Supplementary Information

Streamlining the Chemoenzymatic Synthesis of Complex *N*-Glycans by a Stop and Go Strategy

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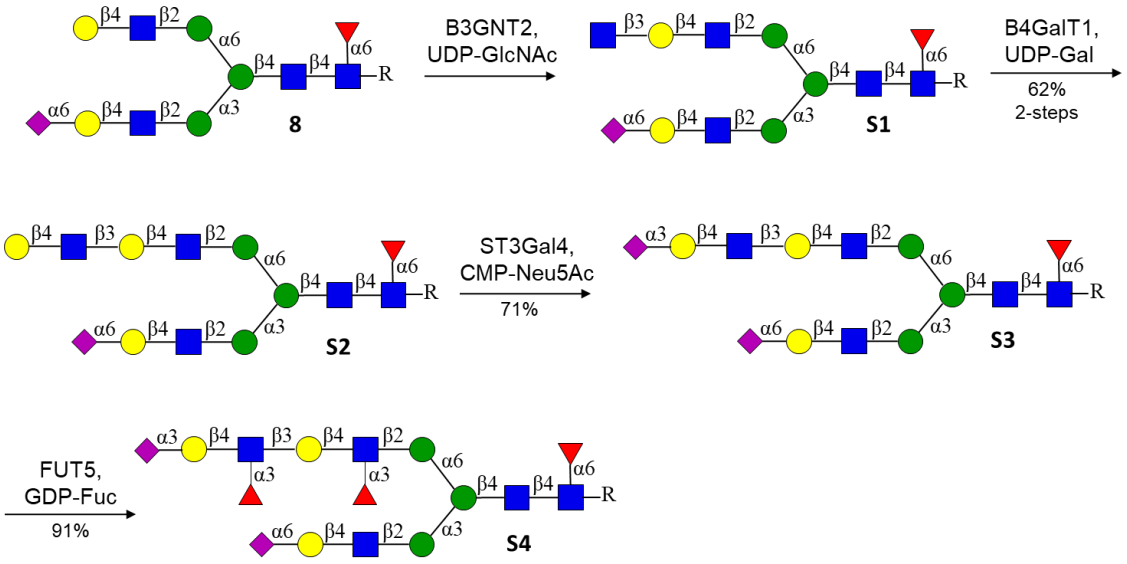
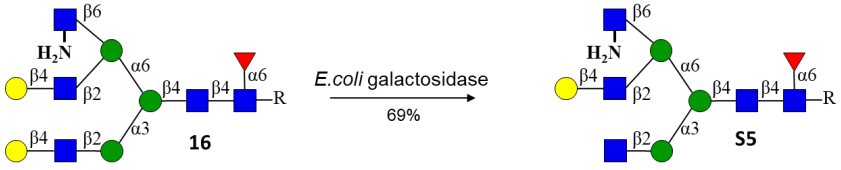
1. Supplementary Figure 1 and 2



Supplementary Fig. 1. Synthesis of asymmetric bi-antennary glycosyl asparagine **S4** from **8**.



Supplementary Fig. 2. Selective galactose removal from tri-antennary glycosyl asparagine **16** using galactosidase from *E. coli.*

2. Experimental Conditions

2a. Materials and methods

^{1}H spectra were recorded on a 600 MHz Varian Inova or an Agilent 900 MHz DD2 spectrometer with a triple resonance (HCN) cryogenically cooled probe spectrometer. Chemical shifts are reported in parts per million (ppm) relative to H1 and C1 of reducing N-acetylglucosamine which were set to δ 5.08 and 78.02 repectivelly as the internal standard. NMR data is represented as follows: Chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, dd = doublet of doublets, m = multiplet and / or multiple resonances, br. = broad signal), *J* coupling, integration, and peak identity. NMR signals were assigned based on ^{1}H NMR, gCOSY, gHSQC, zTOCSY, and NOESY experiments. Enzymatic reactions were monitored by mass spectrometry recorded on an Applied Biosystems SCIEX MALDI TOF / TOF 5800 using 2,5-dihydroxybenzoic acid (DHB) as a matrix or a Shimadzu 20AD UFLC LCMS-IT-TOF. Reagents were purchased from Sigma-Aldrich (unless otherwise noted) and used without further purification. HILIC-HPLC purification of compounds was performed on a Shimadzu 20AD UFLC LCMS-IT-TOF with a Waters XBridge BEH, Amide column, 5 µm, 10 x 250 mm. HPLC grade acetonitrile and water were purchased from Fischer. Uridine 5'-diphosphogalactose diphosphate galactose (UDP-Gal) and cytidine-5'monophospho-N-acetylneuraminc acid (CMP-Neu5Ac) were both purchased from Roche, uridine 5'-diphospho-N-acetylglucosamine (UDP-GlcNAc) was purchased from Sigma-Aldrich, and guanosine 5'-diphospho-β-L-fucose (GDP-Fuc) was purchased from Carbosynth.

2b. Extraction, Isolation and Trimming of SGP

Sialyl Glycopeptide (SGP, 5) Extraction

SGP (**5**) was extracted according to our previously reported procedure[1]. In short, commercially available egg yolk powder (Natural Foods, Inc., 2.27 Kg) was suspended twice in 95% ethanol (4 L) and mechanically stirred for 2 h at room temperature to remove lipids and other organic soluble components. The filtrate was discarded and the insoluble powder was suspended twice in aqueous ethanol (40% *v/v* ethanol, 3 L) solution. The insoluble material was discarded and the filtrate was concentrated under reduced pressure at 40 °C. The resulting translucent liquid was purified using an active carbon / celite column (500 g of active carbon and 500 g celite). Impurities were removed by flushing the column with 3 L of water (0.1% *v/v* TFA), 3 L of 5% acetonitrile in water (0.1% *v/v* TFA), and 3 L 10% acetonitrile in water (0.1% *v/v* TFA). The desired glycopeptide was released from the column using a solution of 25% acetonitrile in water (0.1% *v/v* TFA), and fractions containing the product were pooled and dried under reduced pressure. The resulting white powder was subjected to size-exclusion chromatography (Bio-Rad® P-2, fine particle size 45 – 90 µm, column dimensions 5.0 cm x 80 cm, 250 mL fractions) eluting with 0.1 M ammonium bicarbonate to yield SGP (**5**) as a fluffy, white powder (1.82 g, or 0.8 mg SGP / g egg yolk powder).

Trimming and Modification of SGP to Prepare Glycosyl Asparagine-CBz 1[1] Isolated SGP **5** (319 mg) was dissolved in 5 mL of Tris buffer (100 mM, pH 8.0) containing 5 mM $CaCl_2$. Pronase from *Streptomyces griseus* (Sigma-Aldrich # P5147-1G, 150 mg) was added, and the reaction was incubated for 5 days at 37 °C with shaking. The reaction was monitored by ESI-MS and once complete the mixture was heated at 80 °C for 20 min followed by Pronase removal using an Amicon Ultra-10 (MWCO-10k) centrifugal filter. The filtrate was lyophilized and purified by size-exclusion chromatography (Bio-Rad P-2 BioGel, fine particle size 45 – 90 µm, 2x80 cm), eluting with a 0.1 M ammonium bicarbonate solution. The fractions containing the glycosylated asparagine were pooled, lyophilized, and dissolved in 5 mL of water. To this mixture was added K_2CO_3 (1.1 g), and CBzCl (0.54 g, 3.2 mmol) drop-wise. The heterogeneous mixture was stirred vigorously at room temperature until ESI-MS indicated complete installation of the CBz-protecting group (**6**). The reaction was diluted with water (50 mL) and extracted with ethyl acetate (2x50 mL). The organic phase was discarded, and the aqueous phase was lyophilized and purified by size-exclusion chromatography using P-2 BioGel eluting with a 0.1 M ammonium bicarbonate solution. The fractions containing **6** were pooled, lyophilized, and re-dissolved in 5 mL of sodium acetate buffer (50 mM, pH 5.5) containing 5 mM $CaCl_2$. To this mixture was added neuraminidase from *Clostridium perfringens* (New England Biolabs # P0720L, 40 µL, 2000 units) and the reaction was incubated overnight at 37 °C with shaking at which time, ESI-MS indicated all the sialic acid residues had been removed. The pH of the reaction mixture was adjusted to 4.5 with acetic acid after which, BSA (5 mg) and *β*-galactosidase (200 µL, 800 units:) from *Aspergillus niger* (Megazyme # E-BGLAN) were added. The reaction was incubated at 37 °C with shaking overnight, after which another 150 µL of *β*-galactosidase were added. The reaction was monitored by ESI-MS and once complete galactose removal was observed the enzymes were removed using an Amicon Ultra-10 (MWCO-10k) centrifugal filter. The filtrate was lyophilized and purified by size-exclusion chromatography using P-2 BioGel eluting with a 0.1 M ammonium bicarbonate solution. The fractions containing the trimmed glycosyl asparagine-CBz were pooled, lyophilized, and dissolved in 10 mL of MES buffer (100 mM, pH 7.3). To this mixture BSA (1 mg), calf intestine alkaline phosphatase (CIAP, 100 µL, 2kU/mL), GDP-Fucose (75 mg), and FUT8 (200 µL, 1 mg/mL) were added and the reaction was incubated overnight at 37 °C with shaking. The reaction was lyophilized and purified by size-exclusion chromatography using P-2 BioGel eluting with a 0.1 M ammonium

bicarbonate solution. The fractions containing **1** were pooled, lyophilized, and subjected to HILIC-HPLC (see section 2f) for final purification to give the compound **1** (74 mg, 39%).

2c. Expression and Purification of Enzymes

Recombinant Expression and Purification of PmGlmU

The gene sequence of *Pasteurella multocida N*-acetylglucosamine-1-phosphate uridylyltransferase (PmGlmU) from *Pasteurella multocida* strain P-1059 (ATCC 15742) with a C-terminal His_6-tag[2] were synthesized, ligated into a pET15b plasmid using NdeI and RhoI restriction sites, and transformed into *E. coli* BL21 (DE3) cells by Genscript. *E. coli* BL21 cells harboring the pET15b-PmGlmU plasmid were cultured in LB medium containing ampicillin (100 µg/mL) at 37 °C until an OD_{600nm} of 0.8-1.0 was reached. Protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG, final concentration 100 µM) and cultures where incubated at 20°C with rigorous shaking for 18 h. The cells were harvested by centrifugation (4,000 × g) at 4 °C for 20 min and the resulting pellet was resuspended in lysis buffer (100 mM Tris-HCl, pH = 8, containing 0.1% Triton X-100, lysozyme (100 µg/mL) and DNAse (5 µg/mL)). The cells were lysed by passing the suspension twice through a French Press at 10 000 PSI and 4 °C and the lysate was clarified by centrifugation (10,000 × g) at 4 °C for 45 min. Purification was performed by loading the supernatant onto a Ni-NTA superflow column pre-equilibrated with binding buffer (10 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH = 7.5). The column was washed with washing buffer (40 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH = 7.5) and the PmGlmU enzyme was eluted with elution buffer (200 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH = 7.5). Fractions containing purified PmGlmU enzyme were combined and 10% glycerol was added for storage at 4 °C. From 1 L of culture medium 120-150 mg of PmGlmU was obtained.

Human Glycosyl Transferase Expression and Purification

The catalytic domains of human glycosyl transferases (as shown in the table below) were expressed as soluble, secreted fusion proteins by transient transfection of HEK293 suspension cultures[3,4]. The coding regions were amplified from Mammalian Gene Collection clones, human tissue cDNAs, or generated by gene synthesis by a process that appended a tobacco etch virus (TEV) protease cleavage site[5] to the NH_2-terminal end of the coding region and attL1 and attL2 Gateway adaptor sites were extended on the 5' and 3' terminal ends of the coding region during transfer to pDONR221 vector backbone[4]. The pDONR221 clones were then recombined via LR clonase reaction into a custom Gateway adapted version of the pGEn2 mammalian expression vector[4] to assemble a recombinant coding region comprised of a 25 amino acid NH_2-terminal signal sequence from the *T. cruzi* lysosomal α-mannosidase[6] followed by an 8xHis tag, 17 amino acid AviTag,[7] "superfolder" GFP[8], the nine amino acid sequence encoded by attB1 recombination site, followed by the TEV protease cleavage site and the respective glycosyltransferase catalytic domain coding region.

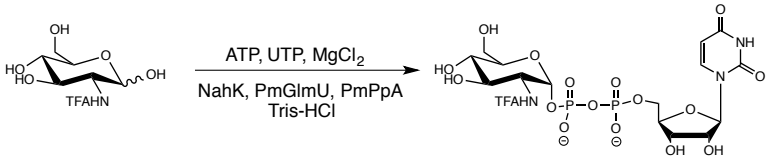
Suspension culture HEK293 cells (Freestyle 293-F cells, Life Technologies, Grand Island, NY) were transfected as previously described[3,4] and the culture supernatant was subjected to Ni^{2+}-NTA superflow chromatography (Qiagen, Valencia, CA). Enzyme preparations were eluted with 300 mM imidazole, concentrated by ultrafiltration, and subjected to gel filtration on a Superdex 75 column (GE Healthcare) preconditioned with a buffer containing 20 mM HEPES, pH 7.0, 100 mM NaCl, 10% glycerol, 0.05% Na azide. Peak fractions were pooled and concentrated to ~1 mg/mL using an ultrafiltration pressure cell membrane (Millipore, Billerica, MA) with a 10 kDa molecular weight cutoff.

Supplementary Table 1: Enzyme expression details

Enzyme	Amino Acid Residues	Uniprot ID	Expression (mg/L)
MGAT4B	41 – 548	Q9UQ53	60
MGAT5	31 – 741	Q09328	11
B3GNT2	35 – 397	Q9NY97	53
B4GALT1	63 – 398	P15291	74
FUT5	40 – 374	Q11128	31
FUT8	41 – 575	Q9BYC5	49
ST6GAL1	75 – 406	P15907	143
ST3GAL4	41 – 333	Q11206	68

2d. UDP-GlcNTFA Preparation

Procedure for the one-pot three-enzyme preparation of UDP-GlcNTFA (4)



4

GlcNTFA[9] (162 mg, 589 µmol), ATP (390 mg, 707 µmol) and UTP (390 mg, 707 µmol) were dissolved in 59 mL of 100 mM Tris-HCl buffer (pH = 8.0) containing 10 mM $MgCl_2$. To this solution was added *Bifidobacterium longum N*-acetylhexosamine 1-kinase (NahK, 14 µg/µmol substrate), *Pasteurella multocida N*-acetylglucosamine-1-phosphate uridylyltransferase (PmGlmU, 17 µg/µmol substrate) and *Pasteurella multocida* inorganic pyrophosphatase (PmPpA, 7 µg/µmol substrate), and the reaction mixture was incubated overnight at 37 °C with gentle shaking. Reaction progress was monitored by ESI-TOF MS, and once complete 59 mL of cold ethanol was added and the mixture was incubated at 4 °C for 1h. The reaction mixture was centrifuged and the supernatant was removed, concentrated, and purified by a P2 BioGel column using 0.1 M NH_4HCO_3 as eluent, followed by silica gel column chromatography (4:2:1 EtOAc/MeOH/H_2O) afforded UDP-GlcNTFA **4** (273 mg, 70%) as a white solid[2]. ^{1}H NMR (500 MHz, D_2O): δ 7.97 (d, *J* = 8.1 Hz, 1H, H6-Uridine), 5.99 (d, *J* = 4.5 Hz, 1H, H1-Ribose), 5.98 (d, *J* = 8.0 Hz, 1H, H5-Uridine), 5.63 (dd, *J* = 7.0, 3.3 Hz, 1H, H1-GlcNTFA), 4.42 – 4.34 (m, 2H, H2-Ribose, H3-Ribose), 4.33 – 4.28 (m, 1H, H4-Ribose), 4.24 (dd, *J* = 4.5, 2.7 Hz, 1H, H5-Ribose), 4.21 (dd, *J* = 5.6, 3.0 Hz, 1H, H5'-Ribose), 4.12 (dt, *J* = 10.8, 2.9 Hz, 1H, H2-GlcNTFA), 4.00-3.95 (m, 2H, H3-GlcNTFA, H4-GlcNTFA), 3.89 (dd, *J* = 12.5, 2.3 Hz, 1H, H6-GlcNTFA), 3.83 (dd, *J* = 12.6, 4.3 Hz, 1H, H6'-GlcNTFA), 3.62-3.59 (m, 1H, H5-GlcNTFA. ^{13}C NMR (76 MHz, D_2O): δ 141.6 (C6-Uridine), 102.5 (C5-Uridine), 93.7 (C1-GlcNTFA), 88.5 (C1-Ribose), 83.0 (C4-Ribose), 73.7 (C2-Ribose), 73.0 (C3-GlcNTFA), 70.1 (C4-GlcNTFA), 69.5 (C3-Ribose), 69.4 (C5-GlcNTFA), 64.9 (C5-Ribose), 60.1 (C6-GlcNTFA), 54.3 (C2-GlcNTFA). ESI-MS m/z calcd for $C_{17}H_{23}F_3N_3O_{17}P_2$, $[M-H]^-$: 660.0460, found 660.0417.

2e. General Protocols for Enzymatic Reactions and Glycosyl Asparagine Modification

General procedure for the installation of β1,3 GlcNAc using B3GNT2
Glycosyl asparagine acceptor (1 eq) and UDP-GlcNAc (1.5 eq) were dissolved to provide a final acceptor concentration of 2 – 5 mM in a HEPES buffered solution (50 mM, pH 7.3) containing KCl (25 mM), $MgCl_2$ (2 mM) and DTT (1 mM). Calf intestine alkaline phosphotase (CIAP, 1% total volume, 1 kU mL^{-1}) and B3GNT2 (1% *wt/wt* relative to acceptor substrate) were added, and the reaction mixture was incubated overnight at 37 °C with gentle shaking. Reaction progress was monitored by MALDI-TOF MS or ESI-TOF MS, and if starting material remained after 18 h another portion of B3GNT2 was added until no starting material could be detected. The reaction mixture was centrifuged over a Nanosep® Omega ultrafiltration device (10 kDa MWCO) to remove reaction proteins and the filtrate was lyophilized. Purification by HILIC HPLC (see section 2f) or P2 size-exclusion column chromatography provided the desired product.

General procedure for the installation of β1,6-GlcNTFA using MGAT5. Glycosyl asparagine acceptor **1** (17.6 mg, 10.2 μmol) and UDP-GlcNTFA (13.5 mg, 20.4 μmol) were dissolved at a final acceptor concentration of 10 mM in a sodium cacodylate buffered solution (100 mM, pH 6.5) containing $MnCl_2$ (10 mM) and BSA (1% total volume, stock solution = 10 mg mL^{-1}). Calf intestine alkaline phosphatase (CIAP, 1% total volume, stock solution = 1kU mL^{-1}) and MGAT5 (40 μg/μmol acceptor) were added, and the reaction mixture was incubated overnight at 37 °C with gentle shaking. Reaction progress was monitored by MALDI-TOF MS and if starting material remained after 18 h another portion of MGAT5 was added until no starting material could be detected. The reaction mixture was centrifuged over a Nanosep® Omega ultrafiltration device (10 kDa MWCO) to remove reaction proteins and the filtrate was lyophilized. Purification by HPLC using a HILIC column (supporting information 2f) provided desired product **14** as a white fluffy solid (18.6 mg, 92%).

General procedure for the installation of β1,4-GlcNTFA using MGAT4B. Glycosyl asparagine acceptor **2** (4.0 mg, 2.1 μmol) and UDP-GlcNTFA (2.75 mg, 4.2 μmol) were dissolved at a final acceptor concentration of 5 mM in a Tris buffered solution (100 mM, pH 7.5) containing $MnCl_2$ (5 mM) and BSA (1% total volume). CIAP (1% total volume) and MGAT4B (400 μg/μmol acceptor) were added, and the reaction mixture was incubated overnight at 37 °C with gentle shaking. Reaction progress was monitored by ESI-TOF MS, and if starting material remained after 18 h another portion of MGAT4B was added until no starting material could be detected. The reaction mixture was centrifuged over a Nanosep® Omega ultrafiltration device (10 kDa MWCO) to remove reaction proteins, and the filtrate was lyophilized. Purification by HPLC using a HILIC column (supporting information 2f) provided the desired product **S6** as a white fluffy solid (3.8 mg, 85%).

General procedure for the installation of β1,4 Gal using B4GALT1
Glycosyl asparagine acceptor (1 eq) and UDP-Gal (1.5 eq per Gal to be added) were dissolved to a provide an acceptor concentration of 2 – 5 mM in a Tris buffered solution (100 mM, pH 7.5) containing $MnCl_2$ (10 mM) and BSA (1% total volume). CIAP (1% volume total) and B4GALT1 (1% *wt/*wt relative to acceptor substrate) were added, and the reaction mixture was incubated overnight at 37 °C with gentle shaking. Reaction progress was monitored by MALDI-TOF MS or ESI-TOF MS, and if starting material remained after 18 h another portion of B4GALT1 was added until no starting material could be detected. The reaction mixture was centrifuged over a Nanosep® Omega ultrafiltration device (10 kDa MWCO) to remove reaction proteins and the filtrate was lyophilized. Purification by HILIC HPLC (see section 2f) or P2 size-exclusion column chromatography provided the desired product.

General procedure for the installation of α1,3 Fuc using FUT5
Glycosyl asparagine acceptor (1 eq) and GDP-Fuc (1.5 eq per Fuc to be added) were dissolved at a final acceptor concentration of 2 – 5 mM in a Tris buffered solution (50 mM, pH 7.3) containing $MnCl_2$ (10 mM). CIAP (1% total volume) and FUT5 (1% *wt/wt*) were added, and the reaction mixture was incubated overnight at 37 °C with gentle shaking. Reaction progress was monitored by MALDI-TOF MS or ESI-TOF MS, and if starting material remained after 18 h another portion of FUT5 was added until no starting material could be detected. The reaction mixture was centrifuged over a Nanosep® Omega ultrafiltration device (10 kDa MWCO) to remove reaction proteins and the filtrate was lyophilized. Purification by HILIC HPLC (see section 2f) or P2 size-exclusion column chromatography provided the desired product.

General Procedure for the installation of α2,3 Neu5Ac using ST3GAL4
Glycosyl asparagine acceptor (1 eq) and CMP-Neu5Ac (1.5 eq) were dissolved at a final acceptor concentration of 2 – 5 mM in a sodium cacodylate buffered solution (50 mM, pH 7.2) containing BSA (1% total volume). CIAP (1% volume total) and ST3GAL4 (1% *wt/wt* relative to acceptor substrate) were added, and the reaction mixture was incubated overnight at 37 °C with gentle shaking. Reaction progress was monitored by ESI-TOF MS, and if starting material remained after 18 h another portion of ST3GAL4 was added until no starting material could be detected. The reaction mixture was centrifuged over a Nanosep® Omega ultrafiltration device (10 kDa MWCO) to remove reaction proteins, and the filtrate was lyophilized. Purification by HILIC HPLC (see section 2f) or P2 size-exclusion column chromatography provided the desired product.

General procedure for the selective installation of terminal α2,6 Neu5Ac using ST6GAL1
Glycosyl asparagine (1 eq) and CMP-Neu5Ac (1.1 eq) were dissolved at a final acceptor concentration of 2 – 5 mM in a sodium cacodylate buffered solution (100 mM, pH 6.5) containing BSA (1% volume total). CIAP (1% volume total) and ST6GAL1 (1% *wt/wt* relative to acceptor substrate) were added, and the reaction mixture was incubated overnight at 37 °C with gentle shaking. The reaction mixture was centrifuged over a Nanosep® Omega ultrafiltration device (10 kDa MWCO) to remove reaction proteins, and the filtrate was lyophilized. Purification by HILIC HPLC (see section 2f) or P2 size-exclusion column chromatography provided the desired product.

General procedure for the selective cleavage of galactose using *E. coli* β-galactosidase[10]
Glycosyl asparagine was dissolved at a concentration of 5 mM in a Tris buffered solution (50 mM, pH 7.3) containing 5 mM $MgCl_2$. To this solution was added 50 U/μmol glycosyl asparagine of *E. coli* β-galactosidase (Sigma-Aldrich #, G5635) and the mixture was incubated overnight at 37 °C. The reaction mixture was centrifuged using a Nanosep® Omega ultrafiltration device (10 kDa MWCO) to remove the enzyme and the filtrate was lyophilized Purification by HILIC HPLC (see section 2f) or P2 size-exclusion column chromatography provided the desired product.

General procedure for removal of TFA protecting group of an *N*-glycan. The GlcNTFA moiety of **S6** was converted to $GlcNH_2$ by dissolving the substrate (3.8 mg, 1.8 μM) in H_2O to a final concentration of 10 mM. The pH of the solution was adjusted to 10 using μL aliquots 1 M NaOH. The reaction mixture was incubated overnight at 37 °C with gentle shaking. Progress of the reaction was monitored by MALDI-TOF MS and once complete the solvent was removed by lyophilization. The reaction was neutralized by μL aliquots of 1 M acetic acid and purified by P2 size-exclusion chromatography eluting with 50 mM ammonium bicarbonate to yield the desired target **2** as a white fluffy solid (3.5 mg, 92%).

General procedure for the conversion of $GlcNH_2$ to $GlcN_3$. Substrate **15** (9.3 mg, 5 μmol, 1 eq) was dissolved in water (1.6 mL) and to this solution was added imidazole-1-sulfonyl azide hydrogen sulfate (13.4 mg, 50 μmol), K_2CO_3 (6.8 mg, 50 μmol) and catalytic $CuSO_4.5H_2O$. The reaction mixture was incubated overnight at 37 °C with gentle shaking. Reaction progress was monitored by MALDI-TOF MS and if starting material remained, an additional $^1/_2$ portion of the imidazole-1-sulfonyl azide hydrogen sulfate, K_2CO_3, and $CuSO_4$ was added until no starting material could be observed. The reaction solvent was removed by lyophilization and the salts were removed by P2 size-exclusion chromatography eluting with 50 mM ammonium bicarbonate to yield **23** as a white fluffy solid (7.2 mg, 76%).

General procedure for reduction of $GlcN_3$. Intermediate **27** (2.3 mg, 0.66 μmol, 1 eq) was dissolved in a solution of 9:1 pyridine / triethylamine to give a final concentration of 5 mM. The mixture was vortexed until all solids dissolved and 10 eq. 1,3-dithiolpropane (0.7 mg, 6.6 μmol, 10 eq) were added in one portion. The reaction mixture was kept at 37 °C was until no azide could be detected by ESI-TOF-MS. Reaction was carried forward to acetylate the amine without further purification.

General procedure for amine acetylation. 18 (1.3 mg, 0.5 μmol, 1 eq) was dissolved in water to a final concentration of 2 mM. The pH was adjusted to 8 using μL aliquots of 1M NaOH. To this solution was added solid AcOSu (0.7 mg, 5 μmol, 10 eq) in one portion. The reaction mixture was vortexed vigorously until all solids were dissolved. The reaction was kept at 37 °C until full acetylation was observed by ESI-TOF-MS. In the event starting amine was detected, additional AcOSu (5 eq) was added until complete conversion was observed. The reaction was lyophilized and purified by HPLC using a HILIC column (supporting information 2f) to afford **19** as a white fluffy solid (0.9 mg, 67%).

2f. General Protocols for HILIC-HPLC Purification

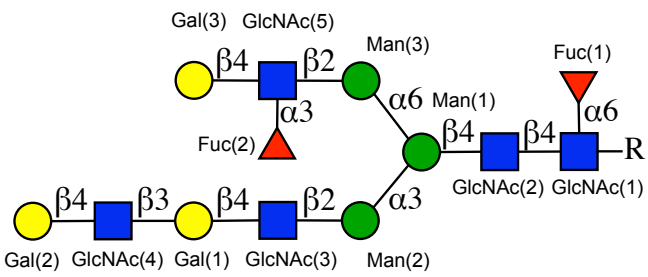
HILIC-HPLC Purification Conditions for Glycosyl Asparagine Targets
Semi-preparative HILIC-HPLC was performed on a Shimadzu LC-ESI-IT-TOF with a Waters XBridge BEH, Amide column, 5 µm, 10 x 250 mm at a flow rate of 2.3 mL/min, injection volume of 100 µL (10-20 mg/mL), with 1% of the flow is diverted to the ESI-MS detector using a splitter. Mobile phase A was 10 mM ammonium formate in water, adjusted to pH 4.5 with formic acid; mobile phase B was 90% aceteonitrile with 10% 10 mM ammonium formate in water (pH = 4.5). The general condition using a linear gradient is as follows:

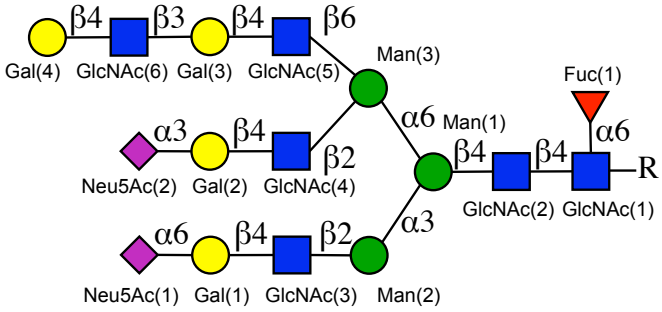
Time (min)	A (%)	B (%)
0	20	80
40	55	45
45	80	20
55	20	80
60	20	80

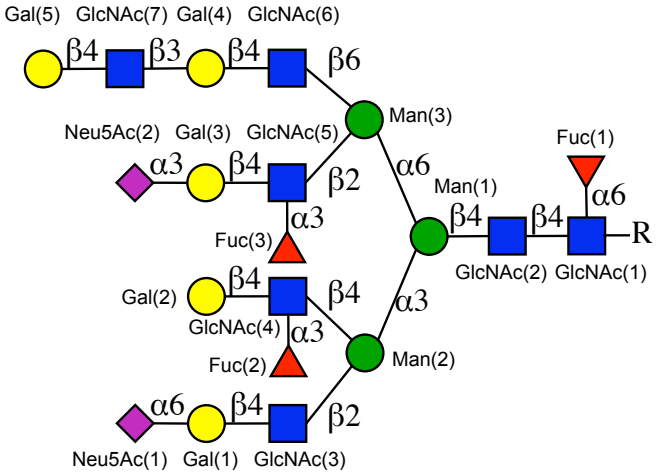
1 was purified using a linear gradient with the following conditions:

Time (min)	A (%)	B (%)
0	20	80
60	40	60
70	50	50
71	80	20
80	80	20
85	20	80
90	20	80

3. NMR Nomenclature

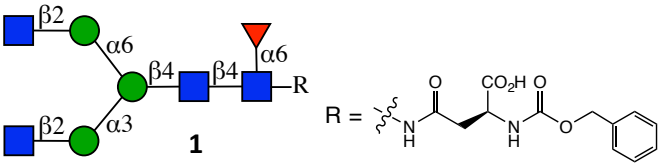






4. Characterization of glycosyl asparagine targets

Compound 1



1

^{1}H (900 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 10.2 Hz, 1H)	3.87	3.78	3.70	N/R[a]	3.90 3.70	-[b]	2.15 -1.95 12H
GlcNAc(2)	4.71 (d, *J* = 8.1 Hz, 1H)	3.82	3.63	3.78	N/R	N/R	-	2.15 -1.95 12H
GlcNAc(3)	4.59 (d, *J* = 8.6 Hz, 1H)	3.74	3.58	3.49	N/R	N/R	-	2.15 -1.95 12H
GlcNAc(4)	4.59 (d, *J* = 8.6 Hz, 1H)	3.74	3.58	3.49	N/R	N/R	-	2.15 -1.95 12H
Man(1)	4.80	4.28 (d, *J* = 2.5 Hz, 1H)	3.80	3.66	N/R	4.00 3.82	-	-
Man(2)	5.15	4.22 (d, *J* = 3.2 Hz, 1H)	3.94	3.78	3.54	N/R	-	-
Man(3)	4.95	4.14	3.93	3.65	3.52	N/R	-	-
Fuc(1)(α6)	4.89 (d, *J* = 3.8 Hz, 1H)	3.82	3.89	3.93	4.14	-	1.21 (d, *J* = 6.6 Hz, 3H)	-

Signal	Proton	Carbon
Aromatic	7.48 7.46	128.6 127.7
$\underline{CH_2}$-Ph	5.17	67.1
NH-$\underline{CH}$-CO_2H	4.53	51.2
C(O)-$\underline{CH_2}$-CH	2.87 2.78	37.1

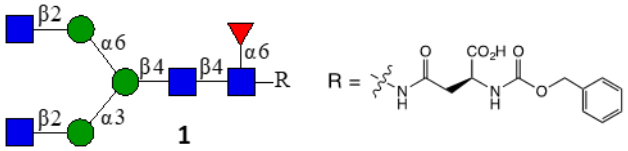
^{13}C (225 MHz, D_2O): δ (ppm)

	C1
GlcNAc(1)	78.0
GlcNAc(2)	100.8
GlcNAc(3)	99.9
GlcNAc(4)	99.9
Man(1)	100.2
Man(2)	99.3
Man(3)	96.9
Fuc(1)(α6)	99.2

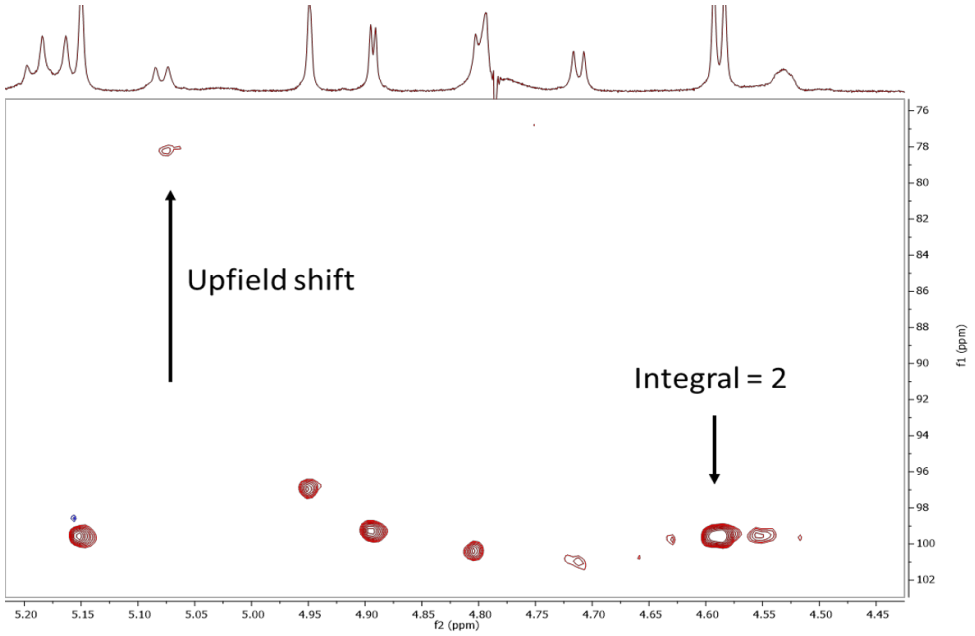
[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{68}H_{104}N_6O_{44}$, $[M-2H]^{2-}$: 854.3048, found 854.2907.

