Supplement

Materials

HS precursor polysaccharide was prepared from E. coli K5 strain.[9] Heparan Sulfate C-5 epimerase, 3-OST1, 6-OST2a, and NDST2 sulfotransferases were all cloned and expressed in baculovirus system.[12, 13, 15, 16, 17] [^{35}S]PAPS and [^{34}S]PAPS were prepared as reported earlier whereas [^{32}S]PAPS was purchased from Calbiochem. All chemicals were purchased from Sigma. ATIII and Factor Xa were from Haematologic Technologies Inc. Chromogenic substrate S-2765 was from Chromogenix. Heparitinase I, II and III were obtained from Seikagagu. APS kinase was a generous gift from Professor I. H. Segel (Univ. of California, Davis).

Expression of Heparan Sulfate Sulfotransferases and Epimerase

HS biosynthetic enzymes were cloned and expressed in baculovirus system as described in earlier work. (Liu, J., Shriver, Z., Blaiklock, P., Yoshida, K., Sasisekharan, R., and Rosenberg, R. D. (1999) J. Biol. Chem. 274, 38155-38162)

Cloning and expression of Epimerase

cDNA cloning of human glucuronyl C5 epimerase

A cDNA clone coding for human C5 epimerase was isolated from a human fetal brain cDNA panel (origene) by screening with PCR primers spanning nucleotides 7-157 of the coding region. A donor plasmid for the preparation of recombinant baculovirus expressing a soluble form of the epimerase was constructed in pFastBac HT plasmid modified by the insertion of honeybee melittin signal peptide ahead of the histidine tag. The construction employed a synthetic oligonucleotide adapter that also encoded amino acids 35-44 of the epimerase and two restriction fragments isolated from the cDNA clone (TaqI to EcoRI and EcoRI to SacI) that incorporate the rest of the epimerase coding.

Baculovirus Expression and Purification of Glucuronyl C5 epimerase

Human glucuronyl C5 epimerase recombinant baculovirus was prepared using the donor and the Bac-to-Bac baculovirus expression system (Life Technologies, Inc.) according to the manufacturer's protocol, except that recombinant bacmid DNA was purified using an endotoxin-free plasmid purification kit (Qiagen, Inc.) and transfection of Sf9 cells was scaled up to employ 15 µg of bacmid DNA and 2.5×10^7 exponentially growing cells in four 100-mm dishes. Medium containing recombinant baculovirus was harvested at 3 days post-transfection and amplified twice for about 65 hours each on Sf9 cells. The resulting high-titer viral stock was stored in aliquots (0.75 ml) sufficient to infect 3.5×10^8 cells, as determined by Western blotting of medium from infected cells using $(his)_4$ antibody (Qiagen). Infected cells were plated in ten 150 mm dishes and incubated at 26°C for 90-96 hours. The pooled medium was centrifuged at 400xg, adjusted to 10 mM in HEPES, titrated to pH 7.4, chilled on ice for 30 minutes and centrifuged at 16,000xg. The clarified pool diluted in half with 10 mM HEPES, pH 7.4, made 1mM in PMSF, and applied to an 8 ml column of ToyoPearl AF heparin 650M. The column was washed with 40 ml of HCG 50 (10 mM HEPES, pH 7.4, 2% glycerol, 0.6% CHAPS, 50 mM NaCl) and eluted with an 80 ml linear gradient of 50 to 600 mM

NaCl in HCG. Aliquots of selected 1ml fractions were analyzed by western blotting for the presence of the histidine tag and for the purity and then adjusted to 500 mM in NaCl, 10 mM in imidazole and concentrated an Amicon YM-10 membrane to about 3 ml.

