Supporting Information

Size-Controlled Chemoenzymatic Synthesis of Homogeneous Oligosaccharides of *Neisseria meningitidis* W Capsular Polysaccharide

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Protein sequence of the recombinant NmSiaDw

MAVIIFVNGIRAVNGLVKSSINTANAFAEEGLDVHLINFVGNITGAEHLYPPFHLHPNVKTSSIIDLFNDIP
ENVSCRNTPFYSIHQQFFKAEYSAHYKHVLMKIESLLSAEDSIIFTHPLQLEMYRLANNDIKSKAKLIVQIH
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DNA sequence of the recombinant NmSiaDw

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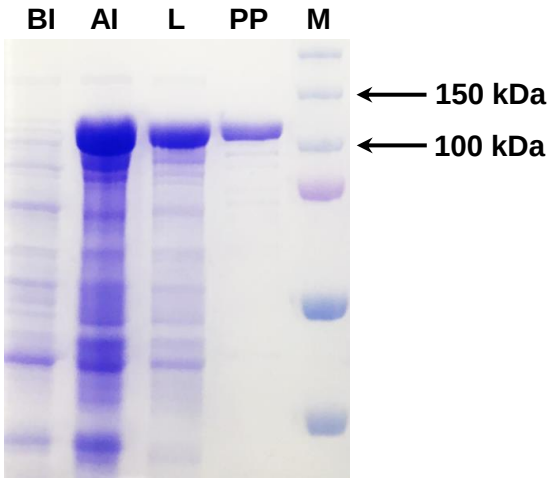


Figure S1. SDS-PAGE (10% Tris-Glycine gel) analysis for the expression and the purification of C-His_6-tagged $NmSiaD_W$ (Calculated MW is 121 kDa). Lanes: BI, whole cell extract before induction; AI, whole cell extract after induction; L, lysate after induction; PP, Ni^{2+}-NTA column purified protein; M, protein markers (Bio-Rad Precision Plus Protein™ Standards, 10–250 kDa).

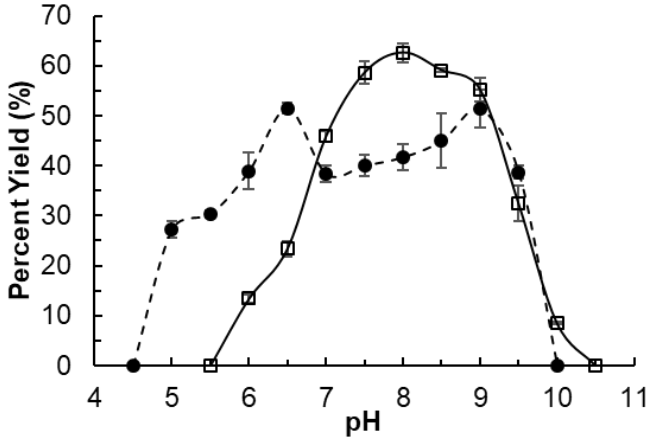


Figure S2. pH profiles of the galactosyltransferase (filled circles with dashed line) and the sialyltransferase (open squares with solid line) activities of $NmSiaD_W$ using **S1** and **G2**, respectively, as acceptor substrates. Buffers used were: Citric acid, pH 3.0–4.5; MES, pH 5.0–6.5; Tris-HCl, pH 7.0–9.0; CAPS, pH 9.5–11.0. Cbz-tagged acceptor substrates **S1** and **G2** as well as the corresponding glycosylated products were detected at $A_{215\ nm}$. Average percent yields of the reactions were plotted against reaction pH values. Error bars represent standard deviations of technical duplicates.

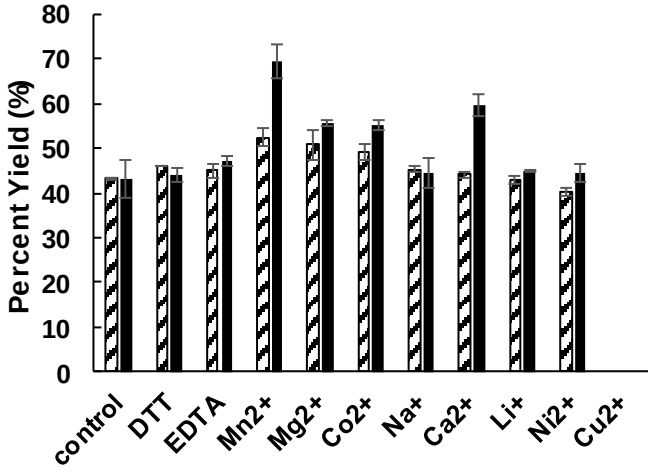


Figure S3. Metal effects on the galactosyltransferase (black bars) and the sialyltransferase (stripe bars) activities of $NmSiaD_W$ using **S1** and **G2**, respectively, as acceptor substrates. Cbz-tagged acceptor substrates **S1** and **G2** as well as the corresponding glycosylated products were detected at $A_{215\ nm}$. Average percent yields of the reactions were plotted against metal ions. Error bars represent standard deviations of technical duplicates.

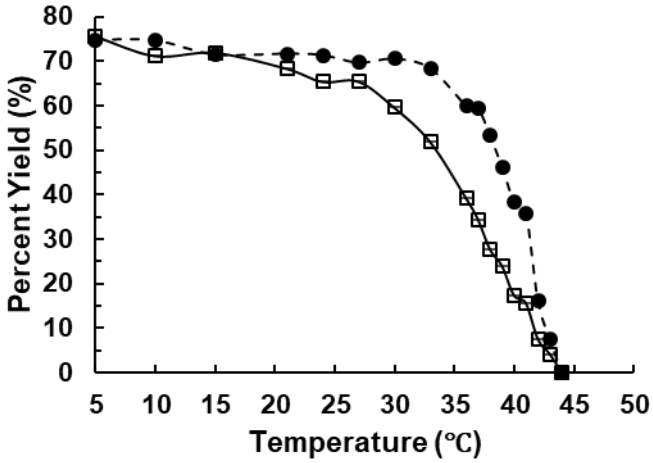


Figure S4. Thermostability of the galactosyltransferase (filled circles with dashed line) and the sialyltransferase (open squares with solid line) activities of $NmSiaD_W$ using **S1** and **G2**, respectively, as acceptor substrates. Cbz-tagged acceptor substrates **S1** and **G2** as well as the corresponding glycosylated products were detected at $A_{215\ nm}$. Average percent yields of the reactions were plotted against incubation temperatures. Error bars represent standard deviations of technical duplicates.

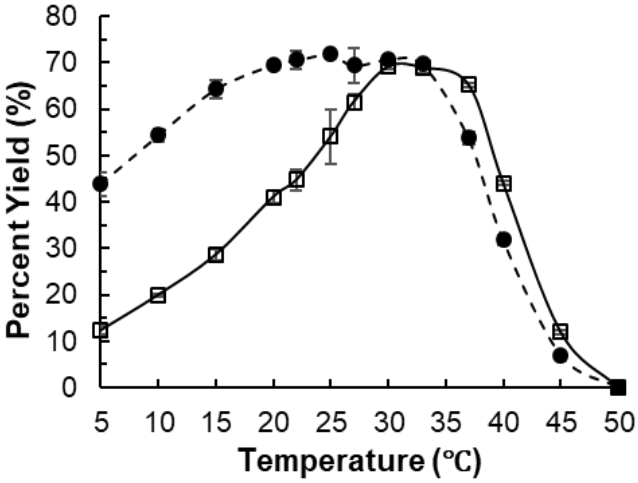
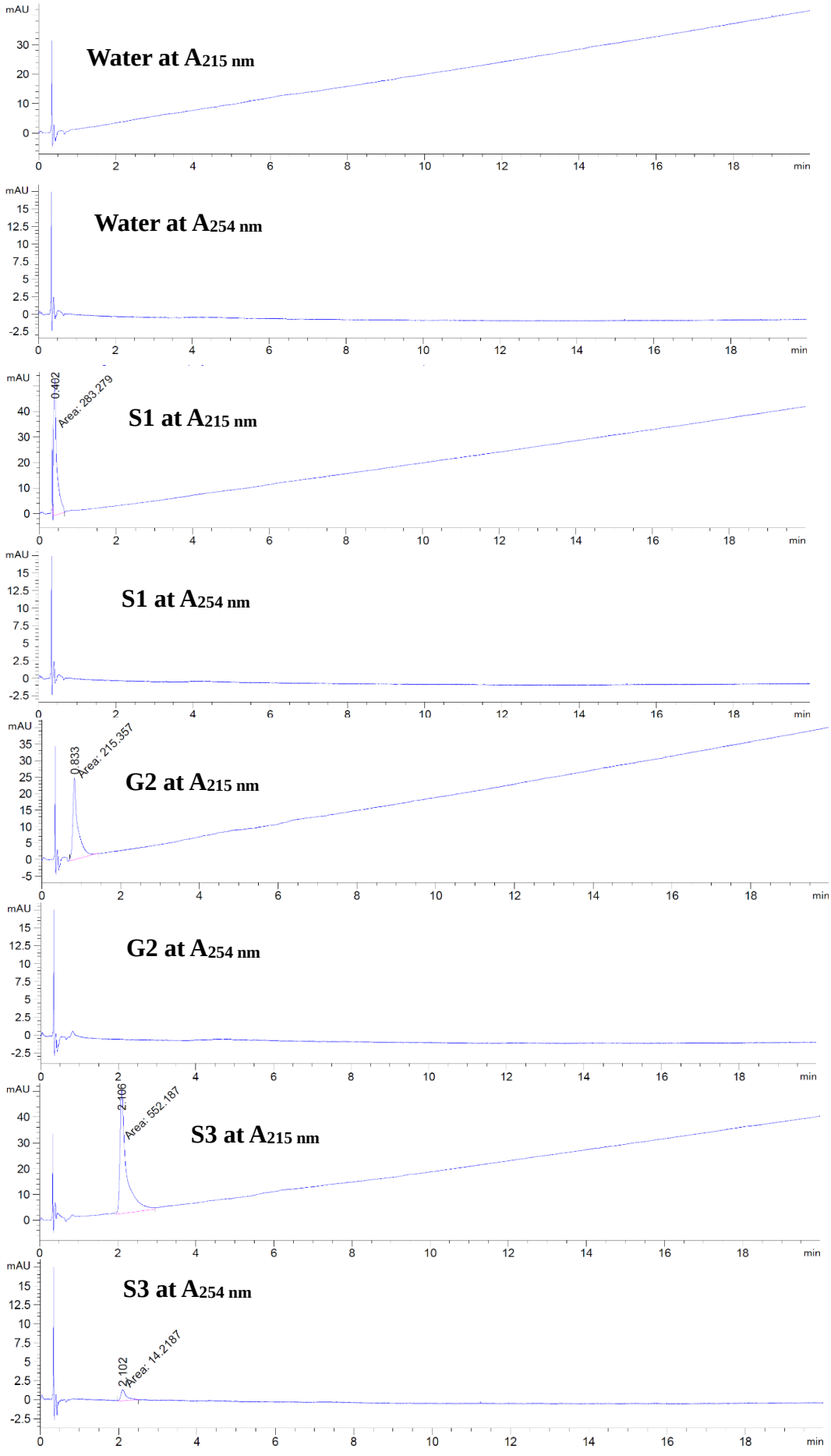
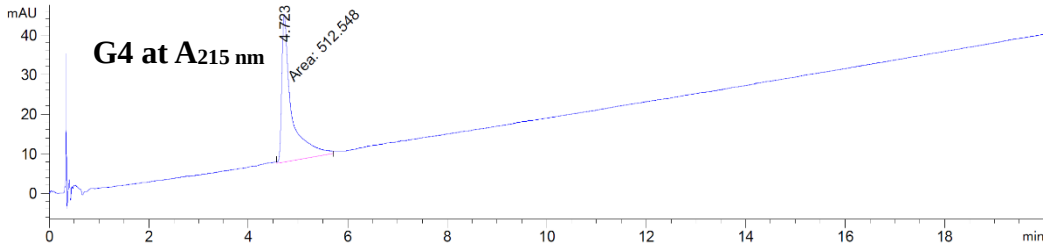
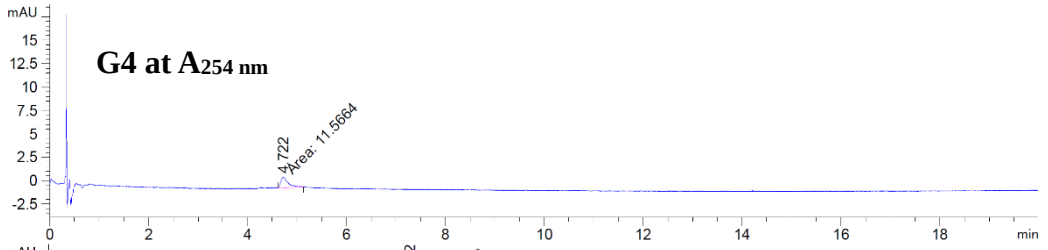
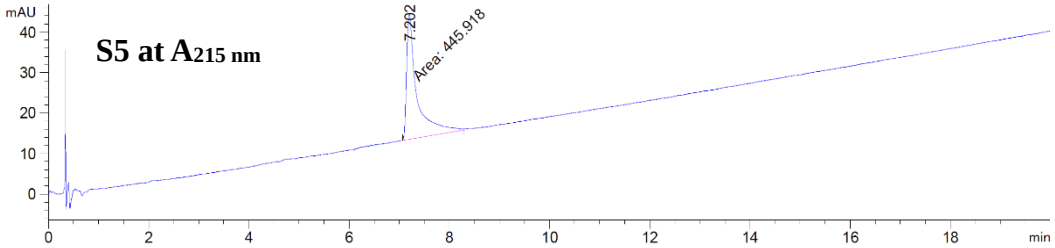


Figure S5. Temperature profiles of the galactosyltransferase (filled circles with dashed line) and the sialyltransferase (open squares with solid line) activities of $NmSiaD_W$ using **S1** and **G2**, respectively, as acceptor substrates. Cbz-tagged acceptor substrates **S1** and **G2** as well as the corresponding glycosylated products were detected at $A_{215\ nm}$. Average percent yields of the reactions were plotted against reaction temperatures. Error bars represent standard deviations of technical duplicates.


Water at A215 nm
Water at A254 nm
0.462
Area: 283.279
S1 at A215 nm
S1 at A254 nm
0.833
Area: 215.357
G2 at A215 nm
G2 at A254 nm
2.106
Area: 552.187
S3 at A215 nm
2.102
Area: 14.2187
S3 at A254 nm
mAU
min


G4 at A215 nm
4.723
Area: 512.548
mAU
min


G4 at A254 nm
4.722
Area: 11.5664
mAU
min


S5 at A215 nm
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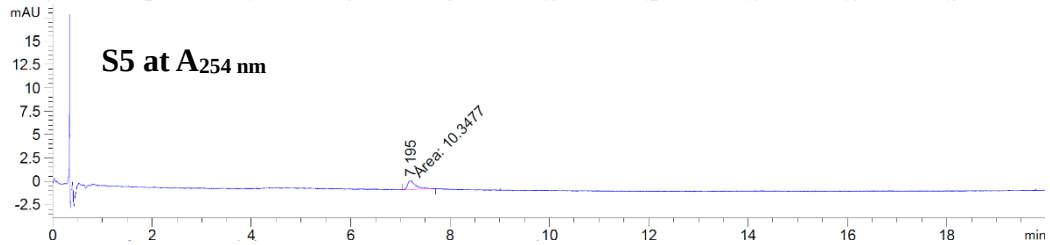
Area: 445.918
mAU
min


S5 at A254 nm
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Area: 10.3477
mAU
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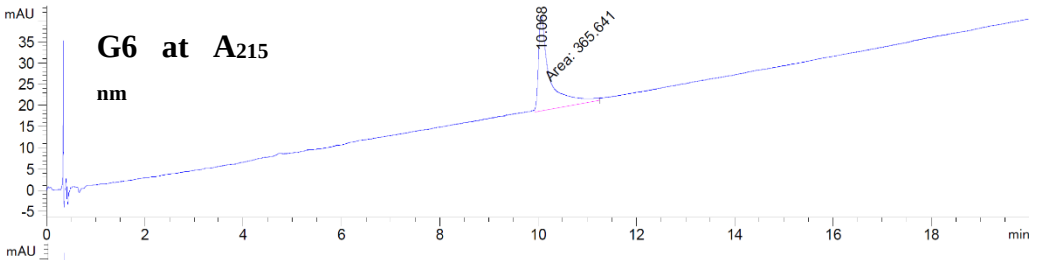
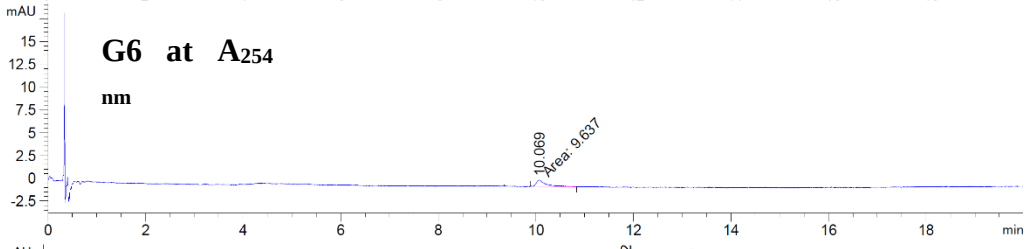

G6 at A215 nm
10.068
Area: 365.641
mAU
min


G6 at A254 nm
10.069
Area: 9.637
mAU
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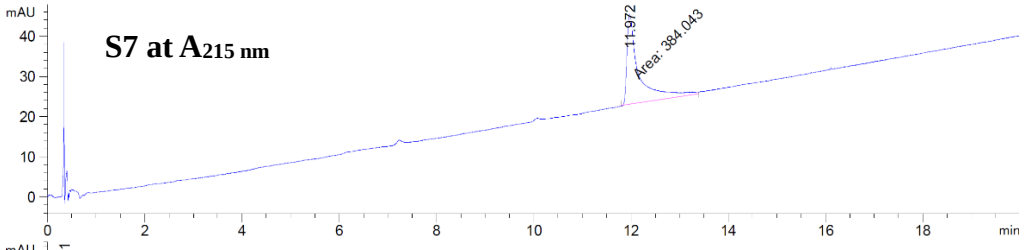
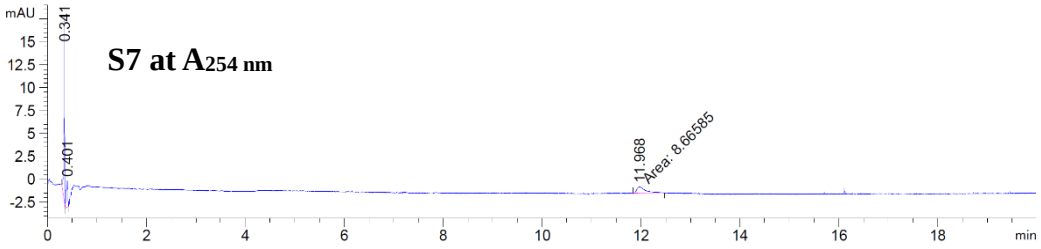
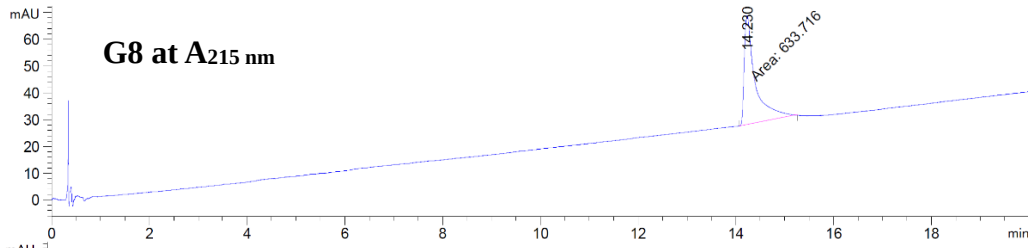

S7 at A215 nm
11.972
Area: 384.043
mAU
min


S7 at A254 nm
0.341
0.401
11.968
Area: 8.66585
mAU
min


G8 at A215 nm
mAU
14.230
Area: 633.716
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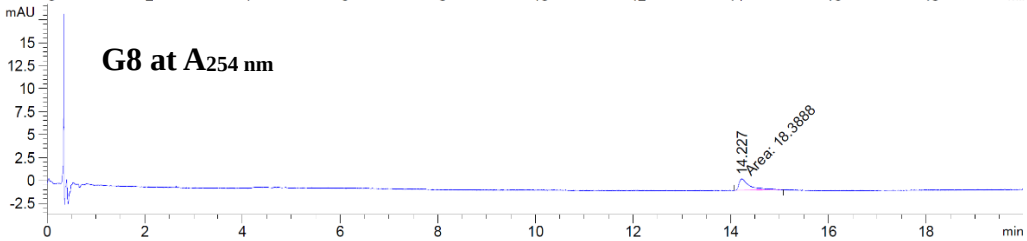
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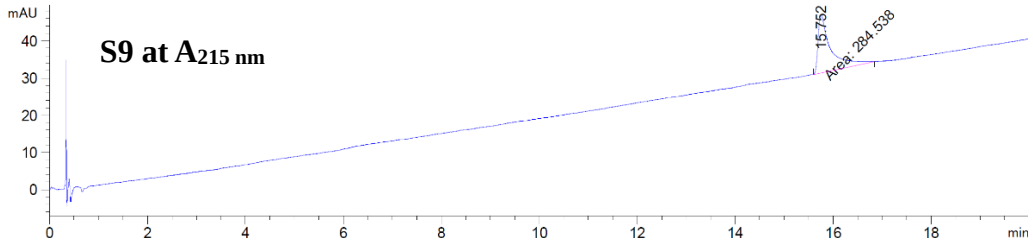
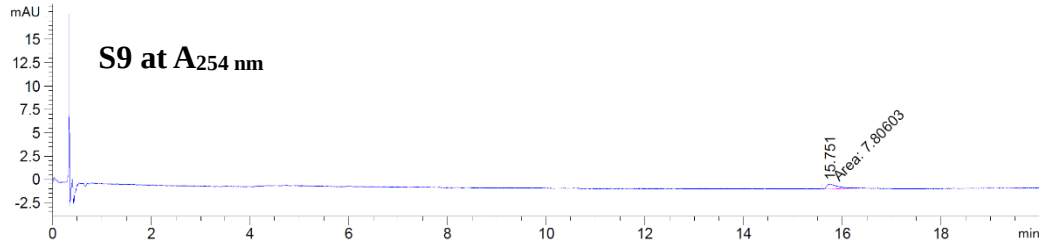
min


S9 at A215 nm
mAU
15.752
Area: 284.538
min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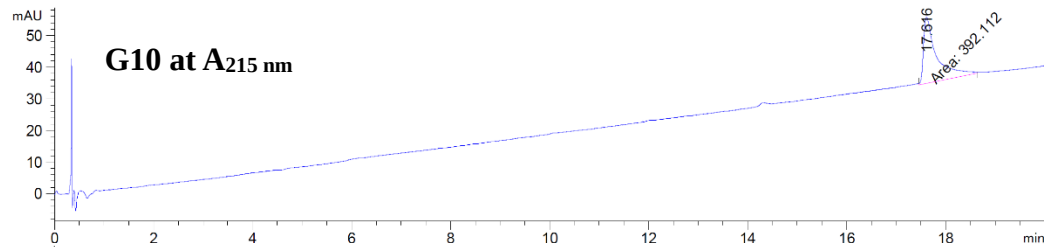
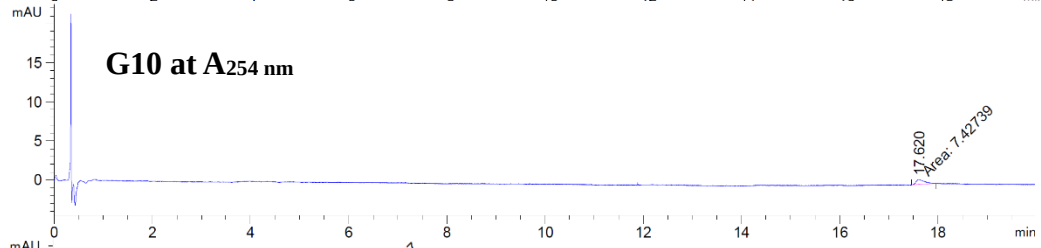
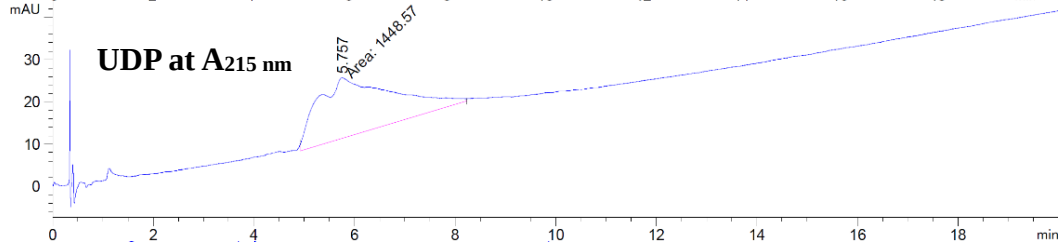
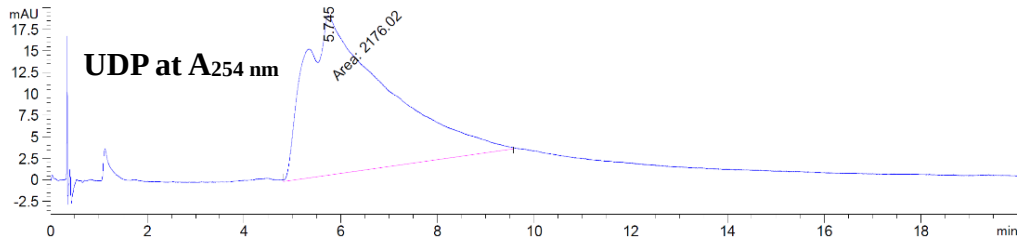

G10 at A215 nm
mAU
17.616
Area: 392.112
min


G10 at A254 nm
mAU
17.620
Area: 7.42739
min


UDP at A215 nm
mAU
5.757
Area: 1448.57
min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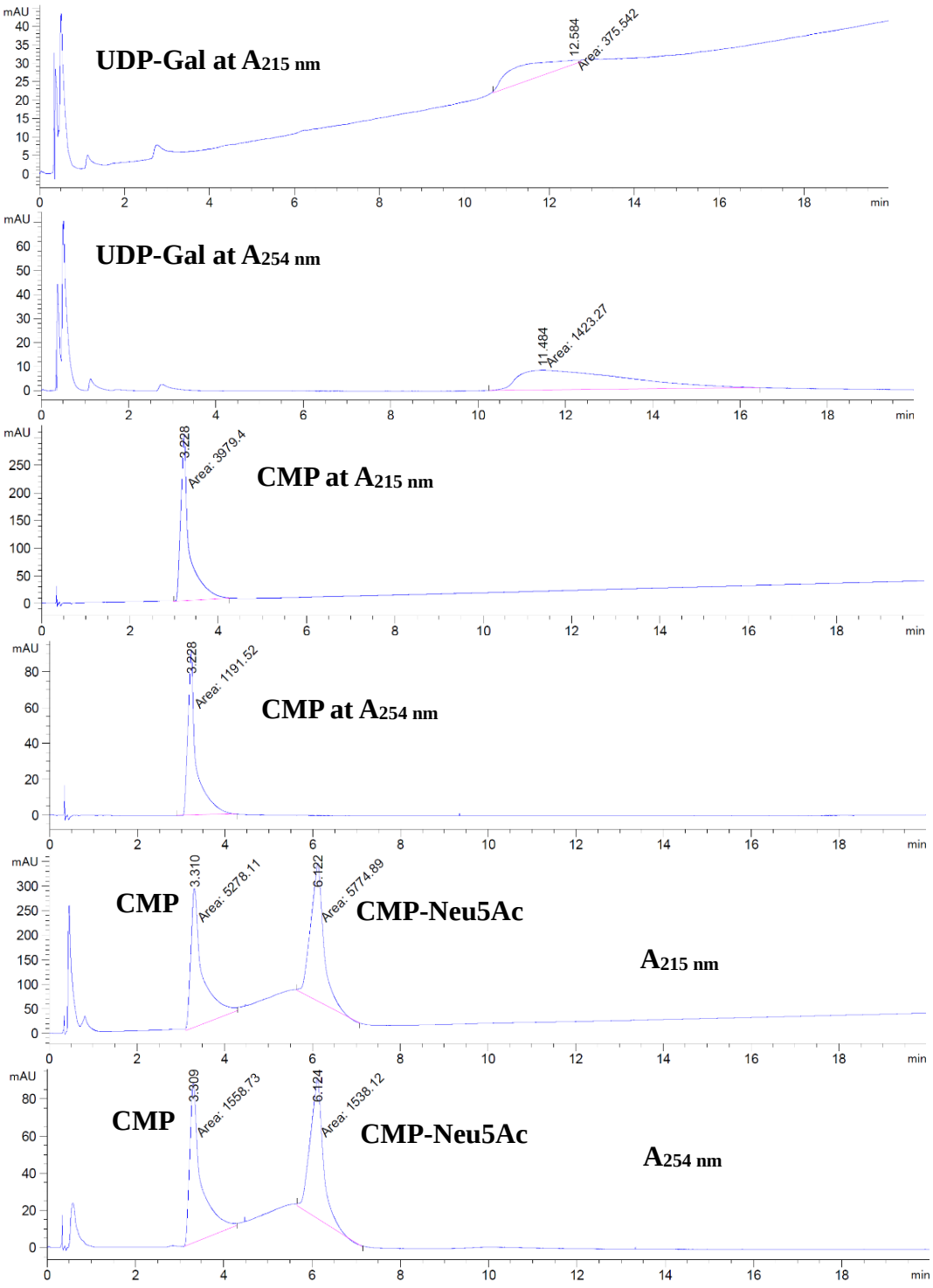


Figure S6. UHPLC chromatograms for mono- and oligosaccharides (**S1**–**G10**, 1 mM) as well as UDP, UDP-Gal, CMP, and CMP + CMP-Neu5Ac (10 mM) monitored at $A_{215\ nm}$ and $A_{254\ nm}$.

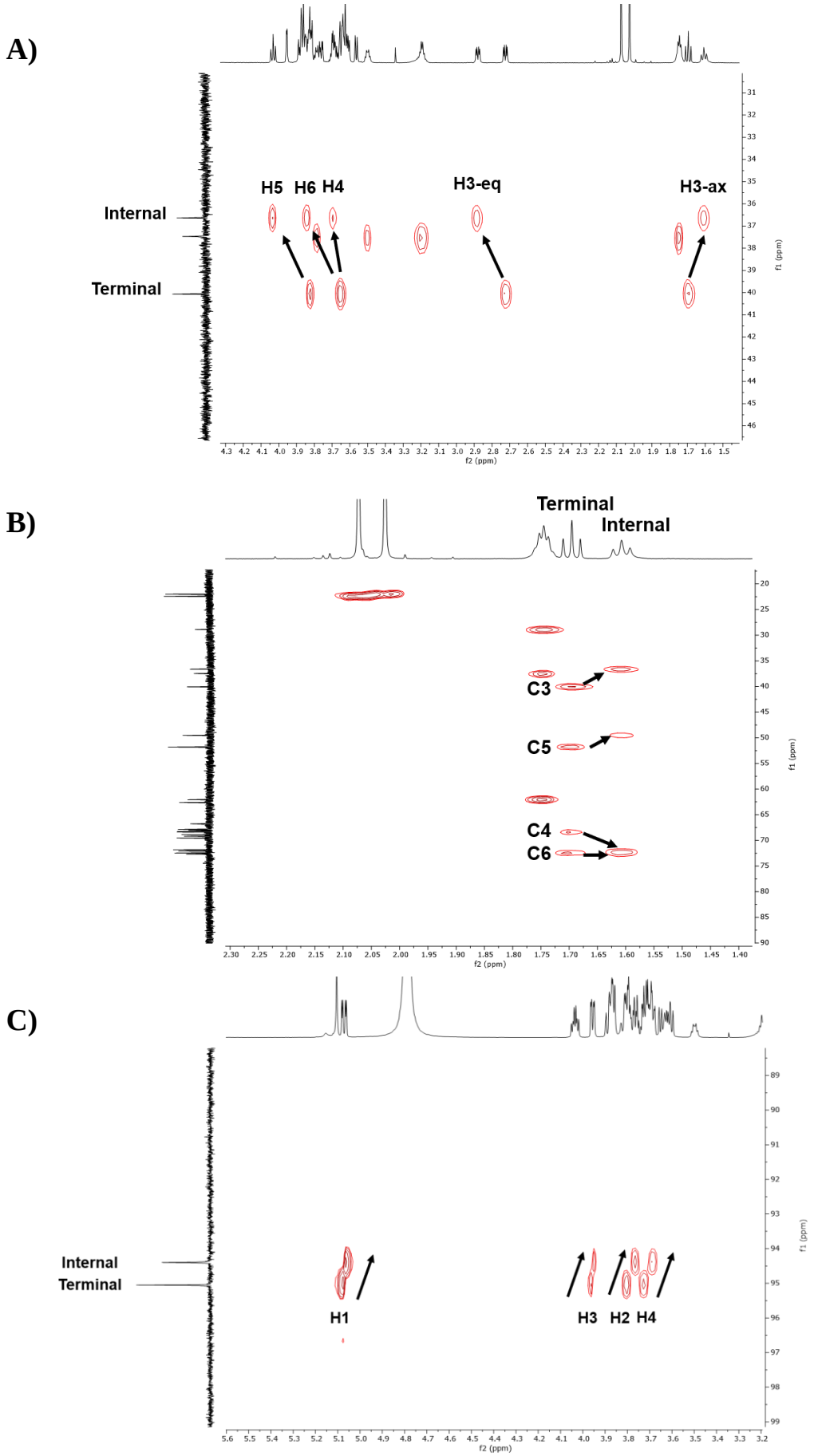
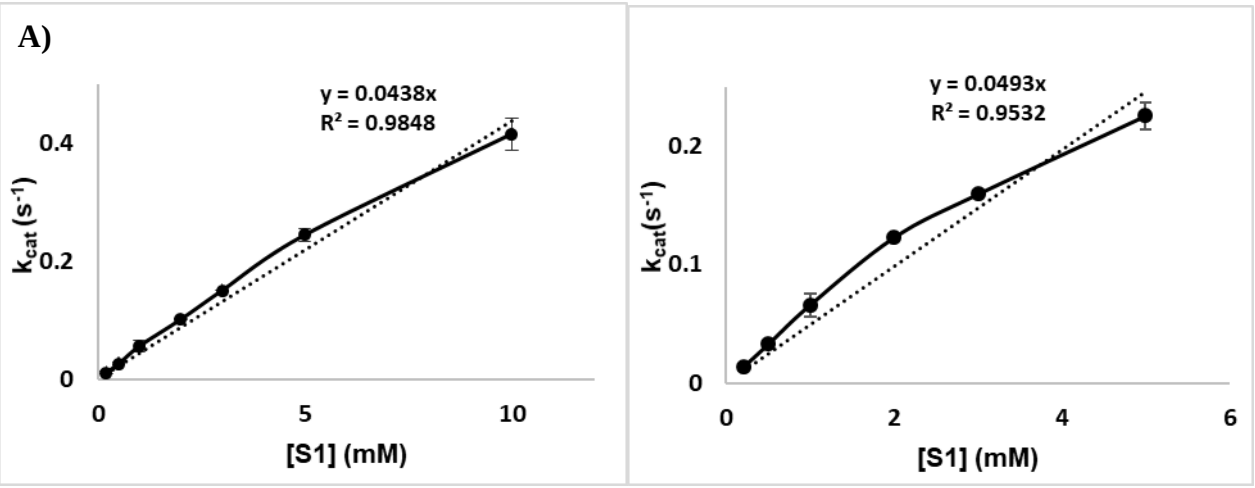


Figure S7. Sections of HSQC-TOCSY 90 ms spectra to compare the chemical shifts for internal and external Neu5Ac residues in sialyltrisaccharide **S3** (**A** and **B**) or Gal residues in galactosyltetrasaccharide **G4** (**C**).