Characterization of 1:

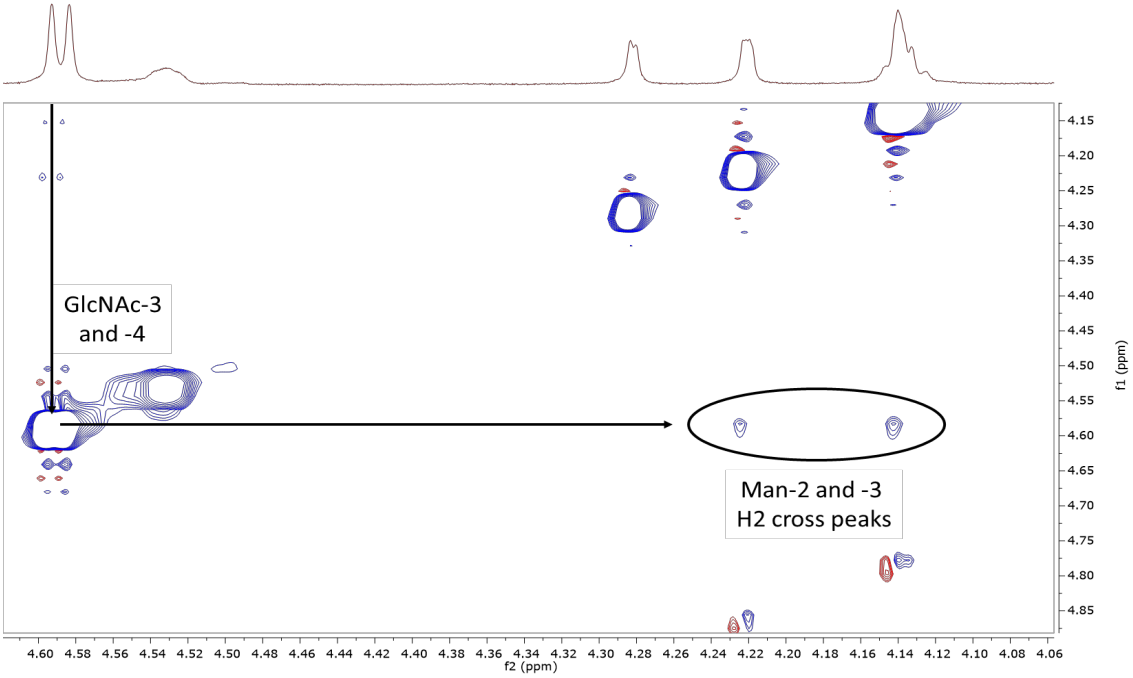


Assignment of anomeric and glycosidic linkages of **1** could be determined using a combination of 1D and 2D NMR experiments. Initial examination of the anomeric region of the ^{13}C-^{1}H HSQC (**Supplementary Fig. 3)** illustrated seven



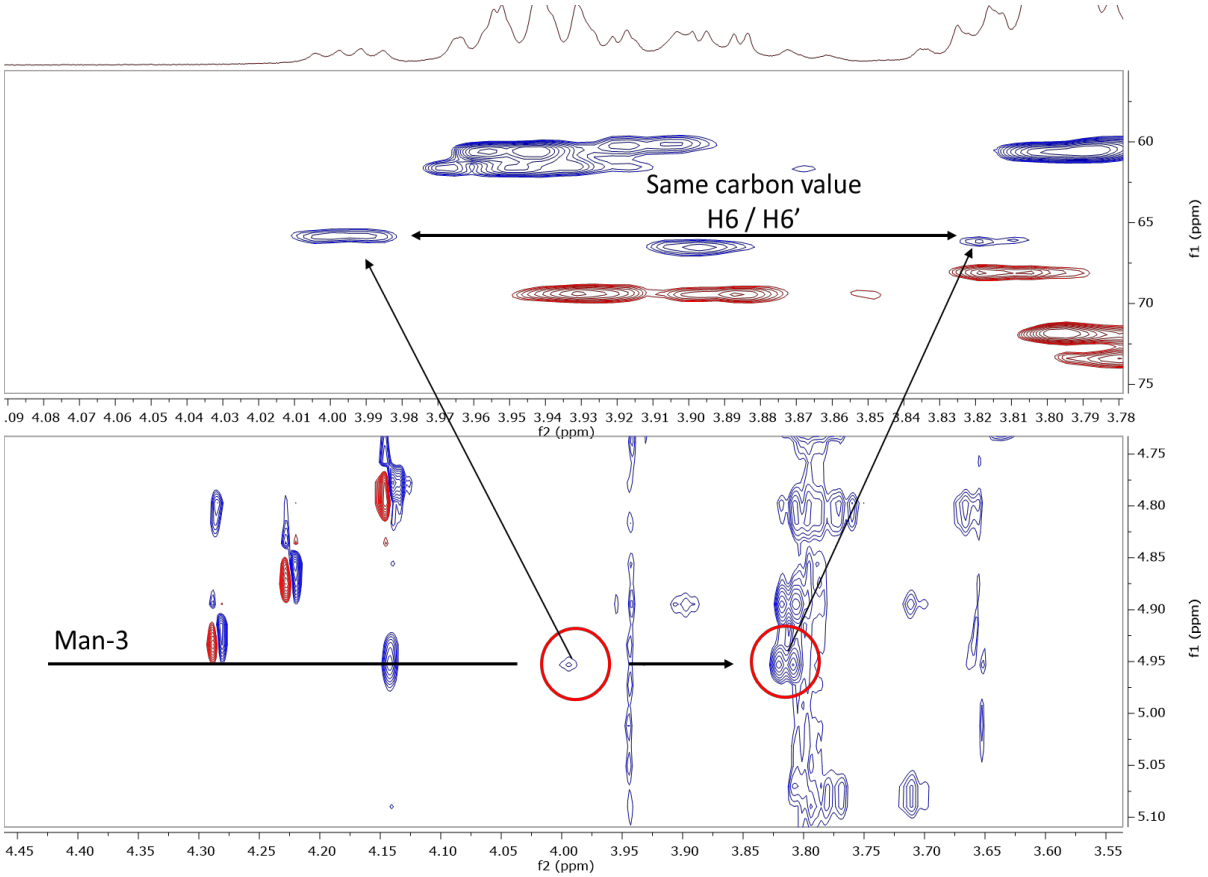
Supplementary Fig. 3. Analysis of anomeric region of **1**.

unique signals. The reducing GlcNAc H1 (GlcNAc-1) could be assigned (δ5.08) due to a significant upfield shift in its carbon value resulting from the anomeric amide aglycone β-linkage. The signal at δ4.59 integrated to a value of two



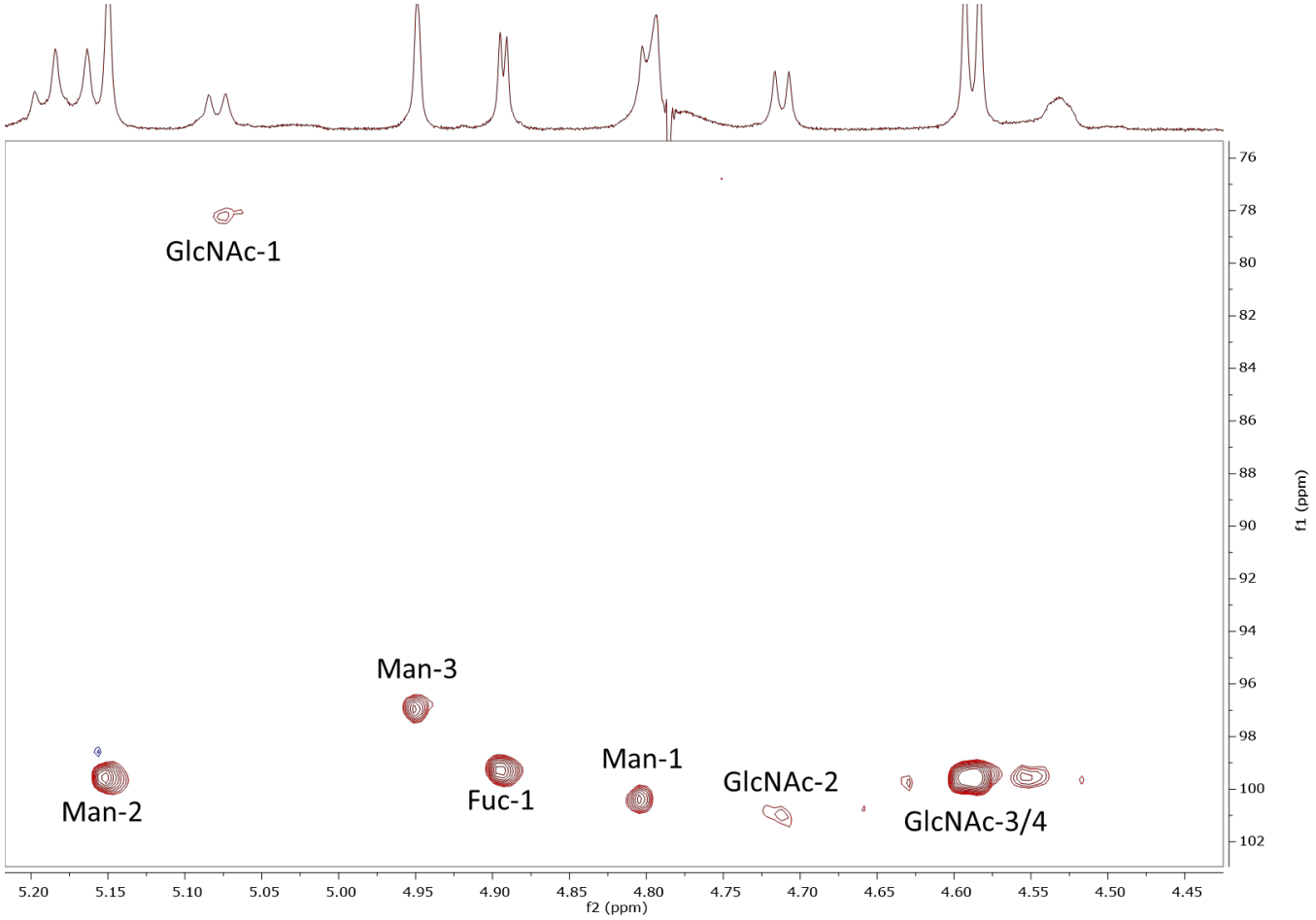
Supplementary Fig. 4. NOESY observed GlcNAc-3/4 anomeric coupling with H2 from Man-2/3

with a coupling constant of 8.6 Hz suggesting this signal corresponded to GlcNAc-3 and -4 as these monosaccharides occupy a similar electronic environment and both have β-linkages which is consistent with the observed *J*-values. Accordingly, it was expected that the H1s from these two GlcNAc residues would have a through space correlation with the H2s from the Man-2 and -3 spin systems. Analysis of the NOESY spectrum (**Supplementary Fig. 4**) showed two



Supplementary Fig. 5. HSQC-NOESY inspection of the anomeric Man-3 signal.

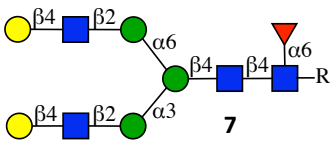
cross peaks at δ4.22 and δ4.14. The downfield nature and small coupling constants of these signals are diagnostic of the equatorial Man H2s. The H2 of Man-1 was assigned by process-of-elimination at δ4.28, and examination of COSY data led us to identify the H1 signal at δ4.80. Having identified Man-1, we could look for through space glycosidic linkage correlations to identify Man-2 and -3 as they are α-linked to the -3 and -6 positions of Man-1, respectively. Indeed, NOESY-HSQC analysis (**Supplementary Fig. 5**) of the anomeric signal at δ4.95 showed a through space correlation with two peaks at δ4.00 and δ3.82 that are associated with the same carbon δ65.79 indicating that this anomeric signal belongs to Man-3 and the through space coupling represents the diastereotopic H6/H6' from Man-1. As such, the anomeric signal at δ5.15 must belong to Man-2. At this point, six of the eight anomic signals had been assigned and we could finish the signal identification by examination of the two remaining doublets located at δ4.89 and δ4.71. Both peaks had an integration of one, but the signal at δ4.89 had a *J*-value of 3.8 Hz (α-linkage) compared to 8.6 Hz (β-linkage) of the δ4.71 peak. As such, we could assign the anomeric signals from Fuc (δ4.89) and GlcNAc-2 (δ4.71). Identification of all anomeric peaks (**Supplementary Fig. 6**) provided the foundation necessary for full assignment of compound **1**. The remaining intra-spin system proton signals were assigned using 2D COSY and TOCSY analysis.



Supplementary Fig. 6. Complete anomeric assignment of **1**.

Compound 7

7 was prepared from **1** (20 mg, 11.7 µmol) using the general procedure for the installation of β1,4 Gal with B4GALT1. After HILIC purification, **7** was obtained as a white solid (15.8 mg, 70%).



7

^{1}H (900 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 9.8 Hz, 1H)	3.87	3.80	3.70	N/R[a]	3.89 3.70	-[b]	2.15 -1.95 12H
GlcNAc(2)	4.71 (d, *J* = 8.2 Hz, 1H)	3.83	3.63	3.77	N/R	N/R	-	2.15 -1.95 12H
GlcNAc(3)	4.62 (d, *J* = 7.9 Hz, 1H)	3.78	3.61	3.76	N/R	N/R	-	2.15 -1.95 12H
GlcNAc(4)	4.62 (d, *J* = 7.9 Hz, 1H)	3.78	3.61	3.76	N/R	N/R	-	2.15 -1.95 12H
Man(1)	4.8	4.28 (d, *J* = 1.6 Hz, 1H)	3.80	3.66	N/R	3.99 3.82	-	-
Man(2)	5.15	4.23 (d, *J* = 2.1 Hz, 1H)	3.94	3.78	3.54	N/R	-	-
Man(3)	4.96	4.14	3.93	3.65	3.53	N/R	-	-
Fuc(1)(α6)	4.89 (d, *J* = 3.7 Hz, 1H)	3.81	3.89	3.93	4.14	-	1.21 (d, *J* = 6.5 Hz, 3H)	-
Gal(1)	4.51 (d, *J* = 7.7 Hz, 1H)	3.58	3.70	3.96	N/R	N/R	-	-
Gal(2)	4.51 (d, *J* = 7.7 Hz, 1H)	3.58	3.70	3.96	N/R	N/R	-	-

Signal	Proton	Carbon
Aromatic	7.49-7.43 (m, 5H)	128.6, 128.2, 127.6
$\underline{CH_2}$-Ph	5.18 (d, *J* = 12.2 Hz, 1H) 5.14 (d, *J* = 12.4 Hz, 1H)	66.9
NH-**$\underline{CH}$**-CO_2H	4.36 (dd, *J* = 9.3, 4.2 Hz, 1H)	52.9
C(O)-**$\underline{CH_2}$**-CH	2.84 (dd, *J* = 15.4, 4.1 Hz, 1H) 2.65 (d, *J* = 15.4, 9.4 Hz, 1H)	38.4

^{13}C (225 MHz, D_2O): δ (ppm)

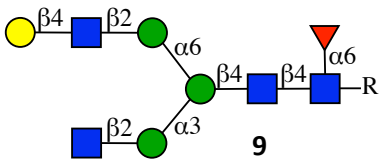
	C1
GlcNAc(1)	78.1
GlcNAc(2)	100.8
GlcNAc(3)	99.3
GlcNAc(4)	99.3
Man(1)	100.4
Man(2)	99.4
Man(3)	96.9
Fuc(1)(α6)	99.2
Gal(1)	102.8
Gal(2)	102.8

[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{80}H_{124}N_6O_{54}$, $[M-2H]^{2-}$: 1016.3576 found 1016.3493.

Compound 9

9 was prepared from **7** (3.2 mg, 1.6 μmol) using the general procedure for the selective cleavage of galactose with *E. coli* β-galactosidase. After HILIC purification, compound **9** was obtained as a white solid (2.0 mg, 67%).



9

^{1}H (900 MHz, D_2O): δ (ppm)

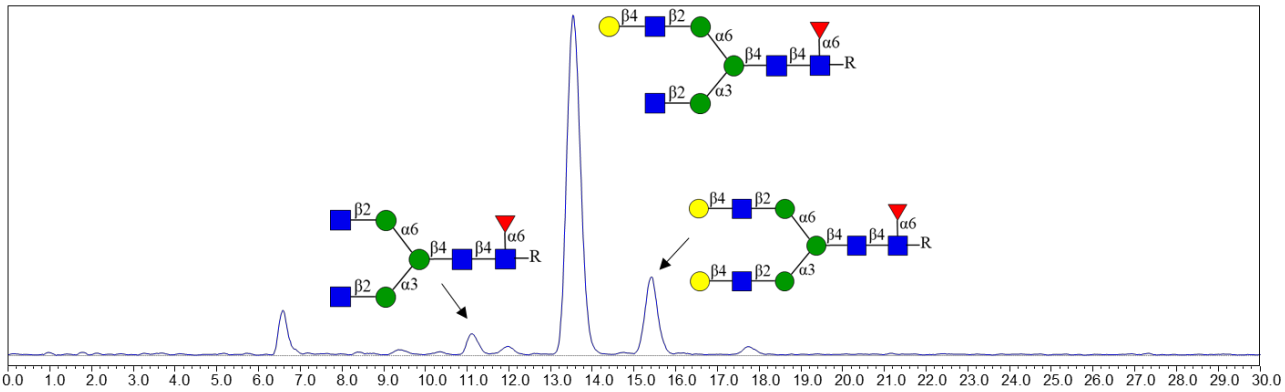
	H1	H2	H3	H4	H5	H6	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 9.3 Hz, 1H)	3.86	3.79	3.70	N/R[a]	3.89 3.69	-[b]	2.15 -1.95 12H
GlcNAc(2)	4.71 (d, *J* = 8.0 Hz, 1H)	3.81	3.63	3.76	N/R	N/R	-	2.15 -1.95 12H
GlcNAc(3)	4.59 (d, *J* = 8.4 Hz, 1H)	3.73	3.59	3.47	N/R	N/R	-	2.15 -1.95 12H
GlcNAc(4)	4.62 (d, *J* = 8.3 Hz, 1H)	3.78	3.60	3.74	N/R	N/R	-	2.15 -1.95 12H
Man(1)	4.80	4.28 (d, *J* = 2.9 Hz,1H)	3.80	3.65	N/R	3.98 3.81	-	-
Man(2)	5.15	4.22 (d, *J* = 3.3 Hz, 1H)	3.94	3.78	3.54	N/R	-	-
Man(3)	4.96	4.14	3.92	3.65	3.53	N/R	-	-
Fuc(1)(α6)	4.89 (d, *J* = 3.8 Hz, 1H)	3.80	3.89	3.92	4.14	-	1.21 (d, *J* = 6.5 Hz, 3H)	-
Gal(1)	4.51 (d, *J* = 7.9 Hz, 1H)	3.59	3.70	3.96	N/R	N/R	-	-

^{13}C (225 MHz, D_2O): δ (ppm)

	C1
GlcNAc(1)	78.1
GlcNAc(2)	100.9
GlcNAc(3)	99.6
GlcNAc(4)	99.4
Man(1)	100.3
Man(2)	99.5
Man(3)	97.0
Fuc(1)(α6)	99.2
Gal(1)	102.9

[a] Not reported
[b] Not applicable

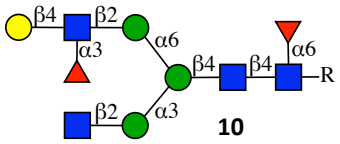
ESI TOF-MS *m/z* calcd for $C_{74}H_{114}N_6O_{49}$, $[M-2H]^{2-}$: 935.3312, found 935.3125.



Supplementary Fig. 7. HILIC purification of β-galactosidase mediated removal for the terminal gal residue on the MGAT arm. After overnight incubation, the major desired product was shown to elute at 13 min with residual starting material and agalactosylated impurities eluting at 15.5 and 11 min, respectively. HILIC conditions described in Section 2f.

Compound 10

10 was prepared from **9** (1.2 mg, 0.64 μmol) using the general procedure for the installation of α1,3 Fuc with FUT5. After HILIC purification, compound **10** was obtained as a white solid (0.9 mg, 71%).



10

^{1}H (600 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 9.6 Hz, 1H)	3.87	3.80	3.69	N/R[a]	3.88 3.69	-[b]	2.15 -1.95 12H
GlcNAc(2)	4.71 (d, *J* = 8.0 Hz, 1H)	3.82	3.63	3.77	N/R	N/R	-	2.15 -1.95 12H
GlcNAc(3)	4.58 (d, *J* = 8.3 Hz, 1H)	3.73	3.59	3.47	N/R	N/R	-	2.15 -1.95 12H
GlcNAc(4)	4.62 (d, *J* = 8.6 Hz, 1H)	3.95	3.89	3.60	N/R	N/R	-	2.15 -1.95 12H
Man(1)	4.81	4.29	3.80	3.67	N/R	N/R	-	-
Man(2)	5.15	4.22 (d, *J* = 3.2 Hz, 1H)	3.93	3.77	3.53	N/R	-	-
Man(3)	4.94	4.13	3.93	3.64	3.51	N/R	-	-
Fuc(1)(α6)	4.89 (d, *J* = 3.5 Hz, 1H)	3.80	3.89	3.92	4.14	-	1.21 (d, *J* = 6.4 Hz, 3H)	-
Fuc(2)	5.16 (d, *J* = 3.9 Hz, 1H)	3.72	3.94	3.82	4.87 (d, *J* = 6.6 Hz, 1H)	-	1.21 (d, *J* = 6.4 Hz, 3H)	-
Gal(1)	4.48 (d, *J* = 7.9 Hz, 1H)	3.53	3.69	3.93	N/R	N/R	-	-

^{13}C (150 MHz, D_2O): δ (ppm)

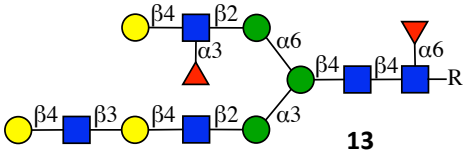
	C1
GlcNAc(1)	78.1
GlcNAc(2)	100.9
GlcNAc(3)	99.5
GlcNAc(4)	99.2
Man(1)	100.5
Man(2)	99.4
Man(3)	96.8
Fuc(1)(α6)	99.3
Fuc(2)	98.4
Gal(1)	101.7

[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{80}H_{124}N_6O_{53}$, $[M\text{-}2H]^{2-}$: 1008.3602, found 1008.3376.

Compound 13

13 was prepared from **10** (0.5 mg, 0.25 µmol) by first installing a β1,4 Gal using the general procedure for B4GALT1. The reaction was monitored by ESI-MS and once complete conversion to compound **11** was observed (*m/z* calcd for $C_{86}H_{134}N_6O_{58}$, $[M-2H]^{2-}$: 1089.3866, found 1089.3758), the reaction mixture was centrifuged using a Nanosep® Omega ultrafiltration device (10 kDa MWCO) and the filtrate was lyophilized. To the resulting crude residue containing **11** a β1,3 GlcNAc was installed using the general procedure for B3GNT2. Once ESI-MS indicated complete conversion to **12** (*m/z* calcd for $C_{94}H_{147}N_7O_{63}$, $[M-2H]^{2-}$: 1191.4279, found 1191.4132), the mixture was centrifuge using a Nanosep® Omega ultrafiltration device (10 kDa MWCO) and lyophilized. To the resulting crude residue containing **12** a β1,4 Gal was installed using the general procedure for B4GALT1. After P2 size-exclusion purification purification, compound **13** was obtained as a white solid (0.5 mg, 79% over 3 steps).



13

^{1}H (900 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 10.0 Hz, 1H)	3.86	3.80	3.70	N/R[a]	N/R	-[b]	2.15 -1.95 15H
GlcNAc(2)	4.71 (d, *J* = 8.4 Hz, 1H)	3.81	3.63	3.77	N/R	N/R	-	2.15 -1.95 15H
GlcNAc(3)	4.61 (d, *J* = 7.7 Hz, 1H)	3.77	3.61	3.75	N/R	N/R	-	2.15 -1.95 15H
GlcNAc(4)	4.74 (d, *J* = 8.4 Hz, 1H)	3.84	3.62	3.77	N/R	N/R	-	2.15 -1.95 15H
GlcNAc(5)	4.62 (d, *J* = 8.4 Hz, 1H)	3.97	3.89	3.60	N/R	N/R	-	2.15 -1.95 15H
Man(1)	4.80	4.29	3.81	3.68	N/R	N/R	-	-
Man(2)	5.15	4.22 (d, *J* = 2.2 Hz, 1H)	3.93	3.78	3.53	N/R	-	-
Man(3)	4.94	4.14	3.93	3.64	3.51	N/R	-	-
Fuc(1)(α6)	4.89 (d, *J* = 4.0 Hz, 1H)	3.81	3.89	3.91	4.14	-	1.21 (d, *J* = 6.5 Hz, 3H)	-
Fuc(2)	5.17 (d, *J* = 3.9 Hz, 1H)	3.73	3.94	3.83	4.87 (d, *J* = 6.6 Hz, 1H)	-	1.21 (d, *J* = 6.5 Hz, 3H)	-
Gal(1)	4.49 (d, *J* = 7.7 Hz, 1H)	3.61	3.76	4.19 (d, *J* = 3.0 Hz, 1H)	N/R	N/R	-	-
Gal(2)	4.51 (d, *J* = 8.0 Hz, 1H)	3.57	3.70	3.96	N/R	N/R	-	-
Gal(3)	4.48 (d, *J* = 7.8 Hz, 1H)	3.53	3.69	3.93	N/R	N/R	-	-

^{13}C (225 MHz, D_2O): δ (ppm)

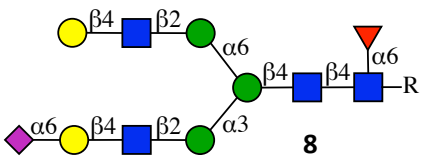
	C1
GlcNAc(1)	78.1
GlcNAc(2)	100.9
GlcNAc(3)	99.4
GlcNAc(4)	99.2
GlcNAc(5)	102.6
Man(1)	100.4
Man(2)	99.4
Man(3)	96.8
Fuc(1)(α6)	99.2
Fuc(2)	98.5
Gal(1)	102.9
Gal(2)	101.7
Gal(3)	102.8

[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{100}H_{157}N_7O_{68}$, $[M-2H]^{2-}$: 1272.4543, found 1272.4438.

Compound 8

8 was prepared from **7** (7.0 mg, 3.44 µmol) using the general procedure for the selective installation of α2,6 Neu5Ac with ST6GAL1. After HILIC purification, compound **8** was obtained as a white solid (5.4 mg, 68%).



