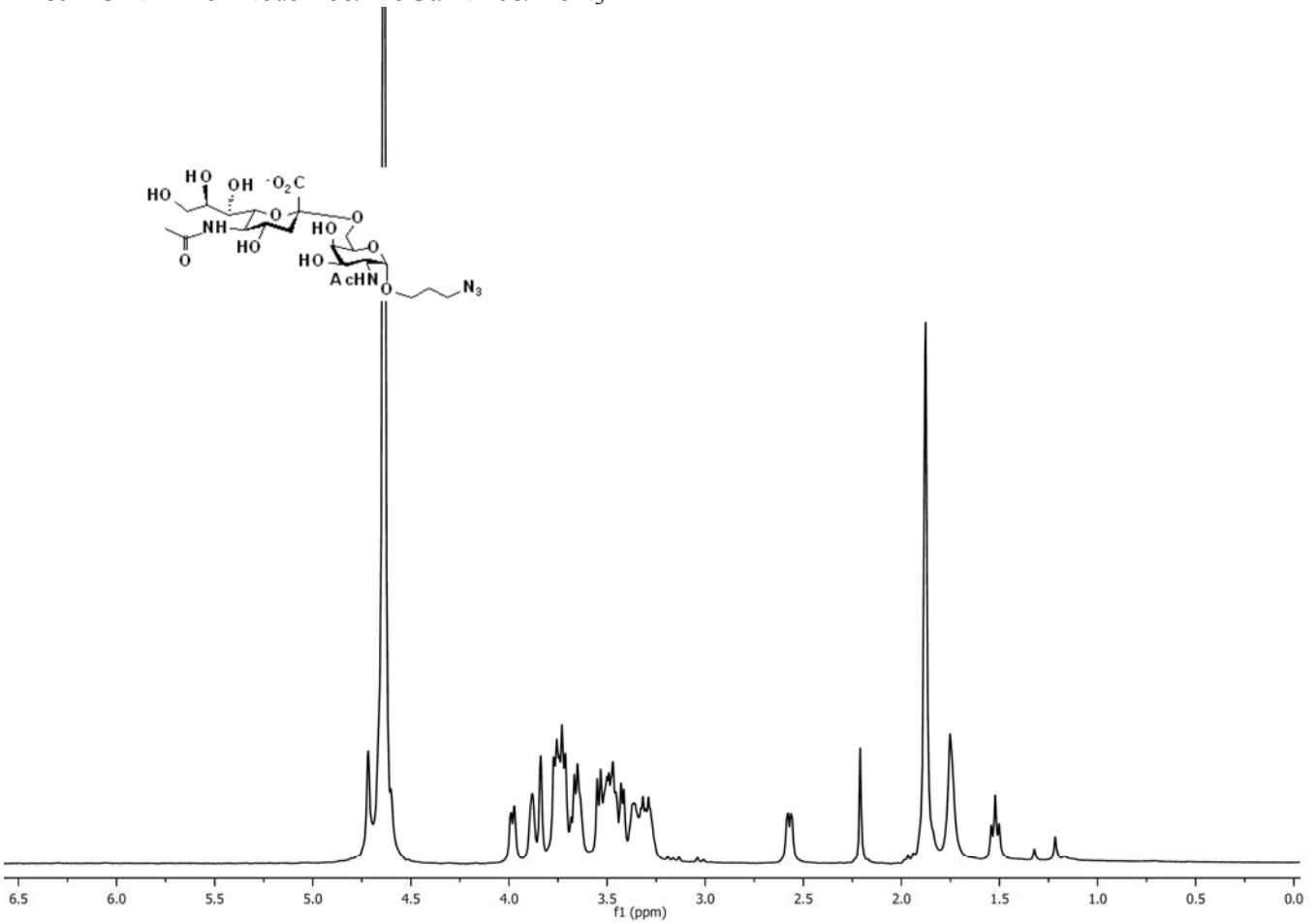
***N*-9-Fluorenylmethoxycarbonyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-2-acetamido-2-deoxy-α-D-galactopyranosyl-L-Serine (24, Neu5Acα2–6GalNAcαSer).** 61 mg, Yield, 72%; white foam. $[\alpha]_D^{24}$ = +42.5° (*c* 0.75, H_2O); ν_{max}/cm^{-1} 3309 (s, OH), 2942 (s, C–H, alkene), 1603 (s, C=O, carboxylic acid), 1558 (m, C=O, amide), 1033 (s, C-N); ^{1}H NMR (600 MHz, D_2O): δ 7.89–7.41 (m, 8H), 4.82 (d, 1H, *J* = 3.6 Hz), 4.67–4.57 (m, 2H), 4.30–3.49 (m, 17H), 2.70 (dd, 1H, *J* = 4.8 and 12.0 Hz), 2.02 (s, 3H), 1.59 (t, 1H, *J* = 12.0 Hz). ^{13}C NMR (150 MHz, D_2O): δ 175.16, 174.85, 174.69, 173.47, 157.59, 144.11, 143.98, 141.12, 128.19, 127.72, 125.14, 125.06, 120.32, 100.49, 97.76, 72.68, 71.87, 69.67, 68.58, 68.51, 68.44, 68.42, 67.60, 66.33, 63.61, 62.74, 61.86, 52.00, 49.82, 47.09, 40.43, 22.17, 20.65. HRMS (ESI) m/z calcd for $C_{37}H_{48}N_3O_{18}$ (M+H) 822.2933, found 822.2936.

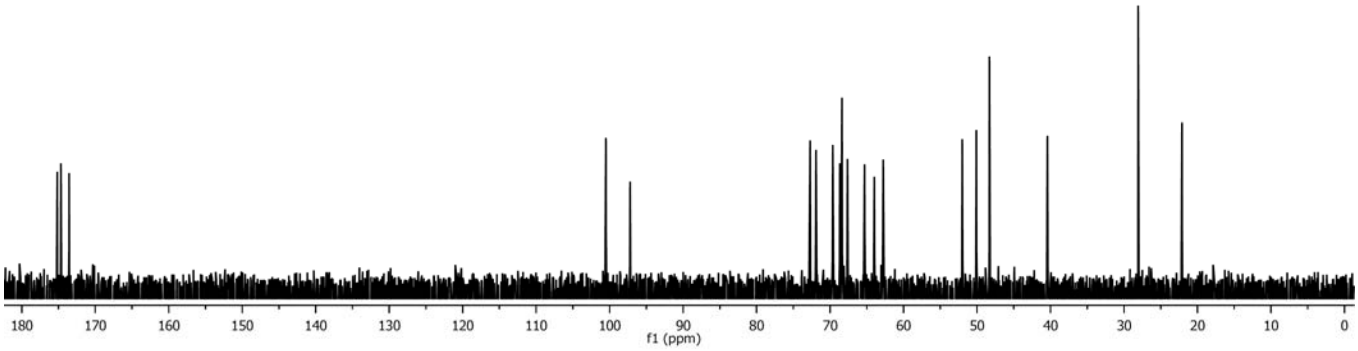
***N*-9-Fluorenylmethoxycarbonyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-2-acetamido-2-deoxy-α-D-galactopyranosyl- L-Threonine (25, Neu5Acα2–6GalNAcαThr).** 40 mg, Yield, 51%; white foam. $[\alpha]_D^{24}$ = +41.2° (*c* 0.75, H_2O); ν_{max}/cm^{-1} 3301 (s, OH), 2932 (s, C–H, alkene), 1600 (s, C=O, carboxylic acid), 1560 (m, C=O, amide), 1032 (s, C-N); ^{1}H NMR (600 MHz, D_2O): δ 7.90–7.41 (m, 8H), 4.94 (d, 1H, *J* = 3.6 Hz), 4.61 (dd, 1H, *J* = 3.0 and 12.0 Hz), 4.30–3.52 (m, 13H), 2.79 (dd, 1H, *J* = 4.8 and 12.6 Hz), 2.03 (s, 3H), 1.93 (s, 3H), 1.70 (t, 1H, *J* = 12.0 Hz), 0.94 (d, 3H, *J* = 6.0 Hz). ^{13}C NMR (100 MHz, D_2O): δ 175.20, 174.79, 174.74, 174.61, 173.48, 158.16, 144.24, 143.73, 141.30, 141.19, 128.35, 128.19, 127.76, 127.72, 125.15, 124.92, 120.25, 100.48, 99.14, 78.08, 72.70, 71.92, 69.81, 68.67, 68.45, 68.42, 67.86, 65.64, 63.74, 62.72, 52.07, 50.02, 47.62, 40.38, 22.34, 22.18, 18.31. HRMS (ESI) m/z calcd for $C_{38}H_{50}N_3O_{18}$ (M+H) 836.3089, found 836.3094.

