SUPPORTING INFORMATION

Improving analytical characterization of glycoconjugate vaccines through combined high-resolution MS and NMR: Application to *Neisseria meningitidis* serogroup B oligosaccharide-peptide glycoconjugates

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Materials and Methods

Materials. An immunogenic 15-residue peptide from tetanus toxoid 830-844 ($TT_{830-844}$) QYI**K**ANS**K**FIGITEL was prepared by solid-phase synthesis in the Facility for Biotechnology Resources (FBR) at the Center for Biologics Evaluation and Research, FDA. α(2,8)-linked N-acetylneuraminic acid trimer (Sia_3) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Sodium periodate, sodium cyanoborohydride and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) and used without further purification, unless otherwise noted. Bio-gel P2 (cat.150-4115) was from Bio-Rad Laboratories, Inc. (California, USA) and the column was packed according to manual instruction.

Sia_3 conjugation to tetanus toxoid $TT_{830-844}$ peptide

Sia_3 was oxidized with sodium periodate ($NaIO_4$, freshly prepared in water) at a $NaIO_4$ to Sia_3 ratio of 1:1 ($NaIO_4$:Sia_3=1.1) in water or 5:1 molar ratio in 0.1M sodium acetate, pH 5.5 on ice for 30 min in the dark to avoid unwanted photo-activated radical reactions.[23] Sodium acetate buffer was prepared using acetic acid–d_3 sodium salt and titrated with acetic acid–d_4 in samples tracked by NMR to ease following the oxidation by NMR. For $NaIO_4$:Sia_3 = 1:1, 1mg of $NaIO_4$ was directly added to 6mg of Sia_3 in 500 μl of water. For $NaIO_4$:Sia_3 = 5:1, 11mg of $NaIO_4$ was directly added to 10mg of Sia_3 in 1.1ml of 0.1M sodium acetate, pH 5.5. One-dimensional (1D) proton NMR experiments were used to monitor the extent of chemical reactions in real time. The oxidized OSs were lyophilized and subsequently purified by gel filtration (P2 resin, Bio- Rad Laboratories) using water as the eluent. Sia_3 and its oxidized forms were analyzed by NMR and MS. For coupling of OSs to $TT_{830-844}$, oxidized (activated) Sia_3 were mixed with $TT_{830-844}$ at a 1:2 molar ratio and incubated for 30 min at room temperature in 0.8 mL of water with or without DMSO. Sodium cyanoborohydride was directly added to the solution and the conjugation reactions were carried out at 37 °C for 1, 2 and 3 days. Subsequently, the reaction mixture was evaluated using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). On day 3, the reaction mixture was directly dialyzed against water using Spectra/Por® 7 Dialysis Membranes, MWCO 1000 (VWR) and lyophilized for NMR and nanoscale liquid chromatography coupled to tandem mass spectrometry **(**NanoLC–MS/MS) analysis. Conjugates were further purified using C18 column (Agilent technologies, CA, USA) for NMR assignment.

MALDI spot preparation and MALDI-TOF-MS analysis

Sia_3, activated Sia_3 or glycoconjugate were loaded onto a stainless steel MALDI-TOF target and allowed to dry. On target lactonization of sialic acid was performed as reported by Galuska et al.[24] Briefly, the dried sample was re-dissolved in 1 µl of 0.5% phosphoric acid, incubated at room temperature until it dried again. Lactonized samples were re-dissolved on-target in 1 µl of a saturated solution of 6-aza-2-thiothymine (ATT, Sigma) in 50% acetonitrile (ACN) or in 1 µl of 2,5 dihydroxybenzoic acid (DHB) in 20% ACN. Samples were allowed to dry at room temperature. MALDI-TOF-MS analysis was performed on an Autoflex instrument (Bruker-Daltonik, Bremen, Germany) equipped with a nitrogen laser and controlled by FlexControl 2.0 software. The instrument was operated in positive–ion mode in both linear and reflectron configurations. Masses were annotated and processed with FlexAnalysis 2.0. External calibration of mass spectra was carried out using maltoheptaose.

Reverse phase NanoLC– MS^E analysis

Characterization of glycoconjugates was performed via a Waters SYNAPT G2 HDMS mass spectrometer (Waters Corporation, Milford, MA) equipped with nano-spray source and nanoAcquity LC system. NanoLC–MS^E collects MS data in an alternating scan mode between low and elevated collision energies. Precursor ion data are collected during the low energy MS scan, and then the collision energy is ramped, increasing over a range of voltages in the dissociation stage to fragment the precursor ions. The data produced during the experiment are used to reconstruct MS/MS spectra without the bias associated with data-dependent ion scanning.[25] Before analysis, the conjugate mixture was dialyzed against water using a 1000 MWCO dialysis for buffer exchange and removal of small chemical products. Glycoconjugates were reconstituted in 0.1% formic acid in water and injected onto a C18 column (HSS T3 nanoACQUITY column 75 µm i.d. × 100 mm, 1.8 µm particle, Waters Corporation) for nanoLC–MS^E analysis.[25] The solvent system consisted of solvent A (100% water, 0.1% FA) and solvent B (100% acetonitrile, 0.1% FA). A Waters nanoAcquity UPLC system was used for automatic sample loading and flow control. The gradient was as follows: 3–50% of solvent B for 47 min, 85% of solvent B for 7 min, and 3% of solvent B for 25 min. The eluent was introduced into the mass spectrometer via an uncoated 15 µm i.d. PicoTip Emitter (New Objective Inc., Woburn, MA). The spray voltage was 3000 V and the mass spectrometer was operated in the positive polarity mode. For MS^E, a low collision energy of 4 V was used for precursor ion scanning