8

^{1}H (900 MHz, D_2O): δ (ppm)

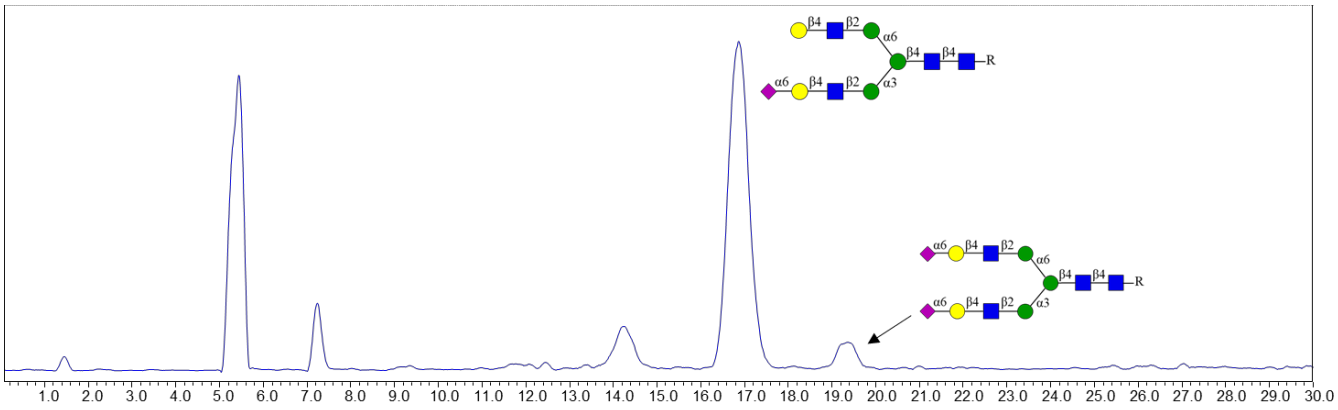
	H1	H2	H3	H4	H5	H6	H7	H8	H9	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 10.0 Hz, 1H)	3.87	3.79	3.71	N/R[a]	N/R	-	-	-	-[b]	2.15 -1.95 15H
GlcNAc(2)	4.71 (d, *J* = 8.4 Hz, 1H)	3.83	3.64	3.78	N/R	N/R	-	-	-	-	2.15 -1.95 15H
GlcNAc(3)	4.64 (d, *J* = 8.1 Hz, 1H)	3.79	3.64	3.69	N/R	N/R	-	-	-	-	2.15 -1.95 15H
GlcNAc(4)	4.62 (d, *J* = 8.3 Hz, 1H)	3.79	3.61	3.76	N/R	N/R	-	-	-	-	2.15 -1.95 15H
Man(1)	4.80	4.29 (d, *J* = 1.9 Hz,1H)	3.81	3.67	N/R	4.00 3.83	-	-	-	-	-
Man(2)	5.17	4.23 (d, *J* = 1.9 Hz, 1H)	3.94	3.78	3.56	N/R	-	-	-	-	-
Man(3)	4.96	4.14	3.93	3.66	3.53	N/R	-	-	-	-	-
Fuc(1)	4.89 (d, *J* = 3.8 Hz, 1H)	3.81	3.90	3.93	4.14	-	-	-	-	1.21 (d, *J* = 6.5 Hz, 3H)	-
Gal(1)	4.48 (d, *J* = 7.9 Hz, 1H)	3.57	3.71	3.97	N/R	N/R	-	-	-	-	-
Gal(2)	4.51 (d, *J* = 7.8 Hz, 1H)	3.58	3.71	3.97	N/R	N/R	-	-	-	-	-
Neu5Ac	-	-	2.70 (dd, *J* = 12.3, 4.6 Hz, 1H) 1.75 (dd, *J* = 12.2 Hz, 1H)	3.70	3.84	N/R	N/R	N/R	N/R	-	2.15 -1.95 15H

^{13}C (225 MHz, D_2O): δ (ppm)

	C1
GlcNAc(1)	78.1
GlcNAc(2)	100.9
GlcNAc(3)	99.3
GlcNAc(4)	99.5
Man(1)	100.4
Man(2)	99.5
Man(3)	97.0
Fuc(1)(α6)	99.2
Gal(1)	103.4
Gal(2)	102.9

[[a]] Not reported
[b] Not applicable

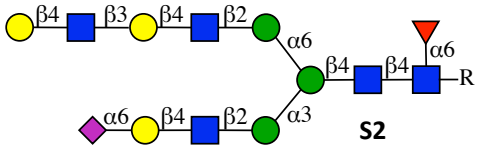
ESI TOF-MS *m/z* calcd for $C_{91}H_{141}N_7O_{62}$, $[M-2H]^{2-}$: 1161.9053, found 1161.9224.



Supplementary Fig. 8. Branch selective sialylation mediated by ST6GAL1. After overnight incubation, the desired major product had a retention time of 16.5 min whereas the over-sialylated product eluted 3 min later at 19.5 min. HILIC purification conditions are described in Section 2f.

Compound S2

S2 was prepared from **8** (1.4 mg, 0.60 µmol) by first installing a β1,3 GlcNAc using the general procedure for B3GNT2. The reaction was monitored by ESI-MS and once complete conversion to **S1** was observed (*m/z* calcd for $C_{99}H_{154}N_8O_{67}$, $[M-2H]^{2-}$: 1263.9267, found 1263.9108), the reaction mixture was centrifuged using a Nanosep® Omega ultrafiltration device (10 kDa MWCO) and the filtrate was lyophilized. To the resulting crude residue containing **S1** a β1,4 Gal was installed using the general procedure for B4GALT1. After HILIC purification, compound **S2** was obtained as a white solid (1.0 mg, 62% over 2 steps).



S2

^{1}H (600 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	H7	H8	H9	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 10.0 Hz, 1H)	3.88	3.79	3.70	N/R[a]	N/R	-	-	-	-[b]	2.15 -1.95 18H
GlcNAc(2)	4.71 (d, *J* = 8.1 Hz, 1H)	3.82	3.63	3.77	N/R	N/R	-	-	-	-	2.15 -1.95 18H
GlcNAc(3)	4.64 (d, *J* = 7.4 Hz, 1H)	3.79	3.63	3.68	N/R	N/R	-	-	-	-	2.15 -1.95 18H
GlcNAc(4)	4.62 (d, *J* = 7.9 Hz, 1H)	3.79	3.60	3.74	N/R	N/R	-	-	-	-	2.15 -1.95 18H
GlcNAc(5)	4.74 (d, *J* = 8.2 Hz, 1H)	3.85	3.62	3.77	N/R	N/R	-	-	-	-	2.15 -1.95 18H
Man(1)	4.81	4.29	3.82	3.67	N/R	4.00 3.82	-	-	-	-	-
Man(2)	5.17	4.23 (d, *J* = 2.0 Hz, 1H)	3.94	3.78	3.55	N/R	-	-	-	-	-
Man(3)	4.96	4.14	3.94	3.65	3.52	N/R	-	-	-	-	-
Fuc(1)	4.89 (d, *J* = 3.9 Hz, 1H)	3.81	3.89	3.93	4.14	-	-	-	-	1.21 (d, *J* = 6.6 Hz, 3H)	-
Gal(1)	4.48 (d, *J* = 8.0 Hz, 1H)	3.57	3.70	3.96	N/R	N/R	-	-	-	-	-
Gal(2)	4.49 (d, *J* = 7.6 Hz, 1H)	3.63	3.76	4.19 (d, *J* = 2.8 Hz, 1H)	N/R	N/R	-	-	-	-	-
Gal(3)	4.51 (d, *J* = 7.8 Hz, 1H)	3.58	3.70	3.96	N/R	N/R	-	-	-	-	-
Neu5Ac	-	-	2.70 (dd, *J* = 12.5, 4.6 Hz, 1H) 1.76 (dd, *J* = 12.4 Hz, 1H)	3.70	3.84	N/R	N/R	N/R	N/R	-	2.15 -1.95 18H

^{13}C (225 MHz, D_2O): δ (ppm)

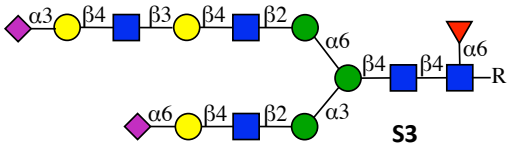
	C1
GlcNAc(1)	78.1
GlcNAc(2)	100.7
GlcNAc(3)	98.9
GlcNAc(4)	99.4
GlcNAc(5)	102.7
Man(1)	100.4
Man(2)	99.5
Man(3)	96.7
Fuc(1)(α6)	99.2
Gal(1)	103.2
Gal(2)	102.9
Gal(3)	102.8

[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{105}H_{164}N_8O_{72}$, $[M-2H]^{2-}$: 1344.9731, found 1344.9492.

Compound S3

S3 was prepared from **S2** (0.5 mg, 0.19 μmol) using the general procedure for the installation of α2,3 Neu5Ac with ST3GAL41. After HILC purification, compound **S3** was obtained as a white solid (0.4 mg, 71%).



S3

^{1}H (900 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	H7	H8	H9	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 10.0 Hz, 1H)	3.87	3.77	3.71	N/R[a]	N/R	-	-	-	-[b]	2.15 -1.95 21H
GlcNAc(2)	4.71 (d, *J* = 8.7 Hz, 1H)	3.83	3.63	3.78	N/R	N/R	-	-	-	-	2.15 -1.95 21H
GlcNAc(3)	4.64 (d, *J* = 8.0 Hz, 1H)	3.78	3.63	3.69	N/R	N/R	-	-	-	-	2.15 -1.95 21H
GlcNAc(4)	4.62 (d, *J* = 8.7 Hz, 1H)	3.78	3.60	3.74	N/R	N/R	-	-	-	-	2.15 -1.95 21H
GlcNAc(5)	4.73 (d, *J* = 8.7 Hz, 1H)	3.84	3.62	3.79	N/R	N/R	-	-	-	-	2.15 -1.95 21H
Man(1)	4.82	4.29	3.81	3.67	N/R	N/R	-	-	-	-	-
Man(2)	5.17	4.23 (d, *J* = 1.8 Hz, 1H)	3.93	3.78	3.55	N/R	-	-	-	-	-
Man(3)	4.96	4.14	3.93	3.65	3.53	N/R	-	-	-	-	-
Fuc(1)	4.89 (d, *J* = 3.3 Hz, 1H)	3.81	3.90	3.94	4.14	-	-	-	-	1.21 (d, *J* = 6.4 Hz, 3H)	-
Gal(1)	4.48 (d, *J* = 8.0 Hz, 1H)	3.57	3.71	3.96	N/R	N/R	-	-	-	-	-
Gal(2)	4.49 (d, *J* = 8.4 Hz, 1H)	3.62	3.76	4.20 (d, *J* = 2.5 Hz, 1H)	N/R	N/R	-	-	-	-	-
Gal(3)	4.59 (d, *J* = 7.7 Hz, 1H)	3.61	3.99	4.16 (d, *J* = 2.7 Hz, 1H)	N/R	N/R	-	-	-	-	-
Neu5Ac(1)	-	-	2.70 (dd, *J* = 12.5, 5.0 Hz, 1H) 1.76 (dd, *J* = 12.2 Hz, 1H)	3.69	3.85	N/R	N/R	N/R	N/R	-	2.15 -1.95 21H
Neu5Ac(2)	-	-	2.79 (dd, *J* = 12.3, 4.5 Hz, 1H) 1.84 (dd, *J* = 12.1 Hz, 1H)	3.72	3.89	N/R	N/R	N/R	N/R	-	2.15 -1.95 21H

^{13}C (225 MHz, D_2O): δ (ppm)

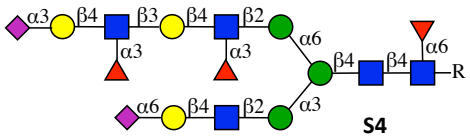
	C1
GlcNAc(1)	78.1
GlcNAc(2)	100.9
GlcNAc(3)	99.2
GlcNAc(4)	99.1
GlcNAc(5)	102.7
Man(1)	100.4
Man(2)	99.4
Man(3)	96.9
Fuc(1)	99.2
Gal(1)	103.4
Gal(2)	102.9
Gal(3)	102.5

[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{116}H_{180}N_9O_{80}$, $[M-3H]^{3-}$: 993.3448, found 993.3376.

Compound S4

S4 was prepared from **S3** (0.2 mg, 0.067 μmol) using the general procedure for the installation of α1,3 Fuc with FUT5. After P2 size-exclusion purification, compound **S4** was obtained as a white solid (0.2 mg, 91%).



S4

^{1}H (600 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	H7	H8	H9	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 9.8 Hz, 1H)	3.87	3.78	3.71	N/R[a]	N/R	-	-	-	-[b]	2.15 -1.95 21H
GlcNAc(2)	4.71 (d, *J* = 7.7 Hz, 1H)	3.82	3.63	3.78	N/R	N/R	-	-	-	-	2.15 -1.95 21H
GlcNAc(3)	4.64 (d, *J* = 7.6 Hz, 1H)	3.79	3.63	3.68	N/R	N/R	-	-	-	-	2.15 -1.95 21H
GlcNAc(4)	4.62 (d, *J* = 7.6 Hz, 1H)	3.98	3.87	3.79	N/R	N/R	-	-	-	-	2.15 -1.95 21H
GlcNAc(5)	4.73 (d, *J* = 8.5 Hz, 1H)	3.99	3.89	3.61	N/R	N/R	-	-	-	-	2.15 -1.95 21H
Man(1)	4.82	4.29	3.81	3.69	N/R	N/R	-	-	-	-	-
Man(2)	5.17	4.23 (d, *J* = 2.3 Hz, 1H)	3.93	3.78	3.55	N/R	-	-	-	-	-
Man(3)	4.94	4.13	3.94	3.64	3.53	N/R	-	-	-	-	-
Fuc(1)	4.89 (d, *J* = 3.8 Hz, 1H)	3.80	3.90	3.93	4.14	-	-	-	-	1.21 (d, *J* = 6.3 Hz, 3H)	-
Fuc(2)	5.16 (d, *J* = 4.0 Hz, 1H)	3.72	3.93	3.82	4.86	-	-	-	-	1.20 (d, *J* = 6.3 Hz, 3H)	-
Fuc(3)	5.16 (d, *J* = 4.0 Hz, 1H)	3.72	3.93	3.82	4.84	-	-	-	-	1.18 (d, *J* = 6.5 Hz, 3H)	-
Gal(1)	4.48 (d, *J* = 8.0 Hz, 1H)	3.57	3.70	3.96	N/R	N/R	-	-	-	-	-
Gal(2)	4.46 (d, *J* = 8.5 Hz, 1H)	3.53	3.74	4.14	N/R	N/R	-	-	-	-	-
Gal(3)	4.57 (d, *J* = 7.9 Hz, 1H)	3.46	3.97	4.13	N/R	N/R	-	-	-	-	-
Neu5Ac(1)	-	-	2.70 (dd, *J* = 12.4, 4.6 Hz, 1H) 1.76 (dd, *J* = 12.1 Hz, 1H)	3.69	3.84	N/R	N/R	N/R	N/R	-	2.15 -1.95 21H
Neu5Ac(2)	-	-	2.80 (dd, *J* = 12.4, 4.7 Hz, 1H) 1.83 (dd, *J* = 12.1 Hz, 1H)	3.71	3.88	N/R	N/R	N/R	N/R	-	2.15 -1.95 21H

^{13}C (150 MHz, D_2O): δ (ppm)

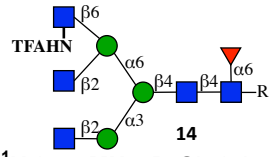
	C1		C1
GlcNAc(1)	78.1	**Fuc(1)**	99.2
GlcNAc(2)	100.8	**Fuc(2)**	98.5
GlcNAc(3)	99.2	**Fuc(3)**	98.5
GlcNAc(4)	99.2	**Gal(1)**	103.5
GlcNAc(5)	102.4	**Gal(2)**	101.7
Man(1)	100.4	**Gal(3)**	101.4
Man(2)	99.6		
Man(3)	96.8		

[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{128}H_{200}N_9O_{88}$, $[M-3H]^{3-}$: 1090.7167, found 1090.7041.

Compound 14

14 was prepared from **1** (17.6 mg, 10.3 μmol) using the general procedure for the installation of β1,6 GlcNTFA using MGAT5. After HILIC purification, compound **14** was obtained as a white solid (18.6 mg, 92%).



14

^{1}H (600 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 9.7 Hz, 1H)	3.86	3.79	3.70	N/R [a]	3.88 3.68	- [b]	2.12 – 1.90 12H
GlcNAc(2)	4.70 (d, *J* = 7.9 Hz, 1H)	3.83	3.78	3.63	N/R	N/R	-	2.12 – 1.90 12H
GlcNAc(3)	4.59 (d, *J* = 8.59, 1H)	3.74	3.60	3.49	N/R	N/R	-	2.12 – 1.90 12H
GlcNAc(4)	4.57 (d, *J* = 8.59, 1H)	3.71	3.59	3.47	N/R	N/R	-	2.12 – 1.90 12H
GlcNTFA	4.65 (d, *J* = 8.4 Hz, 1H)	3.85	3.74	3.53	N/R	N/R	-	-
Man(1)	4.79	4.28	3.80	N/R	N/R	3.93 3.75	-	-
Man(2)	5.16	4.22	3.95	3.79	3.54	N/R	-	-
Man(3)	4.86	4.09	3.91	3.73	3.44	4.30 3.56	-	-
Fuc(1)(α6)	4.89 (d, *J* = 3.7, 1H)	3.81	3.89	3.93	4.13	-	1.19 (d, *J* = 6.5 Hz, 3H)	-

^{13}C (150 MHz, D_2O): δ (ppm)

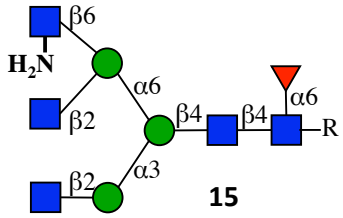
	C1
GlcNAc(1)	78.0
GlcNAc(2)	100.8
GlcNAc(3)	99.5
GlcNAc(4)	99.7
GlcNTFA	100.8
Man(1)	100.2
Man(2)	99.4
Man(3)	99.9
Fuc(1)(α6)	99.2

[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{76}H_{114}F_3N_7O_{49}$, $[M-2H]^{2-}$: 982.8304, found 982.8210.

Compound 15

15 was prepared from **14** (9.8 mg, 5 µmol) using the general procedure for the removal of the TFA protecting group. After P2 size-exclusion purification, **15** was obtained as a white solid (9.0 mg, 96%).

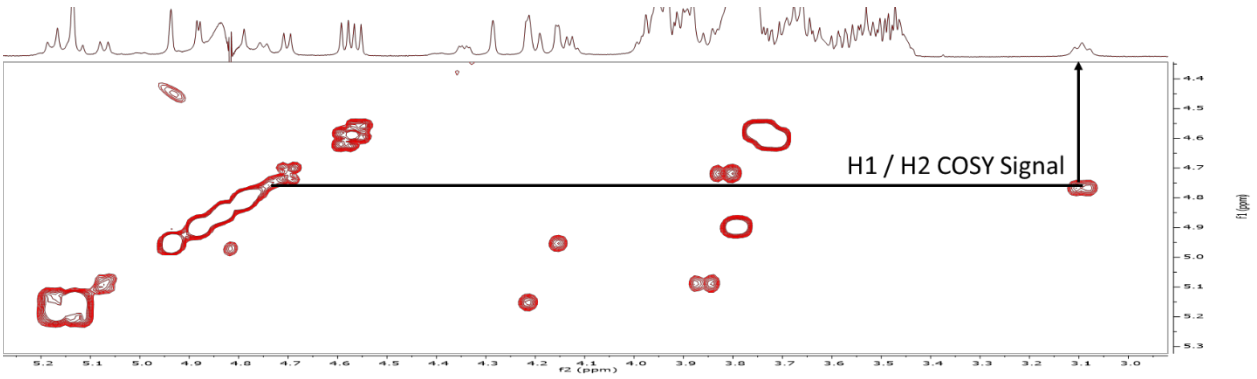


15

^{1}H (600 MHz, D_2O): δ (ppm)

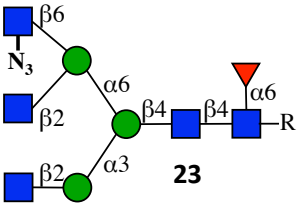
Removal of the trifluoroacetamido (TFA) functionality resulted in a significant change of the electronic environment of H2 on the newly formed $GlcNH_2$ residue. The increased electron density on the amine created an upfield shift in the H2 signal (δ3.09) relative to the H2 signal (δ3.85) of the TFA protected starting material (**14**), which can be observed by the 2D COSY spectrum

ESI TOF-MS *m/z* calcd for $C_{74}H_{115}N_7O_{48}$, $[M-2H]^{2-}$: 934.8392, found 934.8204.



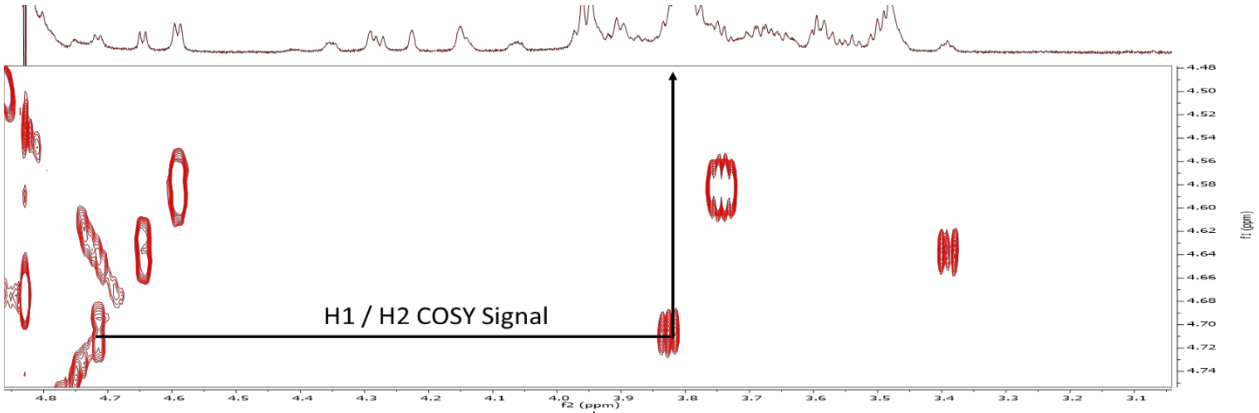
Compound 23

23 was prepared from **15** (6.9 mg, 3.7 µmol) using the general procedure for the installation of azide on $GlcNH_2$. After P2 size-exclusion purification, **23** was obtained as a white solid (5.3 mg, 76%).



^{1}H (600 MHz, D_2O): δ (ppm)

The installation of the azide resulted in a significant electronic change for the $GlcN_3$ H2 signal resulting in a downfield shift in comparison to the H2 (δ3.09) of the $GlcNH_2$ starting material.



MALDI TOF-MS *m/z* calcd for $C_{74}H_{113}N_9O_{48}Na$, $[M+Na]^+$: 947.8344, found 947.8205.

Compound 16

16 was prepared from **15** (3.0 mg, 1.6 μmol) using the general procedure for the installation of β1,4 Gal with B4GALT1. After HILIC purification, compound **16** was obtained as a white solid (2.8 mg, 80%).

^{1}H (900 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 9.8 Hz, 1H)	3.88	3.81	3.77	3.71	3.88 3.70	- [b]	2.20 – 2.05 12H
GlcNAc(2)	4.71 (d, *J* = 7.9 Hz, 1H)	3.83	3.63	3.77	N/R [a]	N/R	-	2.20 – 2.05 12H
GlcNAc(3)	4.62 (d, *J* = 7.2 Hz, 1H)	3.77	3.61	3.77	N/R	N/R	-	2.20 – 2.05 12H
GlcNAc(4)	4.59 (d, *J* = 8.3 Hz, 1H)	3.80	3.60	3.73	N/R	N/R	-	2.20 – 2.05 12H
$GlcNH_2$	4.81	3.16	3.74	3.81	3.54	N/R	-	-
Man(1)	4.80	4.29	3.80	3.66	N/R	3.99 3.81	-	-
Man(2)	5.15	4.23	3.94	3.78	3.54	N/R	-	-
Man(3)	4.95	4.17	3.96	3.84	3.67	4.21 3.93	-	-
Gal(1)	4.50	3.58	3.71	3.97	N/R	N/R	-	-
Gal(2)	4.50	3.58	3.71	3.97	N/R	N/R	-	-
Fuc(1)(α6)	4.89	3.81	3.91	3.93	4.15	-	1.21 (d, *J* = 6.2 Hz, 3H)	-

^{13}C (225 MHz, D_2O): δ (ppm)

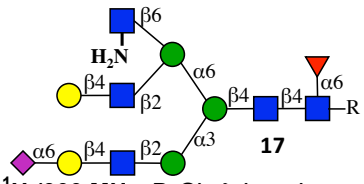
	C1
GlcNAc(1)	78.0
GlcNAc(2)	100.7
GlcNAc(3)	99.2
GlcNAc(4)	99.6
$GlcNH_2$	99.0
Man(1)	100.2
Man(2)	99.3
Man(3)	97.4
Gal(1)	102.8
Gal(2)	102.8
Fuc(1)(α6)	99.2

[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{86}H_{138}N_7O_{58}Na$, $[M+H+Na]^{2+}$: 1109.8975, found 1109.9068.

Compound 17

17 was prepared from **16** (5 mg, 2.3 μmol) using the general procedure for the selective installation of α2,6 Neu5Ac with ST6GAL1. After HILIC purification, compound **17** was obtained as a white solid (4.1 mg, 73%).



17

^{1}H (900 MHz, D_2O): δ (ppm)

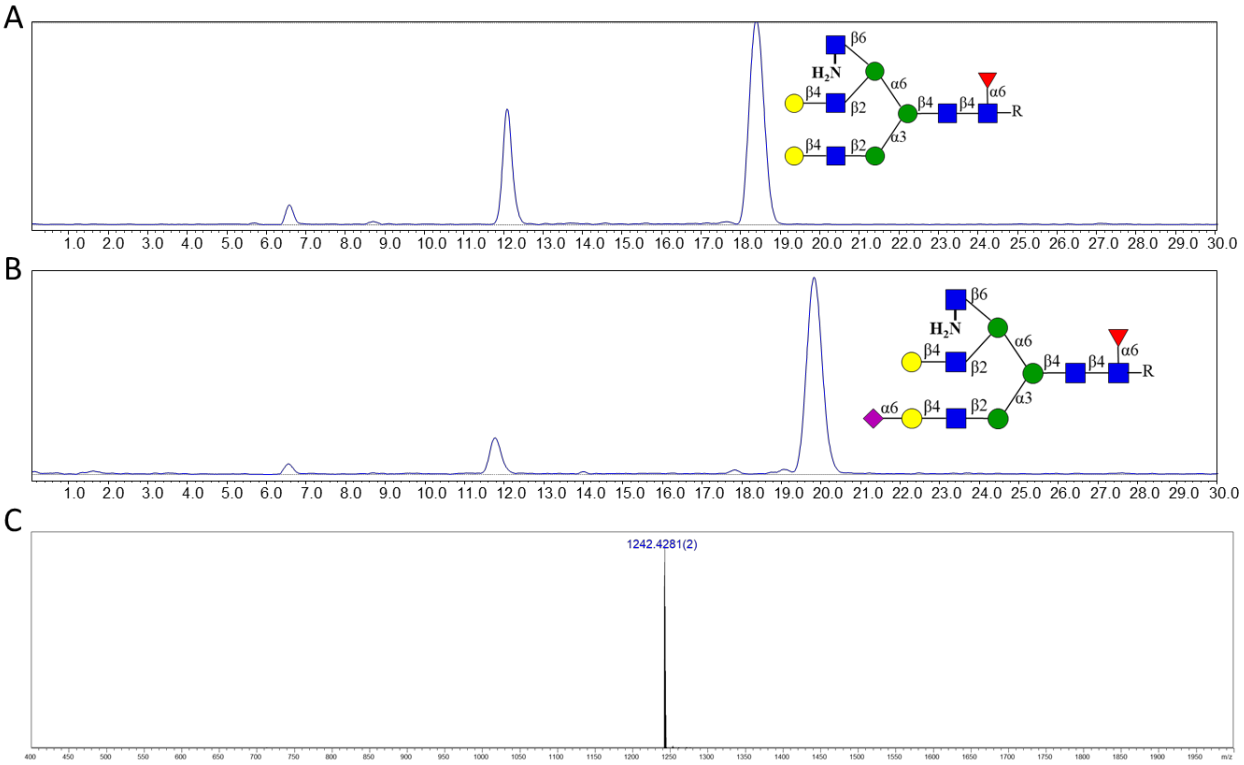
	H1	H2	H3	H4	H5	H6	H7	H8	H9	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 9.7 Hz,1H)	3.85	3.79	3.75	3.70	3.89 3.70	-[b]	-	-	-	2.2 – 1.9 15H
GlcNAc(2)	4.71 (d, *J* = 8.4 Hz, 1H)	3.82	3.62	3.76	N/R[a]	N/R	-	-	-	-	2.2 – 1.9 15H
GlcNAc(3)	4.65 (d, *J* = 8.3 Hz, 1H)	3.77	3.62	3.67	N/R	N/R	-	-	-	-	2.2 – 1.9 15H
GlcNAc(4)	4.59 (d, *J* = 8.4 Hz, 1H)	3.79	3.62	3.81	3.68	N/R	-	-	-	-	2.2 – 1.9 15H
$GlcNH_2$	4.81	3.15 (dd, *J* = 10.7, 8.4 Hz, 1H)	3.72	3.54	N/R	N/R	-	-	-	-	-
Man(1)	4.80	4.29	3.81	3.66	N/R	3.99 3.82	-	-	-	-	-
Man(2)	5.17	4.24 (d, *J* = 3.3 Hz, 1H)	3.93	3.77	3.55	N/R	-	-	-	-	-
Man(3)	4.96 (d, *J* = 1.6 Hz, 1H)	4.16 (d, *J* = 2.2 Hz, 1H)	3.96	3.66	3.84	4.21 3.90	-	-	-	-	-
Gal(1)	4.49 (d, *J* = 7.8 Hz, 1H)	3.56	3.70	3.95	N/R	N/R	-	-	-	-	-
Gal(2)	4.51	3.57	3.70	3.95	N/R	N/R	-	-	-	-	-
Neu5Ac(α6)	-	-	2.69 – eq (dd, *J* = 12.4, 4.7 Hz, 1H) 1.75 – axial (dd, *J* = 12.2 Hz, 1H)	3.69	3.84	N/R	N/R	N/R	N/R	-	2.2 – 1.9 15H
Fuc(1)(α6)	4.89 (d, *J* = 3.8 Hz, 1H)	3.80	3.89	3.90	4.13	-	-	-	-	1.21 (d, *J* = 6.6 Hz, 3H)	-

^{13}C (225 MHz, D_2O): δ (ppm)

	C1
GlcNAc(1)	78.0
GlcNAc(2)	100.8
GlcNAc(3)	99.2
GlcNAc(4)	99.5
$GlcNH_2$	98.9
Man(1)	100.2
Man(2)	99.4
Man(3)	97.4
Gal(1)	103.2
Gal(2)	102.8
Fuc(1)(α6)	99.1

[a] Not reported
[b] Not applicable

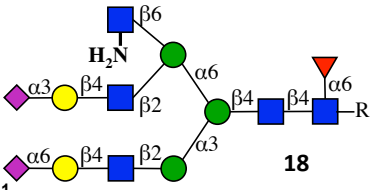
ESI TOF-MS *m/z* calcd for $C_{97}H_{152}N_8O_{66}$, $[M\text{-}2H]^{2-}$: 1242.4398, found 1242.4281.



Supplementary Fig. 9. HILIC purification chromatogram of **17**. A. HILIC trace of the purification of starting material **16**. B. After overnight ST6GAL1 incubation, purification of reaction mixture indicated only the desired mono-sialylated product with significant separation from excess sugar-nucleotides (11.5 min). C. Focused ESI-TOF-MS of mono-sialylated **17**, eluting at 20 min.

Compound 18

18 was prepared from **17** (4.1 mg, 1.6 μmol) using the general procedure for the installation of α2,3 Neu5Ac with ST3GAL4. After HILC purification, compound **18** was obtained as a white solid (3.0 mg, 66%).



