Substrate Specificity of FUT8 and Chemoenzymatic Synthesis of Core-fucosylated Asymmetric *N*-glycans

Angie D. Calderon,[a] Yunpeng Liu,[ab] Xu Li,[a] Xuan Wang,[a] Xi Chen,[c] Lei Li,*[a] and Peng G. Wang*[a]

[a]Department of Chemistry and Center of Diagnostics & Therapeutics, Georgia State University, 50 Decatur St SE, Atlanta, GA 30303

[b]Chemily, LLC, 58 Edgewood Ave NE, Atlanta, GA 30303

[c]Department of Chemistry, University of California, Davis, One Shields Avenue, Davis, CA 95616

Supporting Information

Table of Contents

I. Materials and enzymes

β1,4-galactosyltransferase from bovine milk (B4GALT1) was purchased from Sigma. Thermosensitive Alkaline Phosphatase (FastAP) was purchased from Thermo Scientific. *N*,*N*'-diacetyl-chitobiose (**N01**) was purchased from Sigma, and N-glycans **N02**, **N03**, **N04**, **N05**, **N06** were purchased from V-labs (Covington, LA). Other enzymes including double mutant E271F/R313Y from *Pasteurella multocida* α2,3-sialyltransferase 1 (PmST1m)[1], α2,6-sialyltransferase from *Photobacterium damslae* (Pd2,6ST)[2], C-terminal 66 amino acids truncated *Helicobacter pylori* α1,3-fucosyltransferase (Hpα1,3FT)[3], CMP-sialic acid synthetase from *N. meningitidis* (NmCSS)[4] were expressed and purified as previously described. Enzymes were then desalted against 50 mM Tris-HCl, 100 mM NaCl, and 50% glycerol, and stored at -20 °C for long term use. Sugar nucleotides uridine 5'-diphospho-galactose (UDP-Gal)[5], cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac)[4] and guanosine 5'-diphospho-L-fucose (GDP-Fuc)[6] were prepared as described previously.

II. Expression and purification of human FUT8

Cryopreservation and maintenance of Baculovirus infected Sf9 cells The 50 μL of P1PP1 baculovirus stock and *Sf9* cells for the expression of the FUT8 were kindly provided by Dr. Donald Jarvis from University of Wyoming. For the Titerless Infected-cells Preservation and Scale-up (TIPS) expression method[7], a primary stock of cryopreserved infected cells BIIC was prepared by infecting 25 mL of *Sf9* cells at a concentration of 1×10^6 C/mL with 25 μL of untitered liquid P1PP1 baculovirus stock (an estimated MOI of approximately 3 pfu/cell). The cells were cryopreserved when the average diameter increases by 20-30% of their pre-infected diameter and the viability was above 95%. *Sf9* cells were grown and infected at 28 °C and 150 rpm in complete TNM-FH medium [TNM-FH medium (Sigma-Aldrich), supplemented with 10% Fetal Bovine Serum (FBS) (BioWest), 0.5% antibiotic-antimycotic (Sigma-Aldrich), and 0.1% Pluronic-F68 (Invitrogen)]. The cells were cryopreserved at a density of 1×10^7 C/mL in liquid nitrogen and complete TNM-FH medium and 10% DMSO (Sigma-Aldrich). Cell density was determined by hemocytometer counts, and the cell viability was evaluated by Trypan Blue staining.

FUT8 expression and purification *Sf9* cells were infected at a cell density of 1×10^6 C/mL. The baculovirus titer was estimated using insect cell density at time of infection and a conservative cellular baculovirus maximum production rate of 100 pfu/cell for 1×10^6 C/mL meaning 1×10^8 pfu/mL. BIIC stock was thawed in a 37 °C water bath and diluted 1:100 into cell-free medium, then 10 mL of infected cell-free medium was added to 1 L of uninfected cells (1:1000 dilution)[7]. The medium was collected after 72 h of expression followed by purification. The purification of the secreted protein was accomplished by diluting the medium 1:1 with equilibration buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl) and loading to 2 mL of pre-equilibrated Ni-Sepharose Excell

(GE Healthcare)[8]. The column was rinsed using 200 mL of wash buffer (50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 50 mM imidazole), and finally eluted with 5 mL of elution buffer (50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 250 mM imidazole). The enzyme was desalted and concentrated using Amicon YM-30 ultrafiltration membrane (Millipore) against desalting buffer (50 mM Tris-HCl pH 7.5). Purified FUT8 was confirmed by SDS-PAGE (>90% pure) and western blot (**Figure S1**). Using TIPS method 0.42 mg of human FucT8 were purified from 1 L cultures as determined using Bradford method. The activity of recombinant FUT8 was confirmed by using **N000** as a substrate, monitored by MS (**Figure S2**).

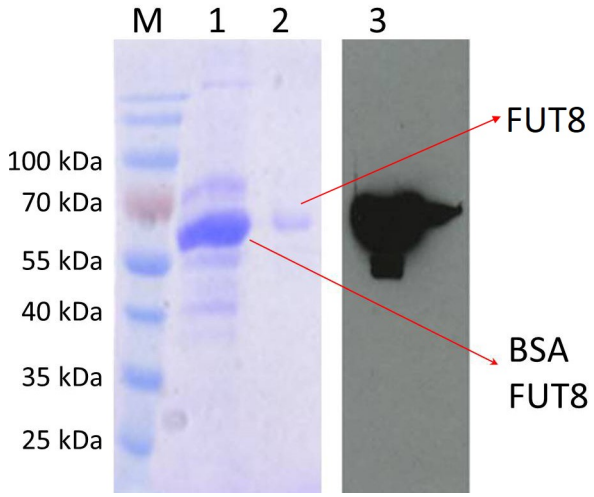


Figure S1. SDS-PAGE and western blot analysis of FUT8. 1) SDS-PAGE of medium after overexpression of FUT8; 2) SDS-PAGE of purified FUT8; and 3) western blot analysis of purified FUT8 using anti-His antibody as the primary antibody. BSA, a major protein component of Fetal Bovine Serum used in the culture medium, has similar molecular weight as that of FUT8.

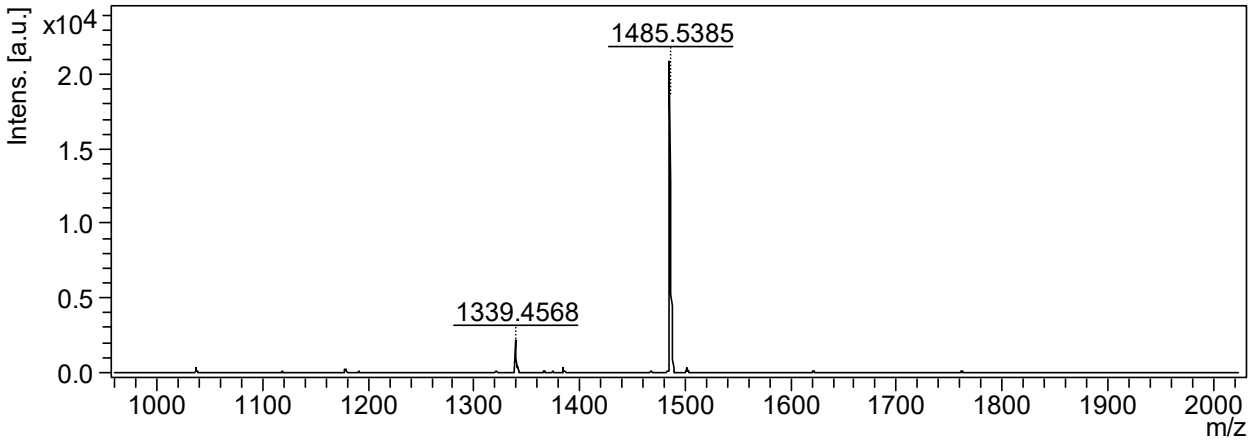


Figure S2. MALDI-MS analysis of FUT8 catalyzed reaction using **N000** as substrate. Majority of N000 (molecular weight 1316.4865, found M+Na 1339.4568) was converted into core-fucosylated one (molecular weight 1462.5444, found M+Na 1485.5385).

III. Substrate specificity study of FUT8

A detailed substrate specificity study of FUT8 was performed using 77 chemically or chemoenzymatically synthesized, or commercially available *N*-glycans (with free reducing end) as acceptors (**Figure S3**). Reactions were performed in a 96 well PCR plate, with each well (20 μL total volume) contains one *N*-glycan substrate (0.3 mM, 2.5 μg to 15.1 μg), GDP-Fuc (1 mM), FUT8 (0.05 mg/mL) and MES buffer (100 mM, pH 7.0). Reactions were incubated at 37 °C for 4 h in an Eppendorf thermocycler (Mastercycler Pro), followed by enzyme inactivation at 95 °C for 20 min. A negative control was also set up using **N000** as substrate without enzyme. After centrifugation (13,000 g for 10 min), the supernatant was analyzed by HPLC using an analytical Waters XBridge BEH amide column (130 Å, 5 μm, 4.6 x 250 mm), detected by an evaporative light scattering detector (Shimadzu ELSD-LTII). Gradient elution (%B: 75– 60% for glycans with less than 9 monosaccharides, 70– 55% for glycans with 9 or more monosaccharides) was performed with Solvent A (100 mM ammonium formate, pH 3.4) and Solvent B (Acetonitrile). The percent yields (average of three replicated reactions, see **Table S1**) were calculated as % = Product peak area/ (Product peak area + Substrate peak area) × 100.

Table S1. Substrate specificity of FUT8. ND, Not Detected. Conversions were monitored by HPLC-ELSD, average of three replicates were shown.

Glycan substrate	Conversion (%)
N04	5.26
N010	75.6
N020	49.8
N030	32.8
N000	91.3
N110	63.4
N210	ND
N211	67.8
N212	69.4
N213	70.4
N214	40.1
N215	34.8
All other N-glycans	ND

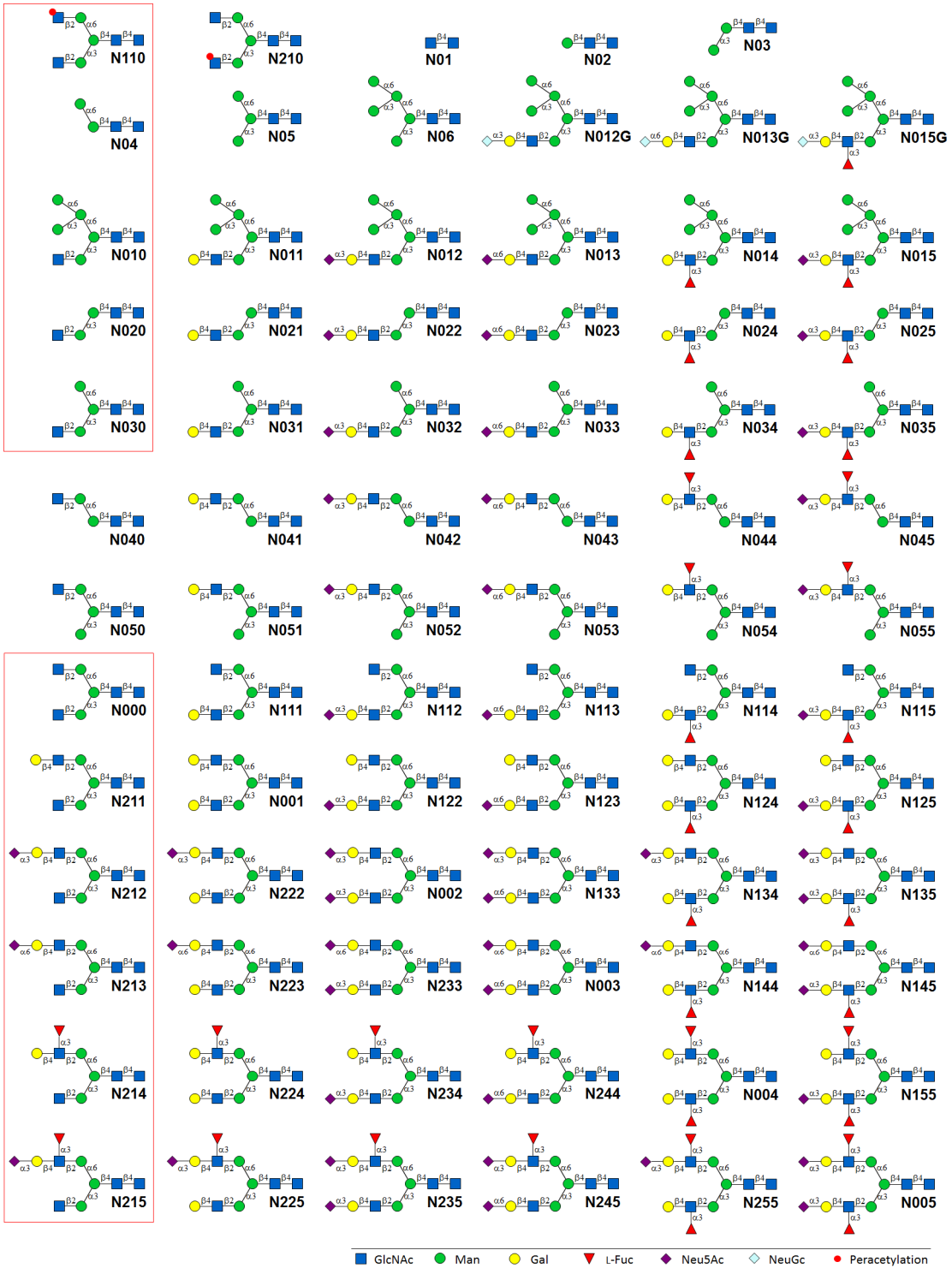


Figure S3. The 77 *N*-glycans used as acceptor for FUT8 substrate specificity study. The preparation and characterization of all substrates were published previously[9]. The ones in the red square were found to be suitable acceptors for FUT8.