Expression and Purification of NDST2

The complete coding sequence of human NDST2 was assembled from portions of two clones isolated from a human fetal brain cDNA library panel (Origene Technologies) using a unique, internal restriction site. A baculovirus donor plasmid was constructed in pFastBacMelHT to give secreted expression of a truncated, chimeric NDST2, in which amino acids 1-45 were replaced with fourteen aminoacid containing a central hexa-histidinyl sequence. This plasmid was transformed into E. coli DH10Bac cells (Invitrogen) to yield recombinant bacmid which, upon transfection into Sf9 insect cells, produced recombinant baculovirus. Expressed protein was purified from the serum-free medium of baculovirus-infected Sf9 cells at ~90 hours post-infection. Medium, diluted in half and adjusted to 10 mM PIPES, pH 7.0, was applied to a heparin affinity column (ToyoPearl AF heparin 650M). The column was eluted with a NaCl gradient, with peak activity emerging at 0.3 M NaCl. The pooled activity peak was adjusted to 0.5 M in NaCl, concentrated by ultrafiltration, and fractionated on NiNTA magnetic agarose beads (Qiagen). Bound protein was eluted with 250 mM imidazole.

Expression and Purification of 3-OST1

3-OST1 recombinant baculovirus was prepared from 3-OST1 baculovirus expression plasmid using the Bac-to-Bac Baculovirus Expression system (Life Technologies, Inc.) according to the manufacturer's protocol. Exponentially growing Sf9 cells (3 to 4 × 10^7 cells/T175, Invitrogen) were infected with 25 µl (2-4 × 107 plaque form units/ml) of recombinant viral stock solution. The cell medium (40 ml/T175 flask) was changed to serum-free medium (SFM-900, Life Technologies, Inc.) 48 h after infection. The medium was harvested every 24 h for 4 days. The harvested medium was centrifuged at 1000 × g for 15 min, and CHAPS was added to a final concentration of 0.6%. This solution was frozen in liquid nitrogen and stored at −80 °C for subsequent purification. The entire purification was carried out at 4 °C. The harvested medium was mixed with Tris-HCl to a final concentration of 10 mM, adjusted to pH 8 with 1 N NaOH, and centrifuged. The supernatant was mixed with an equal volume of cold 10 mM Tris, pH 8.0, and then loaded on a heparin-AF Toyopearl-650 M column (1 × 10 cm, TosoHaas), which was equilibrated with 10 mM Tris, 0.6% CHAPS, 2% glycerol, pH 8.0 (TCG buffer), and 200 mM NaCl, at a flow rate of 4 ml/min. The column was then washed with 80 ml of TCG buffer containing 200 mM NaCl and eluted with a linear gradient of NaCl from 200 to 1000 mM in 80 ml of TCG buffer. The fractions (66 ml) containing 3-OST1 activity were pooled and dialyzed against 200 mM NaCl in TCG buffer using 14,000 MWCO tubing (Spectrum). The dialyzed solution was loaded on a 3',5'-ADP-agarose column (0.5 × 8 cm, Sigma), which was equilibrated with 200 mM NaCl in TCG buffer at a flow rate of 0.2 ml/min. The column was washed with 6 ml of TCG buffer containing 200 mM NaCl and eluted with a linear gradient of NaCl from 200 to 1000 mM in 12 ml of TCG buffer followed by an isocratic elution with 12 ml of 1000 mM NaCl. The fractions (9 ml) containing 3-OST1 activity were pooled. A portion of 3',5'-ADP-agarose column purified material (1 ml) was further fractionated by gel

permeation chromatography-HPLC (GPC-HPLC). The column was equilibrated with a buffer containing 25 mM MOPS, 2% glycerol, 0.6% CHAPS, 1000 mM NaCl, pH 7.0, at a flow rate of 0.5 ml/min at room temperature. The GPC-HPLC purified enzymes were frozen in liquid nitrogen and stored at −80 °C.