18

^{1}H (900 MHz, D_2O): δ (ppm)

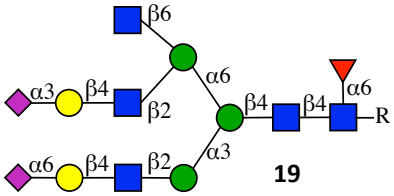
	H1	H2	H3	H4	H5	H6	H7	H8	H9	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J*= 10.9 Hz, 1H)	3.87	3.78	3.72	N/R[a]	N/R	-[b]	-	-	-	2.15 – 2.05 18H
GlcNAc(2)	4.70 (d, *J* = 8.7 Hz, 1H)	3.82	3.63	3.77	N/R	N/R	-	-	-	-	2.15 – 2.05 18H
GlcNAc(3)	4.65 (d, *J*= 8.2 Hz, 1H)	3.78	3.63	3.68	N/R	N/R	-	-	-	-	2.15 – 2.05 18H
GlcNAc(4)	4.59	3.79	3.72	3.79	N/R	N/R	-	-	-	-	2.15 – 2.05 18H
$GlcNH_2$	4.82	3.16	3.73	3.54	N/R	N/R	-	-	-	-	-
Man(1)	4.81	4.29	3.81	3.66	N/R	N/R	-	-	-	-	-
Man(2)	5.16	4.23	3.93	3.78	N/R	N/R	-	-	-	-	-
Man(3)	4.94	4.17	3.94	3.84	N/R	N/R	-	-	-	-	-
Gal(1)	4.49 (d, *J* = 7.7 Hz, 1H)	3.57	3.71	3.97	N/R	N/R	-	-	-	-	-
Gal(2)	4.58	3.61	4.00	4.16	N/R	N/R	-	-	-	-	-
Neu5Ac(α3)	-	-	1.84 – axial (dd, *J* =12.0 Hz, 1H) 2.79 – eq. (dd, *J* = 13.0, 4.6 Hz, 1H)	3.73	3.89	N/R	N/R	N/R	N/R	-	2.15 – 2.05 18H
Neu5Ac(α6)	-	-	1.76 – axial (dd, *J* = 12.3 Hz, 1H) 2.60 – eq. (dd, *J* = 12.9, 4.6 Hz, 1H)	3.70	3.85	N/R	N/R	N/R	N/R	-	2.15 – 2.05 18H
Fuc(1)(α6)	4.90	3.81	3.90	3.80	4.14	-	-	-	-	1.22 (d, *J* = 6.6 Hz, 3H)	-

[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{108}H_{169}N_9O_{74}$, $[M-2H]^{2-}$: 1387.9875 found 1388.0402.

Compound 19

19 was prepared from **18** (1.3 mg, 0.5 μmol) using the general acetylation procedure. After HILIC purification, compound **19** was obtained as a white solid (0.9 mg, 67%).



19

^{1}H (900 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	H7	H8	H9	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 10.1 Hz, 1H)	3.86	3.78	3.62	N/R[a]	N/R[b]	-	-	-	-	2.15 – 2.05 21H
GlcNAc(2)	4.70 (d, *J* = 8.7 Hz, 1H)	3.82	3.63	3.77	N/R	N/R	-	-	-	-	2.15 – 2.05 21H
GlcNAc(3)	4.62	3.74	3.59	3.76	N/R	N/R	-	-	-	-	2.15 – 2.05 21H
GlcNAc(4)	4.64	3.79	3.63	3.68	N/R	N/R	-	-	-	-	2.15 – 2.05 21H
GlcNAc(5)	4.57	3.74	3.60	3.48	N/R	N/R	-	-	-	-	2.15 – 2.05 21H
Man(1)	4.81	4.28	3.81	N/R	N/R	N/R	-	-	-	-	-
Man(2)	5.16	4.23	3.93	3.78	N/R	N/R	-	-	-	-	-
Man(3)	4.94	4.12	3.90	N/R	N/R	N/R	-	-	-	-	-
Gal(1)	4.58	3.60	3.99	4.14	N/R	N/R	-	-	-	-	-
Gal(2)	4.47 (d, *J* = 7.8 Hz, 1H)	3.56	3.70	3.95	N/R	N/R	-	-	-	-	-
Neu5Ac(α3)	-	-	1.84 – axial 2.79 – eq. (dd, *J* = 12.7, 4.7 Hz, 1H)	3.73	3.69	3.89	N/R	N/R	N/R	-	2.15 – 2.05 21H
Neu5Ac(α6)	-	-	1.76 – axial 2.70 – eq.	3.70	3.85	N/R	N/R	N/R	N/R	-	2.15 – 2.05 21H
Fuc(1)(α6)	4.90	3.80	N/R	N/R	4.14	-	-	-	-	1.22 (d, *J* = 6.5 Hz, 3H)	-

^{13}C (225 MHz, D_2O): δ (ppm)

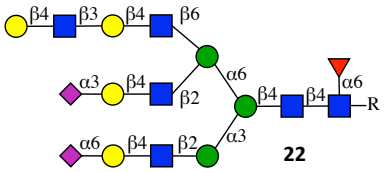
	C1
GlcNAc(1)	78.0
GlcNAc(2)	100.8
GlcNAc(3)	99.4
GlcNAc(4)	99.1
GlcNAc(5)	101.5
Man(1)	100.2
Man(2)	99.4
Man(3)	97.0
Gal(1)	102.5
Gal(2)	103.4
Fuc(1)(α6)	99.1

[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{110}H_{170}N_9O_{75}$, $[M-3H]^{3-}$: 938.9927, found 939.0233.

Compound 22

22 was prepared from **19** (1 mg, 0.35 μmol) by first installing a β1,4 Gal using the general procedure for B4GALT1. The reaction was monitored by ESI-MS and once complete conversion to **20** (*m/z* calcd for $C_{116}H_{181}N_9O_{80}$ $[M-2H]^{2-}$ 1490.0192, found 1490.0528), the reaction mixture was centrifuged using a Nanosep® Omega ultrafiltration device (10 kDa MWCO) and the filtrate was lyophilized. To the resulting crude residue containing **20** was installed a β1,3 GlcNAc using the general procedure for B3GNT2. The reaction was monitored by ESI-MS and once complete conversion to **21** was observed (*m/z* calcd for $C_{124}H_{193}N_{10}O_{85}$ $[M-3H]^{3-}$ 1061.0379, found 1061.0227), the reaction was centrifuged using a Nanosep® Omega ultrafiltration device (10 kDa MWCO) and the filtrate was lyophilized. To the resulting crude reside was installed a β1,4 Gal using the general procedure for B4GALT1 and after P2 size-exclusion purification, compound **22** was obtained as a white solid (0.5 mg, 47% over 3 steps).



22

^{1}H (600 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	H7	H8	H9	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 10 Hz, 1H)	3.87	3.79	3.63[]	N/R[a]	N/R	-[b]	-	-	-	2.15 – 2.03 24H
GlcNAc(2)	4.71	3.81	3.63	3.77	N/R	N/R	-	-	-	-	2.15 – 2.03 24H
GlcNAc(3)	4.61	3.74	3.63	3.77	N/R	N/R	-	-	-	-	2.15 – 2.03 24H
GlcNAc(4)	4.64	3.81	3.64	3.70	N/R	N/R	-	-	-	-	2.15 – 2.03 24H
GlcNAc(5)	4.57 (d, *J* = 7.8 Hz, 2H)	3.80	3.63	3.77	N/R	N/R	-	-	-	-	2.15 – 2.03 24H
GlcNAc(6)	4.74 (d, *J* = 8.2 Hz, 1H)	3.83	3.63	3.78	N/R	N/R	-	-	-	-	2.15 – 2.03 24H
Man(1)	4.80	4.30	3.82	N/R	N/R	N/R	-	-	-	-	-
Man(2)	5.15	4.24	3.94	3.78	N/R	N/R	-	-	-	-	-
Man(3)	4.89	4.28	3.82	N/R	N/R	N/R	-	-	-	-	-
Gal(1)	4.57 (d, *J* = 7.8 Hz, 2H)	3.60	4.00	4.16	N/R	N/R	-	-	-	-	-
Gal(2)	4.46	3.56	3.71	3.97	N/R	N/R	-	-	-	-	-
Gal(3)	4.50	3.62	3.77	4.20	N/R	N/R	-	-	-	-	-
Gal(4)	4.51	3.58	3.71	3.97	N/R	N/R	-	-	-	-	-
Neu5Ac(α3)	-	-	1.84 – axial 2.79 – eq (dd, *J* = 12.7, 4.7 Hz, 1H)	3.71	3.88	N/R	N/R	N/R	N/R	-	2.15 – 2.03 24H
Neu5Ac(α6)	-	-	1.75 – axial (dd, *J* = 12.2 Hz, 1H) 2.70 – eq (dd, *J* = 14.8, 2.7 Hz, 1H)	3.70	3.83	N/R	N/R	N/R	N/R	-	2.15 – 2.03 24H
Fuc(1)(α6)	4.89	3.81	3.90	3.79	4.15	-	-	-	-	1.21 (d, *J* = 6.5 Hz, 3H)	-

^{13}C 150 MHz, D_2O): δ (ppm)

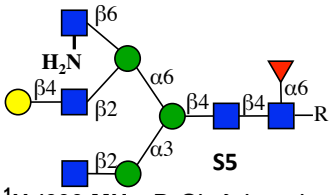
	C1		C1
GlcNAc(1)	78.0	**Man(2)**	99.4
GlcNAc(2)	N/R	**Man(3)**	99.2
GlcNAc(3)	N/R	**Gal(1)**	102.4
GlcNAc(4)	N/R	**Gal(2)**	103.4
GlcNAc(5)	101.5	**Gal(3)**	102.8
GlcNAc(6)	102.7	**Gal(4)**	102.7
Man(1)	100.2	**Fuc(1)(α6)**	99.2

[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{130}H_{203}N_{10}O_{90}$, $[M-3H]^{3-}$: 1114.7211, found 1114.7538.

Compound S5

S5 was prepared from **16** (1.0 mg, 0.5 µmol) using the general procedure for the selective cleavage of galactose with *E. coli* β-galactosidase. After HILIC purification, compound **S5** was obtained as a white solid (0.7 mg, 69%).



S5

^{1}H (600 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 10.1 Hz, 1H)	3.88	3.80	3.72	N/R[a]	3.88 3.70	-[b]	2.15 – 1.95 12H
GlcNAc(2)	4.70 (d, *J* = 8.2 Hz, 1H)	3.82	3.63	3.78	N/R	N/R	-	2.15 – 1.95 12H
GlcNAc(3)	4.59 (d, *J* = 8.4 Hz, 2H)	3.73	3.61	3.49	N/R	N/R	-	2.15 – 1.95 12H
GlcNAc(4)	4.59 (d, *J* = 8.4 Hz, 2H)	3.80	3.61	3.74	N/R	N/R	-	2.15 – 1.95 12H
$GlcNH_2$	4.80	3.17 (dd, *J* = 10.6, 8.5 Hz, 1H)	3.74	3.55	N/R	3.98 3.80	-	-
Man(1)	4.78	4.30	3.81	3.67	N/R	N/R	-	-
Man(2)	5.14	4.23	3.94	3.78	3.55	N/R	-	-
Man(3)	4.95	4.17	3.96	3.85	3.69	N/R	-	-
Gal(1)	4.50 (d, *J* = 7.8 Hz, 1H)	3.59	3.71	3.97	N/R	N/R	-	-
Fuc(1)(α6)	4.89 (d, *J* = 3.7 Hz, 1H)	3.81	3.90	N/R	4.14	-	1.21 (d, *J* = 6.6 Hz, 3H)	-

^{13}C 150 MHz, D_2O): δ (ppm)

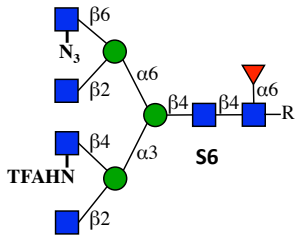
	C1
GlcNAc(1)	78.0
GlcNAc(2)	100.8
GlcNAc(3)	99.5
GlcNAc(4)	99.5
$GlcNH_2$	99.0
Man(1)	100.2
Man(2)	99.6
Man(3)	97.4
Gal(1)	102.8
Fuc(1)(α6)	99.1

[a] Not reported
[b] Not applicable

MALDI-MS *m/z* calcd for $C_{80}H_{127}N_7O_{53}Na$, $[M+Na]^+$: 2056.7350, found 2057.0229.

Compound S6

S6 was prepared from **23** (4.0 mg, 2.1 μmol) using the general procedure for the installation of β1,4-GlcNTFA with MGAT4B. After HILC purification, compound **S6** was obtained as a white solid (3.8 mg, 84%).



S6

^{1}H (600 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 9.7 Hz, 1H)	3.88	3.78	3.72	N/R[a]	3.90 3.70	-[b]	2.21 – 1.94 12H
GlcNAc(2)	4.71	3.83	3.64	3.77 7	N/R	N/R	-	2.21 – 1.94 12H
GlcNAc(3)	4.60	3.75	3.58	3.48	N/R	N/R	-	2.21 – 1.94 12H
GlcNAc(4)	4.60	3.75	3.58	3.48	N/R	N/R	-	2.21 – 1.94 12H
GlcNTFA	4.90	3.19 (dd, *J* = 10.6, 8.4 Hz, 1H)	3.74	3.60	N/R	N/R	-	-
$GlcN_3$	4.65 (d, *J* = 8.1 Hz, 1H)	3.39	3.43	3.79	N/R	N/R	-	-
Man(1)	4.81	4.27	3.81	N/R	N/R	4.06 3.82	-	-
Man(2)	5.15	4.28	3.81	3.59	N/R	N/R	-	-
Man(3)	4.95	4.14	3.92	3.80	3.57	4.27 3.79	-	-
Fuc(1)(α6)	4.90	3.81	3.90	3.94	4.15	-	1.22 (d, *J* = 6.6 Hz, 3H)	-

^{13}C (150 MHz, D_2O): δ (ppm)

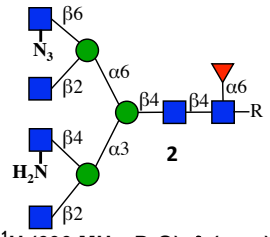
	C1
GlcNAc(1)	78.0
GlcNAc(2)	101.1
GlcNAc(3)	99.9
GlcNAc(4)	99.9
GlcNTFA	97.4
$GlcN_3$	102.0
Man(1)	100.5
Man(2)	99.4
Man(3)	97.4
Fuc(1)(α6)	99.4

[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{82}H_{123}F_3N_{10}O_{53}$, $[M-2H]^{2-}$: 1076.3600, found 1076.3822.

Compound 2

2 was prepared from **S6** (3.8 mg, 1.8 μmol) using the general procedure for the removal of the TFA protecting group. After P2 size-exclusion purification, compound **2** was obtained as a white solid (3.4 mg, 92%).



^{1}H (600 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 9.7 Hz, 1H)	3.87	3.78	3.72	N/R[a]	3.90 3.70	-[b]	2.21 – 1.94 12H
GlcNAc(2)	4.71 (d, *J* = 8.1 Hz, 1H)	3.83	3.64	3.77	N/R	N/R	-	2.21 – 1.94 12H
GlcNAc(3)	4.60	3.74	3.56	3.46	N/R	N/R	-	2.21 – 1.94 (m, 12H)
GlcNAc(4)	4.60	3.74	3.56	3.46	N/R	N/R	-	2.21 – 1.94 (m, 12H)
$GlcNH_2$	4.81	3.11	3.68	3.58	N/R	N/R	-	-
$GlcN_3$	4.65 (d, *J* = 8.1 Hz, 1H)	3.38	3.47	3.79	N/R	N/R	-	-
Man(1)	4.79	4.27	3.81	N/R	N/R	4.06 3.82	-	-
Man(2)	5.15	4.27	3.80	3.58	N/R	N/R	-	-
Man(3)	4.95	4.14	3.92	3.80	3.57	4.27 3.79	-	-
Fuc(1)(α6)	4.90 (d, *J* = 3.1 Hz, 1H)	3.81	3.90	3.94	4.15	-	1.21 (d, *J* = 6.4 Hz, 3H)	-

^{13}C (150 MHz, D_2O): δ (ppm)

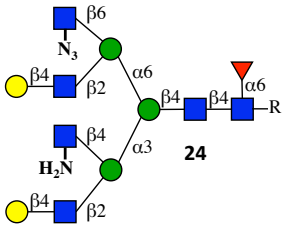
	C1
GlcNAc(1)	78.0
GlcNAc(2)	101.0
GlcNAc(3)	99.9
GlcNAc(4)	99.9
$GlcNH_2$	98.0
$GlcN_3$	101.8
Man(1)	100.4
Man(2)	99.4
Man(3)	97.3
Fuc(1)(α6)	99.3

[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{80}H_{124}N_{10}O_{52}$, $[M-2H]^{2-}$: 1028.3689, found 1028.3575.

Compound 24

24 was prepared from **2** (2.3 mg, 1.1 µmol) using the general procedure for the installation of β1,4 Gal with B4GALT1. After HILC purification, compound **24** was obtained as a white solid (1.9 mg, 73%).



^{1}H (900 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 10.1 Hz, 1H)	3.88	3.78	3.72	N/R[a]	3.90 3.70	-[b]	2.21 – 1.88 12H
GlcNAc(2)	4.71 (d, *J* = 8.1 Hz, 1H)	3.82	3.64	3.77	N/R	N/R	-	2.21 – 1.88 12H
GlcNAc(3)	4.62	3.80	3.61	3.76	N/R	N/R	-	2.21 – 1.88 12H
GlcNAc(4)	4.62	3.80	3.61	3.76	N/R	N/R	-	2.21 – 1.88 12H
$GlcNH_2$	4.90	3.18	3.73	3.56	N/R	N/R	-	-
$GlcN_3$	4.64 (d, *J* = 8.1 Hz, 1H)	3.38	3.48	3.79	N/R	N/R	-	-
Man(1)	4.79	4.28	3.81	N/R	N/R	4.06 3.82	-	-
Man(2)	5.15	4.28	3.81	3.57	N/R	N/R	-	-
Man(3)	4.95	4.14	3.92	3.80	3.57	4.27 3.79	-	-
Gal(1)	4.50	3.58	3.70	3.97	N/R	N/R	-	-
Gal(2)	4.50	3.58	3.70	3.97	N/R	N/R	-	-
Fuc(1)(α6)	4.90	3.81	3.90	3.93	4.14	-	1.22 (d, *J* = 6.5 Hz, 3H)	-

^{13}C (225 MHz, D_2O): δ (ppm)

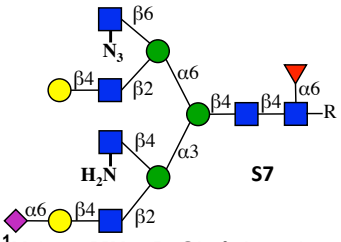
	C1
GlcNAc(1)	78.0
GlcNAc(2)	100.7
GlcNAc(3)	99.3
GlcNAc(4)	99.3
$GlcNH_2$	96.9
$GlcN_3$	101.6
Man(1)	100.3
Man(2)	99.2
Man(3)	96.9
Gal(1)	102.7
Gal(2)	102.7
Fuc(1)(α6)	99.1

[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{92}H_{144}N_{10}O_{62}$, $[M-2H]^{2-}$: 1190.4217, found 1190.4107.

Compound S7

S7 was prepared from **24** (1.9 mg, 0.8 μmol) using the general procedure for the installation of α2,6 Neu5Ac with ST6GAL1. After HILIC purification, compound **S7** was obtained as a white solid (1.9 mg, 79%).



^{1}H (600 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	H7	H8	H9	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 9.6 Hz, 1H)	3.86	3.81	3.71	N/R[a]	N/R	-	-	-	-	2.15 – 1.90 15H
GlcNAc(2)	4.73 (d, *J* = 8.6 Hz, 1H)	3.81	3.64	3.76	N/R	N/R	-	-	-	-	2.15 – 1.90 15H
GlcNAc(3)	4.64 (d, *J* = 8.0 Hz, 1H)	3.81	3.64	3.70	N/R	N/R	-	-	-	-	2.15 – 1.90 15H
GlcNAc(4)	4.61 (d, *J* = 8.5 Hz, 1H)	3.79	3.61	3.74	N/R	N/R	-	-	-	-	2.15 – 1.90 15H
$GlcNH_2$	4.86	3.14	3.70	3.57	N/R	N/R	-	-	-	-	-
$GlcN_3$	4.65	3.39 (d, *J* = 8.4 Hz, 1H)	3.48	3.78	N/R	N/R	-	-	-	-	-
Man(1)	4.81	4.28	N/R	N/R	N/R	N/R	-	-	-	-	-
Man(2)	5.12	4.28	3.80	3.56	N/R	N/R	-	-	-	-	-
Man(3)	4.95	4.15	3.92	N/R	N/R	N/R	-	-	-	-	-
Gal(1)	4.50 (d, *J* = 7.8 Hz, 1H)	3.57	3.71	3.97	N/R	N/R	-	-	-	-	-
Gal(2)	4.47 (d, *J* = 7.8 Hz, 1H)	3.56	3.71	3.97	N/R	N/R	-	-	-	-	-
Fuc(1)(α6)	4.91 (d, *J* = 3.9, 1H)	3.80	3.91	3.93	4.13	-	-	-	-	1.20 (d, *J* = 6.5 Hz, 3H)	-
Neu5Ac(α6)	-	-	1.74 – axial (dd, *J* = 12.1 Hz, 1H) 2.70 – eq. (dd, *J* = 12.2, 4.6 Hz, 1H)	3.69	3.83	N/R	N/R	N/R	N/R	-	2.15 – 1.90 15H

^{13}C (150 MHz, D_2O): δ (ppm)

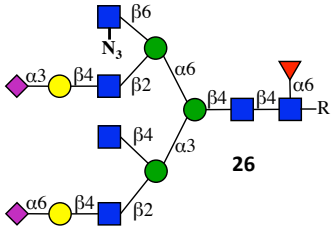
	C1
GlcNAc(1)	78.0
GlcNAc(2)	100.7
GlcNAc(3)	99.3
GlcNAc(4)	99.3
$GlcNH_2$	N/R
$GlcN_3$	101.6
Man(1)	100.3
Man(2)	99.1
Man(3)	N/R
Gal(1)	102.7
Gal(2)	103.3
Fuc(1)(α6)	99.2

[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{103}H_{161}N_{11}O_{70}$, $[M-2H]^{2-}$: 1335.9940, found 1336.0374.

Compound 26

26 was prepared from **S7** (1.0 mg, 0.37 μmol) by first installing the α2,3 Neu5Ac using the general procedure for ST3GAL4. The reaction was monitored by ESI-MS and once complete conversion to **25** was observed (*m/z* calcd for $C_{114}H_{178}N_{12}O_{78}$ $[M-2H]^{2-}$ 1481.5171, found 1481.5016), the reaction mixture was centrifuged using a Nanosep® Omega ultrafiltration device (10 kDa MWCO) and the pH of the filtrate was adjusted to 8 using 1M NaOH. The primary amine was acetylated using the general acetylation procedure and after HILIC purification, compound **26** was obtained as a white solid (0.8 mg, 71% over 2 steps).



26

^{1}H (600 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	H7	H8	H9	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 9.8 Hz, 1H)	3.87	3.79	3.70	N/R [a]	N/R	- [b]	-	-	-	2.20 – 1.93 21H
GlcNAc(2)	4.70 (d, *J* = 8.1 Hz, 1H)	3.81	3.62	3.76	N/R	N/R	-	-	-	-	2.20 – 1.93 21H
GlcNAc(3)	4.61	3.78	3.60	3.74	N/R	N/R	-	-	-	-	2.20 – 1.93 21H
GlcNAc(4)	4.63	3.79	3.70	3.63	N/R	N/R	-	-	-	-	2.20 – 1.93 21H
GlcNAc(5)	4.56	3.78	3.53	3.60	N/R	N/R	-	-	-	-	2.20 – 1.93 21H
$GlcN_3$	4.65	3.38	3.47	3.77	N/R	N/R	-	-	-	-	-
Man(1)	4.80	4/26	N/R	N/R	N/R	N/R	-	-	-	-	-
Man(2)	5.15	4.26	N/R	N/R	N/R	N/R	-	-	-	-	-
Man(3)	4.94	4.16	N/R	N/R	N/R	N/R	-	-	-	-	-
Gal(1)	4.48 (d, *J* = 8.1 Hz, 1H)	3.56	3.71	3.96	N/R	N/R	-	-	-	-	-
Gal(2)	4.58	3.60	4.00	4.16	N/R	N/R	-	-	-	-	-
Fuc(1)(α6)	4.90	3.80	3.90	N/R	4.14	-	-	-	-	1.21 (d, *J* = 6.4 Hz, 3H)	-
Neu5Ac(α3)	-	-	2.79 – eq. (dd, *J* = 12.2, 4.0 Hz, 1H) 1.84 – axial (dd, *J* = 13.2 Hz, 1H)	3.72	3.87	N/R	N/R	N/R	N/R	-	2.20 – 1.93 21H
Neu5Ac(α6)	-	-	2.70 – eq. (dd, *J* = 12.5, 4.1 Hz, 1H) 1.75 – axial (dd, *J* = 13.2 Hz, 1H)	3.69	3.83	N/R	N/R	N/R	N/R	-	2.20 – 1.93 21H

^{13}C (150 MHz, D_2O): δ (ppm)

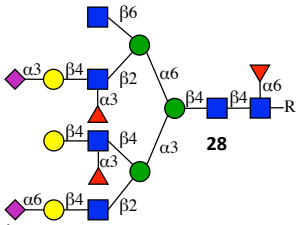
	C1		C1
GlcNAc(1)	78.0	**Gal(1)**	103.4
GlcNAc(2)	100.9	**Gal(2)**	102.5
GlcNAc(3)	99.3	**Fuc(1)(α6)**	99.2
GlcNAc(4)	99.3		
GlcNAc(5)	101.5		
$GlcN_3$	101.7		
Man(1)	100.4		
Man(2)	99.0		
Man(3)	N/R		

[a] Not reported
[b] Not applicable

ESI TOF-MS *m/z* calcd for $C_{116}H_{180}N_{12}O_{79}$, $[M-2H]^{2-}$: 1502.5224, found 1502.5682

Compound 28

28 was prepared from **26** (1.9 mg, 0.66 μmol) by first installing a β1,4 Gal using the general procedure for B4GALT1. The reaction was monitored by ESI-MS and once complete installation of the Gal was observed (*m/z* calcd for $C_{122}H_{189}N_{12}O_{84}$ $[M-3H]^{3-}$ 1055.6979, found 1055.6864), the reaction mixture was centrifuged using a Nanosep® Omega ultrafiltration device (10 kDa MWCO) and the filtrate was lyophilized. To the resulting crude residue two α1,3-Fuc's were installed using the general procedure for FUT5. The reaction was monitored by ESI-MS and once complete conversion to **27** was observed (*m/z* calcd for $C_{134}H_{209}N_{12}O_{92}$ $[M-3H]^{3-}$ 1153.0698, found 1153.0551), the reaction mixture was centrifuged using a Nanosep® Omega ultrafiltration device (10 kDa MWCO) and the filtrate was lyophilized. The azide on the **27** was reduced and the resulting amine was acetylated using the general azide reduction / acetylation conditions, and after P2 size-exclusion purification, compound **28** was obtained as a white solid (1.5 mg, 65% over 4 steps).



^{1}H (600 MHz, D_2O): δ (ppm)

	H1	H2	H3	H4	H5	H6	H7	H8	H9	CH_3	NHAc
GlcNAc(1)	5.08 (d, *J* = 9.5 Hz, 1H)	3.87	3.78	3.70	N/R[a]	N/R	-[b]	-	-	-	2.2 – 2.0 24H
GlcNAc(2)	4.71 (d, *J* = 7.9 Hz, 1H)	3.84	3.64	3.76	N/R	N/R	-	-	-	-	2.2 – 2.0 24H
GlcNAc(3)	4.63	3.81	3.69	3.64	N/R	N/R	-	-	-	-	2.2 – 2.0 24H
GlcNAc(4)	4.72	3.83	3.76	3.64	N/R	N/R	-	-	-	-	2.2 – 2.0 24H
GlcNAc(5)	4.60	4.00	3.91	3.69	N/R	N/R	-	-	-	-	2.2 – 2.0 24H
GlcNAc(6)	4.57	3.75	3.61	3.49	N/R	N/R	-	-	-	-	2.2 – 2.0 24H
Man(1)	4.80	4.25	N/R	N/R	N/R	N/R	-	-	-	-	-
Man(2)	5.15	4.26	N/R	N/R	N/R	N/R	-	-	-	-	-
Man(3)	4.98	4.15	N/R	N/R	N/R	4.22 3.62	-	-	-	-	-
Gal(1)	4.47	3.59	3.70	3.97	N/R	N/R	-	-	-	-	-
Gal(2)	4.49	3.55	3.70	3.94	N/R	N/R	-	-	-	-	-
Gal(3)	4.56	3.57	3.97	4.13	N/R	N/R	-	-	-	-	-
Fuc(1)(α6)	4.89	3.81	3.90	3.93	4.14	-	-	-	-	1.21	-
Fuc(2)(α3)	5.15	3.72	3.83	3.95	4.86	-	-	-	-	1.20	-
Fuc(3)(α3)	5.15	3.72	3.83	3.95	4.86	-	-	-	-	1.20	-
Neu5Ac(α3)	-	-	2.81 -eq 1.83 – axial (dd, *J* = 12.1 Hz, 1H)	3.73	3.88	N/R	N/R	N/R	N/R	-	2.2 – 2.0 24H
Neu5Ac(α6)	-	-	2.71 – eq (dd, *J* = 12.3, 4.5 Hz, 1H) 1.76 – axial (dd, *J* = 12.1 Hz, 1H)	3.70	3.84	N/R	N/R	N/R	N/R	-	2.2 – 2.0 24H

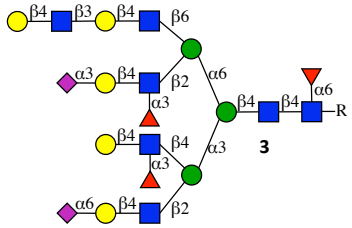
^{13}C (150 MHz, D_2O): δ (ppm)

	C1		C1
GlcNAc(1)	78.0	**Man(3)**	96.7
GlcNAc(2)	10098	**Gal(1)**	103.4
GlcNAc(3)	99.4	**Gal(2)**	101.7
GlcNAc(4)	101.2	**Gal(3)**	101.6
GlcNAc(5)	100.8	**Fuc(1)(α6)**	99.1
GlcNAc(6)	101.5	**Fuc(2)(α3)**	98.6
Man(1)	100.3	**Fuc(3)(α3)**	98.6
Man(2)	98.6		

[a] Not reported
[b] Not applicable

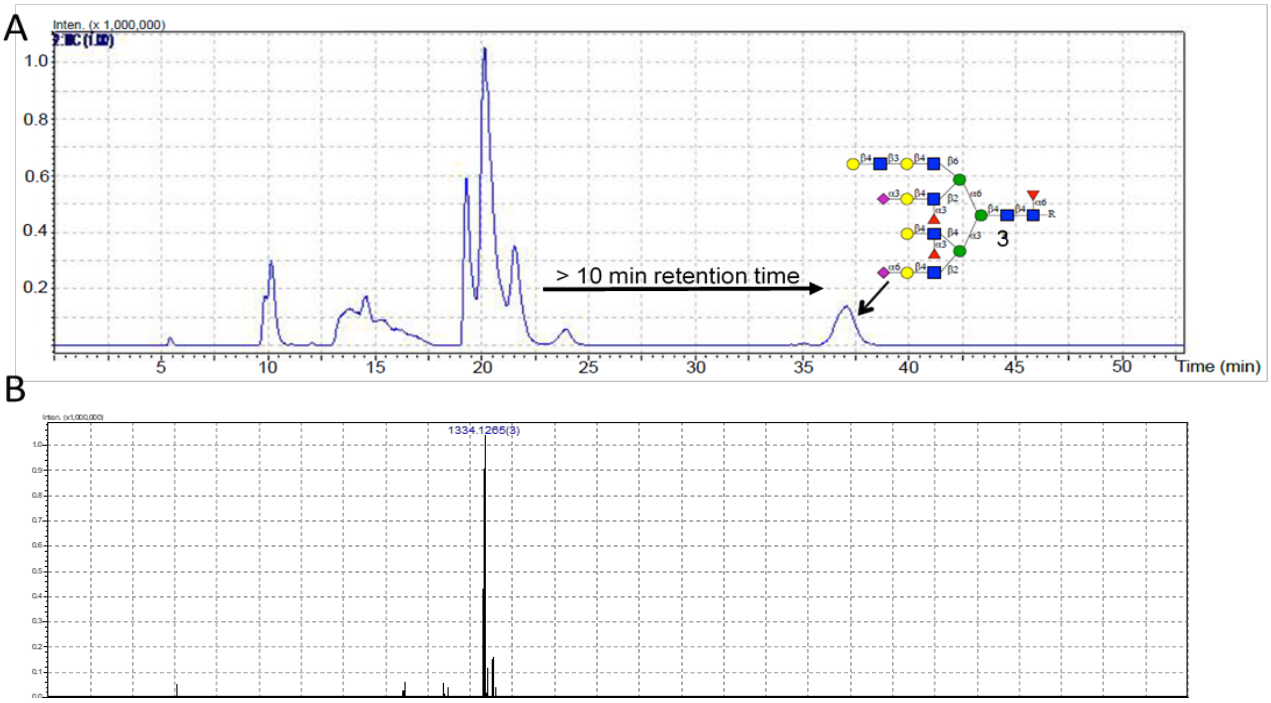
ESI TOF-MS *m/z* calcd for $C_{136}H_{213}N_{10}O_{93}$, $[M-3H]^{3-}$: 1158.0754, found 1158.0666.