A)



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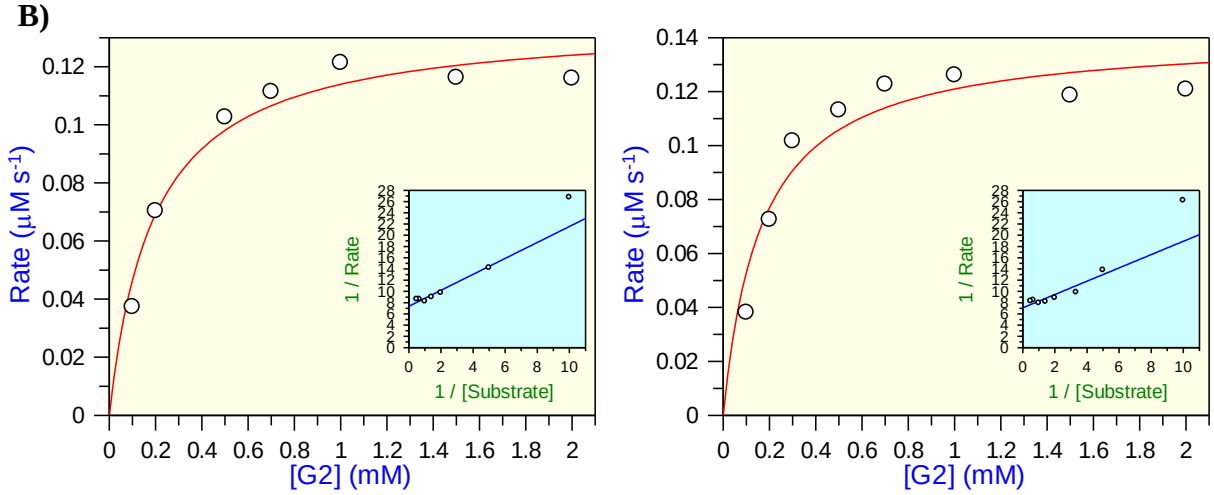

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Km	0.1931	0.0402

Parameter	Value	Std. Error
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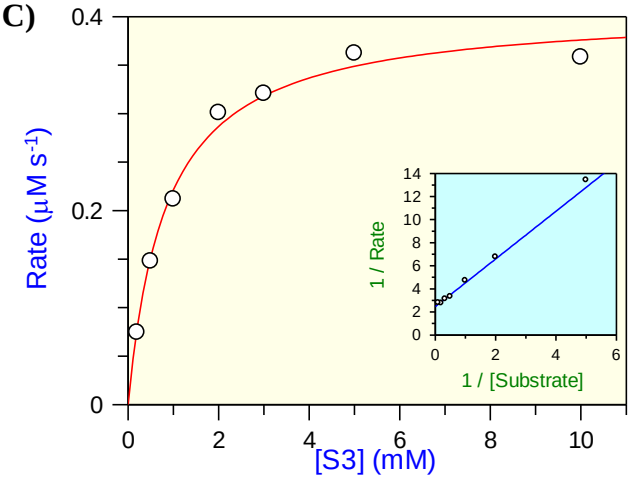
Km	0.1672	0.0445

C)



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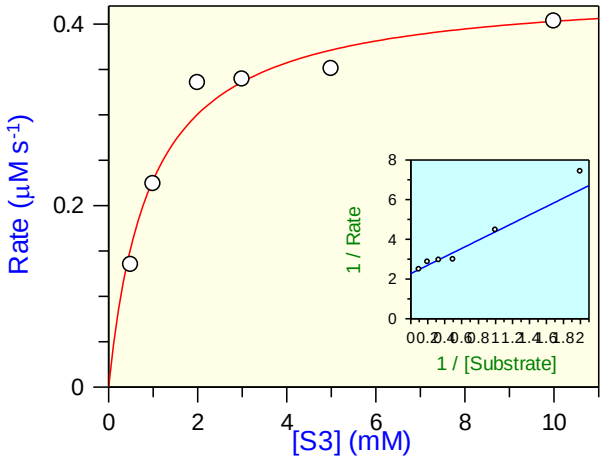

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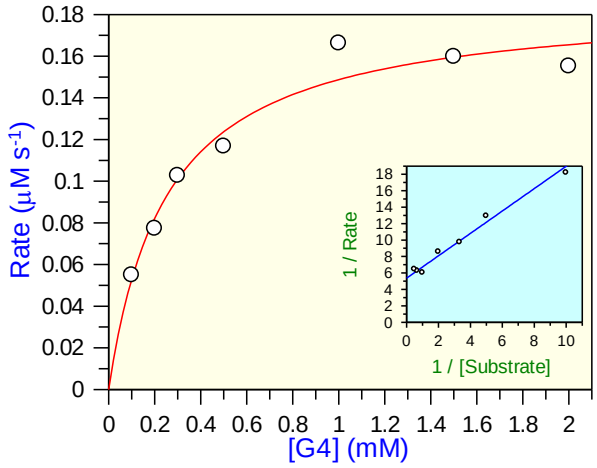
D)



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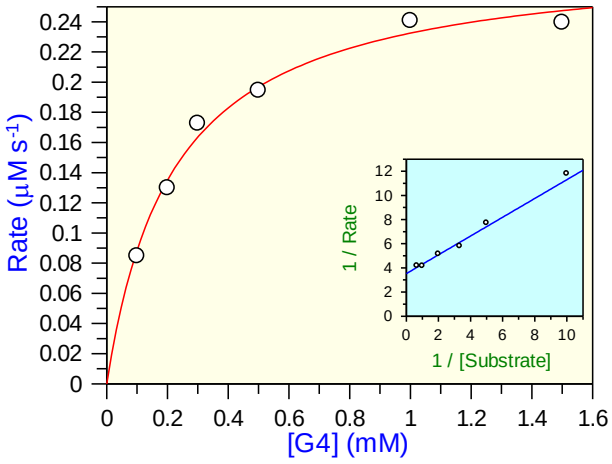
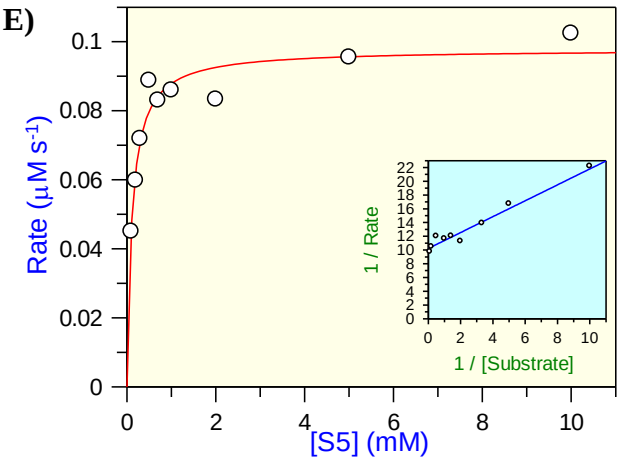
Km	0.2541	0.0488

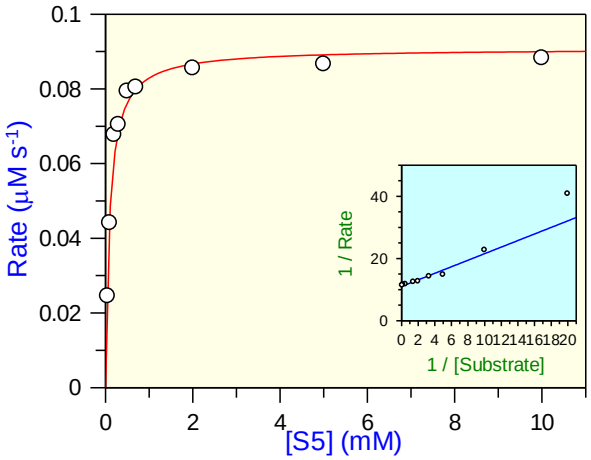


Parameter	Value	Std. Error
Vmax	0.2838	0.0100
Km	0.2207	0.0251

E)

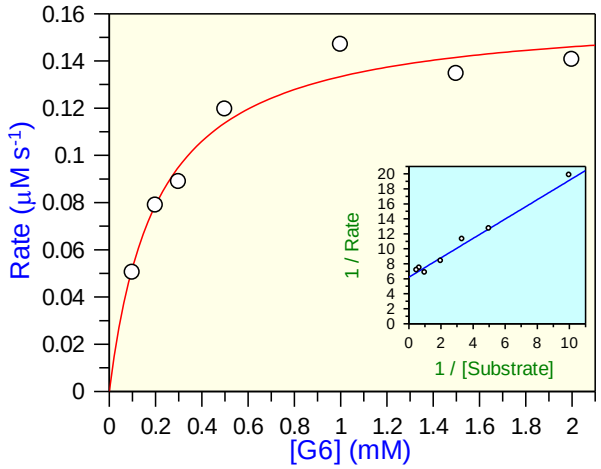


Parameter	Value	Std. Error
Vmax	0.0978	0.0031
Km	0.1131	0.0200

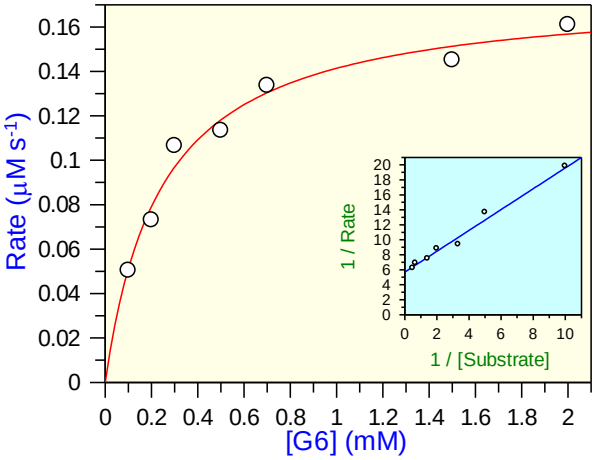


Parameter	Value	Std. Error
Vmax	0.0908	0.0023
Km	0.0957	0.0120

F)

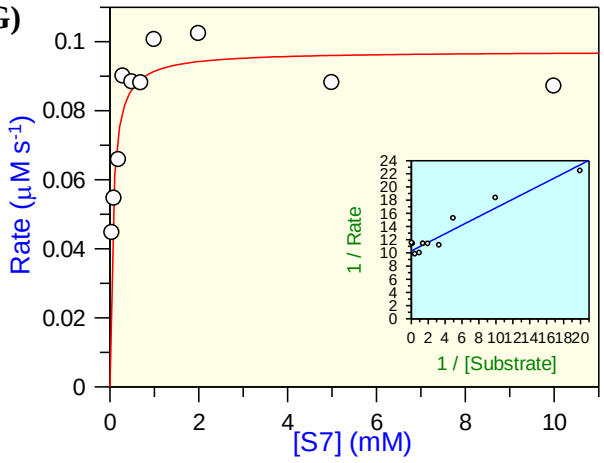


Parameter	Value	Std. Error
Vmax	0.1612	0.0078
Km	0.2087	0.0374

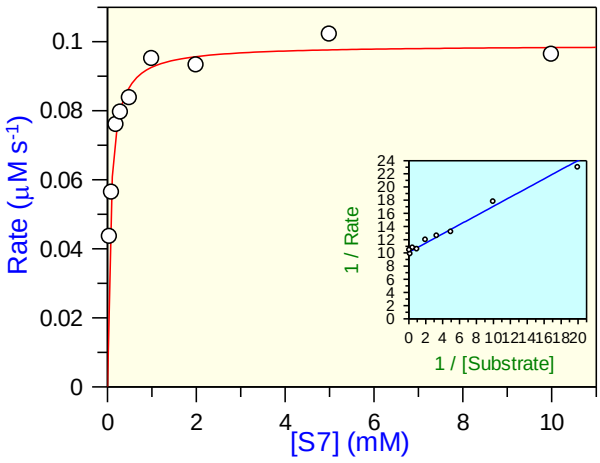


Parameter	Value	Std. Error
Vmax	0.1760	0.0071
Km	0.2449	0.0334

G)

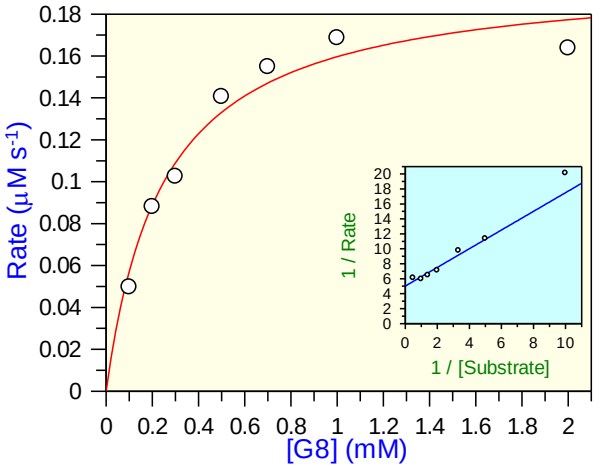


Parameter	Value	Std. Error
Vmax	0.0972	0.0039
Km	0.0637	0.0153

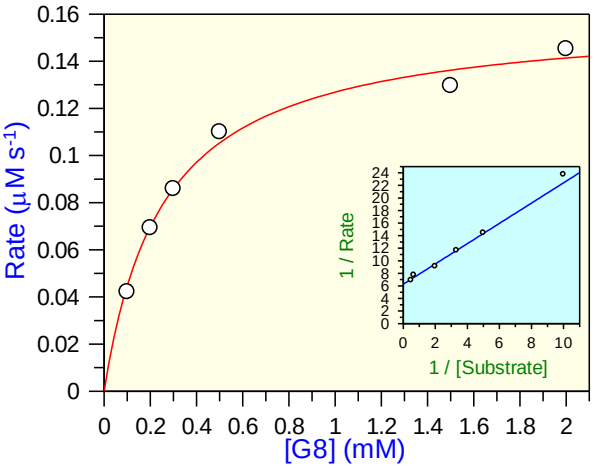


Parameter	Value	Std. Error
Vmax	0.0990	0.0016
Km	0.0689	0.0062

H)

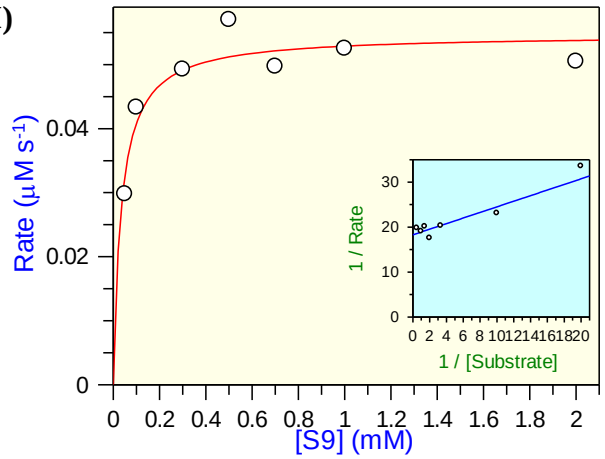


Parameter	Value	Std. Error
Vmax	0.1994	0.0117
Km	0.2491	0.0473

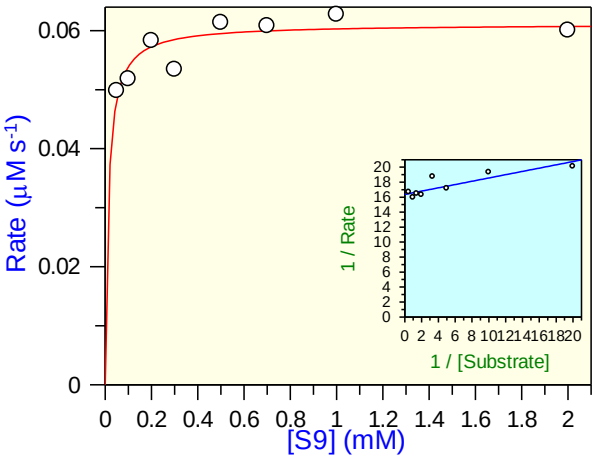


Parameter	Value	Std. Error
Vmax	0.1596	0.0053
Km	0.2575	0.0275

I)

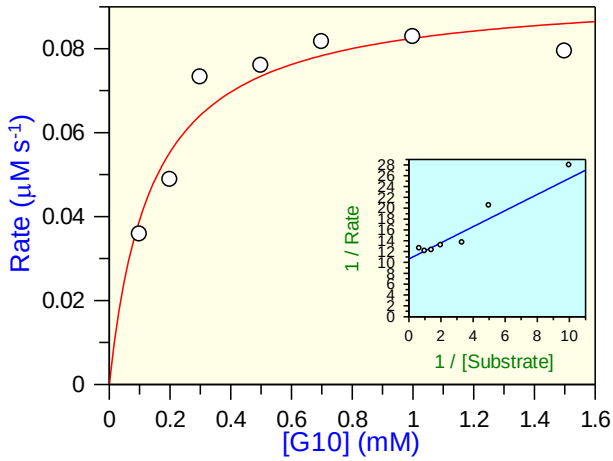


Parameter	Value	Std. Error
Vmax	0.0547	0.0021
Km	0.0341	0.0088

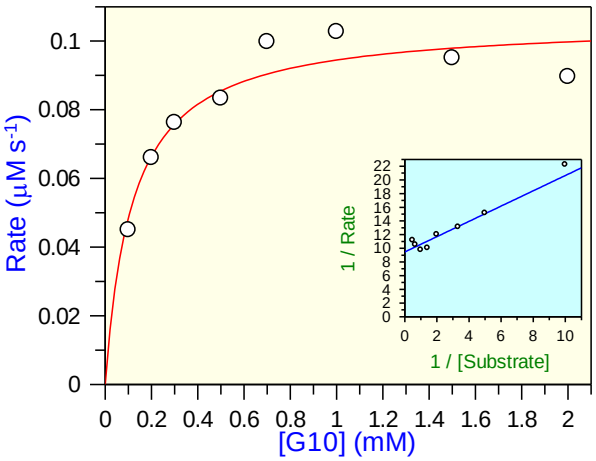


Parameter	Value	Std. Error
Vmax	0.0611	0.0014
Km	0.0134	0.0038

J)



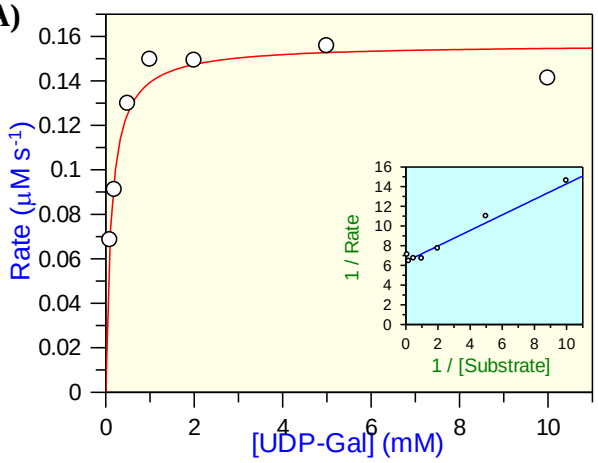
Parameter	Value	Std. Error
Vmax	0.0940	0.0061
Km	0.1398	0.0372

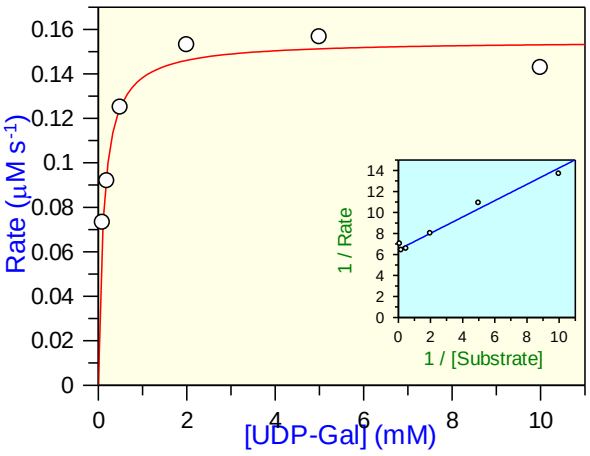


Parameter	Value	Std. Error
Vmax	0.1056	0.0051
Km	0.1180	0.0271

Figure S8. Graphs (**A**–**J**) for determining kinetics parameters using **S1**–**G10** as acceptor substrates, respectively. The unit for V_{max} is μM s^{-1} and the unit for K_m is mM.

A)

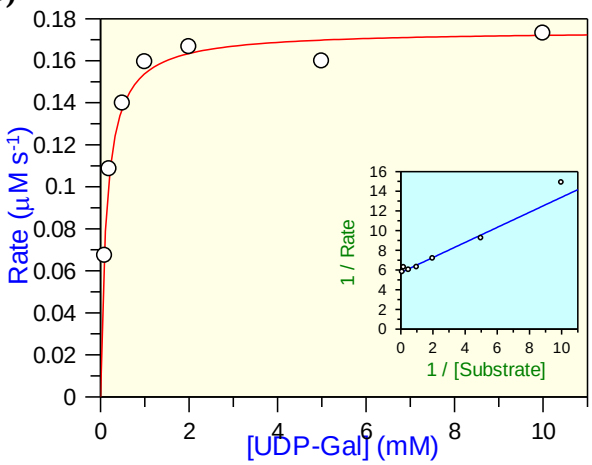


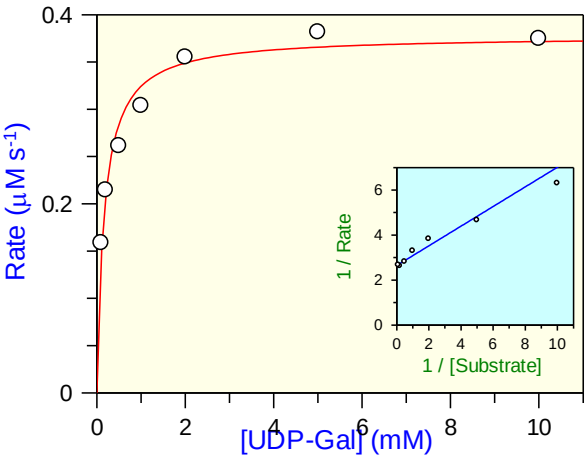


Parameter	Value	Std. Error
Vmax	0.1565	0.0050
Km	0.1235	0.0217

Parameter	Value	Std. Error
Vmax	0.1549	0.0046
Km	0.1205	0.0189

B)

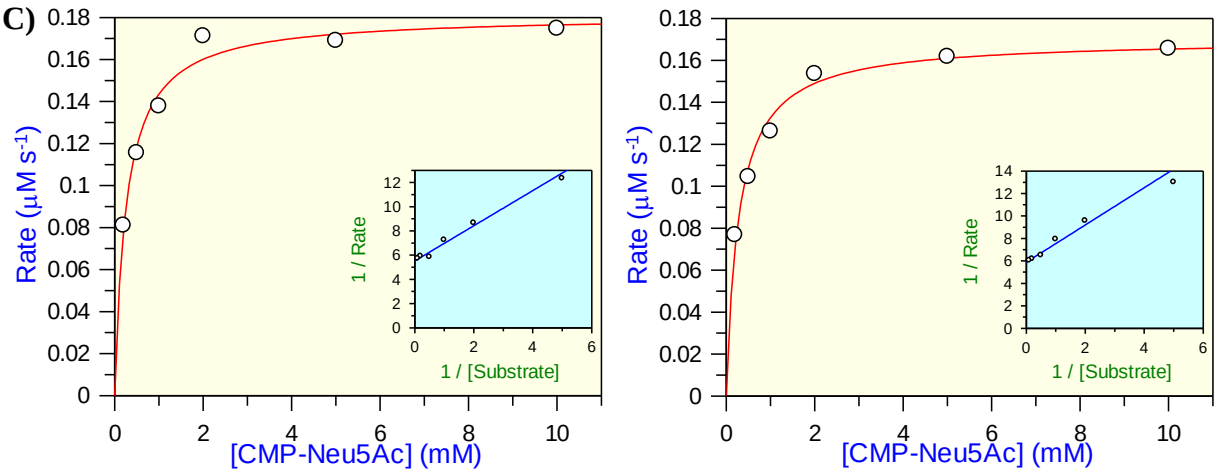




Parameter	Value	Std. Error
Vmax	0.1744	0.0040
Km	0.1335	0.0164

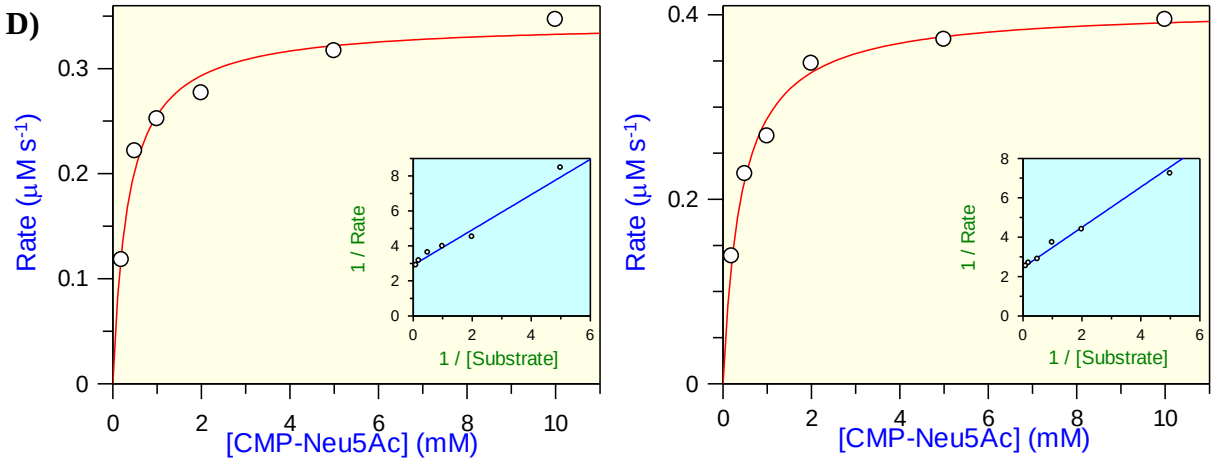
Parameter	Value	Std. Error
Vmax	0.3777	0.0110
Km	0.1650	0.0244

.



Parameter	Value	Std. Error
Vmax	0.1811	0.0049
Km	0.2627	0.0361

Parameter	Value	Std. Error
Vmax	0.1701	0.0040
Km	0.2820	0.0327



Parameter	Value	Std. Error
Vmax	0.3441	0.0116
Km	0.3465	0.0536

Parameter	Value	Std. Error
Vmax	0.4077	0.0096
Km	0.4172	0.0423

Figure S9. Graphs for determining NmSiaD$_W$ kinetics parameters by varying UDP-Gal [using **S3** (**A**) or **S9 (B**) as an acceptor] or CMP-Neu5Ac concentrations [using **G2** (**C**) or **G10** (**D**) as an acceptor]. The unit for V_{max} is μM s^{-1} and the unit for K_M is mM.

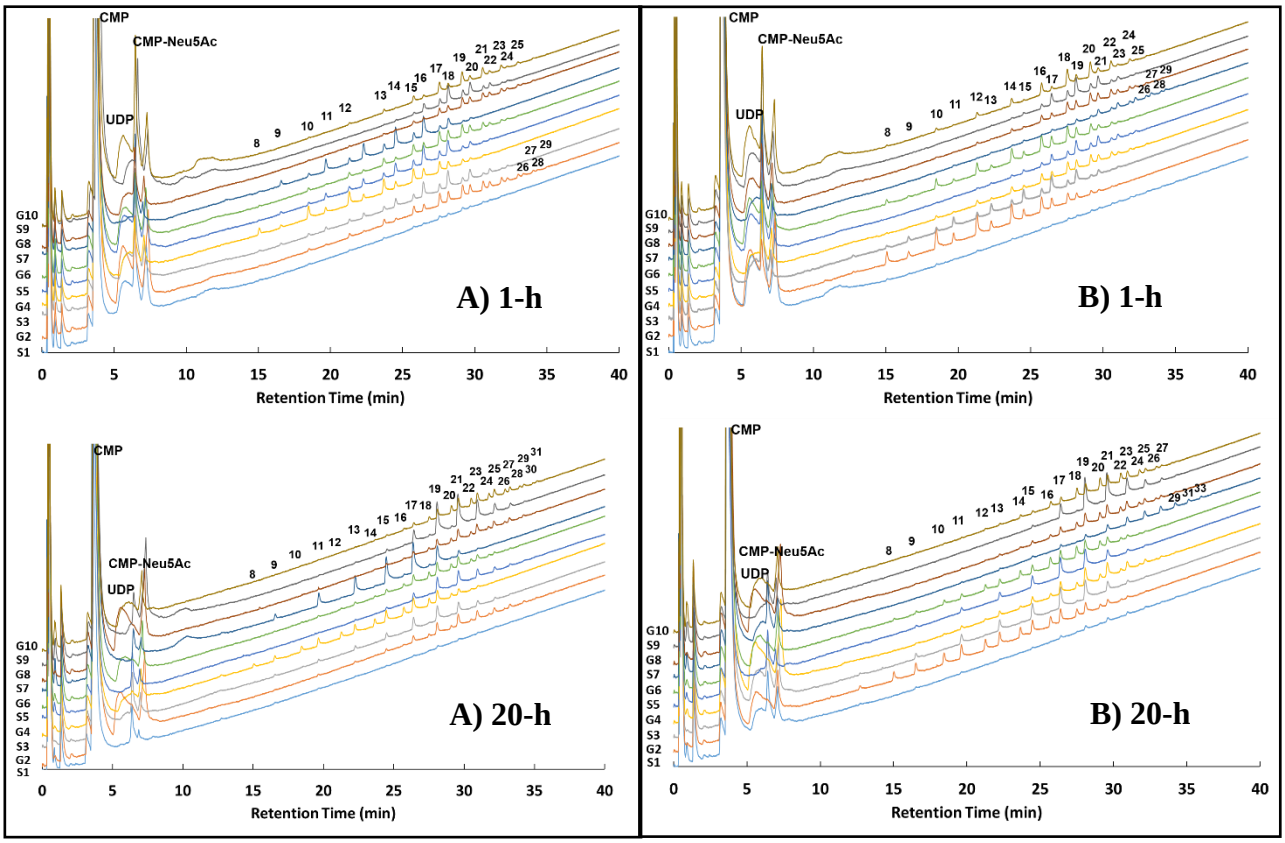


Figure S10. Duplicate results (**A** and **B**) of product profiles for polymerization reactions catalyzed by NmSiaD$_W$ in the presence of 10 equivalents (50 mM) of both UDP-Gal and CMP-Neu5Ac donors using an acceptor substrate (5 mM) selected from oligosaccharides of different sizes (**S1–G10**) with a reaction duration of 1 hour (Top panels) or 20 hours (Bottom panels). Samples were analyzed by ultra-high performance liquid chromatography (UHPLC) at $A_{215\ nm}$ using an AdvanceBio Glycan Map column.

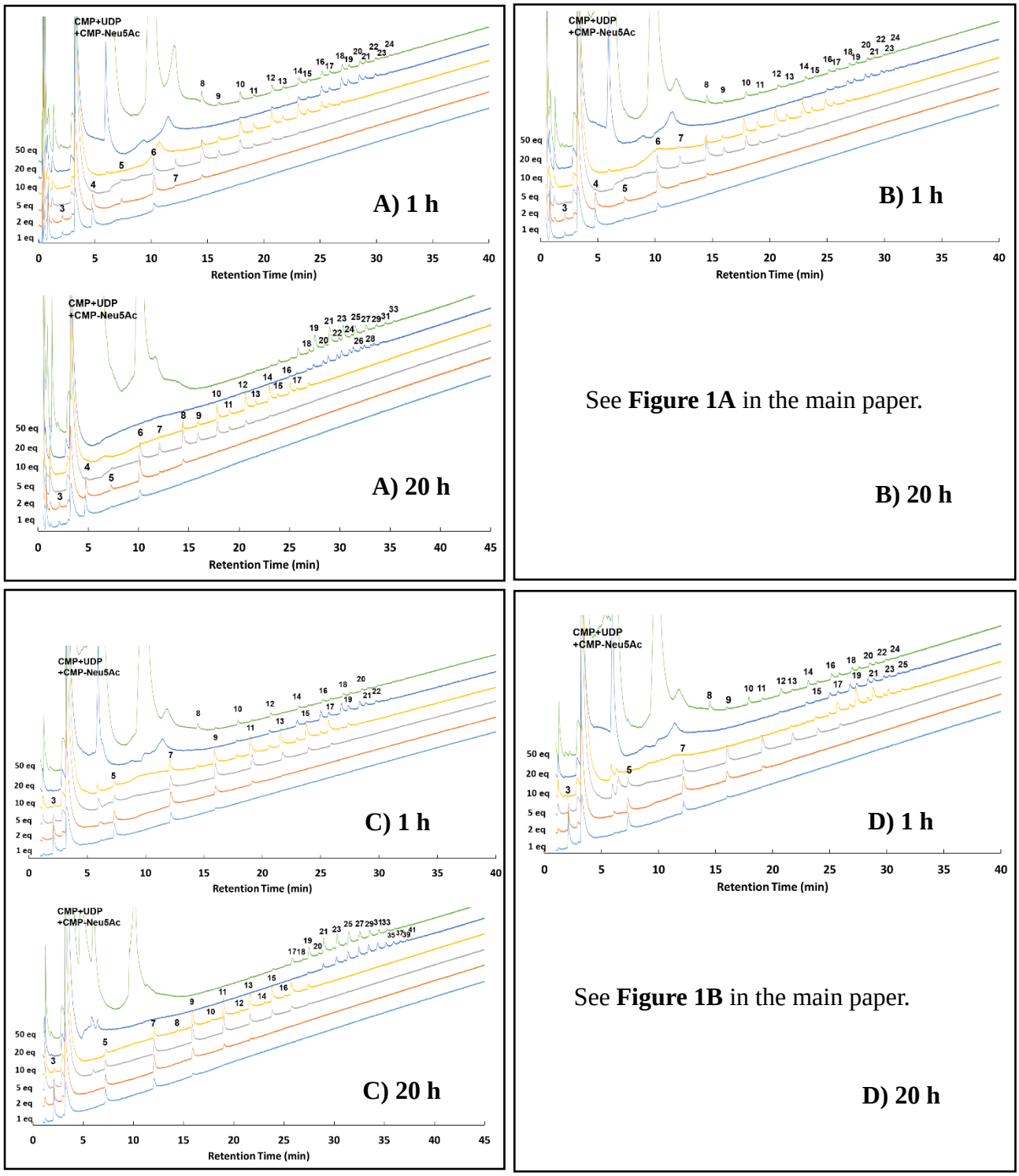


Figure S11. Duplicate results (**A** and **B** or **C** and **D**) of product profiles for polymerization reactions catalyzed by $NmSiaD_W$ in the presence of different equivalents of both UDP-Gal and CMP-Neu5Ac donors (1, 2, 5, 10, 20, or 50 equivalents) using **G2** (**A** and **B**) or **S3** (**C** and **D**) as the acceptor substrate (5 mM) with a reaction duration of 1 hour (Top panels) or 20 hours (Bottom panels).