Expression and Purification of 6-OST2a

Human 6-OST2a recombinant baculovirus was prepared using the pFastBac HT donor plasmid modified by the insertion of honeybee mellitin signal peptide and the Bac-to-Bac baculovirus expression system (Life Technologies, Inc.) according to the manufacturer's protocol, except that recombinant bacmid DNA was purified using an endotoxin-free plasmid purification kit (Qiagen, Inc.) and transfection of Sf9 cells was scaled up to employ 3 µg of bacmid DNA and 6 × 106 exponentially growing cells in a 100-mm dish. At day 3 post-transfection, baculovirus was precipitated from the medium with 10% polyethylene glycol, 0.5 M NaCl at 12,000 × g, re-suspended in 14 ml of medium, and applied to a 100-mm dish seeded with 1.5 × 107 Sf9 cells. Medium from the infected cells was harvested after 90 h of growth at 27 °C, centrifuged at 400 × g, made to 10 mM in Tris, adjusted to pH 8.0, and centrifuged at 4000 × g. Clarified medium was diluted with an equal volume of cold 10 mM Tris-HCl, pH 8.0, and stirred for 30 min with 0.6 ml (packed volume) of Toyopearl 650M chromatographic media (TosoHaas). The heparin-Sepharose was packed into a column (0.4 × 4.75 cm), washed with 5 ml of TCG 50 (10 mM Tris-HCl, pH 8.0, 2% glycerol, 0.6% CHAPS, 50 mM NaCl), eluted with 1.2 ml of TCG 1000 (as above, but 1 M in NaCl) containing 10 mM imidazole, and concentrated to 0.25 ml in a Microcon YM-10 centrifugal filter (Millipore Corp.). Histidine-tagged recombinant enzyme was affinity-purified by mixing the product eluted from heparin-Sepharose for 90 min at 4 °C with nickel-nitrilotriacetic acid magnetic agarose beads (Qiagen, Inc.) magnetically sedimented from 60 µl of suspension. The beads were washed twice with 0.125 ml of TCG 400 containing 20 mM imidazole and eluted twice with 0.03 ml of TCG 400 containing 250 mM imidazole. The combined elution fractions contained ~25% of the sulfotransferase activity present in the starting medium.

Buffer for sulfotransferase reactions

The labeling 2X buffer contains 50 mM MES (pH 7.0), 1% (W/V) triton X-100, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 2.5 mM $CaCl_2$, 0.075 mg/mL protamine chloride, 1.5 mg/mL BSA with or without P40. PAPS stock solution with the final concentration of 20 mg/mL was prepared and stored at -80 °C.

Digestion of oligosaccharides with Heparitinase I, Heparitinase II, and Heparinase

Heparitinase I (EC 4.2.2.8), heparitinase II (no EC number), and heparinase (EC 4.2.2.7) were obtained from Seikagagu; Heparitinase I recognizes the following sequences: GlcNAc/NS±6S(3S?)-GlcA-GlcNAc/NS±6S. Heparitinase II has broad sequence recognition, GlcNAc/NS±6S(3S?)-GlcA/IdoA±2S-GlcNAc/NS±6S. Heparinase recognizes the sequences: GlcNAc/NS±3S±6S-IdoA2S-GlcNAc/NS±6S. Polysaccharides were digested with 1 mU of Hep1, Hep II and Hep III in a total volume of 100 µl of 40 mM Ammonium acetate containing 1mM Calcium chloride buffer (pH

7.0) at 37 °C overnight. A portion of the reaction mixture (10µl) was subjected to heat inactivation at 100°C for 2 min, which was then diluted to 20 µl with double distilled water and loaded onto capillary HPLC coupled to ESI-MS for structural analysis to evaluate disaccharide profile.

Flow Injection Capillary Liquid Chromatography

An Ultimate capillary HPLC workstation (Dionex, Sunnyvale, USA) was used for microseparation. UltiChrom software was used in data acquisition and analysis. A gradient elution was performed, using a binary solvent system composed of water (eluent A) and 70% aqueous methanol (eluent B), both containing 8 mM acetic acid and 5 mM dibutylamine as an ion-pairing agent. HPLC separations were performed on a 0.3 mm × 250 mm C18 polymeric silica column (Vydac, Hesperia, USA). The column temperature was maintained at 25 ºC and the flow rate was set to 5 µL min^{-1}. Sample volumes of 6.3 µL were injected. The chromatographic conditions were optimized for resolution of disaccharides. In brief, non-sulfated disaccharide was eluted with 100% A, single sulfated disaccharides were eluted with 10% B, isocratic elution with 20% B for double sulfated disaccharides, followed by isocratic elution with 35% B for triple sulfated disaccharide. The column was washed and equilibrated by further elution with 100% B for 10 min, returning to 100% A for 10 min at the end of the run. The absorbance of the column eluate was monitored at 232 nm. Alternatively isocratic elution with 0 % B, followed by 40 % B was carried out to determine the total disaccharide composition.