References

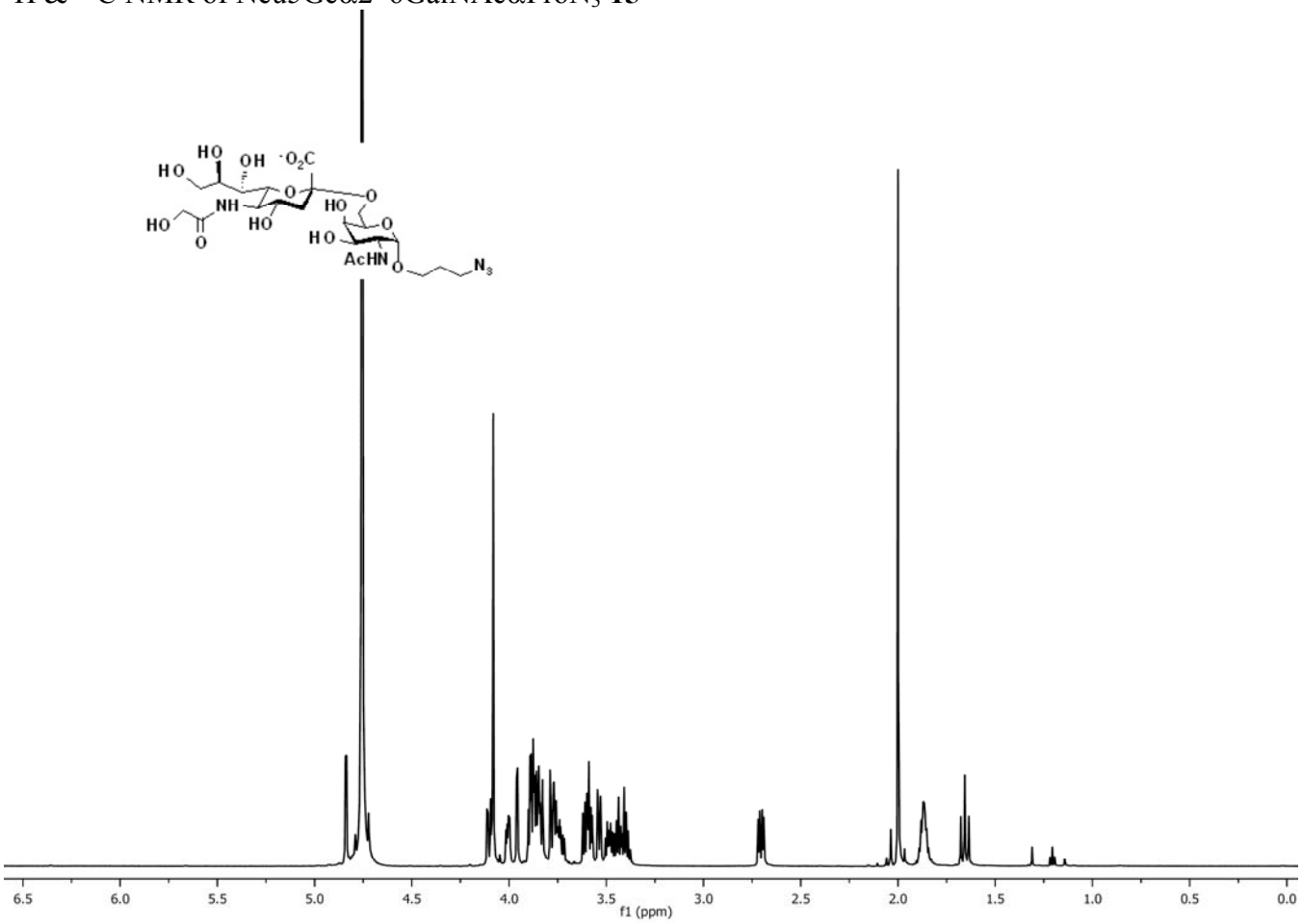
1. M. Sun, Y. Li, H. A. Chokhawala, R. Henning and X. Chen, *Biotechnol. Lett.*, 2008, **30**, 671–676.
2. H. Yu, H. Chokhawala, R. Karpel, H. Yu, B. Wu, J. Zhang, Y. Zhang, Q. Jia and X. Chen, *J. Am. Chem. Soc.*, 2005, **127**, 17618–17619.
3. H. Yu, S. Huang, H. Chokhawala, M. Sun, H. Zheng and X. Chen, *Angew. Chem. Int. Ed. Engl.*, 2006, **45**, 3938–3944.
4. H. Cao, Y. Li, K. Lau, S. Muthana, H. Yu, J. Cheng, H. A. Chokhawala, G. Sugiarto, L. Zhang and X. Chen, *Org. Biomol. Chem.*, 2009, **7**, 5137–5145.
5. H. Yu, V. Thon, K. Lau, L. Cai, Y. Chen, S. Mu, Y. Li, P. G. Wang and X. Chen, *Chem. Commun. (Camb.)*, 2010, **46**, 7507–7509.

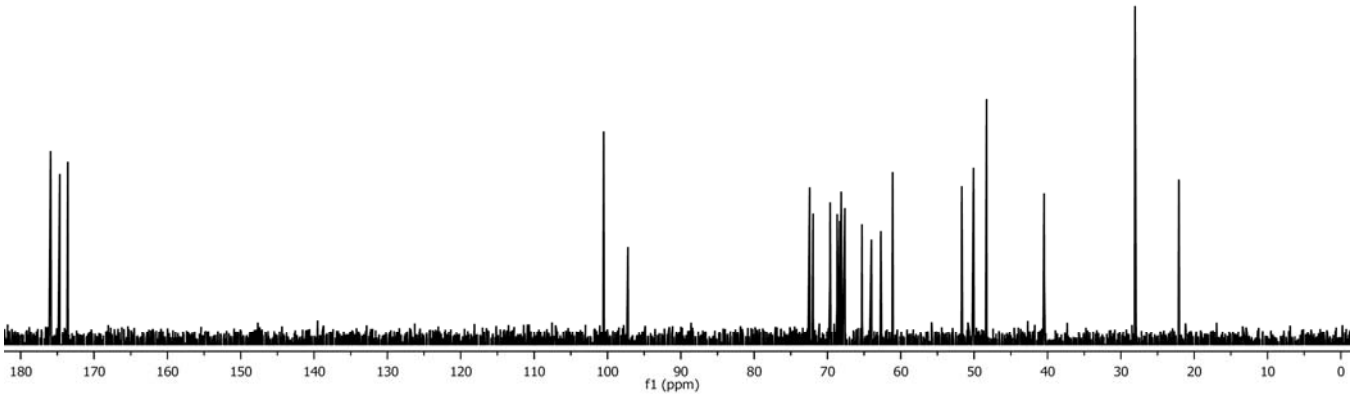
^{1}H & ^{13}C NMR of Neu5Acα2–6GalNAcαProN_3 **14**