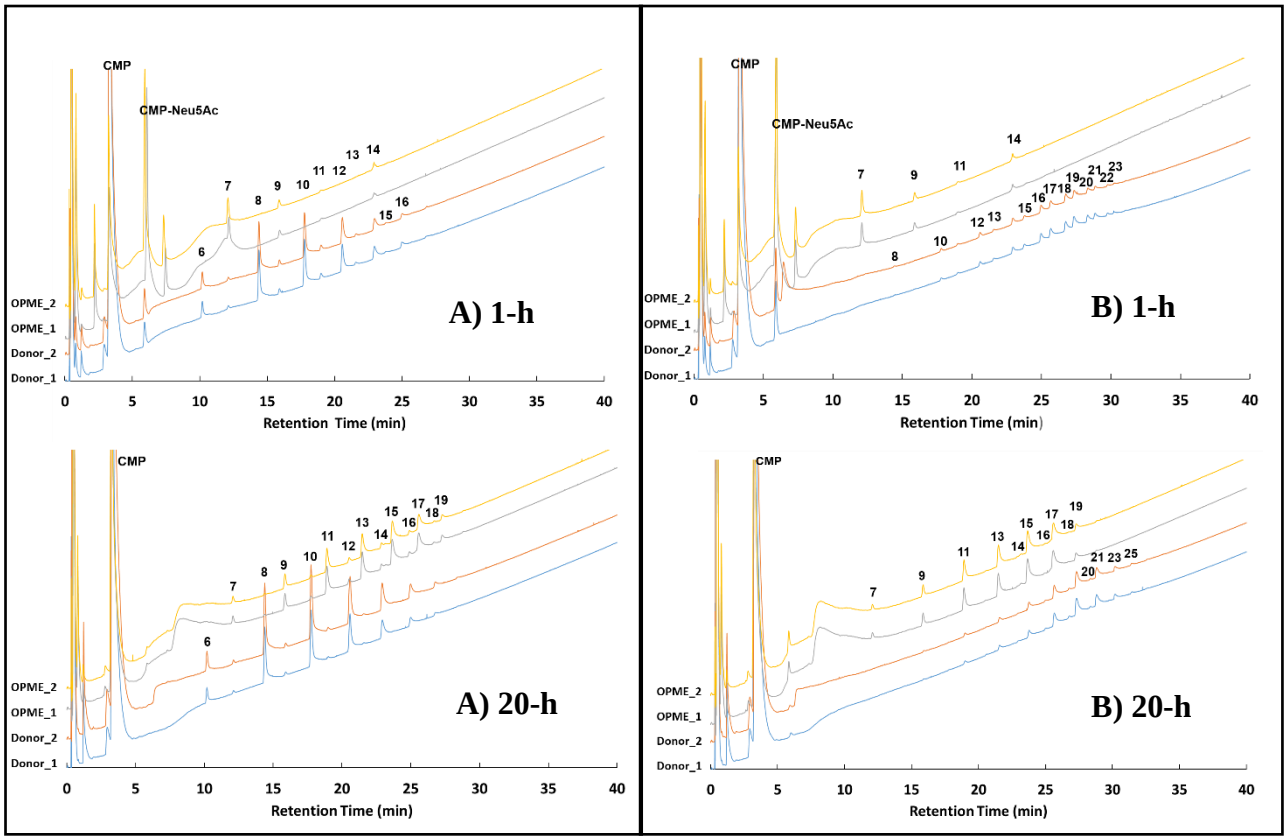


Figure S12. Duplicate results of product profiles for polymerization reactions catalyzed by $NmSiaD_W$ in the presence of 10 equivalents (50 mM) of both UDP-Gal and CMP-Neu5Ac donors or OPME reaction in the presence of 10 equivalents (50 mM) of donor precursors, using 5 mM **G2** (**A**) or **S3** (**B**) as an acceptor for a reaction duration of 1 hour (Top panels) or 20 hours (Bottom panels). Samples were analyzed by ultra-high performance liquid chromatography (UHPLC) at $A_{215\ nm}$ using an AdvanceBio Glycan Map column.

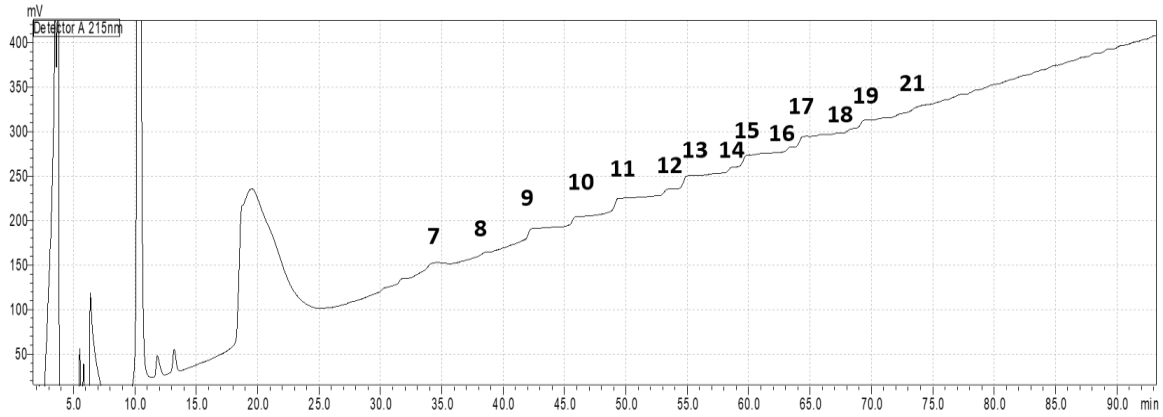
Table S1. Average molecular masses and polydispersity of product profiles of 20-hour reactions using different ratios (1–50 equivalents) of donors versus **G2** (5 mM) in **Figure 1A**. M_n: number average molecular mass; M_w: mass average molecular mass; PDI: polydispersity index, PDI=M_w/M_n.

Donor Equivalents	1	2	5	10	20	50
M_n (g/mol)	951	1310	2135	3072	5325	6050
M_w (g/mol)	1051	1416	2237	3215	5487	6609
PDI	1.11	1.08	1.05	1.05	1.03	1.09

Table S2. Average molecular masses and polydispersity of product profiles of 20-hour reactions using different ratios (1–50 equivalents) of donors versus **S3** (5 mM) in **Figure 1B**. M_n: number average molecular mass; M_w: mass average molecular mass; PDI: polydispersity index, PDI=M_w/M_n.

Donor Equivalents	1	2	5	10	20	50
M_n (g/mol)	1333	1704	2567	4313	7051	7526
M_w (g/mol)	1453	1888	2870	4436	7272	8554
PDI	1.09	1.11	1.12	1.03	1.03	1.14

Table S3. LC-MS analysis of OPME polymerization in the presence of 10 equivalents (50 mM) of donor precursors, using 5 mM of **G2** with a reaction time of 20 hours. DP: degree of polymerization or the number of monosaccharide units in the compound.



DP	Retention Time (min)	Calculated Mass (g/mol) for molecules containing two deprotonated carboxylate groups	Raw Data for *m/z* (2 negative charges)
7	35	1857.63	928.85
8	38	2019.68	1009.85
9	43	2310.78	1155.70
10	47	2472.83	1236.60
11	50	2763.93	1382.10
12	53	2925.98	1462.95
13	55	3217.08	1608.60
14	59	3379.13	1689.60
DP	Retention Time (min)	Calculated Mass (g/mol) for molecules containing three deprotonated carboxylate groups	Raw Data for *m/z* (3 negative charges)
15	60	3669.22	1223.15
16	63	3831.27	1277.20
17	64	4122.37	1374.35
18	68	4284.42	1428.05
19	69	4575.52	1525.35
21	74	5028.67	1675.95

Experimental details

General methods

Chemicals were purchased and used as received without further purification. NMR spectra were recorded using an 800 MHz Bruker Avance III spectrometer in the NMR facility at the University of California, Davis. Chemical shifts are reported in parts per million (ppm) on the δ scale. High resolution electrospray ionization (ESI) mass spectra (HRMS) were obtained using a Thermo Electron LTQ-Orbitrap Hybrid MS in the Mass Spectrometry Facility at the University of California, Davis. UHPLC assays were performed using Agilent 1290 Infinity LC with an EclipsePlus C18 (Rapid Resolution HD, 1.8 μm, 2.1×50 mm, 959757-902) or an AdvanceBio Glycan Map (1.8 μm, 2.1×150 mm, 859700-913) column from Agilent Technologies. Reverse phase chromatography purification of products was performed with a C18 column (ODS-SM, 50 mm, 120 Å, 3.0 × 20 cm) from Yamazen Corporation on a CombiFlash Rf 200i system. Galactose was from Fisher Scientific, Inc. *N*-Acetylneuraminic acid (Neu5Ac) was from Inalco (Italy). Adenosine 5'-triphosphate (ATP), cytosine 5'-triphosphate (CTP) and uridine 5'-triphosphate (UTP) were purchased from Hangzhou Meiya Pharmaceutical Co. Ltd. Recombinant enzymes *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS),[1] *Pasteurella multocida* inorganic pyrophosphatase (PmPpA),[2] *Streptococcus pneumoniae* TIGR4 galactokinase (SpGalK),[3] *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP)[4] were expressed and purified as described previously.

Cloning, protein overexpression and purification

$NmSiaD_W$ synthetic gene (GenBank accession number Y13970) with sequence optimized for expression in *E. coli* was customer synthesized by GeneArt and cloned in pMA-RQ (ampR) vector.

To subclone $NmSiaD_W$ as an C-His_6-tagged fusion protein in pET22b(+) vector, two primers were designed for polymerase chain reaction (PCR) and the sequences were: forward primer 5'-AGCT*CATATG*GCCGTTATTATTTTTGTGAATGGTATTCGTGCCG-3' (NdeI restriction site is italicized) and reverse primer 5'-AGCT*AAGCTT*TTACTTCTCTTGGCCGAAAAACTGGTTTTCAATATCTGC-3' (HindIII restriction site is italicized). PCR was performed in a 50-μL reaction mixture containing plasmid DNA (50 ng), forward and reverse primers (0.5 μM each), 5 × reaction buffer (10 μL), dNTP mixture (0.2 mM), and 1 U of Phusion High-Fidelity DNA Polymerase (New England Biolabs). The reaction mixture was subjected to 30 cycles of amplification with an annealing temperature of 72 °C. The resulting PCR product was purified and digested with NdeI and HindIII restriction enzymes. The purified and digested PCR product was ligated with a predigested pET22b(+) vector and transformed into *E. coli* DH5α cells. Selected clones were grown for minipreps and the purified plasmids were analyzed by DNA sequencing performed by Genewiz.

A plasmid with confirmed sequence was transformed into *Escherichia coli* BL21 (DE3). To express the recombinant $NmSiaD_W$, bacteria were cultivated in 1 L of LB rich medium in the presence of 100 μg/mL ampicillin. The expression was achieved by induction with 0.1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) when $OD_{600\ nm}$ of the culture reached 0.6 followed by incubation at 16 °C for 72 h. Cells were harvested (6000 ×g, 15 min, 16 °C), re-suspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.1% Triton X-100), and the mixture was subjected to sonication (amplitude 60%, 3 s on and 15 s off, 6 min). The supernatant was obtained by centrifugation (4300 ×g, 30 min, 4 °C), loaded onto a Ni^{2+}-NTA affinity column at 4 °C that was pre-equilibrated with 6 column volumes of binding buffer containing Tris-HCl buffer (50 mM, pH 8.0), NaCl (300 mM), and imidazole (5 mM). It was washed with 10 column volumes of binding buffer and 10 column volumes of 10% and 20% elute buffer, respectively, and eluted with elute buffer containing Tris-HCl (50 mM, pH 8.0), NaCl (300 mM), imidazole (150 mM). The purified protein fractions were combined and concentrated. The resulting sample was dialyzed using dialysis buffer (Tris-HCl, 20 mM, pH 8.0 containing 10% glycerol) and stored at 4 °C.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis
Gels were prepared with 12% acrylamide in the presence of 0.1% SDS. Cells and protein samples were incubated in the loading buffer (50 mM Tris-HCl, pH 6.8, 0.1% bromophenol blue, 10% glycerol, 100 mM DTT) for 10 min at 95 °C. Denatured samples were loaded to the gel and the gel was developed at 150 V for 1 h. The gel was then stained with coomassie blue dye (1 g/L) in a solution of acetic acid:methanol:water (=1:4:5 by volume) and de-stained using the same solution without the dye.

pH profile
Assays were performed in duplicate at 30 °C for 20 min in a total volume of 10 μL in a buffer (200 mM) with a pH value in the range of 3.0–11.0 containing a donor substrate (1.2 mM) (UDP-Gal for GalT and CMP-Neu5Ac for SiaT assays), an acceptor substrate (1 mM) (**S1** for GalT and **G2** for SiaT assays), $MgCl_2$ (10 mM), and $NmSiaD_W$ (19.8 μg for GalT and 0.17 μg for SiaT assays). Reactions were quenched by adding 10 μL of pre-chilled ethanol followed by incubation at -20 ℃ for 30 min. The precipitates were removed by centrifugation (11000 ×g, 5 min, 4 °C). Reaction mixtures were assayed using an Agilent 1290 Infinity II LC System with a PDA detector (monitored at 215 nm) and an Eclipse Plus C18 column (Rapid Resolution HD, 1.8 μm, 2.1×50 mm, 959757-902) at 30 °C. An elution solvent of 11% acetonitrile and 89% H_2O containing 0.1% TFA was used for **S1** and 10% acetonitrile and 90% H_2O containing 0.1% TFA was used for **G2**. Buffers used were: Citric acid, pH 3.0–4.5; MES, pH 5.0–6.5; Tris-HCl, pH 7.0–9.0; CAPS, pH 9.5–11.0.

Metal effects
Assays were performed in duplicate at 30 °C for 20 min in a total volume of 10 μL in a buffer (MES, 100 mM, pH 6.5 for GalT and Tris-HCl, 100 mM, pH 8.0 for SiaT assays) containing a donor substrate (1.2 mM) (UDP-Gal for GalT and CMP-Neu5Ac for SiaT assays), an acceptor substrate (1 mM) (**S1** for GalT and **G2** for SiaT assays), $NmSiaD_W$ (19.8 μg for GalT and 0.17 μg for SiaT assays), and the presence of EDTA, DTT, Mg^{2+}, Ca^{2+}, Li^{+}, Na^{+}, Co^{2+}, Cu^{2+}, Mn^{2+}, or Ni^{2+} (10 mM). Reactions were quenched by adding 10 μL of pre-chilled ethanol followed by incubation at -20 ℃ for 30 min. Reaction mixtures were assayed as described above for pH profile studies.

Temperature profile
Assays were performed in duplicate at different temperatures for 20 min in a total volume of 10 μL in a buffer (MES, 100 mM, pH 6.5 for GalT and Tris-HCl, 100 mM, pH 8.0 for SiaT assays) containing a donor substrate (1.2 mM) (UDP-Gal for GalT and CMP-Neu5Ac for SiaT assays), an acceptor substrate (1 mM) (**S1** for GalT and **G2** for SiaT assays), $MgCl_2$ (10 mM), and $NmSiaD_W$ (19.8 μg for GalT and 0.17 μg for SiaT assays). Reactions were quenched by adding 10 μL of pre-chilled ethanol to the reaction mixture followed by incubation at -20 ℃ for 30 min. Products were assayed as described above for pH profile studies.

Thermostability
Enzyme was pre-incubated at a given temperature for 30 min, then put on ice for 10 min. Reactions were performed at 30 ℃ and activity assays were then performed as described above for the temperature profile assays.

Enzyme kinetics by varying acceptor concentrations
For galactosyltransferase acceptors, reactions were performed in duplicate at 30 °C for 10 min in the presence of MES buffer (100 mM, pH 6.5), $MgCl_2$ (10 mM) and 2 mM UDP-Gal with a total volume of 20 μL, and varying concentrations (0.05, 0.1, 0.2, 0.3, 0.5, 0.7, 1.0, 2.0, 5.0 and 10.0 mM) of the acceptor substrate. The concentration of $NmSiaD_W$ varied from 0.011 to 4.049 μM when different acceptors were used. Reactions were quenched by adding 20 μL of pre-chilled ethanol followed by incubation at -20 °C for 30 min. The apparent kinetic parameters were

obtained by fitting the experimental data (the average values of duplicate assay results) into the Michaelis-Menten equation using Grafit 5.0. For sialyltransferase acceptors, reactions were performed in duplicate at 30 °C for 10 min in the presence of 100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and 10 mM CMP-Neu5Ac with a total volume of 20 μL, and varying concentrations (0.1, 0.2, 0.3, 0.5, 0.7, 1.0, 1.5, 2.0, 3.0, 5.0 and 10.0 mM) of the acceptor substrate. The concentration of $NmSiaD_W$ varied from 0.011 to 0.018 μM according to different acceptors. Reactions were quenched by adding 20 μL of pre-chilled ethanol followed by incubation at -20 °C for 30 min. Products were assayed using an Agilent 1290 Infinity II LC System with a PDA detector (monitored at 215 nm) and an Eclipse Plus C18 column (Rapid Resolution HD, 1.8 μm, 2.1×50 mm, 959757-902) or an AdvanceBio Glycan Map column (1.8 μm, 2.1×150 mm, 859700-913) (see **Table S4** below for detailed elution conditions) at 30 °C. The apparent kinetic parameters were obtained by fitting the experimental data (the average values of duplicate assay results) into the Michaelis-Menten equation using Grafit 5.0.

Table S4. Elution conditions for $NmSiaD_W$ kinetics studies with different acceptors (**S1**–**G10**).

Acceptor	[E] (μM)	Column	Solvent A	Solvent B	B%
S1	4.049	Eclipse Plus C18	0.1% TFA in H_2O	Acetonitrile	11
G2	0.014	AdvanceBio Glycan	35 mM NaCl, 0.1% TFA in H_2O	Acetonitrile	88
S3	0.048	AdvanceBio Glycan	35 mM NaCl, 0.1% TFA in H_2O	Acetonitrile	88–84 over 4 min
G4	0.011	Eclipse Plus C18	10 mM tetrabutylammonium, 50 mM ammonium formate, pH 4.5	Acetonitrile	27–34 over 4 min
S5	0.018	AdvanceBio Glycan	35 mM NaCl, 0.1% TFA in H_2O	Acetonitrile	75
G6	0.014	AdvanceBio Glycan	35 mM NaCl, 0.1% TFA in H_2O	Acetonitrile	75
S7	0.018	AdvanceBio Glycan	35 mM NaCl, 0.1% TFA in H_2O	Acetonitrile	77–72 over 5 min
G8	0.018	AdvanceBio Glycan	35 mM NaCl, 0.1% TFA in H_2O	Acetonitrile	72
S9	0.011	AdvanceBio Glycan	35 mM NaCl, 0.1% TFA in H_2O	Acetonitrile	71
G10	0.014	AdvanceBio Glycan	35 mM NaCl, 0.1% TFA in H_2O	Acetonitrile	70

Enzyme kinetics by varying donor concentrations

For varying UDP-Gal concentrations, reactions were performed in duplicate at 30 °C for 10 min in the presence of MES buffer (100 mM, pH 6.5), $MgCl_2$ (10 mM), **S3** or **S9** (2 mM), UDP-Gal (0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 mM), and $NmSiaD_W$ (0.025 μM for **S3**, 0.031 μM for **S9**) in a total volume of 20 μL. For varying CMP-Neu5Ac concentrations, reactions were performed in duplicate at 30 °C for 10 min in the presence of Tris-HCl buffer (100 mM, pH 8.0), $MgCl_2$ (10 mM), **G2** or **G10** (1 mM), CMP-Neu5Ac (0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 mM), and $NmSiaD_W$ (0.025 μM for **G2**, 0.062 μM for **G10**) in a total volume of 20 μL. Data analyses were performed as described above.

Polymerization studies

For studies using acceptor substrates of varied lengths, reactions were performed in duplicate in a total volume of 50 μL at 30 °C in Tris-HCl buffer (100 mM, pH 8.5) containing $MgCl_2$ (10 mM),

UDP-Gal (50 mM), CMP-Neu5Ac (50 mM), an acceptor substrate (5 mM, selected from **S1**–**G10**) and $NmSiaD_W$ (50 μg).

For donor ratio profile studies using **G2** or **S3** as the acceptor substrate, reactions were performed in duplicate in a total volume of 50 μL at 30 °C in Tris-HCl buffer (100 mM, pH 8.5) containing $MgCl_2$ (10 mM), both UDP-Gal and CMP-Neu5Ac (5, 10, 25, 50, 100 and 250 mM), an acceptor substrate **G2** or **S3** (5 mM), and $NmSiaD_W$ (50 μg).

For one-pot multienzyme (OPME) polymerization studies using **G2** or **S3** as the acceptor substrate, reactions were performed in two steps. A donor synthesis reaction was performed in a total volume of 150 μL at 30 °C for 10 hours in Tris-HCl buffer (144 mM, pH 8.5) containing $MgCl_2$ (14.4 mM), CTP (72 mM), Neu5Ac (72 mM), UTP (72 mM), ATP (72 mM), Gal (72 mM), SpGalK (100 μg), BLUSP (50 μg), PmPpA (100 μg) and NmCSS (80 μg). Then polymerization reactions were performed in duplicate in a total volume of 50 μL each at 30 °C containing a reaction mixture of the donor synthesis (35 μL), **G2** or **S3** (5 mM), and $NmSiaD_W$ (50 μg). Samples were taken and quenched at 1 h and 20 h, respectively, by transferring 20 μL of reaction mixture into an equal volume of pre-chilled ethanol followed by incubation at -20 °C for 30 min.

Reaction mixtures were analyzed using UHPLC (monitored at 215 nm) with an AdvanceBio Glycan Map column (a HILIC column from Agilent, 1.8 μm, 2.1×150 mm, 859700-913) at 30 °C. Solvent A (35 mM NaCl, 0.1% TFA in H_2O) and solvent B (acetonitrile) were used to establish an elution gradient, starting with 90% B at 1.300 mL/min and reaching to 40% B at 0.675 mL/min over 50 min. Relative yields were calculated from peak area integration and used to obtain number average molecular weight, weight average molecular weight, and polydispersity index.

Reaction mixtures were analyzed at 30 °C using a Shimadzu LCMS-2020 system (monitored at 215 nm) with a XBridge BEH Amide Column (a HILIC column from Waters, 130 Å, 5 μm, 4.6 × 250 mm). Solvent A (0.1% formic acid in H_2O) and solvent B (acetonitrile) were used to establish an elution gradient, starting with 72.5% B at 1.300 mL/min and reaching to 12.5% B at 0.800 mL/min over 120 min.

Chemical synthesis of Neu5Acα-ProNHCbz (S1)

Synthesis of Neu5Ac methyl ester (3)

N-Acetylneuraminic acid (15.0 g, 0.49 mol) was suspended in dry methanol (200 mL) and Dowex 50WX4 (H^+) resin (10 g) was added. The mixture was stirred at room temperature for overnight. The reaction was monitored by MS and TLC (EtOAc:MeOH:H_2O = 4:2:1, by volume). Upon completion, the resin was removed by filtration, and the filtrate was concentrated *in vacuo* and dried under vacuum to yield **2** as a white solid. The obtained solid was dissolved in anhydrous pyridine (200 mL), followed by the addition of acetic anhydride (70 mL) and 4-dimethylaminopyridine (DMAP, 400 mg). The reaction was stirred at room temperature for overnight and the reaction was monitored by TLC (Hexane:EtOAc = 1:3 by volume). The reaction mixture was diluted with 500 mL of ethyl acetate and extracted with water for three times. The organic layer was dried with anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. The product was purified by silica gel column (hexane:EtOAc = 1:2 to 1:4, by volume) to obtain 22.2 g of the peracetylated product (**3**) with a yield of 86% for two steps.

Synthesis of Neu5Acα-ProNHCbz (S1)

Peracetylated Neu5Ac methyl ester (**3**, 6.0 g, 11.3 mmol) was dissolved in anhydrous dichloromethane (20 mL) in a round bottom flask (200 mL) and the reaction was placed in an ice-water bath. Acetyl chloride (80 mL) was added followed by the addition of anhydrous methanol (2 mL) under Argon and the reaction mixture was stirred for 20 min. The reaction flask was then sealed and the mixture was stirred at room temperature for 2 days. The reaction progress was monitored by TLC analysis (hexane:EtOAc = 1:4, by volume). Upon completion, the reaction mixture was concentrated, co-evaporated with toluene for three times, and dried under vacuum. Without further purification of the crude product, molecular sieves 4 Å (6.0 g), anhydrous

dichloromethane (50 mL), and benzyl *N*-(3-hydroxypropyl) carbamate (4.78 g, 22.8 mmol) were added under argon. The mixture was placed in an ice-water bath and silver triflate (2.90 g, 11.3 mmol) was added. The reaction flask was covered with an aluminum foil and the mixture was stirred at room temperature for overnight. The reaction progress was monitored by TLC analysis (hexane:EtOAc = 1:4, by volume). Upon completion, the reaction mixture was filtered over Celite and washed with DCM. The filtrate was concentrated for purification by silica gel column chromatography (hexane:EtOAc = 1:1 to 1:4, by volume). Fractions were collected, concentrated, and dried under vacuum. (Note, when silver carbonate was used as a promoter instead of silver triflate, the glycosylation yield was much lower and glycal was formed as a major byproduct.)

The glycosylation product (**5**) was dissolved in anhydrous methanol (100 mL). Sodium methoxide was added until the pH was around 9.0, and the reaction mixture was stirred for overnight at r.t. The reaction was monitored by TLC analysis with two different developing solvent systems (EtOAc:hexane = 4:1, by volume, to monitor consumption of starting material; and EtOAc:MeOH:H_2O = 8:2:0.5, by volume, to monitor the formation of the deacetlyated product). Upon completion, the reaction mixture was neutralized by adding Dowex 50WX4 (H^+) resin. The resin was then removed by filtration and the filtrate was concentrated *in vacuo* and dried under vacuum. The product was dissolved in 100 mL of a solvent mixture (water:methanol = 4:1, by volume). The pH of the reaction mixture was adjusted to 9.0 using 2.0 M of NaOH and the mixture was stirred for overnight at r.t. The reaction was monitored by TLC analysis (EtOAc:MeOH:H_2O:AcOH = 7:2:1:0.2, by volume). Up completion, the reaction mixture was neutralized by adding Dowex 50WX4 (H^+) resin. The resin was then removed by filtration, and the filtrate was concentrated *in vacuo*. The crude product was purified by a silica gel column (EtOAc:MeOH:H_2O = 8:2:1, by volume) to produce **S1** (3.79 g, 67% for four steps).

^{1}H NMR (800 MHz, D_2O) δ 7.46–7.37 (m, 5H, Ar-H), 5.08 (s, 2H, O-$\underline{CH_2}$-Ar), 3.87–3.75 (m, 4H, H-8, H-9, H-5, O-$\underline{CH_2}$-CH_2), 3.70–3.64 (m, 2H, H-4, H-9), 3.61 (dd, *J* = 11.9, 6.0 Hz, 1H, H-9), 3.59 (dt, *J* = 9.1, 1.4 Hz, 1H, H-7), 3.50–3.45 (m, 1H, O-$\underline{CH_2}$-CH_2), 3.23-3.11 (m, 2H, CH_2-NH), 2.72 (dd, *J* = 12.5, 4.6 Hz, 1H, H-3_{eq}), 2.03 (d, *J* = 1.2 Hz, 3H, $\underline{CH_3}$-CO), 1.73 (p, *J* = 6.5 Hz, 2H, O-CH_2-$\underline{CH_2}$-CH_2-NH), 1.65 (t, *J* = 12.2 Hz, 1H, H-3). ^{13}C NMR (200 MHz, D_2O) δ 175.06 (COOH), 173.66 (CH_3-$\underline{CO}$), 158.33 (NH-COO), 136.52 (O-CH_2-$\underline{Ar}$), 128.76 (Ar), 128.29 (Ar), 127.60 (Ar), 100.55 (C-2), 72.55 (C-6), 71.70 (C-8), 68.25 (C-4), 68.14 (C-7), 66.76 (O-$\underline{CH_2}$-Ar), 62.50 (C-9), 62.07 (O-$\underline{CH_2}$-CH_2), 51.90 (C-5), 40.35 (C-3), 37.52 (CH_2-NH), 28.92 (O-CH_2-$\underline{CH_2}$-CH_2-NH), 22.01 ($\underline{CH_3}$-CO). HRMS (ESI) m/z calculated for $C_{22}H_{31}N_2O_{11}^-$ (M-H) 499.1928, found 499.1914.

OPME synthesis of galactosyldisaccharide Galα1–4Neu5AcαProNHCbz (G2). A reaction mixture in a total volume of 200 mL containing Tris-HCl buffer (100 mM, pH 8.5), Neu5AcαProNHCbz (**S1**) (1.00 g, 2.0 mmol), galactose (0.72 g, 4.0 mmol), ATP disodium salt (2.42 g, 4.4 mmol), UTP trisodium salt (2.42 g, 4.4 mmol), $MgCl_2$ (20 mM), SpGalK (22 mg), BLUSP (22 mg), PmPpA (22 mg), and $NmSiaD_W$ (22 mg) was incubated in a 250-mL bottle in a shaker (100 rpm) at 30 °C for 98 hrs. The reaction progress was monitored by UHPLC (Eclipse Plus C18, Agilent, 11% Acetonitrile + 0.1% TFA in water, monitored at 215 nm). When an optimal yield was achieved, pre-chilled ethanol (200 mL) was added and the resulting mixture was incubated at 4 °C for 30 min. The precipitates were removed by centrifugation (4300 × g, 30 min, 4 °C). The supernatant was concentrated and purified by a C18 column in a CombiFlash Rf 200i system with a gradient of water with 0.1% TFA (v/v) and acetonitrile (0-100% acetonitrile) for elution. Fractions containing the product were collected, neutralized, concentrated, and further purified by a C18 column to produce disaccharide Galα1–4Neu5AcαProNHCbz (**G2**) as a sodium salt (1.26 g, 92%).

^{1}H NMR (800 MHz, D_2O) δ 7.46–7.37 (m, 5H, Ar-H), 5.09 (s, 2H, O-$\underline{CH_2}$-Ar), 5.08 (d, *J* = 4.0 Hz, 1H, H''-1), 4.04 (t, *J* = 10.3 Hz, 1H, H'-5), 3.96 (d, *J* = 3.3 Hz, 1H, H''-3), 3.86 (td, *J* = 6.3, 3.1 Hz, 1H, H'-8), 3.84–3.77 (m, 5H, H'-5, H'-9, H''-2, H''-5, O-$\underline{CH_2}$-CH_2), 3.75–3.68 (m, 4H, H'-4, H''-4, H''-6), 3.64–3.59 (m, 2H, H'-7, H'-9), 3.52-3.47 (m, 1H, O-$\underline{CH_2}$-CH_2), 3.23–3.14 (m, 2H,

CH_2-NH), 2.88 (dd, *J* = 12.5, 4.7 Hz, 1H, H'-3_{eq}), 2.03 (d, *J* = 1.6 Hz, 3H, CH_3-CO), 1.74 (p, *J* = 6.6 Hz, 2H, O-CH_2-$\underline{CH_2}$-CH_2-NH), 1.62 (t, *J* = 12.0 Hz, 1H, H'-3_{ax}). ^{13}C NMR (200 MHz, D_2O) δ 174.32 (COOH), 173.49 (CH_3-$\underline{CO}$), 158.34 (NH-COO), 136.53 (O-CH_2-$\underline{Ar}$), 128.76 (Ar), 128.28 (Ar), 127.58 (Ar), 100.60 (C'-2), 94.72 (C''-1), 72.82 (C'-4), 72.19 (C'-6), 71.77 (C'-8), 71.02 (C''-5), 69.36 (C''-4), 69.05 (C''-3), 68.04 (C'-7), 67.88 (C''-2), 66.76 (O-$\underline{CH_2}$-Ar), 62.48 (C'-9), 62.07 (O-$\underline{CH_2}$-CH_2), 60.73 (C''-6), 49.53 (C'-5), 37.47 (CH_2-NH), 36.73 (C'-3), 28.91 (O-CH_2-$\underline{CH_2}$-CH_2-NH), 22.11 ($\underline{CH_3}$-CO). HRMS (ESI) m/z calculated for $C_{28}H_{41}N_2O_{16}^-$ (M-H) 661.2456, found 661.2458.

OPME synthesis of sialyltrisaccharide Neu5Acα2–6Galα1–4Neu5AcαProNHCbz (S3). A reaction mixture in a total volume of 64 mL containing Tris-HCl buffer (100 mM, pH 8.5), galactosyldisaccharide **G2** (426 mg, 0.64 mmol), Neu5Ac (260 mg, 0.83 mmol), CTP disodium salt (445 mg, 0.83 mmol), $MgCl_2$ (20 mM), NmCSS (4 mg), and $NmSiaD_W$ (4 mg) was incubated in a 250-mL bottle in a shaker (100 rpm) at 30 °C for 15 hrs. The reaction progress was monitored by UHPLC (AdvanceBio Glycan Map, Agilent, 87% Acetonitrile + 0.1% TFA in water, monitored at 215 nm). When an optimal yield was achieved, pre-chilled ethanol (64 mL) was added and the resulting mixture was incubated at 4 °C for 30 min. Procedures for centrifugation, concentration, purification, collection and neutralization were similar to that described above for **G2** to produce sialyltrisaccharide **S3** as a sodium salt (620 mg, 96%).

^{1}H NMR (800 MHz, D_2O) δ 7.49–7.35 (m, 5H, Ar-H), 5.11 (s, 2H, O-$\underline{CH_2}$-Ar), 5.06 (d, *J* = 3.9 Hz, 1H, H''-1), 4.03 (t, *J* = 10.3 Hz, 1H, H'-5), 3.96 (d, *J* = 3.4 Hz, 1H, H''-3), 3.90–3.74 (m, 10H), 3.73–3.59 (m, 8H), 3.56 (dd, *J* = 9.0, 1.7 Hz, 1H), 3.50 (dt, *J* = 10.6, 6.1 Hz, 1H, O-$\underline{CH_2}$-CH_2), 3.24–3.15 (m, 2H, CH_2-NH), 2.88 (dd, *J* = 12.5, 4.7 Hz, 1H, H'-3_{eq}), 2.73 (dd, *J* = 12.4, 4.7 Hz, 1H, H'''-3_{eq}), 2.07 (s, 3H, H'-CH_3-CO), 2.03 (s, 3H, H'''-CH_3-CO), 1.74 (p, *J* = 6.8 Hz, 2H, O-CH_2-$\underline{CH_2}$-CH_2-NH), 1.70 (t, *J* = 12.2 Hz, 1H, H'''-3_{ax}), 1.61 (t, *J* = 12.0 Hz, 1H, H'-3_{ax}). ^{13}C NMR (200 MHz, D_2O) δ 174.97, 174.49, 173.48, 173.32, 158.37 (NH-COO), 136.56 (O-CH_2-$\underline{Ar}$), 128.75 (Ar), 128.27 (Ar), 127.56 (Ar), 100.59 (C'-2), 100.11 (C'''-2), 94.54 (C''-1), 72.69 (C'-4), 72.46 (C'''-6), 72.09, 71.83, 69.52, 69.19, 68.89 (C''-3), 68.37 (C'''-4), 68.27, 68.03, 67.84 (C''-2), 66.76 (O-$\underline{CH_2}$-Ar), 62.62, 62.60, 62.52 (C''-6), 62.06 (O-$\underline{CH_2}$-CH_2), 51.79 (C'''-5), 49.52 (C'-5), 40.06 (C'''-3), 37.47 (CH_2-NH), 36.64 (C'-3), 28.89 (O-CH_2-$\underline{CH_2}$-CH_2-NH), 22.42 (H'-CH_3-CO), 22.00 (H'''-CH_3-CO). HRMS (ESI) m/z calculated for $C_{39}H_{58}N_3O_{24}^-$ (M-H) 952.3410, found 952.3390.

OPME synthesis of galactosyltetrasaccharide Galα1–4Neu5Acα2–6Galα1–4Neu5AcαProNHCbz (G4). A reaction mixture in a total volume of 53 mL containing Tris-HCl buffer (100 mM, pH 8.5), sialyltrisaccharide **S3** (528 mg, 0.53 mmol), galactose (124 mg, 0.67 mmol), ATP disodium salt (380 mg, 0.67 mmol), UTP trisodium salt (379 mg, 0.67 mmol), $MgCl_2$ (20 mM), SpGalK (4.8 mg), BLUSP (4.8 mg), PmPpA (4.8 mg) and $NmSiaD_W$ (2.4 mg) was incubated in a 125-mL bottle in a shaker (100 rpm) at 30 °C for 16 hrs. The reaction progress was monitored by UHPLC (AdvanceBio Glycan Map, Agilent, 80% Acetonitrile + 0.1% TFA in water, monitored at 215 nm). When an optimal yield was achieved, pre-chilled ethanol (53 mL) was added and the resulting mixture was incubated at 4 °C for 30 min. Procedures for centrifugation, concentration, purification, collection and neutralization were similar to that described above for **G2** to produce galactosyltetrasaccharide **G4** as a sodium salt (556 mg, 91%).