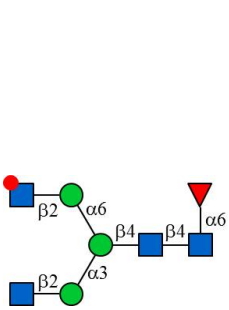
IV. General methods for glycan synthesis and purification

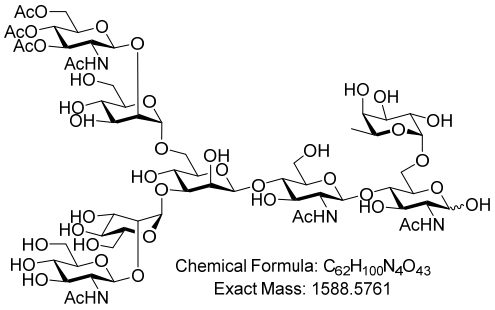
Reactions catalyzed by B4GALT1, Hpα1,3FT, PmST1m and Pd2,6ST were performed and monitored as we described previously[9]. Reactions catalyzed by FUT8 were performed in 100 mM MES buffer (pH 7.0), containing 2 mM glycan acceptor, 4 mM GDP-Fuc donor, FastAP (10 U/mL) and 0.1 mg/mL FUT8. Reactions were allowed to proceed for 6 h to overnight at 37 °C until substrates were totally converted to products (monitored by HPLC-ELSD as described above). The reactions were then quenched by freezing in -80 °C for 30 min, followed with concentration by lyophilization. HPLC-A_{210nm} was then used to purify target glycans using a semi-preparative amide column (130 Å, 5 μm, 10 mm × 250 mm). The running conditions are solvent A: 100 mM ammonium formate (for glycans with Neu5Ac residues) or water (for glycans without Neu5Ac residues), pH 3.4; solvent B: acetonitrile; flow rate: 4 mL/min; B%: 70-55% within 25 min. Products containing portions were then concentrated and lyophilized for characterization. The purity of each glycan was confirmed by HPLC-ELSD using an analytical Waters amide column (130 Å, 5 μm, 4.6 mm × 250 mm). The running conditions are solvent A: 100 mM ammonium formate, pH 3.4; solvent B: acetonitrile; flow rate: 1 mL/min; B%: 65-50% within 25 min.

The molecular weight of each N-glycans was confirmed by MALDI-MS performed on UltrafleXtreme MALDI TOF/TOF Mass Spectrometer (Bruker). Scan range of MS^1 was according to the molecular weight of N-glycans, and reflector mode was used for N-glycan analysis. Mass spectra were obtained in both positive and negative extraction mode with the following voltage settings: ion source 1 (19.0 kV), ion source 2 (15.9 kV), and lens (9.3 kV). The reflector voltage was set to 20 kV. The laser was pulsed at 7 Hz and the pulsed ion extraction time was set to 400 ns. The laser power was kept in the 25–40% range. Structures of key intermediates and asymmetric N-glycans with core-fucosylation were confirmed by ^{1}H NMR.

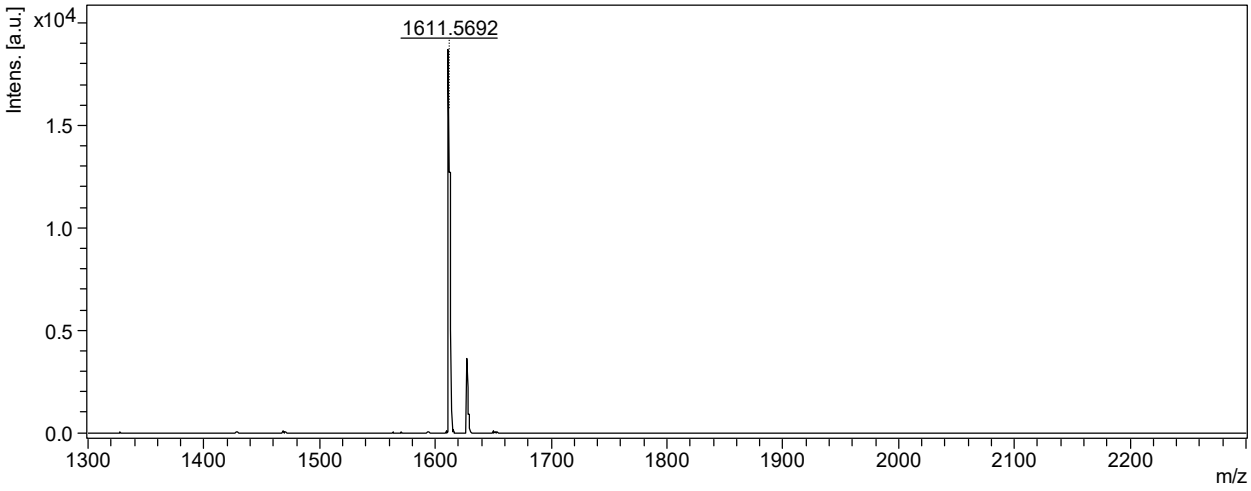
V. HPLC profiles and MS data of purified N-glycans

N6110 (6.9 mg)

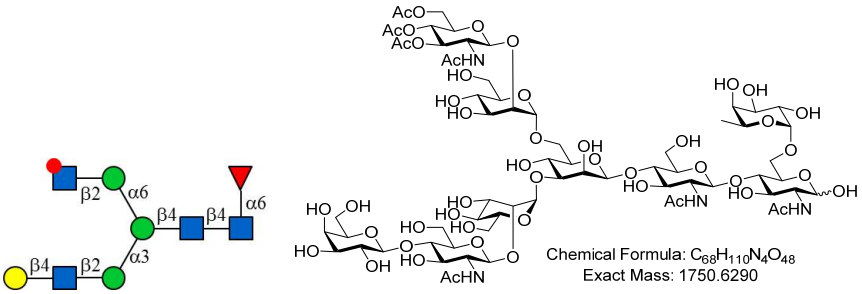




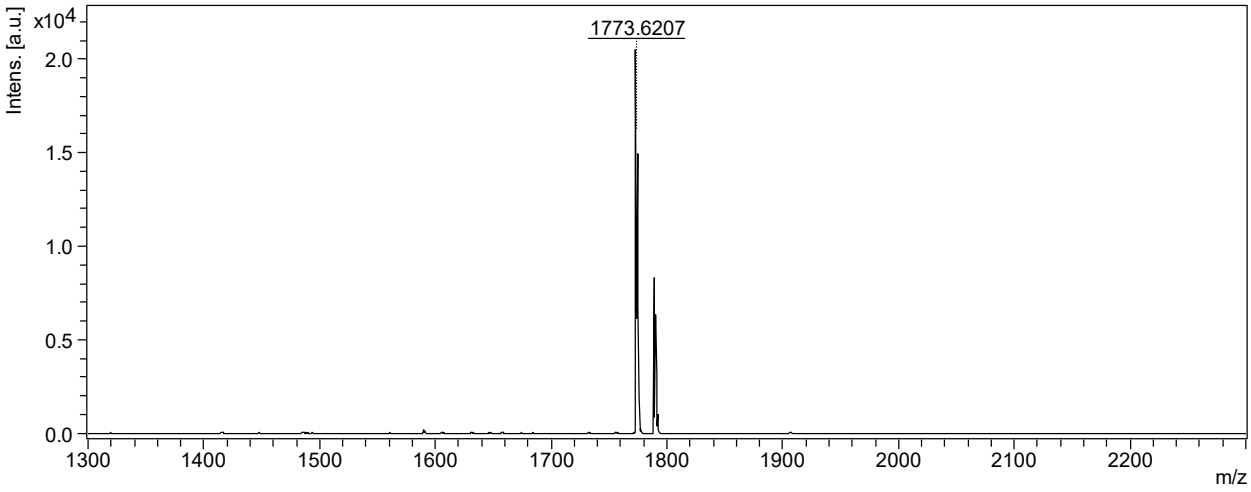
MALDI-MS, calculated: 1588.5761; found $[M+Na]^+$: 1611.5692, $[M+K]^+$: 1627.1403



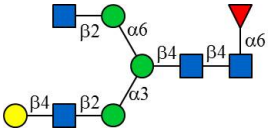
N6111a (3.8 mg)

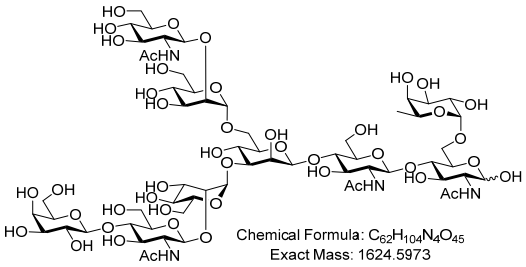


MALDI-MS, calculated: 1750.6290; found $[M+Na]^+$: 1773.6207, $[M+K]^+$: 1789.2301

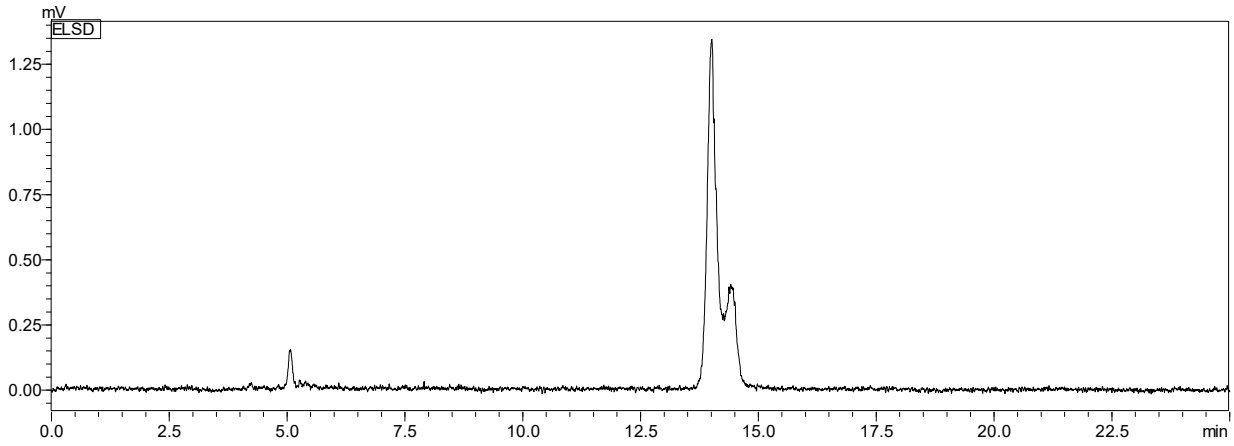


N6111 (3.5 mg)

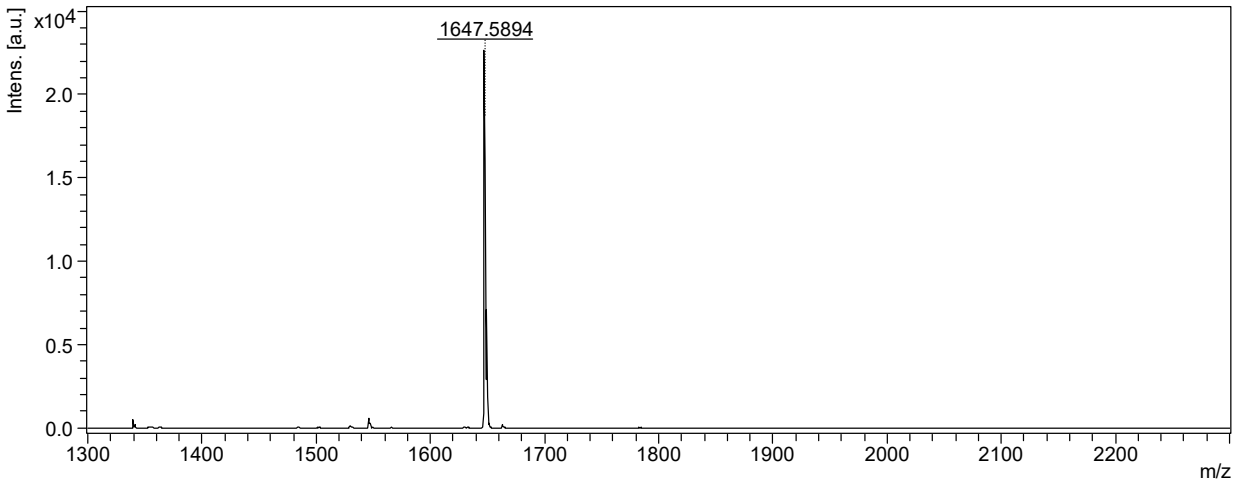




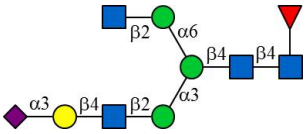
HPLC-ELSD, T_R = 14.01 min



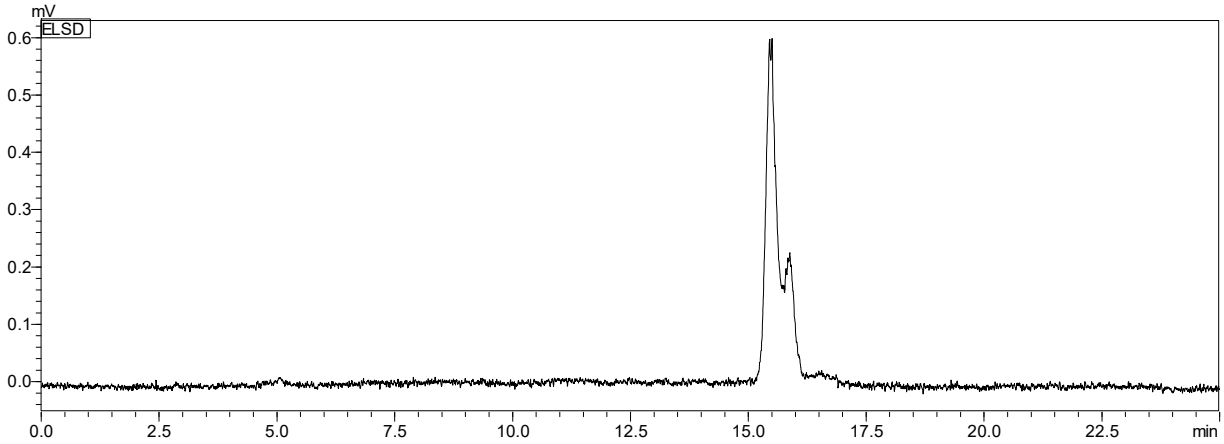
MALDI-MS, calculated: 1624.5973; found $[M+Na]^+$: 1647.5894



