Supporting Information

Cloning, expression, and purification of *Pasteurella multocida* UDP-glucose dehydrogenase (PmUgd)

Cloning of PmUgd-His$_6$ from *Pasteurella multocida* strain P-1059 (ATCC#15742). Forward primer 5'-GATCCATATG AAGAAAATTACAATTGCTGGGGC-3' (*Nde*I restriction site is underlined) and reverse primer 5'-CCGCTCGAG AGCATCACCGCCAAAAATATCTCTTG-3' (*Xho*I restriction site is underlined) were used for polymerase chain reaction (PCR) amplification of the full-length gene PmUgd from *Pm* strain P-1059 (ATCC#15742). PCRs were performed in a reaction mixture of 50 μL containing genomic DNA (10 ng), forward and reverse primers (0.2 μM each), 10 × Herculase buffer (5 μL), dNTP mixture (0.2 mM), and 5 U (1 μL) of Herculase-enhanced DNA polymerase. The reaction mixture was subjected to 30 cycles of amplification. After heating at 96°C for 2 min, 30 cycles including denature at 96 °C for 20 sec., annealing at 55 °C for 30 sec. and elongation at 72 °C for 1 min were carried out, followed by a final elongation at 72 °C for 7 min. The DNA obtained was digested with *Nde*I and *Xho*I and ligated with pET22b(+) that was pre-cut by the same pair of restriction enzymes. The ligated product was transformed into electrocompetent *E. coli* DH5α cells. Positive plasmids were selected and subsequently transformed into BL21 (DE3) chemically competent cells. DNA sequencing and deduced protein sequence showed that the obtained PmUgd has the same protein sequence as reported in GenBank accession number WP_005756855.

Overexpression of PmUgd-His$_6$. *E. coli* BL21 (DE3) harboring the recombinant plasmid was grown in LB rich medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) containing 100 μg/mL of ampicillin until the $OD_{600\ nm}$ reached 0.8–1.0. The expression of the protein was induced by adding 0.1 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) followed by incubation at 20 °C for 20 hours with vigorous shaking at 250 rpm in a C25KC incubator shaker (New Brunswick Scientific, Edison, NJ). Bacterial cells were harvested by centrifugation at 3,696 × *g* for 30 min at 4 °C in a Sorvall Legend RT centrifuge with a hanging bucket rotor. The cell pellet was resuspended in 20 mL of lysis buffer (pH 8.0, 100 mM Tris-HCl containing 0.1% Triton X-100) per liter cell culture. Lysozyme (1 mg/L culture) and DNaseI (50 μg/L culture) were then added to the cell suspension. After the mixture was incubated at 37 °C for 1 hour with vigorous shaking, cell lysate was separate from the inclusion bodies and other cellular debris by centrifugation (Sorvall RC-5B centrifuge with a S5-34 rotor) at 7,000 × *g* for 30 min at 4 °C.

Purification of PmUgd-His$_6$. Purification of the His$_6$-tagged protein from the lysate was achieved using an AKTA FPLC system (GE Healthcare) equipped with a HisTrap™ FF 5 mL column. The column was pre-equilibrated with 8 column volumes of binding buffer (5 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5) prior to the loading of the lysate. After loading the sample, the column was washed with 8 column volumes of washing buffer (40 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5). Protein elution was carried out with 8 column volumes of elute buffer (200 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5). The fractions containing the purified enzyme were collected and stored at 4 °C.

SDS-PAGE analysis of PmUgd-His$_6$. SDS-PAGE was performed in 12% Tris-glycine gels using Bio-Rad Mini-protein III cell gel electrophoresis unit (Bio-Rad, Hercules, CA) at DC = 120 V. Bio-Rad Low range SDS-PAGE standards were used as molecular weight standards. Gels were stained with Coomassie Blue (Figure S1).

Optimize storage condition of PmUgd-His$_6$. In order to efficiently apply PmUgd-His$_6$ in chemoenzymatic synthesis, an optimal storage condition is necessary. To do so, 1 mL of purified PmUgd-His$_6$ was dialyzed against Tris-HCl buffer (20 mM, pH 7.5), frozen, and dried using a lyophilizer. In addition, 6 mL of purified PmUgd-His$_6$ was be dialyzed against Tris-HCl buffer (20

mM, pH 7.5) containing 10% glycerol. After the dialysis, the dialyzed PmUgd-His_6 (1 mL each) was stored at 4 °C or -20 °C. For the remaining 4 mL of the dialyzed enzyme, glycerol was added to 1 mL of aliquots to make 20%, 30%, 40%, and 50% of glycerol solutions, respectively. The solutions were stored at -20 °C. The activity of each PmUgd-His_6 preparation in converting UDP-Glc to UDP-GlcA was tested after 1 day, 1 week, 2 weeks, 3 weeks, and 4 weeks using small scale assays. Enzyme solutions containing 40% or 50% glycerol with storage at -20 °C were found to be the best among all storage conditions tested.

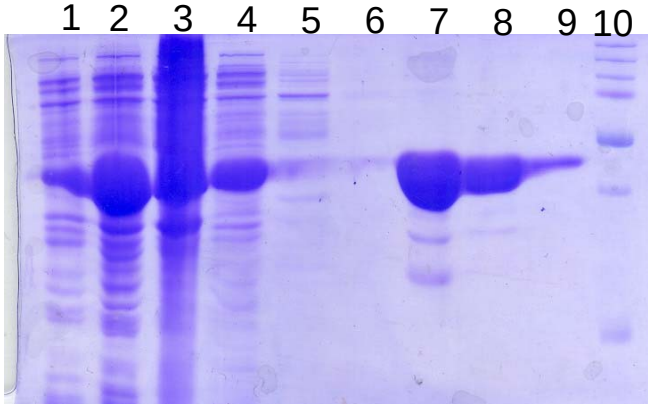


Figure S1. SDS-PAGE (12% Tris-Glycine gel) analysis of PmUgd-His_6. Lanes: 1, whole cells, before IPTG induction; 2, whole cells, after IPTG induction; 3, inclusion bodies, after induction; 4, lysate, after induction; 5, wash fraction 1; 6, wash fraction 2; 7, elute fraction 1; 8, elute fraction 2; 9, elute fraction 3; 10, protein standards (Low range SDS-PAGE Standards, Bio-Rad).

DNA sequence of PmUgd-His_6 (**Note:** The sequence for His_6-tag is underlined)

ATGAAGAAAATTACAATTGCTGGGGCTGGCTATGTTGGTTTATCCAATGCAGTATTATTAGCTCAACA
CCACAATGTGATCTTATTAGATATTGATCAAAATAAAGTTGATTTAATTAATAATAAAAAATCGCCCA
TCACAGATAAAGAAATCGAAGATTTCTTACAAAATAAATCACTGACAATGATGGCAACAACAGATAAA
GAAGTGGCATTAAAAAACGCAGACTTTGTCATCATCGCAACGCCAACAGACTATAATACCGAAACAGG
TTATTTTAATACATCCACTGTTGAAGCTGTCATTGAACAAACCCTTTCAATCAATCCACAAGCAACGA
TTATTATAAAATCAACGATTCCCGTTGGTTTTACCGAAAAAATGCGTGAGAAATTTCATACCAAGAAC
ATTATTTTTTCTCCTGAGTTTTTAAGAGAAGGAAAAGCACTTCATGACAATTTGTTTCCAAGCAGAAT
TATTGTTGGCAGTACTTCTTATCAAGCAAAAGTATTTGCCGATATGTTGACACAGTGTGCCAGAAAAA
AAGATGTAACTGTTTTATTTACACACAATACTGAGGCTGAAGCTGTTAAATTATTTGCAAATACGTAT
CTCGCAATGCGAGTTGCCTTTTTTAATGAATTAGATACTTATGCGAGTCTTCACCATTTAAATACAAA
AGACATTATCAATGGTATTTCTACTGATCCTCGCATTGGTACACACTACAATAACCCAAGTTTCGGCT
ATGGCGGTTATTGTTTACCCAAAGACACTAAACAGTTACTGGCTAACTATGCTGACGTACCTCAAAAT
CTCATTGAAGCCATTGTCAAATCTAATGAAACCAGAAAACGTTTCATTACTCATGATGTATTAAATAA
GAAACCTAAAACTGTTGGTATTTATCGTTTAATCATGAAGTCAGGTTCTGATAACTTCAGAGCTTCTG
CTATTCTCGATATTATGCCGCATCTCAAAGAAAACGGTGTTGAGATTGTGATTTATGAGCCAACCTTA
AATCAACAGGCATTTGAGGACTACCCCGTTATTAATCAACTCTCTGAATTTATTAATCGCTCTGATGT
CATTCTCGCTAATCGTTCTGAGCCAGATTTAAATCAATGTTCCCATAAAATCTATACAAGAGATATTT
TTGGCGGTGATGCTCTCGAGCACCACCACCACCACCACTGA

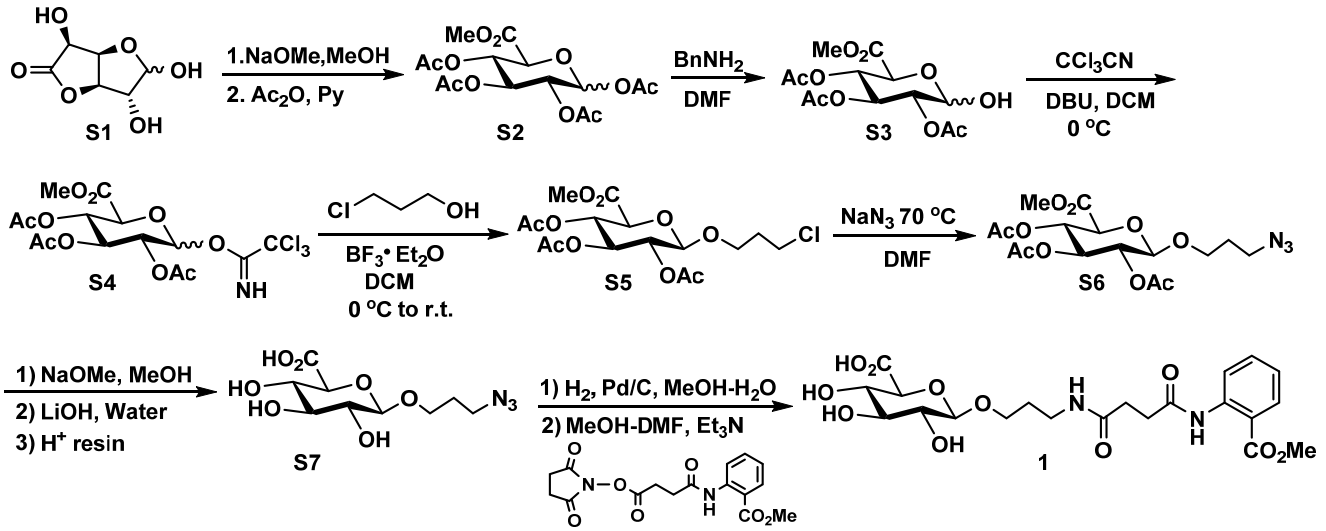
Protein sequence of PmUgd-His_6 (**Note:** The sequence for His_6-tag is underlined)

MKKITIAGAGYVGLSNAVLLAQHHNVILLDIDQNKVDLINNKKSPITDKEIEDFLQNKSLTMMATTDK
EVALKNADFVIIATPTDYNTETGYFNTSTVEAVIEQTLSINPQATIIIKSTIPVGFTEKMREKFHTKN
IIFSPEFLREGKALHDNLFPSRIIVGSTSYQAKVFADMLTQCARKKDVTVLFTHNTEAEAVKLFANTY
LAMRVAFFNELDTYASLHHLNTKDIINGISTDPRIGTHYNNPSFGYGGYCLPKDTKQLLANYADVPQN
LIEAIVKSNETRKRFITHDVLNKKPKTVGIYRLIMKSGSDNFRASAILDIMPHLKENGVEIVIYEPTL
NQQAFEDYPVINQLSEFINRSDVILANRSEPDLNQCSHKIYTRDIFGGDALEHHHHHH

General methods for compound purification and characterization

Chemicals were purchased and used without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian VNMRS 600 MHz and Bruker Avance 800 MHz spectrometers. MALDI-TOF analysis of samples was carried out using an Applied Biosystems 4700 MALDI TOF/TOF and high resolution electrospray ionization (HR-ESI) mass spectra were obtained using Thermo Electron LTQ-Orbitrap Hybrid MS at the Mass Spectrometry Facility in the University of California, Davis. Silica gel 60 Å (Sorbent Technologies) was used for silica gel column chromatography. Analytical thin-layer chromatography (Sorbent Technologies) was performed on silica gel plates using anisaldehyde sugar stain for detection. Gel filtration chromatography was performed with a column (100 cm × 2.5 cm) packed with BioGel P-2 Fine resins. ATP, UTP, GlcNAc, Glc-1-P, NAD^{+}, and glucuronolactone were purchased from Sigma. GlcNTFA, GlcNAc6N_3, UDP-GlcNGc, UDP-GlcNAz, UDP-GlcNAc6NGc were synthesized as described previously.[1] NanK_ATCC55813,[2] PmGlmU,[1] PmPpA,[1, 3] and PmHS2[4] were overexpressed as reported.

Chemical synthesis of GlcAβ2AA (1)



Synthesis of methyl 1,2,3,4-tetra-*O*-acetyl-D-glucopyranuronate **S2**:

Glucuronolactone **S1** (2.0 g, 11.3 mmol) was dissolved in dry MeOH (12 mL) under N_2. To the solution, 20 mg of sodium methoxide was added. The reaction was stirred at room temperature for 3 h, and MeOH was removed *in vacuo*. The resulting syrup was dried under high-vacuum. The above product was dissolved in pyridine (10 mL) and acetic anhydride (8 mL) under N_2 at 0 °C. The reaction was stirred for overnight with temperature slowly increased from 0 °C to room temperature. The mixture was concentrated and purified by silica gel column chromatography (Hexane:EtOAc: = 1:1 by volume) to produce a white solid **S2** in 67% yield. β-isomer: ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.76 (d, *J* = 7.8 Hz, 1H), 5.30 (t, *J* = 9.6 Hz, 1H), 5.25 (t, *J* = 9.6 Hz, 1H), 5.13 (t, *J* = 7.8 Hz, 1H), 4.17 (d, *J* = 9.6 Hz, 1H), 3.73 (s, 3H), 2.10 (s, 3H), 2.03 (s, 6H), 2.02 (s, 3H). ^{13}C NMR (150 MHz, D_2O) δ 170.13, 169.65, 169.53, 168.61, 167.37, 88.90, 70.51, 69.24, 69.07, 69.00, 53.17, 20.95, 20.79, 20.61, 20.55. α-isomer: ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.39 (d, *J* = 3.6 Hz, 1H), 5.51 (t, *J* = 10.2 Hz, 1H), 5.22 (t, *J* = 10.2 Hz, 1H), 5.12 (dd, *J* = 10.2, 3.6 Hz, 1H), 4.41 (d, *J* = 10.2 Hz, 1H), 3.74 (s, 3H), 2.15 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 2.01 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.04, 169.56, 169.32, 168.98, 166.95, 91.48, 73.11, 71.94, 70.27, 69.06, 53.18, 20.93, 20.72, 20.70, 20.63.