followed by an elevated collision energy ramping from 15 to 45 V for fragment ion scanning. The scan time was 0.9 s. An auxiliary pump was used to spray a solution of 200 fmol/µL Glu-fibrinopeptide B in 50:50 methanol/water with 1% acetic acid for mass calibration (lock mass channel) at a flow rate of 500 nL/min and sampling every 30 s. LC/MS data was interpreted using BiopharmaLynx 1.3x (Waters).

NMR measurements

Sample concentrations used to record NMR spectra were: 2 mg/mL for the peptide, 3 mg/mL for the conjugate and 10 mg/mL for Sia_3. Sia_3 samples were dissolved and recorded in 20 mM phosphate buffer pH 6.0 in 100% D_2O or 90%/10% H_2O/D_2O. $TT_{830-844}$, glycoconjugate $TT_{830-844}$-Sia_3 were reconstituted in 20 mM phosphate buffer at pH=5.7 with 10% D_2O. All NMR measurements were performed on Avance III 500 MHz spectrometer equipped with a *z*-gradient QXI probe. Typical acquisition parameters were: Acquisition times: F2 = 292 ms, F1 = 50.9 ms, F2 res = 3.42 Hz/pt, F1 res = 19.65 Hz/pt. One-dimensional (1D) proton experiments were recorded with a 3-9-19 WATERGATE for water suppression,[26] using the Bruker pulse sequence *p3919gp*. ^{1}H-^{1}H total correlation spectroscopy (TOCSY) using DIPSI-2 as the mixing sequence,[27] with mixing times of 15 ms and 90 ms, and nuclear Overhauser enhancement spectroscopy (NOESY) with a mixing time of 120 ms, were recorded with Bruker pulse sequences *dipsi2etgpsi19* and *noesyfpgpph19*, respectively. ^{1}H-^{13}C HSQC-TOCSY (Heteronuclear Single Quantum Coherence-TOCSY) and HSQC were recorded with Bruker pulse sequences *hsqcdietgpsi* and *hsqcetgpsi*, respectively. All spectra were collected at 298K in D_2O with 20mM phosphate buffer 6.0 unless otherwise noted. Spectra were referenced by setting the water signal at 4.77 ppm relative to external DSS (2,2-dimethylsilapentane-5-sulfonic acid). A recycle delay ≥ 5 T_1s was used to acquire the data. The temperature was set 298K using the DSS-HOD chemical shift difference. CCPNMR 2.0 (http://www.ccpn.ac.uk/v2-software/software, UK) analysis and Topspin 3.2 (Bruker, Billerica MA) were used for peak assignment and data processing, respectively.

Table S1. ^{1}H and ^{13}C chemical shifts of Sia_3

	III	II	I
position	**^{1}H (^{13}C)**	**^{1}H (^{13}C)**	**^{1}H (^{13}C)**
H-3a	1.74 (40.4)	1.66 (40.8)	1.77 (39.2)
H-3e	2.78 (40.4)	2.71 (40.8)	2.20 (39.2)
H-4	3.68 (68.5)	3.56 (68.0)	3.99 (67.5)
H-5	3.84 (51.7)	3.80 (52.4)	3.89 (52.4)
H-6	3.63 (72.6)	3.57 (73.6)	3.86 (70.8)
H-7	3.60 (68.2)	3.85 (69.1)	3.79 (67.8)
H-8	3.92 (71.7)	4.12 (78.2)	4.03 (75.8)
H-9	3.65 (62.6)	4.15 (61.2)	3.95 (61.1)
H-9'	3.90 (62.6)	3.68 (61.2)	3.74 (61.1)

At 25 °C, referenced to water

Table S2 ^{1}H and ^{13}C chemical shifts of $TT_{830-844}$.