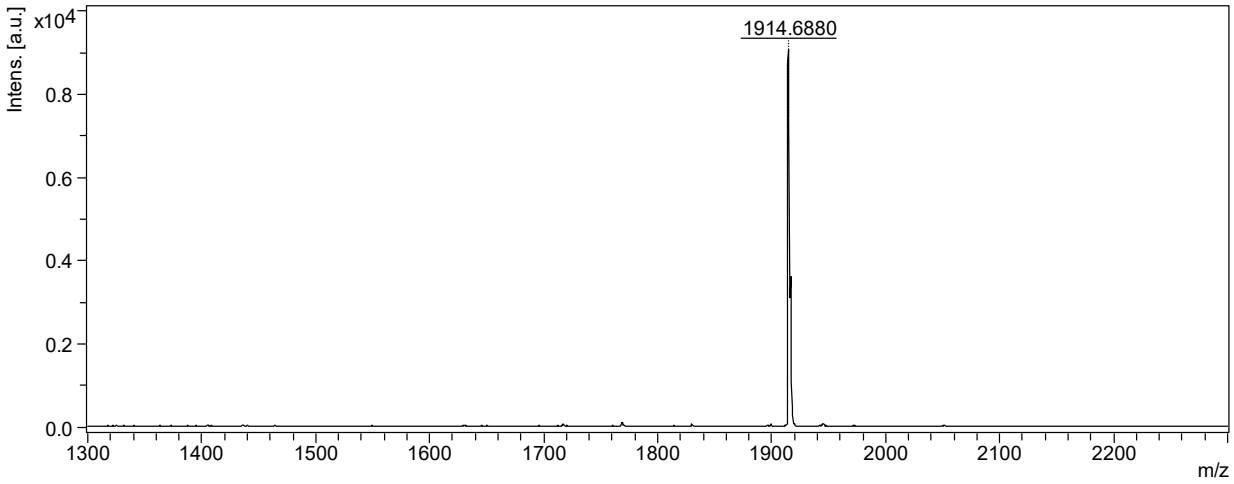
N6112 (1.0 mg)



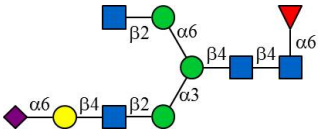
HPLC-ELSD, T_R = 15.45 min



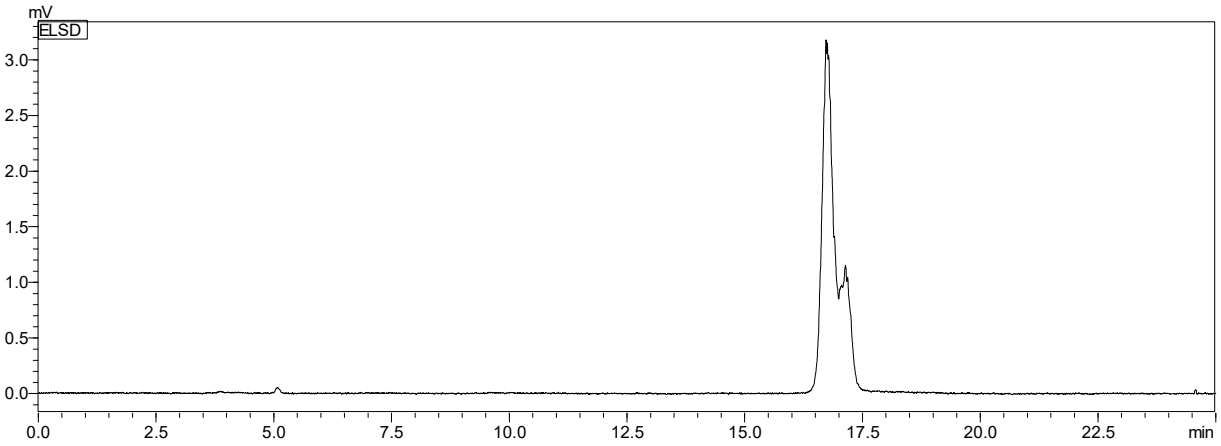
MALDI-MS, calculated: 1915.6927; found $[M-H]^-$: 1914.6880



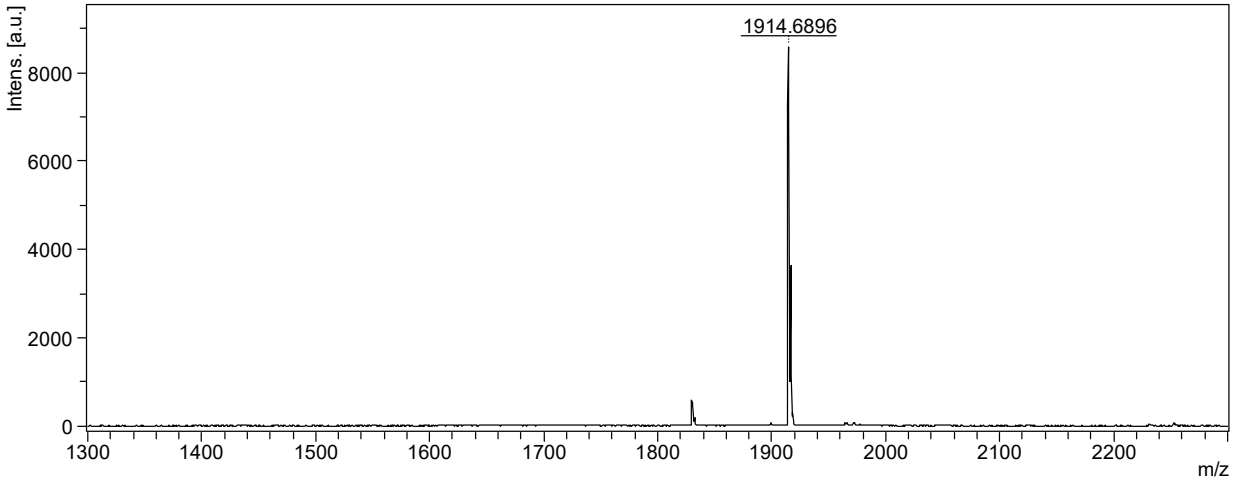
N6113 (1.5 mg)



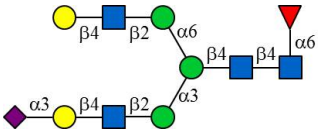
HPLC-ELSD, T_R = 16.73 min



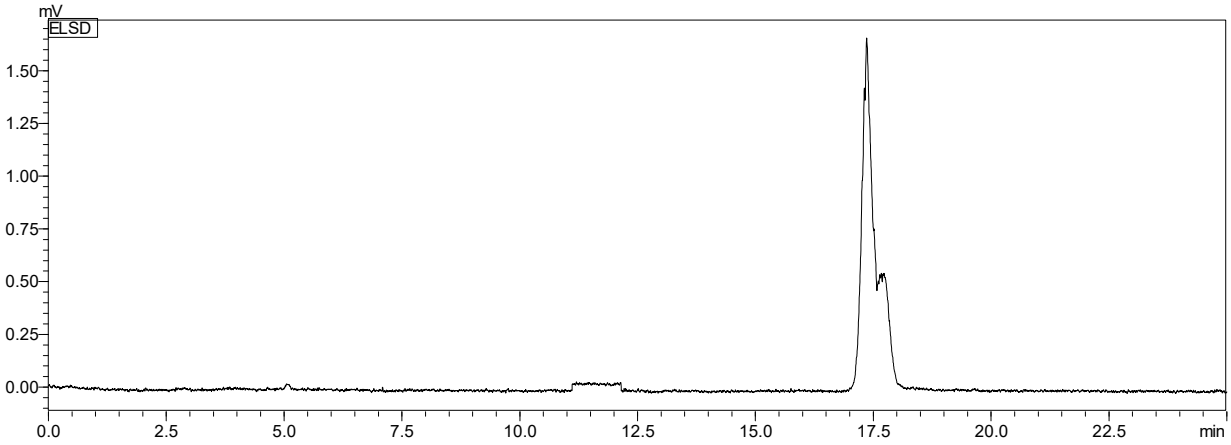
MALDI-MS, calculated: 1915.6927; found $[M-H]^-$: 1914.6896



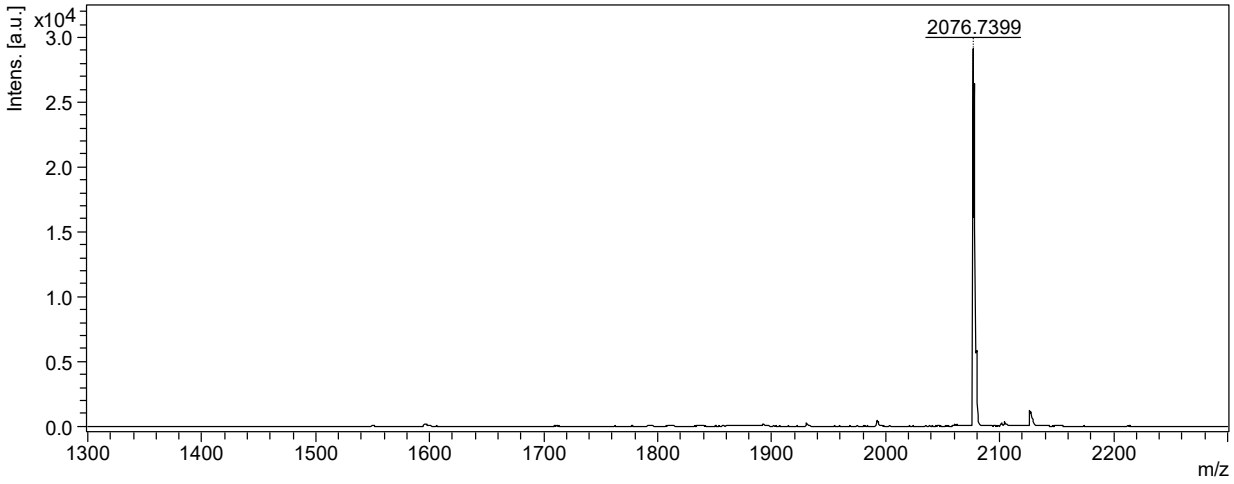
N6122 (0.8 mg)



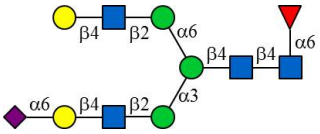
HPLC-ELSD, T_R = 17.36 min



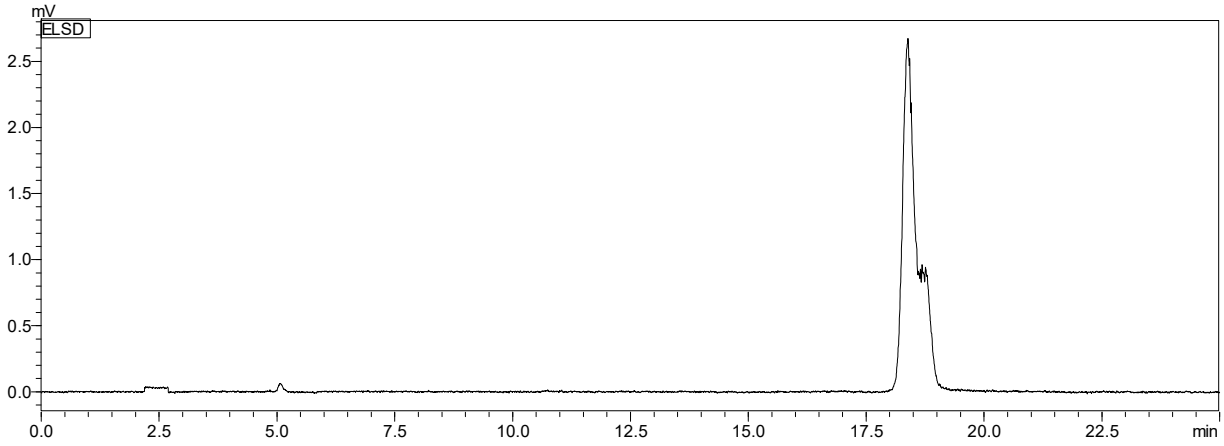
MALDI-MS, calculated: 2077.7455; found $[M-H]^-$: 2076.7399