^{1}H NMR (800 MHz, D_2O) δ 7.49–7.37 (m, 5H, Ar-H), 5.10 (s, 2H, O-$\underline{CH_2}$-Ar), 5.08 (d, *J* = 4.0 Hz, 1H, H''''-1), 5.06 (d, *J* = 3.9 Hz, 1H, H''-1), 4.04 (td, *J* = 10.3, 5.9 Hz, 2H, H'-5, H'''-5), 3.96 (dd, *J* = 10.1, 3.4 Hz, 2H, H''-3, H''''-3), 3.90–3.59 (m, 23H), 3.50 (dt, *J* = 10.9, 6.2 Hz, 1H, O-$\underline{CH_2}$-CH_2), 3.23–3.15 (m, 2H, CH_2-NH), 2.88 (td, *J* = 12.1, 4.6 Hz, 2H, H'-3_{eq}, H'''-3_{eq}), 2.08 (s, 3H, H'-CH_3-CO), 2.03 (s, 3H, H'''-CH_3-CO), 1.75 (h, *J* = 7.4, 6.9 Hz, 2H, O-CH_2-$\underline{CH_2}$-CH_2-NH), 1.66 (t, *J* = 12.1 Hz, 1H, H'''-3_{ax}), 1.61 (t, *J* = 12.0 Hz, 1H, H'-3_{ax}). ^{13}C NMR (200 MHz, D_2O) δ

174.49, 174.30, 173.34, 173.18, 158.36 (NH-COO), 136.57 (O-CH_2-Ar), 128.76 (Ar), 128.27 (Ar), 127.56 (Ar), 100.62 (C'-2), 100.23 (C'''-2), 95.05 (C''''-1), 94.40 (C''-1), 73.23, 72.49, 72.19, 72.09, 71.91, 71.79, 71.02, 69.56, 69.23, 69.21, 69.04 (C''''-3), 68.94 (C''-3), 68.17, 67.96, 67.86, 67.80, 66.76 (O-CH_2-Ar), 62.86, 62.60, 62.46, 62.06 (O-CH_2-CH_2), 60.72 (C''''-4), 49.54 (C'-5), 49.46 (C'''-5), 37.48 (CH_2-NH), 36.77 (C'''-3), 36.57(C'-3), 28.90 (O-CH_2-CH_2-CH_2-NH), 22.43 (H'-CH_3-CO), 22.11 (H'''-CH_3-CO). HRMS (ESI) m/z calculated for $C_{45}H_{68}N_3O_{29}^-$ (M-H) 1114.3939, found 1114.3918.

OPME synthesis of sialylpentasaccharide Neu5Acα2(–6Galα1–4Neu5Acα2)$_2$–ProNHCbz (S5). A reaction mixture in a total volume of 43 mL containing Tris-HCl buffer (100 mM, pH 8.5), galactosyltetrasaccharide **G4** (502 mg, 0.43 mmol), Neu5Ac (175 mg, 0.56 mmol), CTP disodium salt (300 mg, 0.56 mmol), $MgCl_2$ (20 mM), NmCSS (1.0 mg) and $NmSiaD_W$ (2.0 mg) was incubated in a 125-mL bottle in a shaker (100 rpm) at 30 °C for 17 hrs. The reaction progress was monitored by UHPLC (AdvanceBio Glycan Map, Agilent, 75% Acetonitrile + 0.1% TFA in water, monitored at 215 nm). When an optimal yield was achieved, pre-chilled ethanol (43 mL) was added and the resulting mixture was incubated at 4 °C for 30 min. Procedures for centrifugation, concentration, purification, collection and neutralization were similar to that described above for **G2** to produce sialylpentasaccharide **S5** as a sodium salt (512 mg, 83%).

^{1}H NMR (800 MHz, D_2O) δ 7.46–7.37 (m, 5H, Ar-H), 5.10 (s, 2H, O-CH_2-Ar), 5.06 (d, *J* = 3.7 Hz, 2H, $H^{II,IV}$-1), 4.03 (td, *J* = 10.2, 5.7 Hz, 2H, $H^{I,III}$-5), 3.96 (d, *J* = 3.4 Hz, 2H, $H^{II,IV}$-3), 3.92–3.59 (m, 29H), 3.58–3.54 (m, 1H), 3.50 (dt, J = 11.6, 6.2 Hz, 1H, O-CH_2-CH_2), 3.24–3.17 (m, 2H, CH_2-NH), 2.88 (dt, *J* = 12.5, 4.4 Hz, 2H, $H^{I,III}$-3eq), 2.72 (dd, J = 12.4, 4.7 Hz, 1H, H^V-3eq), 2.08 (s, 6H, $H^{I,III}$-CH_3-CO), 2.03 (s, 3H, H^V-CH_3-CO), 1.74 (p, *J* = 6.8 Hz, 2H, O-CH_2-CH_2-CH_2-NH), 1.70 (t, *J* = 12.2 Hz, 1H, H^V-3ax), 1.63 (dt, *J* = 30.6, 12.0 Hz, 2H, $H^{I,III}$-3_{ax}). ^{13}C NMR (200 MHz, D_2O) δ 174.98, 174.51, 174.48, 173.49, 173.39, 173.06, 158.37, 136.56, 128.77, 128.28, 127.57, 100.58, 100.22, 100.14, 94.88, 94.61, 73.11, 72.76, 72.45, 72.11, 72.08, 72.04, 72.01, 71.84, 71.82, 69.59, 69.56, 69.19, 69.08, 68.91, 68.90, 68.38, 68.29, 68.18, 67.98, 67.85, 67.78, 66.76, 62.76, 62.66, 62.64, 62.63, 62.50, 62.46, 62.06, 51.80, 49.56, 49.47, 40.04, 37.48, 36.67, 28.91, 22.48, 22.02. HRMS (ESI) m/z calculated for $C_{56}H_{85}N_4O_{37}^-$ (M-H) 1405.4893, found 1405.4896.

OPME synthesis of galactosylhexasaccharide Galα1(–4Neu5Acα2–6Galα1)$_2$–4Neu5AcαProNHCbz (G6). A reaction mixture in a total volume of 31 mL containing Tris-HCl buffer (100 mM, pH 8.5), sialylpentasaccharide **S5** (450 mg, 0.31 mmol), galactose (73 mg, 0.40 mmol), ATP disodium salt (222 mg, 0.40 mmol), UTP trisodium salt (220 mg, 0.40 mmol), $MgCl_2$ (20 mM), SpGalK (2.4 mg), BLUSP (2.4 mg), PmPpA (2.4 mg), and $NmSiaD_W$ (1.2 mg) was incubated in a bottle (125 mL) in a shaker (100 rpm) at 30 °C for 16 hrs. The product formation was monitored by UHPLC (AdvanceBio Glycan Map, Agilent, 75% Acetonitrile + 0.1% TFA in water, monitored at 215 nm). When an optimal yield was achieved, pre-chilled ethanol (31 mL) was added and the resulting mixture was incubated at 4 °C for 30 min. Procedures for centrifugation, concentration, purification, collection and neutralization were similar to that described above for **G2** to produce galactosylhexasaccharide **G6** as a sodium salt (440 mg, 88%).

^{1}H NMR (800 MHz, D_2O) δ 7.53–7.29 (m, 5H, Ar-H), 5.11 (s, 2H, O-CH_2-Ar), 5.08 (d, *J* = 4.0 Hz, 1H, H^{VI}-1), 5.05 (d, *J* = 3.9 Hz, 2H, $H^{II,IV}$-1), 4.03 (ddt, *J* = 10.3, 6.6, 3.3 Hz, 3H, $H^{I,III,V}$-5), 3.98–3.94 (m, 3H, $H^{II,IV,VI}$-3), 3.91–3.57 (m, 34H), 3.50 (dt, *J* = 11.1, 6.3 Hz, 1H, O-CH_2-CH_2), 3.24–3.16 (m, 2H, CH_2-NH), 2.87 (ddd, *J* = 12.4, 7.8, 4.8 Hz, 3H, $H^{I,III,V}$-3_{eq}), 2.08 (s, 6H, $H^{I,III}$-CH_3-CO), 2.02 (s, 3H, H^V-CH_3-CO), 1.74 (p, *J* = 6.9 Hz, 2H, O-CH_2-CH_2-CH_2-NH), 1.69–1.58 (m, 3H, $H^{I,III,V}$-3_{ax}). ^{13}C NMR (200 MHz, D_2O) δ 174.52, 174.47, 174.29, 173.38, 173.20, 173.08, 158.37, 136.57, 128.76, 128.27, 127.56, 100.57, 100.22, 95.04, 94.79, 94.64, 73.23, 72.99, 72.79, 72.16, 72.10, 72.07, 71.94, 71.88, 71.82, 71.01, 69.59, 69.23, 69.18, 69.09, 69.05, 68.97, 68.89, 68.19, 68.11, 67.98, 67.84, 67.81, 67.78, 66.76, 62.90, 62.77, 62.60, 62.58, 62.51, 62.06,

60.71, 49.55, 49.47, 49.46, 37.48, 36.74, 36.69, 36.63, 28.90, 22.47, 22.11. HRMS (ESI) m/z calculated for $C_{62}H_{95}N_4O_{42}^-$ (M-H) 1567.5421, found 1567.5397.

OPME synthesis of sialylheptasaccharide Neu5Acα2(–6Galα1–4Neu5Acα2)$_3$–ProNHCbz (S7). A reaction mixture in a total volume of 25 mL containing Tris-HCl buffer (100 mM, pH 8.5), galactosylhexasaccharide **G6** (390 mg, 0.25 mmol), Neu5Ac (102 mg, 0.33 mmol), CTP disodium salt (175 mg, 0.33 mmol), $MgCl_2$ (20 mM), NmCSS (0.8 mg), and $NmSiaD_W$ (1.6 mg) was incubated in a 50-mL centrifuge tube in a shaker (100 rpm) at 30 °C for 16 hrs. The product formation was monitored by UHPLC (AdvanceBio Glycan Map, Agilent, 72% Acetonitrile + 0.1% TFA in water, monitored at 215 nm). When an optimal yield was achieved, pre-chilled ethanol (25 mL) was added and the resulting mixture was incubated at 4 °C for 30 min. Procedures for centrifugation, concentration, purification, collection and neutralization were similar to that described above for **G2** to produce sialylheptasaccharide **S7** as a sodium salt (418 mg, 90%).

^{1}H NMR (800 MHz, D_2O) δ 7.49–7.36 (m, 5H, Ar-H), 5.11 (s, 2H, O-$\underline{CH_2}$-Ar), 5.05 (q, *J* = 3.7 Hz, 3H, $H^{II,IV,VI}$-1), 4.03 (td, *J* = 10.3, 3.6 Hz, 3H, $H^{I,III,V}$-5), 3.96 (d, *J* = 3.4 Hz, 3H, $H^{II,IV,VI}$-3), 3.91–3.60 (m, 39H), 3.56 (dd, J = 11.9, 3.2 Hz, 2H), 3.50 (dt, *J* = 10.7, 6.2 Hz, 1H, O-$\underline{CH_2}$-CH_2), 3.23–3.16 (m, 2H, CH_2-NH), 2.87 (dt, *J* = 12.7, 5.6 Hz, 3H, $H^{I,III,V}$-3eq), 2.72 (dd, *J* = 12.4, 4.7 Hz, 1H, H^{VII}-3eq), 2.08 (s, 9H, $H^{I,III,V}$-CH_3-CO), 2.03 (s, 3H, H^{VII}-CH_3-CO), 1.74 (p, *J* = 7.0 Hz, 2H, O-CH_2-$\underline{CH_2}$-CH_2-NH), 1.70 (t, *J* = 12.2 Hz, 1H, H^{VII}-3_{ax}), 1.63 (dt, *J* = 33.5, 12.0 Hz, 3H, $H^{I,III,V}$-3_{ax}).^{13}C NMR (200 MHz, D_2O) δ 174.96, 174.52, 174.49, 174.46, 173.48, 173.09, 173.05, 158.37, 136.57, 128.76, 128.27, 127.56, 100.57, 100.23, 100.21, 100.13, 95.07, 94.88, 94.67, 73.33, 73.11, 72.81, 72.44, 72.11, 72.08, 72.05, 71.96, 71.95, 71.82, 69.62, 69.59, 69.55, 69.17, 69.09, 69.03, 68.91, 68.39, 68.28, 68.19, 68.12, 67.97, 67.84, 67.80, 67.76, 66.76, 62.80, 62.65, 62.63, 62.61, 62.51, 62.06, 51.80, 49.56, 49.48, 40.04, 37.47, 36.77, 36.71, 36.64, 28.89, 22.51, 22.47, 22.00. HRMS (ESI) m/z calculated for $C_{73}H_{112}N_5O_{50}^-$ (M-H) 1858.6375, found 1858.6323.

OPME synthesis of galactosyloctasaccharide Galα1(–4Neu5Acα2–6Galα1)$_3$–4Neu5AcαProNHCbz (G8). A reaction mixture in a total volume of 19 mL containing Tris-HCl buffer (100 mM, pH 8.5), sialylheptasaccharide **S7** (370 mg, 0.19 mmol), galactose (47 mg, 0.25 mmol), ATP disodium salt (143 mg, 0.25 mmol), UTP trisodium salt (143 mg, 0.25 mmol), $MgCl_2$ (20 mM), SpGalK (1.8 mg), BLUSP (1.8 mg), PmPpA (1.8 mg), and $NmSiaD_W$ (0.9 mg) was incubated in a 50-mL centrifuge tube in a shaker (100 rpm) at 30 °C for 16 hrs. The product formation was monitored by UHPLC (AdvanceBio Glycan Map, Agilent, 72% Acetonitrile + 0.1% TFA in water, monitored at 215 nm). When an optimal yield was achieved, pre-chilled ethanol (19 mL) was added and the resulting mixture was incubated at 4 °C for 30 min. Procedures for centrifugation, concentration, purification, collection and neutralization were similar to that described above for **G2** to produce galactosyloctasaccharide **G8** as a sodium salt (380 mg, 95%).

^{1}H NMR (800 MHz, D_2O) δ 7.46–7.37 (m, 5H, Ar-H), 5.11 (s, 2H, O-$\underline{CH_2}$-Ar), 5.07 (d, J = 4.0 Hz, 1H, H^{VIII}-1), 5.05 (dd, *J* = 6.8, 4.0 Hz, 3H, $H^{II,IV,VI}$-1), 4.06–4.00 (m, 4H, $H^{I,III,V,VII}$-5), 3.96 (t, *J* = 3.9 Hz, 4H, $H^{II,IV,VI,VIII}$-3), 3.90–3.58 (m, 45H), 3.49 (dd, *J* = 10.6, 5.7 Hz, 1H, O-$\underline{CH_2}$-CH_2), 3.23–3.17 (m, 2H, CH_2-NH), 2.90–2.84 (m, 4H, $H^{I,III,V,VII}$-3eq), 2.08 (s, 9H, $H^{I,III,V}$-CH_3-CO), 2.02 (s, 3H, H^{VII}-CH_3-CO), 1.74 (p, *J* = 6.8 Hz, 2H, O-CH_2-$\underline{CH_2}$-CH_2-NH), 1.68–1.58 (m, 4H, $H^{I,III,V,VII}$-3_{ax}). ^{13}C NMR (200 MHz, D_2O) δ 174.52, 174.50, 174.46, 174.29, 173.20, 173.08, 158.37, 136.57, 128.76, 128.27, 127.56, 100.57, 100.23, 100.21, 95.08, 95.05, 94.77, 94.68, 73.33, 73.24, 72.97, 72.81, 72.16, 72.11, 72.08, 72.05, 71.95, 71.91, 71.88, 71.82, 71.01, 69.62, 69.59, 69.23, 69.17, 69.11, 69.05, 69.03, 68.97, 68.90, 68.19, 68.13, 67.97, 67.84, 67.81, 67.76, 66.75, 62.89, 62.80, 62.61, 62.59, 62.51, 62.06, 60.71, 49.56, 49.48, 49.46, 37.47, 36.78, 36.74, 36.72, 36.71, 36.59, 28.89, 22.51, 22.47, 22.11. HRMS (ESI) m/z calculated for $C_{79}H_{121}N_5O_{55}^{2-}$ (M/2-H) 1009.8413, found 1009.8401.

OPME synthesis of sialylnonasaccharide Neu5Acα2(–6Galα1–4Neu5Acα2)$_4$–ProNHCbz (S9). A reaction mixture in a total volume of 15 mL containing Tris-HCl buffer (100 mM, pH 8.5), galactosyloctasaccharide **G8** (310 mg, 0.15 mmol), Neu5Ac (61 mg, 0.20 mmol), CTP disodium salt (105 mg, 0.20 mmol), $MgCl_2$ (20 mM), NmCSS (0.5 mg) and $NmSiaD_W$ (1.0 mg) was incubated in a 50-mL centrifuge tube in a shaker (100 rpm) at 30 °C for 16 hrs. The product formation was monitored by UHPLC (AdvanceBio Glycan Map, Agilent, 71% Acetonitrile + 0.1% TFA in water, monitored at 215 nm). When an optimal yield was achieved, pre-chilled ethanol (15 mL) was added and the resulting mixture was incubated at 4 °C for 30 min. Procedures for centrifugation, concentration, purification, collection and neutralization were similar to that described above for **G2** to produce sialylnonasaccharide **S9** as a sodium salt (301 mg, 84%).

^{1}H NMR (800 MHz, D_2O) δ 7.46–7.38 (m, 5H, Ar-H), 5.11 (s, 2H, O-$\underline{CH_2}$-Ar), 5.05 (q, *J* = 3.9 Hz, 4H, $H^{II,IV,VI,VIII}$-1), 4.03 (td, *J* = 10.3, 4.2 Hz, 4H, $H^{I,III,V,VII}$-5), 3.96 (d, *J* = 3.5 Hz, 4H, $H^{II,IV,VI,VIII}$-3), 3.92–3.75 (m, 28H), 3.73–3.59 (m, 23H), 3.56 (dd, *J* = 8.8, 1.8 Hz, 1H), 3.50 (dt, *J* = 11.1, 6.4 Hz, 1H, O-$\underline{CH_2}$-CH_2), 3.23–3.14 (m, 2H, CH_2-NH), 2.90–2.84 (m, 4H, $H^{I,III,V,VII}$-3eq), 2.72 (dd, *J* = 12.5, 4.6 Hz, 1H, H^{IX}-3eq), 2.08 (s, 12H, $H^{I,III,V,VII}$-CH_3-CO), 2.03 (s, 3H, H^{IX}-CH_3-CO), 1.74 (p, *J* = 6.9 Hz, 2H, O-CH_2-$\underline{CH_2}$-CH_2-NH), 1.65 (ddt, *J* = 36.8, 33.3, 12.1 Hz, 5H, $H^{I,III,V,VII,IX}$-3_{ax}). ^{13}C NMR (200 MHz, D_2O) δ 174.96, 174.53, 174.51, 174.48, 174.46, 173.48, 173.09, 173.06, 158.37, 136.57, 128.76, 128.27, 127.56, 100.57, 100.23, 100.21, 100.13, 95.09, 95.04, 94.87, 94.68, 73.34, 73.30, 73.10, 72.81, 72.45, 72.11, 72.11, 72.06, 71.97, 71.95, 71.92, 71.82, 69.62, 69.62, 69.59, 69.55, 69.17, 69.09, 69.05, 69.03, 68.91, 68.39, 68.29, 68.19, 68.14, 68.12, 67.97, 67.84, 67.79, 67.78, 67.76, 66.76, 62.84, 62.79, 62.61, 62.51, 62.06, 51.80, 49.56, 49.48, 40.04, 37.47, 36.78, 36.71, 36.64, 28.89, 23.23, 22.51, 22.47, 22.01. HRMS (ESI) m/z calculated for $C_{90}H_{138}N_6O_{63}^{2-}$ (M/2-H) 1155.3890, found 1155.3855.

OPME synthesis of galactosyldecasaccharide Galα1(–4Neu5Acα2–6Galα1)$_4$–4Neu5AcαProNHCbz (G10). A reaction mixture in a total volume of 10 mL containing Tris-HCl buffer (100 mM, pH 8.5), sialylnonasaccharide **S9** (250 mg, 0.10 mmol), galactose (25 mg, 0.13 mmol), ATP disodium salt (75 mg, 0.13 mmol), UTP trisodium salt (75 mg, 0.13 mmol), $MgCl_2$ (20 mM), SpGalK (1.0 mg), BLUSP (1.0 mg), PmPpA (1.0 mg), and $NmSiaD_W$ (0.5 mg) was incubated in a 50-mL centrifuge tube in a shaker (100 rpm) at 30 °C for 16 hrs. The product formation was monitored by UHPLC (AdvanceBio Glycan Map, Agilent, 70% acetonitrile + 0.1% TFA in water, monitored at 215 nm). When an optimal yield was achieved, pre-chilled ethanol (10 mL) was added and the resulting mixture was incubated at 4 °C for 30 min. Procedures for centrifugation, concentration, purification, collection and neutralization were similar to that described above for **G2** to produce galactosyldecasaccharide **G10** as a sodium salt (221 mg, 83%).

^{1}H NMR (800 MHz, D_2O) δ 7.54–7.35 (m, 5H, Ar-H), 5.11–5.04 (m, 7H, O-$\underline{CH_2}$-Ar, $H^{II,IV,VI,VIII,X}$-1), 4.05 (t, *J* = 10.1 Hz, 5H, $H^{I,III,V,VII,IX}$-5), 3.95 (dd, *J* = 6.2, 3.4 Hz, 5H, $H^{II,IV,VI,VIII,X}$-3), 3.91–3.58 (m, 56H), 3.55–3.50 (m, 1H, O-$\underline{CH_2}$-CH_2), 3.24–3.13 (m, 2H, CH_2-NH), 2.95–2.78 (m, 5H, $H^{I,III,V,VII,IX}$-3eq), 2.06 (s, 12H, $H^{I,III,V,VII}$-CH_3-CO), 2.02 (s, 3H, H^{IX}-CH_3-CO), 1.77–1.71 (m, 2H, O-CH_2-$\underline{CH_2}$-CH_2-NH), 1.71–1.60 (m, 5H, $H^{I,III,V,VII,IX}$-3_{ax}). ^{13}C NMR (200 MHz, D_2O) δ 174.46, 174.35, 171.70, 171.24, 171.16, 171.13, 158.34, 136.54, 128.75, 128.29, 127.57, 99.17, 94.73, 94.41, 94.36, 94.28, 72.37, 72.24, 71.88, 71.75, 71.70, 71.17, 71.05, 71.00, 70.98, 70.93, 69.54, 69.52, 69.31, 69.24, 69.22, 69.02, 68.13, 68.11, 68.04, 67.87, 67.79, 67.77, 67.76, 66.76, 63.21, 62.88, 62.84, 62.81, 62.06, 60.72, 49.34, 49.28, 37.36, 35.99, 35.54, 28.77, 22.33, 22.32, 22.11. HRMS (ESI) m/z calculated for $C_{96}H_{148}N_6O_{68}^{2-}$ (M/2-H) 1236.4153, found 1236.4109.

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(1) Yu, H.; Yu, H.; Karpel, R.; Chen, X. Chemoenzymatic Synthesis of CMP-Sialic Acid Derivatives by A One-Pot Two-Enzyme System: Comparison of Substrate Flexibility of Three Microbial CMP-Sialic Acid Synthetases. *Bioorg. Med. Chem.* **2004,** *12*, 6427-6435.

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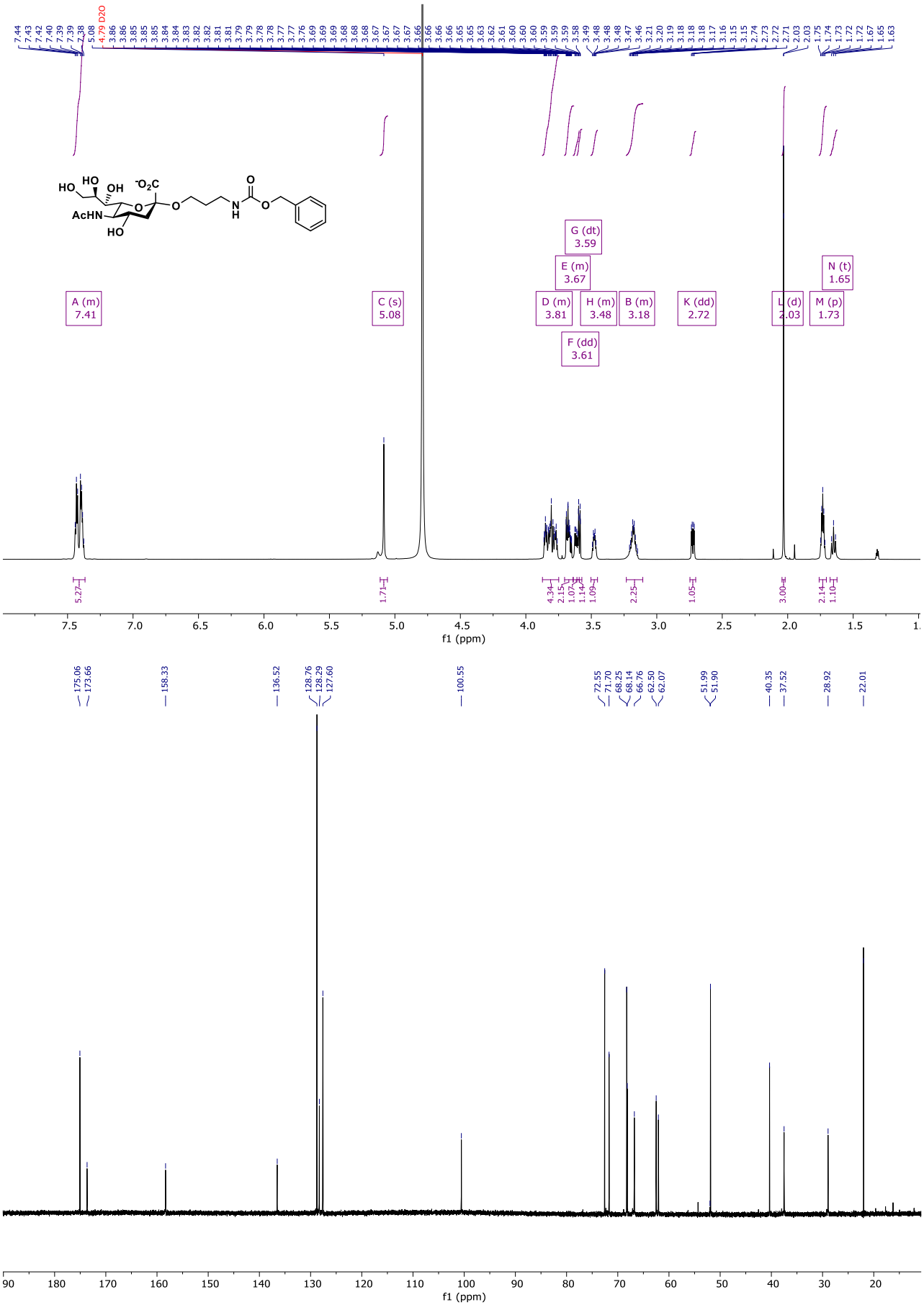
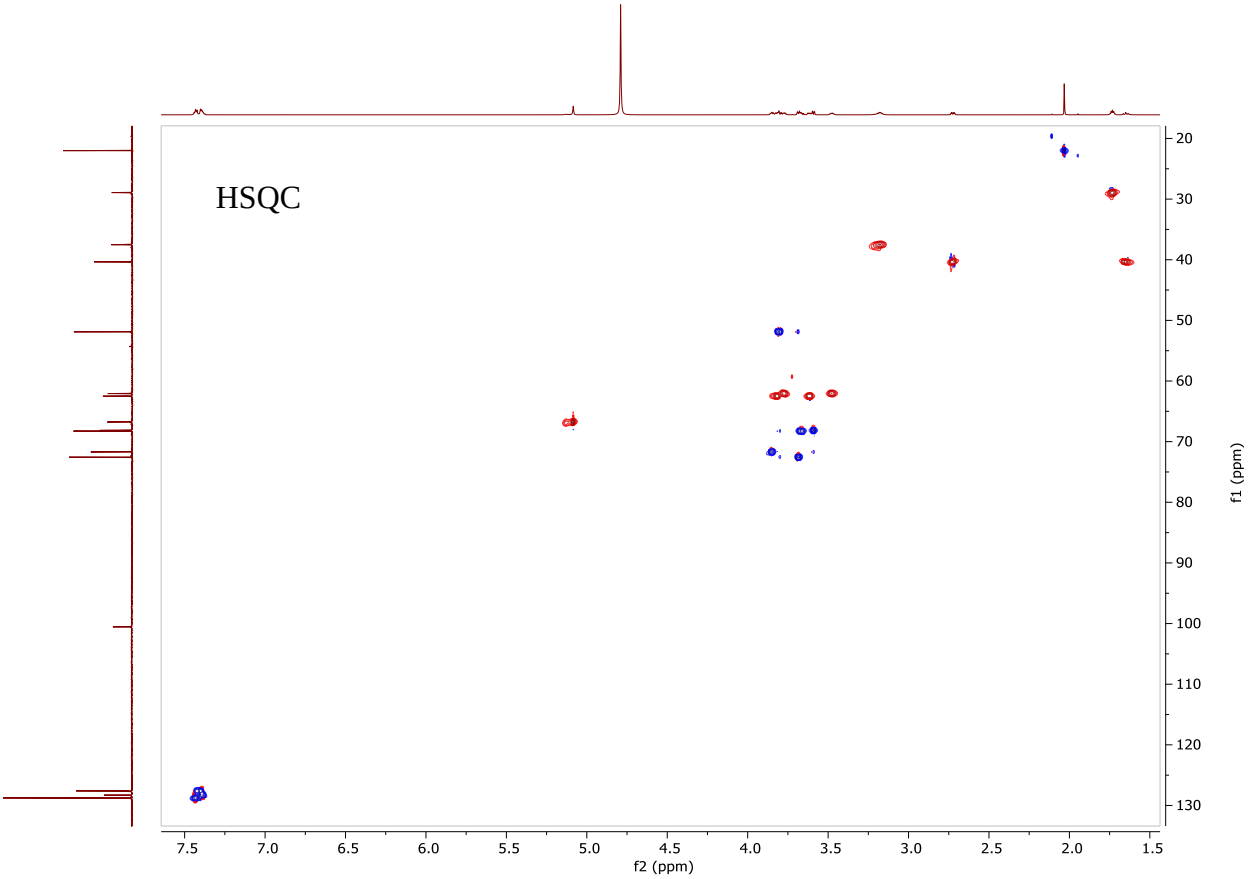
^{1}H, ^{13}C, HSQC, HSQC-TOCSY (90 ms and 10 ms) NMR spectra of sialylmonosaccharide Neu5AcαProNHCbz (**S1**)