Synthesis of methyl 2,3,4-tri-*O*-acetyl-D-glucopyranuronate **S3**:

Methyl 1,2,3,4-tetra-*O*-acetyl-D-glucopyranuronate **S2** (1.2 g, 3.2 mmol) was dissolved in dry DMF (10 mL) under N_2. To the solution, benzylamine (0.42 mL, 3.8 mmol) was added. The mixture was stirred at room temperature for 16 h. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (Hexane:EtOAc = 1:2 by volume) to produce a white solid **S3** in 84% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.50–5.55 (m, 1H), 5.11–5.27 (m, 1H), 4.85–4.91 (m, 1H), 5.39–4.56 (m, 2H), 3.70–3.72 (m, 3H), 1.99–2.05 (m, 9H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.68, 170.45, 170.34, 170.30, 169.94, 169.81, 168.77, 167.83, 95.59, 90.36, 72.98, 72.66, 71.81, 70.97, 69.73, 69.61, 69.34, 68.12, 53.21, 53.11, 20.85, 20.84, 20.77, 20.70, 20.66, 20.65.

Synthesis of methyl 2,3,4-tetra-*O*-acetyl-1-*O*-(3-chloropropyl)-β-D-glucopyranuronate **S5**:

Methyl 2,3,4-tri-*O*-acetyl-D-glucopyranuronate **S3** (800 mg, 2.4 mmol) was dissolved in 8 mL of dichloromethane. Trichloroacetonitrile (1.3 mL, 12 mmol) was added under N_2. After being cooled down to 0 °C, 1,8-diazabicyclo[5.4.0] undee-7-ene (1,8-DBU) was added in a drop-wise manner until the color of solution was changed to brown. The reaction mixture was allowed to stir for 1 h before being concentrated to form a sticky dark brown residue. The silica gel column chromatography (Hexane:EtOAc = 3:2 by volume) produced a white-colored product **S4** in 88% yield. To the mixture of **S4** (200 mg, 0.42 mmol) and MS 4Å, 8 mL of dichloromethane was added, followed by the addition of 3-chloropropanol (0.25 mL, 2.1 mmol). The mixture was stirred for 30 min at room temperature under N_2. After being cooled down to 0 °C, boron trifluoride ether complex (0.06 mL, 0.42 mmol) was added in a drop-wise manner. The reaction was stirred at 0 °C for 3 h. After the TLC showed the completion of the reaction, the mixture was filtered and the filtrate was washed with saturated $NaHCO_3$. The organic layer was rotavapored to produce a crude residue which was purified by silica gel column chromatography (Hexane:EtOAc: = 3:2 by volume) to provide product **S5** in 64% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.17–5.28 (m, 2H), 4.97–5.00 (dd, *J* = 9.6, 7.8 Hz, 1H), 4.53–4.54 (d, *J* = 7.8 Hz, 1H), 4.02–4.04 (d, *J* = 9.6 Hz, 1H), 3.99–4.01 (dd, *J* = 9.6, 4.8 Hz, 1H), 3.27 (s, 3H), 3.66–3.70 (m, 1H), 3.57–3.59 (m, 2H), 2.05–2.09 (m, 1H), 2.04 (s, 3H), 2.01 (s, 3H), 2.00 (s, 3H), 1.91–1.95 (m, 1H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.30, 169.59, 169.52, 167.37, 101.21, 72.78, 72.14, 71.33, 69.62, 66.79, 53.13, 41.48, 32.29, 20.83, 20.82, 20.71.

Synthesis of methyl 2,3,4-tetra-*O*-acetyl-1-*O*-(3-azidopropyl)-β-D-glucopyranuronate **S6**:

Methyl 2,3,4-tetra-*O*-acetyl-1-*O*-(3-chloropropyl)-β-D-glucopyranuronate **S5** (412 mg, 1.0 mmol) was dissolved in 10 mL of DMF. To the solution, sodium azide (325 mg, 5.0 mmol) was added. The reaction was stirred at 65 °C for overnight. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (Hexane:EtOAc: = 3:2 by volume) to produce a white solid in 92% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.19–5.27 (m, 2H), 4.99–5.02 (t, *J* = 7.8 Hz, 1H), 4.54–4.55 (d, *J* = 7.8 Hz, 1H), 4.02–4.04 (d, *J* = 9.6 Hz, 1H), 3.94–3.98 (m, 1H), 3.75 (s, 3H), 3.58–3.62 (m, 1H), 3.32–3.39 (m, 2H), 2.04 (s, 3H), 2.01 (s, 3H), 1.78–1.89 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.27, 169.53, 169.40, 167.34, 101.01, 72.82, 72.23, 71.39, 69.60, 66.92, 53.09, 48.10, 29.11, 20.81, 20.79, 20.68.

Synthesis of 1-*O*-(3-azidoopropyl)-β-D-glucopyranuronic acid **S7**:

Methyl 2,3,4-tetra-*O*-acetyl-1-*O*-(3-azidoopropyl)-β-D-glucopyranuronate **S6** (350 mg, 0.84 mmol) was dissolved in 5 mL of MeOH. To the solution, sodium methoxide was added until the pH reached

9.5. The reaction mixture was stirred at room temperature for 1 h. After the TLC showed the completion of the reaction, potassium hydroxide (60 mg, 2.52 mmol) and water (10 mL) were added. After being stirred at room temperature for 3 h, the reaction mixture was neutralized with DOWEX HCR-W2 (H^+) resin, filtered, and concentrated. The residue was purified by silica gel column chromatography (EtOAc:MeOH:H_2O = 6:2:1 by volume) to produce a white solid **S7** in 79% yield. ^{1}H NMR (600 MHz, D_2O) δ 4.45–4.47 (d, *J* = 7.8 Hz, 1H), 3.95–3.99 (m, 1H), 3.81–3.82 (d, *J* = 9.0 Hz, 1H), 3.71–3.75 (m, 1H), 3.49–3.54 (m, 2H), 3.42–3.45 (t, *J* = 6.6 Hz, 2H), 3.28–3.31 (t, *J* = 8.4 Hz, 1H), 1.86–1.91 (m, 2H). ^{13}C NMR (150 MHz, D_2O) δ 175.17, 102.36, 75.91, 75.64, 73.06, 71.86, 67.57, 48.05, 28.39.

Synthesis of GlcAβ2AA (**1**)

1-*O*-(3-Azidopropyl)-β-D-glucopyranuronic acid **S7** (100 mg, 0.44 mmol) was dissolved in MeOH (10 mL) and 20 mg of Pd/C was added. The mixture was shaken under H_2 gas (4 Bar) for 1 hour, filtered, and concentrated. The residue obtained was dried in high-vacuum and dissolved in 10 mL of DMF-MeOH (1:1 by volume). Dry triethylamine (61 μL) was added under N_2. Then 2-(methoxycarbonyl) succinanilic acid NHS ester[5] (2AA-OSu, 306 mg, 0.88 mmol) was added at 0 °C. The reaction mixture was stirred at room temperature for overnight and was concentrated by rotary evaporation. The residue obtained was purified by silica gel column chromatography (EtOAc:MeOH:H_2O = 8:2:1 by volume) to produce a white solid (**1**) in 83% yield. ^{1}H NMR (600 MHz, D_2O) δ 7.88–7.89 (d, *J* = 8.4 Hz, 1H), 7.79–7.80 (d, *J* = 7.8 Hz, 1H), 7.48–7.51 (t, *J* = 7.8 Hz, 1H), 7.12–7.15 (t, *J* = 7.8 Hz, 1H), 4.27–4.29 (d, *J* = 7.8 Hz, 1H), 3.83–3.86 (m, 1H), 3.81 (s, 3H), 3.58–3.60 (d, *J* = 9.6 Hz, 1H), 3.53–3.57 (m, 1H), 3.40–3.47 (m, 2H), 3.24–3.30 (m, 2H), 3.18–3.22 (m, 1H), 2.61–2.64 (t, *J* = 7.2 Hz, 2H), 2.52–2.54 (t, *J* = 7.2 Hz, 2H), 1.72–1.77 (m, 2H). ^{13}C NMR (150 MHz, D_2O) δ 175.17, 174.18, 172.66, 168.75, 137.89, 134.16, 130.81, 124.41, 121.83, 118.36, 101.96, 75.66, 75.46, 72.83, 71.68, 67.35, 52.63, 36.04, 32.61, 30.83, 28.23.

Yields and characterization of disaccharides 5–7 synthesized by one-pot four-enzyme GlcNAc-activation and transfer system.

GlcNAcα1–4GlcAβ2AA (5). Yield: 95%; white foam. ^{1}H NMR (600 MHz, D_2O) δ 7.95–7.96 (d, *J* = 8.4 Hz, 1H), 7.81–7.83 (d, *J* = 8.4 Hz, 1H), 7.61–7.64 (t, *J* = 7.8 Hz, 1H), 7.29–7.32 (t, *J* = 8.4 Hz, 1H), 5.38–5.39 (d, *J* = 3.6 Hz, 1H), 4.32–4.33 (d, *J* = 7.8 Hz, 1H), 3.89 (s, 3H), 3.79–3.88 (m, 4H), 3.70–3.73 (m, 4H), 3.60–3.63 (m, 1H), 3.55–3.58 (m, 1H), 3.45–3.48 (t, *J* = 9.6 Hz, 1H), 3.20–3.32 (m, 3H), 2.73–2.75 (t, *J* = 6.6 Hz, 2H), 2.58–2.61 (t, *J* = 7.2 Hz, 2H), 2.04 (s, 3H), 1.73–1.78 (m, 2H). ^{13}C NMR (150 MHz, D_2O) δ 175.15, 174.61, 174.51, 173.61, 169.24, 137.16, 134.12, 130.99, 125.45, 123.57, 121.10, 102.21, 96.98, 76.96, 76.69, 75.90, 73.56, 72.01, 70.86, 69.80, 67.58, 60.22, 53.83, 52.93, 36.23, 32.59, 31.20, 28.40, 22.06. HRMS (ESI) *m/z* calcd for $C_{29}H_{41}N_3O_{16}$ (M+H) 688.2560, found 688.2563.

GlcNTFAα1–4GlcAβ2AA (6). Yield: 84%; white foam. ^{1}H NMR (600 MHz, D_2O) δ 7.95–7.97 (d, *J* = 7.8 Hz, 1H), 7.88–7.89 (d, *J* = 7.8 Hz, 1H), 7.62–7.65 (t, *J* = 7.8 Hz, 1H), 7.30–7.32 (t, *J* = 7.8 Hz, 1H), 5.50–5.51 (d, *J* = 3.6 Hz, 1H), 4.34–4.35 (d, *J* = 7.8 Hz, 1H), 4.02–4.04 (dd, *J* = 10.8, 4.2 Hz, 1H), 3.91 (s, 3H), 3.83–3.88 (m, 4H), 3.75–3.77 (m, 3H), 3.63–3.66 (m, 1H), 3.57–3.61 (m, 1H), 3.51–3.55 (t, *J* = 9.6 Hz, 1H), 3.23–3.34 (m, 3H), 2.74–2.76 (t, *J* = 6.6 Hz, 2H), 2.61–2.63 (t, *J* = 7.2 Hz, 2H), 1.77–1.81 (m, 2H). ^{13}C NMR (150 MHz, D_2O) δ 175.15, 174.63, 173.50, 169.26, 159.45 (q, *J* = 37.6 Hz), 137.49, 134.28, 131.09, 125.33, 123.27, 120.52, 117.02 (q, *J* = 284.7 Hz), 102.29, 96.51, 76.90, 76.73, 76.13, 73.61, 72.21, 70.40, 69.85, 67.73, 60.32, 54.54, 53.00, 36.34, 32.75, 31.26, 28.51. HRMS (ESI) *m/z* calcd for $C_{29}H_{38}F_3N_3O_{16}$ (M+H) 742.2277, found 742.2284.

GlcNAc6N$_3$α1–4GlcAβ2AA (7). Yield: 89%; white foam. ^{1}H NMR (600 MHz, D_2O) δ 7.97–7.98 (d, *J* = 7.8 Hz, 1H), 7.82–7.84 (d, *J* = 8.4 Hz, 1H), 7.63–7.66 (t, *J* = 7.2 Hz, 1H), 7.32–7.34 (t, *J* = 7.2 Hz, 1H), 5.40–5.41 (d, *J* = 3.6 Hz, 1H), 4.34–4.35 (d, *J* = 7.8 Hz, 1H), 3.91 (s, 3H), 3.83–3.90 (m, 3H), 3.70–3.73 (m, 3H), 3.57–3.63 (m, 4H), 3.47–3.51 (t, *J* = 9.6 Hz, 1H), 3.22–3.33 (m, 3H), 2.74–2.77 (t, *J* = 6.6 Hz, 2H), 2.60–2.62 (t, *J* = 7.2 Hz, 2H), 2.05 (s, 3H), 1.75–1.79 (m, 2H). ^{13}C NMR (150 MHz, D_2O) δ 175.06, 174.64, 174.53, 173.69, 169.28, 137.07, 134.10, 130.99, 125.54, 123.75, 121.38, 102.21, 97.07, 76.88, 76.80, 76.04, 73.57, 70.85, 70.65, 70.44, 67.60, 53.76, 52.94, 50.62, 36.26, 32.58, 31.23, 28.41, 22.07. HRMS (ESI) *m/z* calcd for $C_{29}H_{40}N_6O_{15}$ (M+H) 713.2625, found 713.2630.

Yields and characterization of disaccharides 11–13 synthesized by PmHS2-catalyzed reaction.

GlcNGcα1–4GlcAβ2AA (11). Yield: 92%; white foam. ^{1}H NMR (600 MHz, D_2O) δ 7.97–7.98 (d, *J* = 7.8 Hz, 1H), 7.81–7.82 (d, *J* = 8.4 Hz, 1H), 7.62–7.65 (t, *J* = 7.2 Hz, 1H), 7.31–7.34 (t, *J* = 8.4 Hz, 1H), 5.39–5.40 (d, *J* = 3.6 Hz, 1H), 4.32–4.33 (d, *J* = 7.8 Hz, 1H), 4.13 (s, 2H), 3.94–3.96 (dd, *J* = 10.8, 4.2 Hz, 1H), 3.90 (s, 3H), 3,77–3.86 (m, 4H), 3.71–3.75 (m, 3H), 3.61–3.64 (m, 1H), 3.55–3.59 (m, 1H), 3.48–3.51 (t, *J* = 9.6 Hz, 1H), 3.20–3.30 (m, 3H), 2.74–2.76 (t, *J* = 6.6 Hz, 2H), 2.59–2.61 (t, *J* = 7.2 Hz, 2H), 1.74–1.78 (m, 2H). ^{13}C NMR (150 MHz, D_2O) δ 175.18, 175.12, 174.62, 173.67, 169.26, 137.05, 134.08, 130.97, 125.52, 123.72, 121.35, 102.19, 97.05, 76.93, 76.66, 76.03, 73.48, 72.06, 70.81, 69.72, 67.57, 61.03, 60.17, 53.45, 52.92, 36.22, 32.56, 31.20, 28.38. HRMS (ESI) *m/z* calcd for $C_{29}H_{41}N_3O_{17}$ (M+H) 704.2509, found 704.2516.

GlcNAzα1–4GlcAβ2AA (12). Yield: 91%; white foam. ^{1}H NMR (600 MHz, D_2O) δ 7.97–7.99 (d, *J* = 7.8 Hz, 1H), 7.82–7.83 (d, *J* = 7.8 Hz, 1H), 7.64–7.66 (t, *J* = 7.2 Hz, 1H), 7.33–7.35 (t, *J* = 7.8 Hz, 1H), 5.41–5.42 (d, *J* = 3.6 Hz, 1H), 4.33–4.34 (d, *J* = 7.8 Hz, 1H), 4.08 (s, 2H), 3.95–3.97 (dd, *J* = 7.8, 3.6 Hz, 1H), 3.91 (s, 3H), 3.82–3.86 (m, 1H), 3.73–3.80 (m, 6H), 3.62–3.65 (m, 1H), 3.56–3.60 (m, 1H), 3.48–3.51 (t, *J* = 9.0 Hz, 1H), 3.22–3.33 (m, 3H), 2.75–2.77 (t, *J* = 6.6 Hz, 2H), 2.60–2.62 (t, *J* = 6.6 Hz, 2H), 1.75–1.79 (m, 2H). ^{13}C NMR (150 MHz, D_2O) δ 175.15, 174.64, 173.71, 170.83, 169.28, 137.04, 134.09, 130.98, 125.56, 123.77, 121.44, 102.21, 96.88, 76.90, 76.70, 75.99, 73.52, 72.06, 70.72, 69.77, 67.60, 60.20, 53.87, 52.93, 51.93, 36.24, 32.57, 31.23, 28.40. HRMS (ESI) *m/z* calcd for $C_{29}H_{40}N_6O_{16}$ (M+H) 729.2574, found 729.2582.