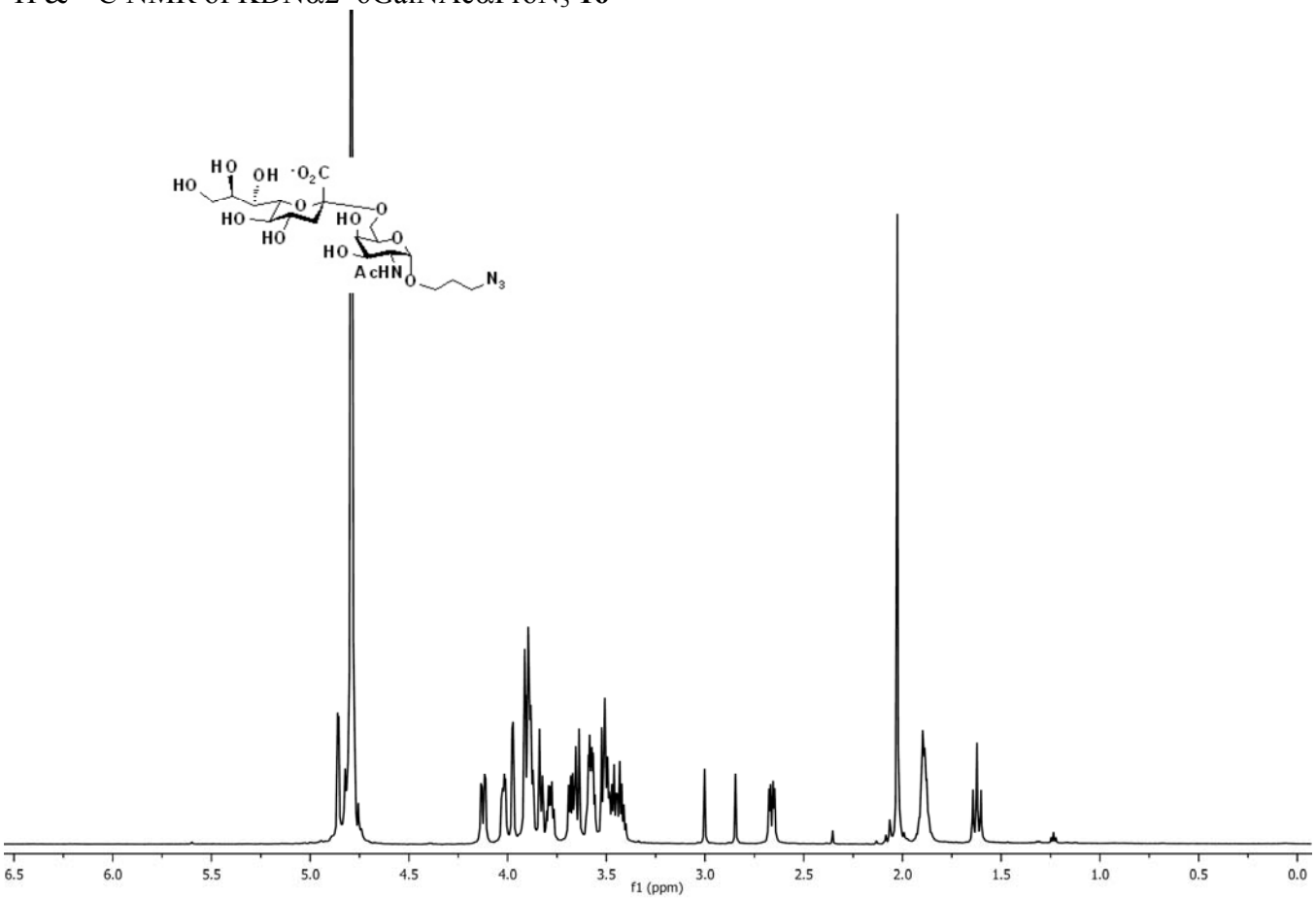


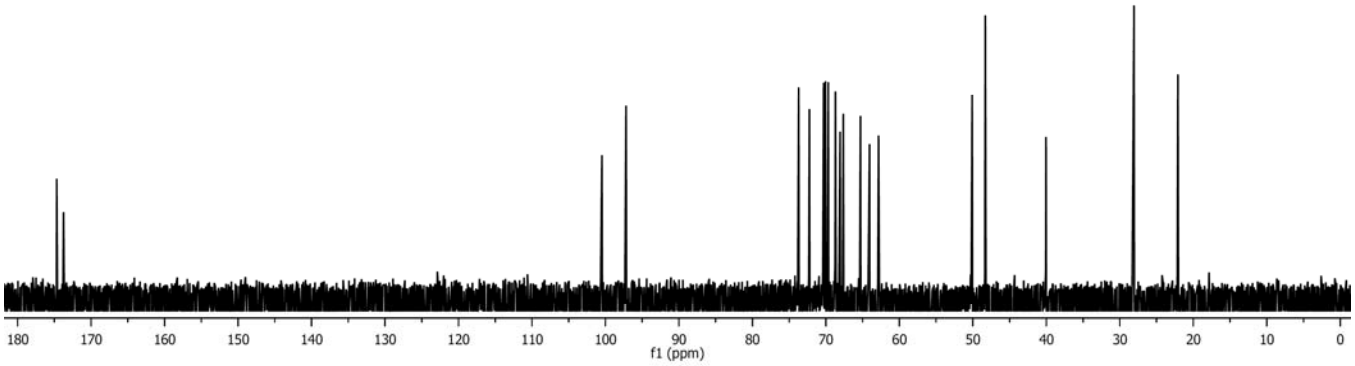
^{1}H & ^{13}C NMR of Neu5Gcα2–6GalNAcαProN_3 **15**



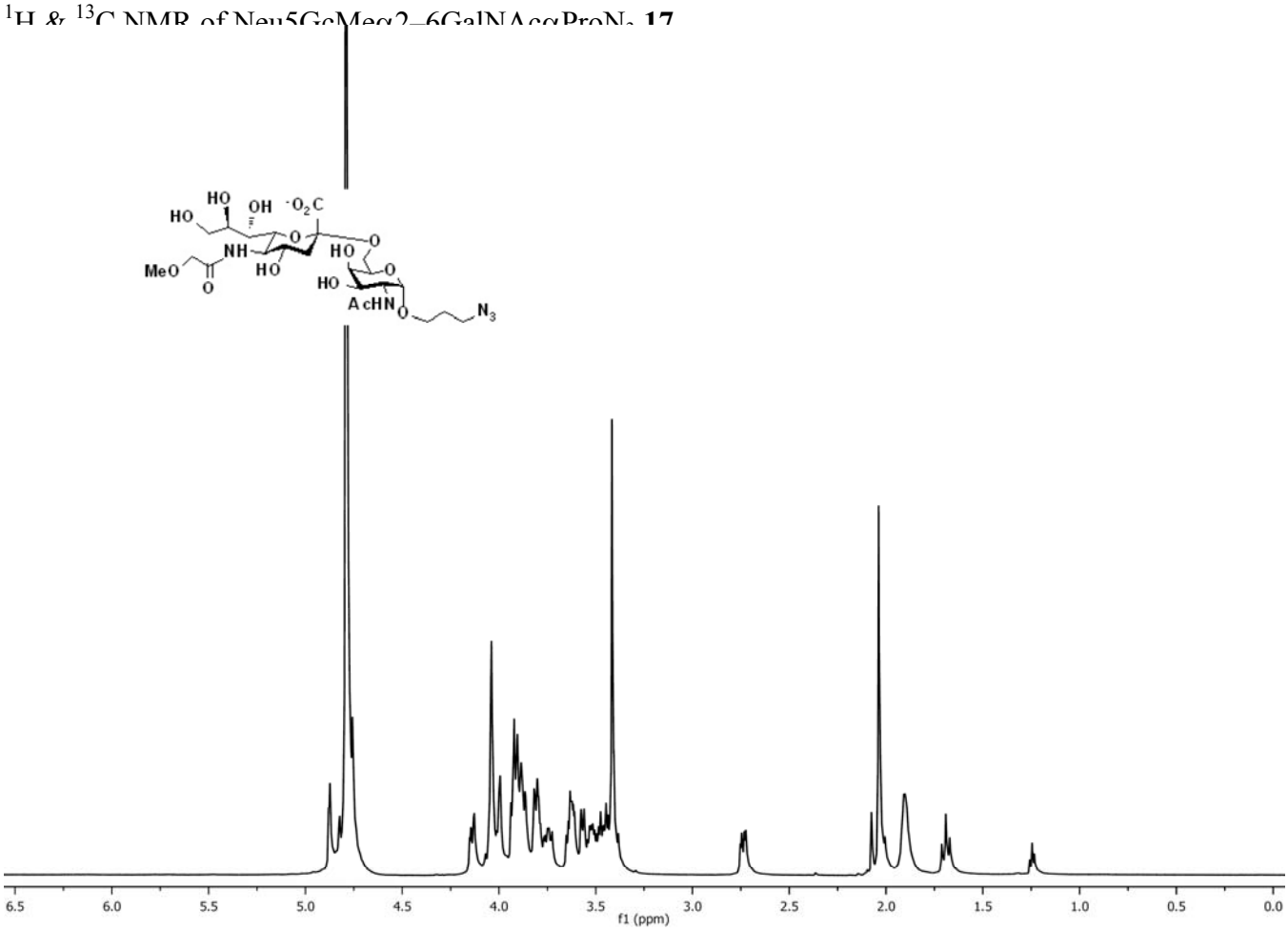


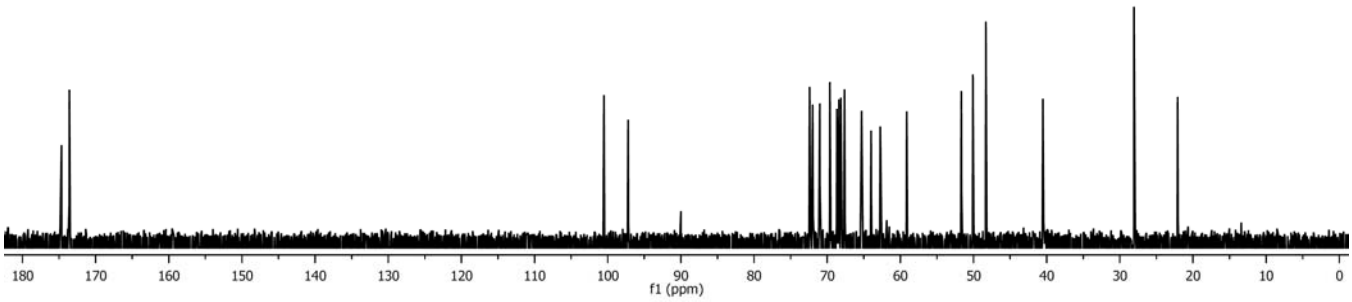
^{1}H & ^{13}C NMR of KDNα2–6GalNAcαProN_3 **16**