Amino Acid	NH	H_α ^{1}H (^{13}C)	H_β ^{1}H (^{13}C)	H_γ ^{1}H (^{13}C)	H_δ, H_ε ^{1}H (^{13}C)	Others
Q1	8.42	4.03(52.4)	2.38 (30.1)	2.11 (26.9)		δ NH_2 6.84, 7.55
Y2	8.23	4.6 (55.0)	3.1,2.87 (35.9)			2,6H 7.15, 3,5H 6.84
I3	8.28	4.10(57.7)	1.79 (36.3)	1.15, 1.43 (24.4)		γ,δ CH_3 0.83
K4	8.37	4.25(53.8)	1.79 (30.3)	1.41 (22.1)	1.70 (26.6), 2.98 (39.4)	
A5	8.35	4.26(49.9)	1.37 (16.4)			
N6	8.43	4.66 (50.6)	2.8, 2.85 (36.1)			γ NH_2 7.59, 6.84
S7	8.20	4.37(56.0)	3.8, 3.86 (61.1)			
K8	8.22	4.22(53.8)	1.64 (30.2)	1.26 (21.9)	1.60 (26.3), 3.1 (39.4)	
F9	8.18	4.63(54.9)	2.98, 3.09 (36.8)			2,6H 7.22, 3,5H 7.31
I10	7.99	4.25(58.4)	1.88 (36.3)	1.43 (24.5)		γ,δ CH_3 0.9
G11	8.03	3.88(42.4)				
I12	7.99	4.12(58.5)	1.80 (36.0)	1.17 (24.5)		γ,δ CH_3 0.8
T13	8.34	4.35(59.2)	4.17 (67.1)	1.17 (18.8)		
E14	8.34	4.30(53.7)	2.05,1.89 (27.7)	2.22 (33.6)		
L15	7.87	4.16(54.0)	1.55 (40.6)	1.55 (40.6)		δ CH_3 0.83, 0.88

Table S3 ^{1}H and ^{13}C chemical shifts of Sia_3-III-7CHO

	III	II	I
position	**^{1}H (^{13}C)**	**^{1}H (^{13}C)**	**^{1}H (^{13}C)**
H-3a	2.05 (33.0)	1.62 (41.0)	1.77 (39.2)
H-3e	2.12 (33.0)	2.69 (41.0)	2.20 (39.2)
H-4	3.97 (65.4)	3.54 (68.2)	3.98 (67.5)
H-5	4.01 (47.6)	3.75 (52.4)	3.87 (52.4)
H-6	3.59 (76.4)	3.49 (73.4)	3.88 (70.9)
H-7	5.90 (97.6)	4.15 (69.4)	3.78 (68.0)
H-8	NA	3.70 (79.6)	4.04 (76.0)
H-9	NA	4.64 (70.1)	3.96 (61.1)
H-9'	NA	3.84 (70.1)	3.73 (61.1)

At 25 °C, referenced to water

Table S4 ^{1}H and ^{13}C chemical shifts of $TT_{830-844}$ -Sia_3-III-7CHO. Changes in ^{1}H chemical shift for amino acids are signified in bold font, indicating that they are impacted by Sia_3 substitution.

Amino Acid	NH	H_α ^{1}H (^{13}C)	H_β ^{1}H (^{13}C)	H_γ ^{1}H (^{13}C)	H_δ, H_ε ^{1}H (^{13}C)	Others
Q1	-	-	-	-		δ NH_2 6.84, 7.55
Y2	-	4.6 (55.0)	3.1, 2.87 (36.4)			2,6H 7.15, 3,5H 6.84
I3	**8.28**	**4.10 (58.3)**	**1.79 (36.3)**	**1.15, 1.43 (24.4)**		γ,δ CH_3 0.83
K4	**8.37**	**4.25 (53.8)**	**1.79 (30.3)**	**1.41 (22.1)**	**1.70 (26.6), 2.98 (39.4)**	
A5	8.35	4.26 (49.9)	1.37 (16.4)			
N6	8.43	4.66 (50.6)	2.8, 2.85 (36.1)			γ NH_2 7.59, 6.84
S7	8.20	4.37 (56.0)	3.8,3.86 (61.1)			
K8	**8.22**	**4.22 (53.8)**	**1.64 (30.2)**	**1.26 (21.9)**	**1.60 (26.3), 3.1 (39.4)**	
F9	8.18	4.63 (54.9)	2.98, 3.09 (36.8)			2,6H 7.22, 3,5H 7.31
I10	7.99	4.25 (58.4)	1.88 (36.3)	1.43(24.5)		γ,δ CH_3 0.9
G11	8.03	3.88 (42.4)				
I12	7.99	4.12 (58.5)	1.80 (36.0)	1.17 (24.5)		γ,δ CH_3 0.8
T13	8.34	4.35 (59.2)	4.17 (67.1)	1.17 (18.8)		
E14	8.34	4.30 (53.7)	2.05, 1.89 (27.7)	2.22 (33.6)		
L15	7.87	4.16 (54.0)	1.55 (40.6)	1.55 (40.6)		δ CH_3 0.83, 0.88