GlcNAc6NGcα1–4GlcAβ2AA (13). Yield: 74%; white foam. ^{1}H NMR (600 MHz, D_2O) δ 7.98–7.99 (d, *J* = 7.8 Hz, 1H), 7.82–7.83 (d, *J* = 7.8 Hz, 1H), 7.64–7.66 (t, *J* = 7.8 Hz, 1H), 7.33–7.35 (t, *J* = 7.8 Hz, 1H), 5.31–5.32 (d, *J* = 3.6 Hz, 1H), 4.33–4.35 (d, *J* = 8.4 Hz, 1H), 4.12 (s, 2H), 3.91 (s, 3H), 3.81–3.90 (m, 4H), 3.68–3.74 (m, 3H), 3.56–3.63 (m, 3H), 3.50–3.53 (dd, *J* = 13.8, 2.4 Hz, 1H), 3.22–3.32 (m, 3H), 2.76–2.78 (t, *J* = 6.6 Hz, 2H), 2.60–2.63 (t, *J* = 7.2 Hz, 2H), 2.04 (s, 3H), 1.75–1.79 (m, 2H). ^{13}C NMR (150 MHz, D_2O) δ 175.48, 175.29, 174.65, 174.55, 173.71, 169.28, 137.03, 134.09, 130.99, 125.57, 123.81, 121.48, 102.16, 97.49, 77.08, 76.86, 76.73, 73.57, 71.51, 70.72, 70.51, 67.57, 61.18, 53.77, 52.94, 39.62, 36.24, 32.57, 31.24, 28.41, 22.08. HRMS (ESI) *m/z* calcd for $C_{31}H_{44}N_4O_{17}$ (M+H) 745.2780, found 745.2787.

HPLC and MALDI-MS analyses of trisaccharides 14–19 synthesized by small scale one-pot three-enzyme GlcA activation and transfer system.

Diluted (100-fold dilution) reaction mixtures were 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 (maintained at 30 °C throughout the experiment), 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 10% acetonitrile. The fluorescent compounds

were detected by excitation at 305 nm and emission at 415 nm. The MS data of the products were acquired using MALDI-TOF MS.

Preparative-scale preparation of trisaccharide 15 in a one-pot three-enzyme system.

Disaccharide GlcNTFAα1–4GlcAβ2AA (**6**) (30 mg, 1 eq.), Glc-1-P (1.2 eq), UTP (1.5 eq) and NAD^+ (2.4 eq.) were dissolved in water in a 15 mL centrifuge tube containing Tris-HCl buffer (100 mM, pH 7.0) and $MgCl_2$ (10 mM). After the addition of EcGalU (1 mg), PmUgd (3 mg), and PmHS2 (4.5 mg), water was added to bring the volume of the reaction mixture to 8 mL. The reaction mixture was incubated in an isotherm incubator for 12 h at 37 °C with gentle shaking. Product formation was monitored by TLC (EtOAc:MeOH:H_2O = 3:2:1 by volume) with *p*-anisaldehyde sugar staining. The reaction was stopped by adding the same volume of ice-cold ethanol and the mixture was incubated at 4 °C for 30 min. The mixture was centrifuged. The supernatant was concentrated and passed through a BioGel P-2 gel filtration column to obtain the desired product. The trisaccharide was further purified by silica gel column chromatography (EtOAc:MeOH:H_2O = 4:2:1 by volume) to obtain a white solid GlcAβ1–4GlcNTFAα1–4GlcAβ2AA (**15**) in 87% yield. ^{1}H NMR (600 MHz, D_2O) δ 7.96–7.97 (d, *J* = 7.8 Hz, 1H), 7.80–7.82 (d, *J* = 8.4 Hz, 1H), 7.61–7.64 (t, *J* = 7.8 Hz, 1H), 7.31–7.33 (t, *J* = 7.2 Hz, 1H), 5.44–5.45 (d, *J* = 3.6 Hz, 1H), 4.94–4.51 (d, *J* = 7.8 Hz, 1H), 4.30–4.31 (d, *J* = 7.8 Hz, 1H), 3.99–4.01 (dd, *J* = 11.4, 3.6 Hz, 1H), 3.94–3.97 (m, 1H), 3.90 (s, 3H), 3.80–3.85 (m, 4H), 3.70–3.75 (m, 4H), 3.57–3.60 (m, 1H), 3.53–3.56 (m, 1H), 3.48–3.52 (m, 2H), 3.35–3.37 (t, *J* = 7.8 Hz, 1H), 3.20–3.31 (m, 3H), 2,73–2.76 (t, *J* = 7.2 Hz, 2H), 2.58–2.61 (t, *J* = 7.2 Hz, 2H), 1.73–1.77 (m, 2H). ^{13}C NMR (150 MHz, D_2O) δ 174.88 (2C), 174.40, 173.45, 169.04, 159.11 (q, *J* = 37.7 Hz), 136.81, 133.86, 130.75, 125.31, 123.50, 121.14, 116.69 (q, *J* = 284.6 Hz), 102.27, 101.95, 96.01, 78.16, 76.60, 76.50, 76.02, 75.75, 75.05, 73.31, 72.82, 71.68, 70.58, 68.59, 67.36, 59.22, 53.85, 52.70, 36.00, 32.34, 30.99, 28.16. HRMS (ESI) *m/z* calcd for $C_{35}H_{46}F_3N_3O_{22}$ (M+H) 918.2603, found 918.2613.

One-pot four-enzyme synthesis of tetrasaccharide 20.

Trisaccharide GlcAβ1–4GlcNTFAα1–4GlcAβ2AA (**15**) (30 mg, 1 eq.), $GlcNAc6N_3$ (1.5 eq.), ATP (1.8 eq.), and UTP (1.8 eq.) were dissolved in water in a 15 mL centrifuge tube containing MES buffer (100 mM, pH 6.5) and $MgCl_2$ (10 mM). After the addition of NanK_ATCC55813 (2.5 mg), PmGlmU (3 mg), PmPpA (1.5 mg), and PmHS2 (4 mg), water was added to bring the volume of the reaction mixture to 6.5 mL. The reaction mixture was incubated in an isotherm incubator for 18 h at 37 °C with gentle shaking. Product formation was monitored by TLC (EtOAc:MeOH:H_2O = 4:2:1 by volume) with *p*-anisaldehyde sugar staining. The reaction was stopped by adding the same volume of ice-cold ethanol and the mixture was incubated at 4 °C for 30 min. The mixture was centrifuged. The supernatant was concentrated and passed through a BioGel P-2 gel filtration column to obtain the desired product. The tetrasaccharide was further purified by silica gel column chromatography (EtOAc:MeOH:H_2O = 5:2:1 by volume) to obtain tetrasaccharide $GlcNAc6N_3$α1–4GlcAβ1–4GlcNTFAα1–4GlcAβ2AA (**20**) as a white solid in 93% yield. ^{1}H NMR (600 MHz, D_2O) δ 7.94–7.96 (d, *J* = 7.8 Hz, 1H), 7.81–7.83 (d, *J* = 8.4 Hz, 1H), 7.60–7.63 (t, *J* = 7.8 Hz, 1H), 7.29–7.31 (t, *J* = 7.2 Hz, 1H), 5.43–5.44 (d, *J* = 3.6 Hz, 1H), 5.40–5.41 (d, *J* = 4.2 Hz, 1H), 4.47–4.49 (d, *J* = 7.8 Hz, 1H), 4.29–4.31 (d, *J* = 8.4 Hz, 1H), 3.93–4.00 (m, 2H), 3.88–3.90 (m, 4H), 3.78–3.86 (m, 6H), 3.66–3.75 (m, 6H), 3.61–3.62 (d, *J* = 2.4 Hz, 2H), 3.57–3.60 (m, 1H), 3.53–3.56 (m, 1H), 3.45–3.48 (t, *J* = 9.0 Hz, 1H), 3.34–3.36 (t, *J* = 7.8 Hz, 1H), 3.19–3.30 (m, 3H), 2.72–2.74 (t, *J* = 6.6 Hz, 2H), 2.57–2.60 (t, *J* = 6.6 Hz, 2H), 2.03 (s, 3H), 1.72–1.76 (m, 2H). ^{13}C NMR (150 MHz, D_2O) δ 174.85, 174.78, 174.36, 174.29, 173.36, 169.00, 159.08 (q, *J* = 284.4 Hz), 136.91, 133.88, 130.75, 125.20, 123.30, 120.83, 116.67 (q, *J* = 37.7 Hz), 102.29, 101.93, 96.83, 96.00, 78.08, 76.57, 76.46, 76.30, 76.28, 76.01, 75.77,

73.36, 73.29, 70.65, 70.55, 70.35, 70.22, 68.47, 67.35, 59.17, 53.76, 53.47, 52.67, 50.38, 35.98, 32.35, 30.95, 28.15, 21.79. HRMS (ESI) *m/z* calcd for $C_{43}H_{58}F_3N_7O_{26}$ (M+H) 1146.3462, found 1146.3478.

Chemical synthesis of tetrasaccharides 21–24.

GlcNAc6N$_3$α1–4GlcAβ1–4GlcNH$_2$α1–4GlcAβ2AA' (21). Tetrasaccharide GlcNAc6N$_3$α1–4GlcAβ1–4GlcNTFAα1–4GlcAβ2AA (**20**) (30 mg, 0.029 mmol) was dissolved in 8 mL of H_2O. The pH of the solution was adjusted to 10 by adding 2 N NaOH (aq.). After being vigorously stirred at room temperature for 1.5 h, the reaction mixture was neutralized with DOWEX HCR-W2 (H^+) resin, filtered, and concentrated. The residue was purified by silica gel column chromatography (EtOAc:MeOH:H_2O = 4:2:1 by volume) to obtain tetrasaccharide GlcNAc6N$_3$α1–4GlcAβ1–4GlcNH$_2$α1–4GlcAβ2AA' (**21**) as a white solid in 81% yield. ^{1}H NMR (600 MHz, D_2O) δ 8.12–8.13 (d, *J* = 7.8 Hz, 1H), 7.85–7.87 (d, *J* = 7.8 Hz, 1H), 7.48–7.50 (t, *J* = 7.2 Hz, 1H), 7.20–7.22 (t, *J* = 7.8 Hz, 1H), 5.59–5.60 (d, *J* = 3.6 Hz, 1H), 5.41–5.40 (d, *J* = 4.2 Hz, 1H), 4.45–4.47 (d, *J* = 7.8 Hz, 1H), 4.27–4.28(d, *J* = 7.8 Hz, 1H), 3.88–3.94 (m, 2H), 3.79–3.88 (m, 6H), 3.66–3.78 (m, 8H), 3.62–3.64 (m, 2H), 3.46–3.52 (m, 2H), 3.34–3.37 (t, *J* = 7.8 Hz, 1H), 3.19–3.33 (m, 3H), 2.73–2.75 (t, *J* = 6.6 Hz, 2H), 2.60–2.62 (t, *J* = 6.6 Hz, 2H), 2.03 (s, 3H), 1.71–1.75 (m, 2H). ^{13}C NMR (150 MHz, D_2O) δ 174.89, 174.87, 174.67, 174.47, 174.29, 172.74, 137.25, 131.59, 130.48, 125.10, 124.00, 120.70, 102.27, 101.92, 96.84, 95.32, 77.32, 76.22, 76.15, 76.10, 76.07, 75.79, 73.38, 73.03, 70.90, 70.65, 70.34, 70.20, 68.24, 68.22, 67.30, 58.92, 53.82, 53.45, 50.38, 35.90, 33.19, 31.28, 28.10, 21.77. HRMS (ESI) *m/z* calcd for $C_{40}H_{58}N_7O_{25}$ (M+H) 1036.3482, found 1036.3497.

GlcNAc6N$_3$α1–4GlcAβ1–4GlcNSα1–4GlcAβ2AA' (22). Tetrasaccharide GlcNAc6N$_3$α1–4GlcAβ1–4GlcNH$_2$α1–4GlcAβ2AA' (**21**) (20 mg, 0.018 mmol) was dissolved in 10 mL of H_2O. The pH of the solution was adjusted to 9.5 by adding 2 N NaOH (aq). Sulfur trioxide-pyridine complex (58 mg, 0.36 mmol) was added in three equal portions during 35 minutes intervals at room temperature, and the pH was maintained at 9.5 throughout the whole process using 2 N NaOH (aq). After being stirred at room temperature for 24 h, the reaction mixture was neutralized with DOWEX HCR-W2 (H^+) resin, filtered, and concentrated. The process was repeated for three times and the product was purified using silica gel column chromatography (EtOAc:MeOH:H_2O = 5:2:1 by volume) to obtain tetrasaccharide GlcNAc6N$_3$α1–4GlcAβ1–4GlcNSα1–4GlcAβ2AA' (**22**) as a light yellow solid in 70% yield. ^{1}H NMR (800 MHz, D_2O) δ 8.13–8.14 (d, *J* = 8.0 Hz, 1H), 7.87–7.88 (d, *J* = 7.2 Hz, 1H), 7.52–7.50 (t, *J* = 8.0 Hz, 1H), 7.22–7.24 (t, *J* = 7.2 Hz, 1H), 5.60–5.61 (d, *J* = 4.0 Hz, 1H), 5.42–5.43 (d, *J* = 3.2 Hz, 1H), 4.50–4.51 (d, *J* = 8.0 Hz, 1H), 4.34–4.35 (d, *J* = 8.0 Hz, 1H), 3.86–3.92 (m, 3H), 3.80–3.83 (m, 4H), 3.73–3.79 (m, 4H), 3.68–3.72 (m, 4H), 3.64–3.65 (d, *J* = 3.2 Hz, 2H), 3.56–3.53 (m, 1H), 3.48–3.50 (t, *J* = 9.6 Hz, 1H), 3.36–3.39 (t, *J* = 8.0 Hz, 1H), 3.27–3.32(m, 1H), 3.19–3.28 (m, 4H), 2.75–2.77 (t, *J* = 7.2 Hz, 2H), 2.62–2.63 (t, *J* = 7.2 Hz, 2H), 2.04 (s, 3H), 1.75–1.77 (m, 1H). ^{13}C NMR (200 MHz, D_2O) δ 174.89, 174.51, 174.32, 174.30, 172.82, 172.78, 137.13, 131.55, 130.41, 125.21, 124.02, 120.77, 102.11, 101.88, 96.88, 96.79, 77.70, 76.48, 76.29, 76.15, 76.11, 75.77, 73.28, 72.54, 72.51, 70.58, 70.30, 70.21, 70.15, 69.38, 67.25, 59.16, 57.45, 53.43, 50.30, 35.89, 33.09, 31.19, 28.09, 21.73. HRMS (ESI) *m/z* calcd for $C_{40}H_{57}N_7O_{28}S$ (M+H) 1116.3051, found 1116.3076.