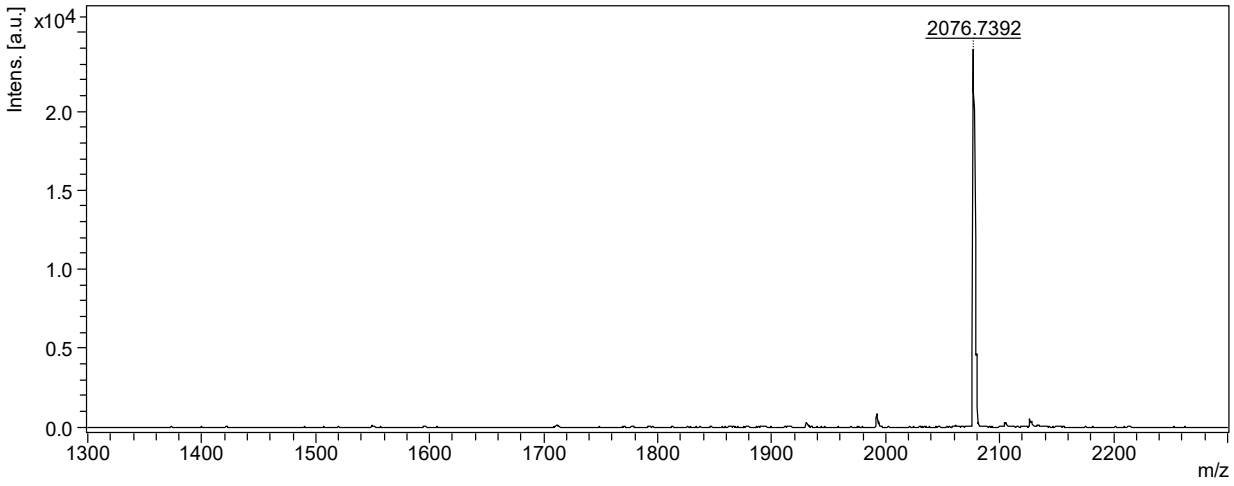
N6123 (1.2 mg)



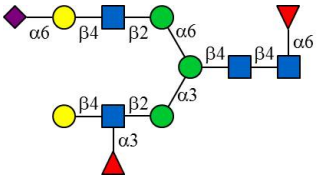
HPLC-ELSD, T_R = 18.36 min



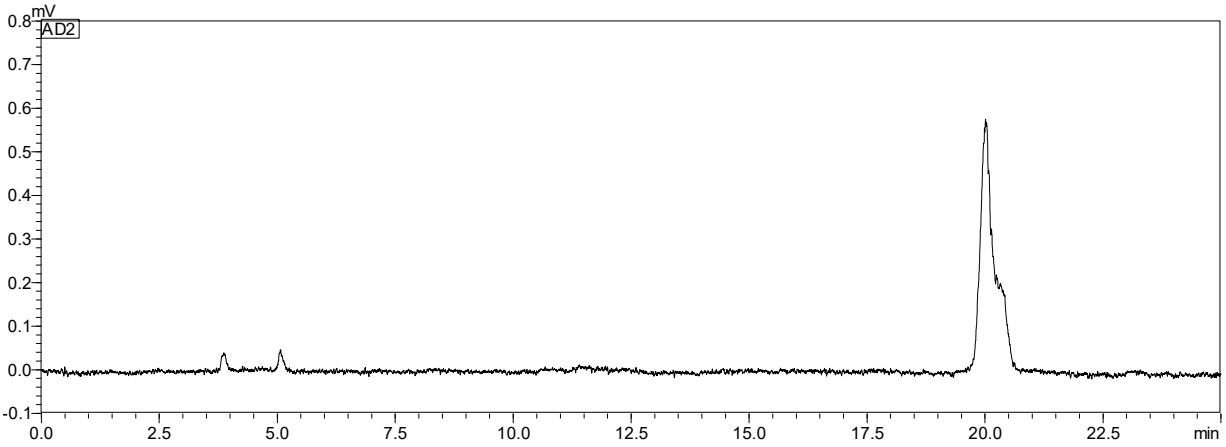
MALDI-MS, calculated: 2077.7455; found $[M-H]^-$: 2076.7392



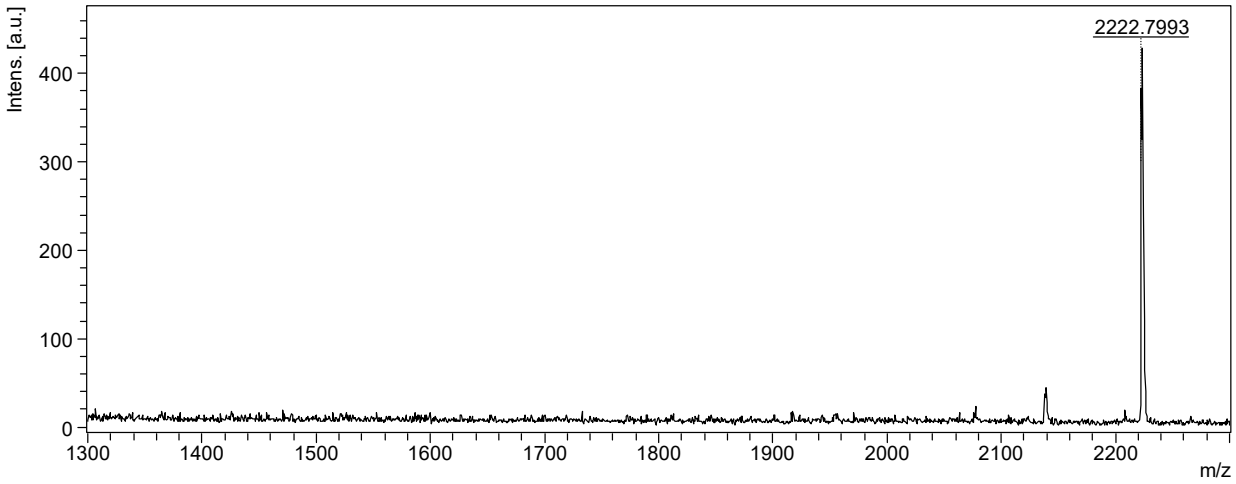
N6144 (0.8 mg)



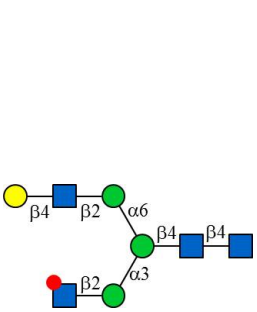
HPLC-ELSD, T_R = 20.12 min

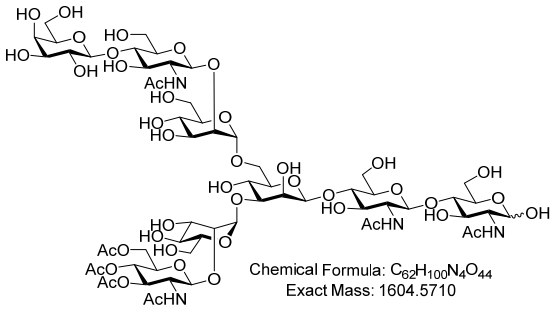


MALDI-MS, calculated: 2223.8034; found $[M-H]^-$: 2222.7993

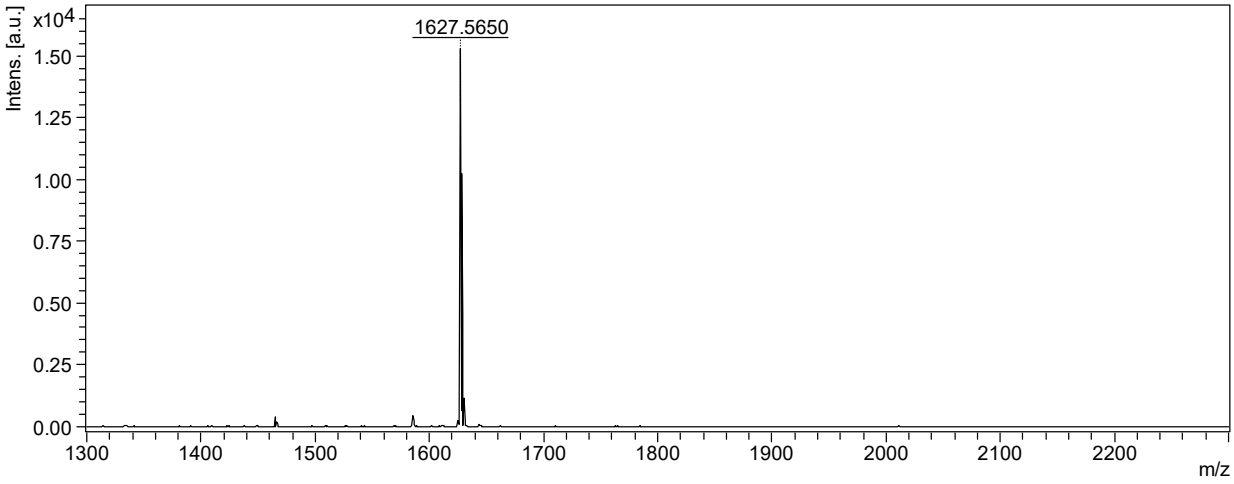


N211a (4.6 mg)

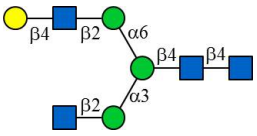




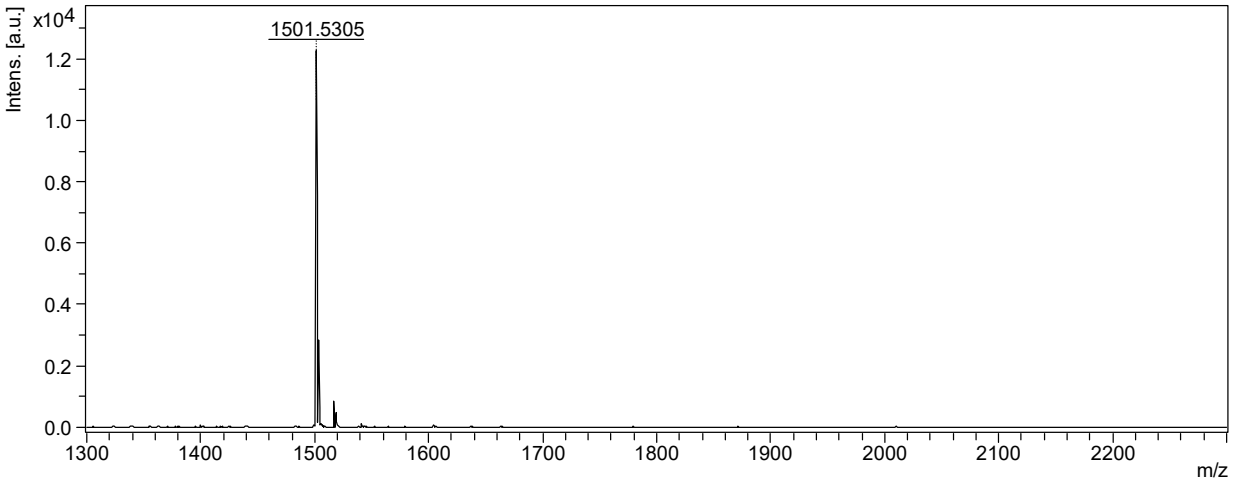
MALDI-MS, calculated: 1604.5710; found $[M+Na]^+$: 1627.5650



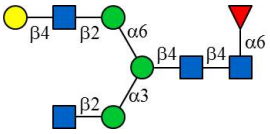
N211 (4.4 mg)



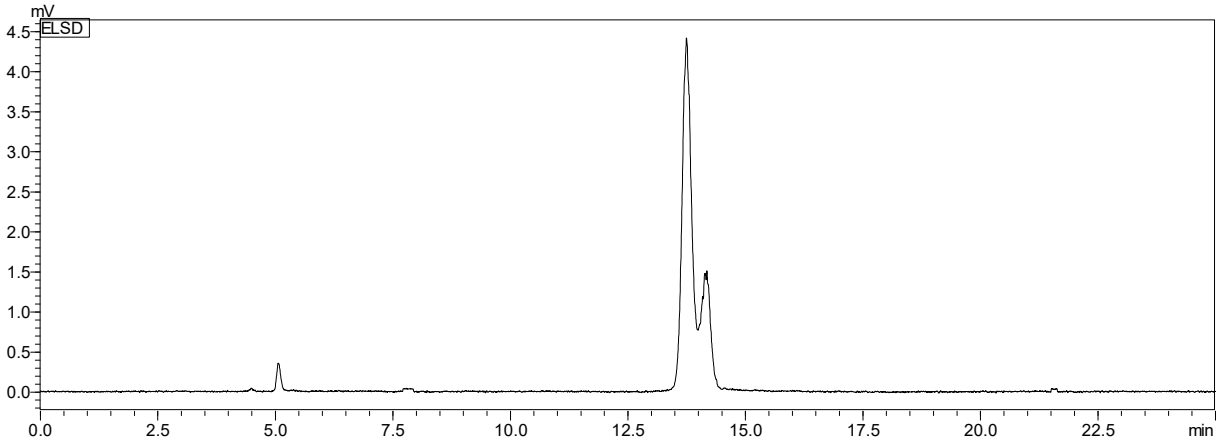
MALDI-MS, calculated: 1478.5393; found $[M+Na]^+$: 1501.5305, $[M+K]^+$: 1517.5030



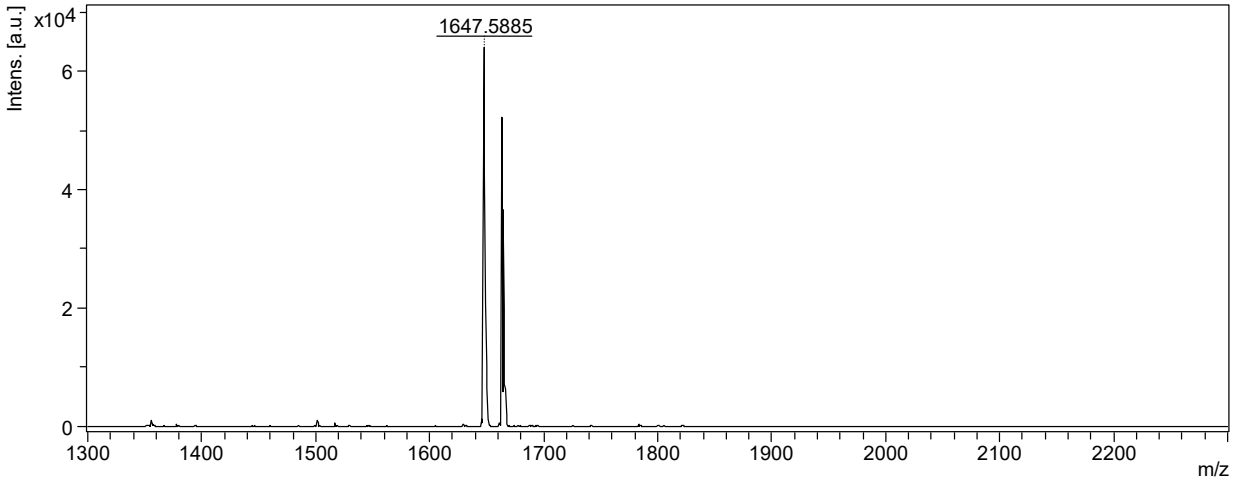
N6211 (4.0 mg)