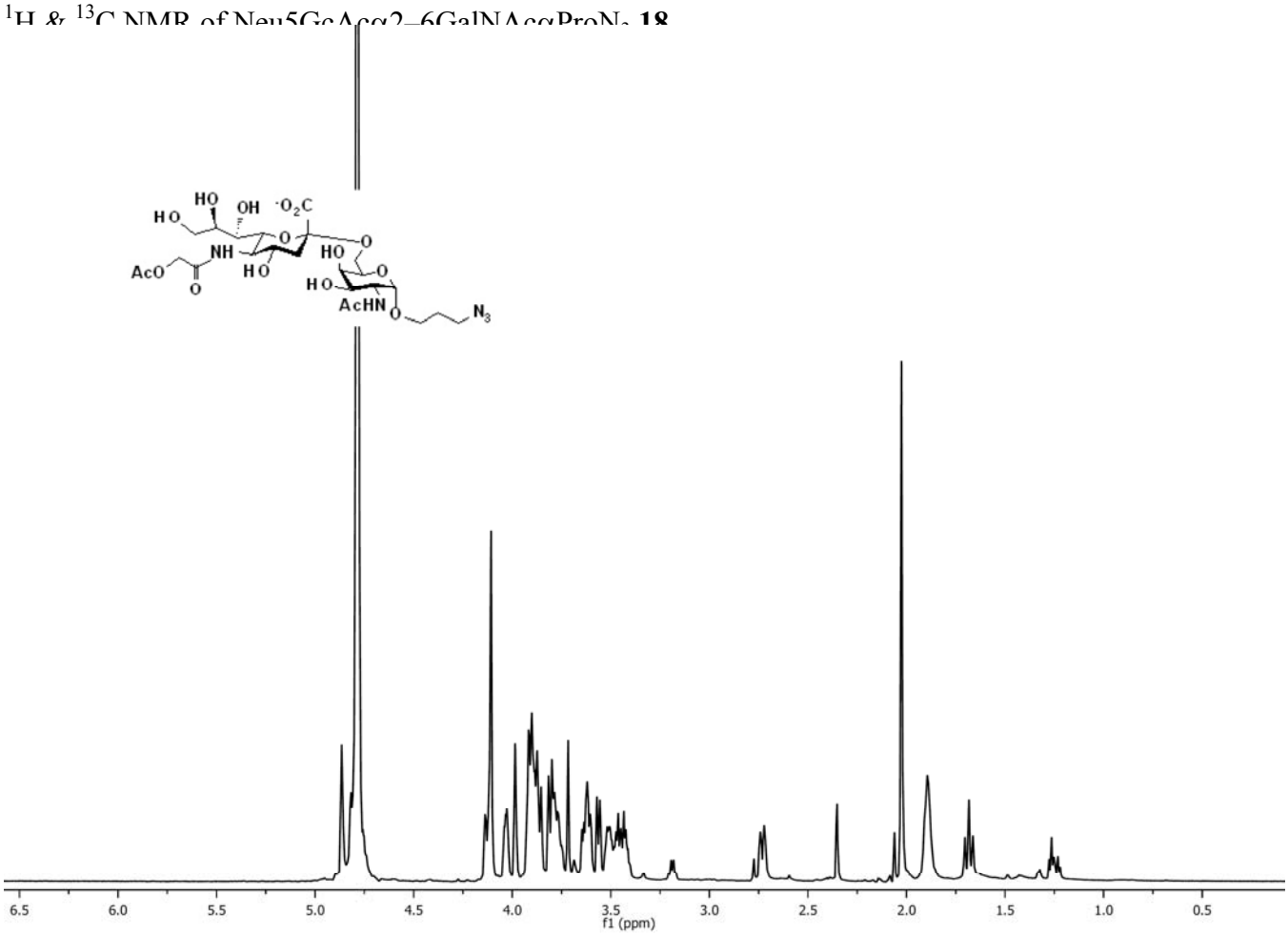


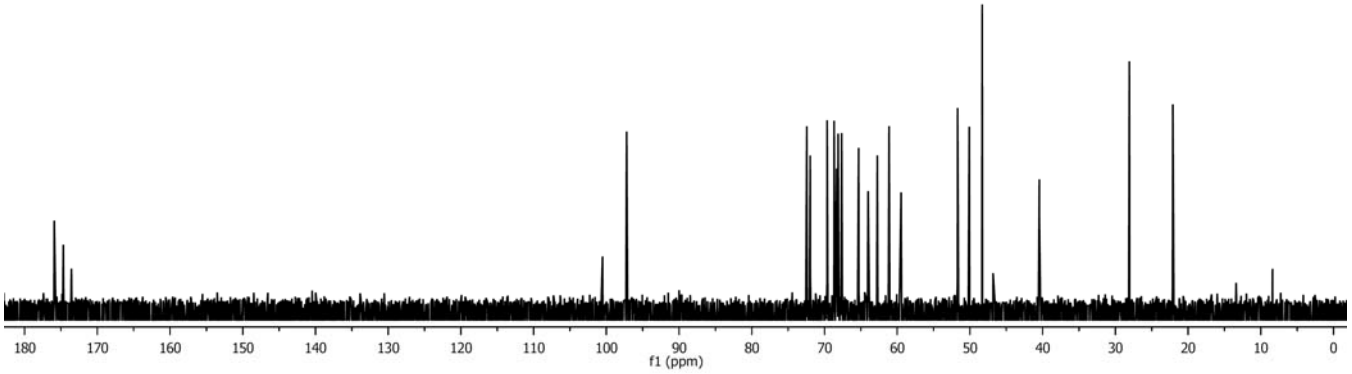
^{1}H & ^{13}C NMR of Neu5GcMeα2–6GalNAcαProN$_3$ **17**