GlcNAc6NH$_2$α1–4GlcAβ1–4GlcNSα1–4GlcAβ2AA' (23). Tetrasaccharide GlcNAc6N$_3$α1–4GlcAβ1–4GlcNSα1–4GlcAβ2AA' **22** (17 mg, 0.015 mmol) was dissolved in 10 mL of H_2O. MeOH (1:1 by volume) and 20 mg of Pd/C were then added. The mixture was shaken under H_2 gas (4 Bar) for 1 h, filtered, and concentrated to produce **23** as a white solid in quantitative yield. ^{1}H NMR (800 MHz, D_2O) δ 8.04–8.05 (d, *J* = 8.0 Hz, 1H), 8.02–8.03 (d, *J* = 8.0 Hz, 1H), 7.64–7.67 (t, *J* = 8.0 Hz, 1H), 7.32–7.34 (t, *J* = 7.2 Hz, 1H), 5.56–5.57 (d, *J* = 4.0 Hz, 1H), 5.34–5.35 (d, *J* = 4.0 Hz, 1H), 4.56–4.57 (d, *J* = 8.0 Hz, 1H), 4.34–4.35 (d, *J* = 8.0 Hz, 1H), 3.92–3.95 (m, 3H), 3.88–3.90 (dd, *J* = 12.0 Hz, 1H),

3.76–3.84 (m, 4H), 3.68–3.75 (m, 5H), 3.55–3.58 (m, 1H), 3.42–3.45 (dd, *J* = 13.6, 3.2 Hz, 1H), 3.35–3.38 (m, 2H), 3.30–3.32 (m, 2H), 3.19–3.27 (m, 4H), 3.12–3.15 (dd, *J* = 12.8, 8.8 Hz, 1H), 2.77–2.79 (t, *J* = 6.4 Hz, 2H), 2.61–2.62 (t, *J* = 6.4 Hz, 2H), 2.05 (s, 3H), 1.75–1.78 (m, 2H). ^{13}C NMR (200 MHz, D_2O) δ 174.77, 174.47, 174.42, 173.26, 172.64, 170.76, 137.73, 133.92, 131.18, 127.27, 124.85, 122.56, 102.18, 102.87, 97.64, 97.48, 77.66, 77.32, 76.56, 75.76, 75.73, 75.70, 74.58, 73.42, 72.45, 71.70, 70.65, 70.08, 69.22, 68.16, 67.53, 59.22, 57.60, 53.33, 40.22, 35.88, 32.75, 31.12, 28.13, 21.78. HRMS (ESI) *m/z* calcd for $C_{40}H_{60}N_5O_{28}S$ (M+H) 1090.3146, found 1090.3171.

GlcNAc6NSα1–4GlcAβ1–4GlcNSα1–4GlcAβ2AA' (24). Tetrasaccharide GlcNAc6NH$_2$α1–4GlcAβ1–4GlcNSα1–4GlcAβ2AA' **23** (14 mg, 0.013 mmol) was dissolved in 5 mL of H_2O. The pH of the solution was adjusted to 9.5 by adding 2 N NaOH. Sulfur trioxide-pyridine complex (30 mg, 0.18 mmol) was added in three equal portions during 1 hour intervals at room temperature. The pH was maintained at 9.5 throughout the whole process by adding 2 N NaOH. After being stirred at room temperature for overnight, the reaction mixture was neutralized with DOWEX HCR-W2 (H^+) resin, filtered, and concentrated. The process was repeated for three times and the product was purified by preparative HPLC using C18 column to provide white solid **24** in 65% yield. ^{1}H NMR (800 MHz, D_2O) δ 8.06–8.07 (d, *J* = 8.0 Hz, 1H), 8.03–8.04 (d, *J* = 8.0 Hz, 1H), 7.66–7.68 (t, *J* = 7.2 Hz, 1H), 7.33–7.34 (d, *J* = 7.2 Hz, 1H), 5.54–5.55 (d, *J* = 3.2 Hz, 1H), 5.35–5.36 (d, *J* = 3.2 Hz, 1H), 4.61–4.62 (d, *J* = 8.0 Hz, 1H), 4.34–4.35 (d, *J* = 8.0 Hz, 1H), 4.11–4.12 (d, *J* = 9.6 Hz, 1H), 3.81–3.93 (m, 4H), 3.73–3.81 (m, 5H), 3.64–3.71 (m, 5H), 3.55–3.58 (m, 1H), 3.50–3.53 (t, *J* = 9.6 Hz, 1H), 3.39–3.41 (t, *J* = 8.0 Hz, 1H), 3.28–3.33 (m, 3H), 3.21–3.27 (m, 3H), 2.77–2.79 (t, *J* = 7.2 Hz, 2H), 2.61–2.62 (t, *J* = 6.4 Hz, 2H), 2.05 (s, 3H), 1.75–1.78 (m, 2H). ^{13}C NMR (200 MHz, D_2O) δ 174.48, 174.34, 173.25, 171.88, 171.81, 170.41, 137.88, 134.18, 131.28, 127.18, 124.87, 122.59, 102.27, 102.04, 97.78, 97.46, 77.88, 76.83, 76.46, 75.65, 75.49, 73.91, 73.88, 73.14, 72.41, 70.83, 70.69, 70.46, 70.13, 69.14, 67.62, 59.21, 57.67, 53.42, 43.29, 35.85, 32.71, 31.09, 28.13, 21.81. HRMS (ESI) *m/z* calcd for $C_{40}H_{60}N_5O_{31}S_2$ (M+H) 1170.2714, found 1170.2730.

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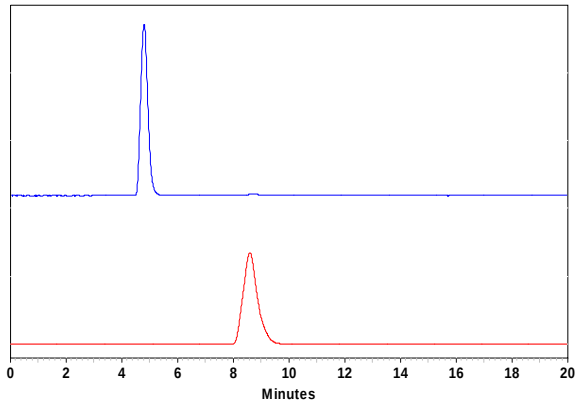
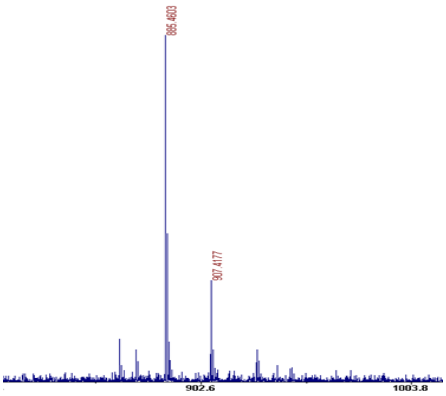
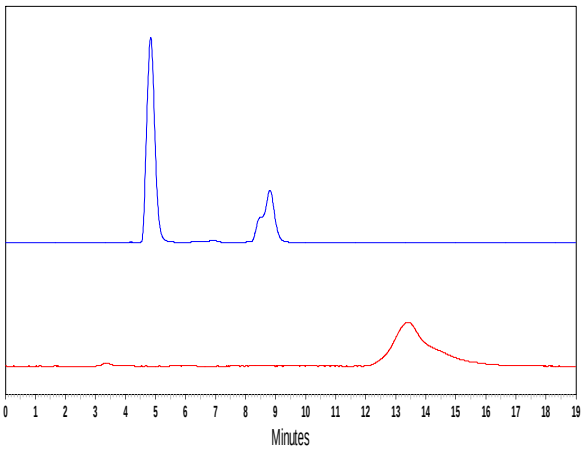
Figure S2. HPLC and MS analysis for trisaccharides **23–28**. Small scale one-pot three-enzyme reactions are shown in the blue color and the corresponding disaccharide standards are shown in the red color. The MS of trisaccharides are detected in MALDI-TOF MS.

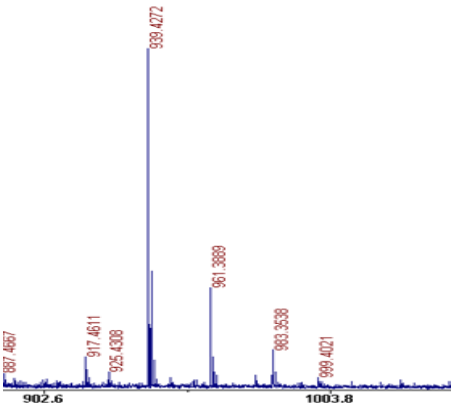
Trisaccharide GlcAβ1–4GlcNAcα1–4GlcAβ2AA (**14**)



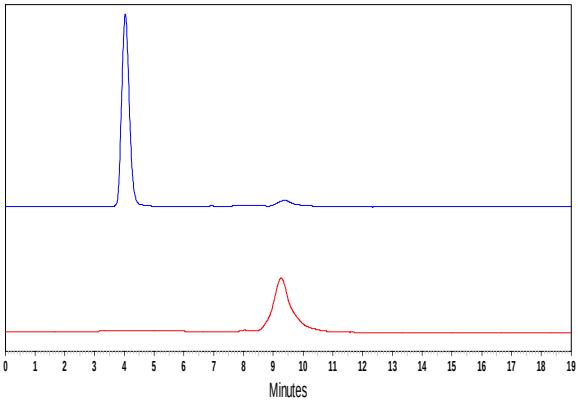


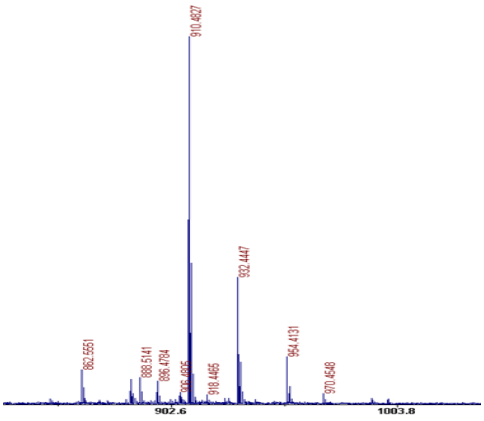
Trisaccharide GlcAβ1–4GlcNTFAα1–4GlcAβ2AA (**15**)



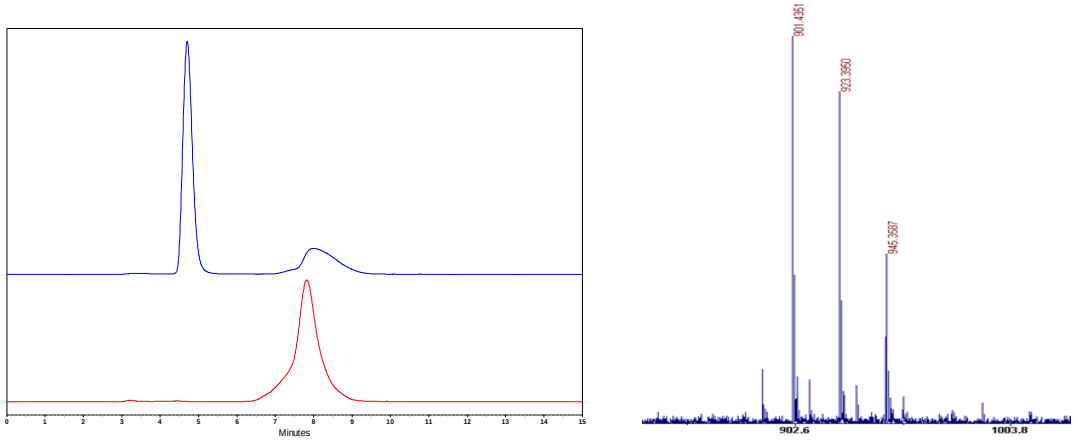


Trisaccharide GlcAβ1–4GlcNAc6N_3α1–4GlcAβ2AA (**16**)

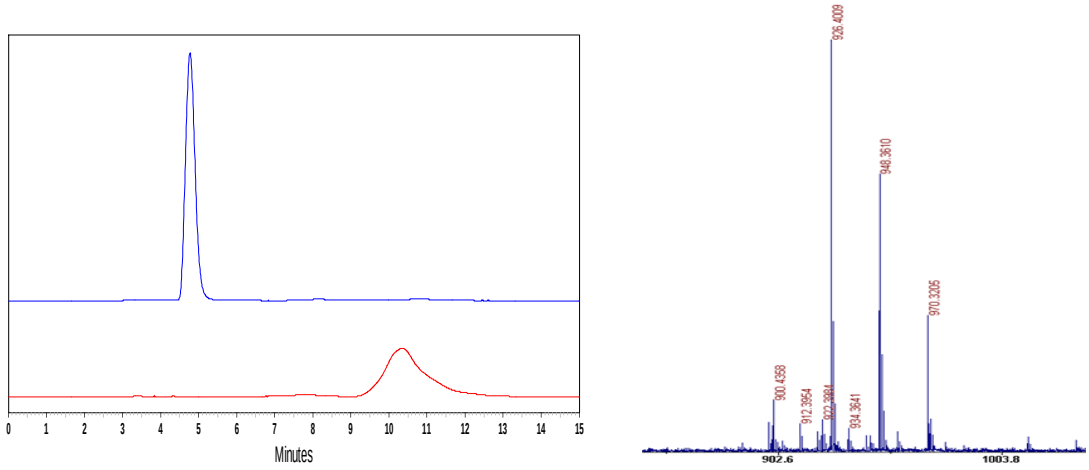




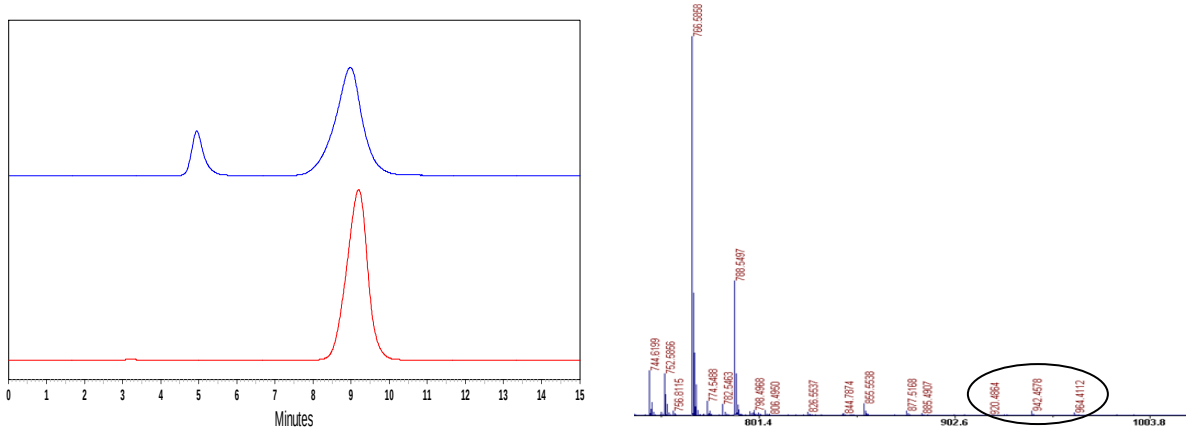
Trisaccharides GlcAβ1–4GlcNGcα1–4GlcAβ2AA (**17**)