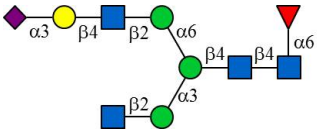
HPLC-ELSD, T_R = 13.75 min



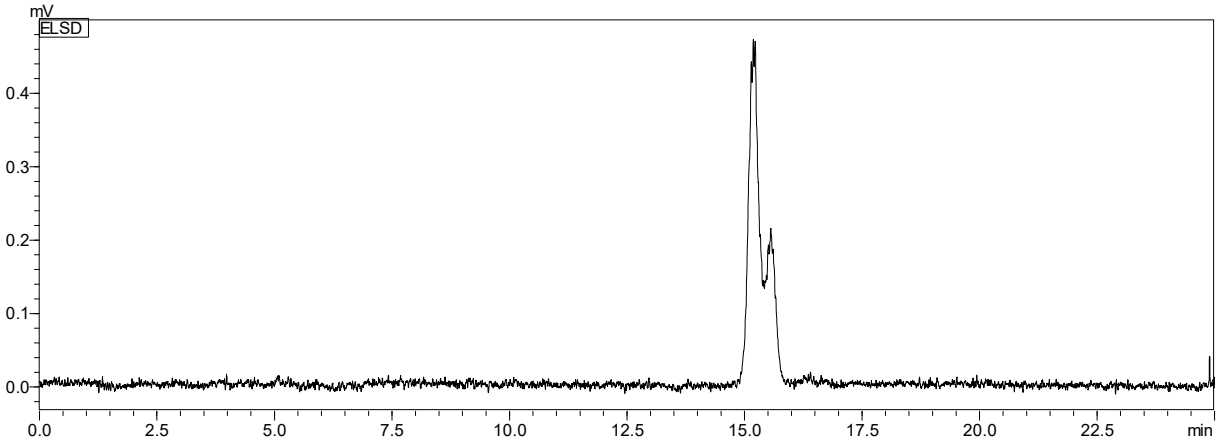
MALDI-MS, calculated: 1624.5973; found $[M+Na]^+$: 1647.5885, $[M+K]^+$: 1663.5652



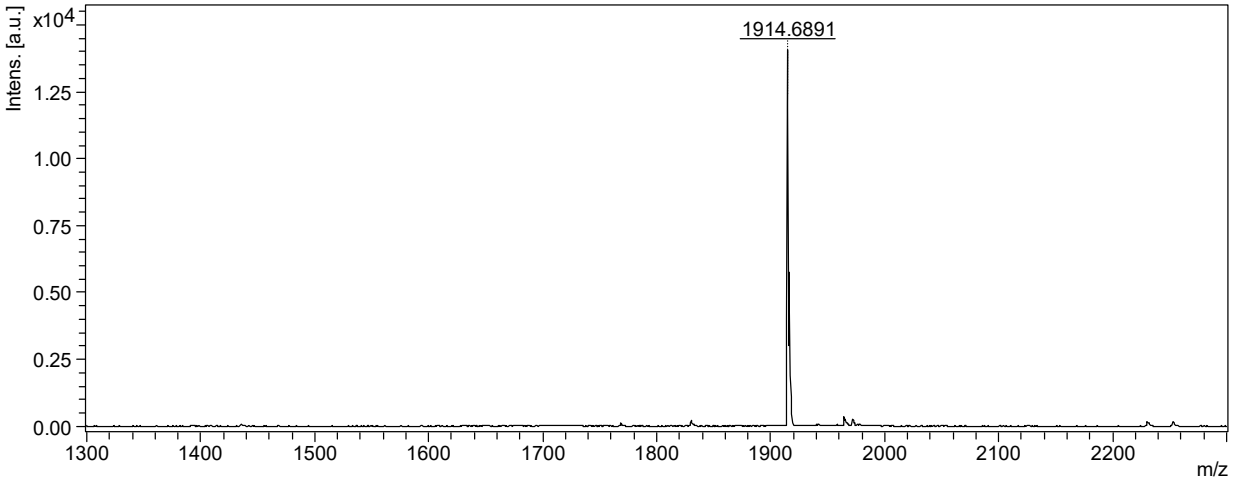
N6212 (1.3 mg)



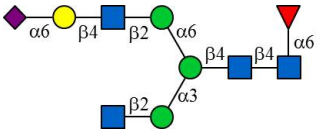
HPLC-ELSD, T_R = 15.19 min



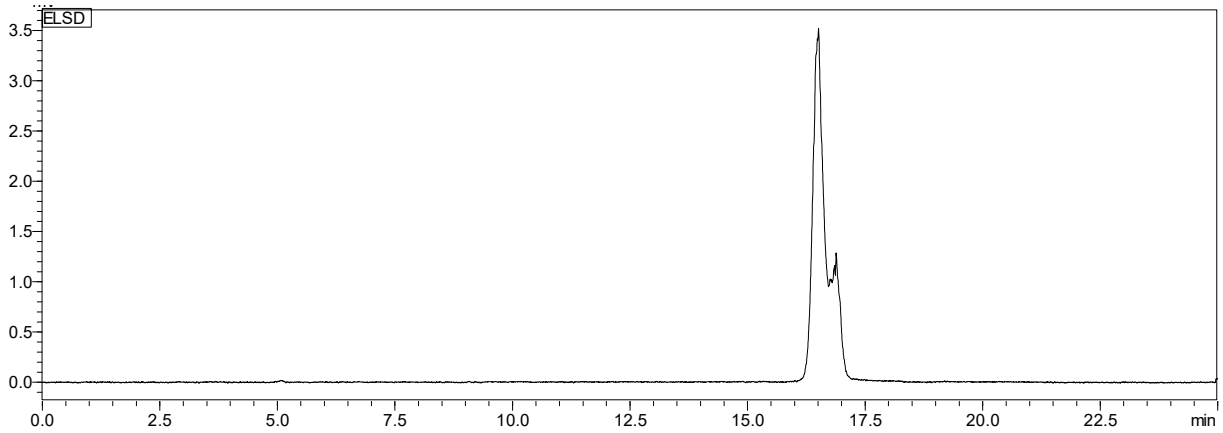
MALDI-MS, calculated: 1915.6927; found $[M-H]^-$: 1914.6891



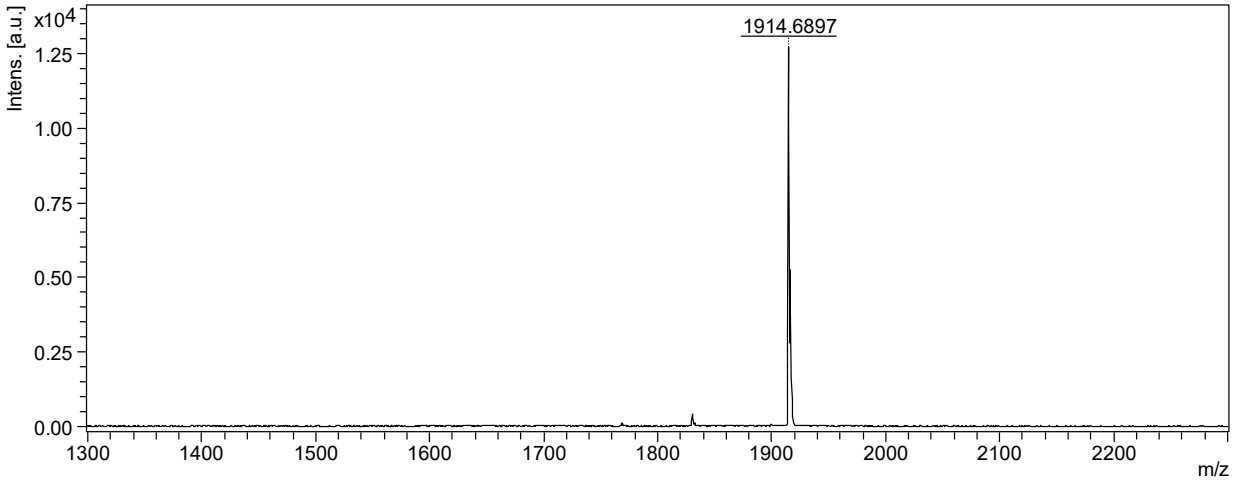
N6213 (1.8 mg)



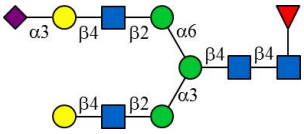
HPLC-ELSD, T_R = 16.51 min



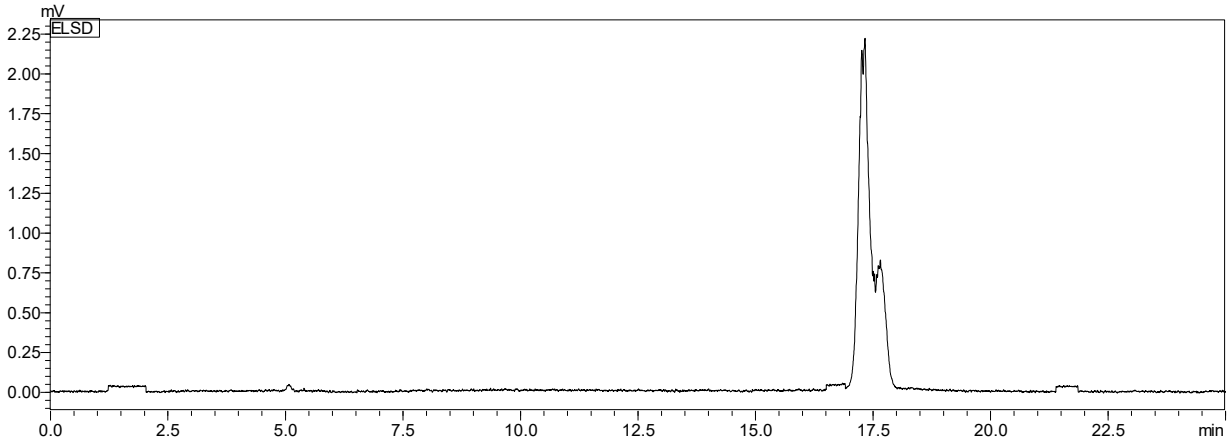
MALDI-MS, calculated: 1915.6927; found $[M-H]^-$: 1914.6897



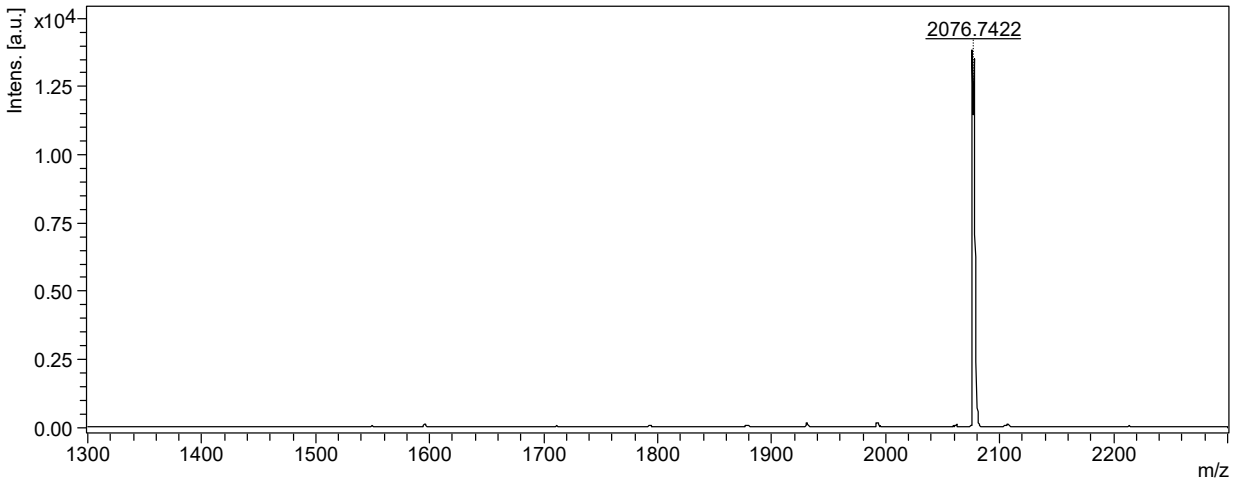
N6222 (0.9 mg)



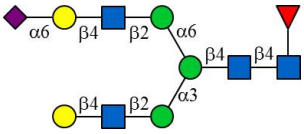
HPLC-ELSD, T_R = 17.32 min



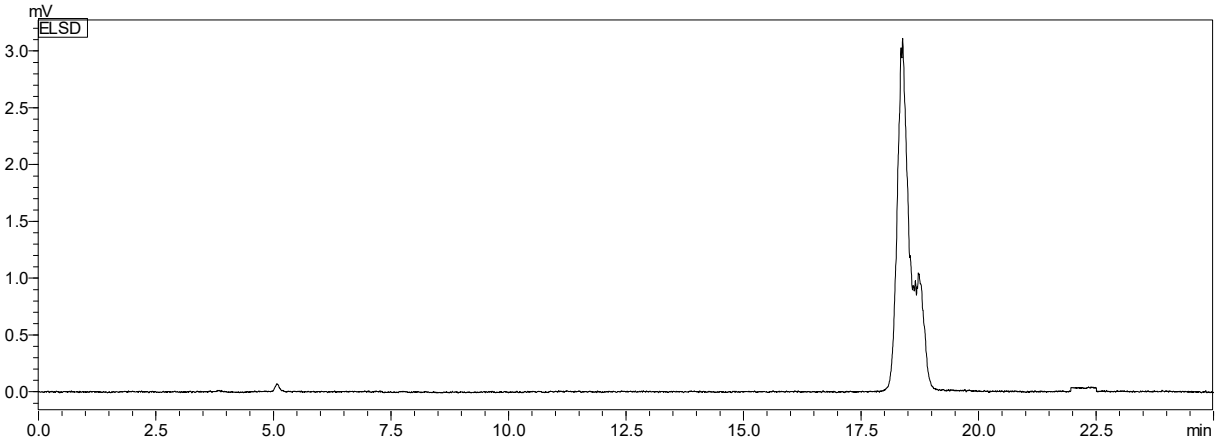
MALDI-MS, calculated: 2077.7455; found $[M-H]^-$: 2076.7422