Compound 3



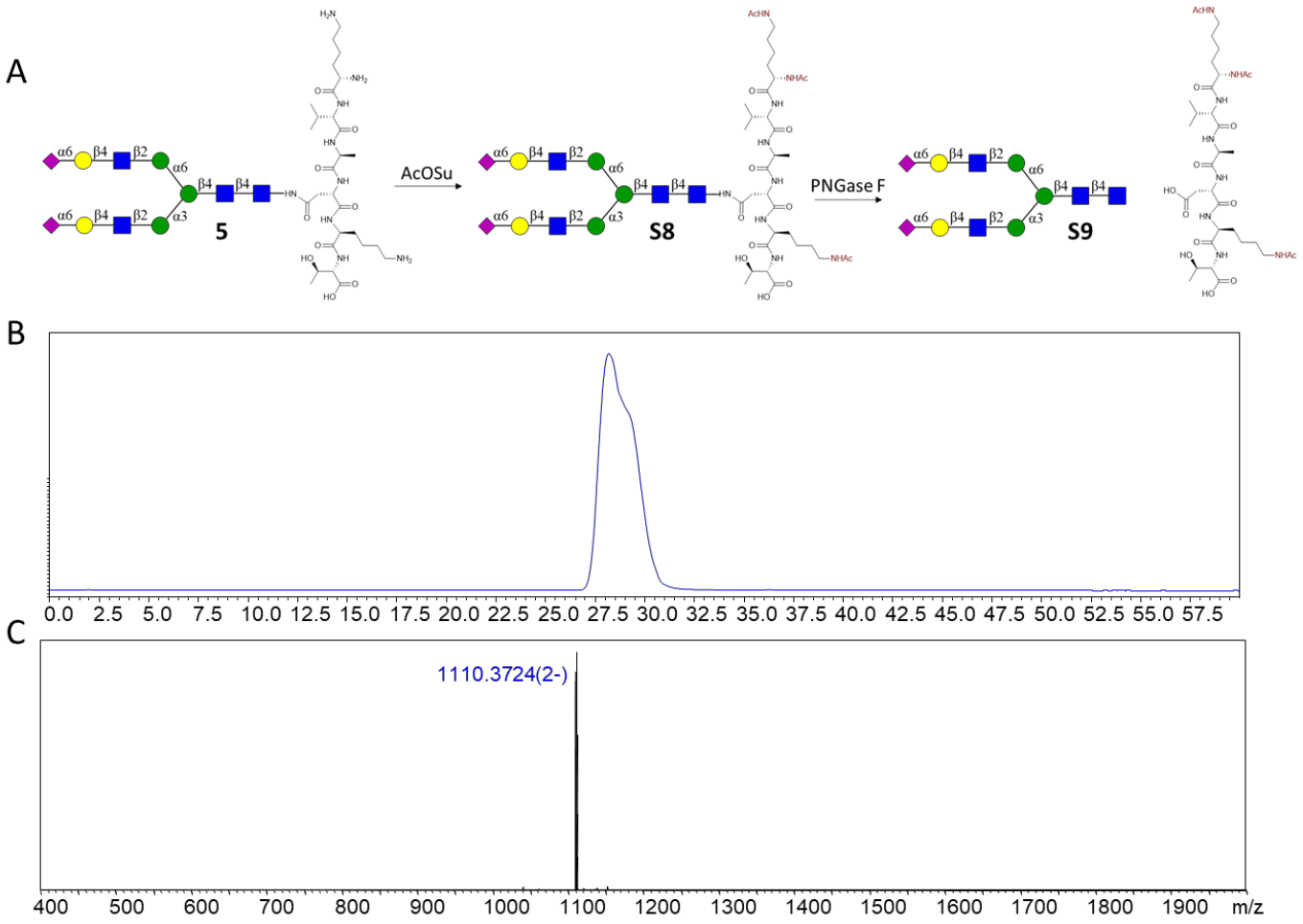
28 (150 µg, 0.04 µmol) was treated B4GALT1 according to the general procedure resulting in the installation of a β1,4 Gal moiety to provide **29**. The reaction was monitored by ESI-MS and once complete installation of the Gal was observed (*m/z* calcd for $C_{142}H_{223}N_{10}O_{98}$ $[M\text{-}3H]^{3-}$ 1212.4275, found 1212.4110), reaction proteins were removed via centrifugation over a Nanosep® Omega ultrafiltration device (10 kDa MWCO) followed by lyophilization of the filtrate. The resulting residue was subjected to enzymatic extension using B3GNT2 according to the general procedure to install a β1,3 GlcNAc moiety. The reaction was monitored by ESI-MS and once complete installation of the GlcNAc was observed (*m/z* calcd for $C_{150}H_{236}N_{11}O_{103}$ $[M\text{-}3H]^{3-}$ 1280.1206, found 1280.1098), the reaction was purified by centrifugation over a Nanosep® Omega ultrafiltration device (10 kDa MWCO) and the resulting filtrate was lyophilized. A β1,4 Gal residue was finally added using B4GALT1 according to the general procedure to provide 20-mer **3** as detected by ESI-MS (*m/z* calcd for $C_{156}H_{246}N_{11}O_{108}$ $[M\text{-}3H]^{3-}$ 1334.1382, found 1334.1265).

Analytical HPLC-MS was performed on a Shimadzu LC-ESI-IT-TOF with a Waters XBridge BEH, Amide column, 2.5 µm, 130 Å, 2.1 x 150 mm using ESI detection at a flow rate of 0.10 mL/min and a linear gradient as described in section 2f. Product containing fractions were lyophilized to yield **3** as a white solid (120 µg, 0.03 µmol, 3-steps 70% yield).

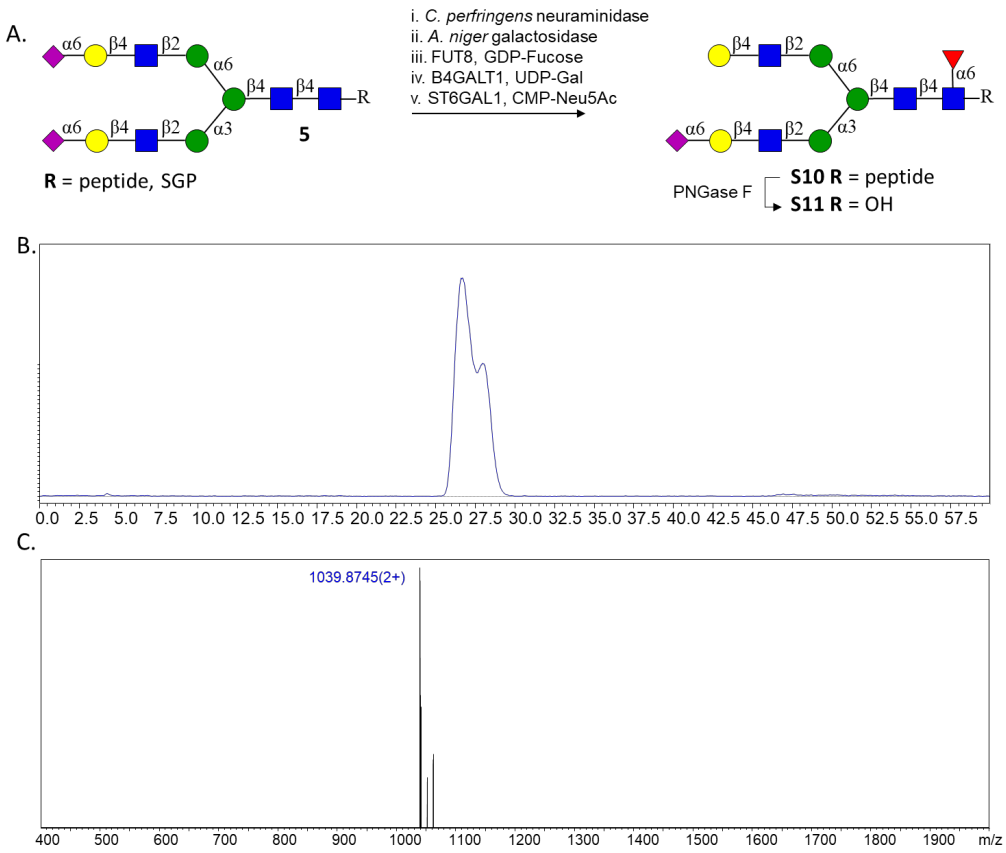


Supplementary Fig.10: Analytical HILIC-HPLC purification of tetra-antennary product **3**.
A. HPLC-MS chromatogram showing significant retention time differences between desired product **3**, and reaction components such as excess sugar nucleotides.
B. Focused ESI-MS chromatogram of product **3** at 37 min.

5. PNGase F can remove peptide of SGP after acetylation of the lysine residues.



Supplementary Fig. 11. 5 A. (50 µg, 0.02 µmol) was dissolved in aqueous NH_4HCO_3 (100 µL, 0.1M) and to the solution was added solid AcOSu (0.5 mg, 2.81 µmol). The reaction was agitated at room temperature for 8 h at which time, ESI-MS confirmed complete conversion to **S8** (*m/z* calcd for $C_{112}H_{187}N_{15}O_{70}$ $[M-2H]^{2-}$ 1,494.0931, found 1494.0824). The pH of the solution was adjusted to 8 using µL aliquots of NaOH (0.1M), and to this solution was added PNGase F (20 µg). The reaction mixture was incubated at 37 °C for 8 h after which, ESI-MS showed complete hydrolysis of the glycopeptide to provide free-reducing glycan **S9** (*m/z* calcd for $C_{84}H_{136}N_6O_{62}$ $[M-2H]^{2-}$ 1110.3843, found 1110.3724). B. HILIC trace of **S9**. Shouldering of peak at 28 min is indicative of α/β-anomers. C. Focused ESI-MS of product peak at 28 min.



Supplementary Fig. 12. Synthesis of asymmetric bi-antennary glycan **S11** with a free-reducing terminus to be used as an analytical standard A. Starting from **5** with full peptide, **S10** was obtained through neuraminidase and galactosidase treatment, core-fucolysation using FUT8, and bis-galactosylation using B4GALT1, and branch selective ST6GAL1 mediated sialylation. PNGase F treatment of mono-sialylated **S10** provided the desired free-reducing product, **S11** (52%, 5-steps). B. LC-MS chromatograph of the purified **S11** displaying two partially overlapping peaks representing the α and β anomer. C. Focused ESI-MS of the peak at 26 min showing expected mass of the product, 1039.8745 $^{2+}$.

6. Microarray Procedure

6a. Materials and Methods

All compounds were printed on NHS-activated Nexterion® slides purchased from Schott using a Scienion S3 non-contact microarray printer. Biotinylated plant lectins *Aleuria aurantia* (AAL) and *Sambucus nigra* agglutinin (SNA) were purchased from Vector Labs. Human galectin-3 and -9 were purchased from R&D Systems. Biotinylated mouse anti-galectin-3 and -9 antibodies were purchased from PeproTech, and streptavidin-AlexaFluor® 635 conjugate was purchased from Thermo Fisher. Stained slides were analyzed using a GenePix 4000B plate reader manufactured by Axon Instruments.

Individual targets were dissolved in a sodium phosphate buffer (pH 9.0, 250 mM) and were printed in replicates of six at a concentration of 100 μM, spot volume ~400 pL, and each slide contained 24 subarrays (3 x 8). Post printing, slides were incubated in a humidity chamber for 24 h and then blocked for 1 h with a 5 mM ethanolamine in a Tris buffer (pH 9.0, 50 mM). Blocked slides were rinsed with DI water, spun dry, and kept in a desiccator at room temperature for future use.

6b. General Procedure for Hydrogenolysis Mediated CBz Deprotection. **13** (0.5 mg, 0.2 μmol) was dissolved in a 10% *t*-butanol/water solution (400 μL) and to this was added a palladium hydroxide on carbon slurry (0.4 mg, 20% *wt* suspended in 100 μL *t*-burtanol/water solution). The reaction was stirred vigorously under an atmosphere of hydrogen (1 atm) and monitored by ESI-MS until no starting material could be detected. Once complete, the reaction was filtered through a Whatman ® syringe filter (0.2 micron) to remove the catalyst and the filtrate was lyophilized to yield **A20** (0.4 mg, 80%) as a white fluffy solid.

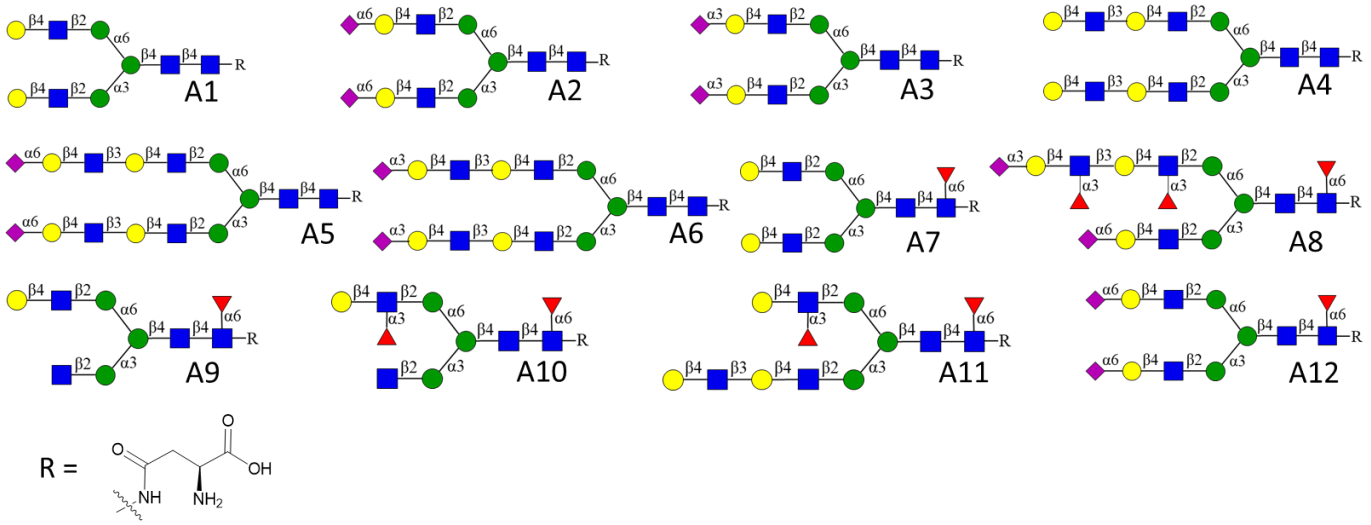
6c. Screening Procedure

Plant Lectins: Screening solutions of biotinylated AAL and SNA were created by dissolving an appropriate amount of stock solution in a TSM binding buffer (TSMBB, 20 mM Tris HCl, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.05% Tween, and 1 g/L BSA, pH 7.4) so that the final lectin concentrations were 1 µg/mL and 20 µg/mL, respectively. Plant lectin screening solution (100 µL) was added to each subarray and was allowed to incubate at room temperature, in the dark, for 1 h, after which the slide was washed consecutively with TSM wash buffer (TSMWB, 20 mM Tris Cl, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.05% Tween, pH 7.4), TSM buffer (20 mM Tris Cl, 50 mM NaCl, 2 mM $CaCl_2$, 2 mM, and $MgCl_2$, pH 7.4), DI water, and spun dry as described in the literature.[11] To each washed subarray was added AlexaFluor® 635 in PBS (100 µL, 5 µg/mL) and the slide was incubated at room temperature, in the dark, for 30 min. After labelling, the slide was washed as described above and kept in the dark prior to analysis.

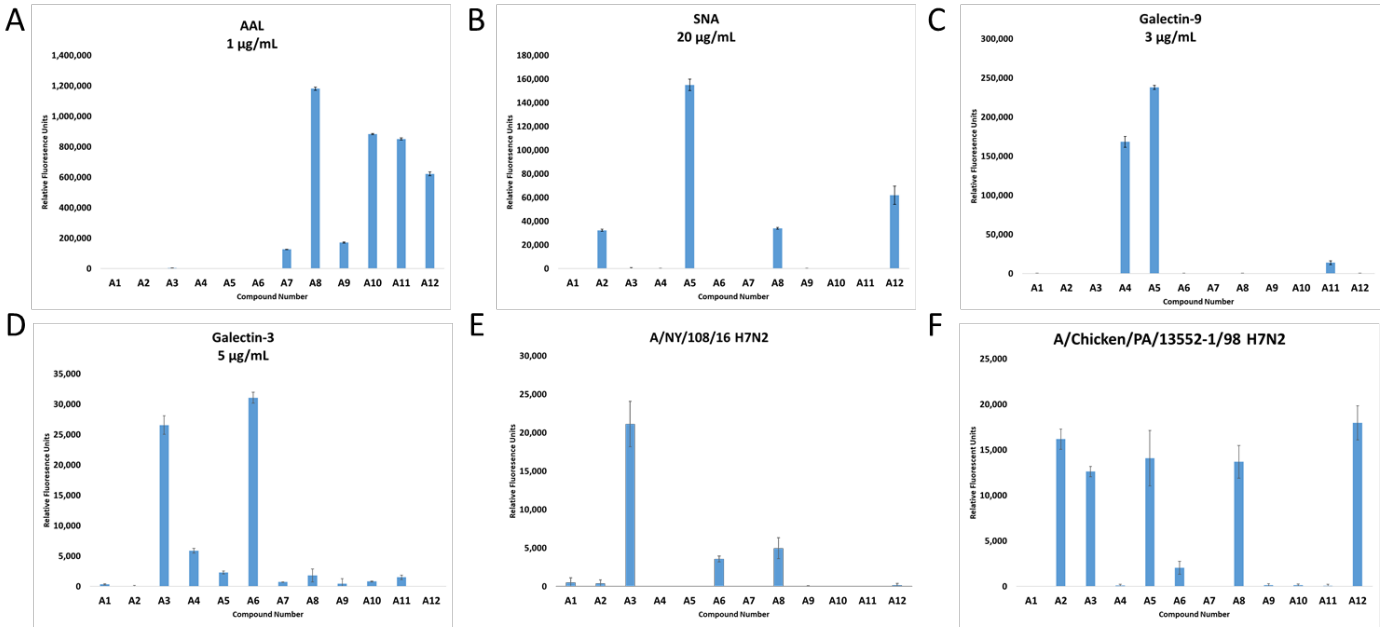
Galectin-3 and -9: Galectin-3 and -9 solutions were made by dissolving the lectin in a TBS binding buffer (TBSBB, 25 mM Tris, 0.15 M NaCl, 0.1 % BSA, and 0.05% Tween, pH 7.2) so that the final concentrations were 5 µg/mL and 3 µg/mL, respectively. Antibody and fluorophore solutions (5 µg/mL) were created by dissolving biotinylated anti-Gal-3 and -9 antibodies and the AlexaFluor in TBSBB. For galectin staining, the screening solution (100 µL) was added to a subarray and was incubated at room temperature, in the dark, for 1 h. After this time, the lectin was washed away using the plant lectin protocol. To the dried plate, anti-Gal-3 and -9 antibodies (100 µL) were added and incubated at room temperature, in the dark, for 1 h. Following plate washing, streptavidin fluorophore (100 µL, 5 µg/mL) was added and incubated for 30 min after which, the slide was washed, dried, and screened.

Influenza viruses: AlexaFluor 633 labeled inactivated avian influenza viruses were provided as a gift from Dr. Tompkins, College of Veterinary Medicine, University of Georgia, Athens. Screening solutions were prepared by diluting virus stock solution in TSMBB to give a final hemagglutination units (HAU) value of $2x10^3$/mL. 100 µL of each virus was added to a subarray, and the slide was incubated at 4 °C, in the dark, for 30 min. After 30 min, the virus was washed away and the slide was screened using the same protocol as described for the plant lectins.

6d. Microarray Library



6e. Microarray Data



Supplementary Fig. 13. Microarray data of four carbohydrate binding proteins and two avian influenza viruses. A. AAL is a general fucose recognizing lectin; only targets containing an α1,3 and/or α1,6-core fucose (**A7 – A12**) were recognized. B. SNA is selective for Neu5Ac terminating structures, specifically those with α2,6 linkage (**A5**), but not α2,3 (**A6**) linkages. C. Galectin-9 is an eosinophil chemoattractant that has preference for extended poly-LacNAc motifs. High responsiveness was observed for symmetric, di-LacNAc structure (**A4**) with additional accommodation for the di-LacNAc target terminating in α2,6-Neu5Ac (**A5**). Interestingly, **A11** endowed with a di-LacNAc motif on the MGAT1 branch and a Le^x epitope at the MGAT2 position did not provide a similar response. D. Galectin-3 is a secreted by macrophages and is associated with immune regulation, the inflammatory process, and cancer. This protein is known to bind with LacNAc / poly-LacNAc targets capped with α2,3 Neu5Ac (**A3, A6**), and to a lesser extent the α2,6 Neu5Ac isomer (**A5**)[12]. Although poly-LacNAc has been shown to favorably bind to Gal-3, **A11** does not provide a signal (similar to Gal-9 screening) suggesting the di-LacNAc asymmetric presentation is not ideal for protein recognition. E. A/NY/108/16 H7N2 virus showed binding to α2,3-Neu5Ac terminating structures, including sialyl Le^x (**A3**, **A6**, **A8**), but not to α2,6-Neu5Ac terminating structures (**A2**, **A12**). F. A/chicken/PA/13552-1/98 H7N2 virus showed broader binding ability recognizing both α2,3 and α2,6 sialylated structures (**A2, A3, A5**), however the addition of an α1,3 fucose to yield SLe^x (**A8**) was not permitted.

7. References

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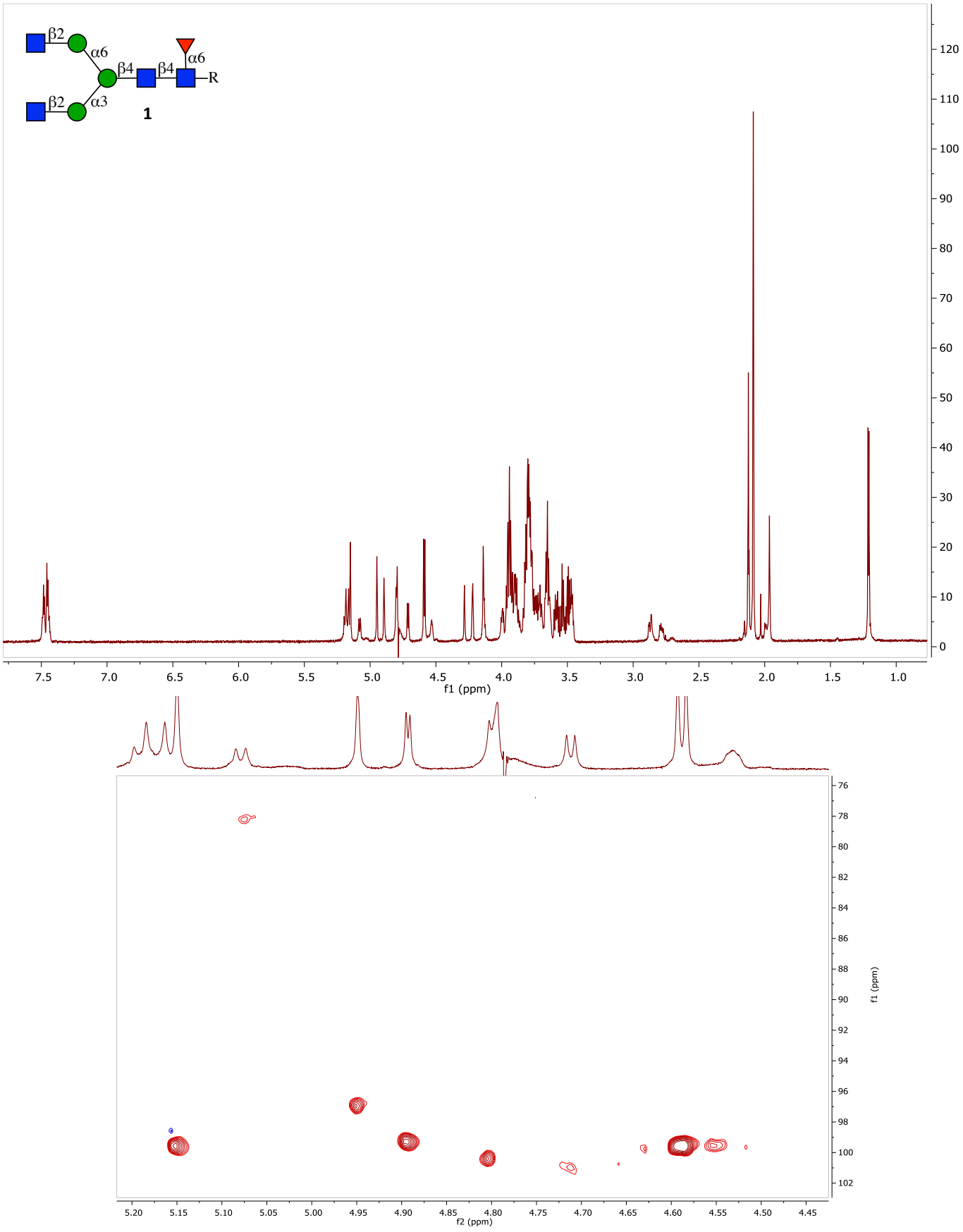
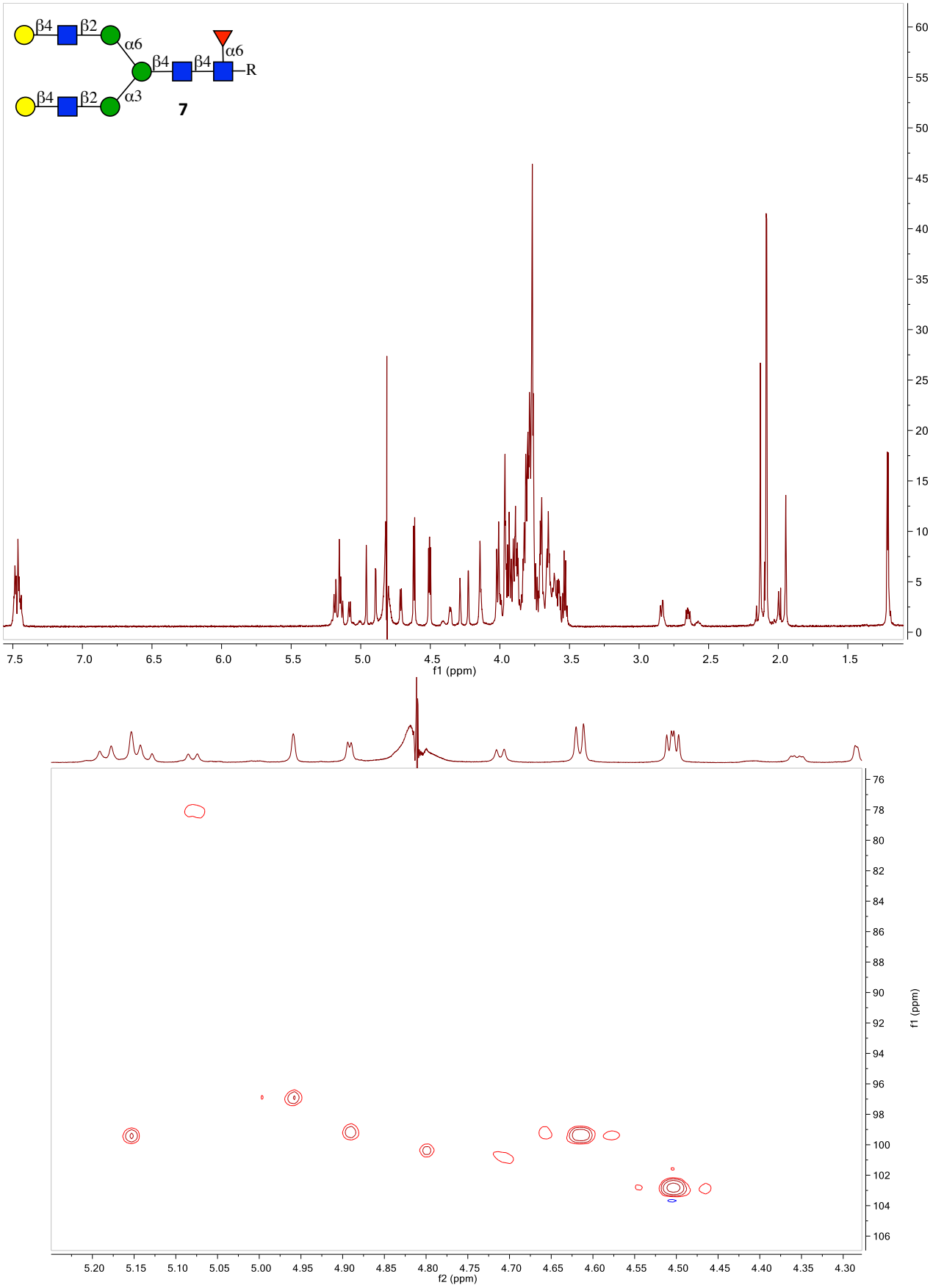
8. NMR Spectra