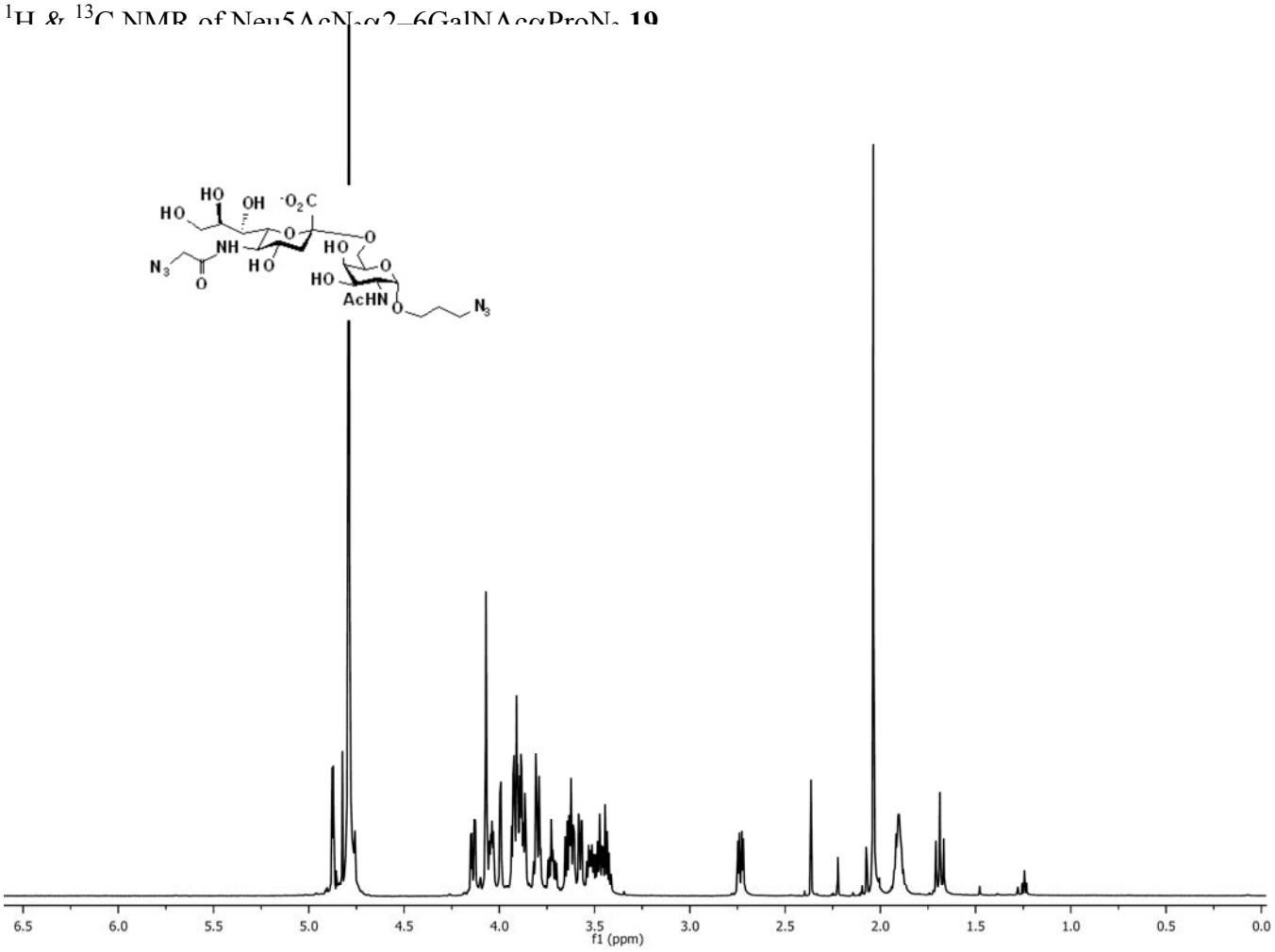


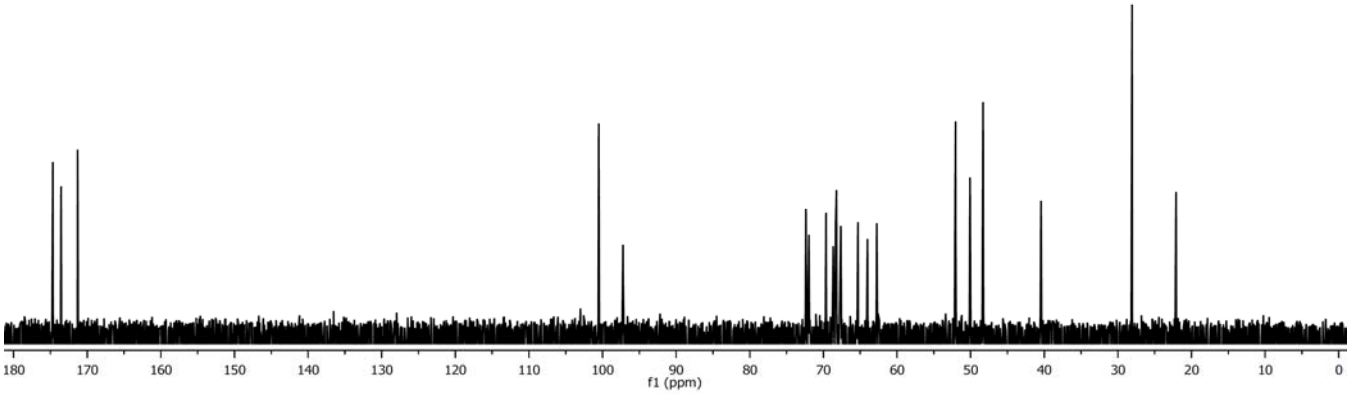
^{1}H & ^{13}C NMR of Neu5GcAcα2–6GalNAcαProN$_3$ **18**



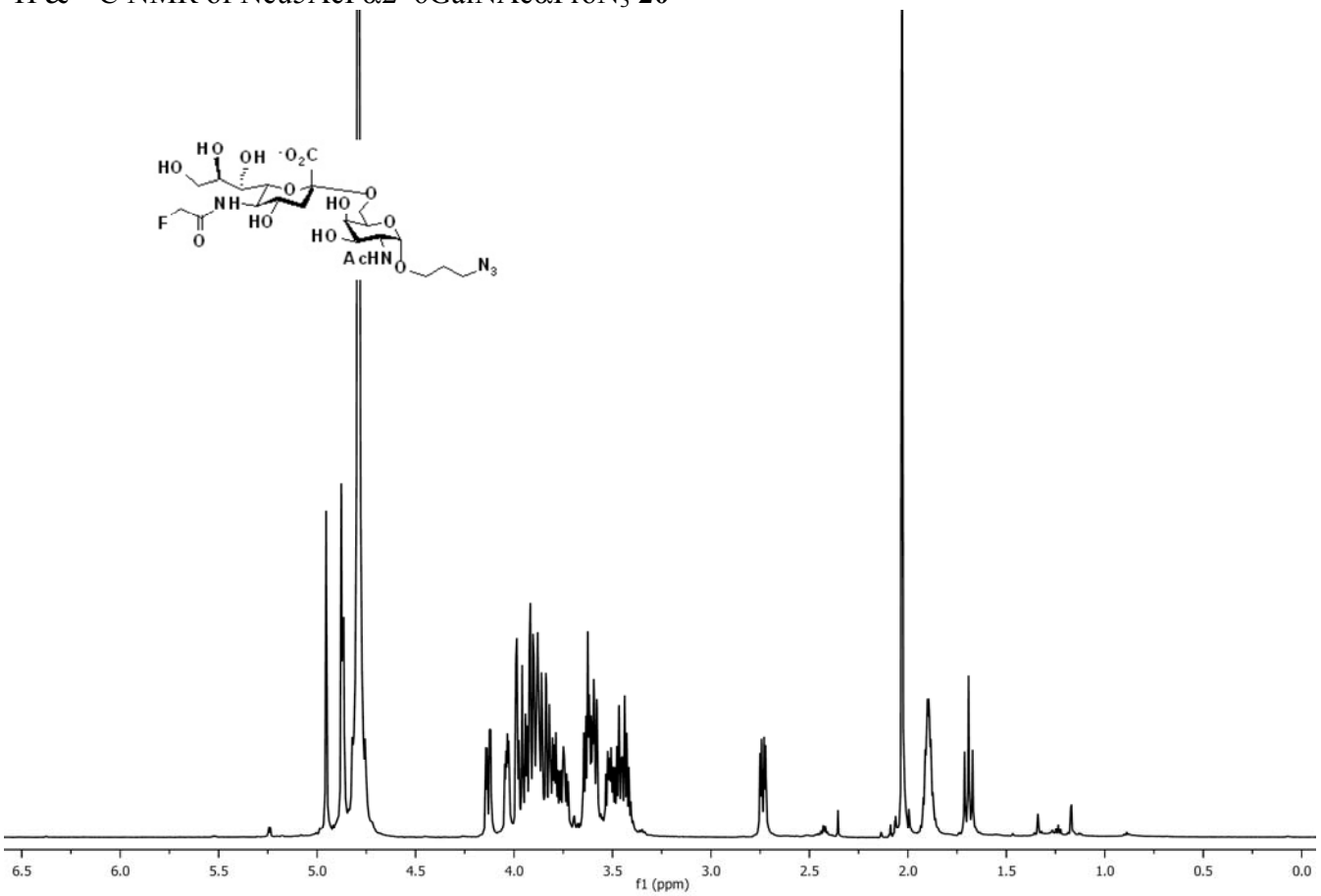


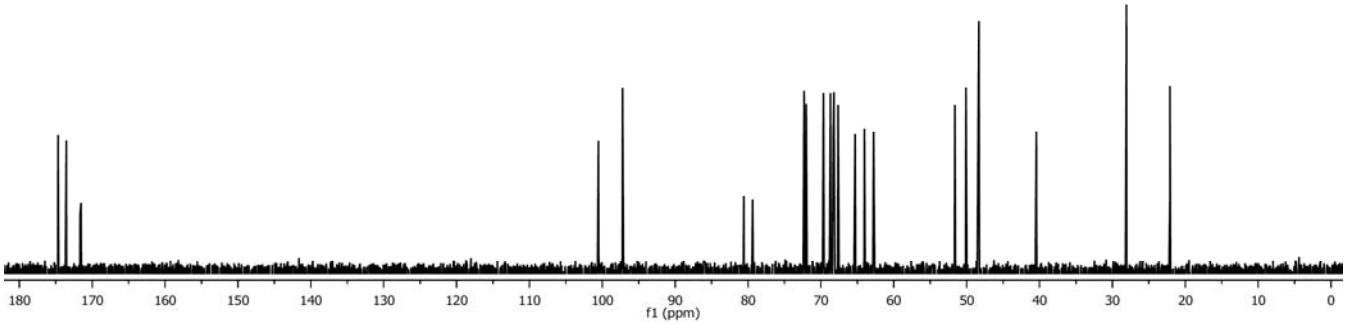
^{1}H & ^{13}C NMR of Neu5AcN$_3$α2–6GalNAcαProN$_3$ **19**