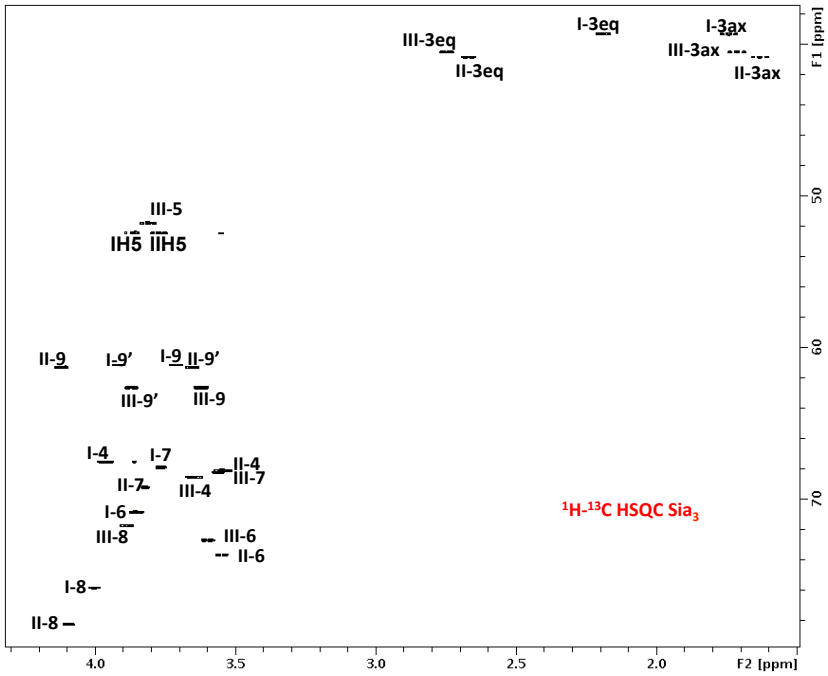
At 25 °C, referenced to water

Table S5 ^{1}H and ^{13}C chemical shifts of Sia_3-III-8CHO

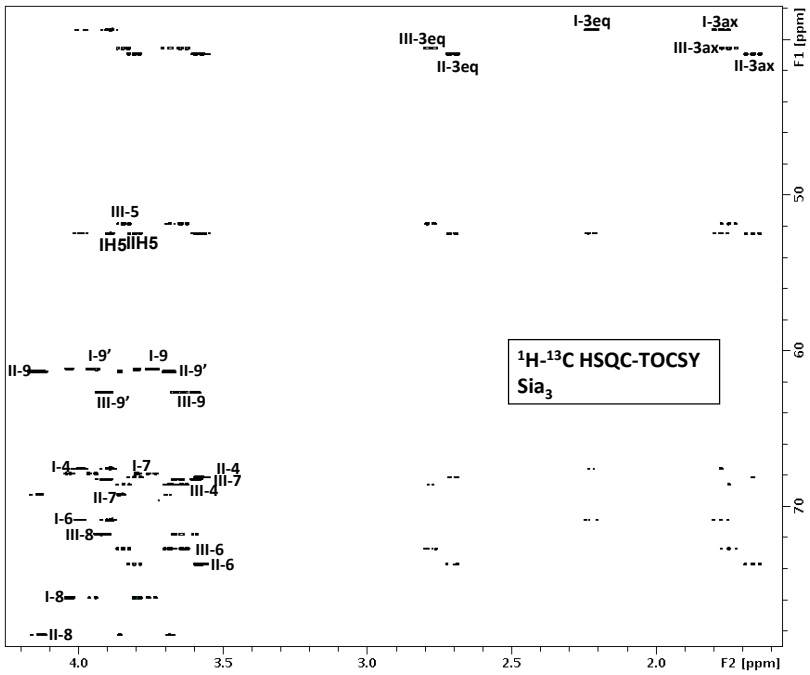
	III	II	I
position	**^{1}H (^{13}C)**	**^{1}H (^{13}C)**	**^{1}H (^{13}C)**
H-3a	1.72 (40.4)	1.65 (40.9)	1.77 (39.2)
H-3e	2.72 (40.4)	2.70 (40.9)	2.20 (39.2)
H-4	3.65 (68.2)	3.56 (68.1)	3.99 (67.5)
H-5	3.84 (51.8)	3.77 (52.4)	3.89 (52.4)
H-6	3.78 (72.8)	3.55 (73.8)	3.86 (70.8)
H-7	3.40 (71.9)	3.86 (69.3)	3.78 (67.8)
H-8	5.19 (90.3) 9.68 (-)	4.11 (78.3)	4.03 (75.6)
H-9	NA	4.15 (61.3)	3.95 (61.1)
H-9'	NA	3.67 (61.3)	3.74 (61.1)