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α6
β4
β4
α6
R
β4
β2
α3
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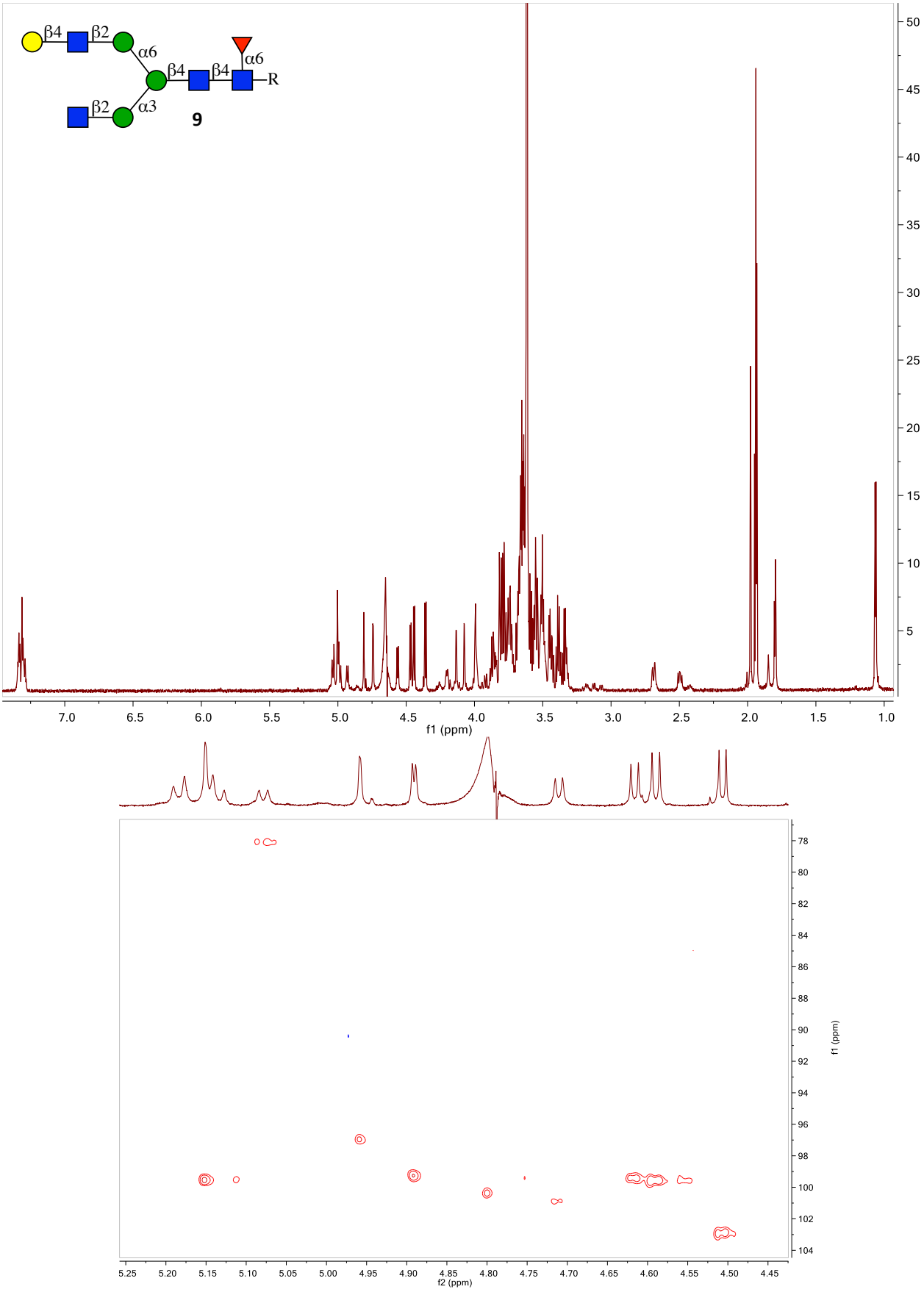
f2 (ppm)
f1 (ppm)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β4
β4
α6
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β2
α3
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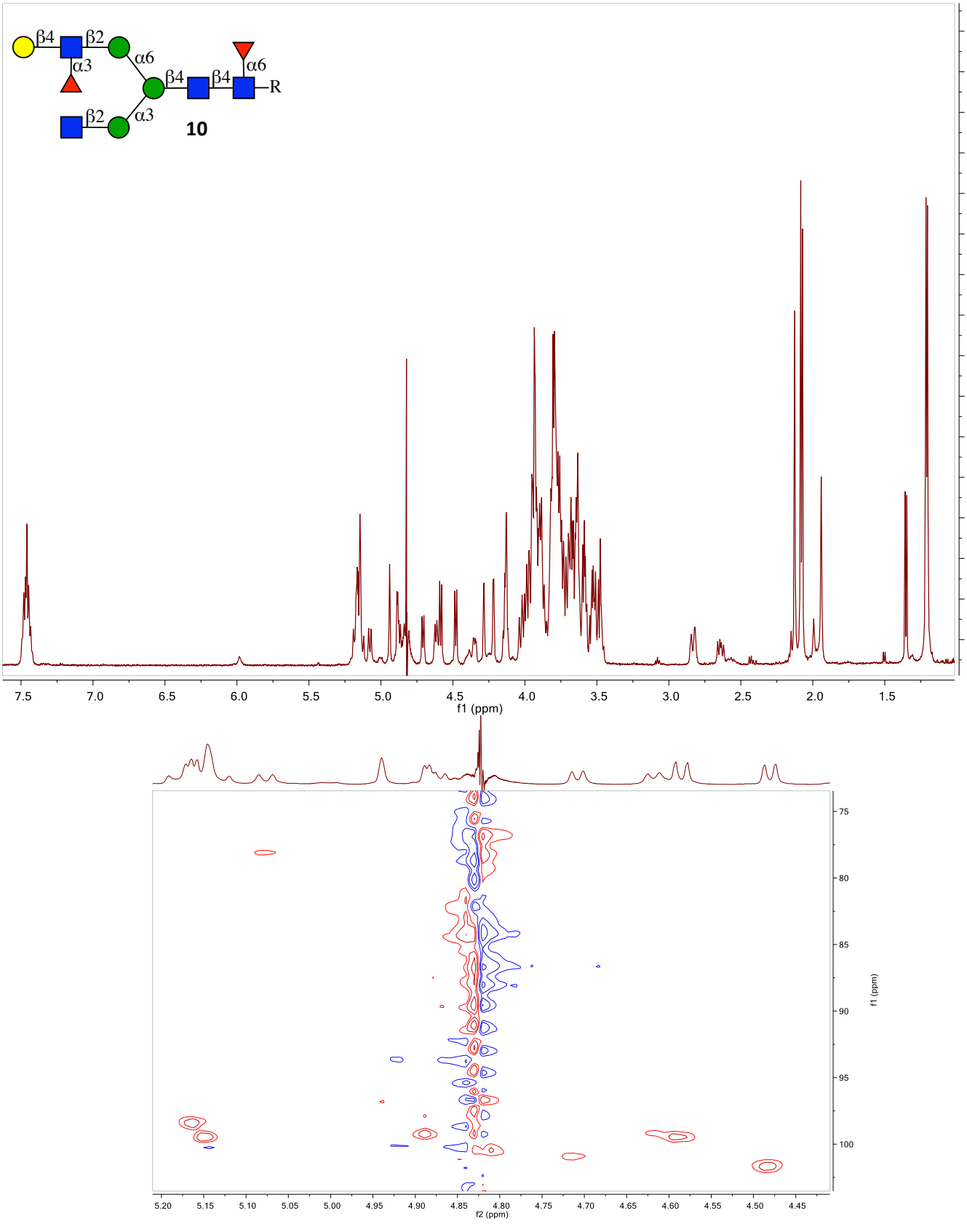
f1 (ppm)
f2 (ppm)
f1 (ppm)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α6
β4
β4
α6
R
β2
α3
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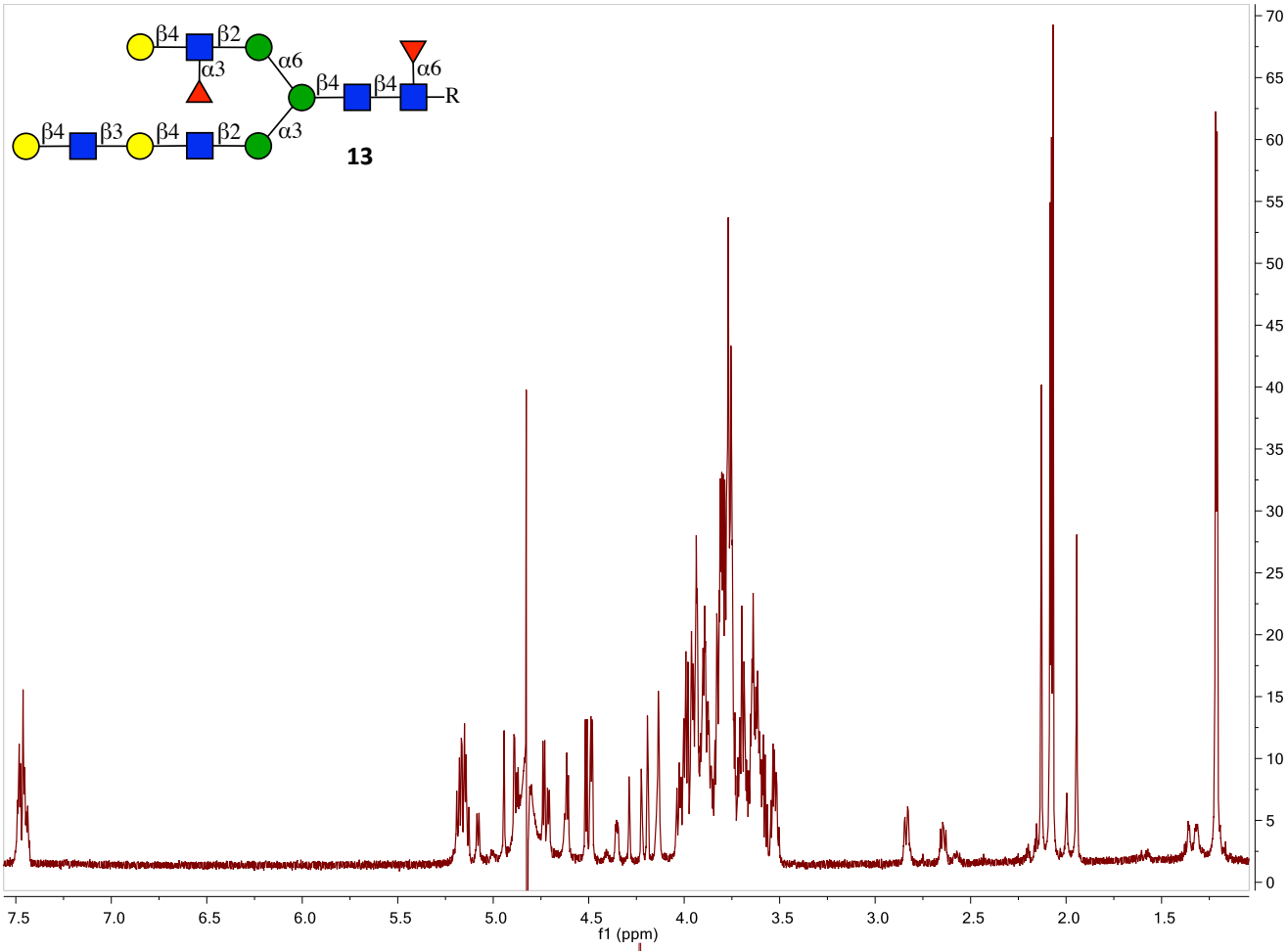
f2 (ppm)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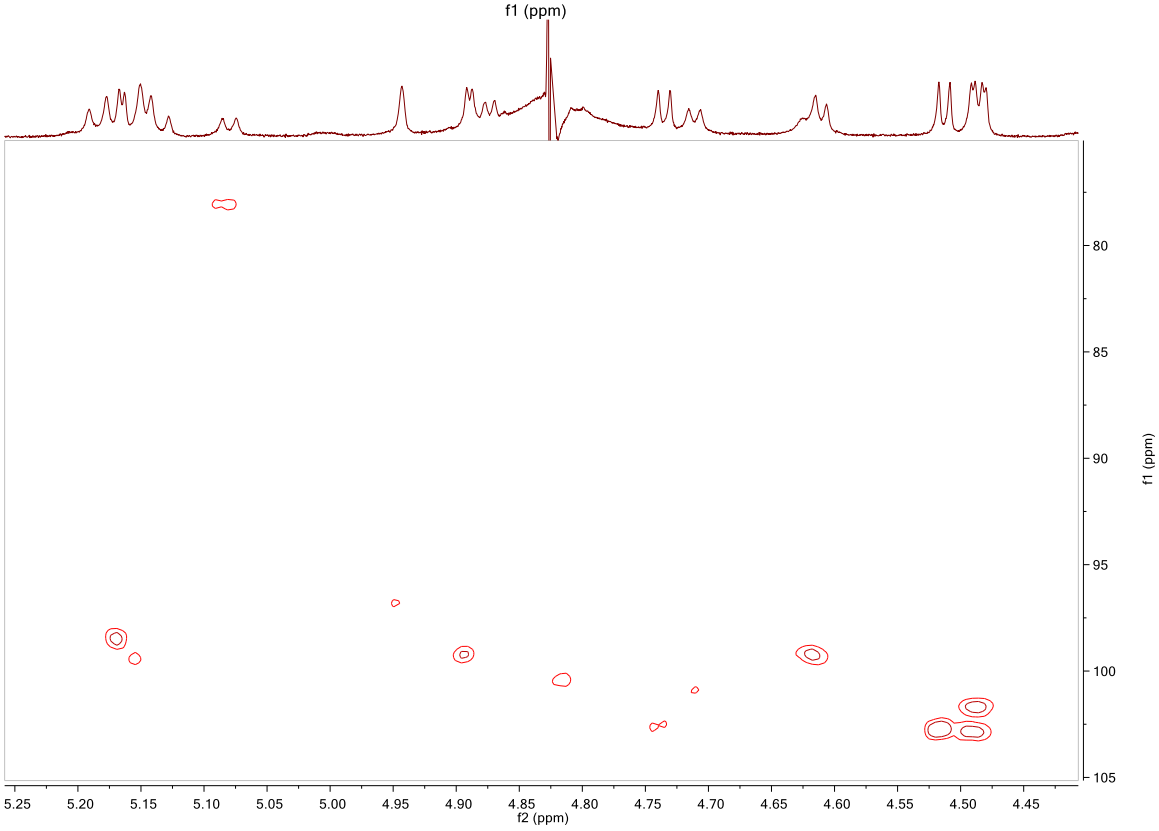
α6
β4
β4
α6
R
β4
β3
β4
β2
α3
13
f1 (ppm)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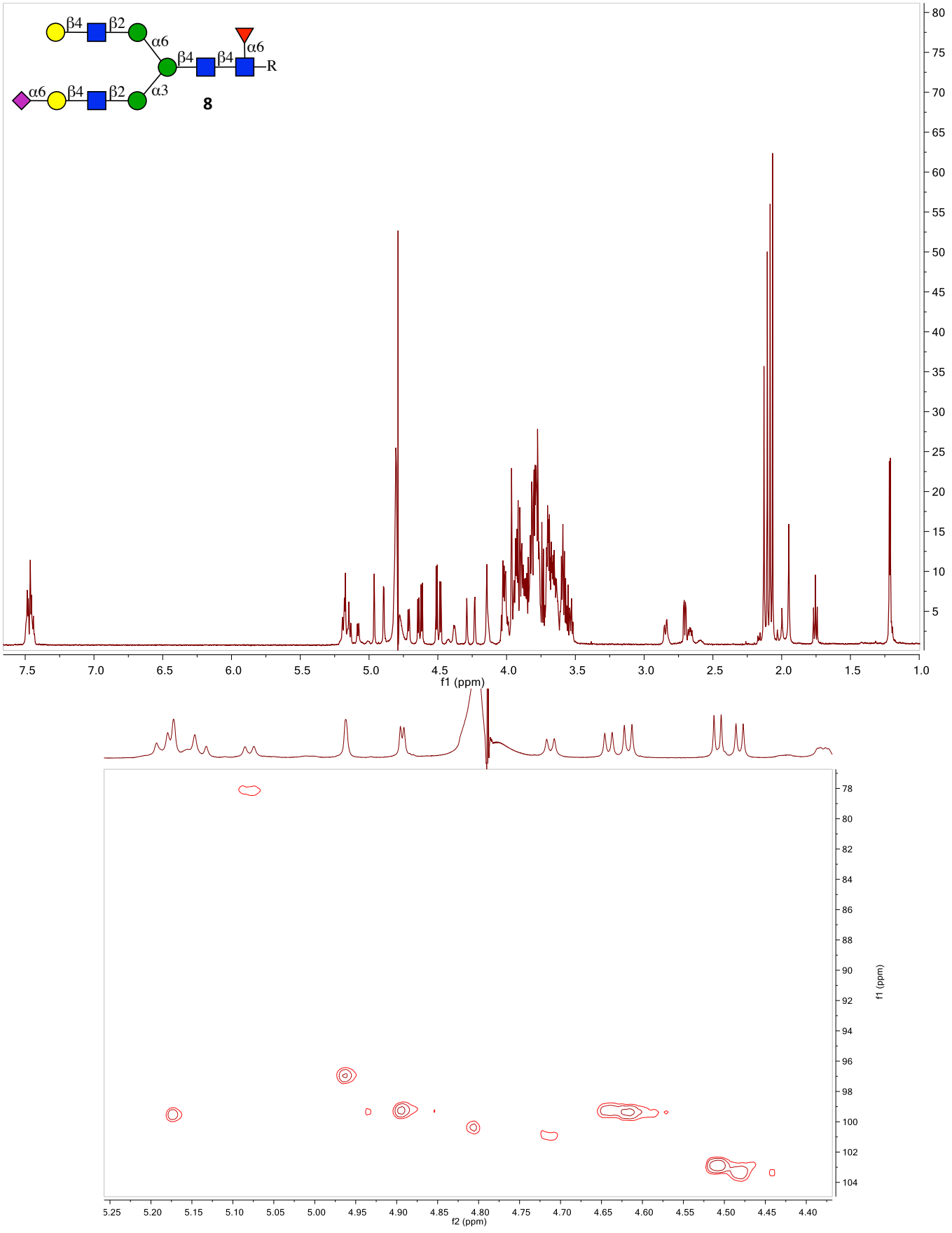
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β4
α6
R
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β2
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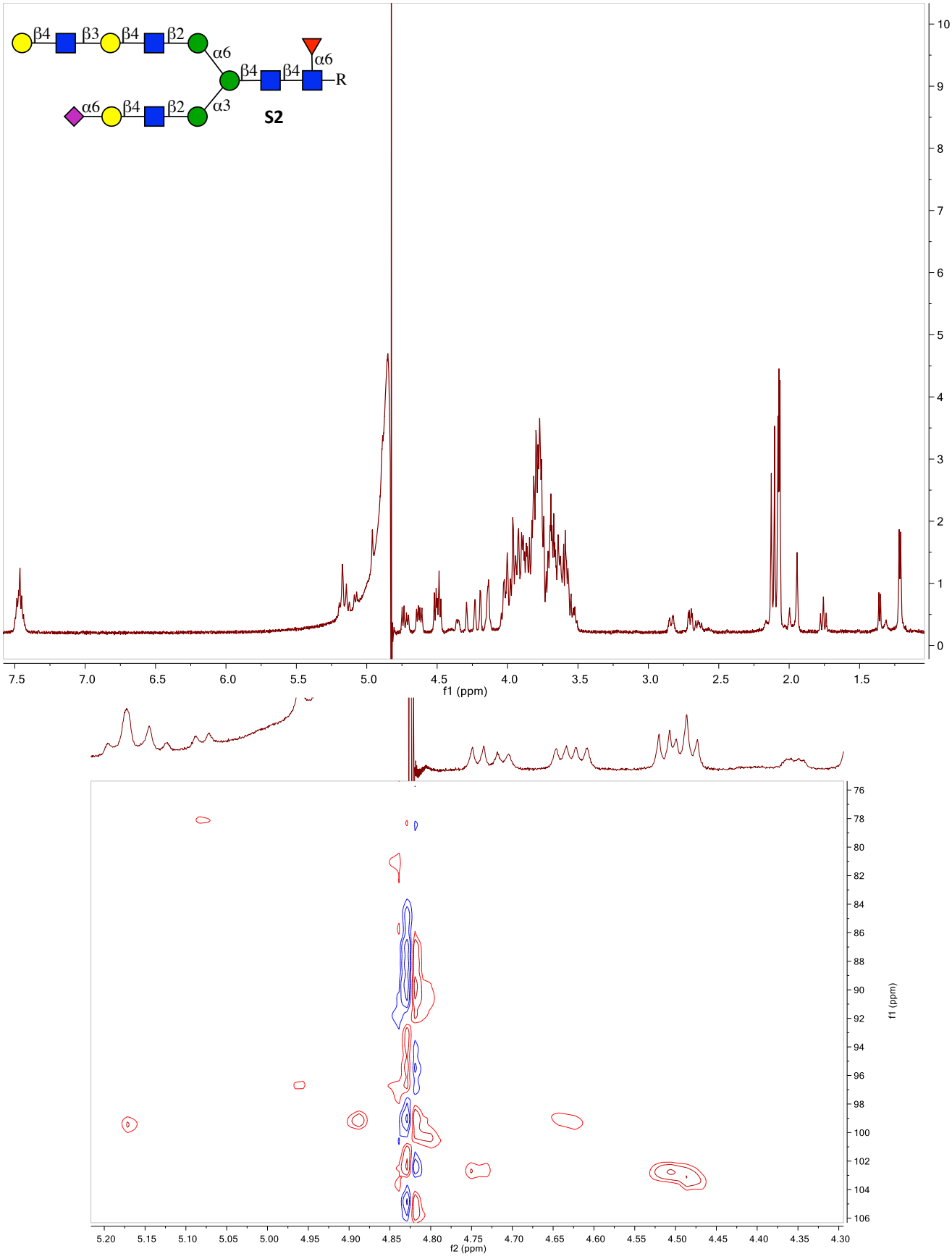
f1 (ppm)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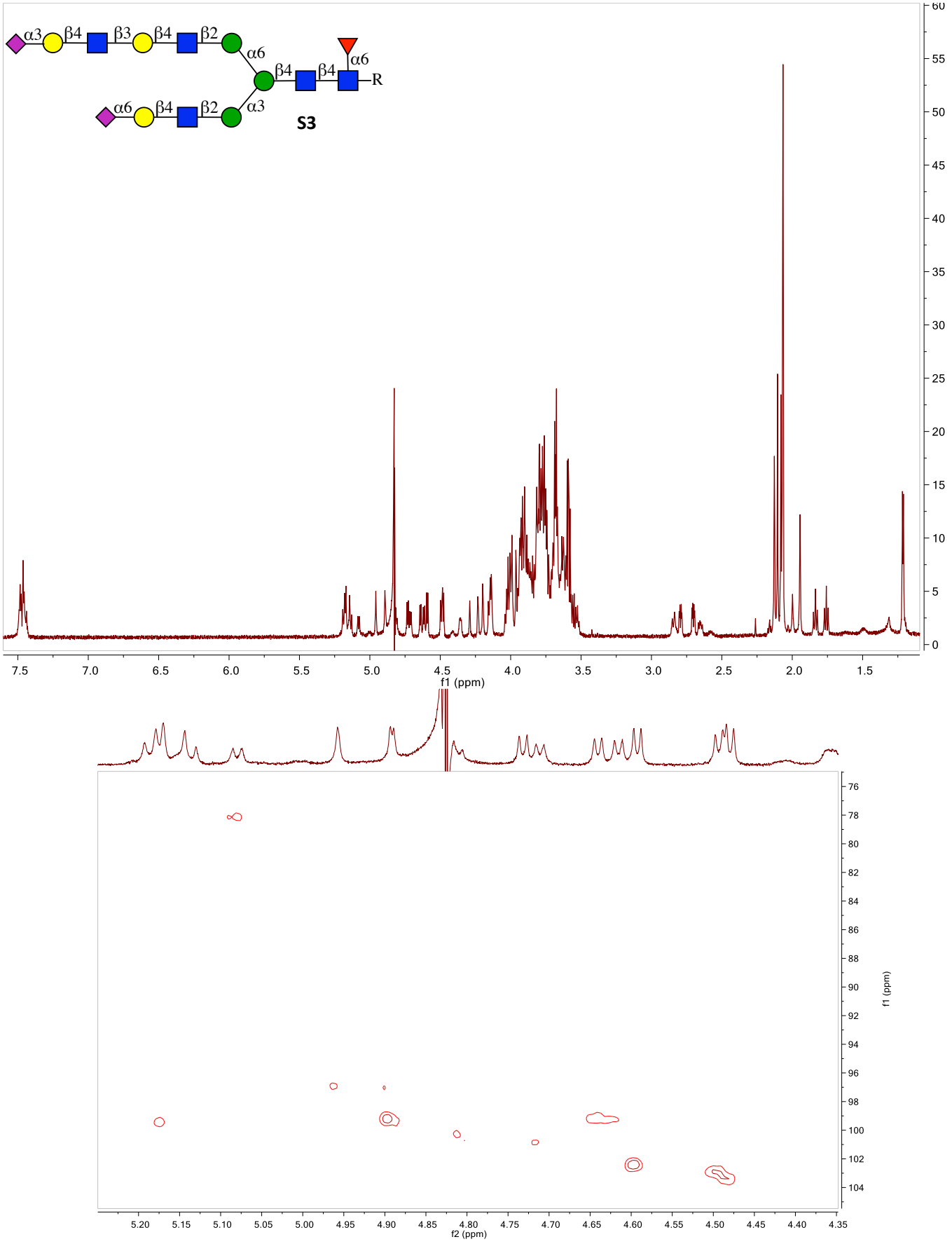
β3
β4
β2
α6
β4
β4
α6
R
α6
β4
β2
α3
S2
f1 (ppm)
f2 (ppm)
f1 (ppm)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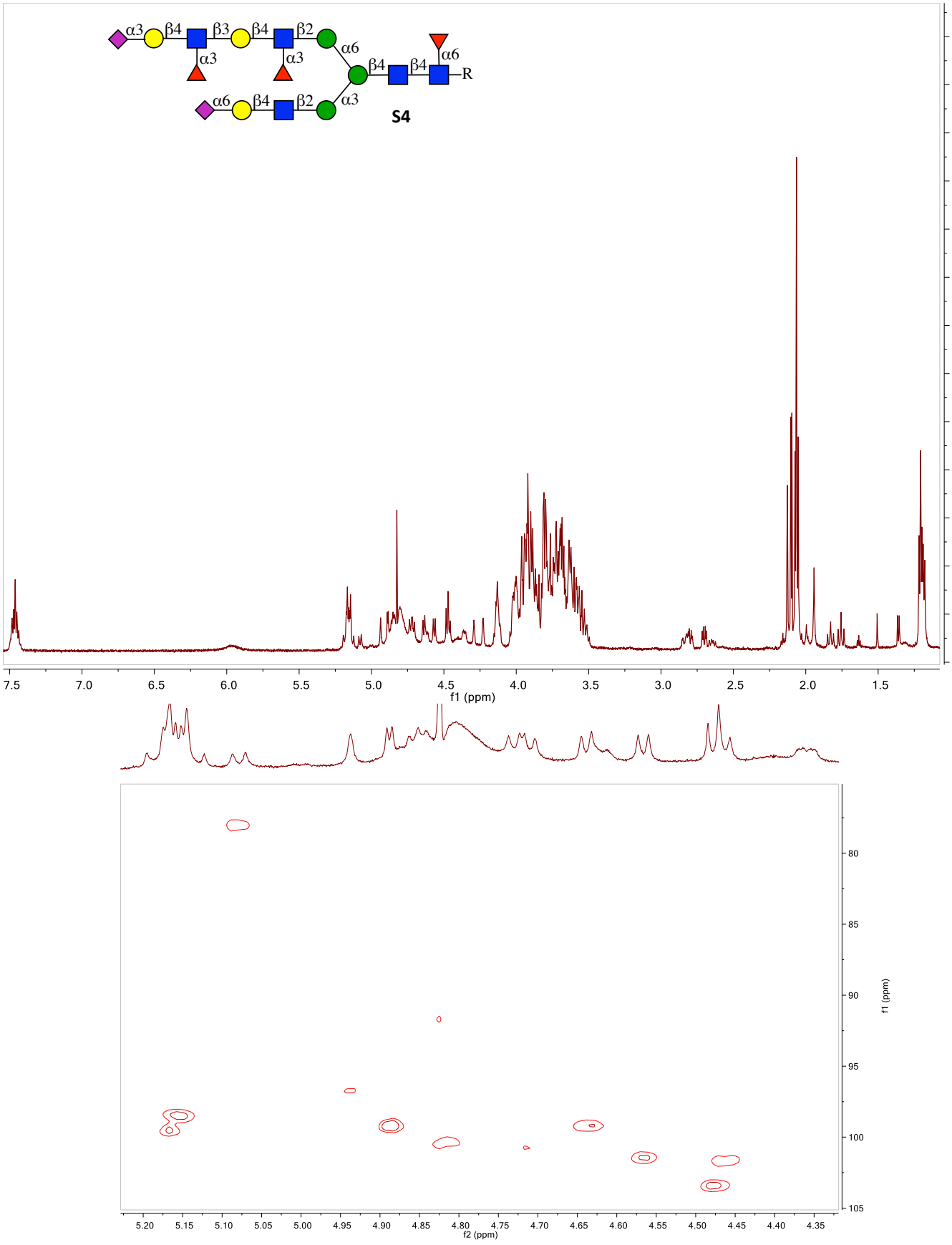
α6 β4 β2 α3
β4 β4 α6 R
S3
f1 (ppm)
f2 (ppm)
f1 (ppm)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α3 α3 β4 β4 α6
α6 β4 β2 α3 R
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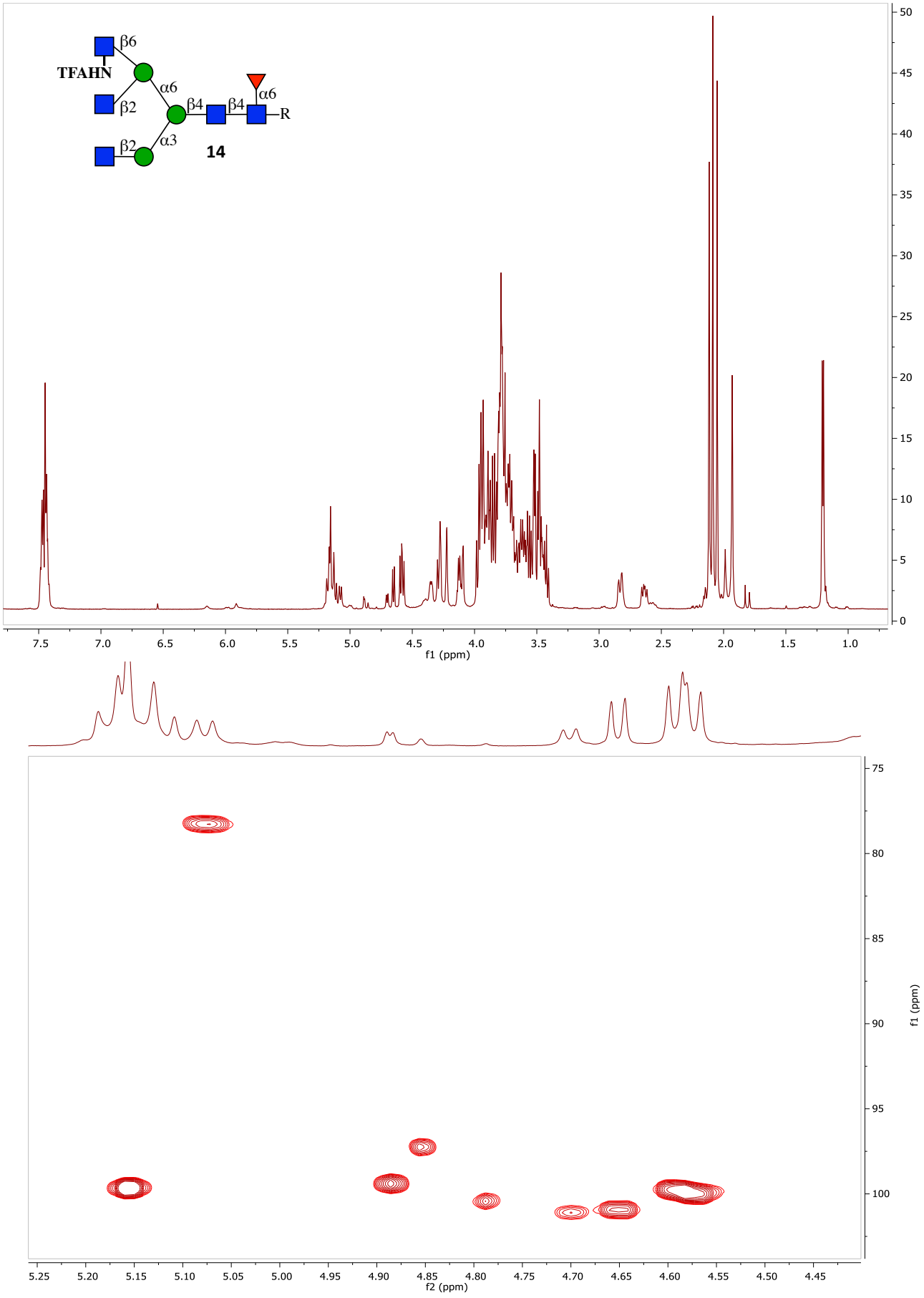
f1 (ppm)
f2 (ppm)
f1 (ppm)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β4
α6
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β2
α3
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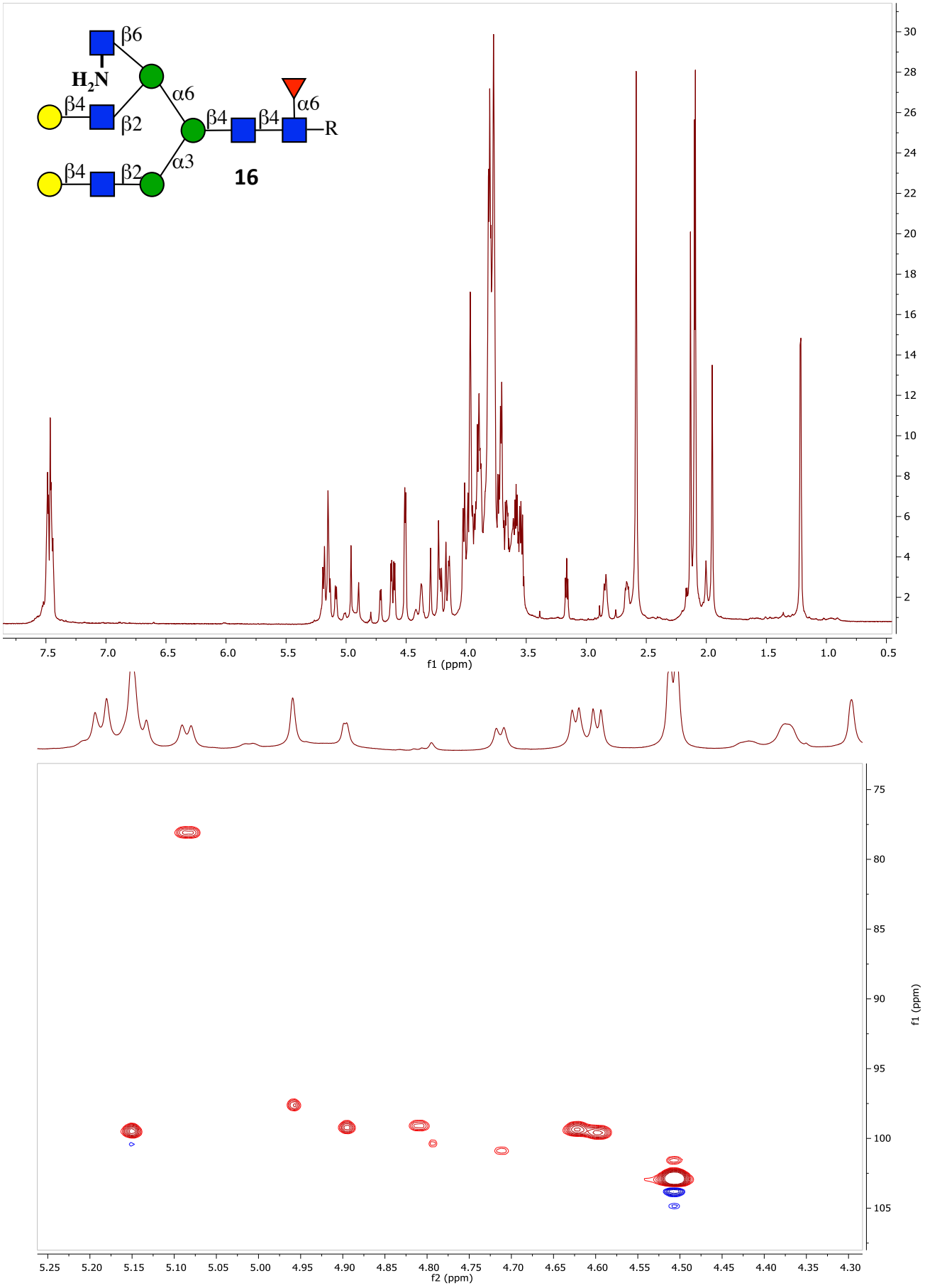
f2 (ppm)
f1 (ppm)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β4
β2
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β4
β4
α6
R
β4
β2
α3
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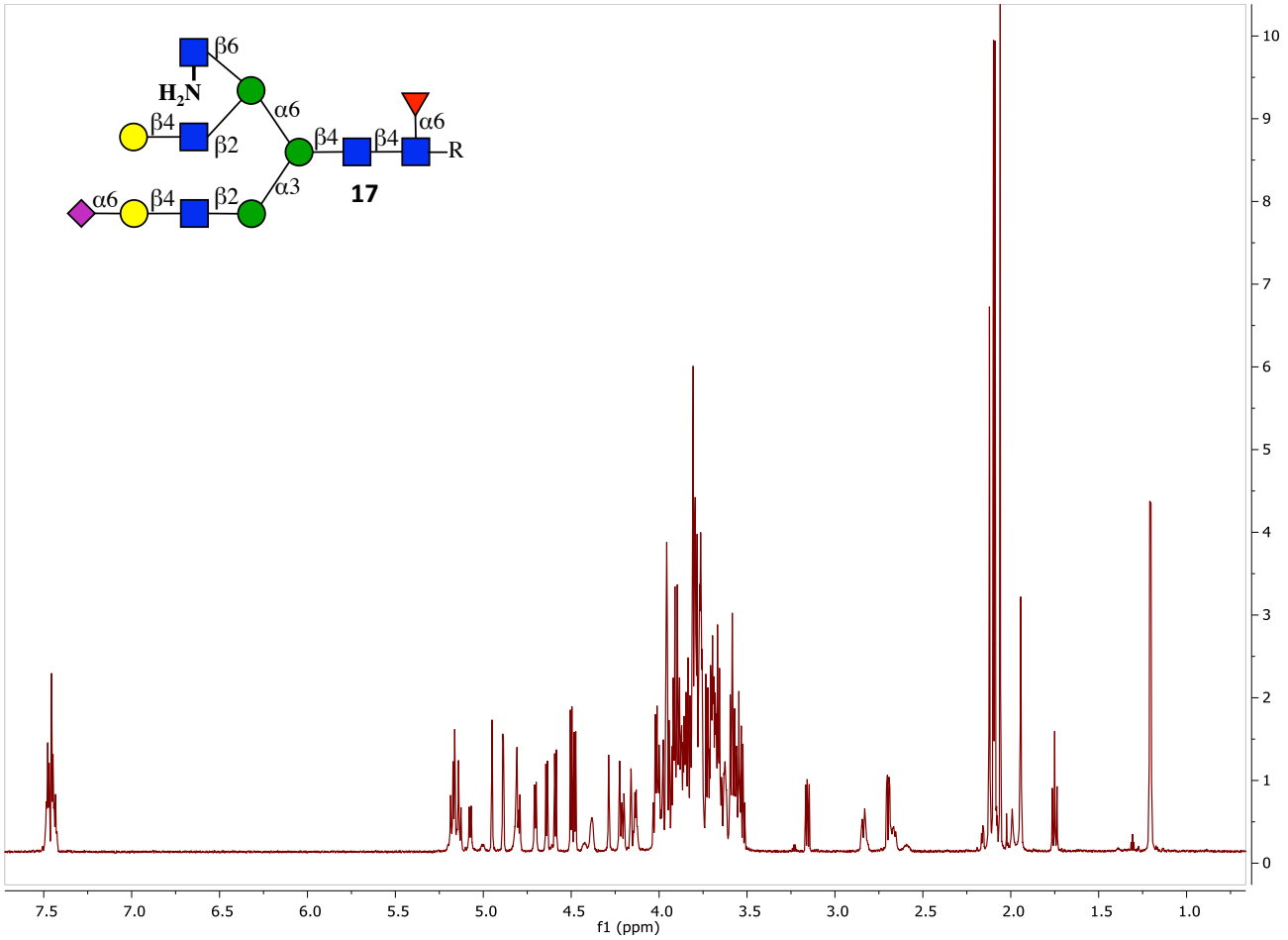
f2 (ppm)
f1 (ppm)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α6
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β4
β4
α6
R
α6
β4
β2
α3
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10
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7.0
6.5
6.0
5.5
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4.5
4.0
3.5
3.0
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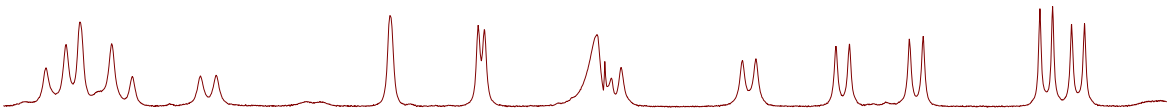
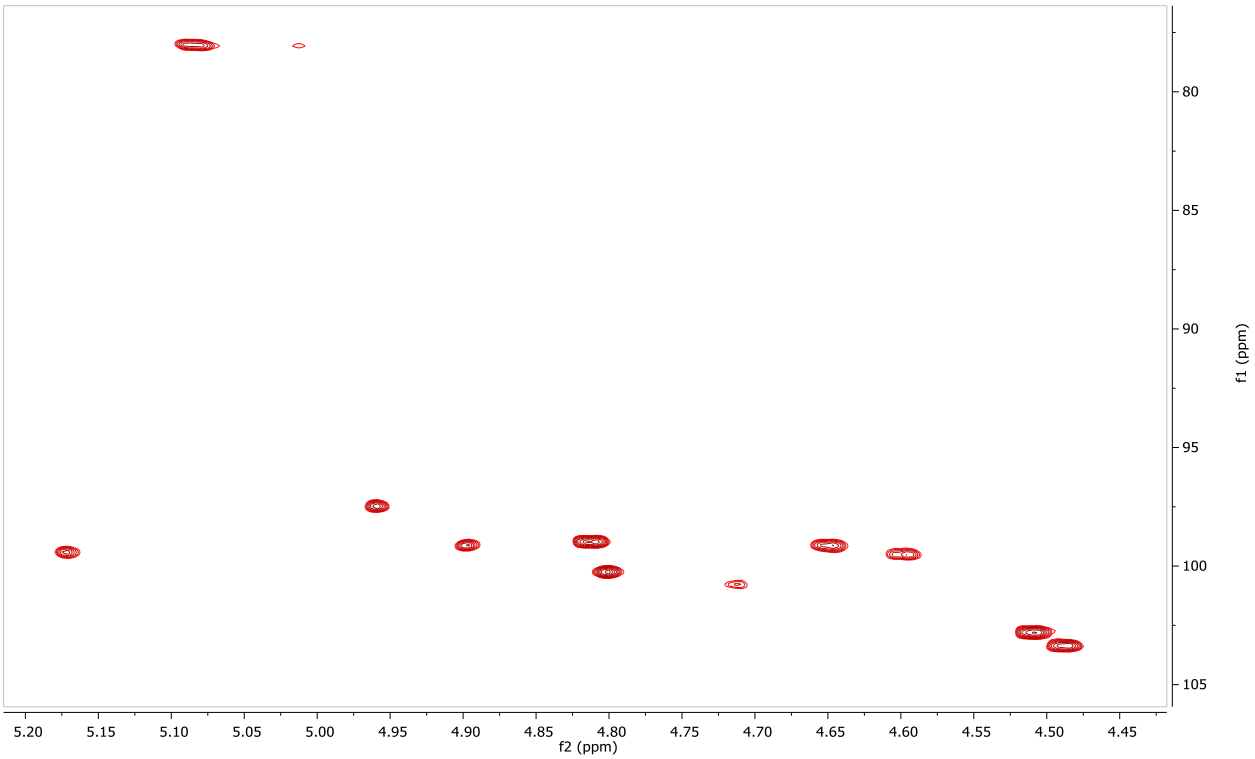
2.0
1.5
1.0
f1 (ppm)