Mass Spectrometry

Mass spectra were acquired on a Mariner BioSpectrometry Workstation ESI time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, MA). In the negative-ion mode, the instrument was calibrated with bis-trifluoromethyl benzoic acid, heptadecafluorononanoic acid, and perfluorotetradecanoic acid. Nitrogen was used as a desolvation gas as well as a nebulizer. Conditions for ESI-MS were as follows: nebulizer flow 0.75 L/min, nozzle temperature 140°C, drying gas (N_2) flow 1.2 L/min, spray tip potential 2.8 kV, nozzle potential 70 V, and skimmer potential 12 V. Negative ion spectra were generated by scanning the range of *m*/*z* 40-2000. During analyses, the indicated vacuum was 1.9×10^{-6} Torr.

Enzymatic modification with recombinant enzymes: NDST2, C5 Epimerase, 6-OST2a, and 3-OST1

The labeling 2x buffer contains 50 mM MES (pH 7.0), 1% (W/V) triton X-100, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 2.5 mM $CaCl_2$, 0.075 mg/ml protamine chloride, 1.5 mg/ml BSA or 25 mM HEPES, 40 mM $CaCl_2$, pH 6.5. For a 2500 µl reaction, the following were assembled: polysaccharide (final concentration was 1mM equivalent of unmodified disaccharide), 1250 µl of 2x buffer, sulfotransferase or epimerase, [^{35}S]PAPS (~ $1.0x10^7$ cpm) or [^{32}S]PAPS (final concentration of 20 µM) , and water was added to make volume 2.5 ml. The reaction was incubated at 37°C for 12 hrs, then diluted to 5 ml with DEAE wash buffer and purified on DEAE column. Alternatively, the reaction was stopped by heating at 70°C and the reaction mixture was centrifuged at 10,000 *g* for 3 min and the supernatant was used for gel mobility shift analysis. Modified

polysaccharide was digested with heparitinases I, II and III and was analyzed by capillary HPLC-ESI-TOF-MS.

Table 1. Enzymes used, their concentration and the relative times employed

Enzymes	Concentration	Quantity	Time
NDST-2	188±25.4 μg/mL	1 μL	Over night (~12 hours)
C5 epimerase	25 μg/mL	1 μL + 1 μL	
6-OST-2a	366±30 μg/mL	1 μL	Over night (~12 hours)
3-OST-1	4 μg/mL	1 μL	

Gel mobility shift assay

Heparin–ATIII binding buffer contained 12% glycerol, 20 mM Tris-HCl (pH 7.9), 100 mM KCl, 1 mM EDTA, and 1 mM DTT. For a typical 20 μl binding reaction, radiolabeled polysaccharide (~ 10,000 cpm) was mixed with AT-III (1 μg) in the binding buffer. The reaction mixture was incubated at room temperature (23°C) for 20 min and was then applied to a 4.5% native polyacrylamide gel (with 0.1% of bis-acrylamide). The gel buffer was 10 mM Tris (pH 7.4) and 1 mM EDTA, and the electrophoresis buffer was 40 mM Tris (pH 8.0), 40 mM acetic acid, 1 mM EDTA. The gel was run at 6 volts/cm for 1–2 h with an SE 250 Mighty Small II gel apparatus (Hoefer Scientific Instruments, San Francisco). After electrophoresis, the gel was transferred to 3 MM paper and dried under vacuum. The dried gel was autoradiographed by a PhosphorImager 445SI (Molecular Dynamics, Sunnyvale, CA). The image was analyzed with NIH Image 1.60 and the band intensities were evaluated.