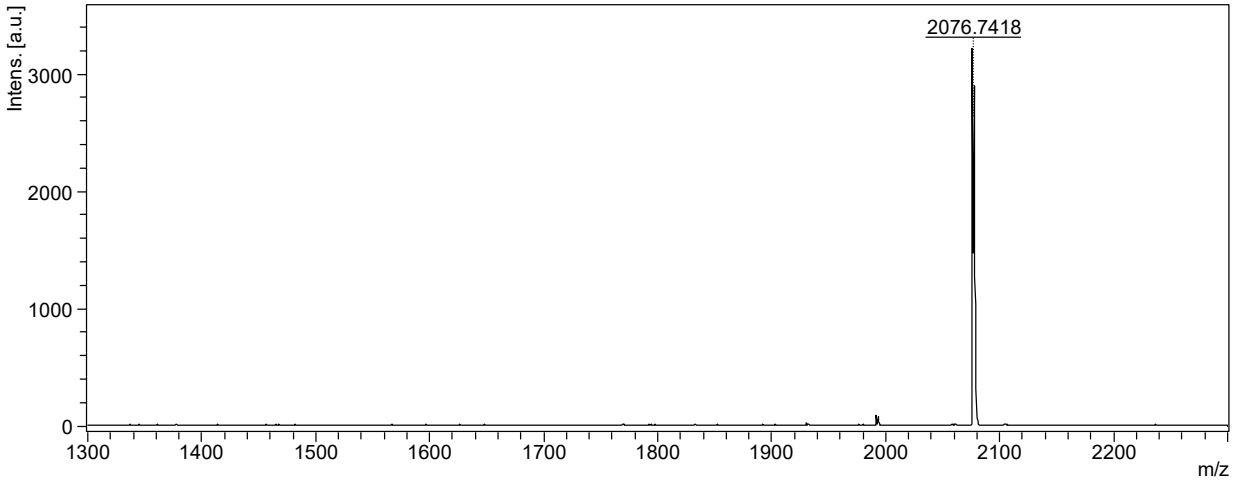
N6223 (1.3 mg)



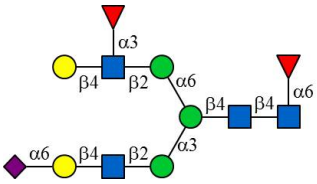
HPLC-ELSD, T_R = 18.39 min



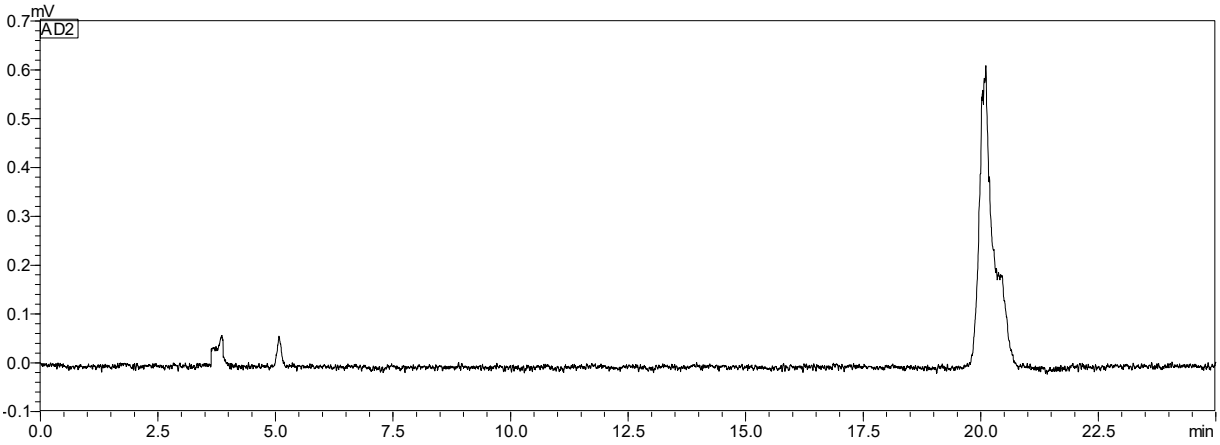
MALDI-MS, calculated: 2077.7455; found $[M-H]^-$: 2076.7418



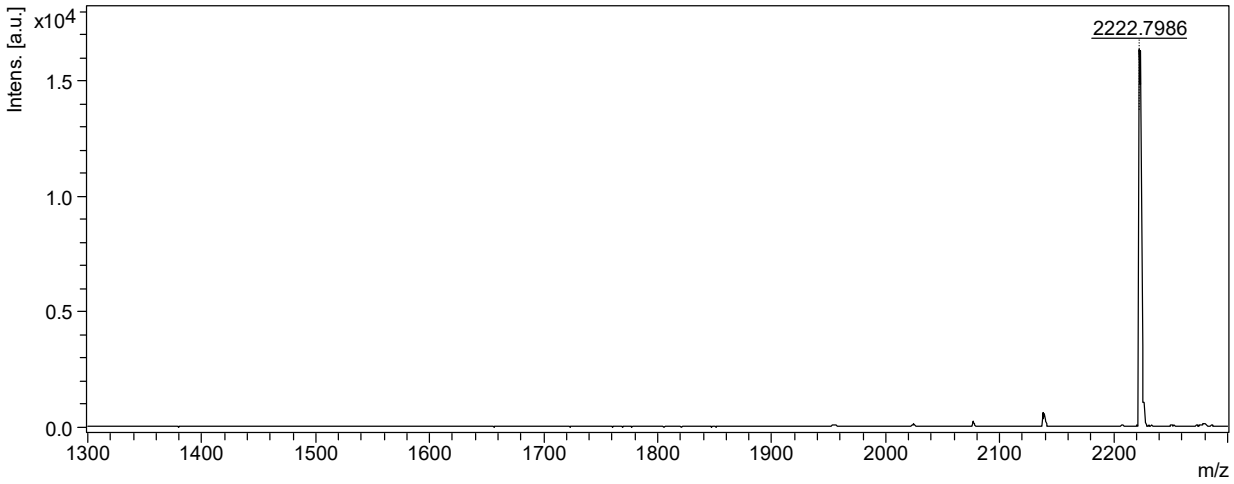
N6244 (0.9 mg)



HPLC-ELSD, T_R = 20.11 min

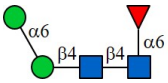


MALDI-MS, calculated: 2223.8034; found $[M-H]^-$: 2222.7986

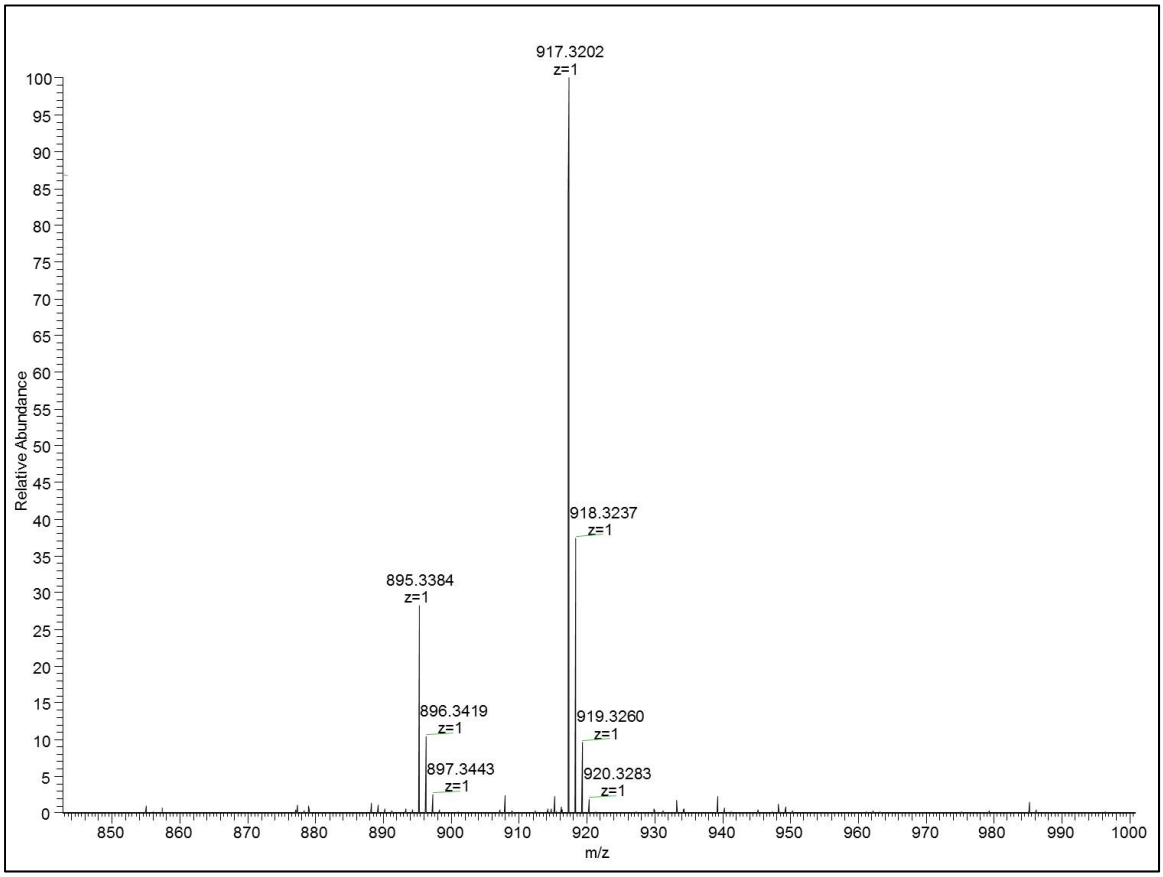


VI. ESI-MS and ^{1}H NMR data of purified N604

N604 (0.4 mg)



ESI-MS, calculated: 894.3329; found $[M+H]^+$: 895.3384, $[M+Na]^+$: 917.3202

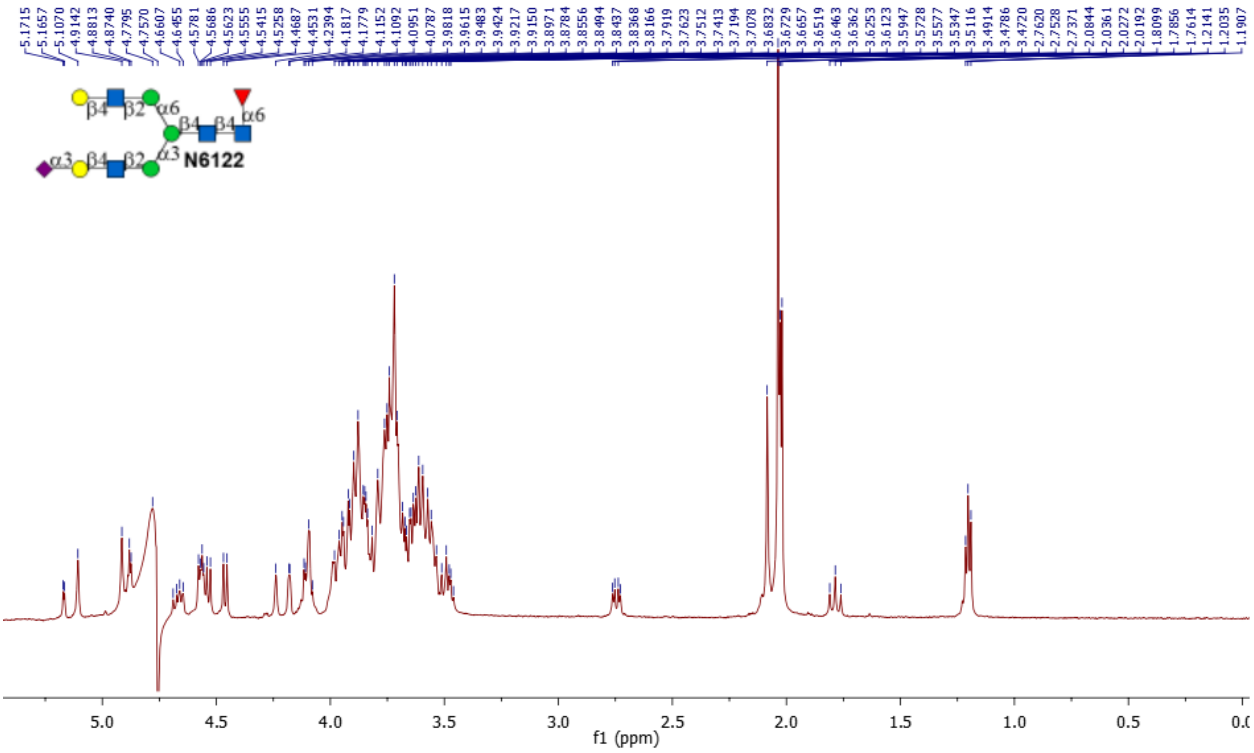


^{1}H NMR of **N604**

^{1}H NMR (D_2O, 500 MHz): δ 6.18 (s, 1 H), 6.17 (s, 1 H),4.65 (s, 1 H), 4.41 (brs, 2 H), 4.20-4.30 (m, 5 H), 4.08-4.12 (m, 2 H), 4.56-4.00 (m, 20 H), 2.12 (s, 3 H, NHAc), 2.07 (s, 3 H, NHAc), 1.25 (t, J = 5.4 Hz, 3 H, Fuc-CH_3)