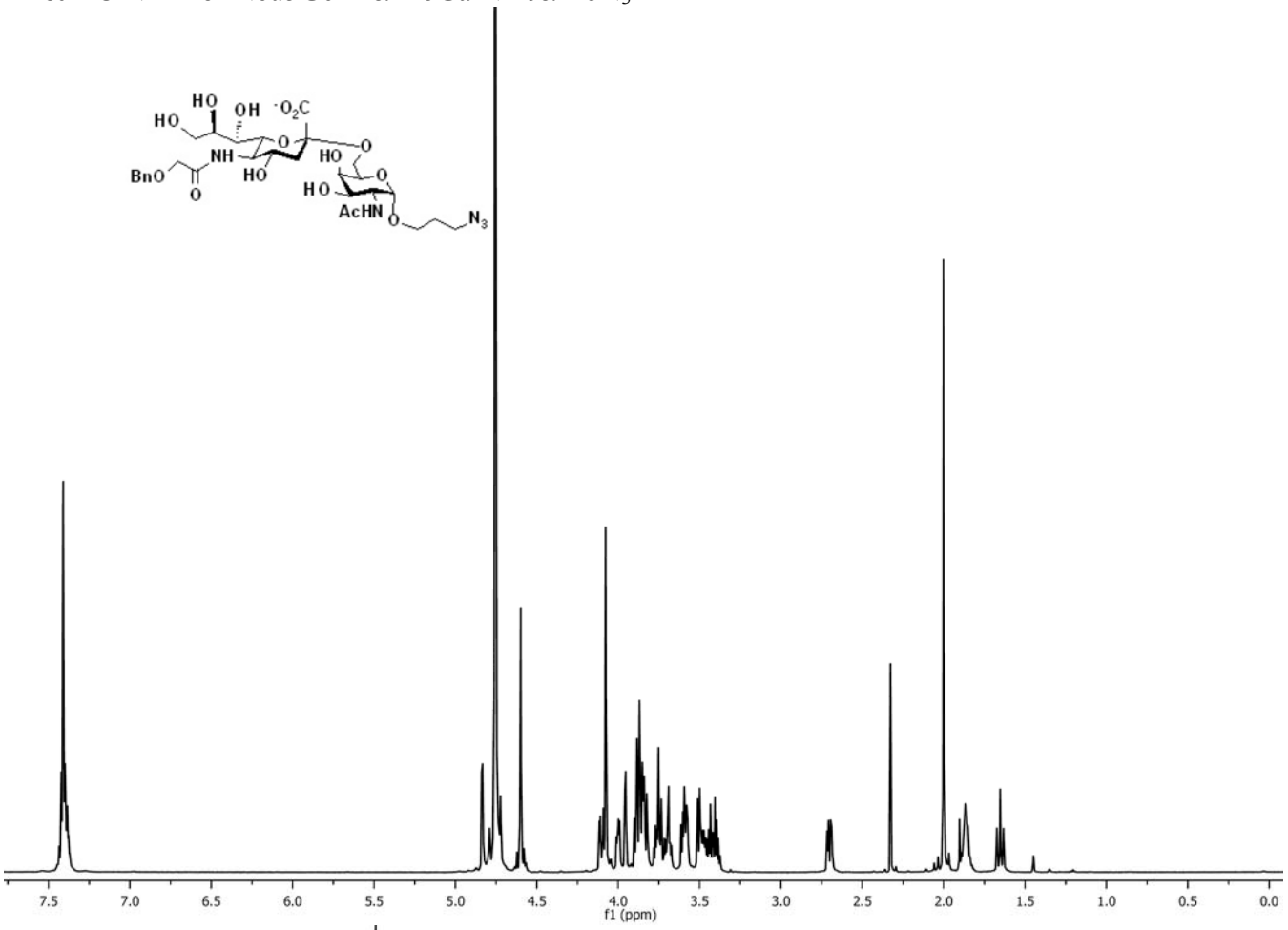


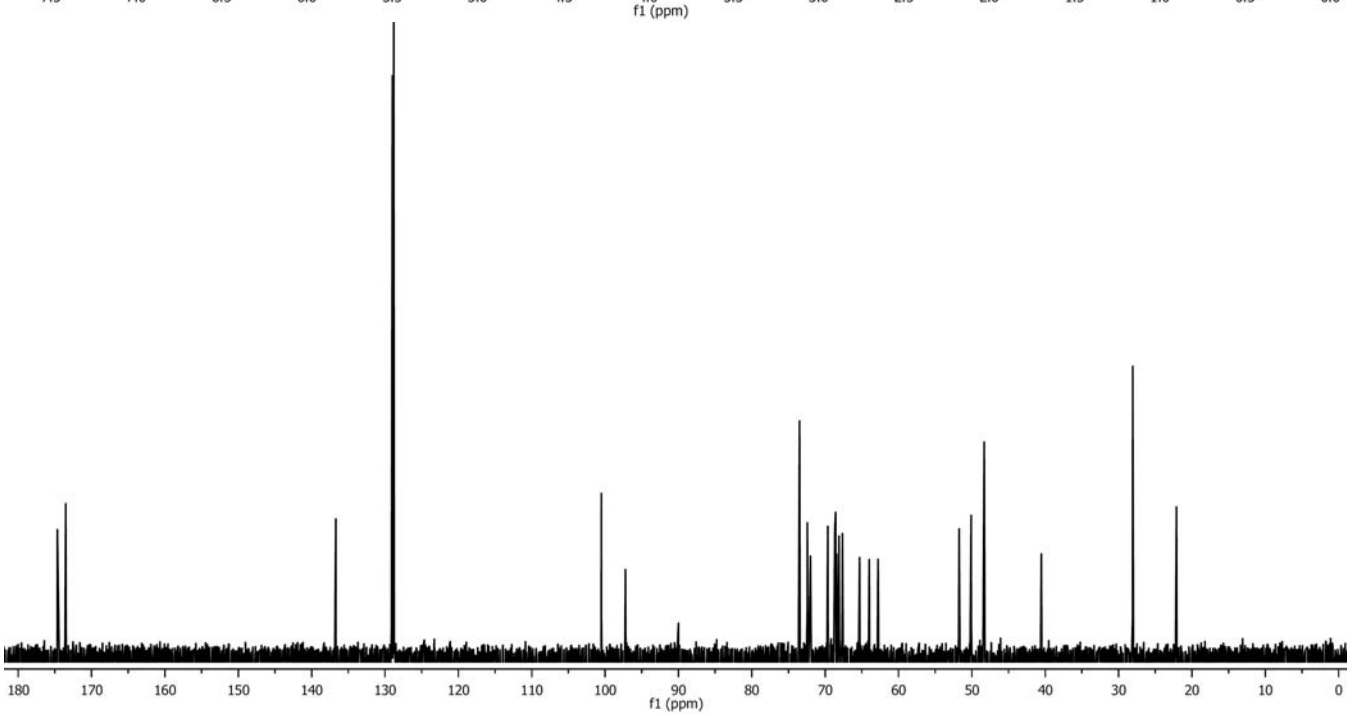
^{1}H & ^{13}C NMR of Neu5AcFα2–6GalNAcαProN$_3$ **20**