Factor Xa assay

Human factor Xa (10.4 mg/ml 50% glycerol, 820 units/mg) was used for assay. Factor Xa was diluted 1:200 with PBS containing 1 mg of bovine serum albumin (4 units/ml and 15 units/ml, respectively). ATIII (2.5 mg/ml) was diluted 1:200 to give a 2×10^{-7} M stock solution. The chromogenic substrate S-2765 was from Chromogenix and the stock solution of 1 mM with 1 mg/ml Polybrene in water was prepared. Heparin (174 international units/mg, Sigma) was used as a standard. Mitrin was used for factor Xa (≦10 ng). The protocol involved adding 25 μl of ATIII (2×10^{-7} M) to 25 μl of a serial dilution of heparin standards or Mitrin in Tris-EDTA (50 mM Tris, 7.5 mM EDTA, and 175 mM NaCl (pH 8.4)) buffer. The reaction was incubated at 37 °C for 75 s. Factor Xa (25 μl, 4 units/ml) was added. After incubating at 37 °C for 195 s, 25 μl of S-2765 was added. The absorbance at 405 nm was read every minute for 10 min using a Beckman UV spectrometer.

Table 2. Disaccharide compositional analysis of Mitrin obtained from total ion chromatogram (LC/MS) (see the following Figure A)[#]

Disaccharides	Percentage
ΔU-GlcNAc	< 1%
ΔU-GlcNAc6S (peak a)	9 %
ΔU-GlcNS (peak b)	10 %
ΔU-GlcNS6S (peak c)	31 %
ΔU-GlcNS6S3S (peak d)	48 %

([#] Disaccharide constituents of resistant tetrasaccharides [peak d, peak e and peak f of Figure A] were also taken into account to calculate the percentage of each disaccharide, which is approximately equivalent to each of sulfotransferase mediated modification)

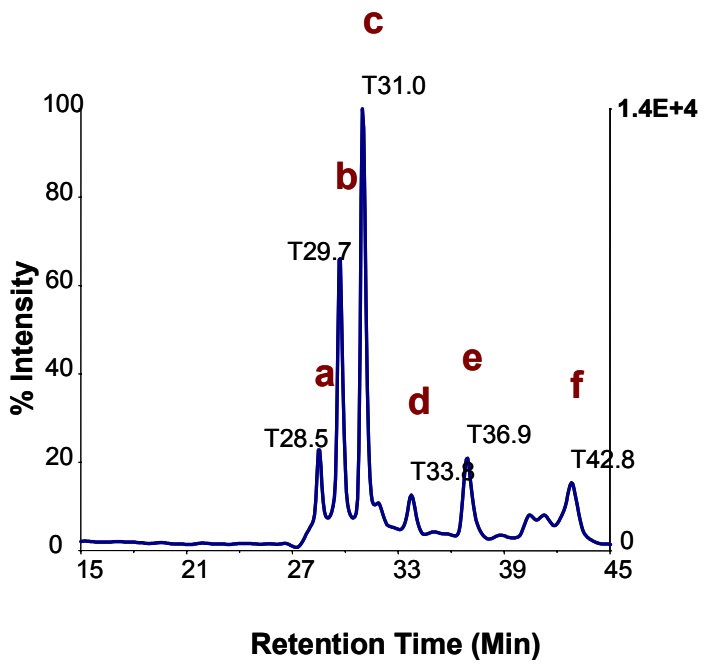
The percentage modification by each biosynthetic enzyme

N-sulfation ~ 90 %
Epimerization* ~ 70 %
6-O sulfation ~ 87 %
3-O sulfation ~ 50 %