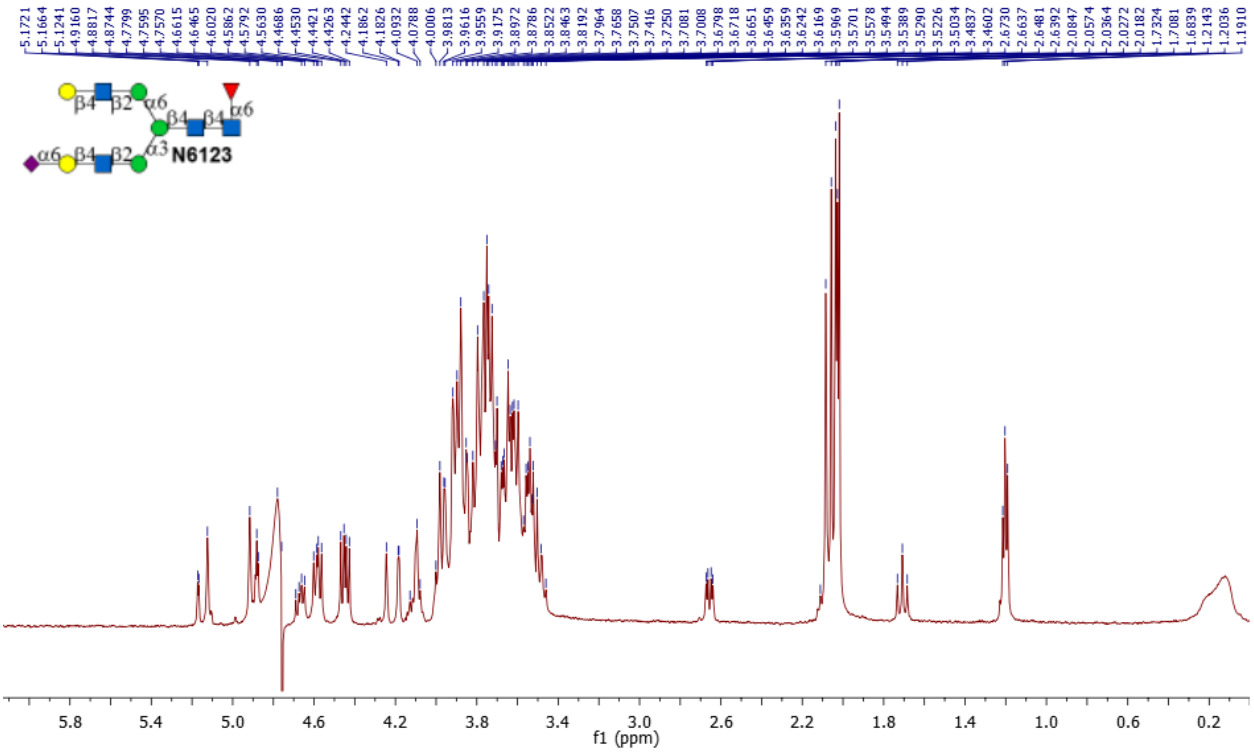
VII. NMR spectra and data of purified core-fucosylated N-glycans

^{1}H NMR of **N6122**



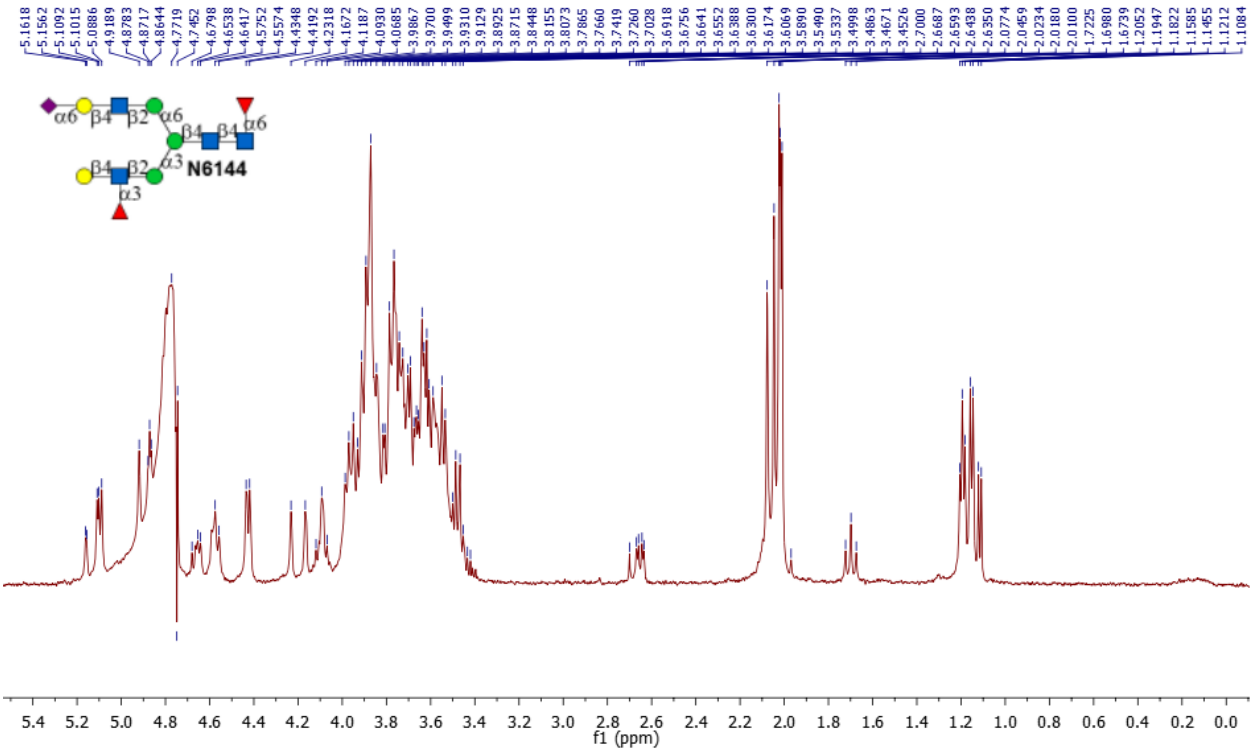
^{1}H NMR (D_2O, 500 MHz): δ 5.17 (d, J = 2.9 Hz, 0.5 H, GlcNAc-1 H1 of α isomer), 5.11 (s, 1 H, Man2 H-1), 4.91 (s, 1 H, Man3 H-1), 4.88 (d, J = 3.7 Hz, 0.5 H, Fuc H-1 of α isomer), 4.87 (d, J = 3.7 Hz, 0.5 H, Fuc H-1 of β isomer), 4.75-4.80 (overlap with D_2O, 1 H, Manβ H-1), 4.65-4.68 (m, 1.5 H, GlcNAc-1 H-1 of β isomer, GlcNAc-2 H-1), 4.57 (d, J = 7.9 Hz, 1 H, GlcNAc-3 H-1), 4.56 (d, 1 H, J = 6.6 Hz, 1 H, GlcNAc-4 H-1), 4.53 (d, J = 7.9 Hz, 1 H, Gal-1 H-1), 4.46 (d, 1 H, J = 7.8 Hz, 1 H, Gal-2 H-1), 4.24 (brs, 1 H), 4.18 (d, J = 1.9 Hz, 1 H), 4.08-4.11 (m, 3 H), 3.46-4.00 (m, 60 H), 2.75 (dd, J = 12.5, 4.6 Hz, 1 H, Neu5Ac H-3eq), 2.08 (s, 3 H, Ac), 2.04 (s, 6 H, Ac), 2.03 (s, 3 H, Ac),2.02 (s, 3 H, Ac), 1.79 (t, J = 12.5 Hz, 1 H, Neu5Ac H-3ax), 1.21 (d, J = 6.4 Hz, 1.5 H, Fuc H-6), 1.20 (d, J = 6.4 Hz, 1.5 H, Fuc H-6)

^{1}H NMR of **N6123**



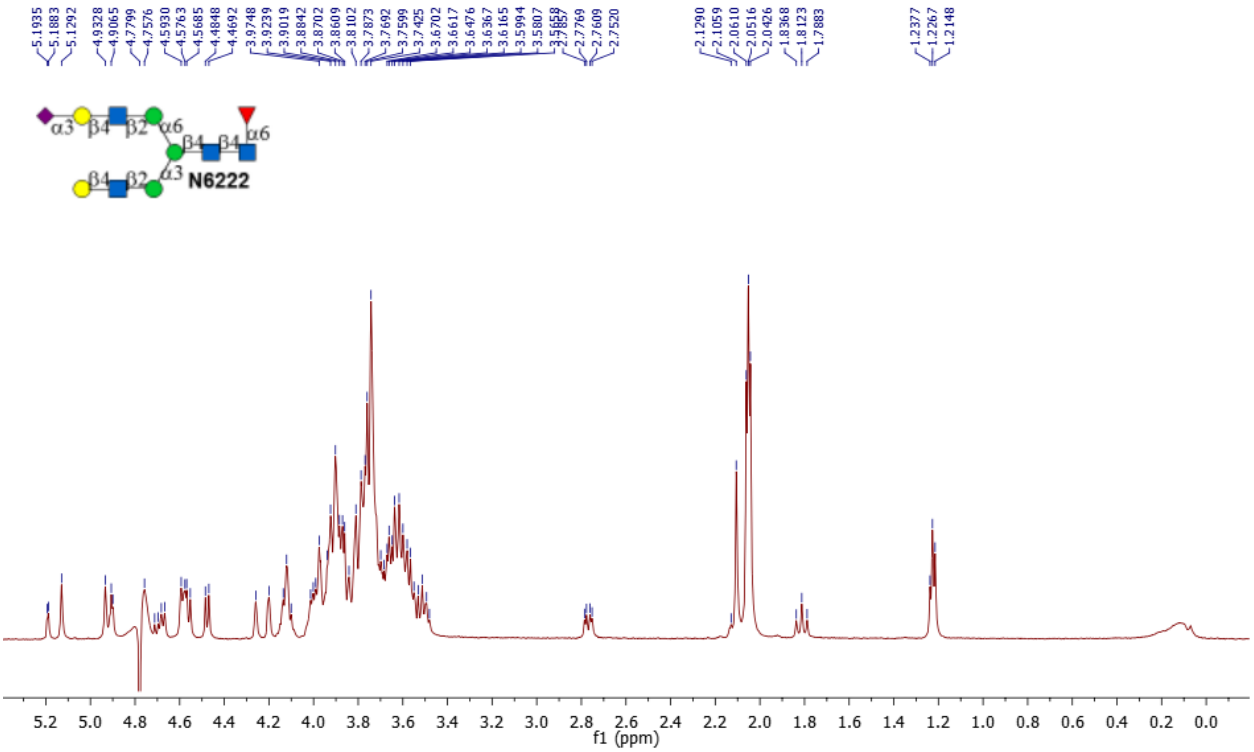
^{1}H NMR (D_2O, 500 MHz): δ 5.17 (d, J = 2.9 Hz, 0.5 H, GlcNAc-1 H-1 of α isomer), 5.12 (s, 1 H, Man2 H-1), 4.92 (s, 1 H, Man3 H-1), 4.88 (d, J = 3.7 Hz, 0.5 H, Fuc H-1 of α isomer), 4.87 (d, J = 3.7 Hz, 0.5 H, Fuc H-1 of β isomer), 4.75-4.85 (overlap with D_2O, 1 H, Manβ H-1), 4.65-4.69 (m, 1.5 H, GlcNAc-1 H-1 of β isomer, GlcNAc-2 H-1), 4.59 (d, J = 7.9 Hz, 1 H, GlcNAc-3 H-1), 4.57 (d, J = 7.8 Hz, 1 H, GlcNAc-4 H-1), 4.46 (d, 1 H, J = 7.8 Hz, 1 H, Gal-1 H-1), 4.43 (d, 1 H, J = 7.9 Hz, 1 H, Gal-2 H-1), 4.24 (brs, 1 H), 4.18 (d, J = 1.8 Hz, 1 H), 4.07-4.09 (m, 2 H), 3.46-4.00 (m, 61 H), 2.66 (dd, J = 12.5, 4.7 Hz, 1 H, Neu5Ac H-3eq), 2.08 (s, 3 H, Ac), 2.06 (s, 3 H, Ac), 2.04 (s, 3 H, Ac),2.03 (s, 3 H, Ac), 2.02 (s, 3 H, Ac), 1.71 (t, J = 12.5 Hz, 1 H, Neu5Ac H-3ax), 1.21 (d, J = 6.3 Hz, 1.5 H, Fuc H-6), 1.20 (d, J = 6.3 Hz, 1.5 H, Fuc H-6)