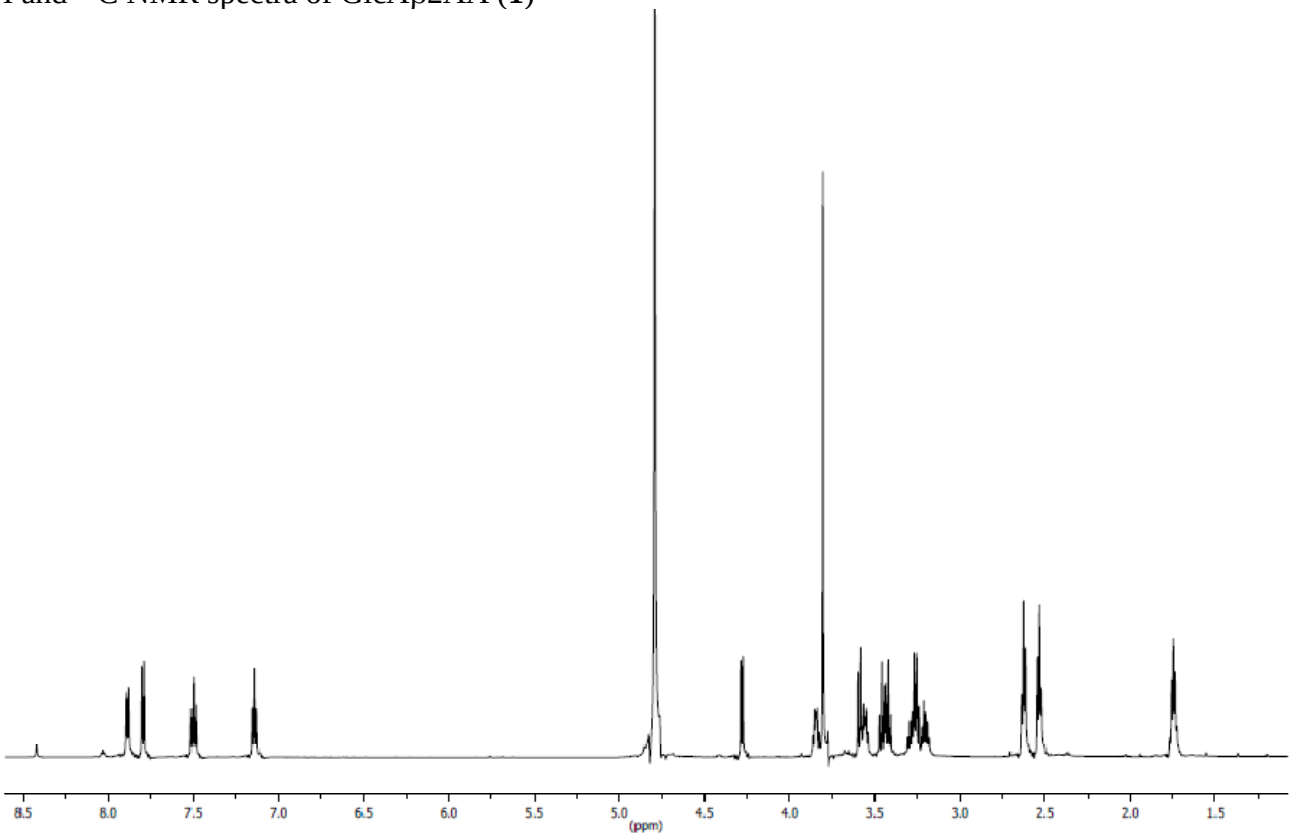
Trisaccharides GlcAβ1–4GlcNAzα1–4GlcAβ2AA (**18**)

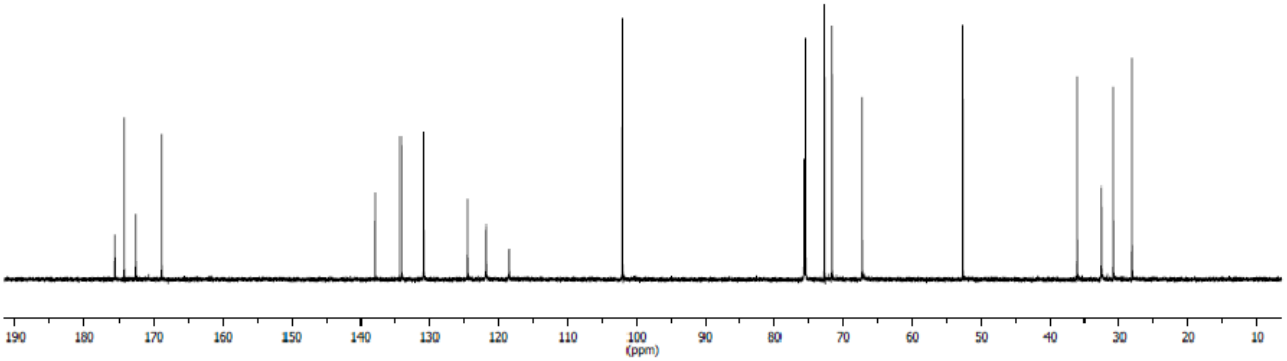


Trisaccharides GlcAβ1–4GlcNAc6NGcα1–4GlcAβ2AA (**19**)

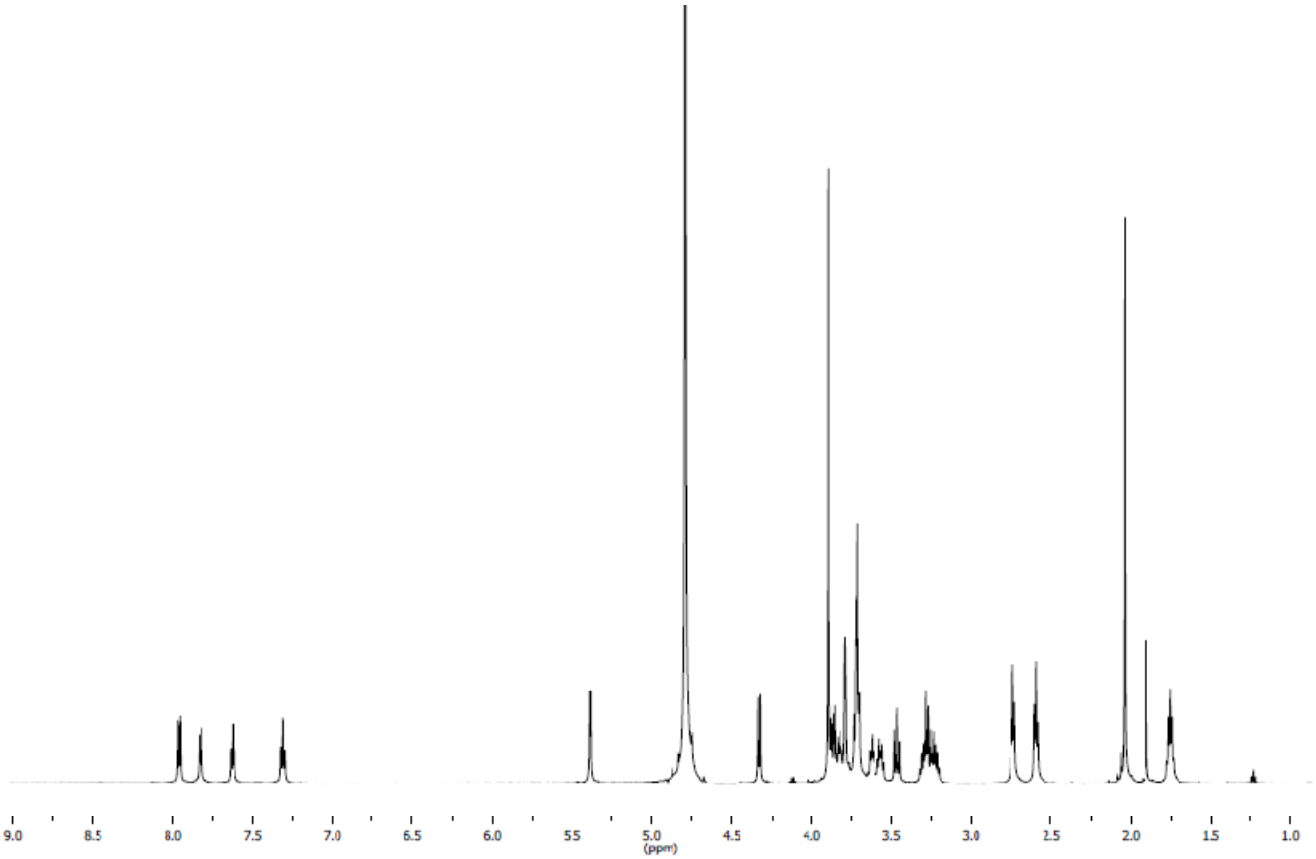


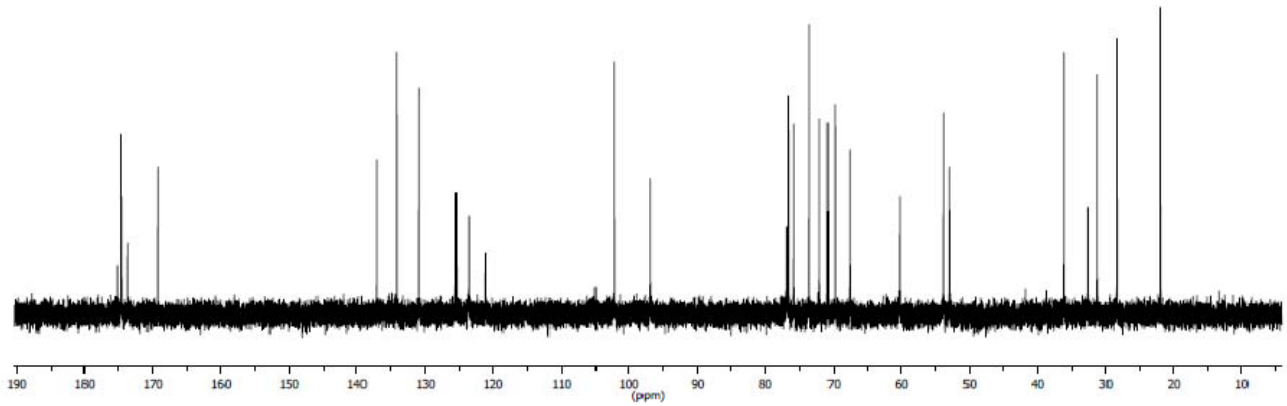
^{1}H and ^{13}C NMR spectra of GlcAβ2AA (**1**)



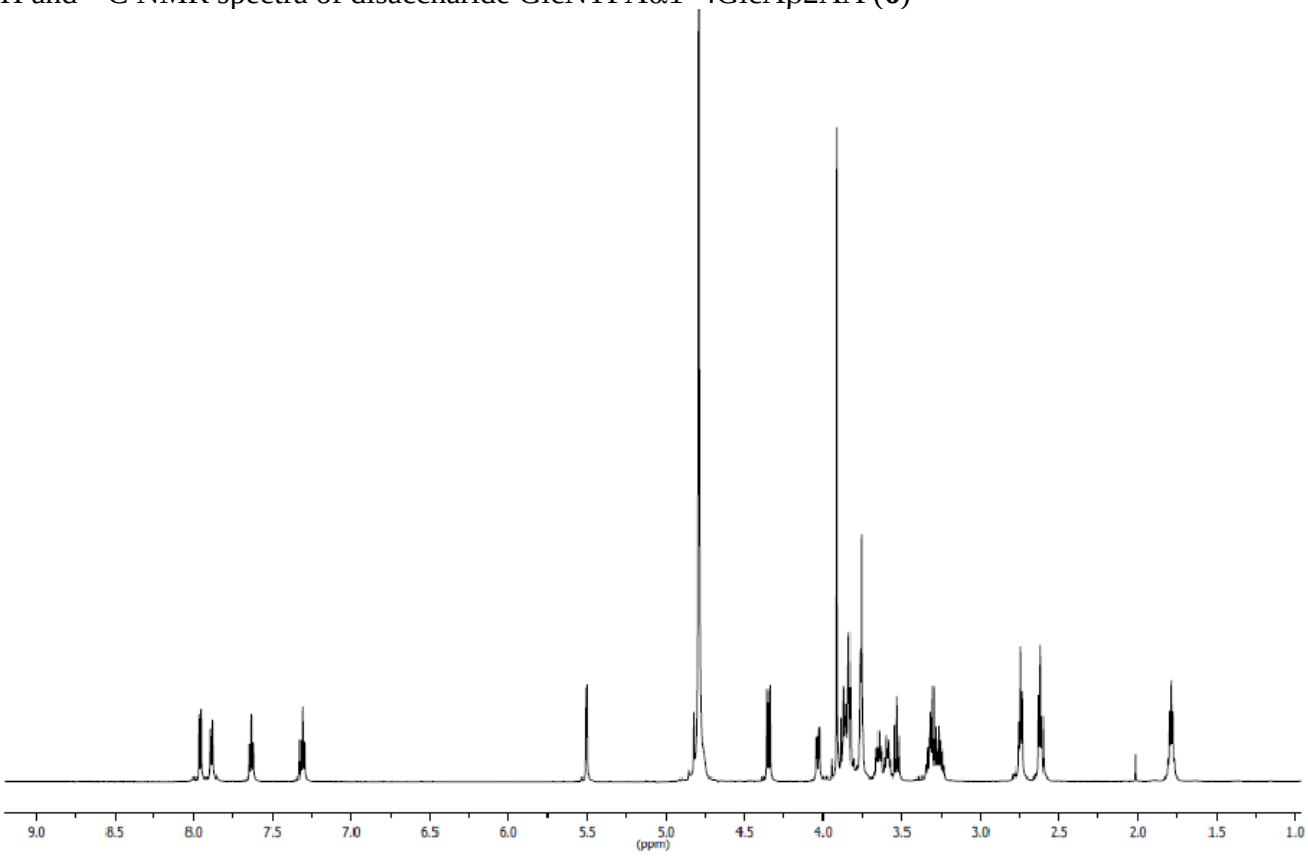


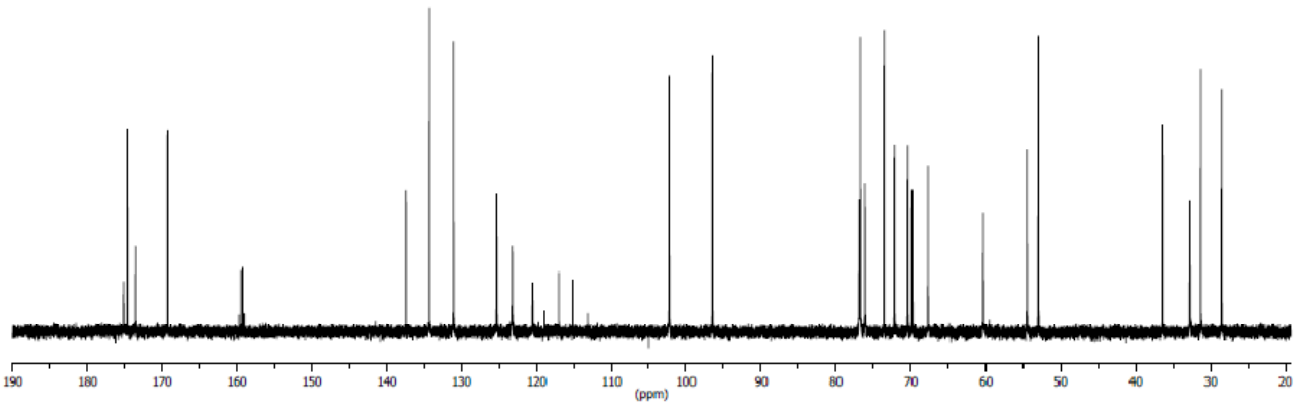
^{1}H and ^{13}C NMR spectra of disaccharide GlcNAcα1–4GlcAβ2AA (**5**)