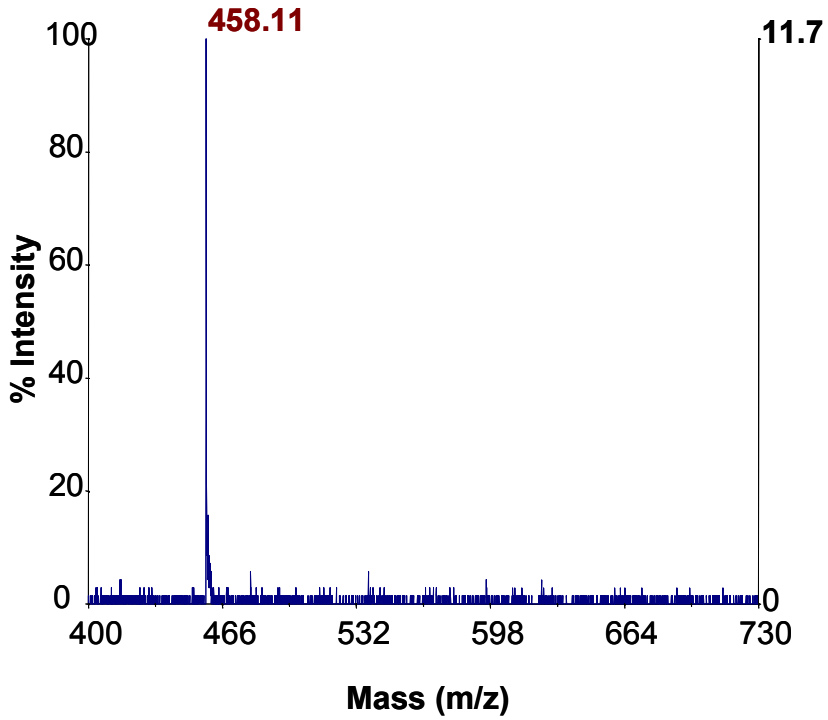
(* percentage conversion of uronic acid by epimerase is calculated from radioactive labeling of polysaccharide **2** with PAP^{35}S catalyzed by 6-OST2a or 6-OST1 followed by nitrous acid degradation to obtain disaccharides: Ido-AnMan_R6S, Glu-AnMan_R6S. The ratio of two disaccharides is an indirect measure of the percentage conversion of uronic acids catalyzed by epimerase. Sincere there is no difference in the mass at disaccharide level following epimerase action, one has to rely on this indirect biochemical method rather than utilizing the most accurate mass spectrometry)