^{1}H NMR of **N6144**



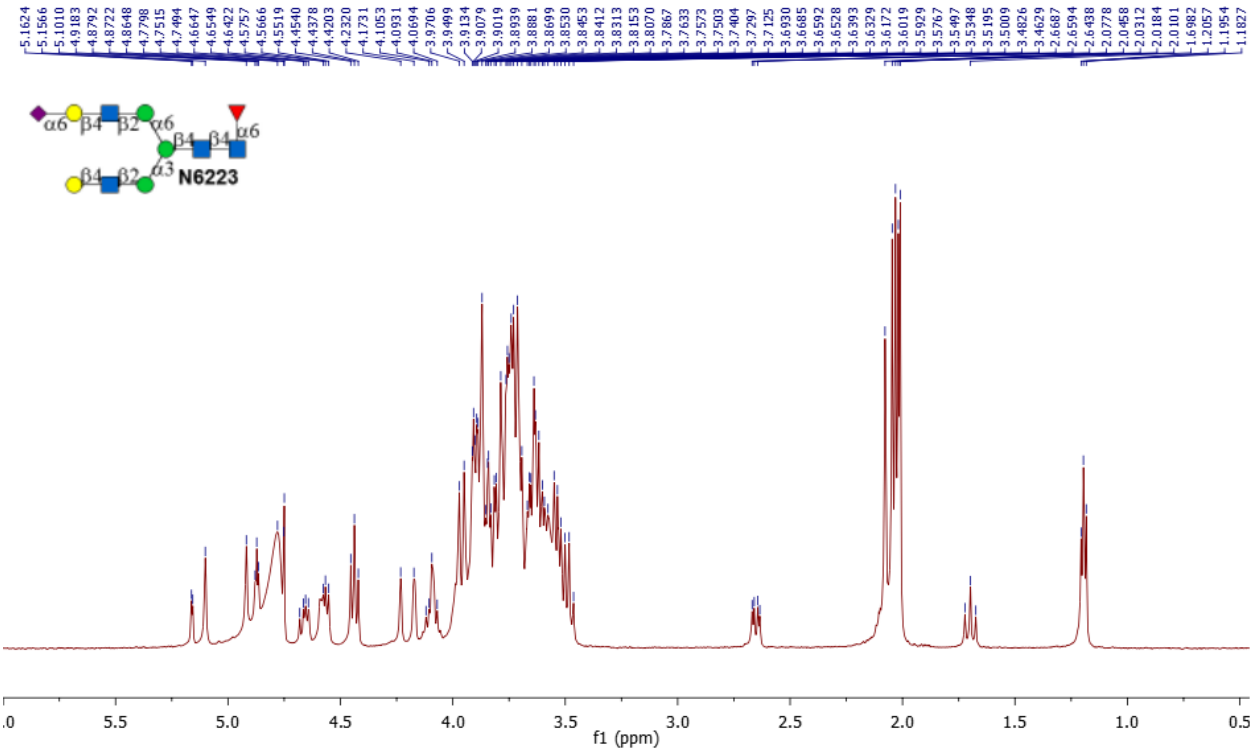
^{1}H NMR (D_2O, 500 MHz): δ 5.16 (d, J = 2.8 Hz, 0.5 H, GlcNAc-1 H1 of α isomer), 5.10 (d, J = 3.9 Hz, 1 H, Fuc-2 H-1), 5.09 (s, 1 H, Man2 H-1), 4.92 (s, 1 H, Man3 H-1), 4.87 (d, J = 3.7 Hz, 0.5 H, Fuc H-1 of α isomer), 4.86 (d, J = 3.7 Hz, 0.5 H, Fuc H-1 of β isomer), 4.75-4.83 (overlap with D_2O, 1 H, Manβ H-1), 4.65-4.68 (m, 1.5 H, GlcNAc-1 H-1 of β isomer, GlcNAc-2 H-1), 4.56-4.58 (m, 2 H, GlcNAc-3 H-1, GlcNAc-4 H-1), 4.41-4.43 (m, 2 H, Gal-1 H-1, Gal-2 H-1), 4.23 (brs, 1 H), 4.17 (d, J = 1.9 Hz, 1 H), 4.07-4.12 (m, 3 H), 3.42-3.99 (m, 64 H), 2.65 (dd, J = 12.5, 4.7 Hz, 1 H, Neu5Ac H-3eq), 2.08 (s, 3 H, Ac), 2.05 (s, 3 H, Ac), 2.02 (s, 3 H, Ac), 2.01 (s, 3 H, Ac), 2.00 (s, 3 H), 1.70 (t, J = 12.5 Hz, 1 H, Neu5Ac H-3ax), 1.21 (d, J = 6.3 Hz, 1.5 H, Fuc H-6), 1.20 (d, J = 6.3 Hz, 1.5 H, Fuc H-6), 1.15 (d, J = 6.5 Hz, 2 H, Fuc H-6), 1.11 (d, J = 6.5 Hz, 1 H, Fuc H-6).

^{1}H NMR of **N6222**



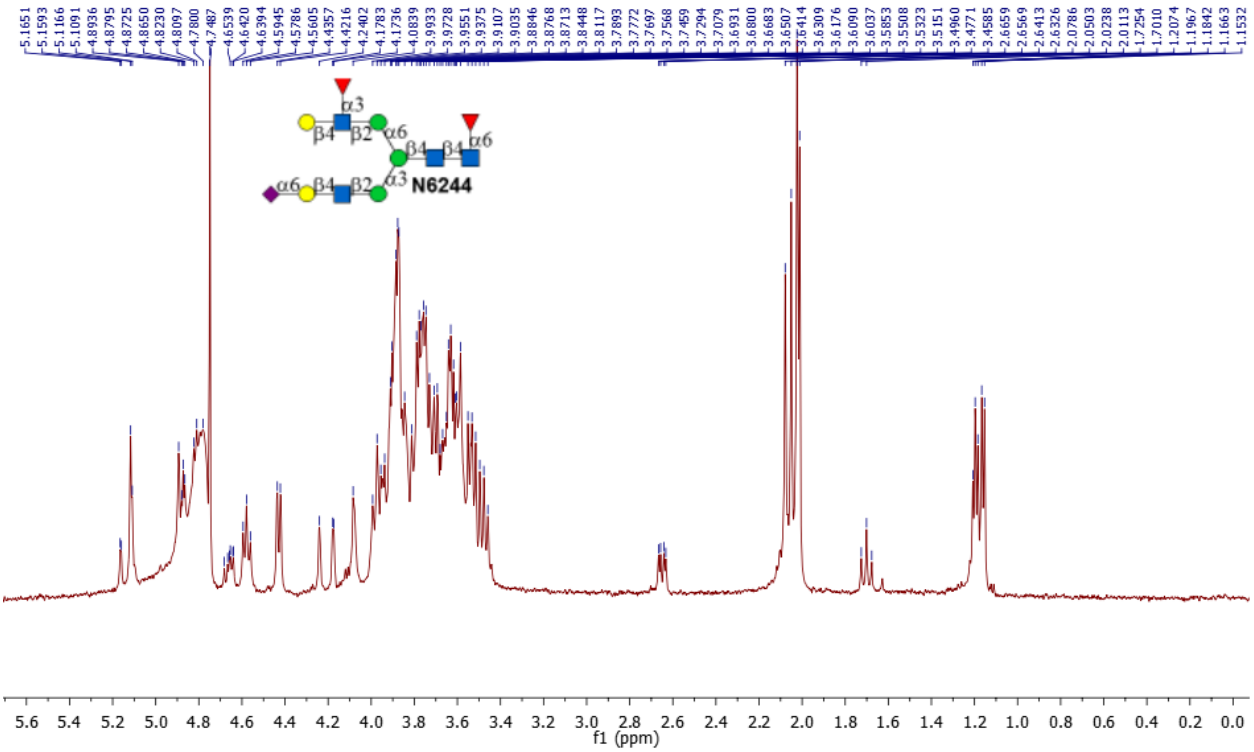
^{1}H NMR (D_2O, 500 MHz): δ 5.19 (d, J = 2.6 Hz, 0.5 H, GlcNAc-1 H-1 of α isomer), 5.13 (s, 1 H, Man2 H-1), 4.93 (s, 1 H, Man3 H-1), 4.90 (d, J = 3.5 Hz, 0.5 H, Fuc H-1 of α isomer), 4.89 (d, J = 3.5 Hz, 0.5 H, Fuc H-1 of β isomer), 4.75-4.80 (overlap with D_2O, 1 H, Manβ H-1), 4.70 (d, J = 8.0 Hz, 0.5 H, GlcNAc-1 H-1 of β isomer), 4.67 (d, J = 7.7 Hz, 1 H, GlcNAc-2 H-1), 4.55-4.59 (m, 3 H, GlcNAc-3 H-1, GlcNAc-4 H-1, Gal-1 H-1), 4.47 (d, 1 H, J = 7.8 Hz, 1 H, Gal-2 H-1), 4.26 (brs, 1 H), 4.20 (brs, 1 H), 4.10-4.14 (m, 3 H), 3.46-4.00 (m, 60 H), 2.77 (dd, J = 12.4, 4.4 Hz, 1 H, Neu5Ac H-3eq), 2.11 (s, 3 H, Ac), 2.06 (s, 3 H, Ac), 2.05 (s, 6 H, Ac),2.04 (s, 3 H, Ac), 1.81 (t, J = 12.4 Hz, 1 H, Neu5Ac H-3ax), 1.22 (d, J = 6.0 Hz, 1.5 H, Fuc H-6), 1.21 (d, J = 6.0 Hz, 1.5 H, Fuc H-6)

^{1}H NMR of **N6223**



^{1}H NMR (D_2O, 500 MHz): δ 5.14 (d, J = 2.9 Hz, 0.5 H, GlcNAc-1 H-1 of α isomer), 5.10 (s, 1 H, Man2 H-1), 4.92 (s, 1 H, Man3 H-1), 4.88 (d, J = 3.7 Hz, 0.5 H, Fuc H-1 of α isomer), 4.87 (d, J = 3.7 Hz, 0.5 H, Fuc H-1 of β isomer), 4.75-4.85 (overlap with D_2O, 1 H, Manβ H-1), 4.64-4.68 (m, 1.5 H, GlcNAc-1 H-1 of β isomer, GlcNAc-2 H-1), 4.55-4.58 (m, 2 H, GlcNAc-3 H-1, GlcNAc-4 H-1), 4.42-4.45 (m, 2 H, Gal-1 H-1, Gal-2 H-1), 4.23 (s, 1 H), 4.17 (brs, 1 H), 4.07-4.12 (m, 2 H), 3.46-4.00 (m, 61 H), 2.65 (dd, J = 12.5, 4.7 Hz, 1 H, Neu5Ac H-3eq), 2.08 (s, 3 H, Ac), 2.04 (s, 3 H, Ac), 2.03 (s, 3 H, Ac), 2.02 (s, 3 H, Ac), 2.01 (s, 3 H, Ac), 1.70 (t, J = 12.5 Hz, 1 H, Neu5Ac H-3ax), 1.20 (d, J = 6.4 Hz, 1.5 H, Fuc H-6), 1.19 (d, J = 6.4 Hz, 1.5 H, Fuc H-6)

^{1}H NMR of **N6244**



^{1}H NMR (D_2O, 500 MHz): δ 5.16 (d, J = 2.9 Hz, 0.6 H, GlcNAc-1 H-1 of α isomer), 5.11 (s, 1 H, Man2 H-1), 5.10 (d, J = 3.8 Hz, 1 H, Fuc-2 H-1), 4.89 (s, 1 H, Man3 H-1), 4.87 (d, J = 3.8 Hz, 0.4 H, Fuc H-1 of α isomer), 4.86 (d, J = 3.8 Hz, 0.6 H, Fuc H-1 of β isomer), 4.75-4.83 (overlap with D_2O, 1 H, Manβ H-1), 4.64-4.68 (m, 1.4 H, GlcNAc-1 H-1 of β isomer, GlcNAc-2 H-1), 4.56-4.59 (m, 2 H, GlcNAc-3 H-1, GlcNAc-4 H-1), 4.42-4.44 (m, 2 H, Gal-1 H-1, Gal-2 H-1), 4.24 (brs, 1 H), 4.17 (d, J = 2.4 Hz, 1 H), 4.06-4.09 (m, 3 H), 3.46-4.00 (m, 64 H), 2.65 (dd, J = 12.3, 4.5 Hz, 1 H, Neu5Ac H-3eq), 2.08 (s, 3 H, Ac), 2.05 (s, 3 H, Ac), 2.02 (s, 6 H, Ac), 2.01 (s, 3 H, Ac), 1.70 (t, J = 12.3 Hz, 1 H, Neu5Ac H-3ax), 1.20 (d, J = 6.3 Hz, 1.2 H, Fuc H-6), 1.20 (d, J = 6.3 Hz, 1.8 H, Fuc H-6), 1.15 (d, J = 6.6 Hz, 3 H, Fuc H-6).

VIII. References

1. G. Sugiarto, K. Lau, Y. Li, Z. Khedri, H. Yu, D. T. Le and X. Chen, *Mol. Biosyst.*, 2011, **7**, 3021.
2. H. Yu, S. Huang, H. Chokhawala, M. Sun, H. Zheng and X. Chen, *Angew. Chem. Int. Ed. Engl.*, 2006, **45**, 3938.
3. S. W. Lin, T. M. Yuan, J. R. Li and C. H. Lin, *Biochemistry*, 2006, **45**, 8108.
4. H. Yu, H. Yu, R. Karpel and X. Chen, *Bioorg. Med. Chem.*, 2004, **12**, 6427.
5. M. M. Muthana, J. Qu, Y. Li, L. Zhang, H. Yu, L. Ding, H. Malekan and X. Chen, *Chem. Commun.*, 2012, **48**, 2728.
6. G. Zhao, W. Guan, L. Cai and P. G. Wang, *Nat. Protoc.*, 2010, **5**, 636.
7. D.J. Wasilko, S.E. Lee, K.J. Stutzman-Engwall, B.A. Reitz, T.L. Emmons, K.J. Mathis, M.J. Bienkowski, A.g. Tomasselli and H.D. Fischer, *Protein Express. Purif.*, 2009, **65**, 122.
8. H. Ihara, Y. Ikeda, N. Taniguchi, *Glycobiology*, 2006, **16**, 909.
9. L. Li, Y. Liu, C. Ma, J. Qu, A.D. Calderon, B. Wu, N. Wei, X. Wang, Y. Guo, Z. Xiao, J. Song, G. Sugiarto, Y. Li, Yu. Hai, C. Xi and P.G. Wang, *Chem. Sci.*, 2015, **6**, 5652.