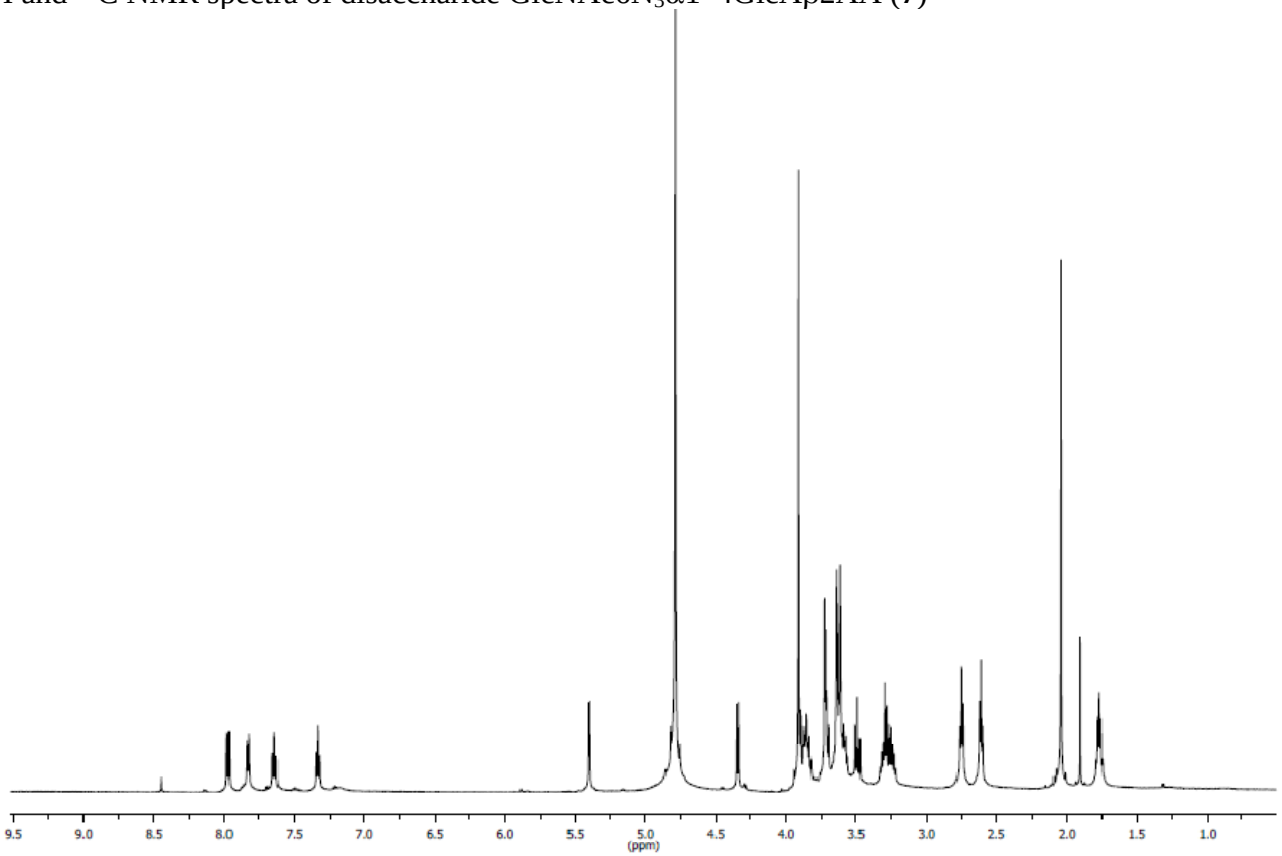


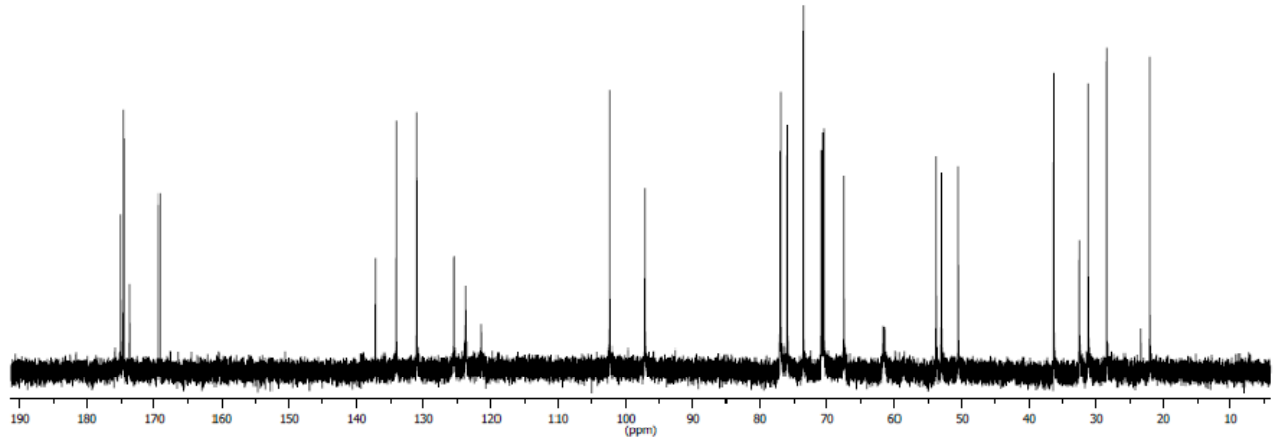
^{1}H and ^{13}C NMR spectra of disaccharide GlcNTFAα1–4GlcAβ2AA (**6**)



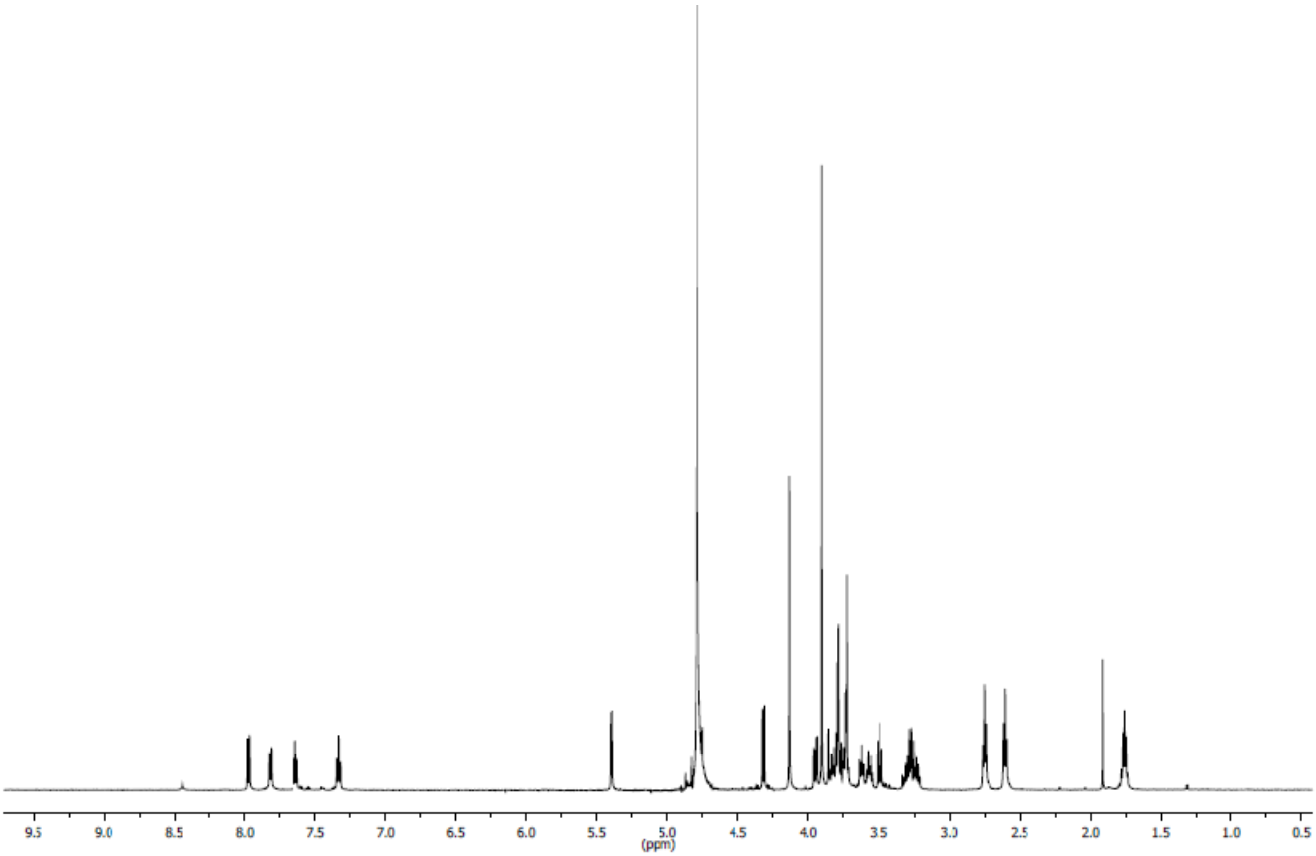


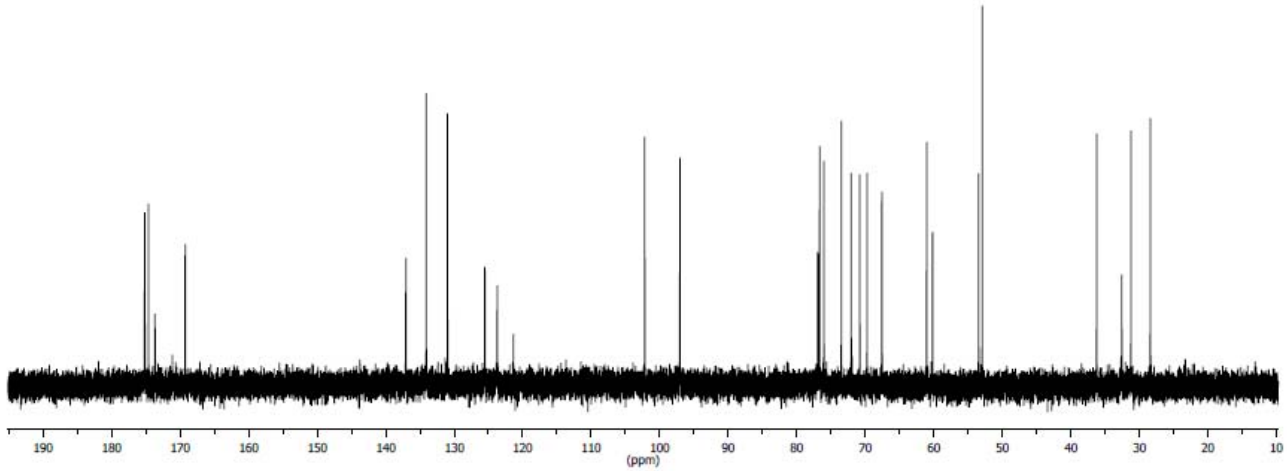
^{1}H and ^{13}C NMR spectra of disaccharide GlcNAc6N$_3$α1–4GlcAβ2AA (**7**)



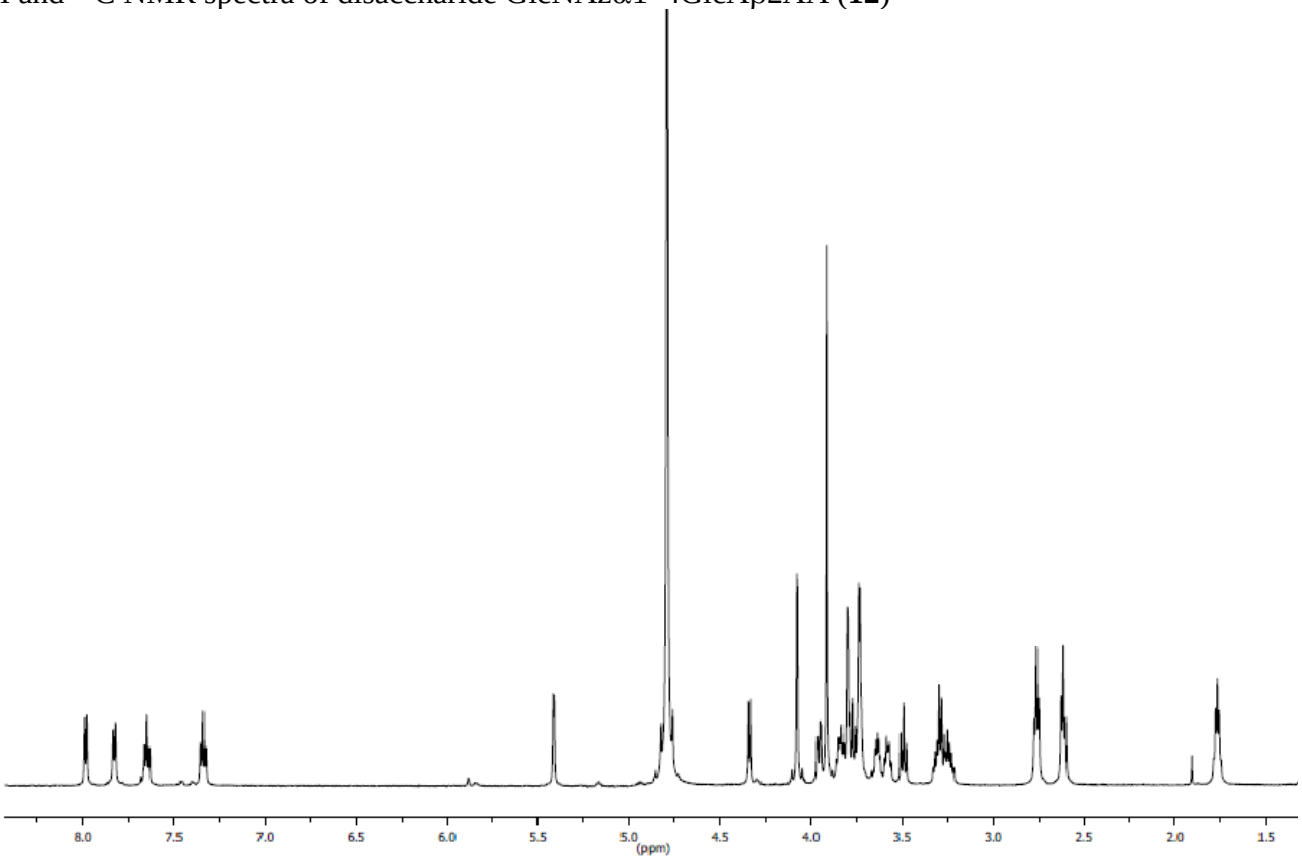


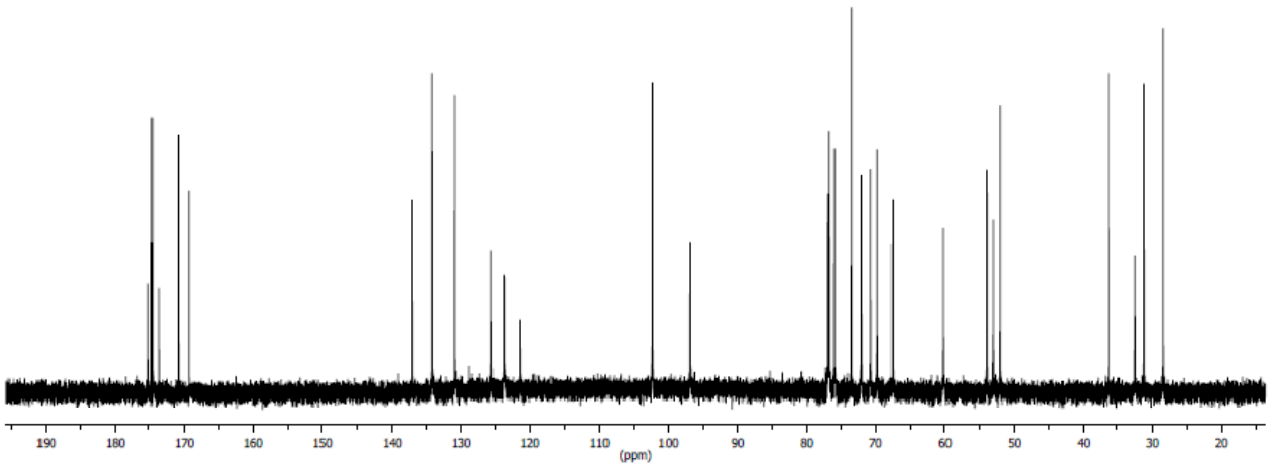
^{1}H and ^{13}C NMR spectra of disaccharide GlcNGcα1–4GlcAβ2AA (**11**)