At 25 °C, referenced to water

Figure S1



A. ^{1}H-^{13}C HSQC spectrum of Sia$_3$ and its assignments.



B. ^{1}H-^{13}C HSQC-TOCSY spectrum Sia$_3$ and its assignments based on their cross peaks

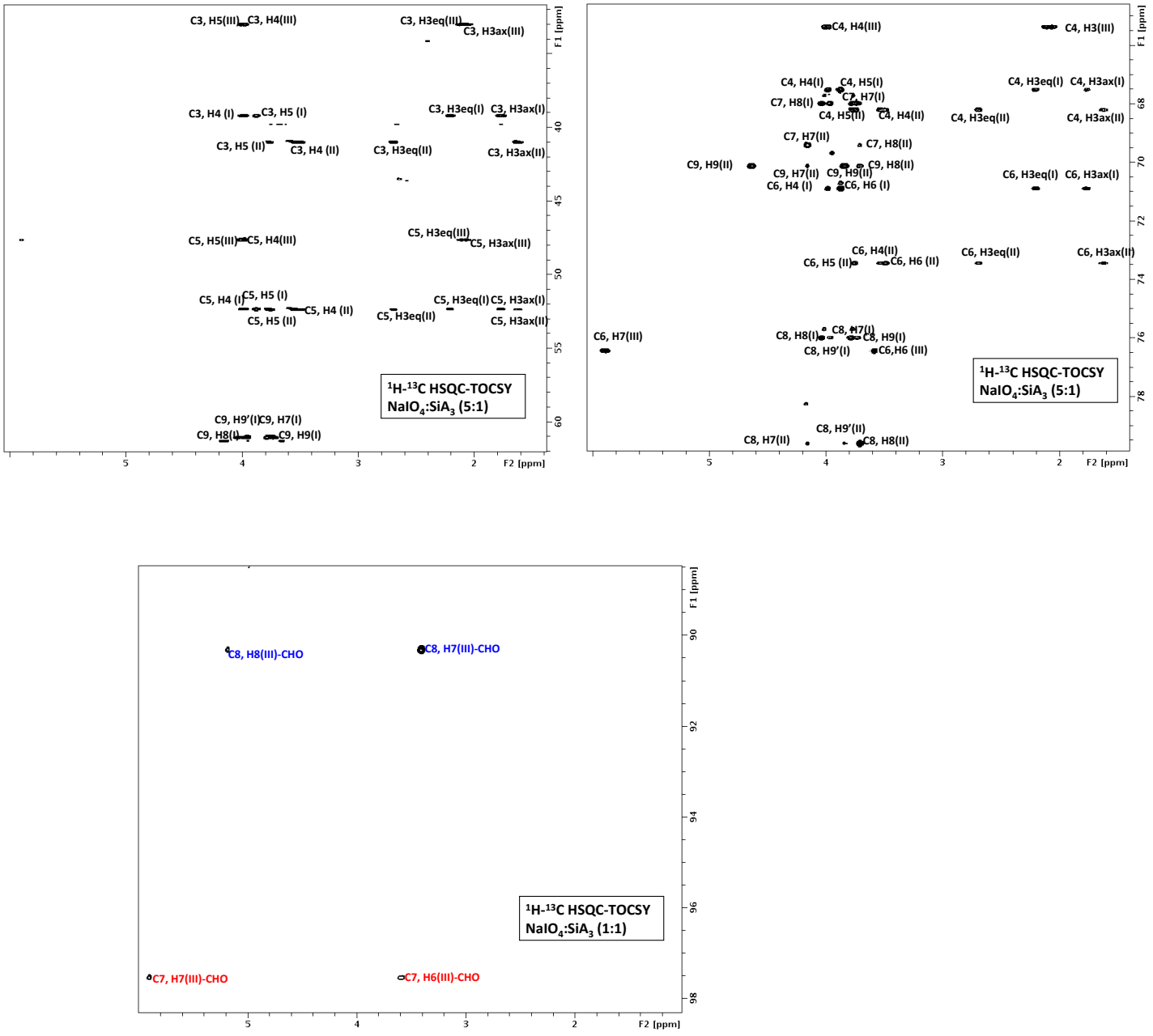
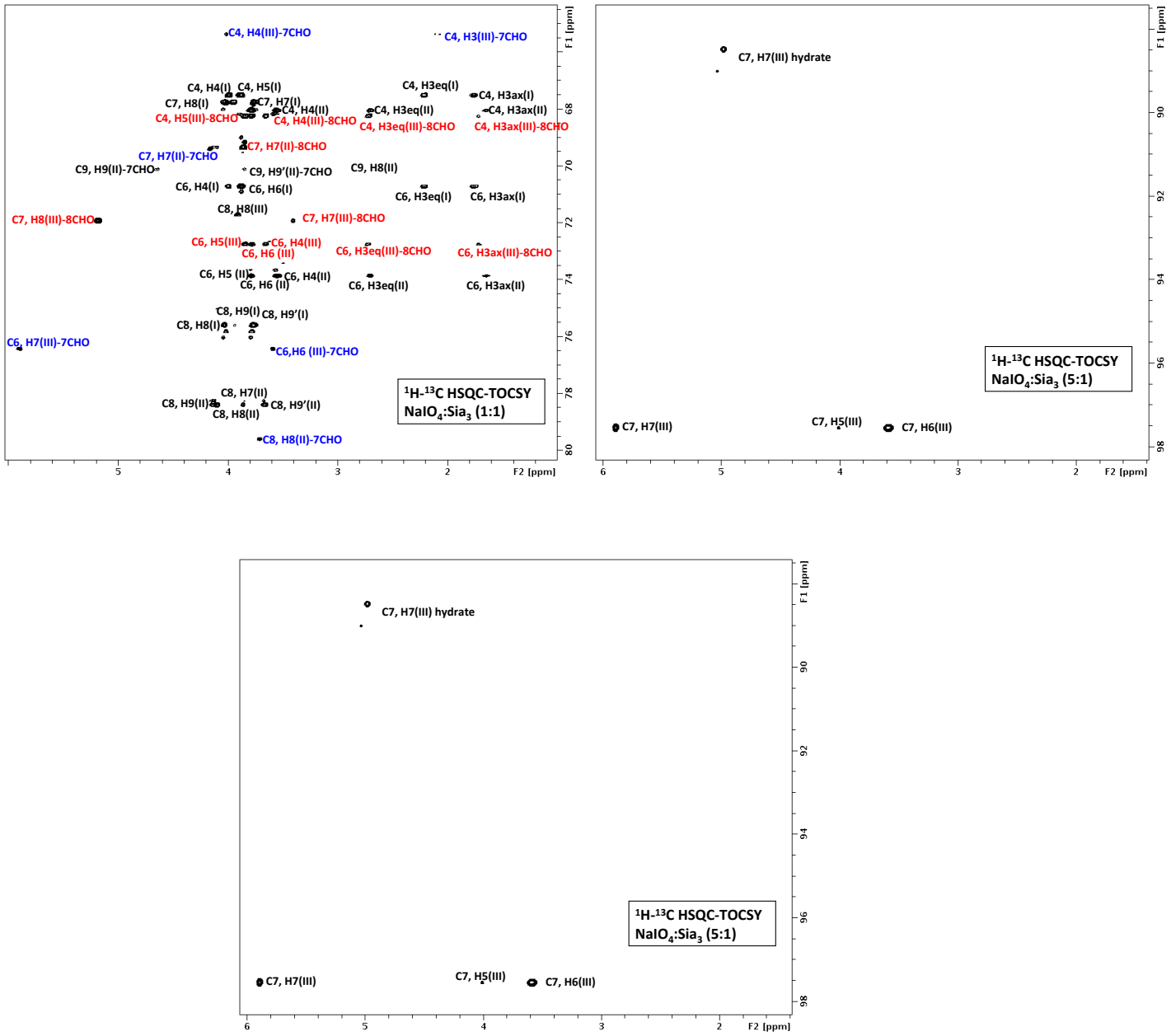