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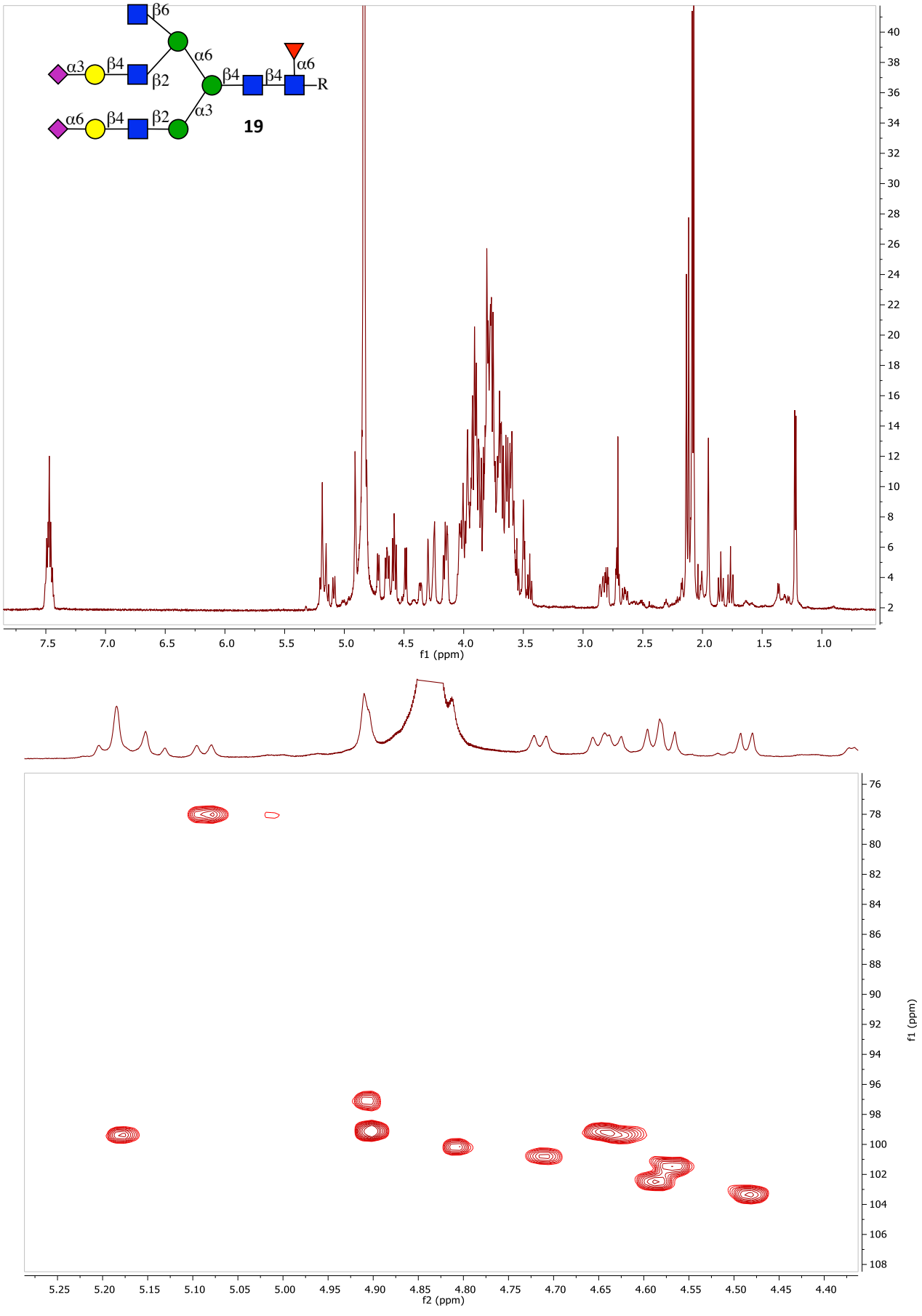
f2 (ppm)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α6
α3
β4
β2
β4
β4
α6
R
α6
β4
β2
α3
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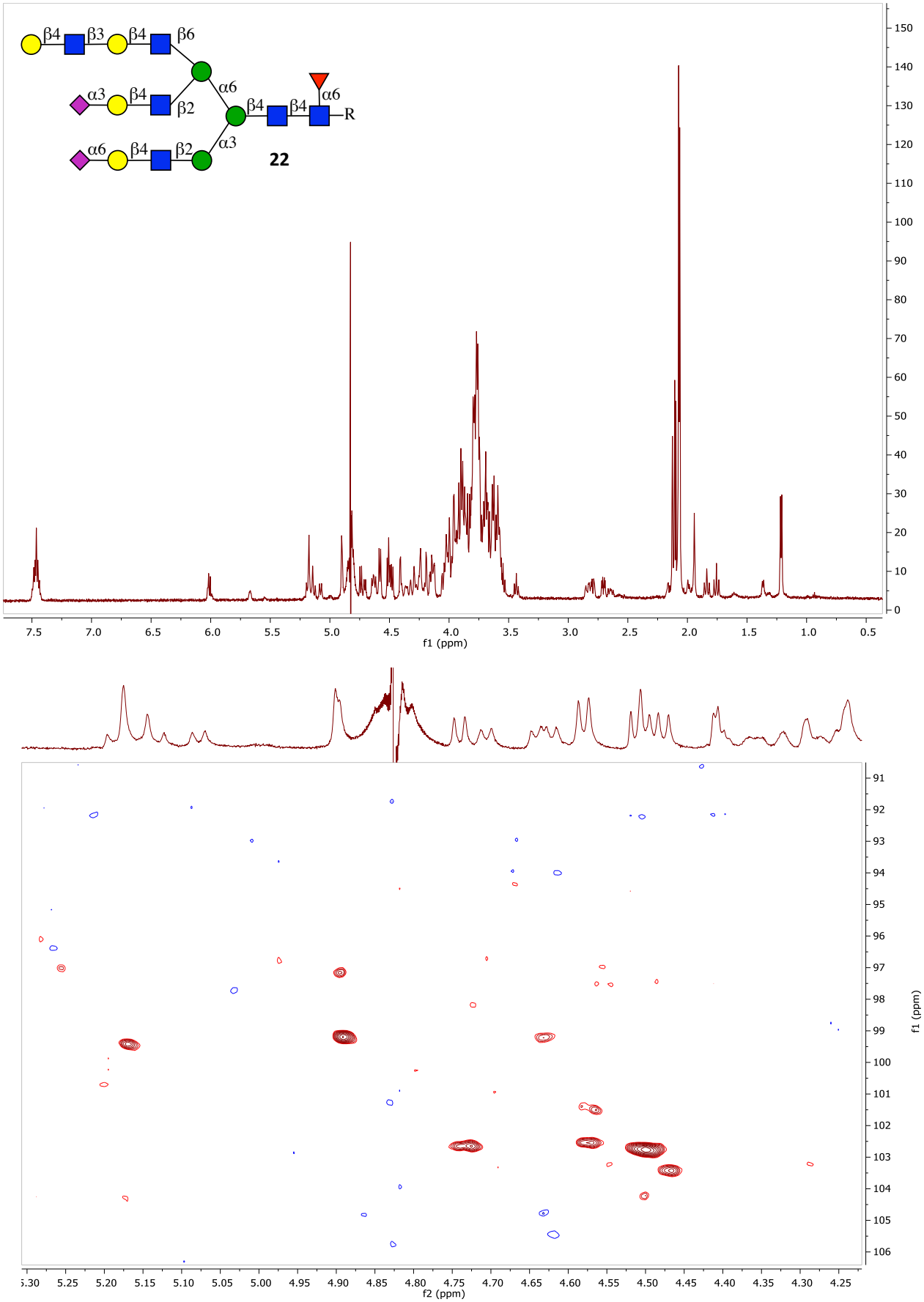
f2 (ppm)
f1 (ppm)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β3
β4
β6
α3
β4
α6
β2
β4
β4
α6
R
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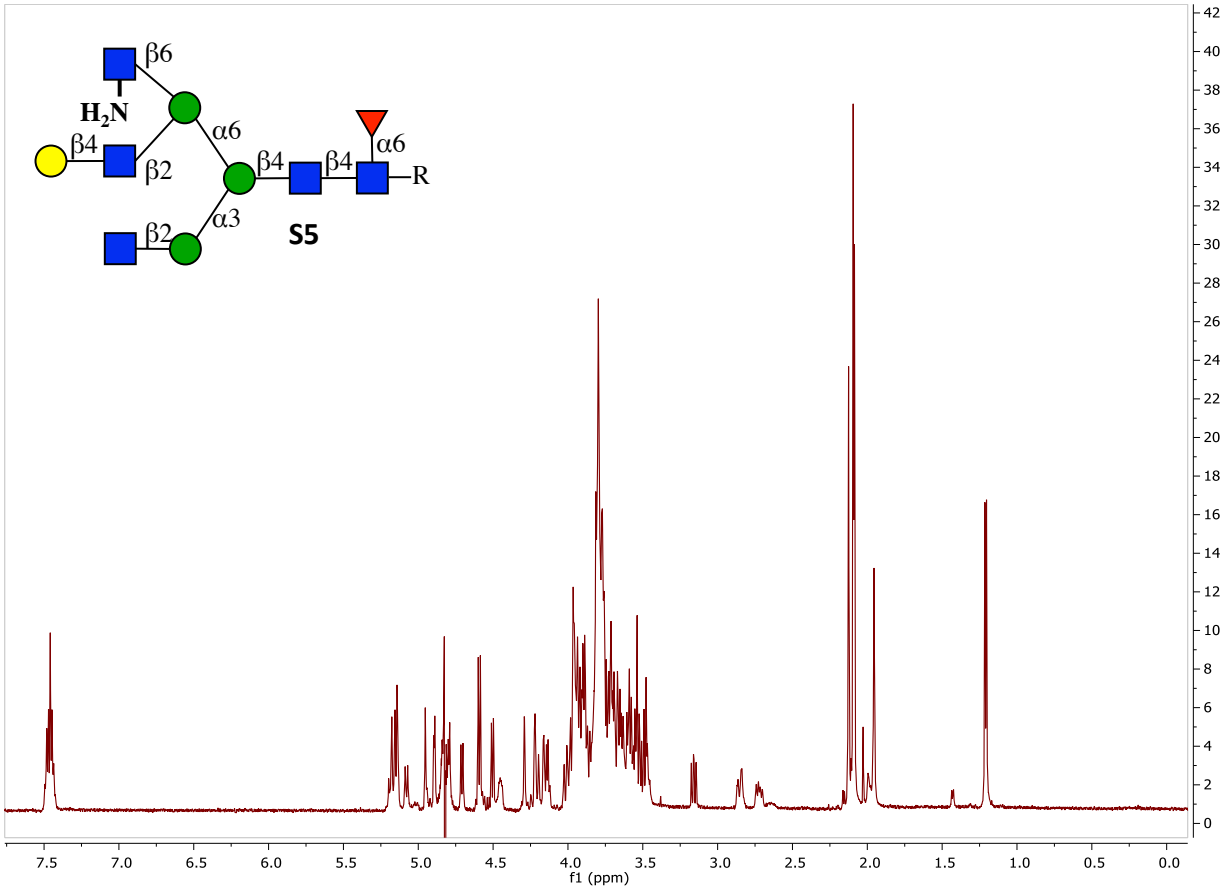
β4
β2
α3
22
f1 (ppm)
f2 (ppm)
f1 (ppm)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β4
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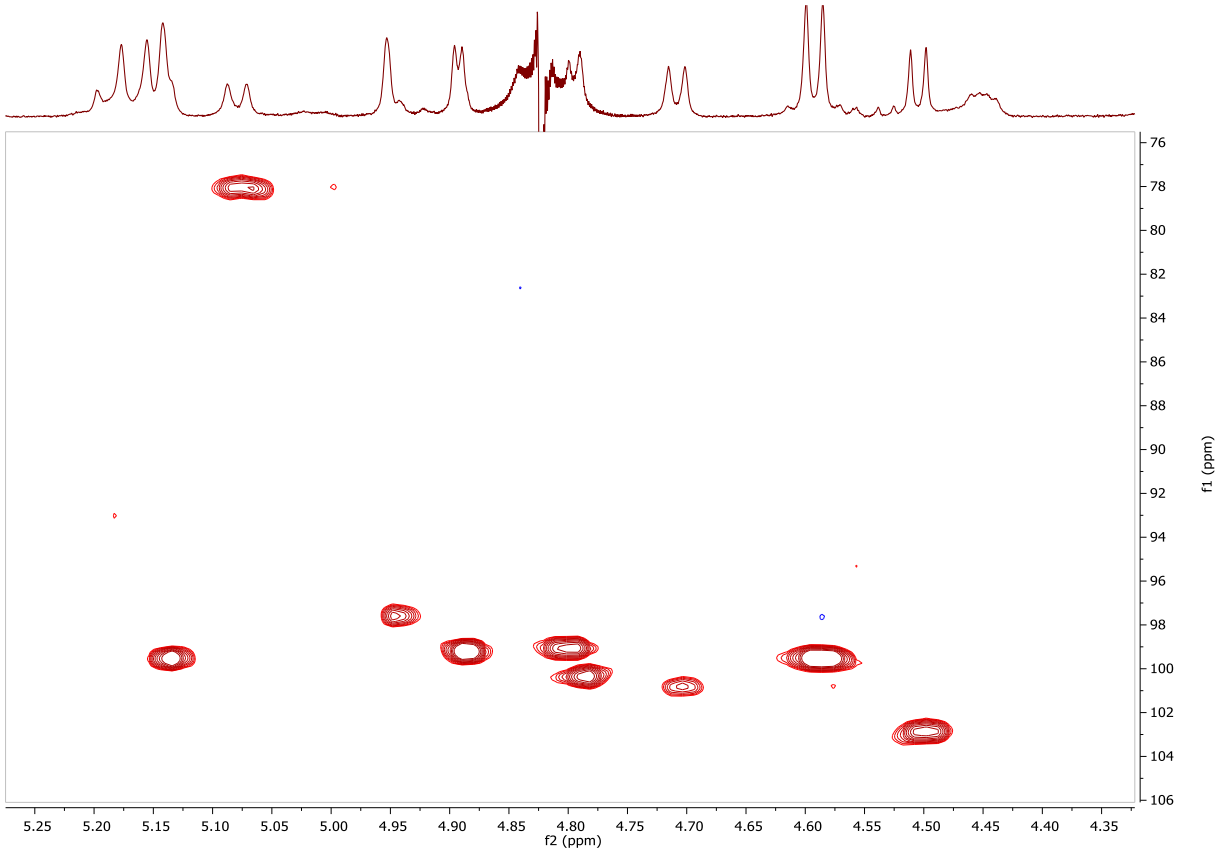
R
β2
S5
f1 (ppm)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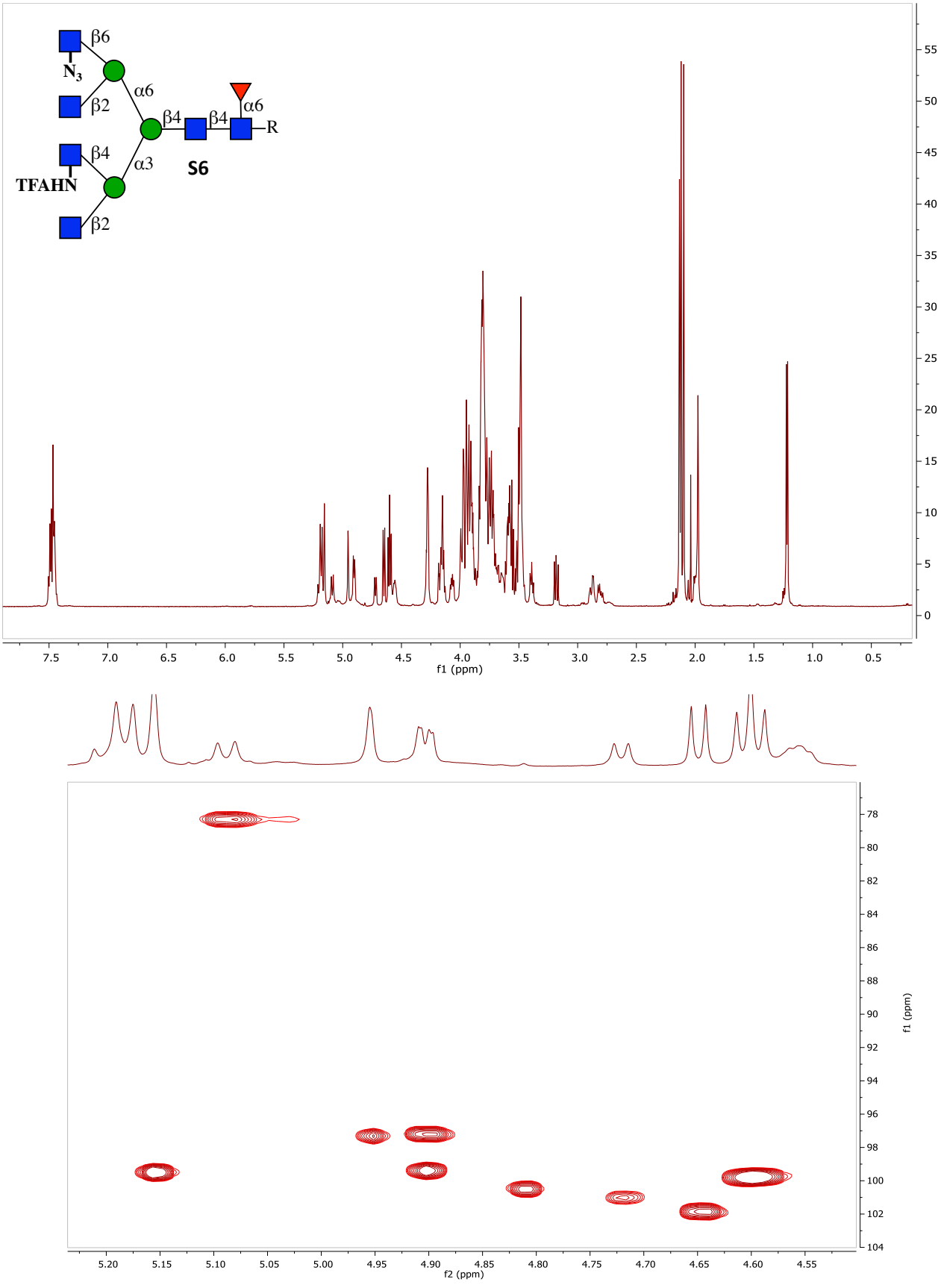
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β2
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β4
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R
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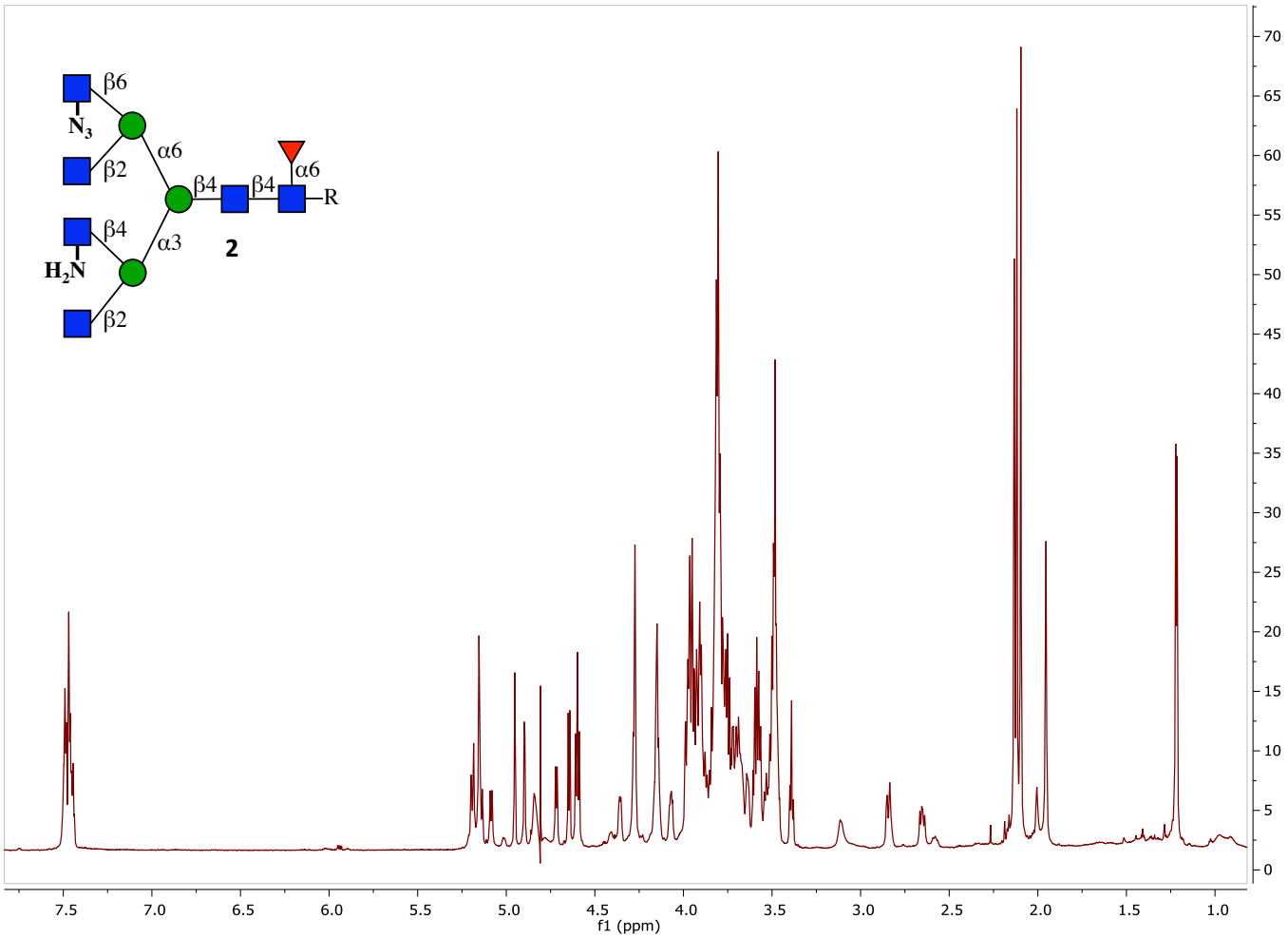
β2
S6
f1 (ppm)
f2 (ppm)
f1 (ppm)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N3
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α6
β4
β4
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R
β4
α3
H2N
β2
2
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
7.5
7.0
6.5
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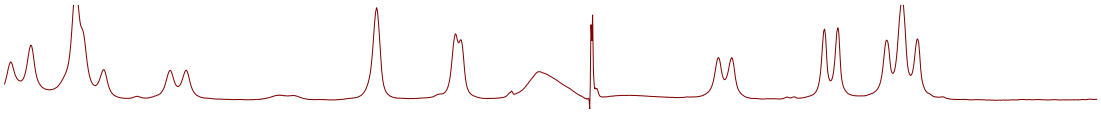
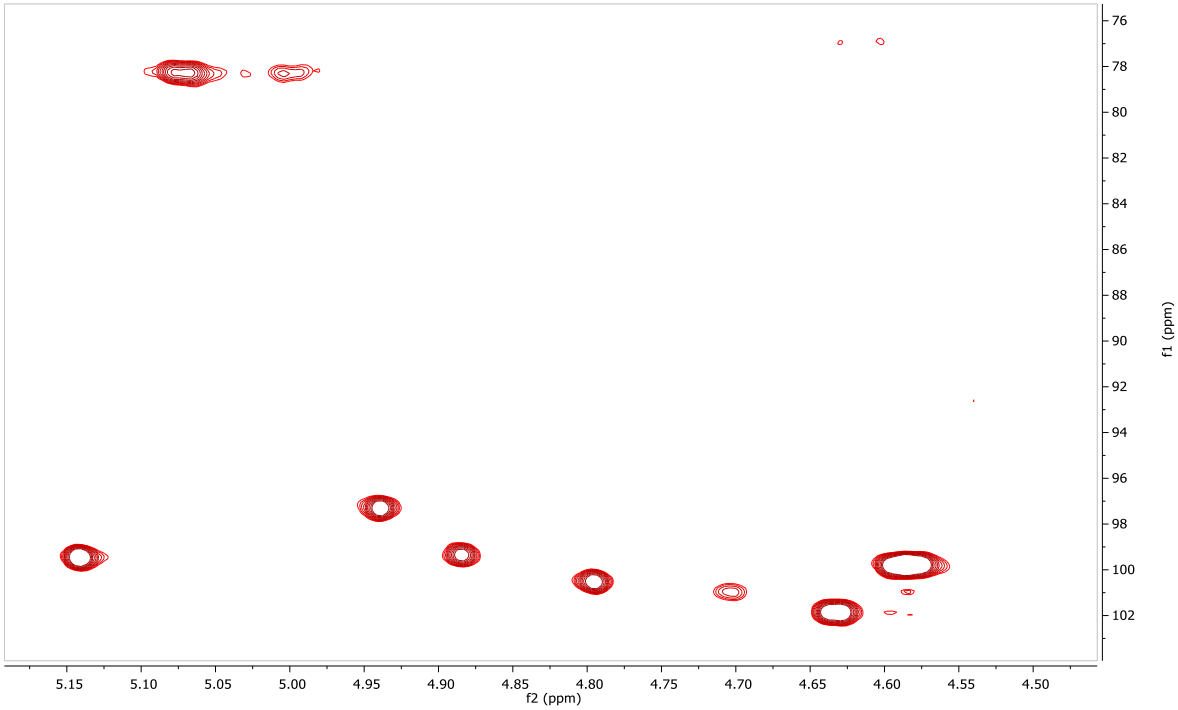
1.0
f1 (ppm)