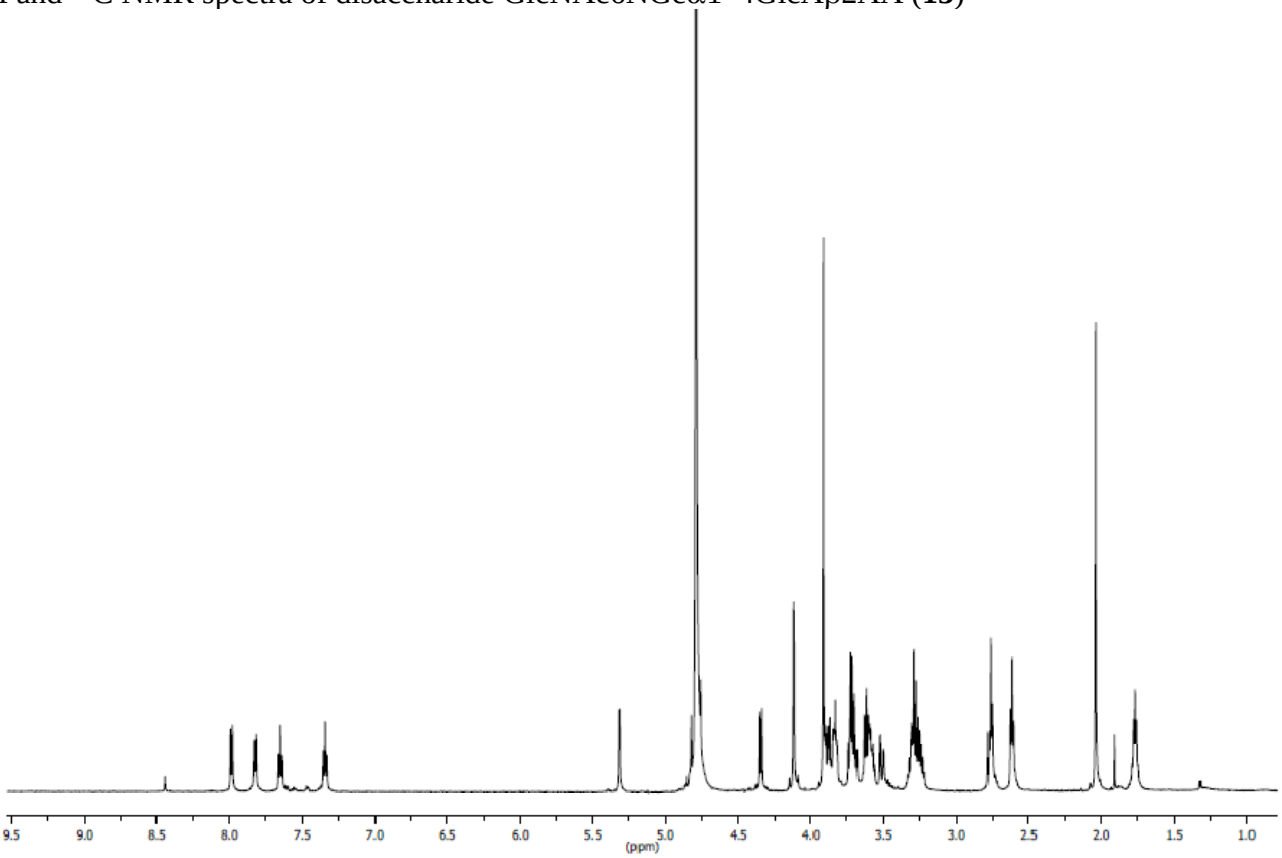


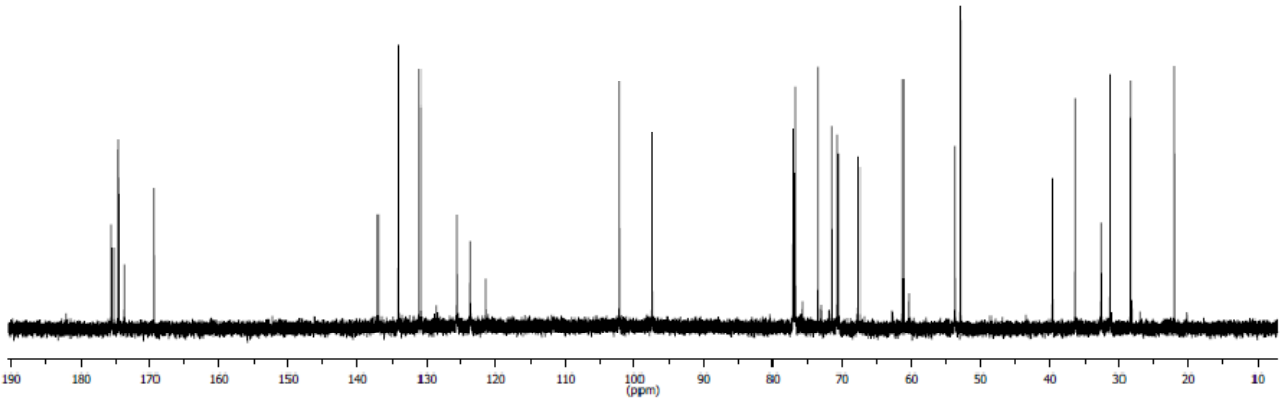
^{1}H and ^{13}C NMR spectra of disaccharide GlcNAzα1–4GlcAβ2AA (**12**)



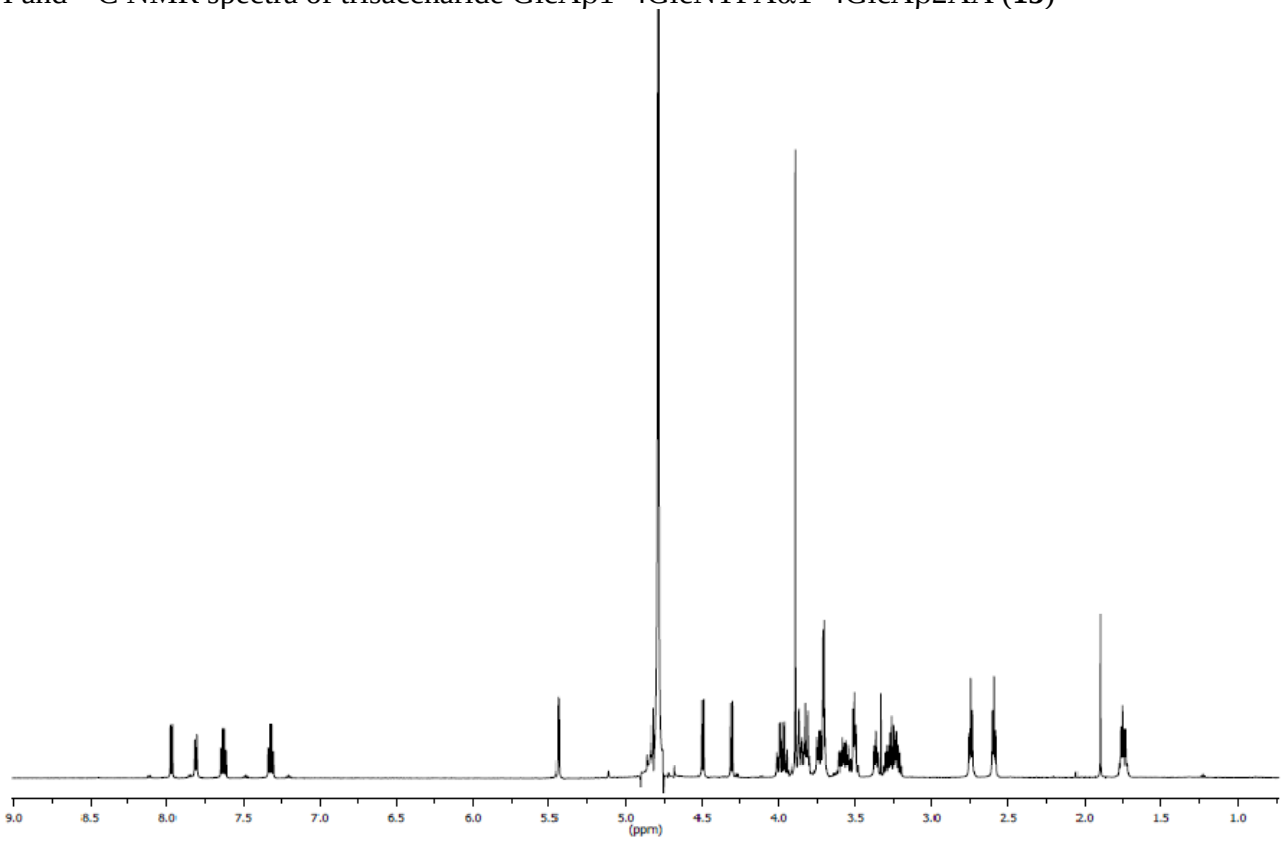


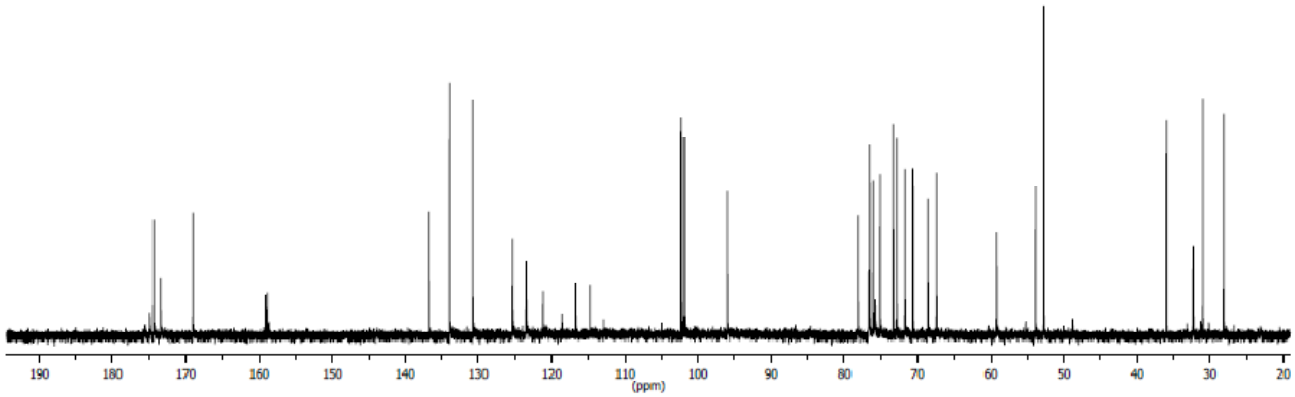
^{1}H and ^{13}C NMR spectra of disaccharide GlcNAc6NGcα1–4GlcAβ2AA (**13**)



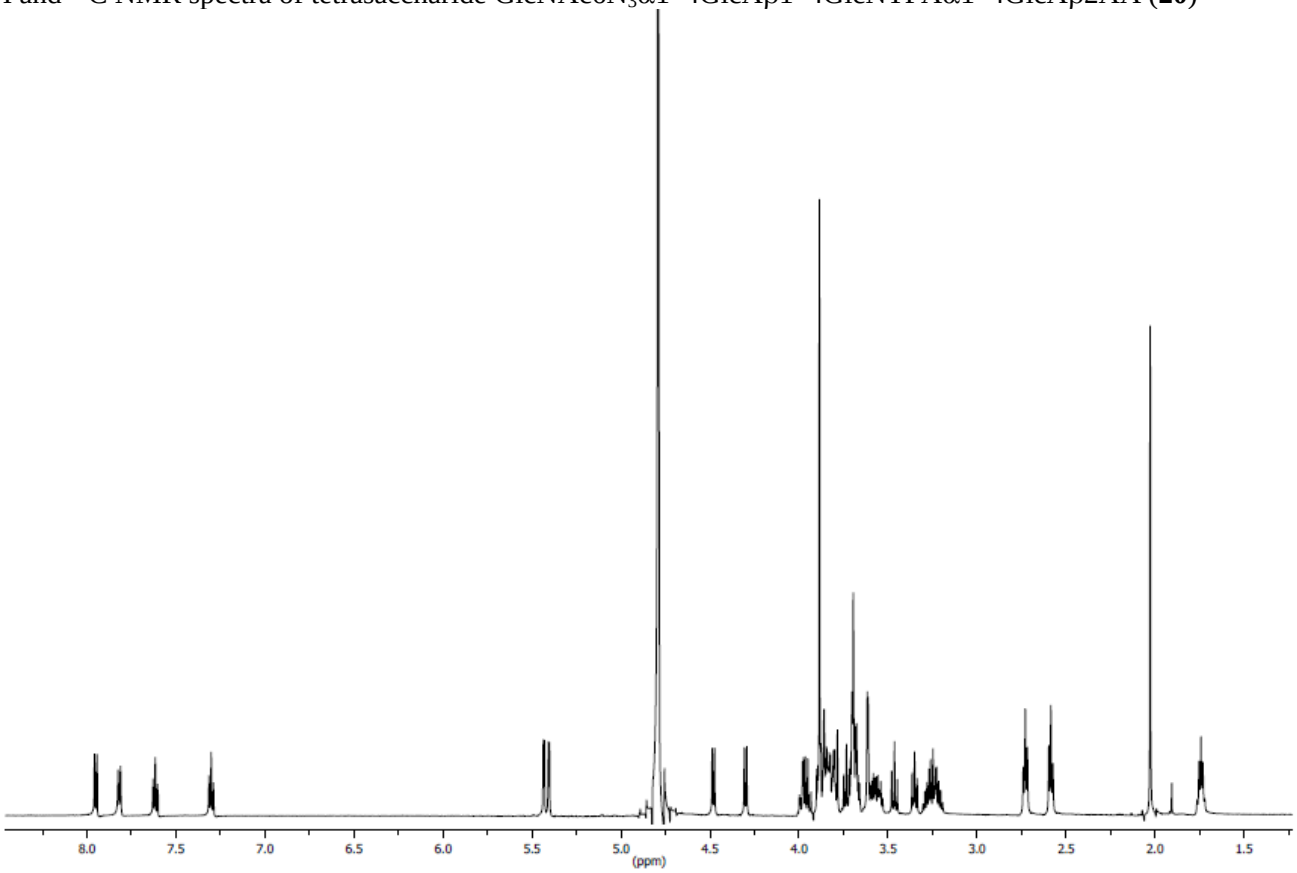


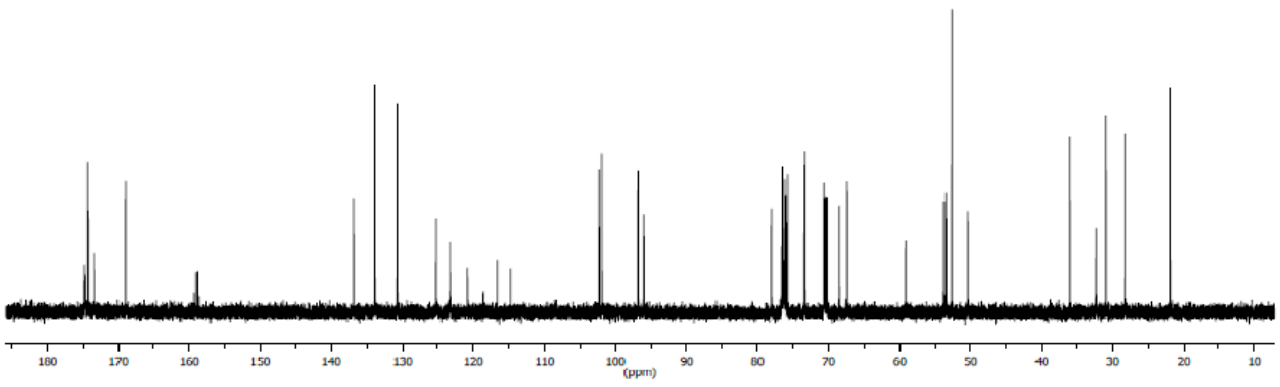
^{1}H and ^{13}C NMR spectra of trisaccharide GlcAβ1–4GlcNTFAα1–4GlcAβ2AA (**15**)