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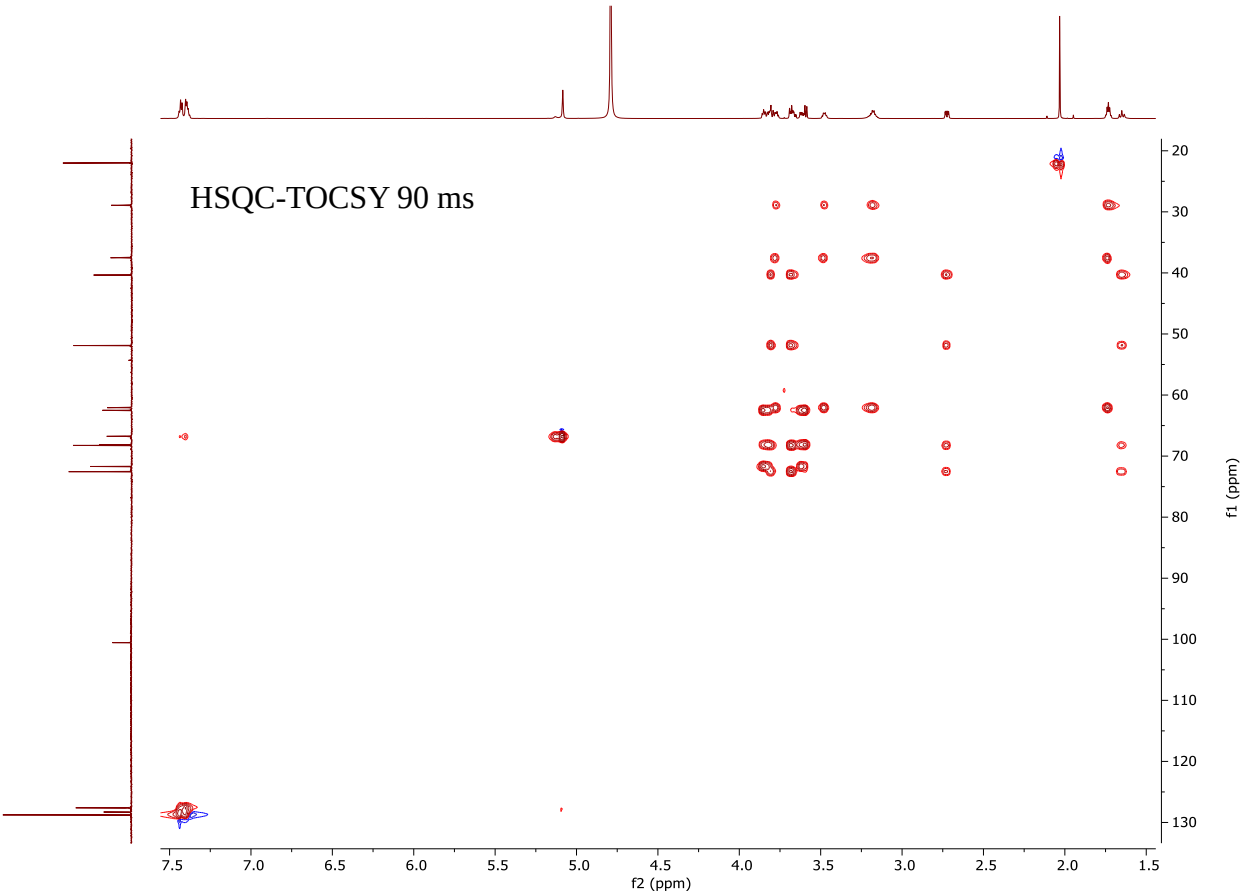
f1 (ppm)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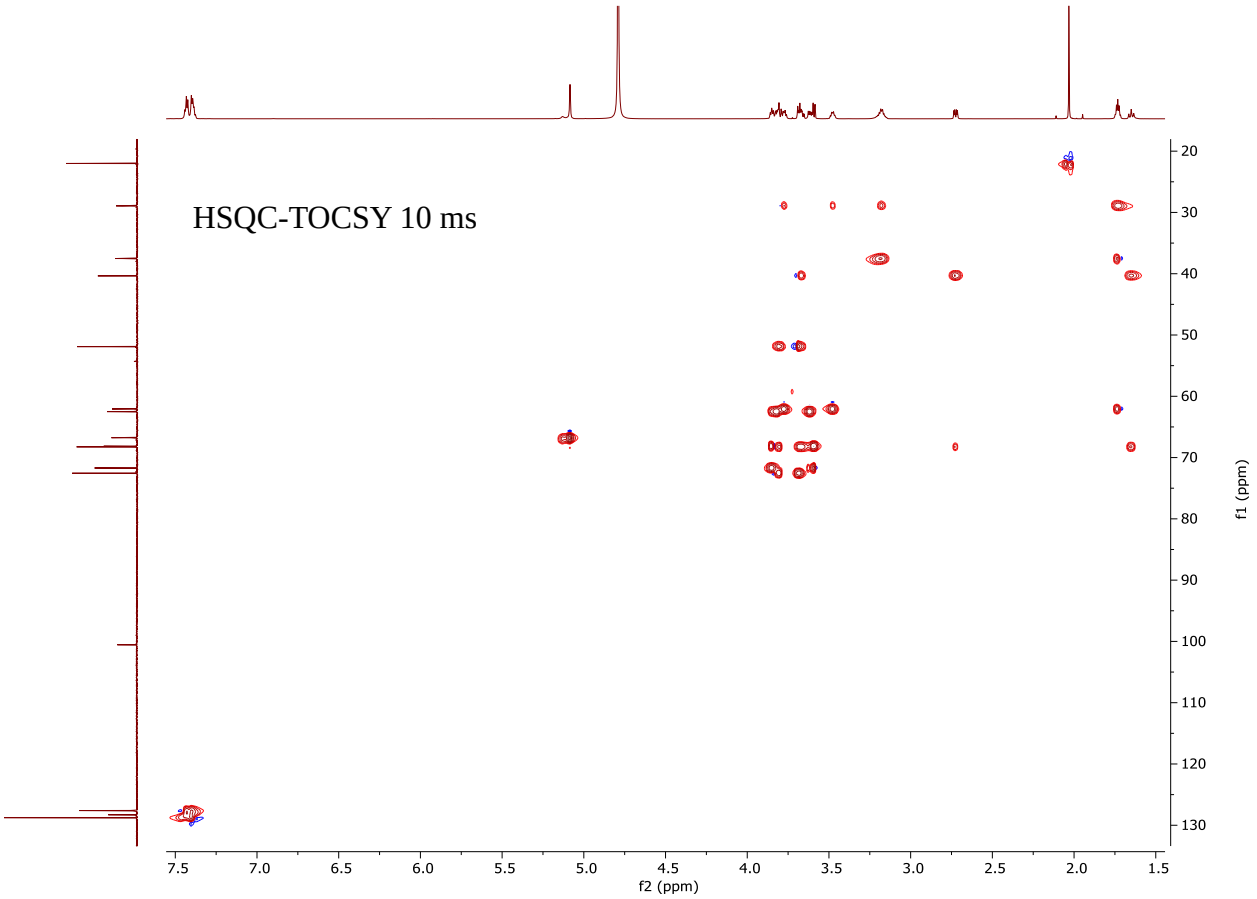
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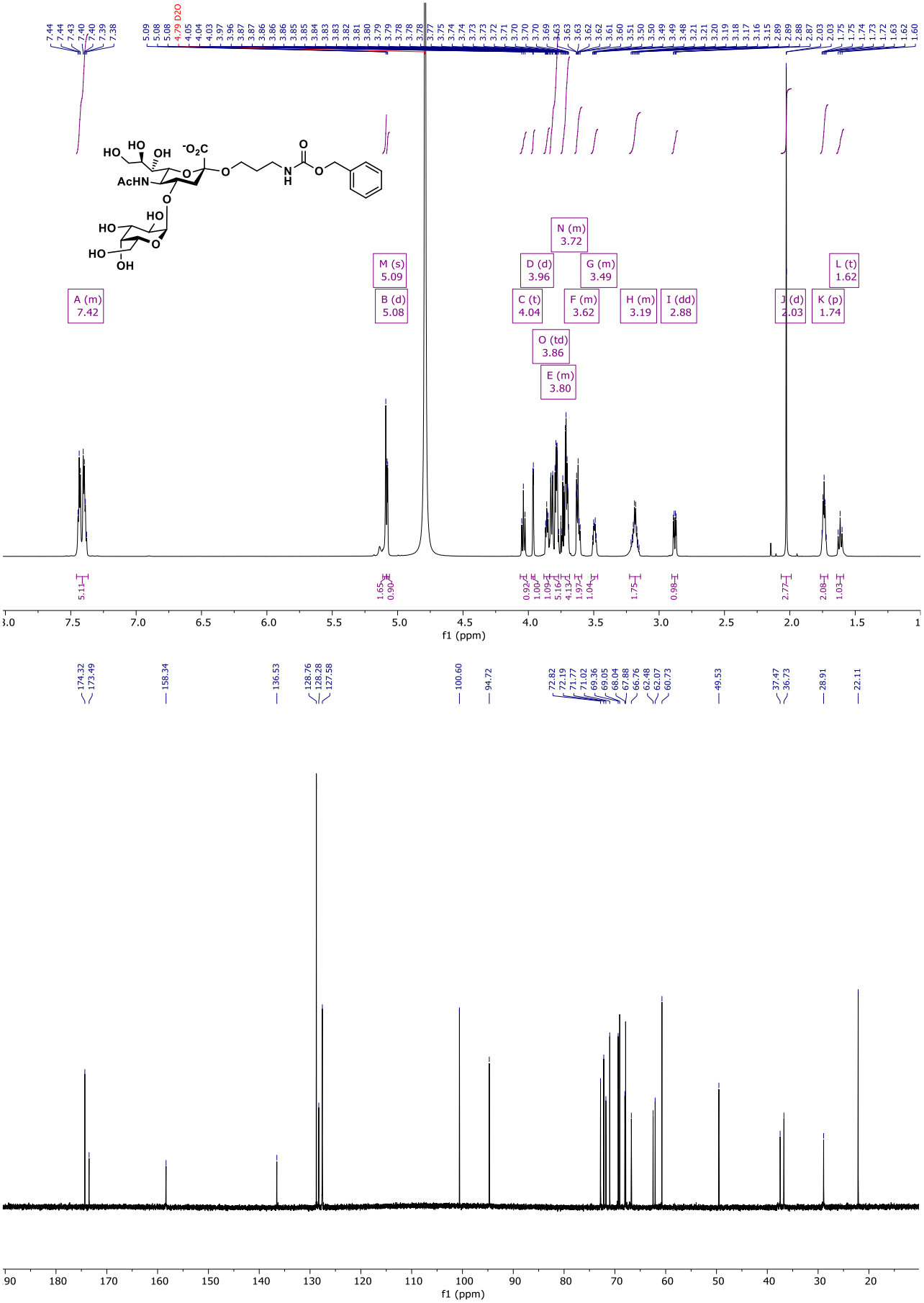
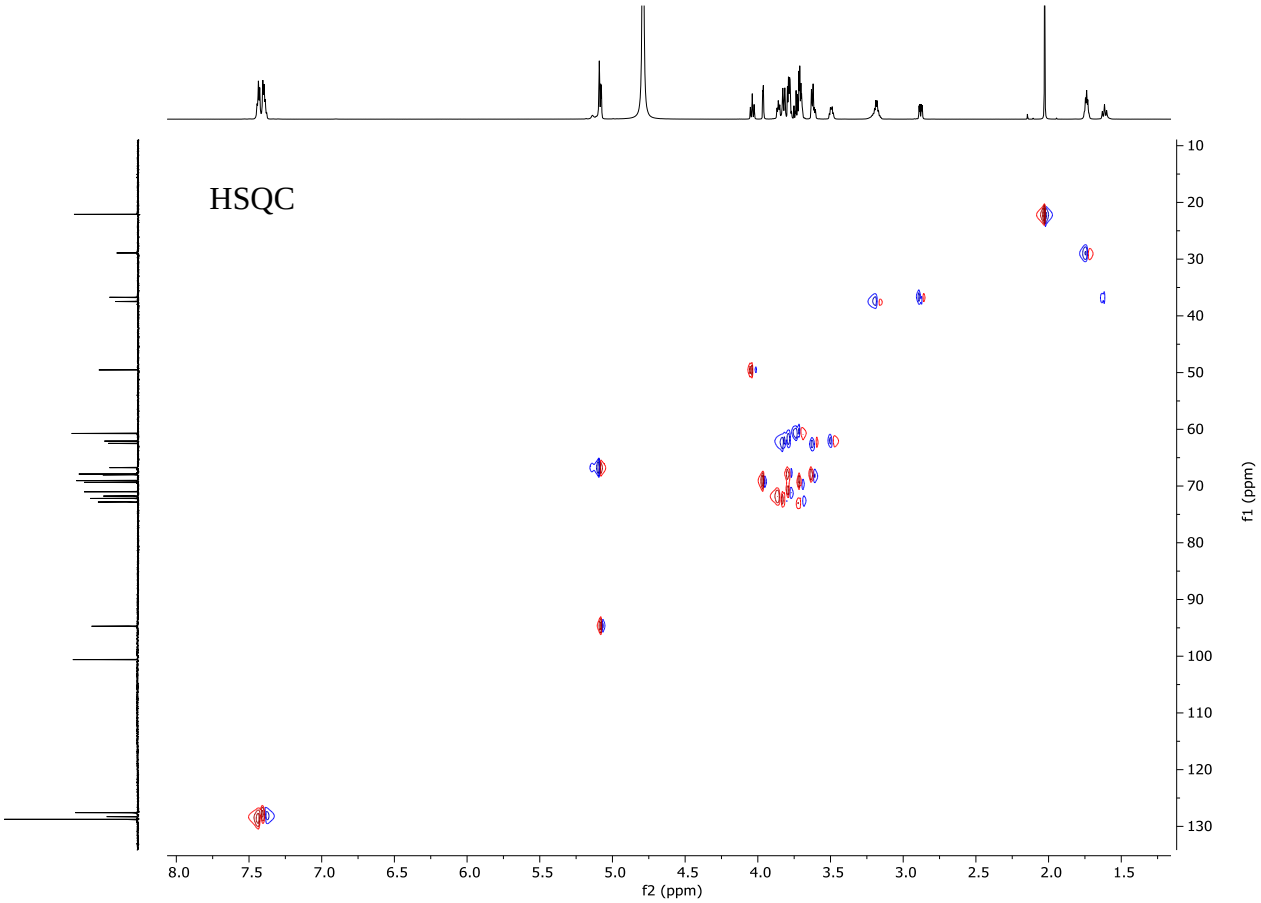
^{1}H, ^{13}C, HSQC, HSQC-TOCSY (90 ms and 10 ms) NMR spectra of galactosyldisaccharide Galα1–4Neu5AcαProNHCbz (**G2**)

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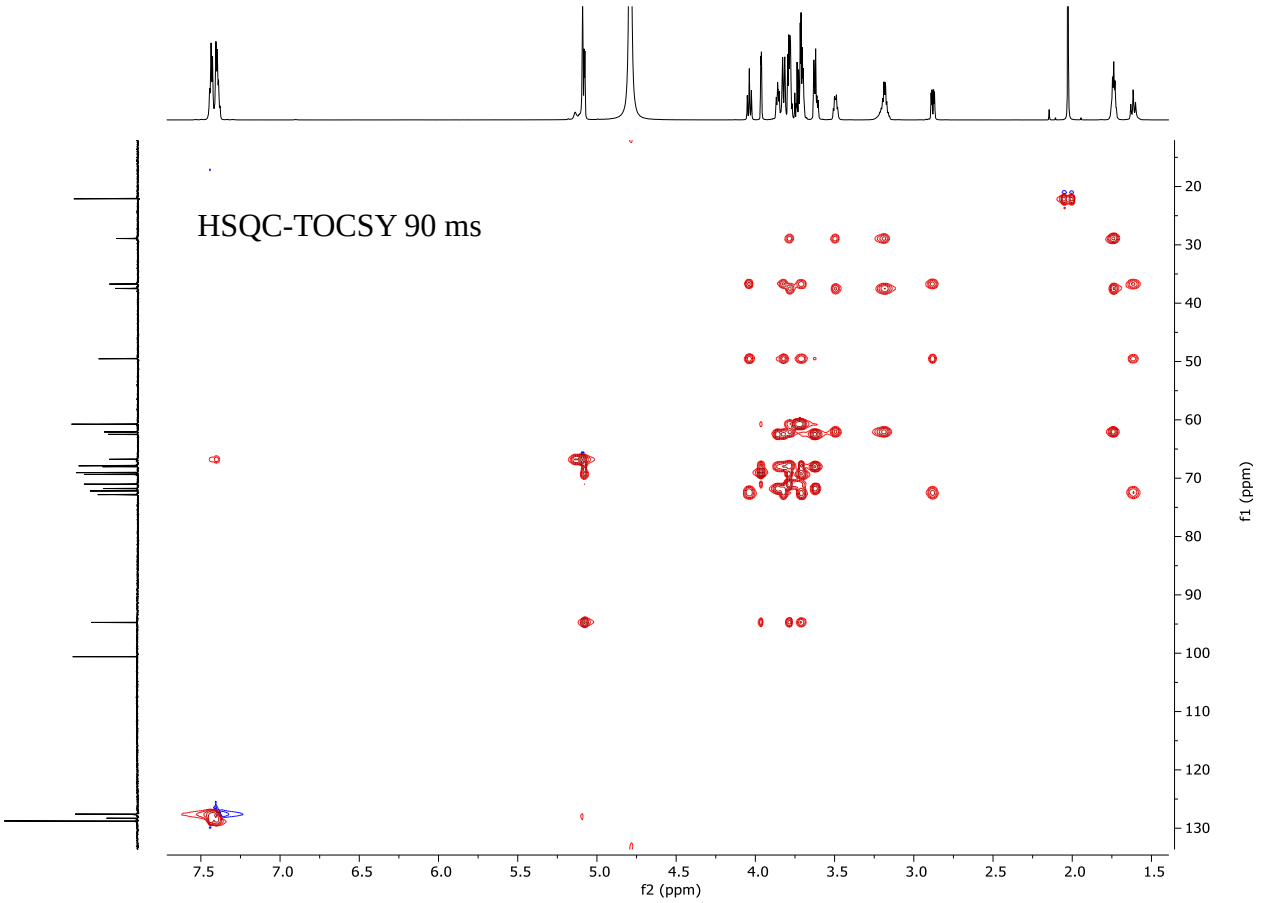
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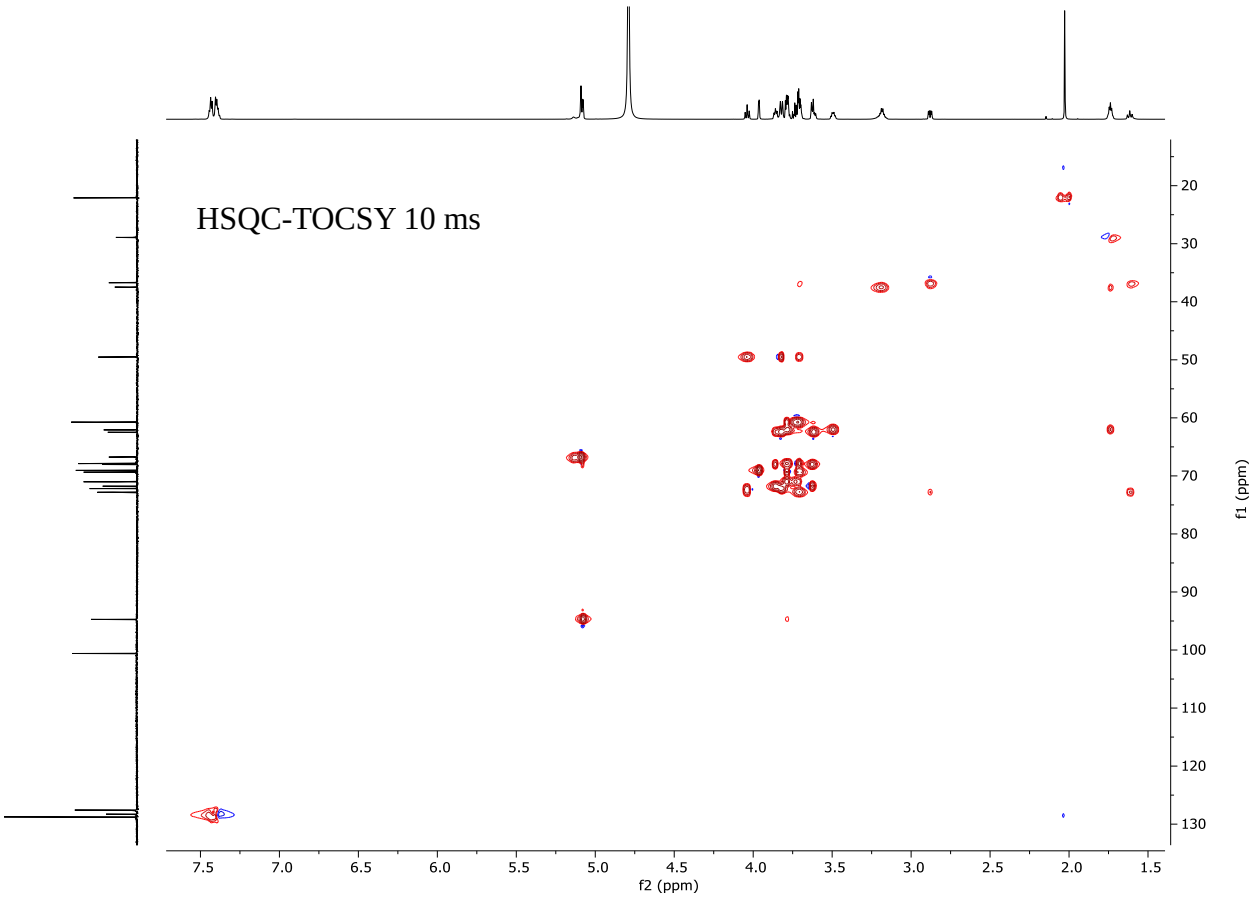
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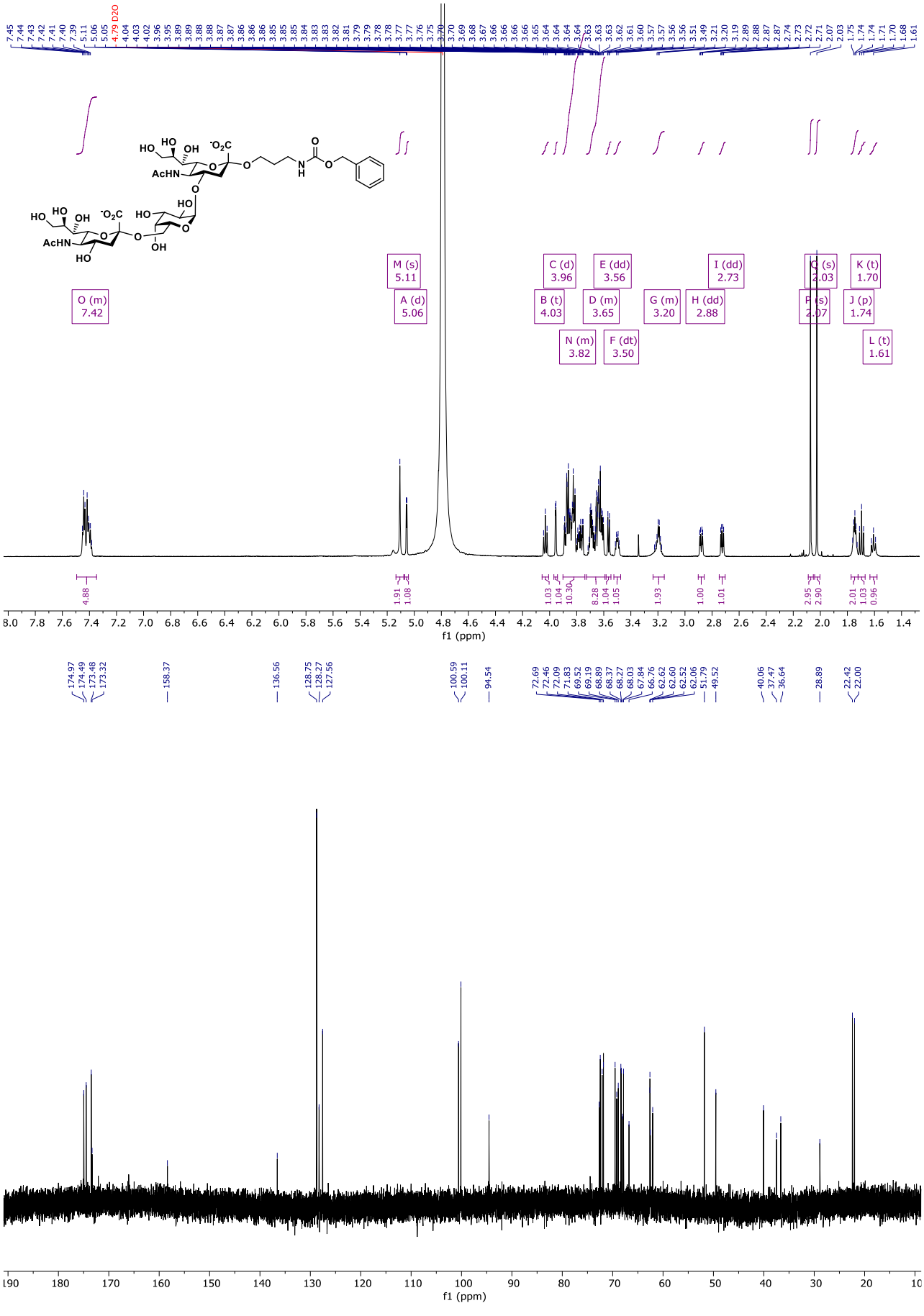
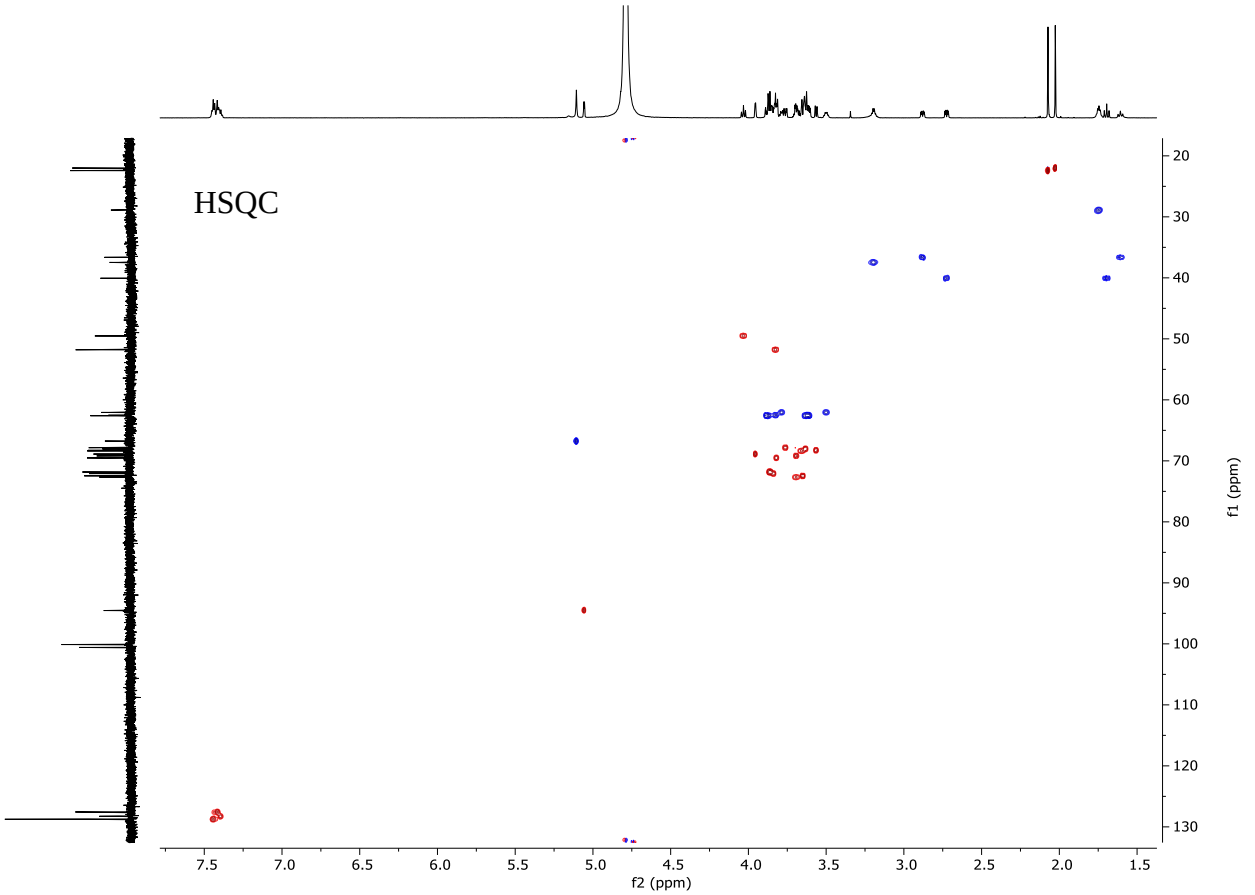
f2 (ppm)
f1 (ppm)

^{1}H, ^{13}C, HSQC, HSQC-TOCSY (90 ms and 10 ms) NMR spectra of sialyltrisaccharide Neu5Acα2–6Galα1–4Neu5AcαProNHCbz (**S3**)

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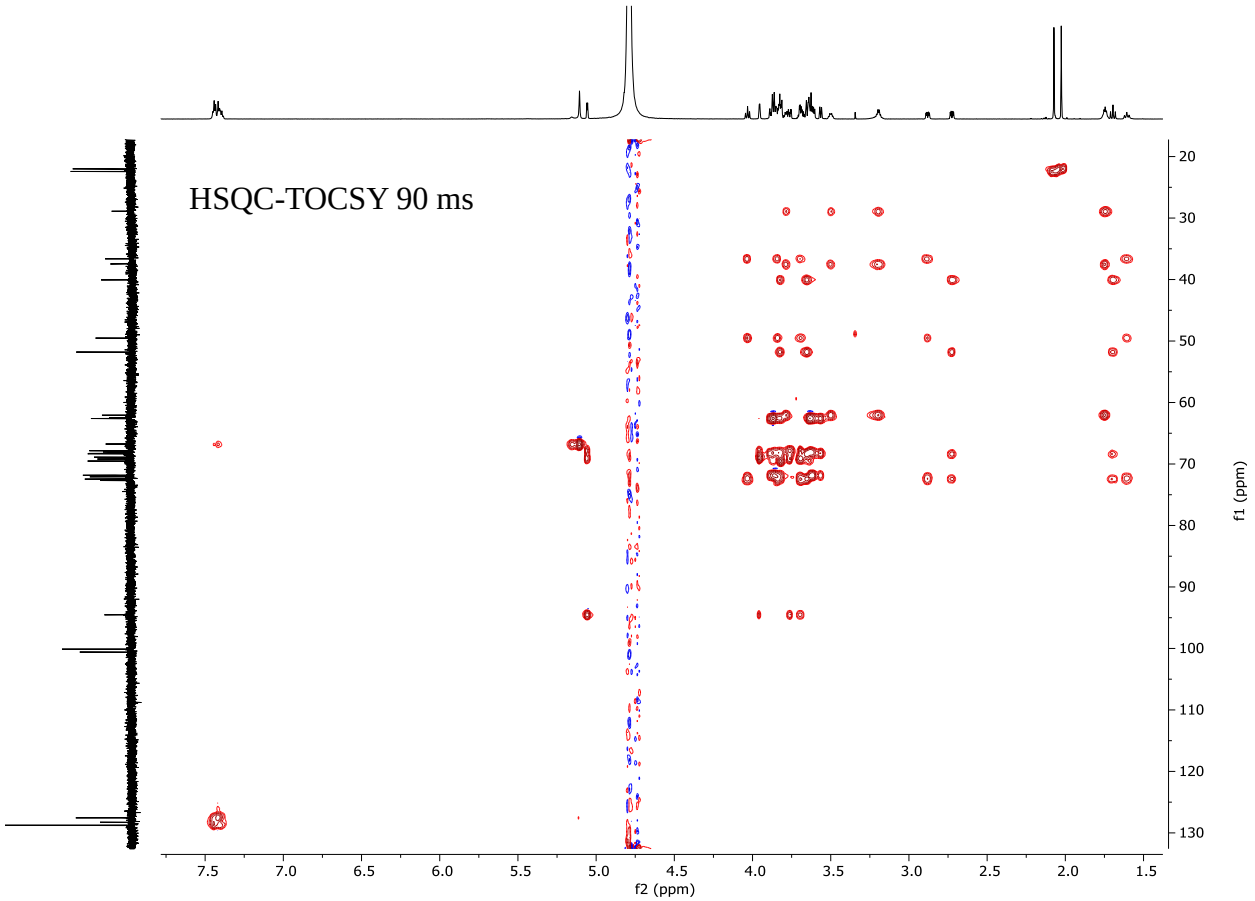
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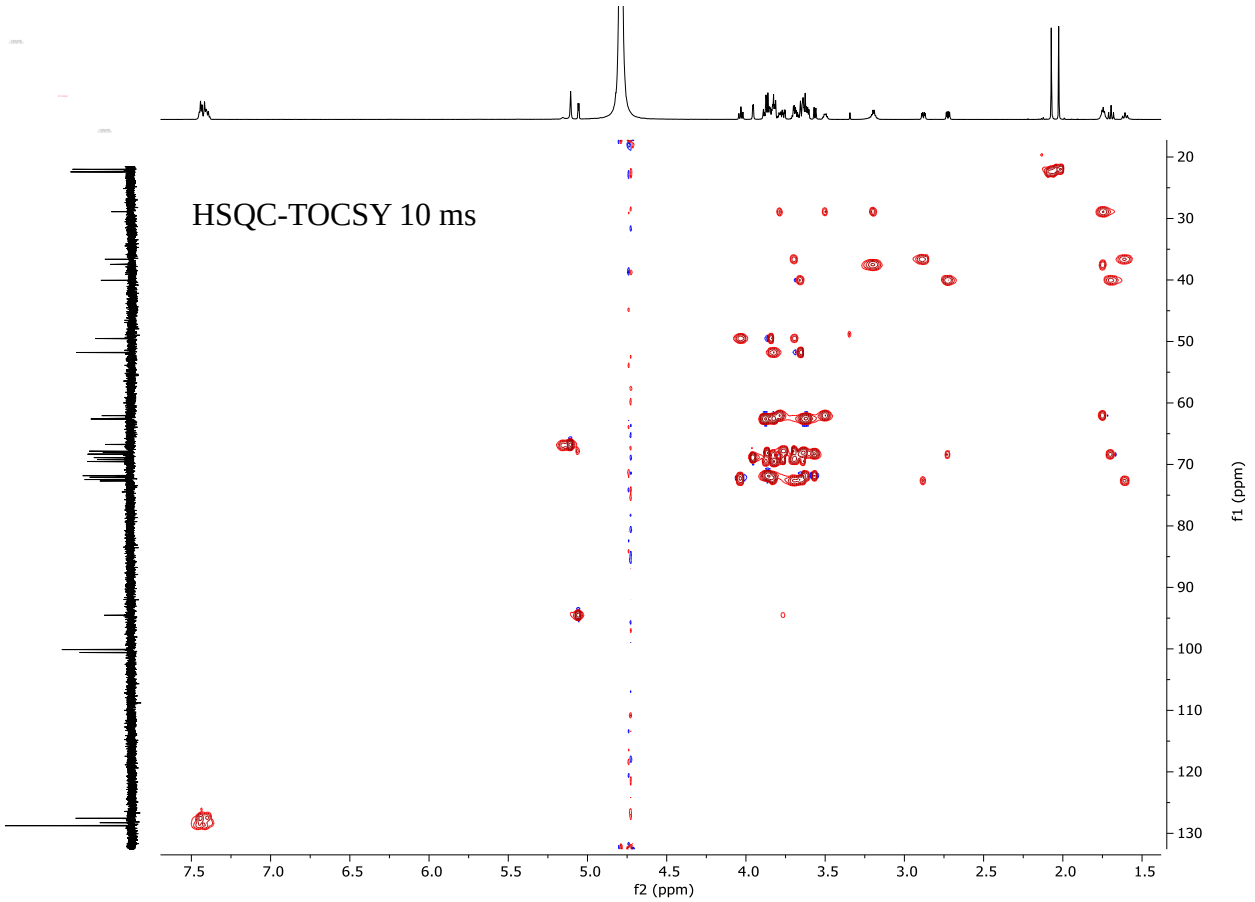
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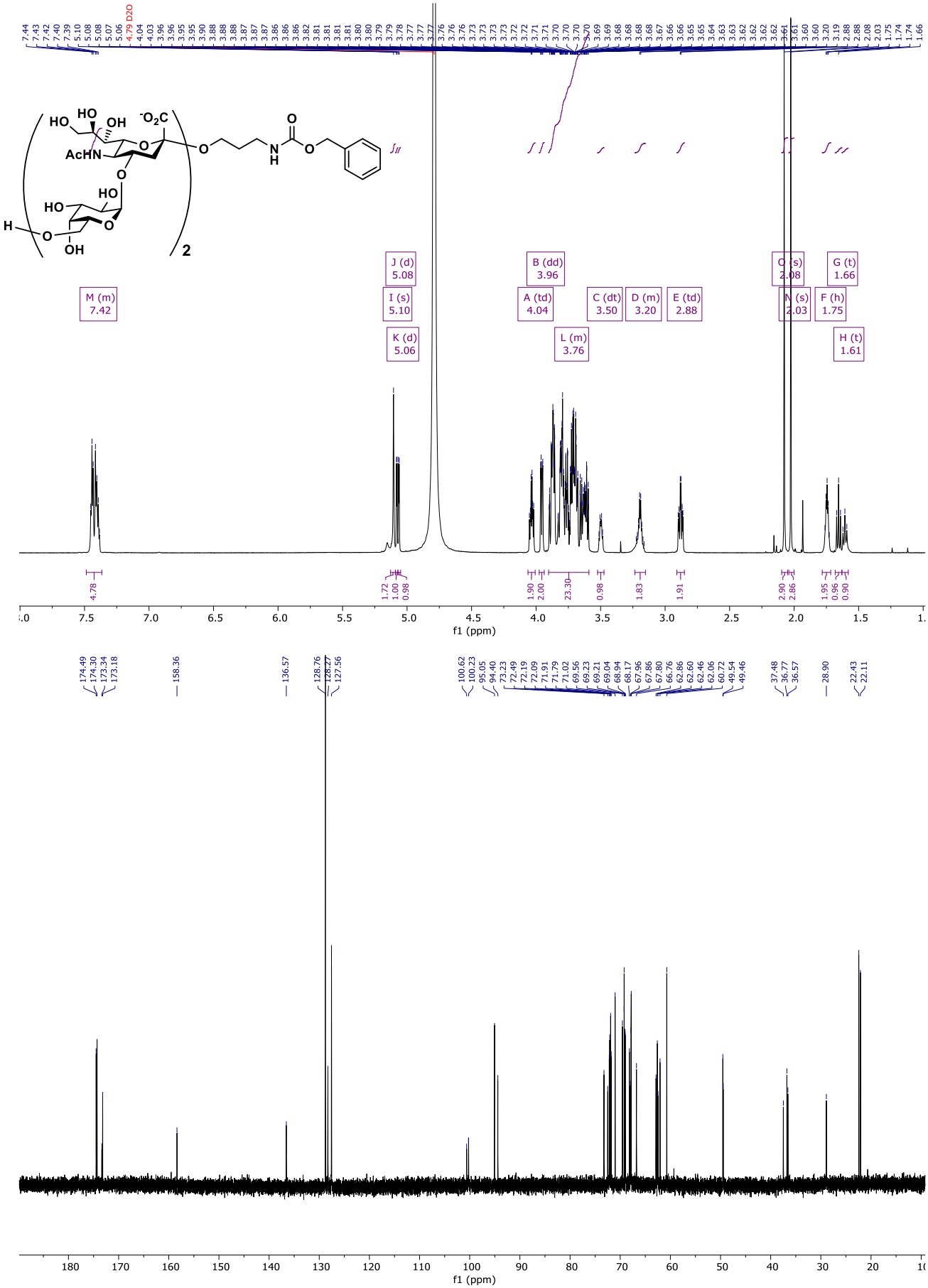
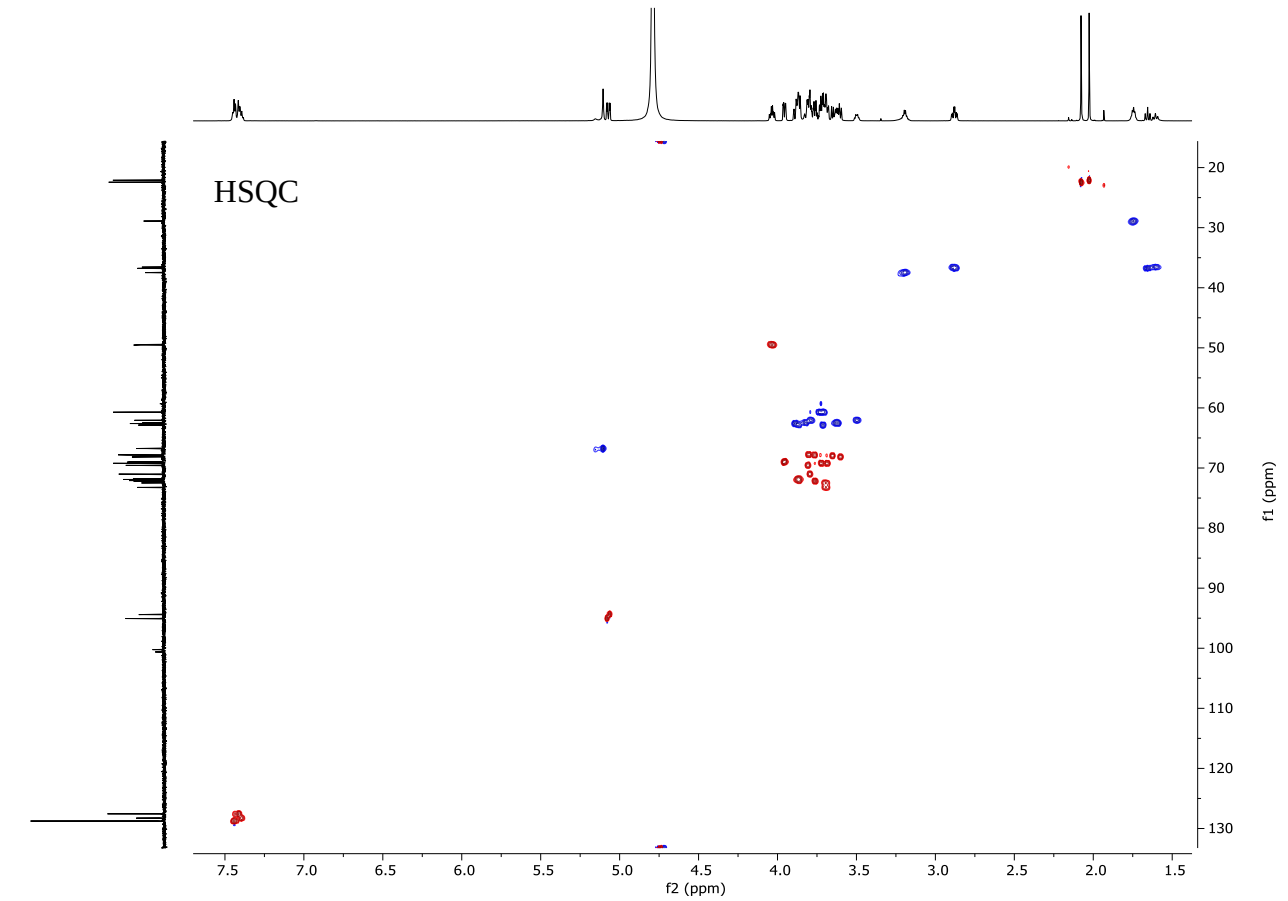
f1 (ppm)