Figure S2A-C. ^{1}H-^{13}C HSQC-TOCSY experiments and assignments of Sia$_3$-III-7CHO



S2D-F. ^{1}H-^{13}C HSQC-TOCSY experiments and assignments of Sia$_3$-III-8CHO.

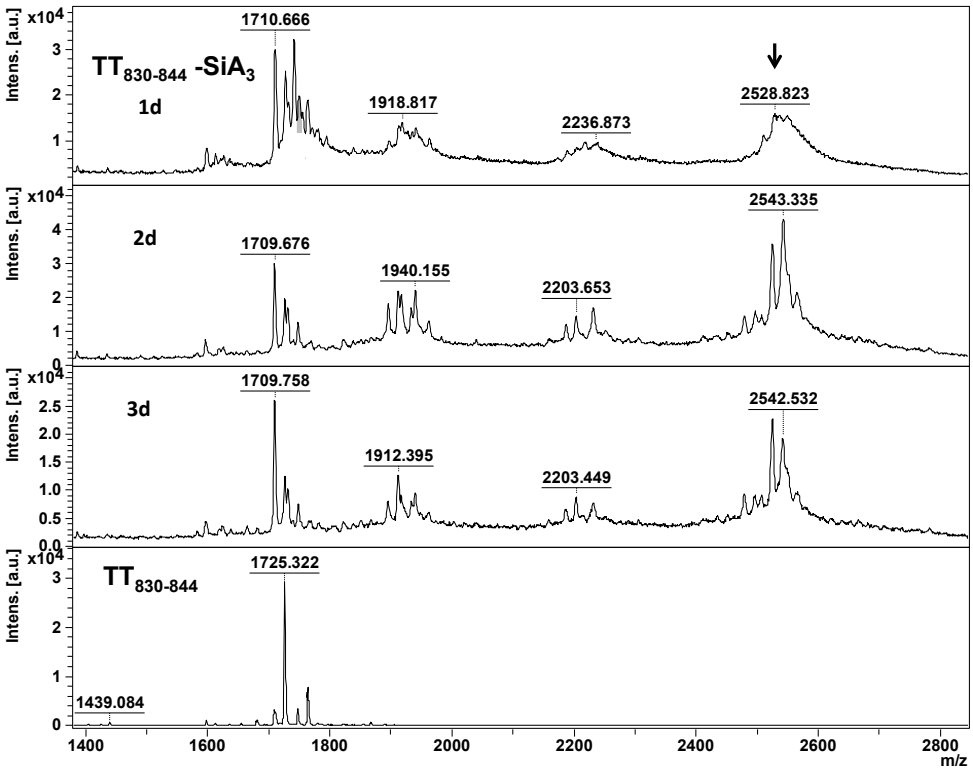
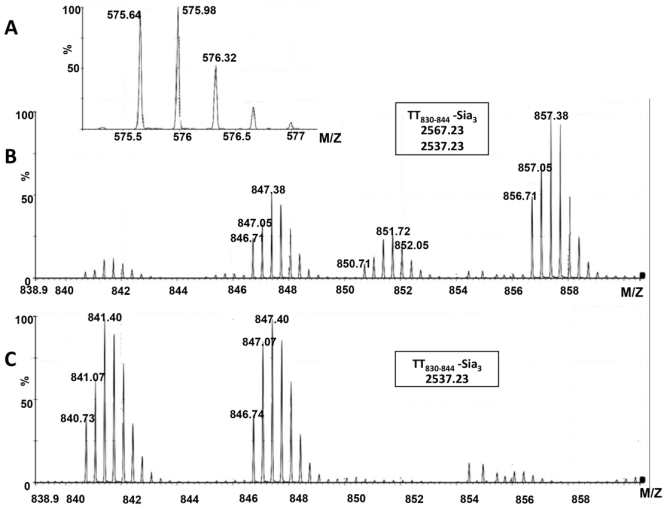