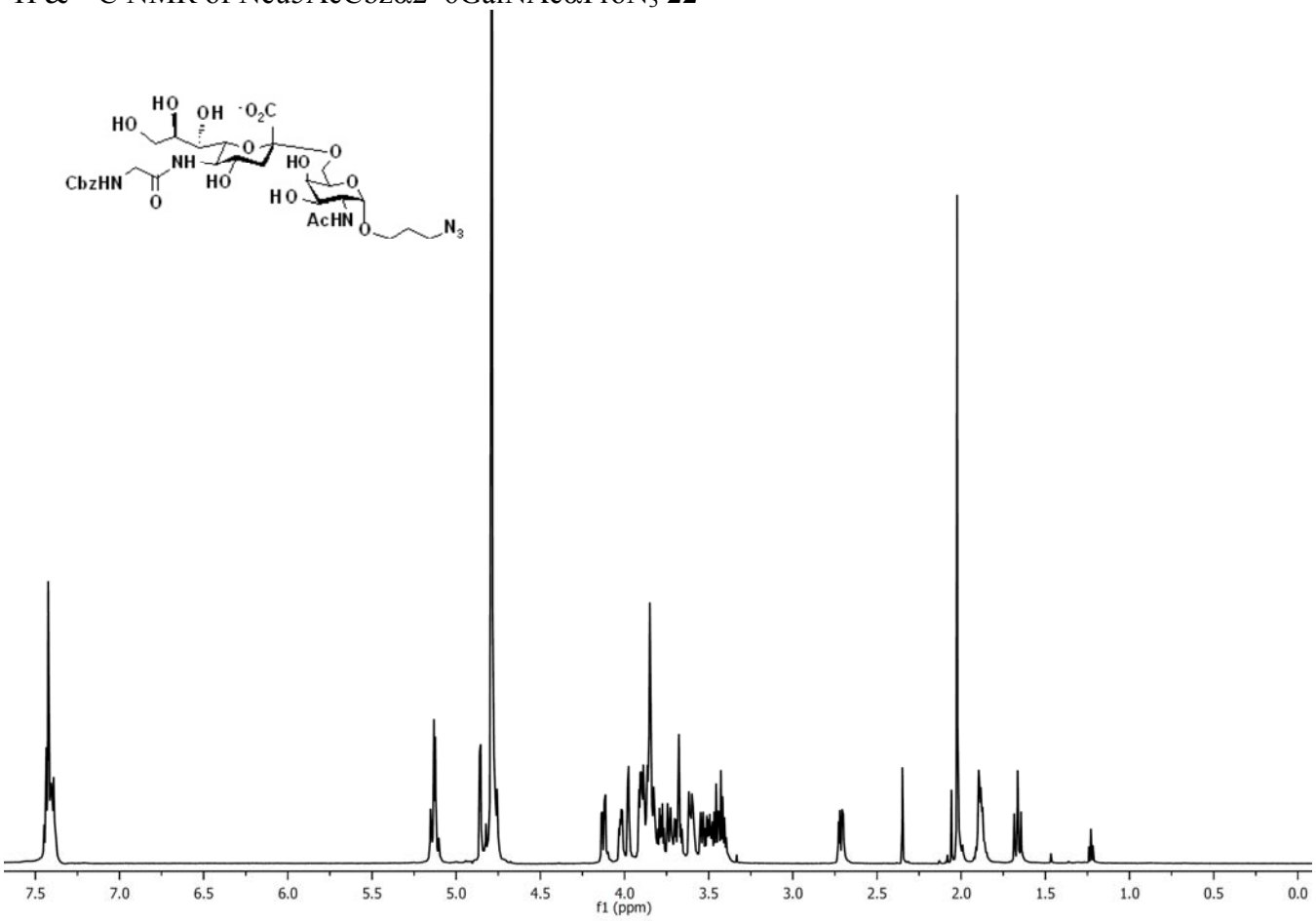


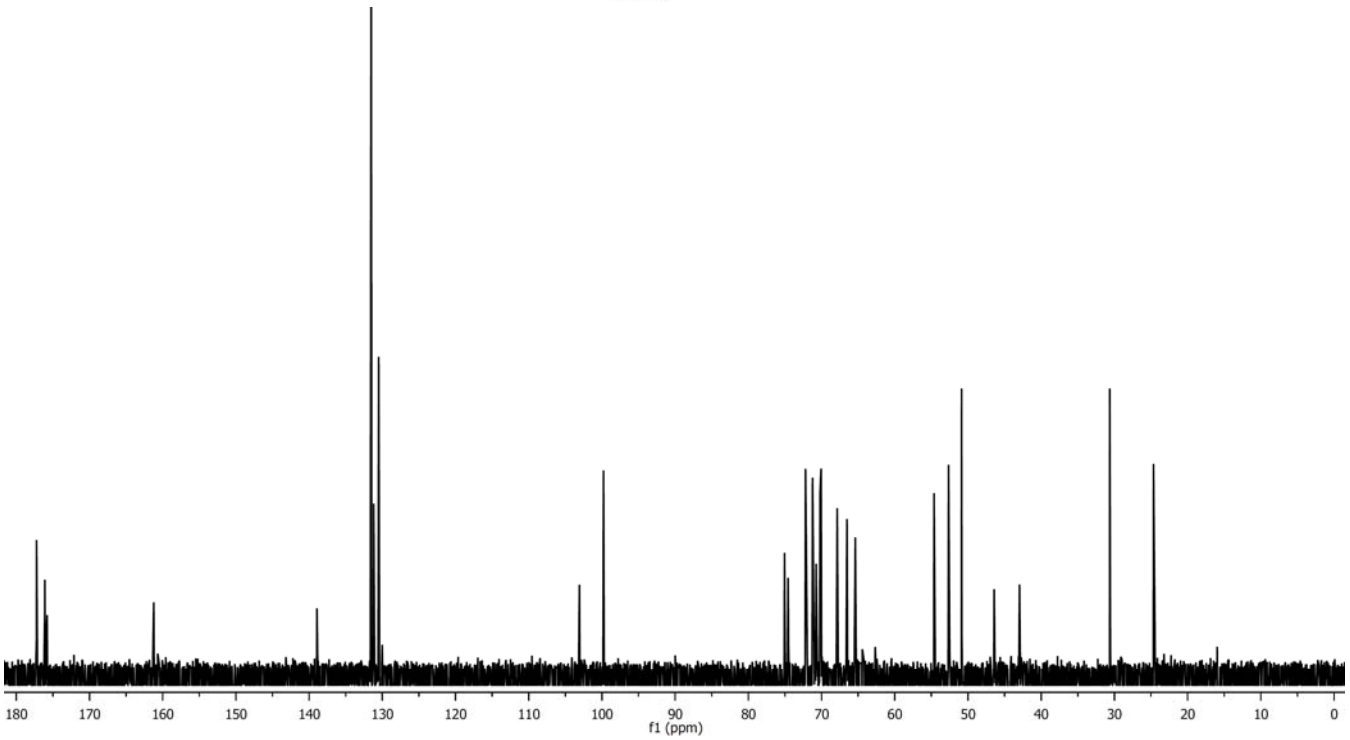
^{1}H & ^{13}C NMR of Neu5GcBnα2–6GalNAcαProN$_3$ **21**