Total Ion Chromatogram of Heparitinase Digested Polysaccharide 3

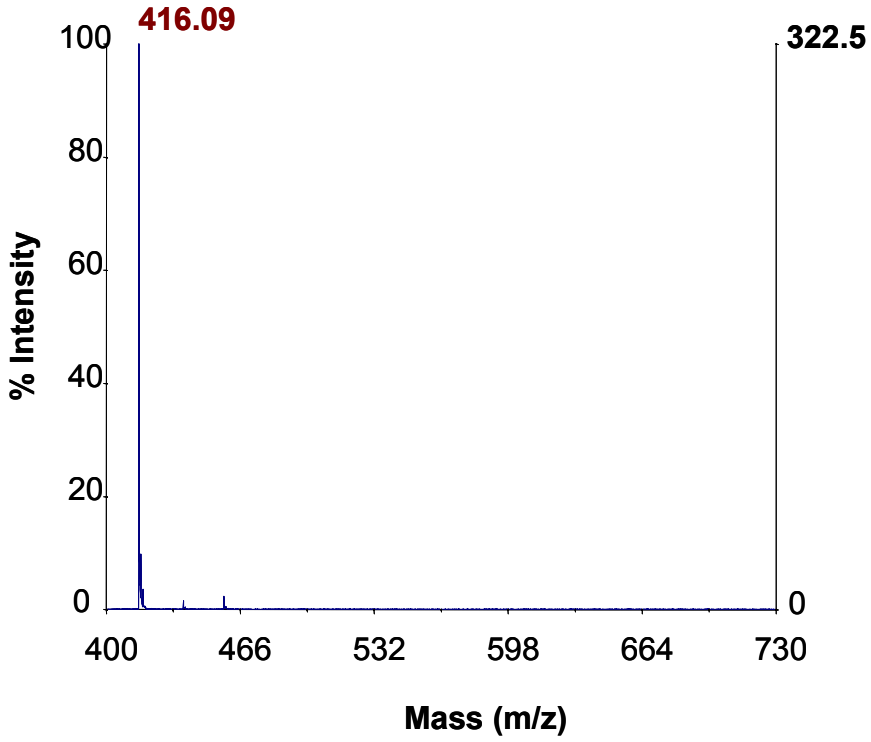
Figure A



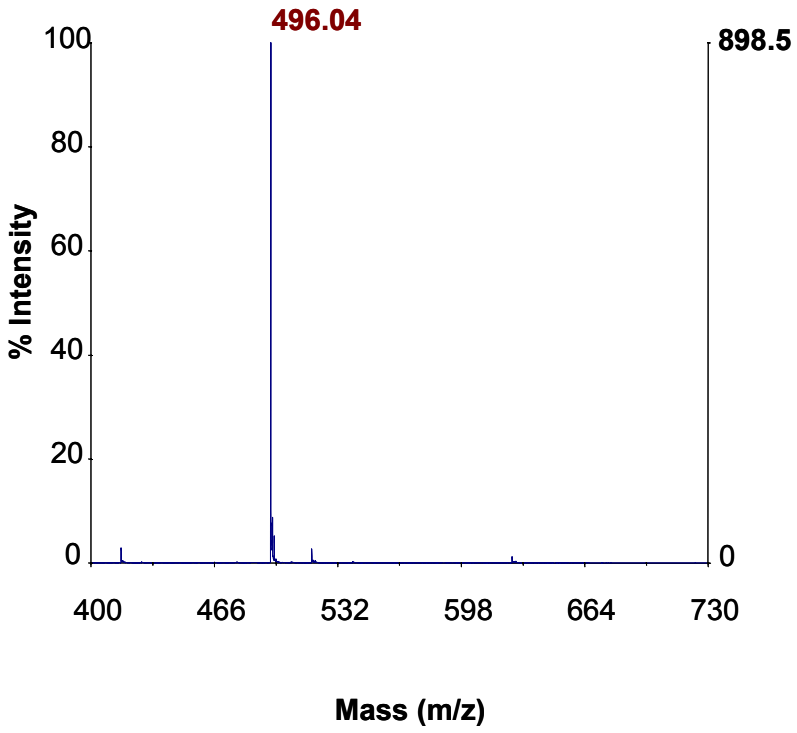
Mass Spectrum of Disaccharide ΔU-GlcNAc6S



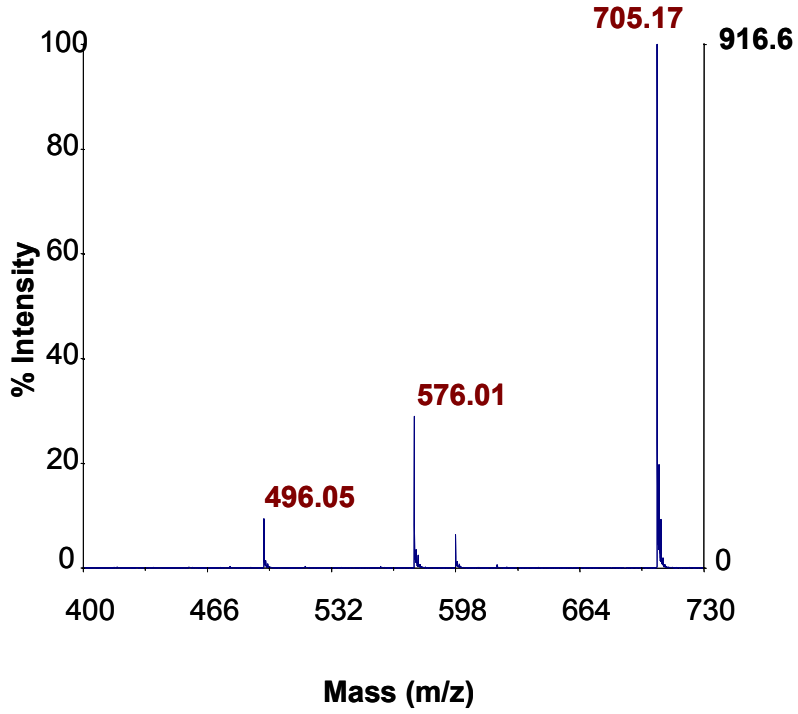
Mass Spectrum of Disaccharide ΔU-GlcNS (peak a)