76
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94
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100
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5.10
5.05
5.00
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4.75
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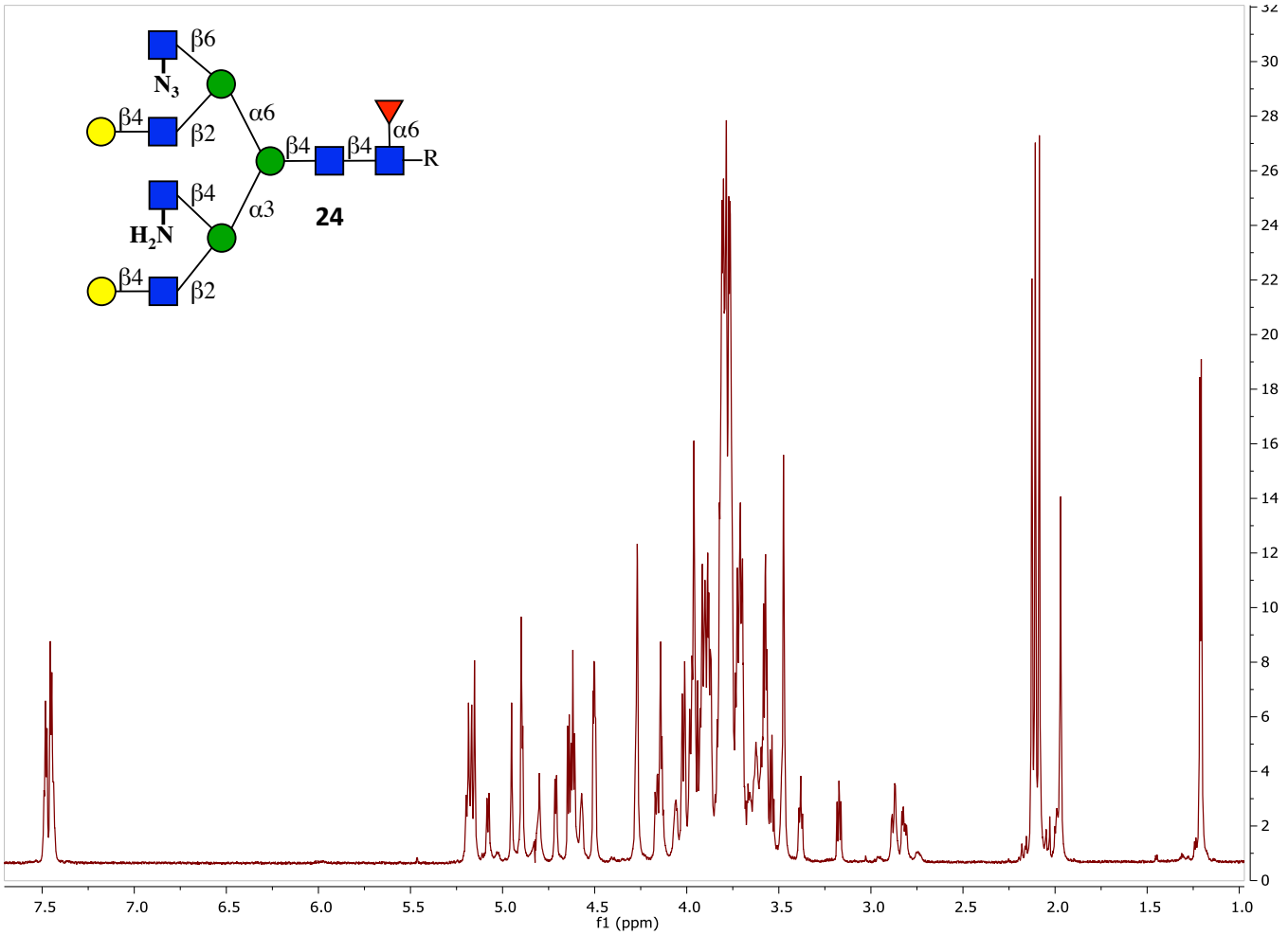
4.65
4.60
4.55
4.50
f2 (ppm)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β4
β2
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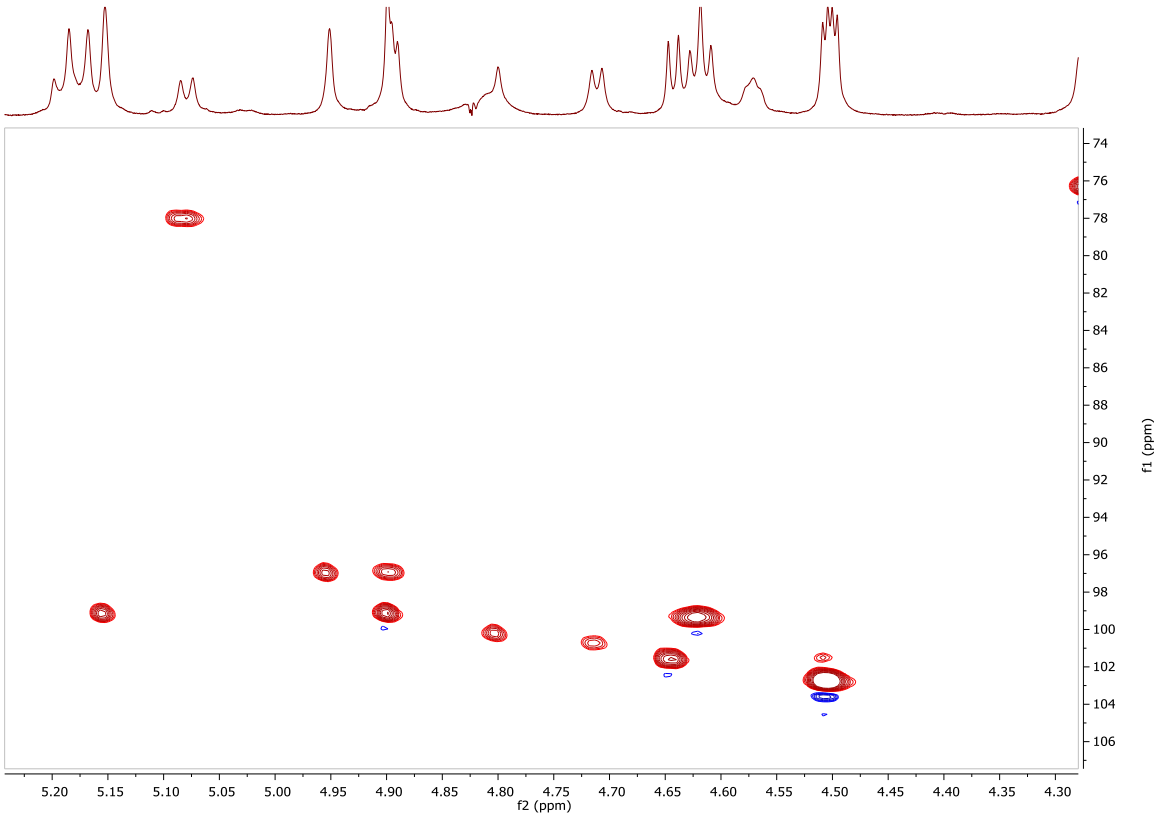
β4
β4
α6
R
β4
α3
H2N
β4
β2
24
f1 (ppm)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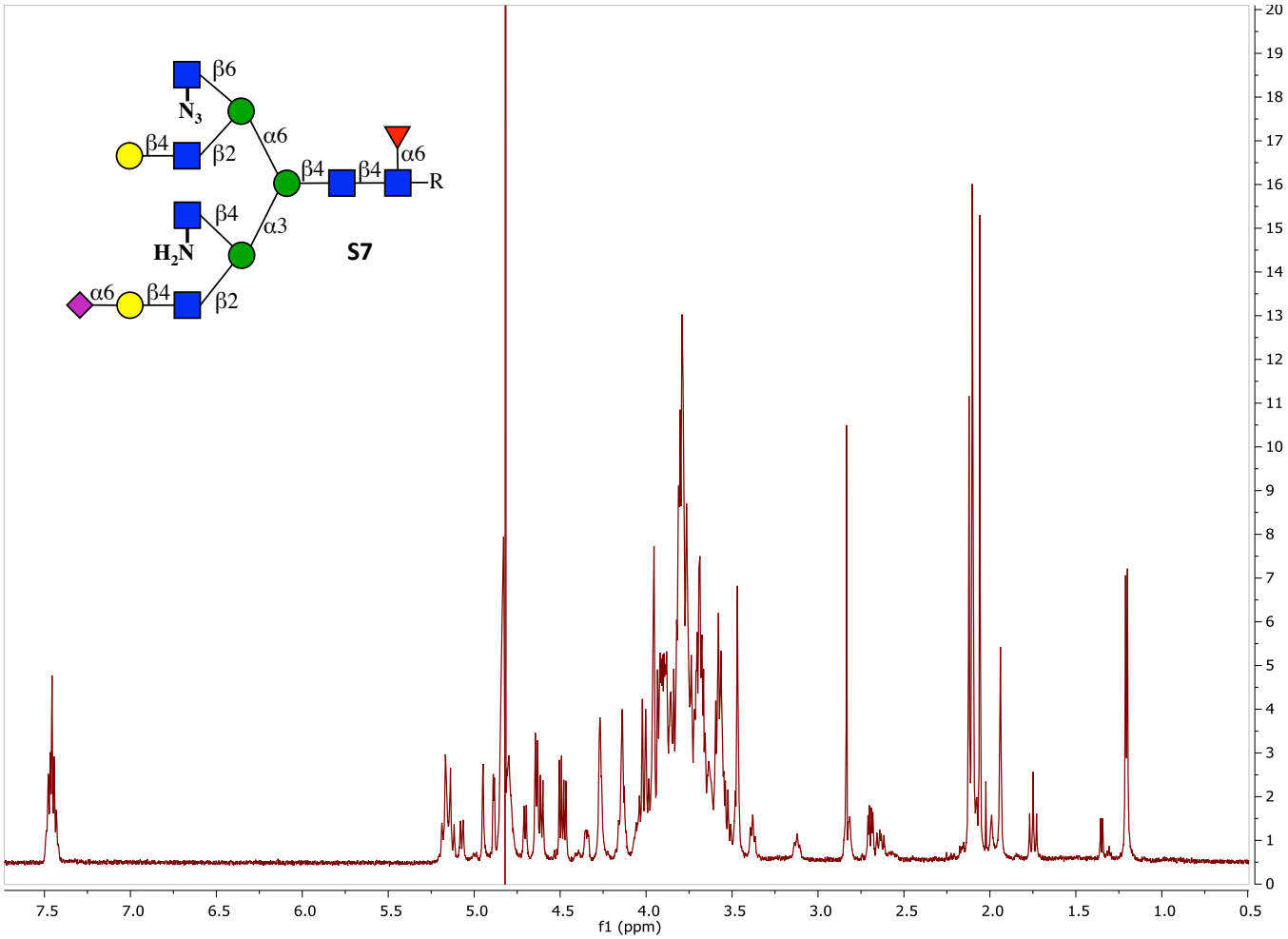
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S7
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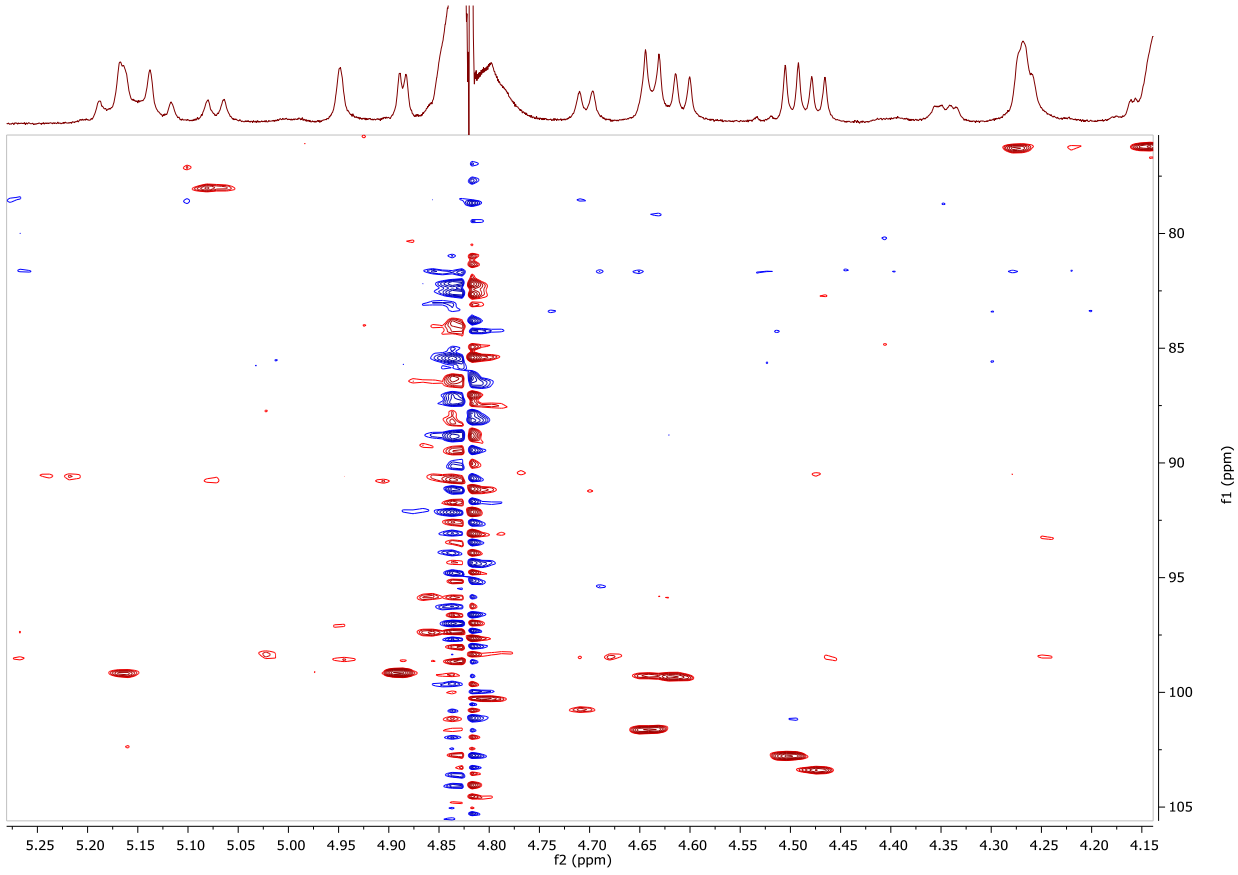
β2
f1 (ppm)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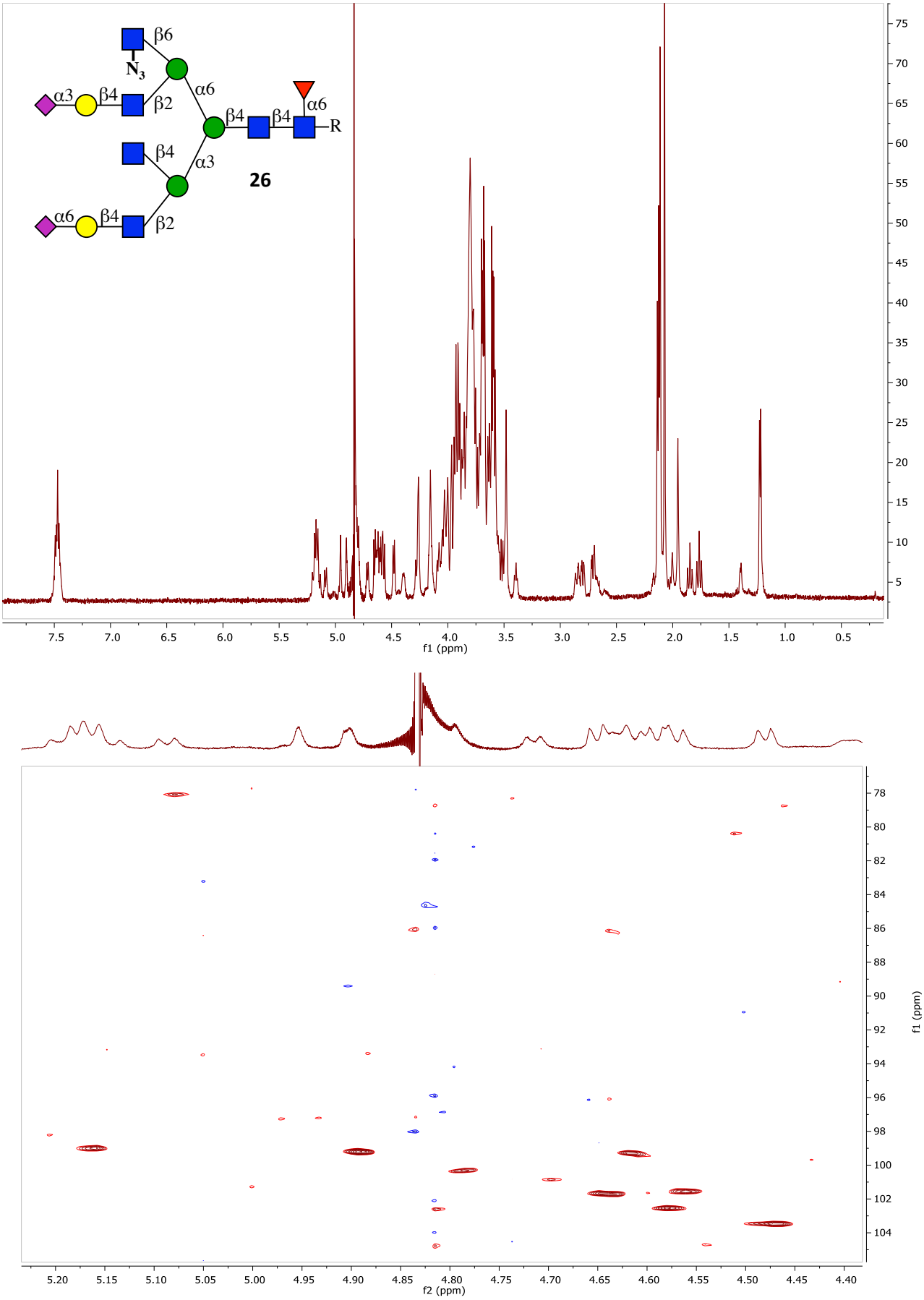
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β4
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α3
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α6
β4
β2
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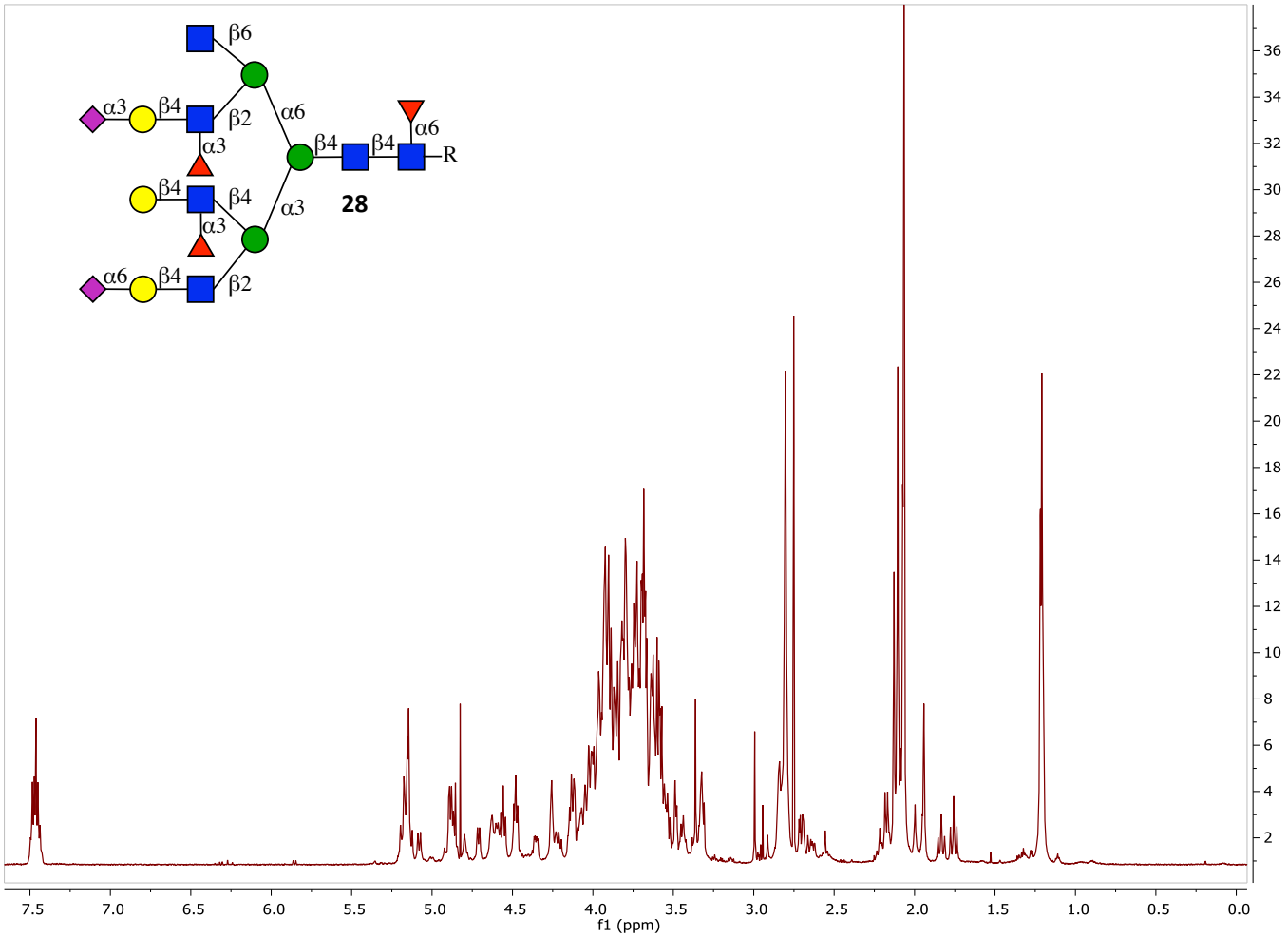
f2 (ppm)
f1 (ppm)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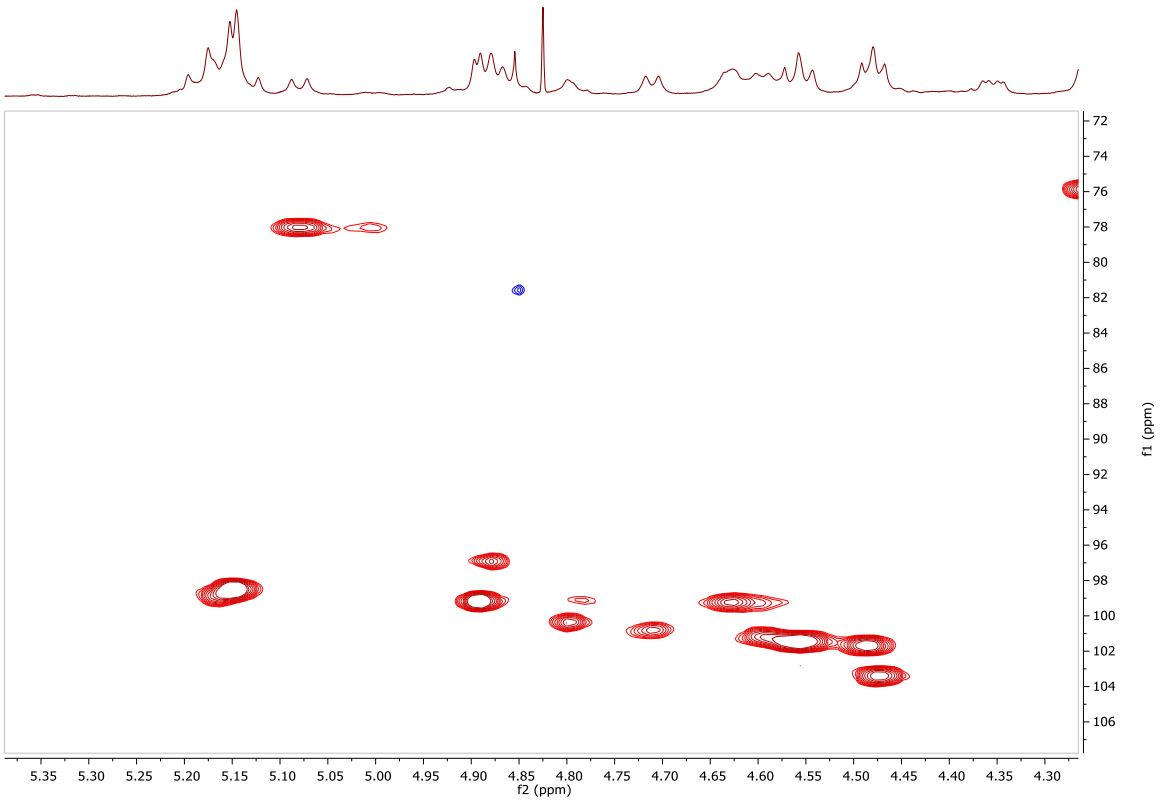
β4
β2
f1 (ppm)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