^{1}H, ^{13}C, HSQC, HSQC-TOCSY (90 ms and 10 ms) NMR spectra of galactosyltetrasaccharide Galα1–4Neu5Acα2–6Galα1–4Neu5AcαProNHCbz (**G4**)

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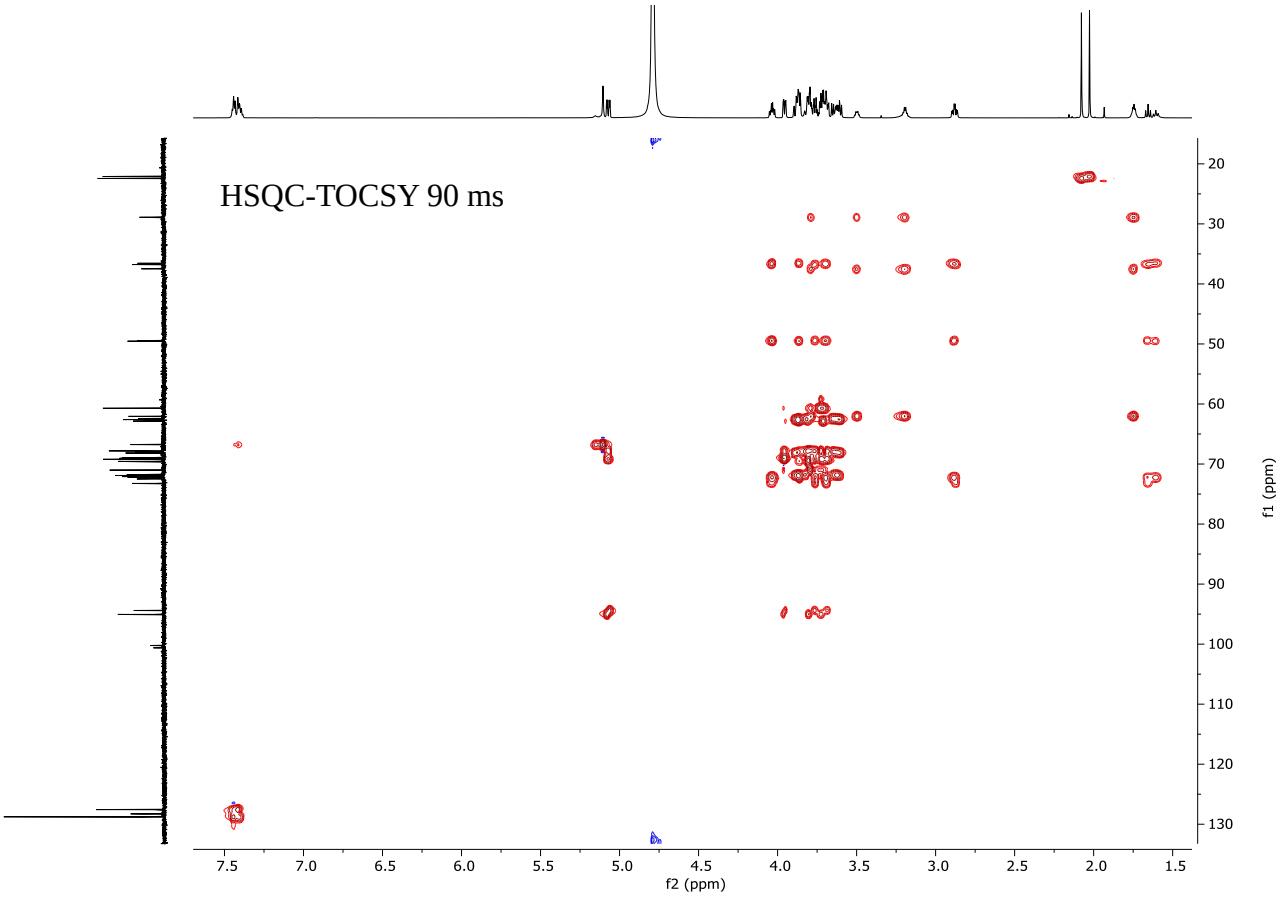
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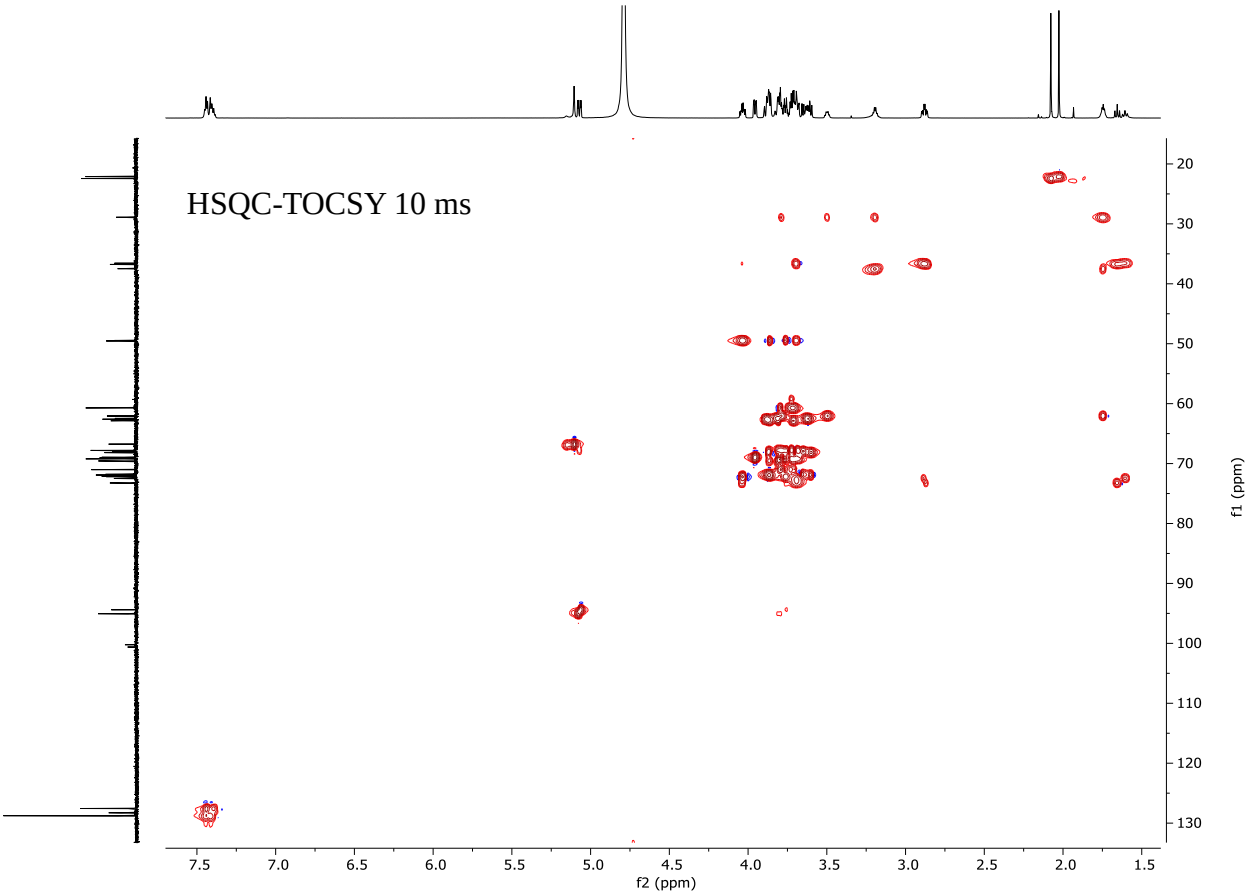
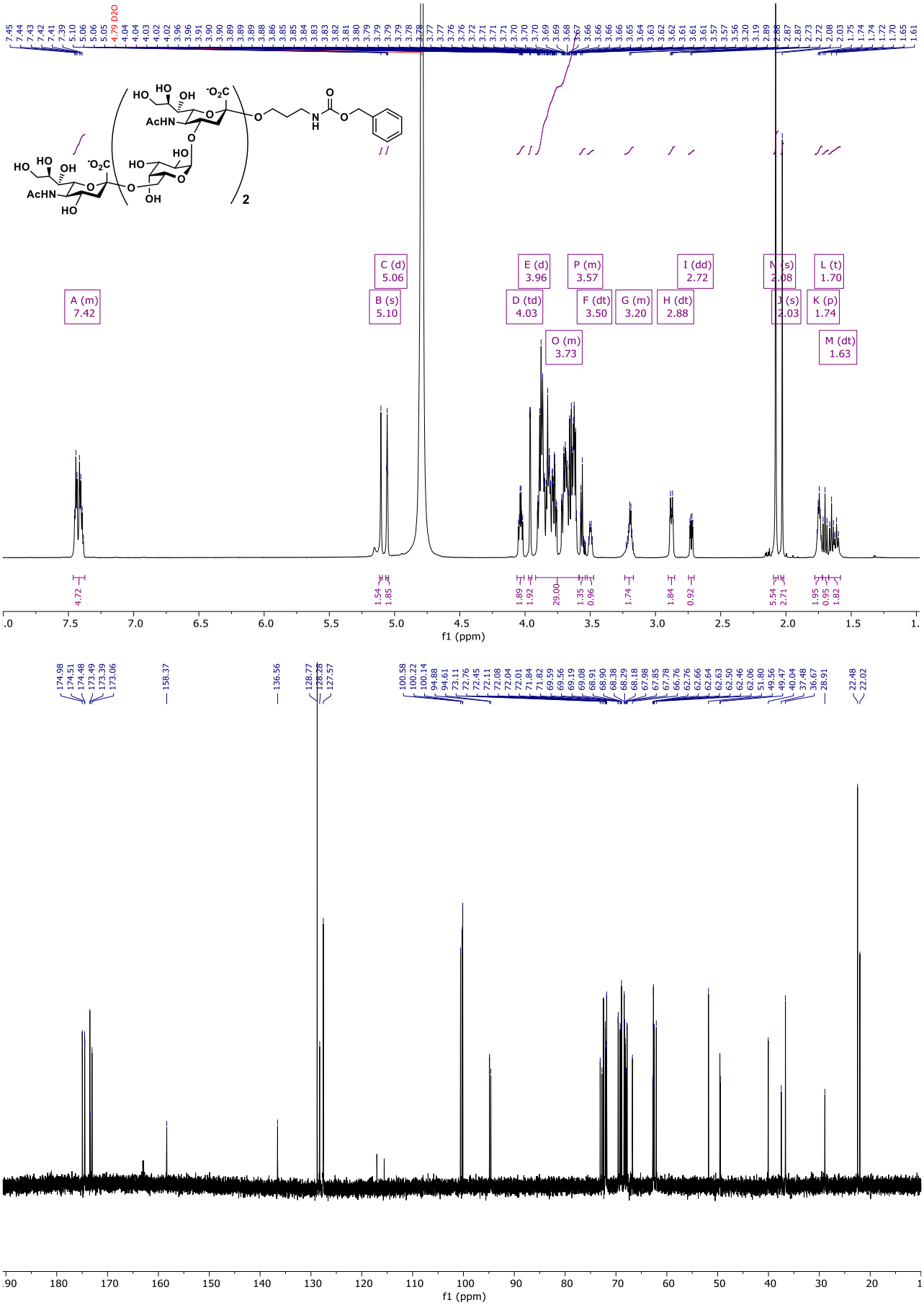
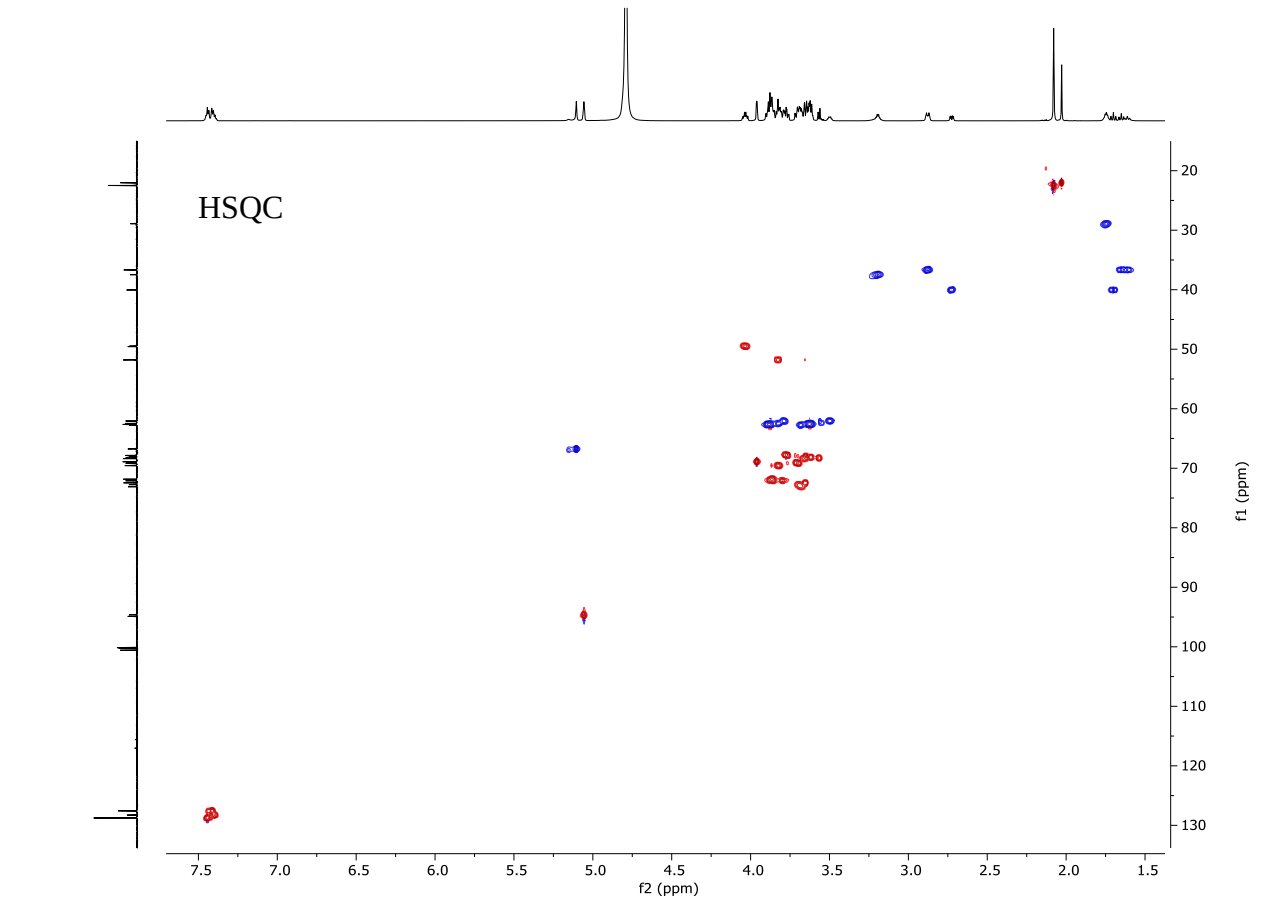
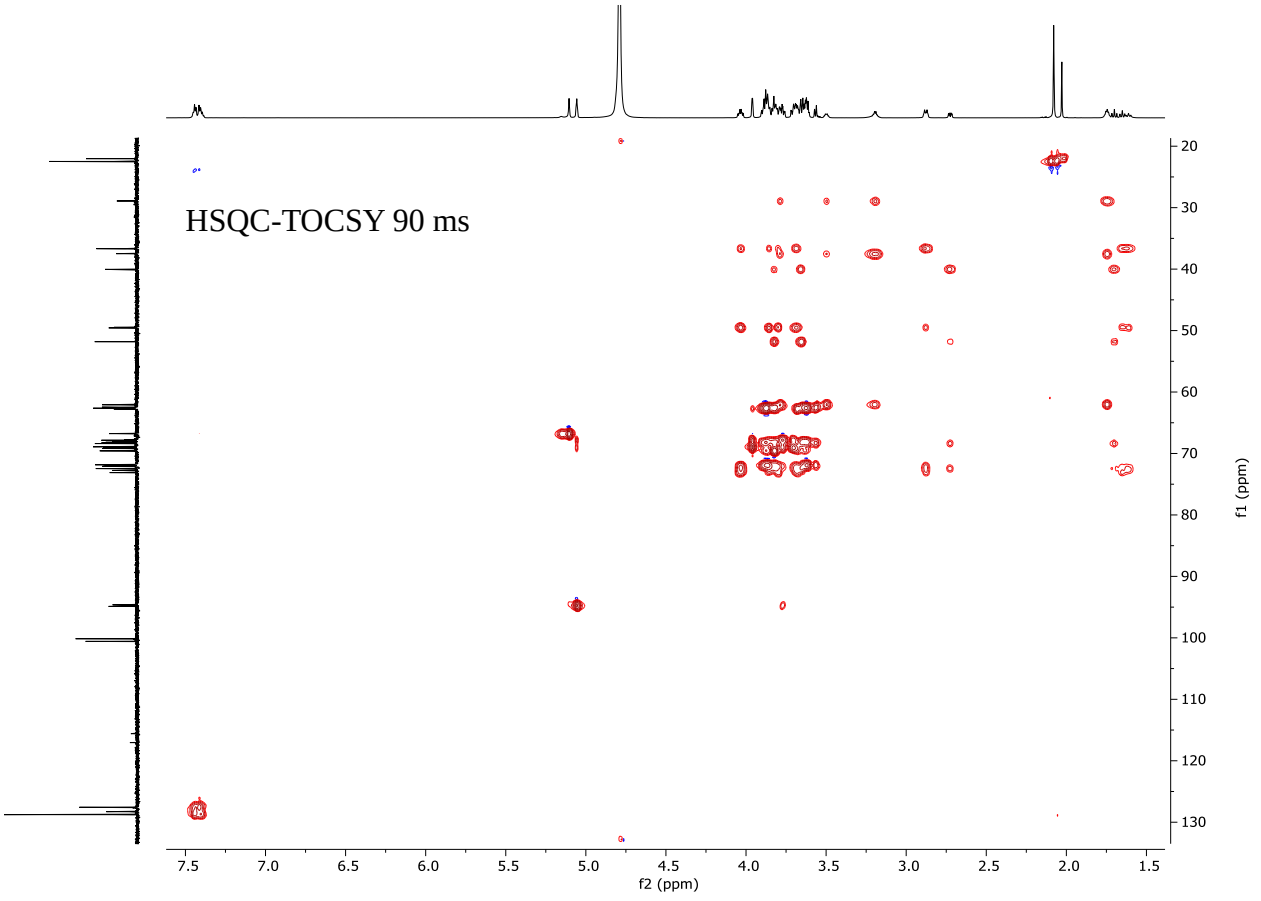
f2 (ppm)
f1 (ppm)

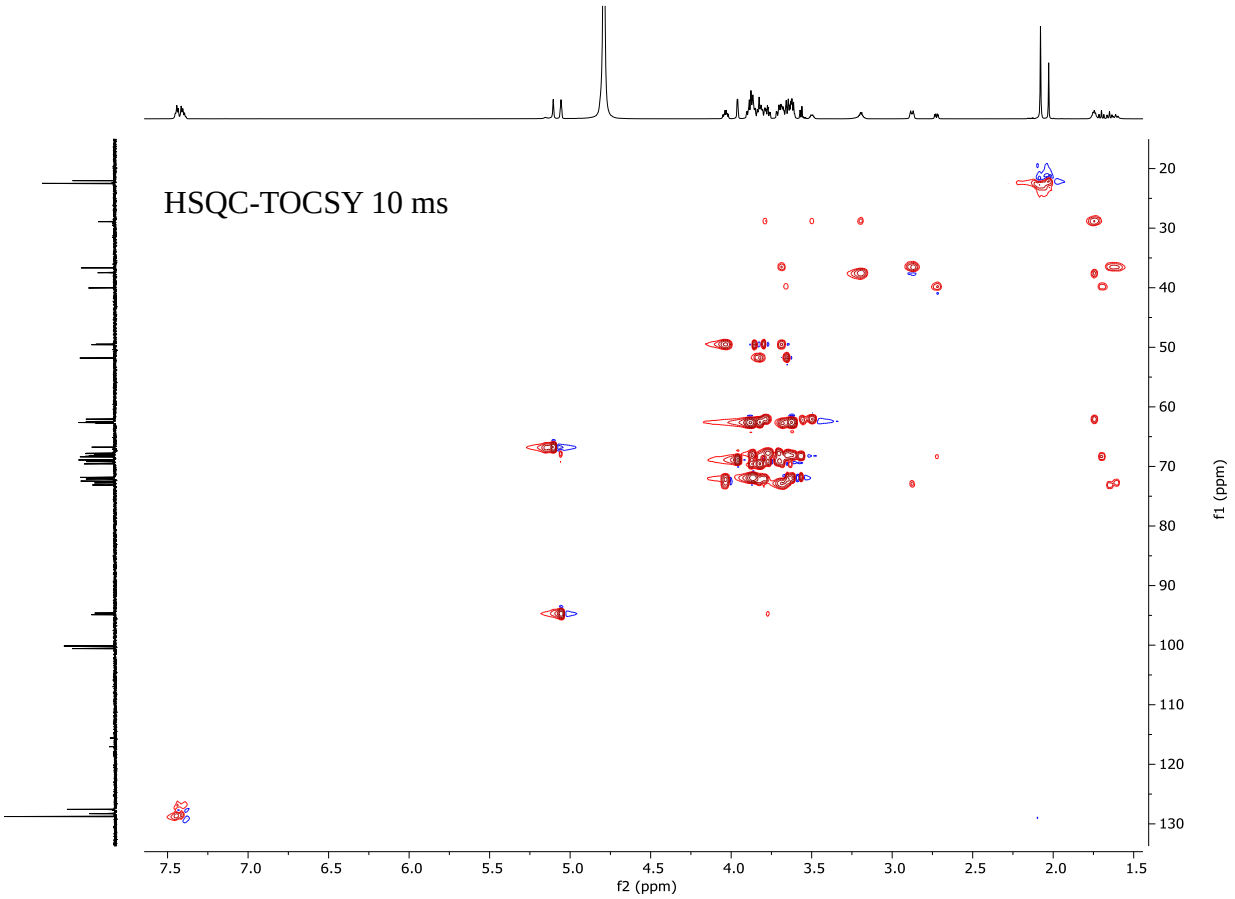

HSQC-TOCSY 10 ms
f2 (ppm)
f1 (ppm)

^{1}H, ^{13}C, HSQC, HSQC-TOCSY (90 ms and 10 ms) NMR spectra of sialylpentasaccharide Neu5Acα2(–6Galα1–4Neu5Acα2)$_2$–ProNHCbz (**S5**)

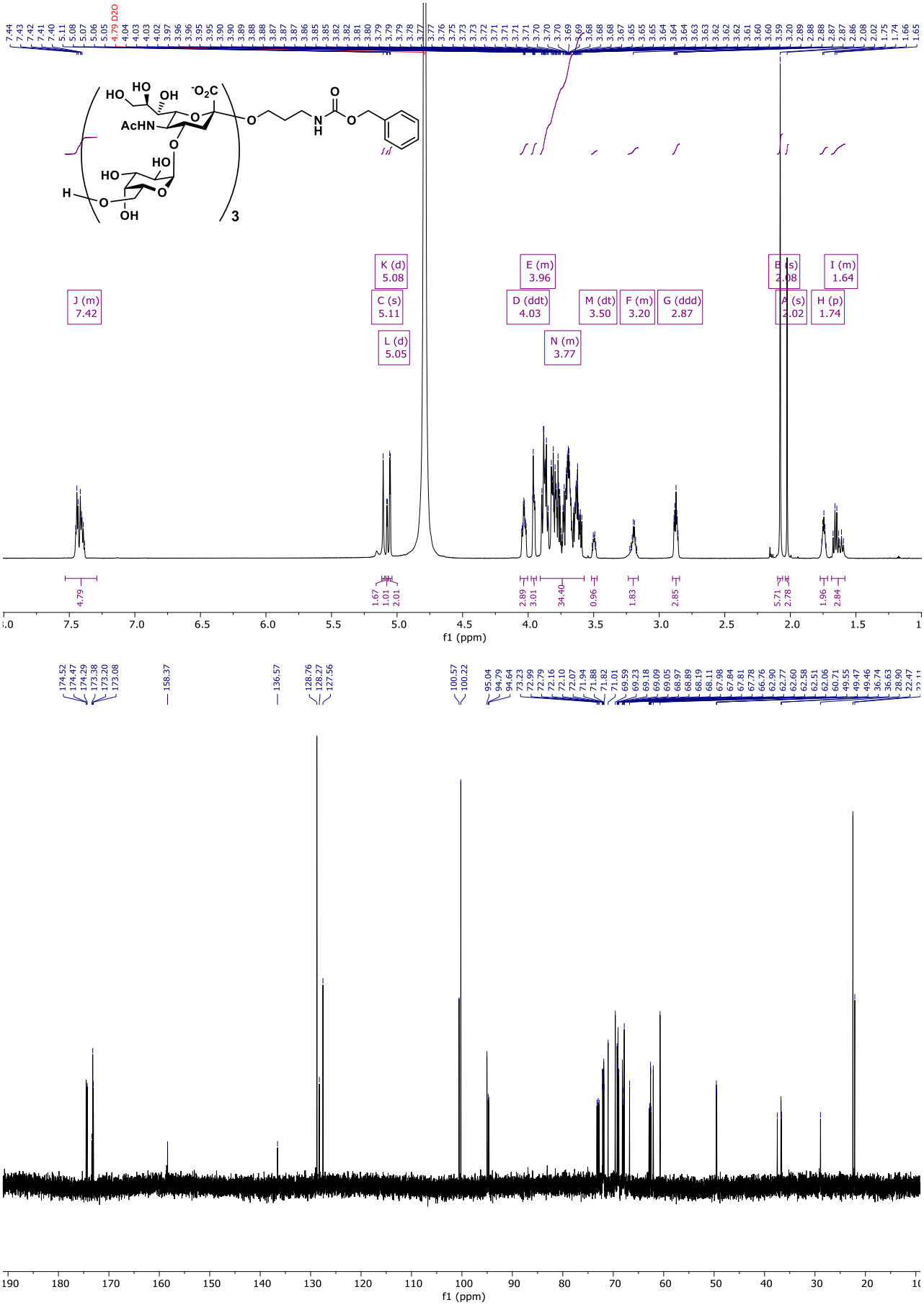


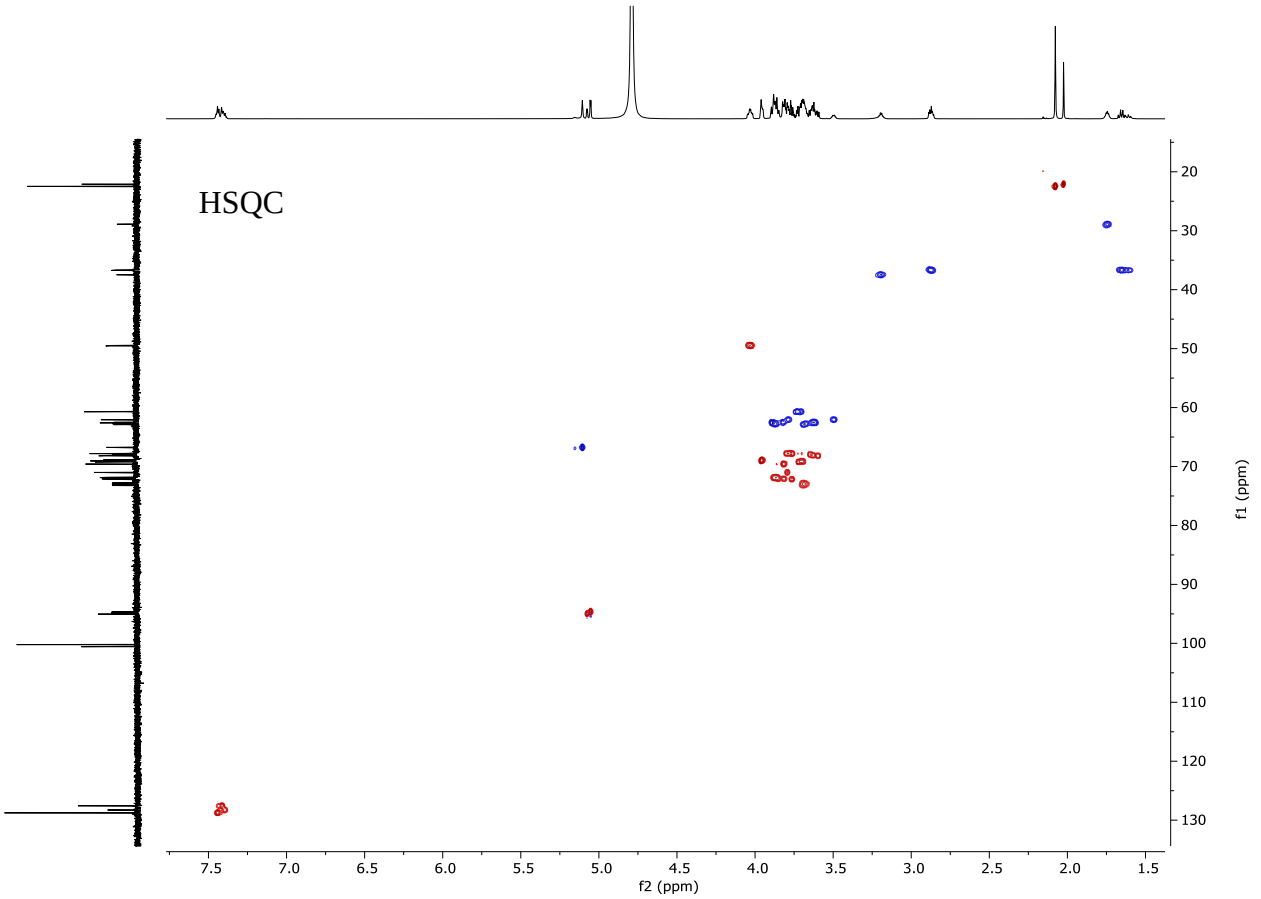

HSQC
f2 (ppm)
f1 (ppm)

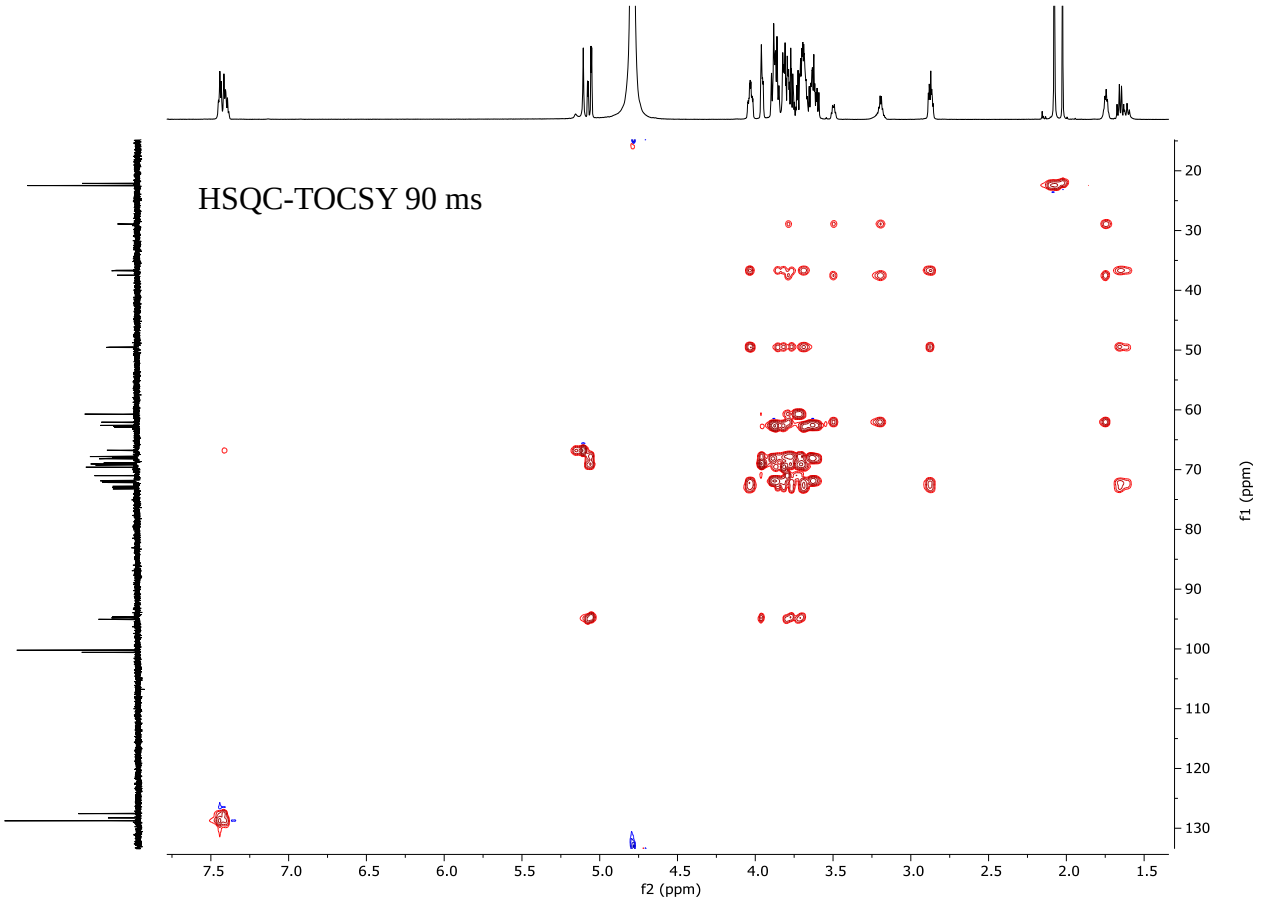

HSQC-TOCSY 90 ms
f2 (ppm)
f1 (ppm)