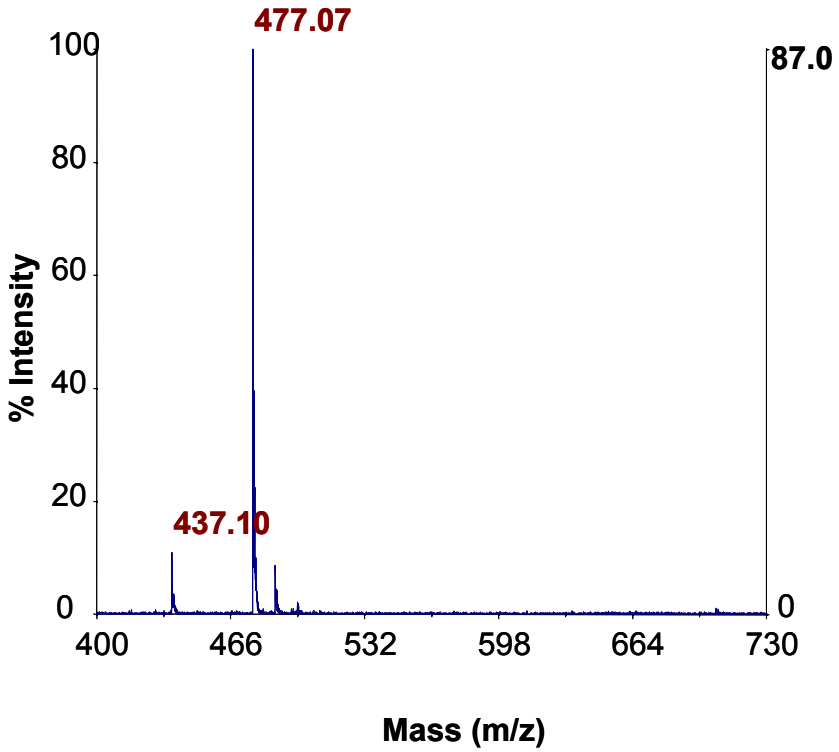
Mass Spectrum of Disaccharide ΔU-GlcNS6S (peak b)



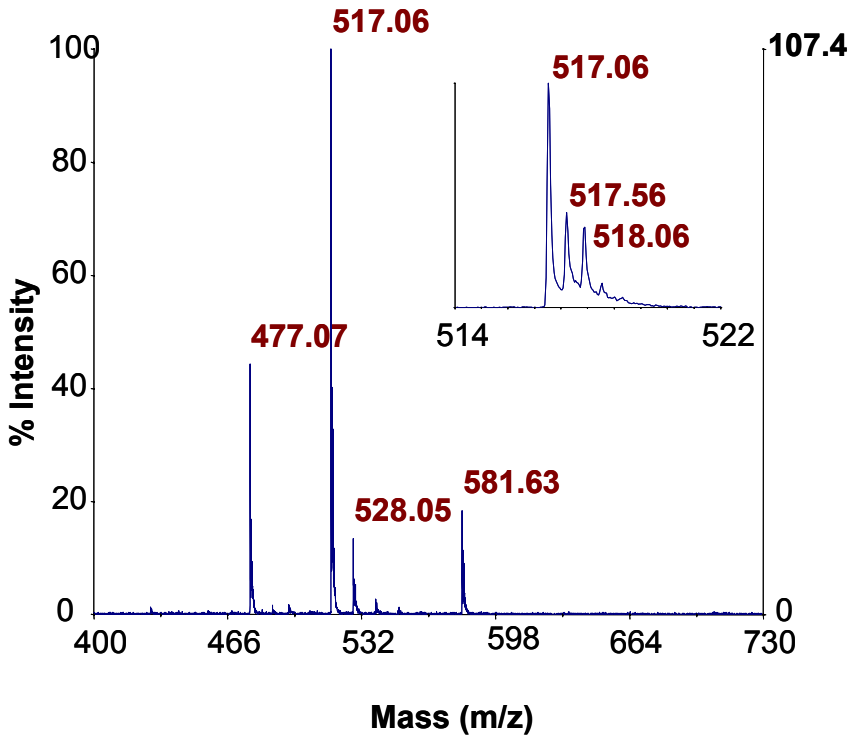
Mass Spectrum of Disaccharide ΔU-GlcNS6S3S (peak c)
(major peak was observed with DBA adduction)



Mass Spectrum of Tetrasaccharide (peak d)



Mass Spectrum of Tetrasaccharide (peak e)



Mass Spectrum of Tetrasaccharide (peak f)