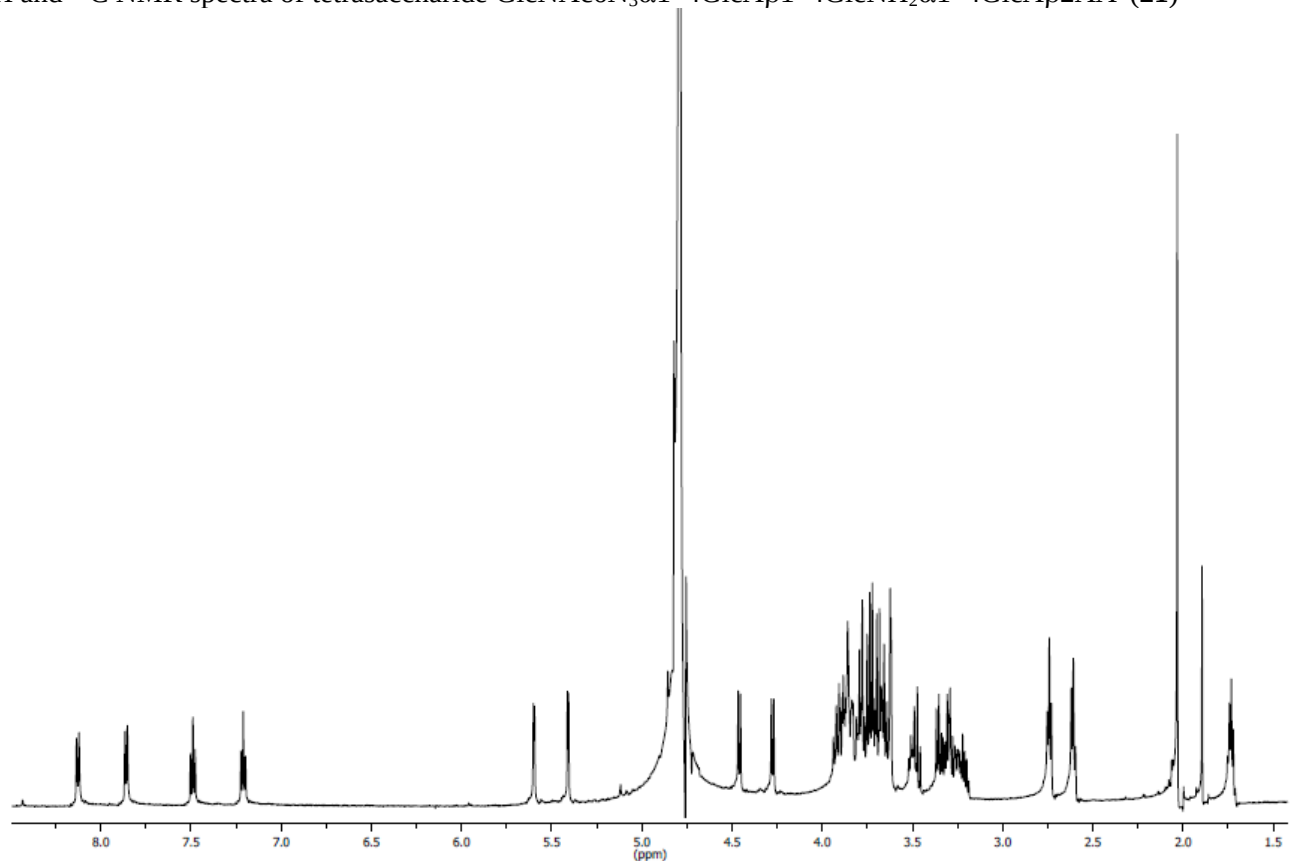


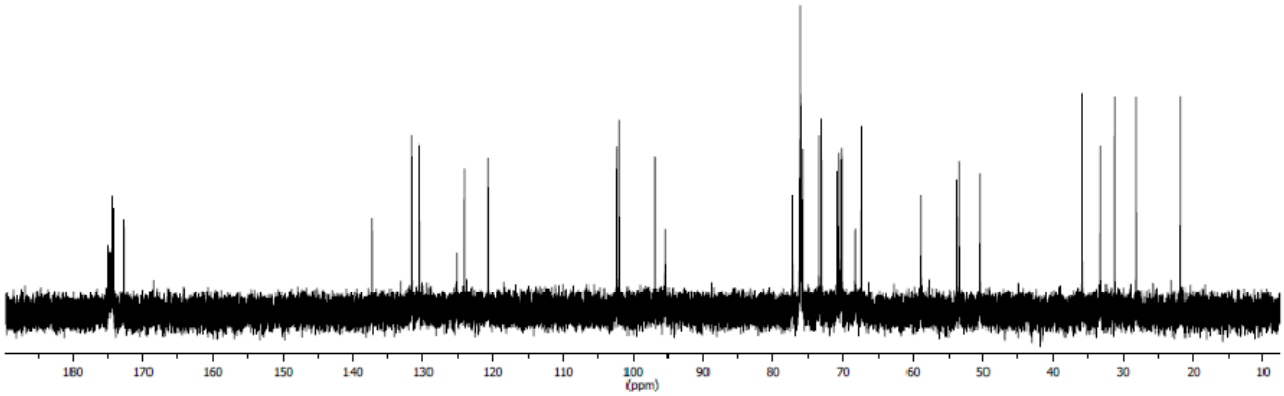
^{1}H and ^{13}C NMR spectra of tetrasaccharide GlcNAc6N$_3$α1–4GlcAβ1–4GlcNTFAα1–4GlcAβ2AA (**20**)



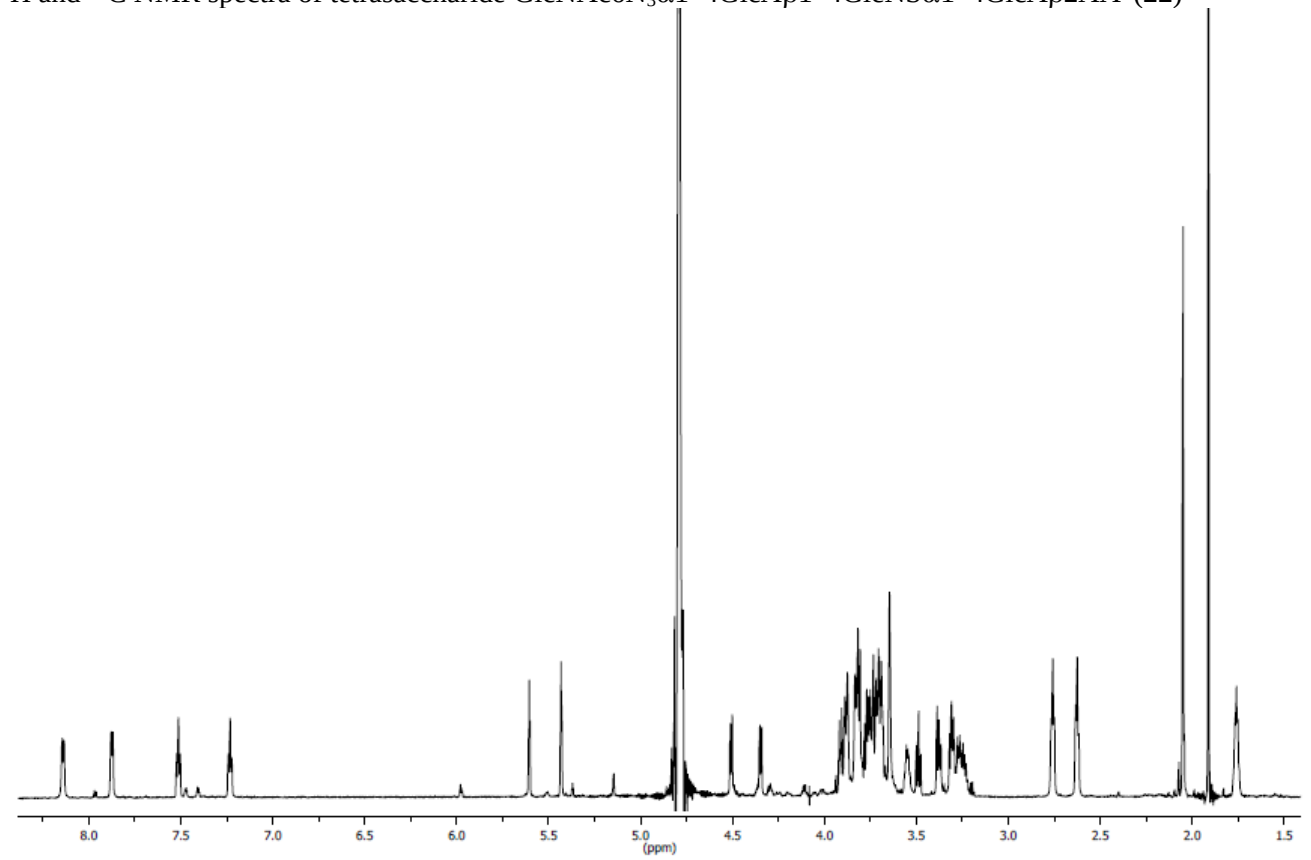


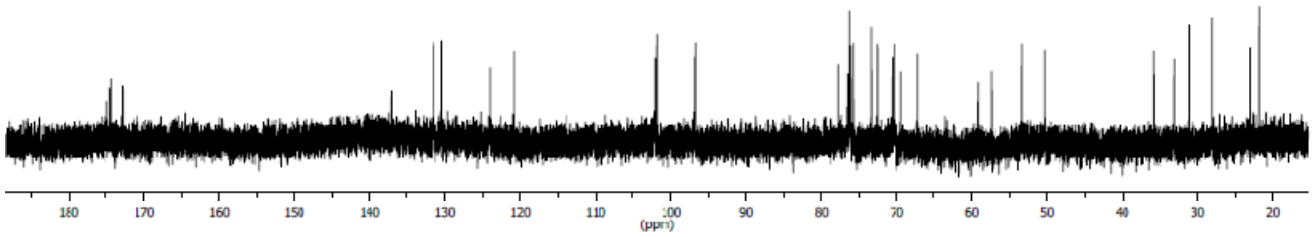
^{1}H and ^{13}C NMR spectra of tetrasaccharide GlcNAc6N_3α1–4GlcAβ1–4GlcNH_2α1–4GlcAβ2AA' (**21**)



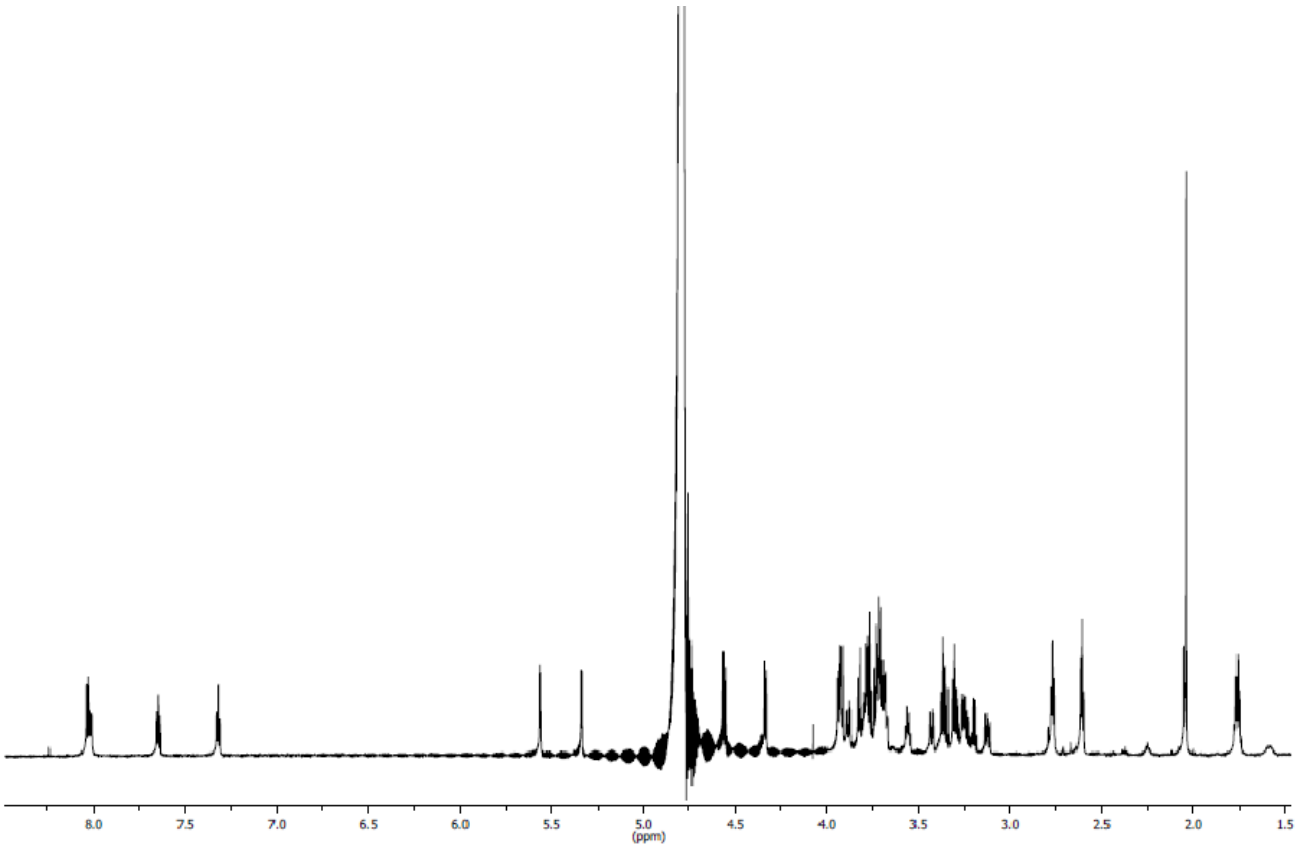


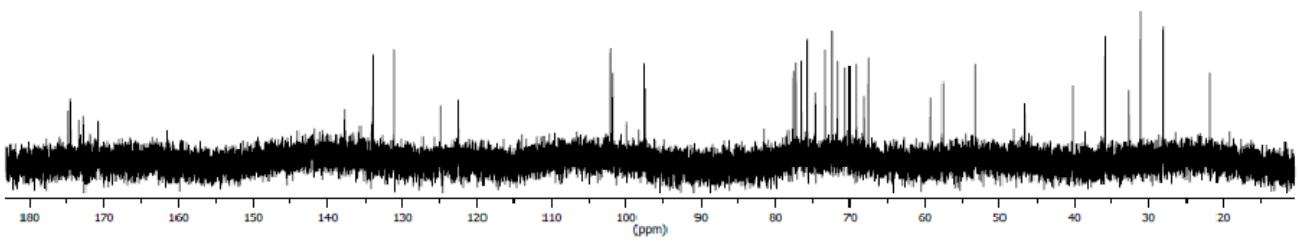
^{1}H and ^{13}C NMR spectra of tetrasaccharide GlcNAc6N$_3$α1–4GlcAβ1–4GlcNSα1–4GlcAβ2AA' (**22**)





^{1}H and ^{13}C NMR spectra of tetrasaccharide GlcNAc6NH_2α1–4GlcAβ1–4GlcNSα1–4GlcAβ2AA' (**23**)





^{1}H and ^{13}C NMR spectra for tetrasaccharide GlcNAc6NSα1–4GlcAβ1–4GlcNSα1–4GlcAβ2AA' (**24**)