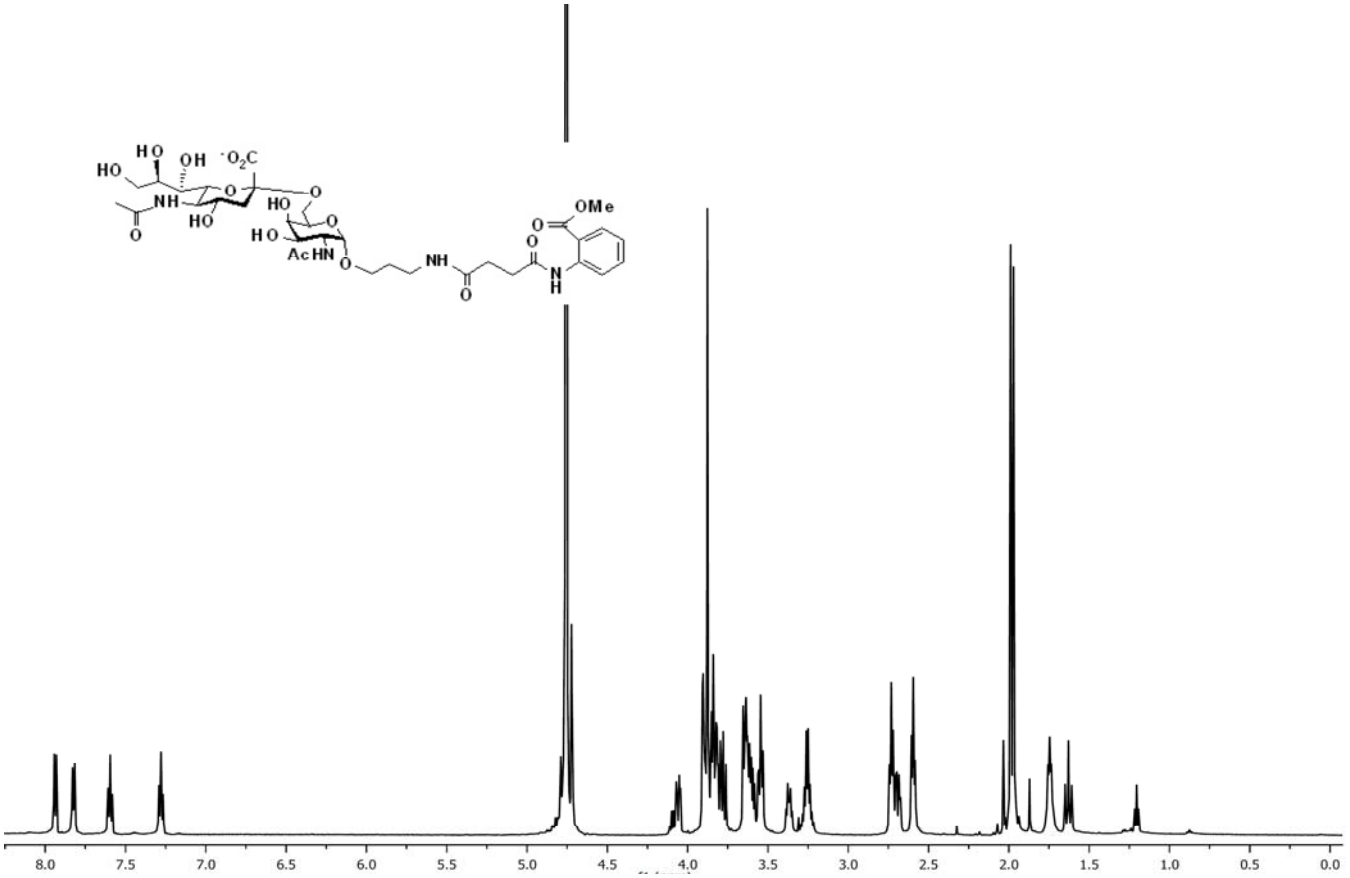


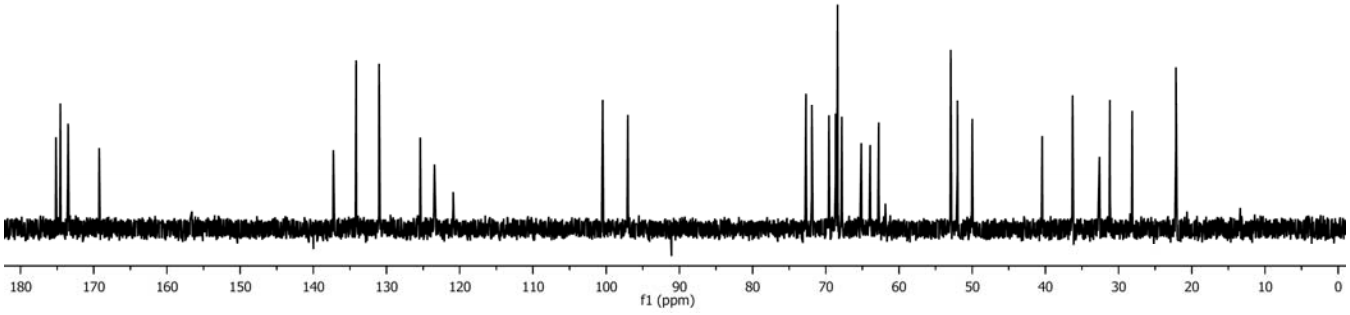
^{1}H & ^{13}C NMR of Neu5AcCbzα2–6GalNAcαProN_3 **22**



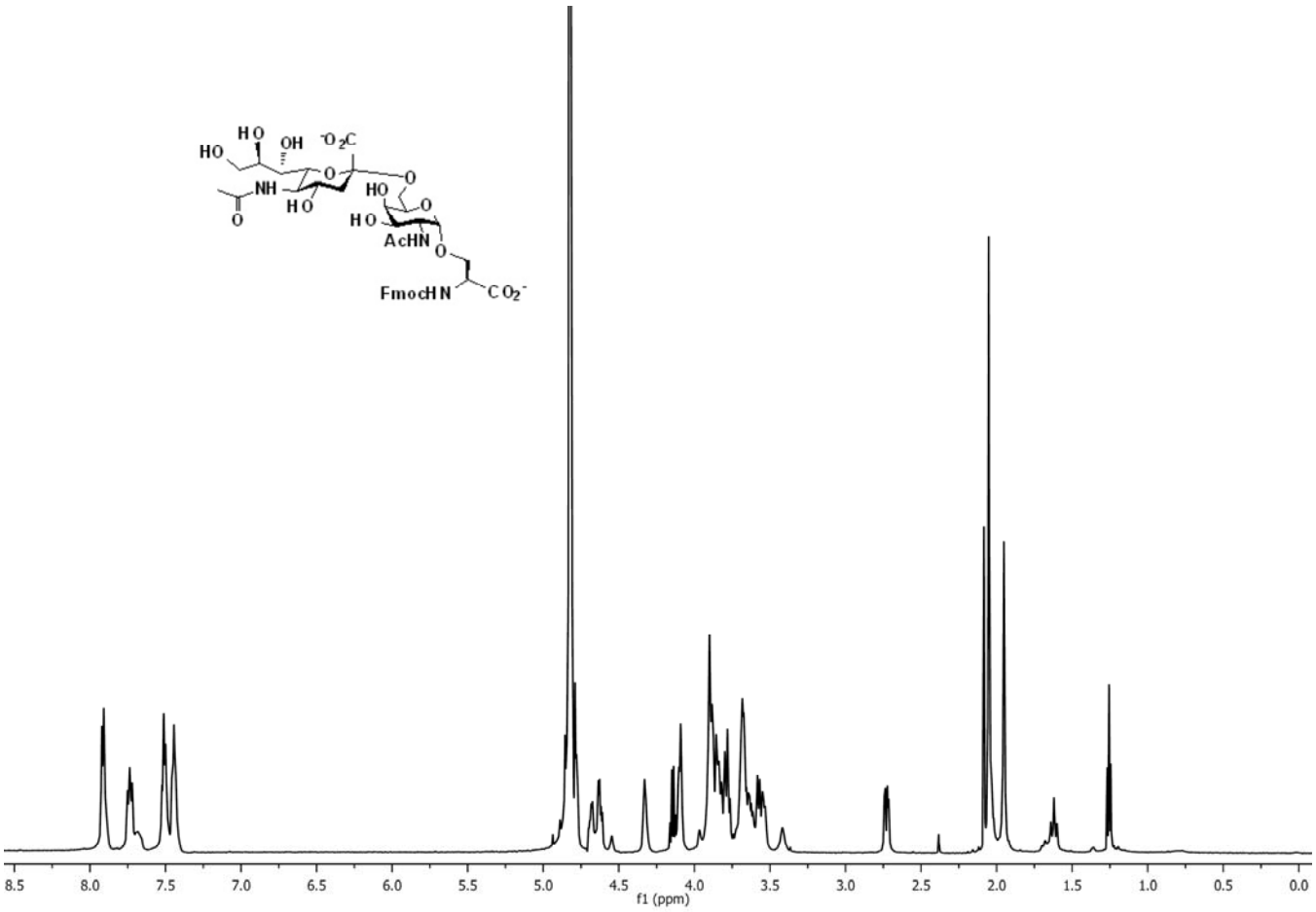


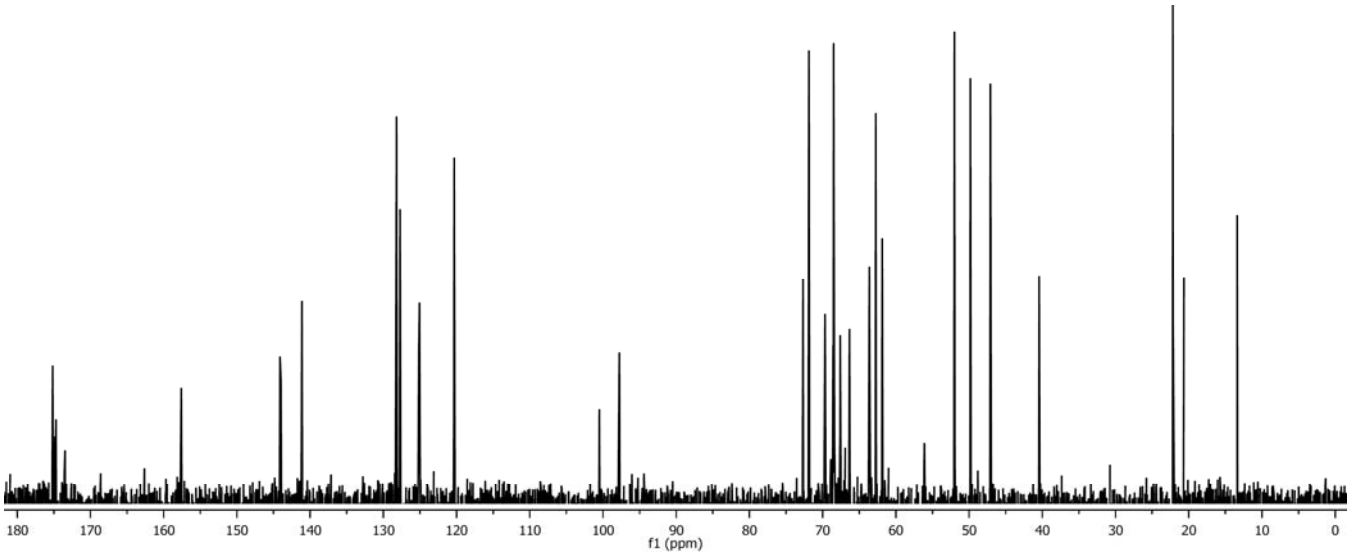
^{1}H & ^{13}C NMR of sialoside Neu5Acα2–6GalNAcα2AA **23**





^{1}H & ^{13}C NMR of sialoside Neu5Acα2–6GalNAcαSer **24**





^{1}H & ^{13}C NMR of sialoside Neu5Acα2–6GalNAcαThr **25**