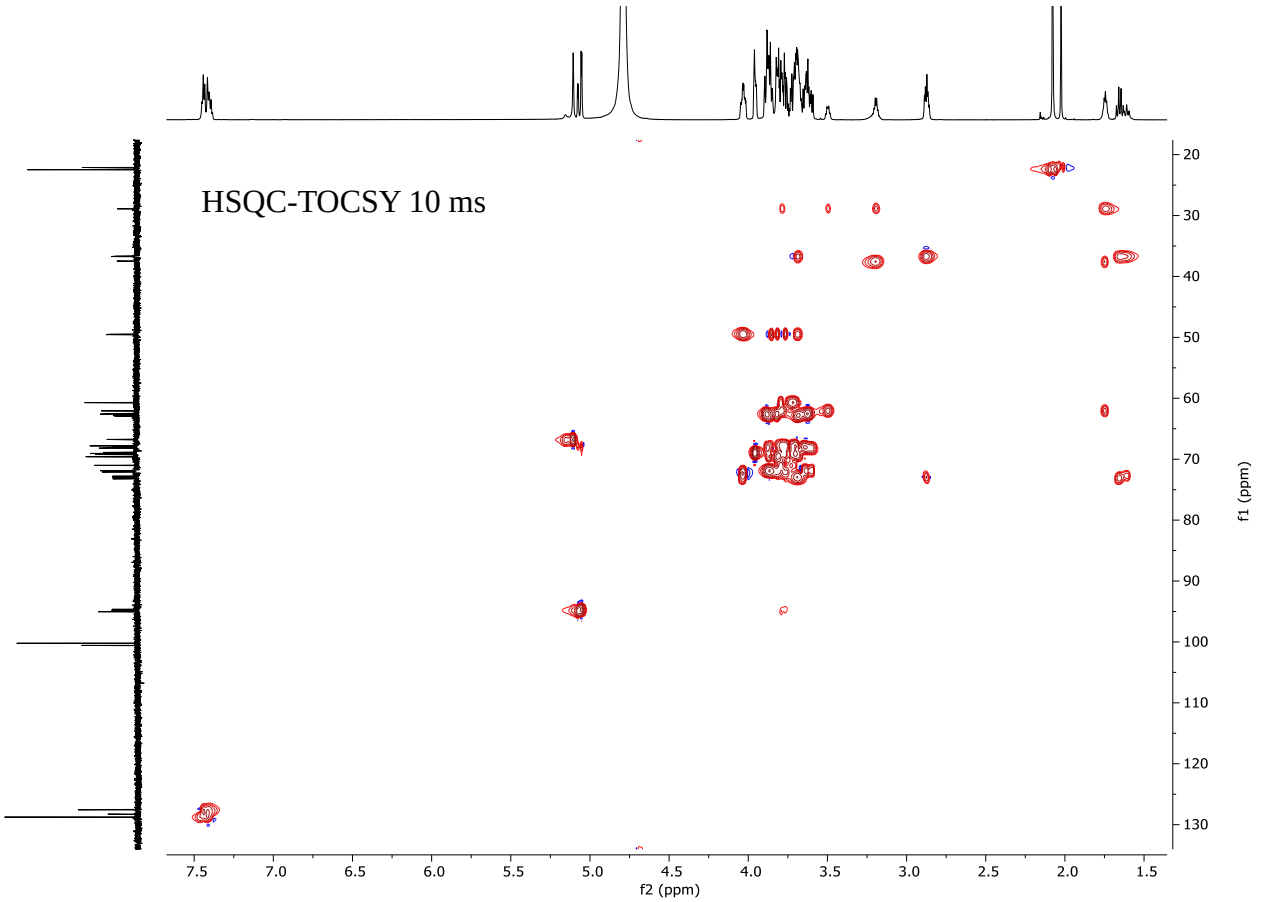

HSQC-TOCSY 10 ms
f2 (ppm)
f1 (ppm)

^{1}H, ^{13}C, HSQC, HSQC-TOCSY (90 ms and 10 ms) NMR spectra of galactosylhexasaccharide Galα1(–4Neu5Acα2–6Galα1)$_2$–4Neu5AcαProNHCbz (**G6**)

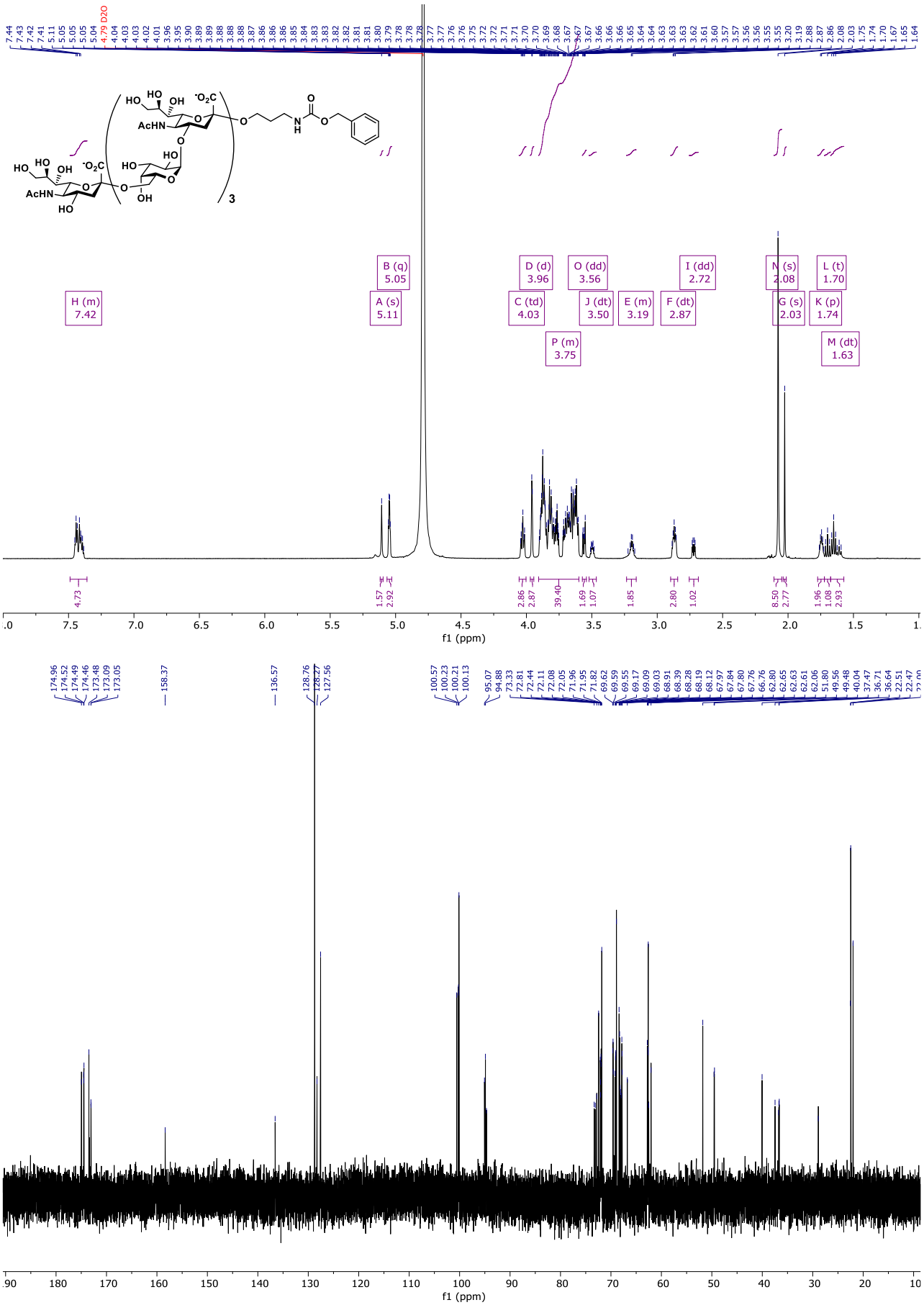


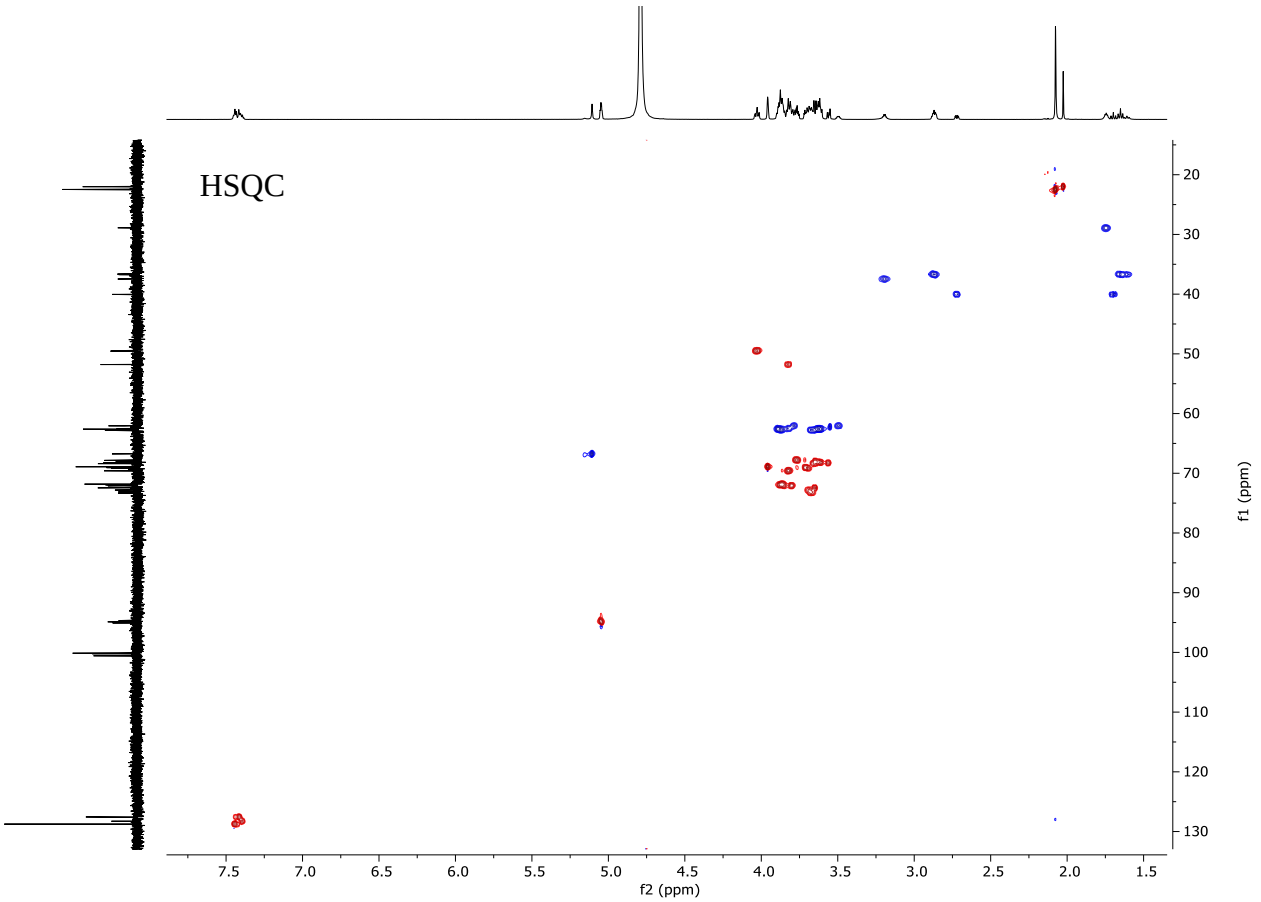

HSQC
f2 (ppm)
f1 (ppm)

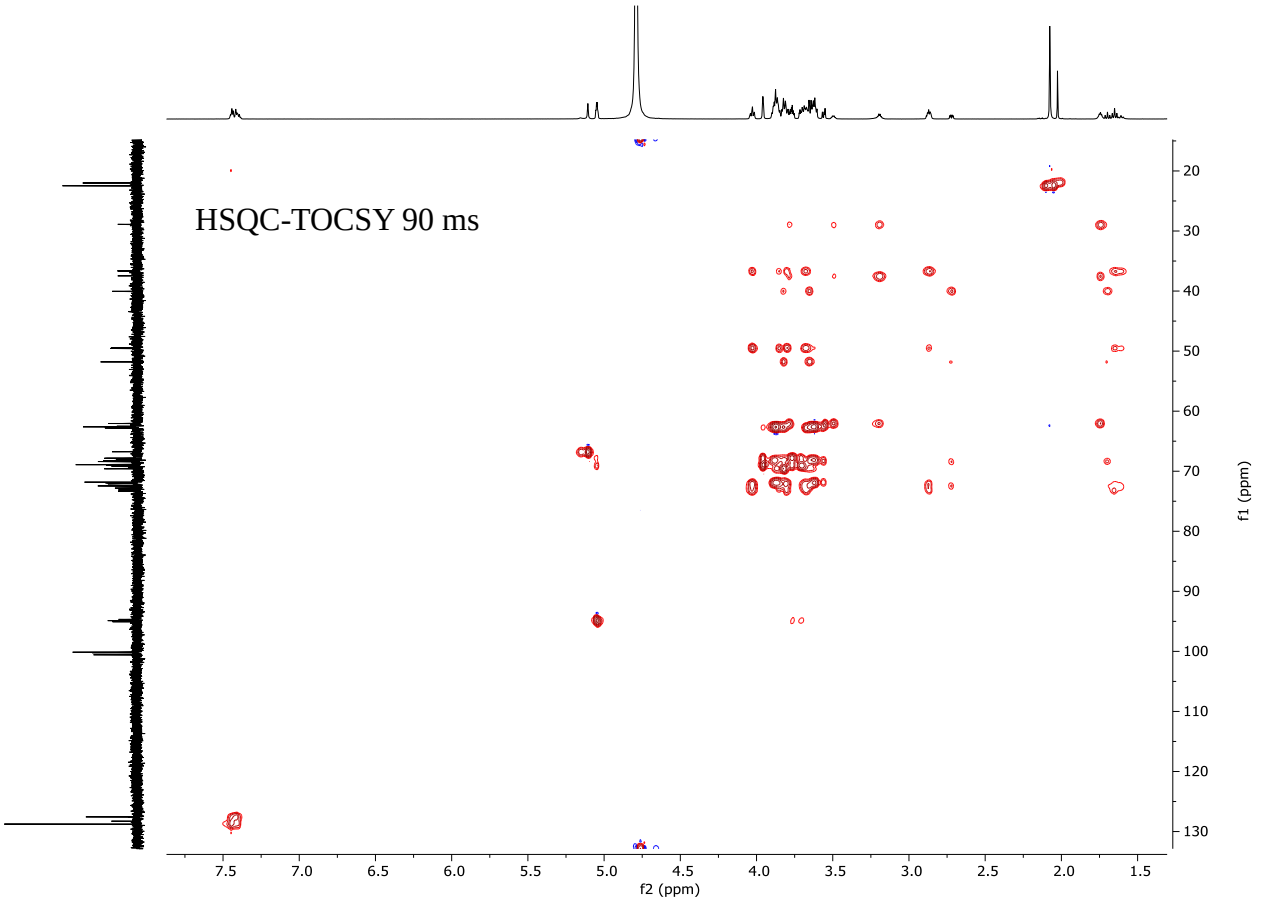

HSQC-TOCSY 90 ms
f2 (ppm)
f1 (ppm)

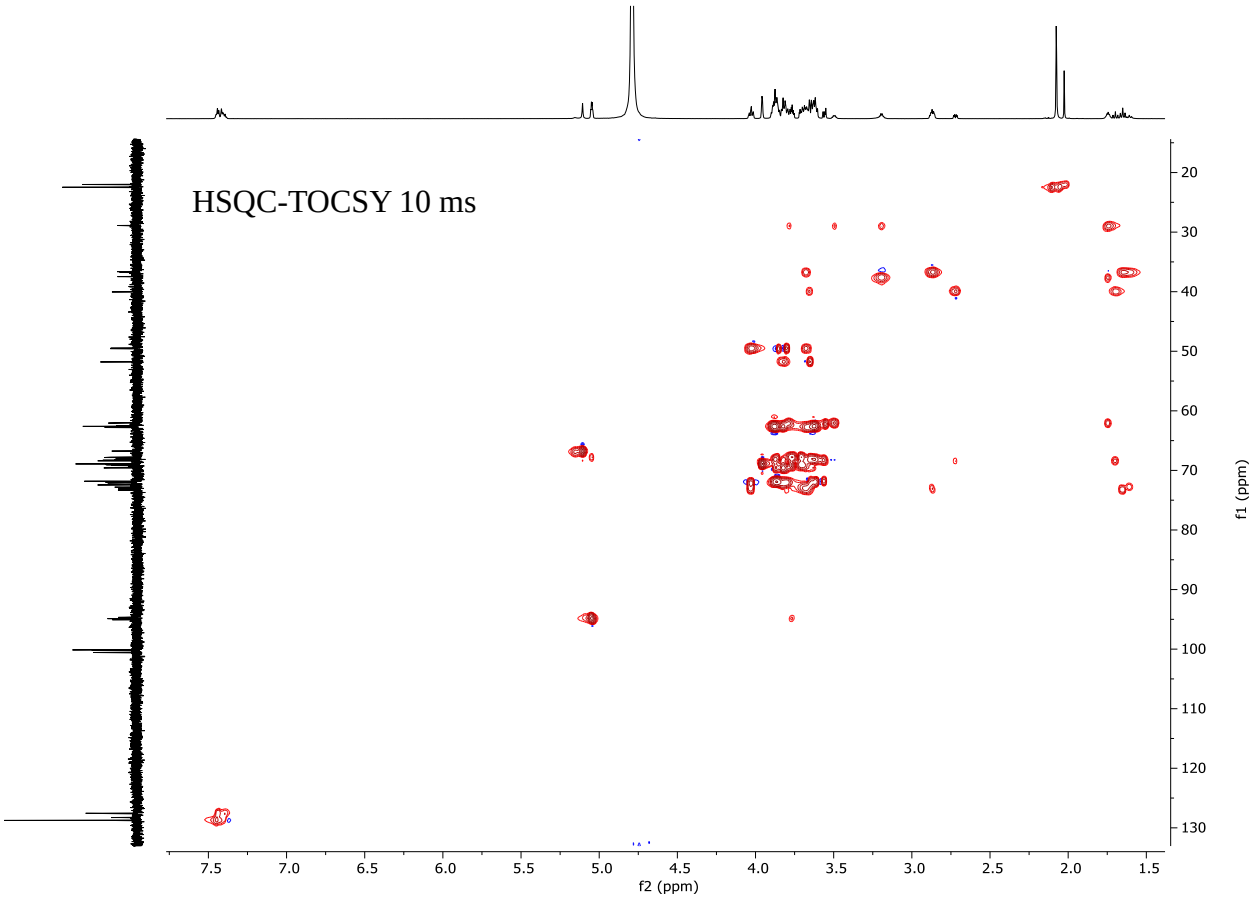

HSQC-TOCSY 10 ms
f2 (ppm)
f1 (ppm)

^{1}H, ^{13}C, HSQC, HSQC-TOCSY (90 ms and 10 ms) NMR spectra of sialylheptasaccharide Neu5Acα2(–6Galα1–4Neu5Acα2)$_3$–ProNHCbz (**S7**)

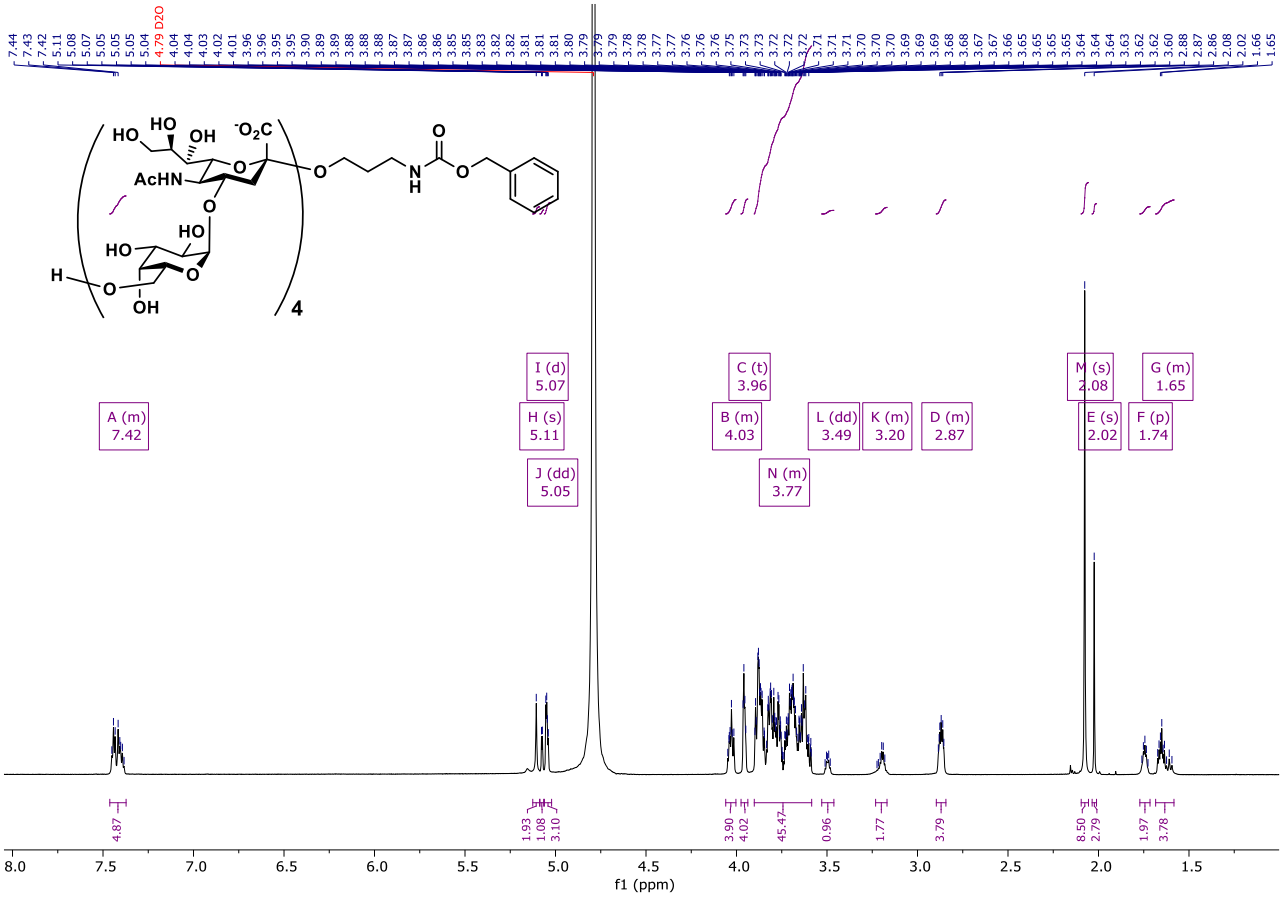


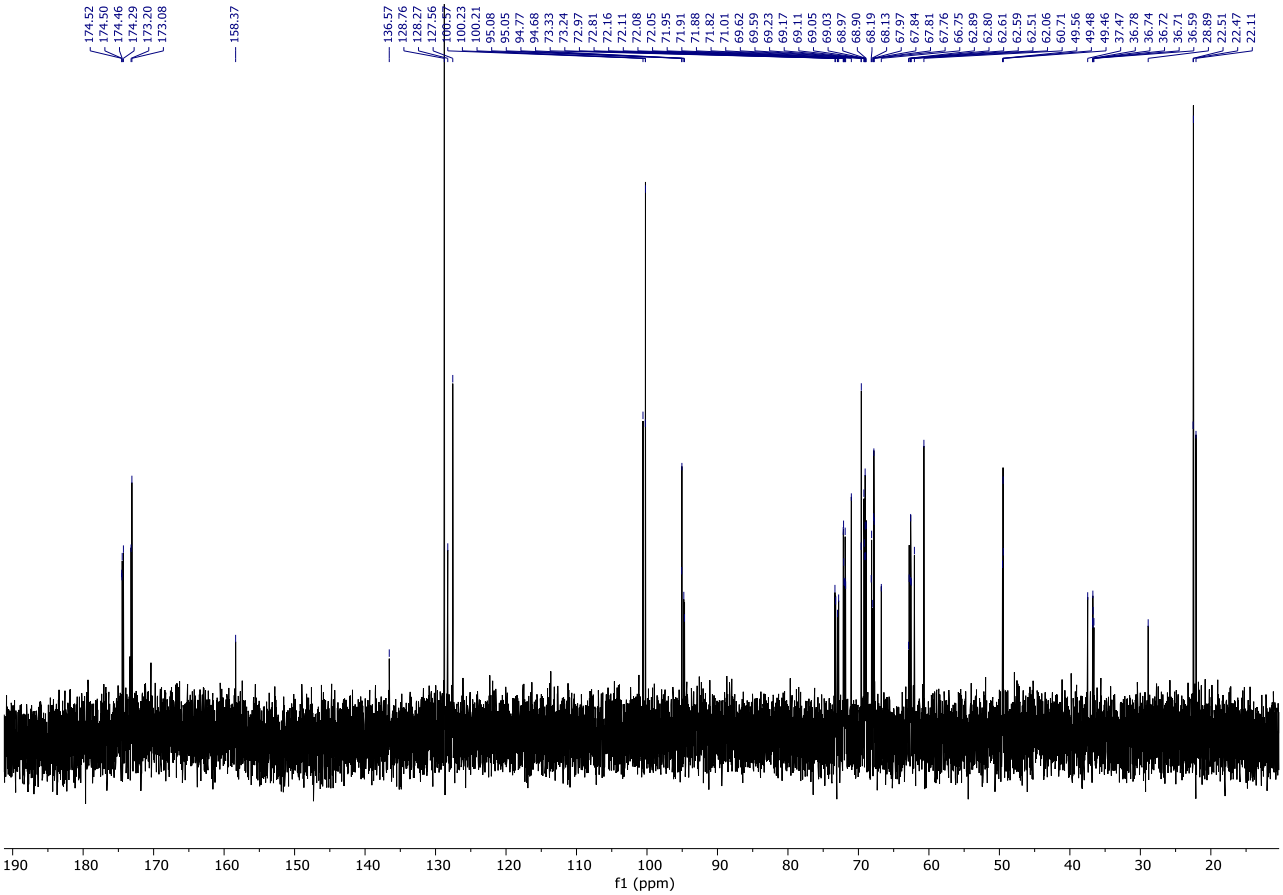

HSQC
f2 (ppm)
f1 (ppm)

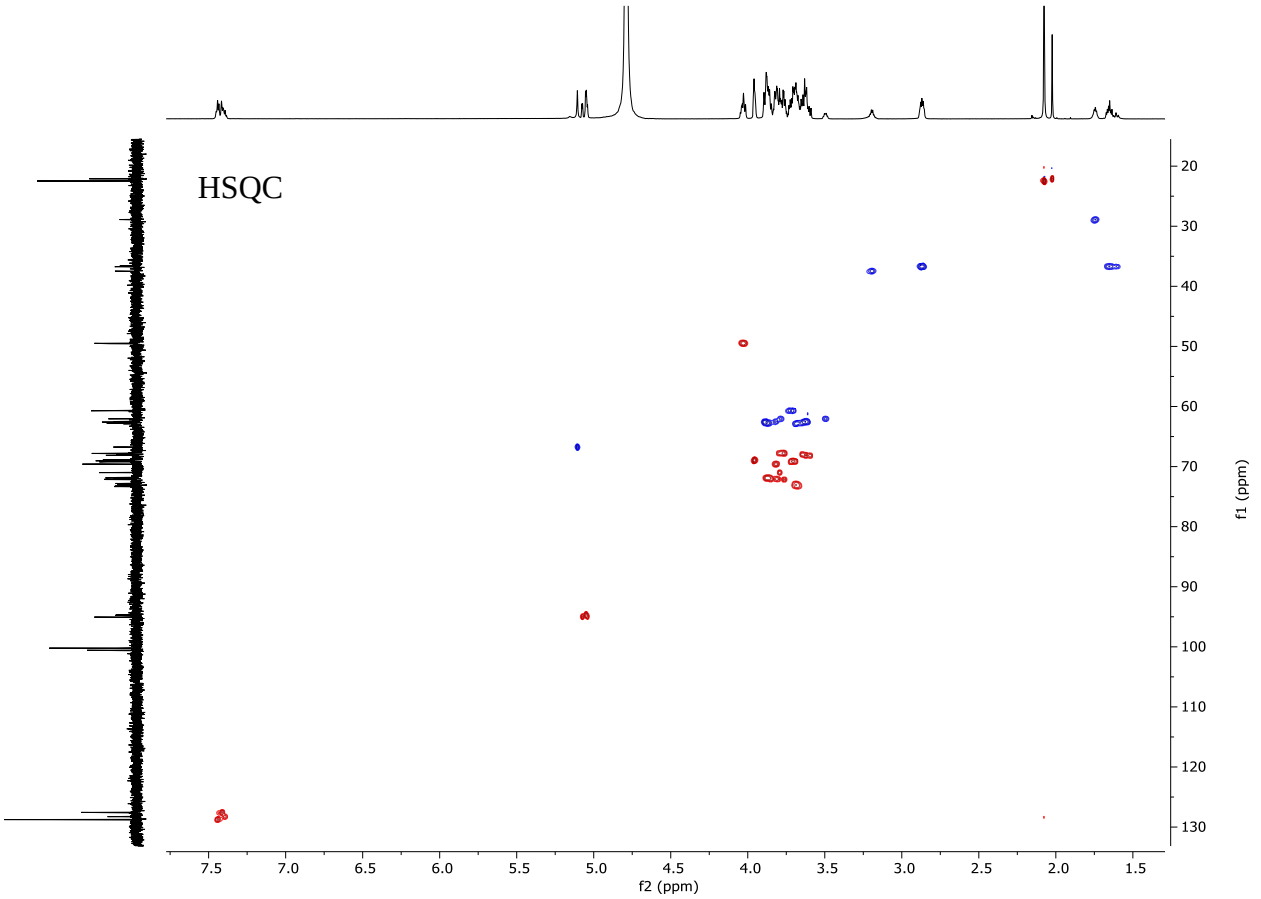

HSQC-TOCSY 90 ms
f2 (ppm)
f1 (ppm)

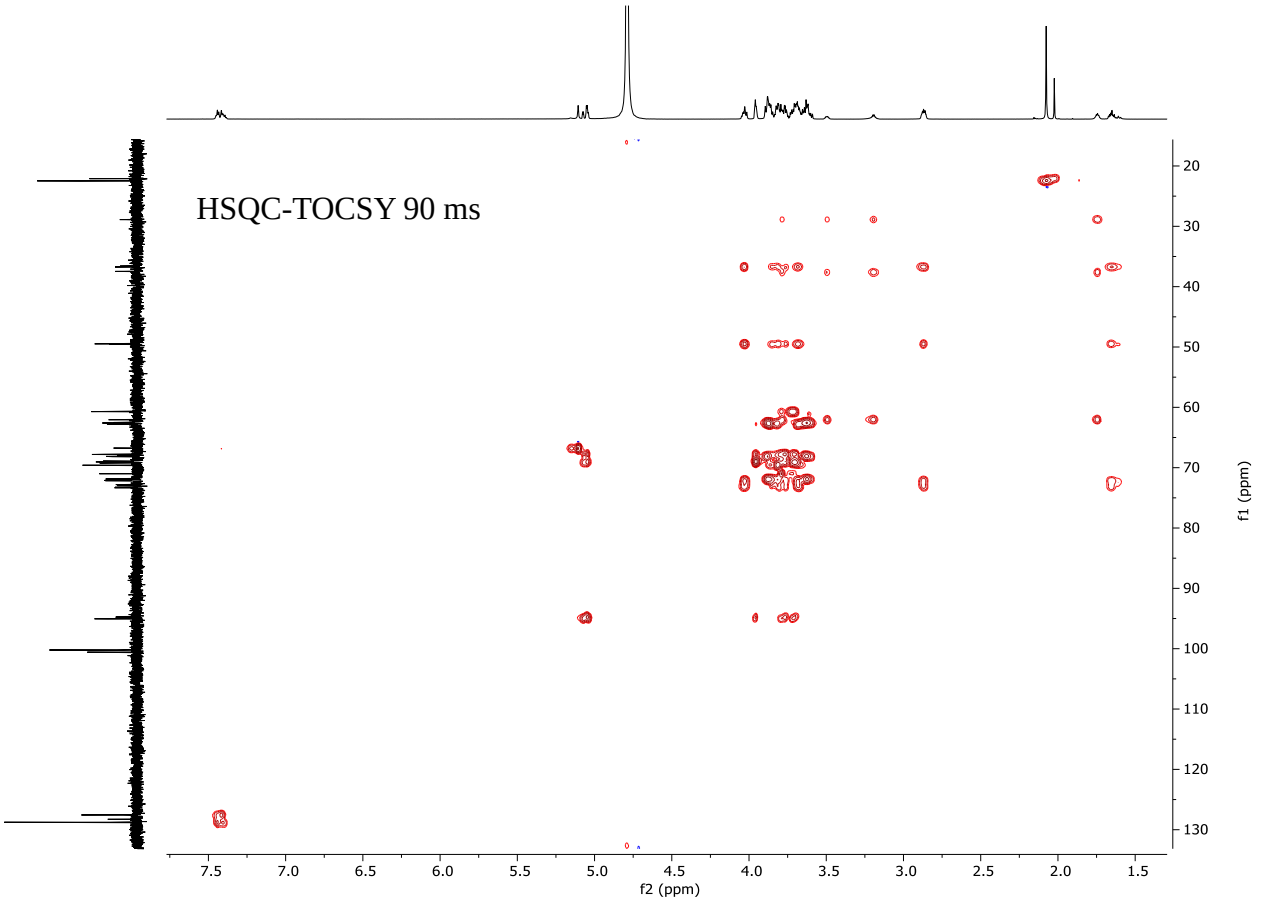

HSQC-TOCSY 10 ms
f2 (ppm)
f1 (ppm)