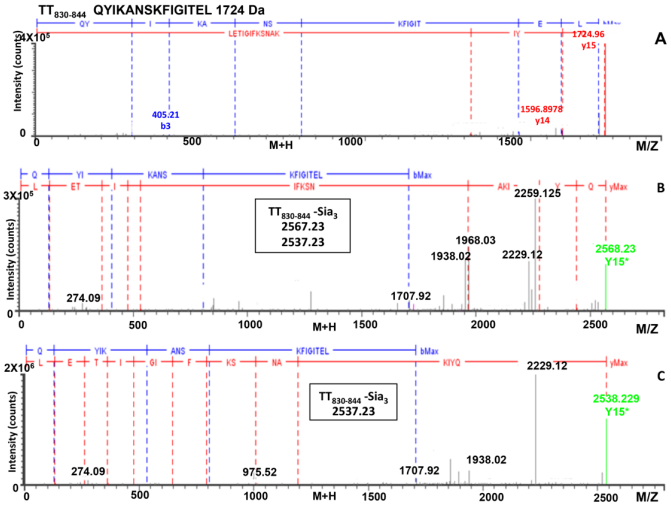
Figure S3 Real time measurement of chemical reaction on day 0, 1, 2, 3 days using MALDI-TOF MS.

Figure S4



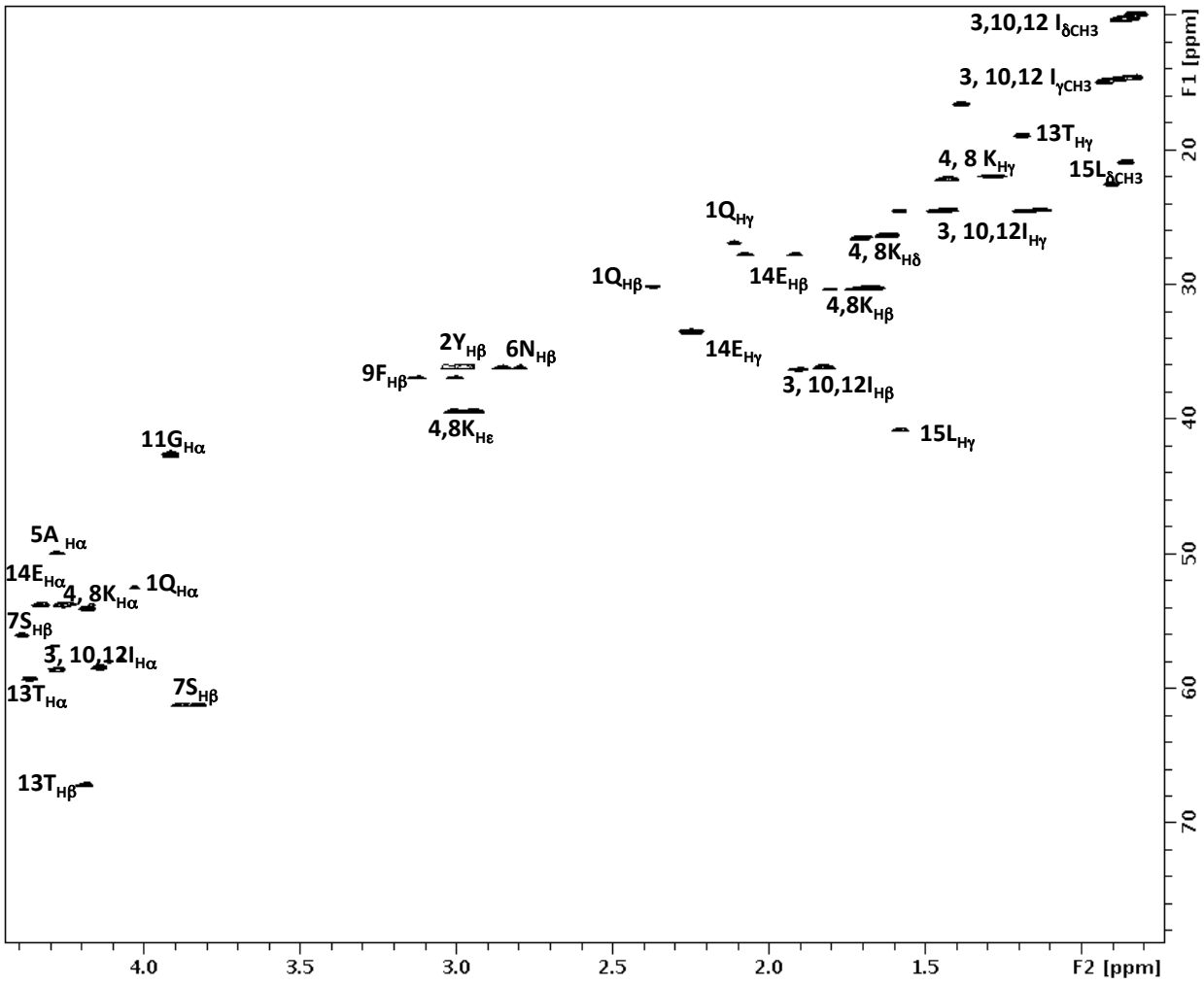
A. ESI LC-MS spectrum of $TT_{830\text{-}844}$ shows a triply charged fragment $(M + 3H)^{+3}$ at m/z 575.95. **B.** At a molar ratio of 1:1 sodium periodate:Sia_3, $TT_{830\text{-}844}$ - Sia_3 is triply charged $(M + 3H)^{+3}$ with fragments at m/z 856.7115 and 846.7094, which correspond to aldehyde formation at III-8 and III-7 positions, respectively. **C.** At a molar ratio of 5:1 sodium periodate: Sia_3, $TT_{830\text{-}844}$ - Sia_3 has only one triply charged fragment $(M + 3H)^{+3}$ at m/z 846.7447.