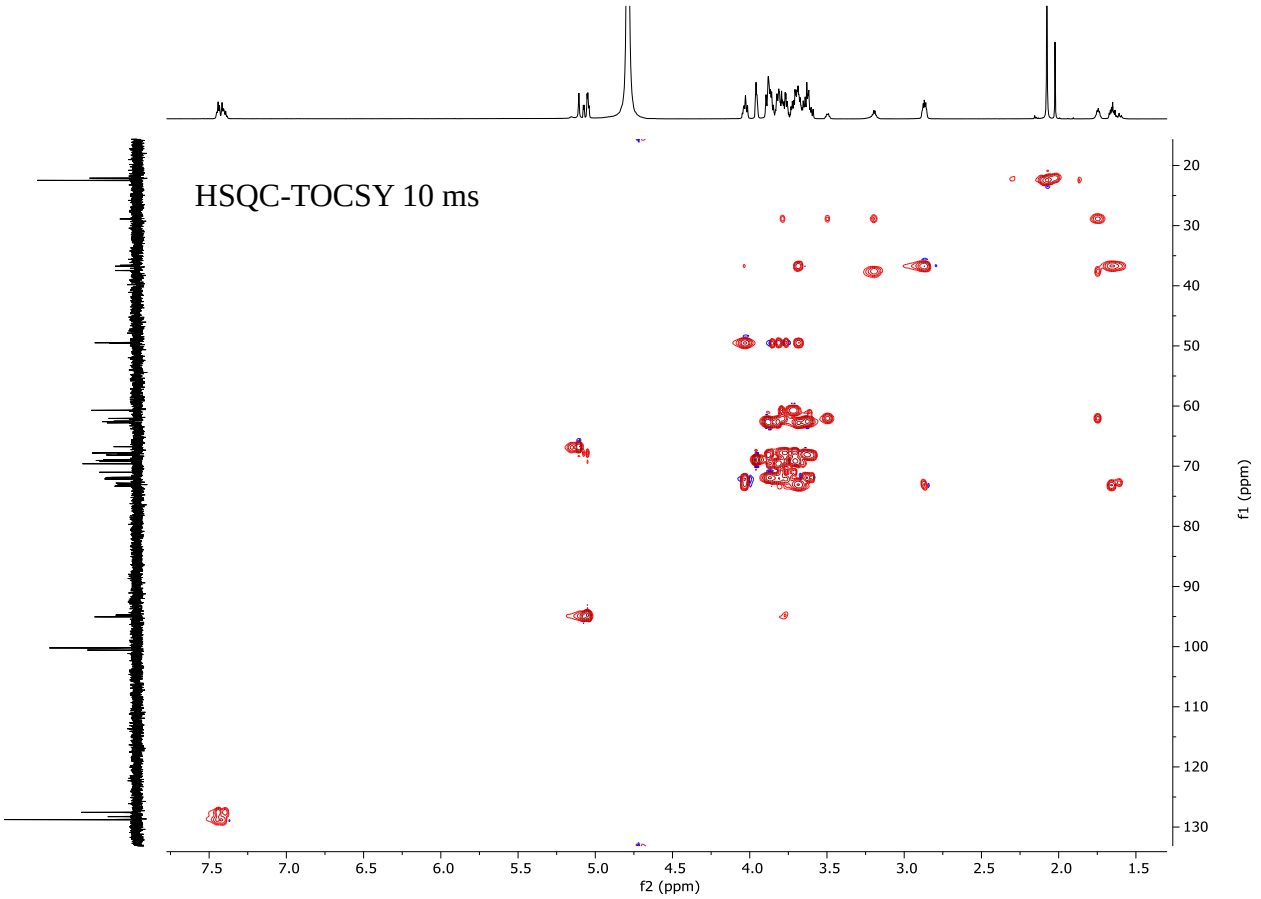
^{1}H, ^{13}C, HSQC, HSQC-TOCSY (90 ms and 10 ms) NMR spectra of galactosyloctasaccharide Galα1(–4Neu5Acα2–6Galα1)$_3$–4Neu5AcαProNHCbz (**G8**)



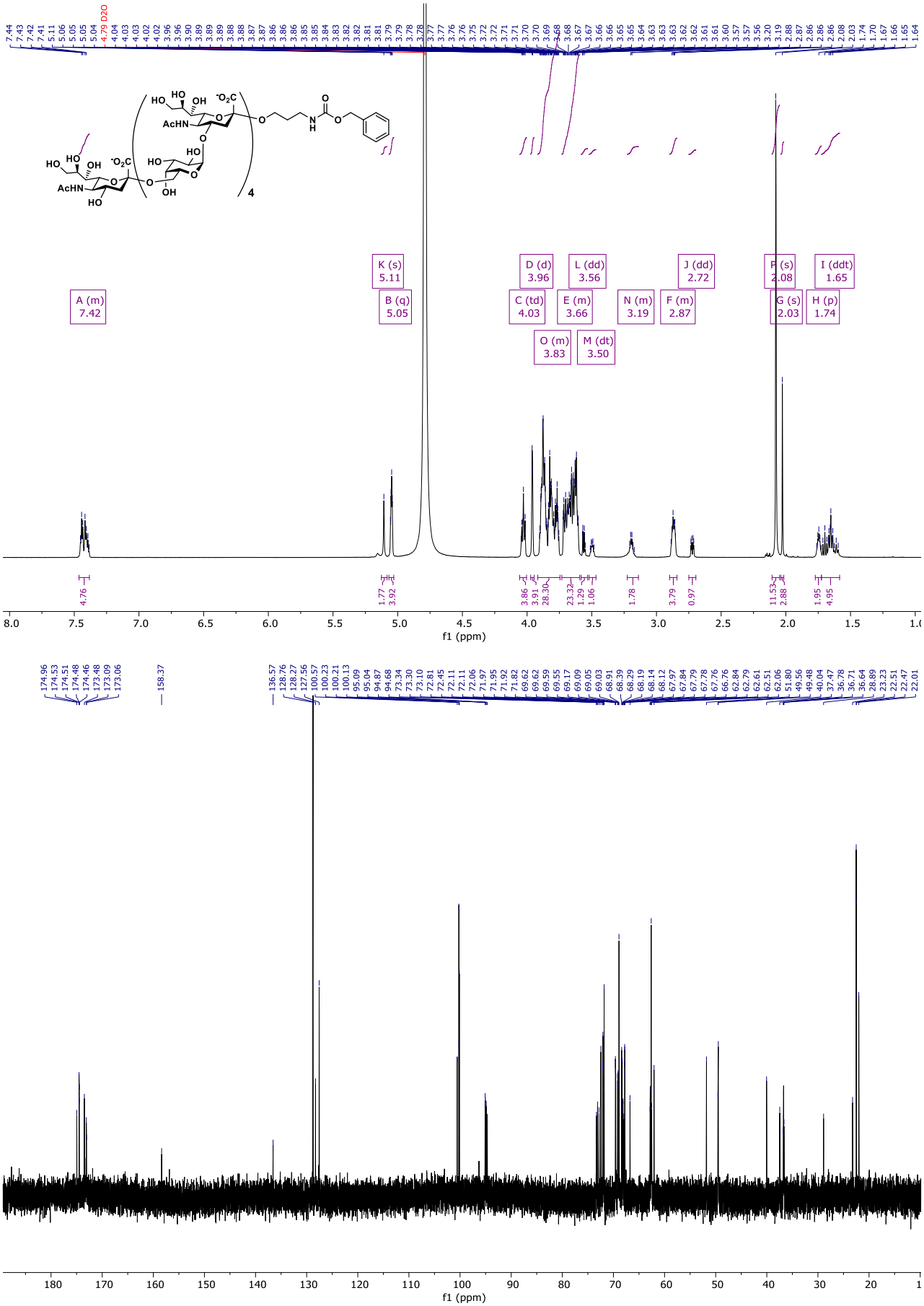


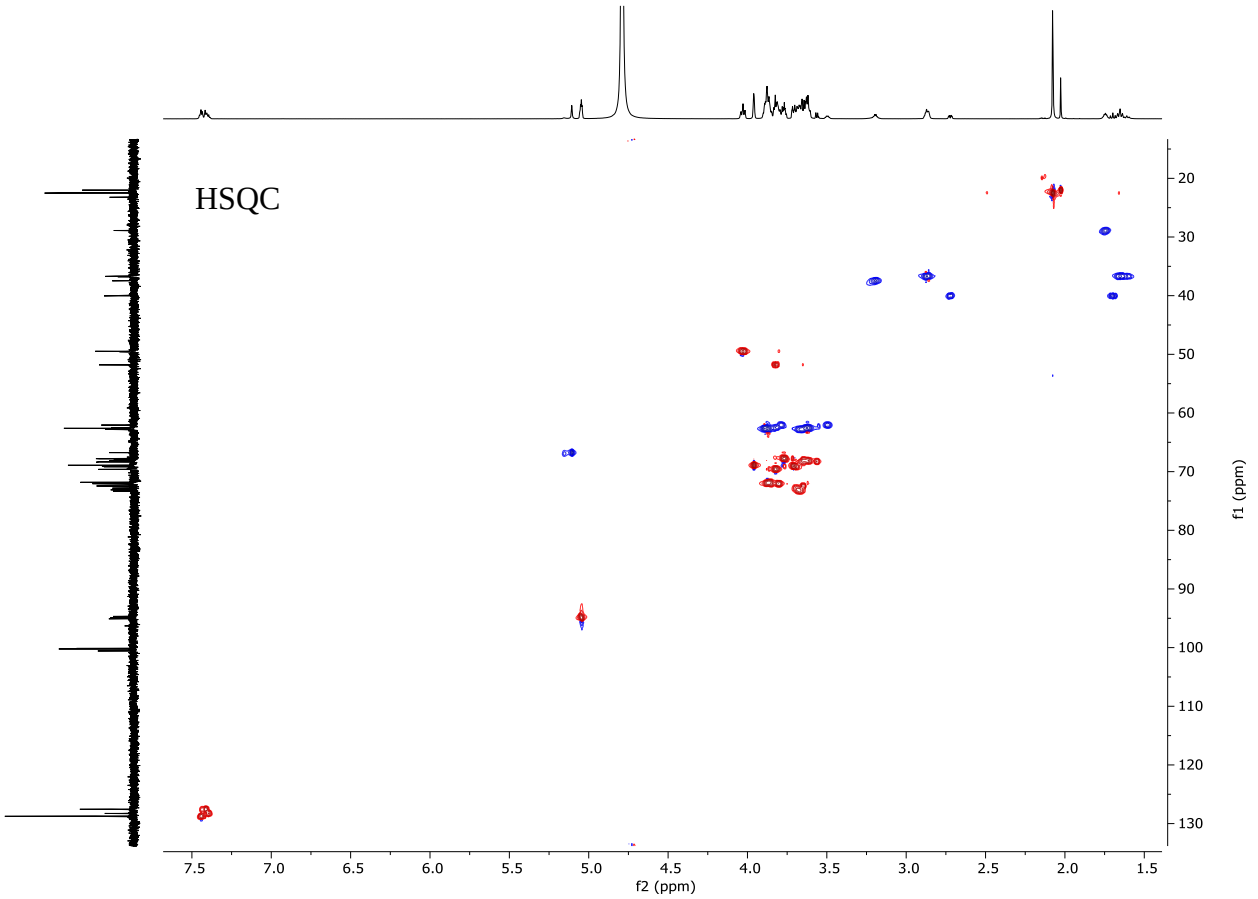

HSQC
f2 (ppm)
f1 (ppm)

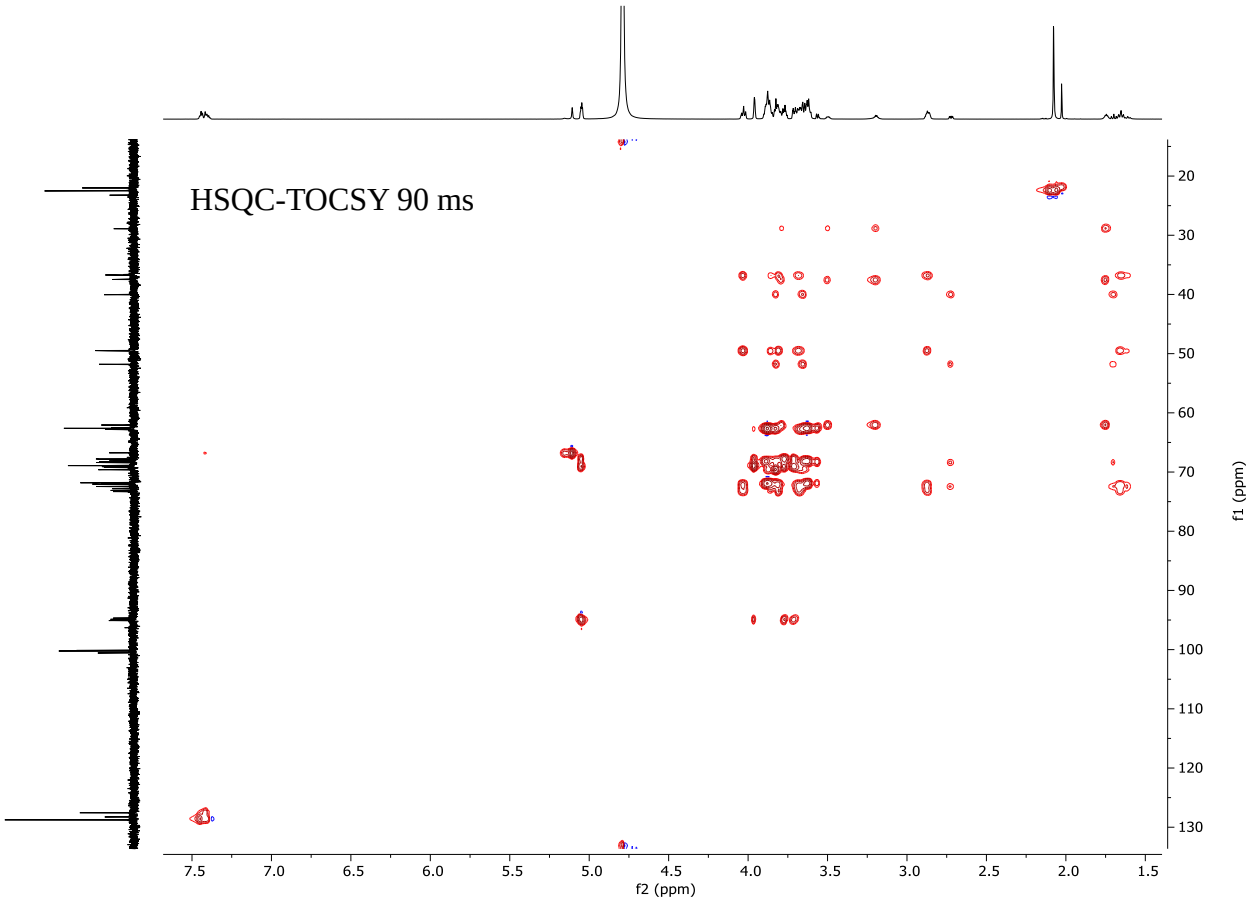

HSQC-TOCSY 90 ms
f2 (ppm)
f1 (ppm)

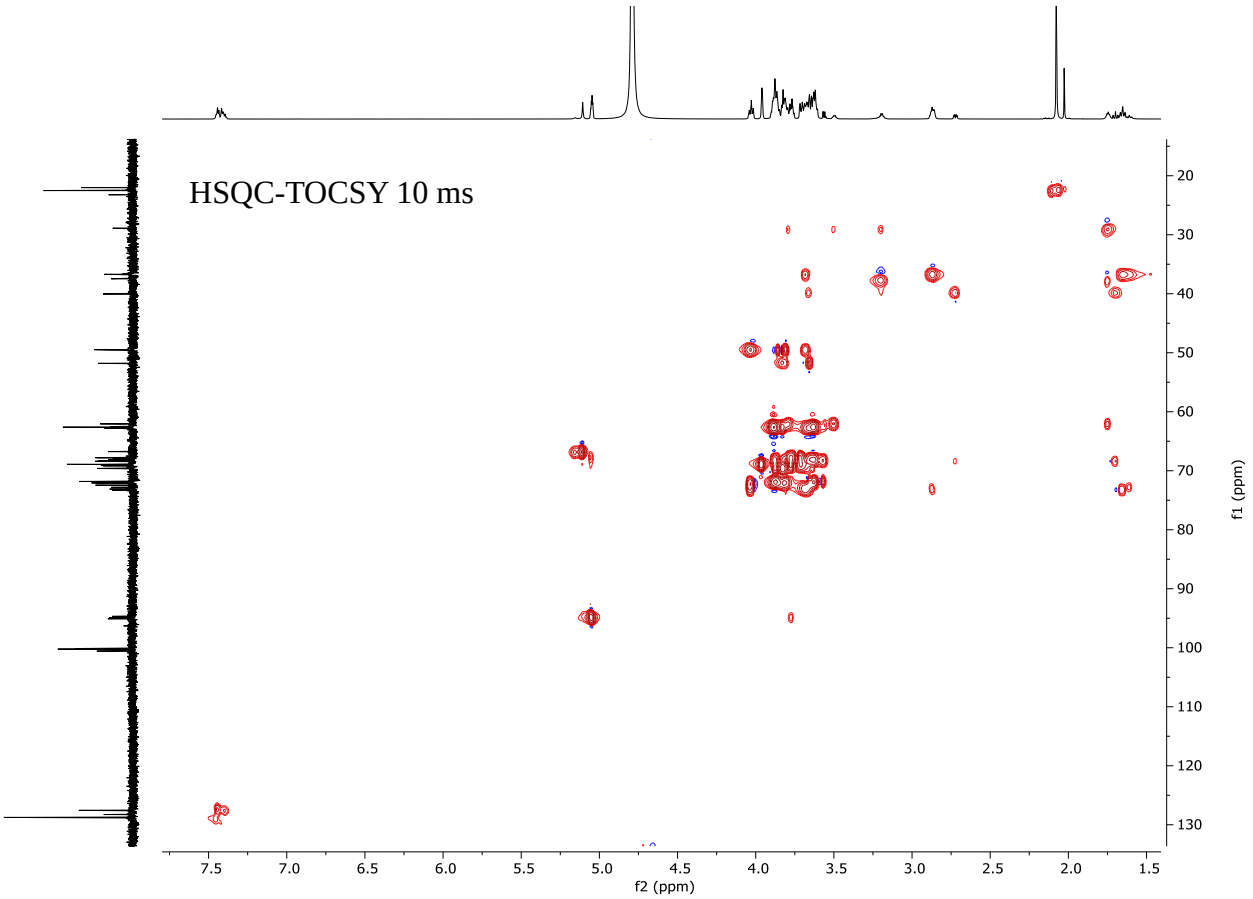

HSQC-TOCSY 10 ms
f2 (ppm)
f1 (ppm)

^{1}H, ^{13}C, HSQC, HSQC-TOCSY (90 ms and 10 ms) NMR spectra of sialylnonasaccharide Neu5Acα2(–6Galα1–4Neu5Acα2)$_4$–ProNHCbz (**S9**)

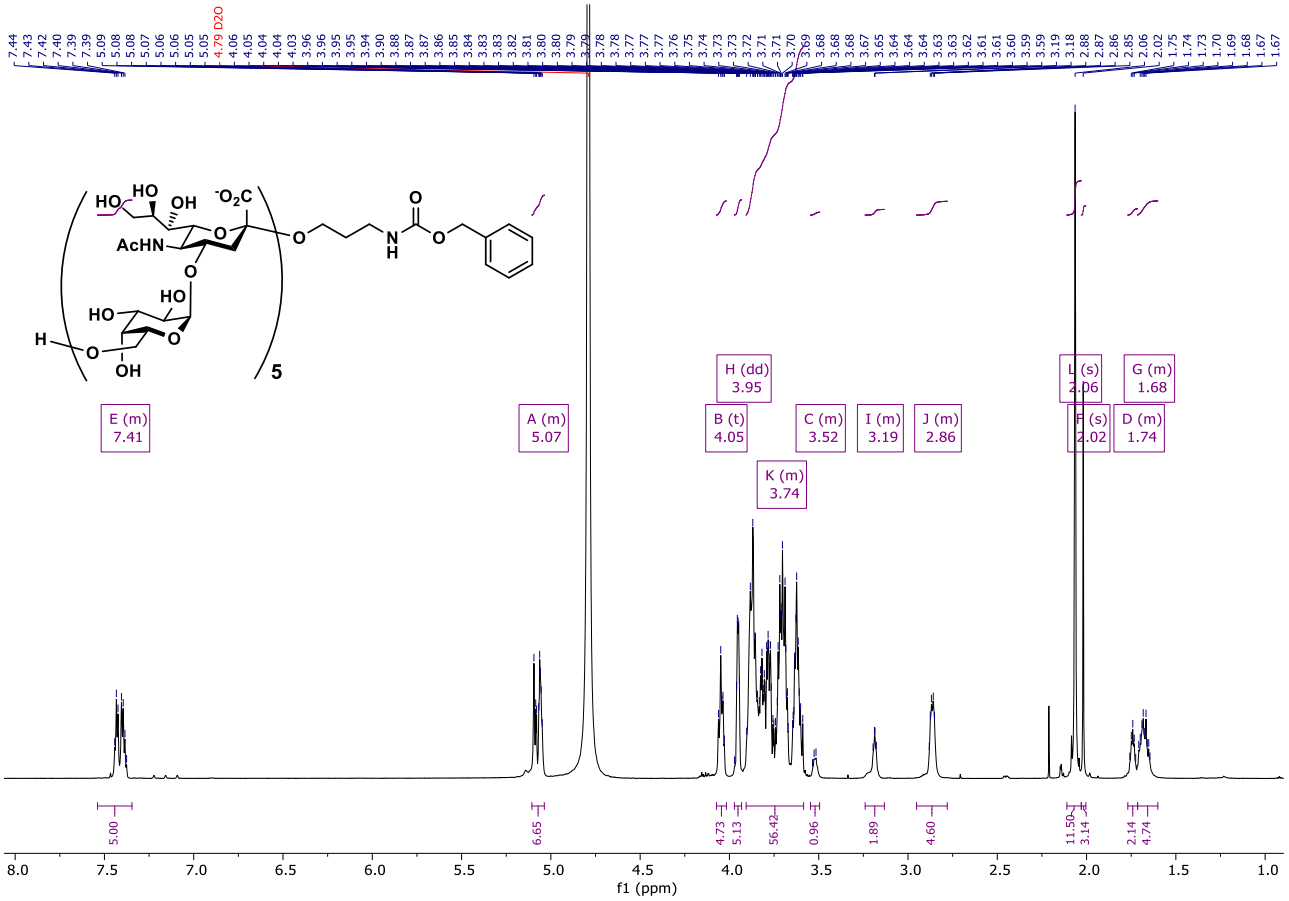


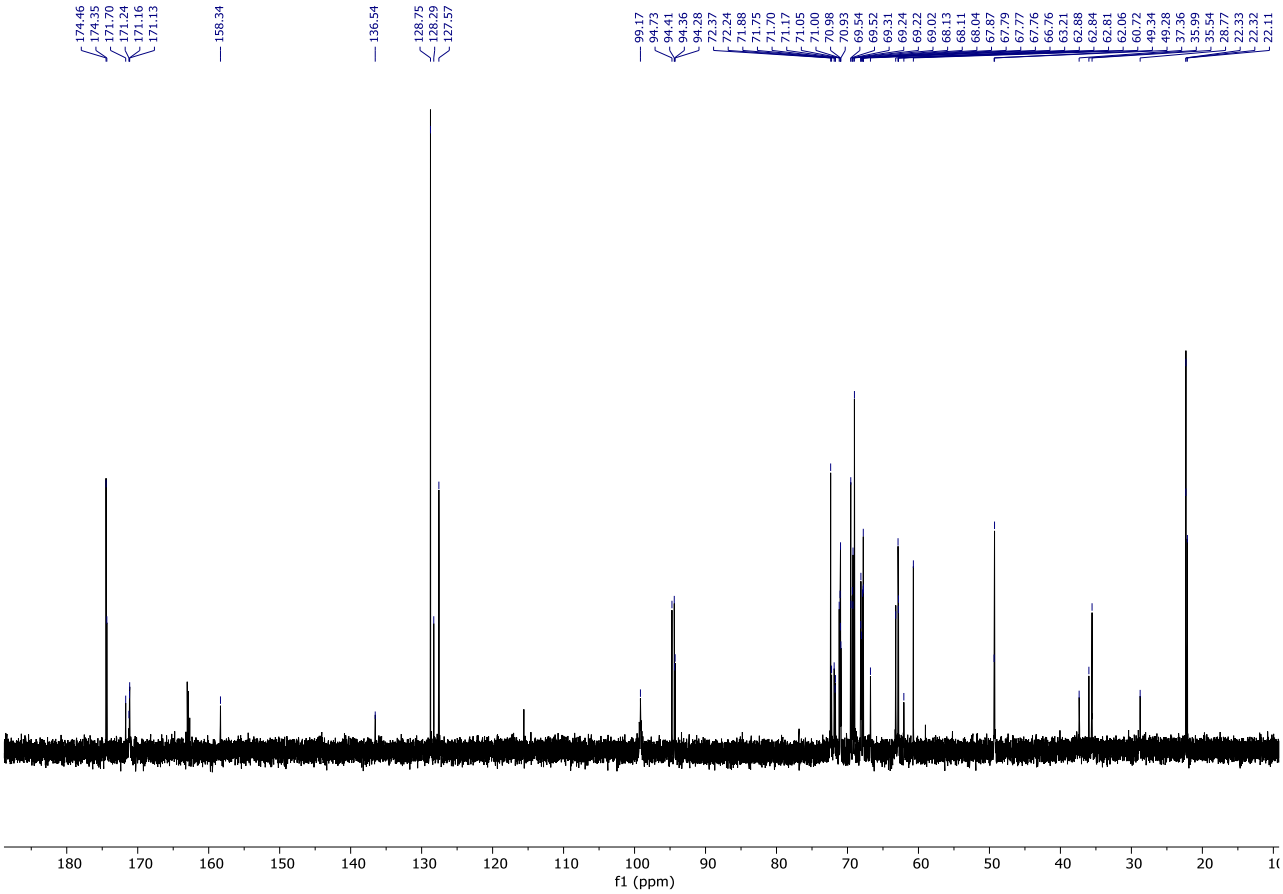

HSQC
f2 (ppm)
f1 (ppm)

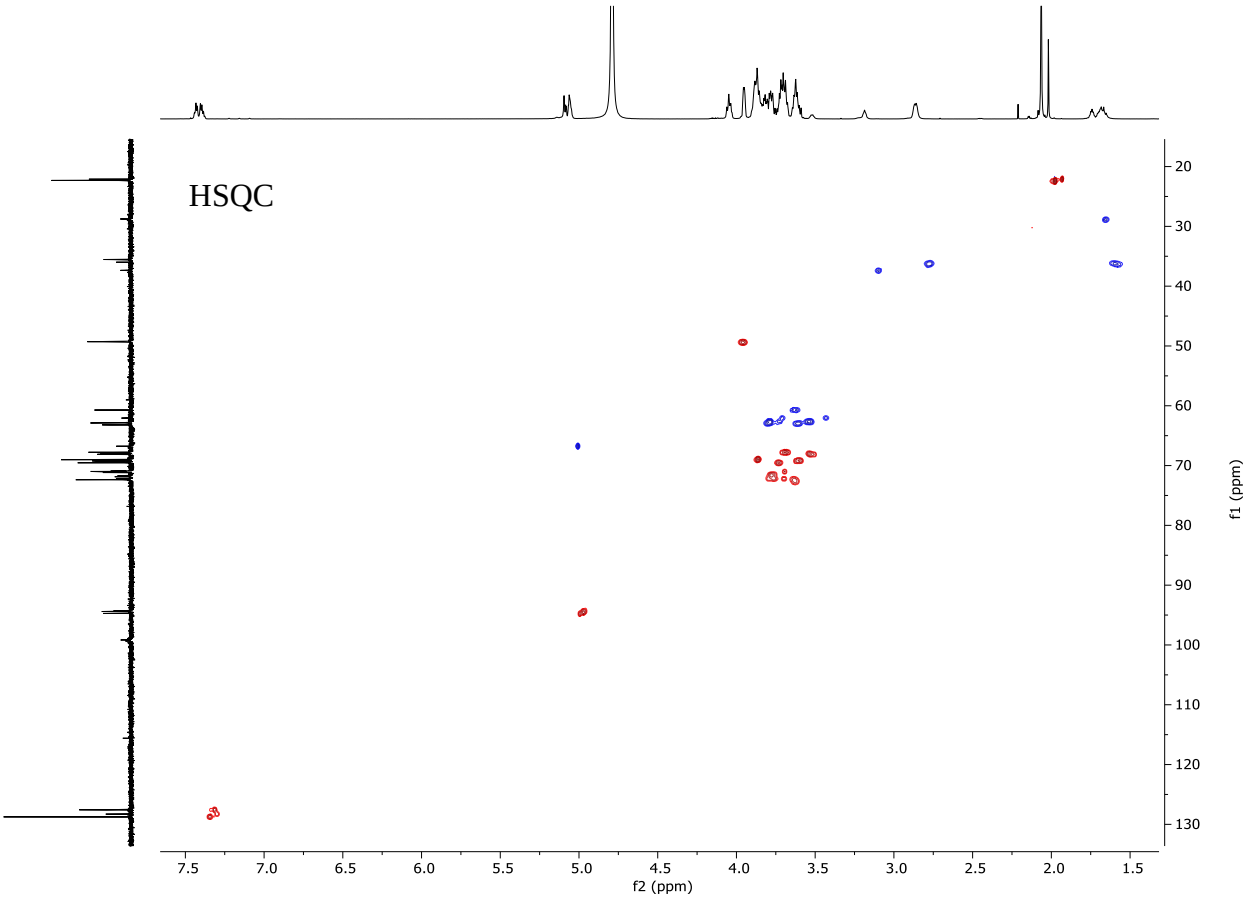

HSQC-TOCSY 90 ms
f2 (ppm)
f1 (ppm)

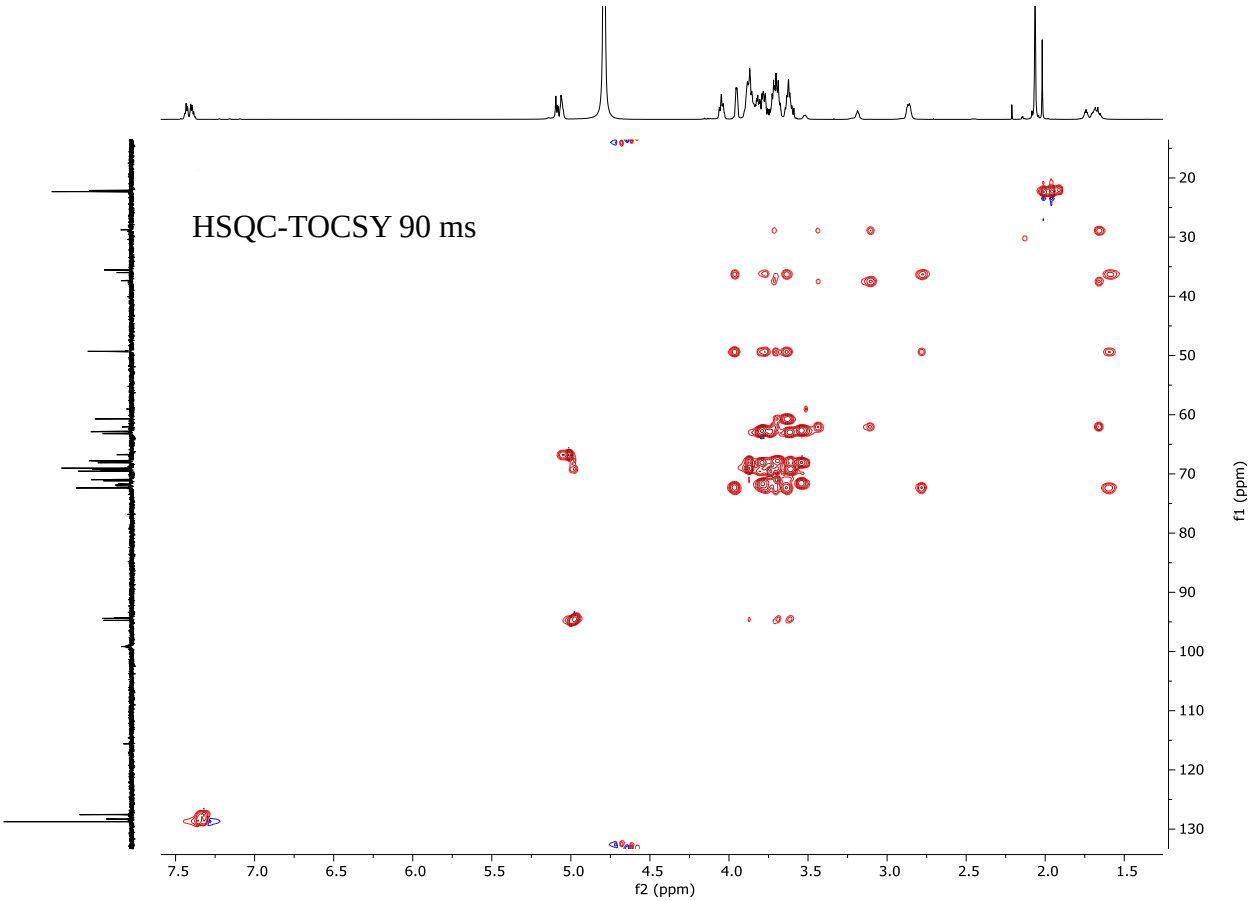

HSQC-TOCSY 10 ms
f2 (ppm)
f1 (ppm)

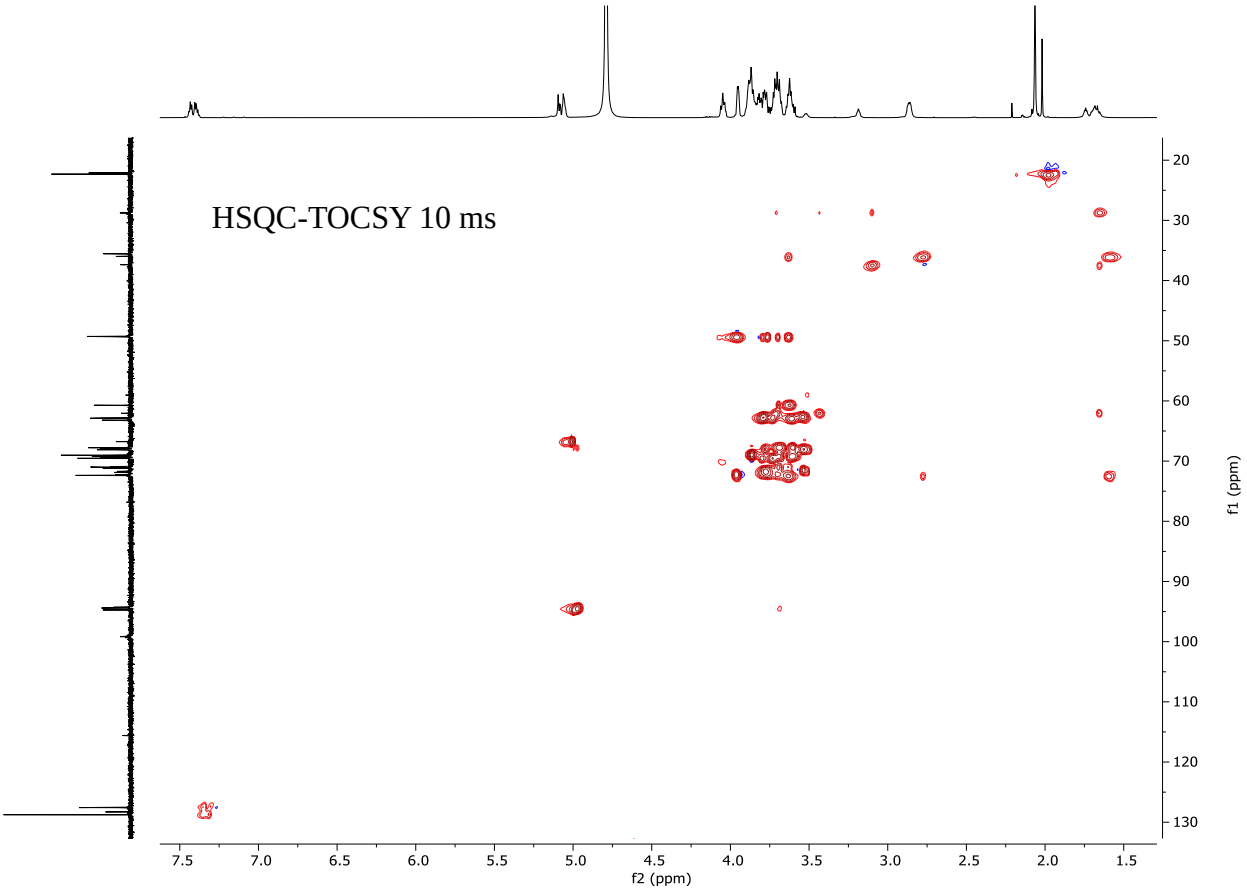
^{1}H, ^{13}C, HSQC, HSQC-TOCSY (90 ms and 10 ms) NMR spectra of galactosyldecasaccharide Galα1(–4Neu5Acα2–6Galα1)$_4$–4Neu5AcαProNHCbz (**G10**)






HSQC
f2 (ppm)
f1 (ppm)


HSQC-TOCSY 90 ms
f2 (ppm)
f1 (ppm)


HSQC-TOCSY 10 ms
f2 (ppm)
f1 (ppm)