Figure S5.



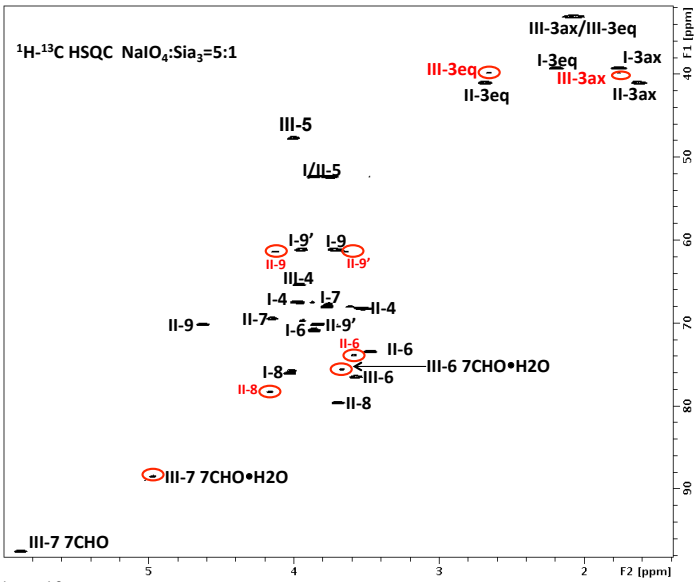
ESI LC MS^E data for $TT_{830-844}$ and the conjugates resulting from conjugation to differentially oxidized Sia_3 starting material. **A.** Fragmentation analysis of $TT_{830-844}$ by nanoLC-ESI-MS^E showing that the analysis can be used to identify $TT_{830-844}$. The figure shows the expected mass for the peptide at 1724.96 amu and predicted peptide fragments at the blue and red dotted lines annotated by their expected mass. **B.** Fragmentation analysis of $TT_{830-844}$ - Sia_3 by nanoLC-ESI-MS^E, showing that the two possible conjugates co-elute. One conjugate has the expected mass at 2568.23 amu while the second conjugate has an expected mass of 2538.23 amu. Predicted peptide fragments at the blue and red dotted lines annotated by their expected mass. Fragment ions m/z at 2259.12 (loss of 309amu), 1968.03 (loss of 291amu), and 1707.92 (loss of 260amu) indicate the fragmentation of sialic acid residues of $TT_{830-844}$-Sia_3 at 2568.23amu, whereas fragment ions m/z at 2229.10(loss of 309amu), and 1938.02 (loss of 291amu) indicated the loss of sialic acids of $TT_{830-844}$-Sia_3 at 2538.23amu. **C.** Fragmentation analysis of $TT_{830-844}$ - Sia_3 conjugate by nanoLC-ESI-MS^E, showing the expected mass for conjugate at 2538.23 amu and predicted peptide fragments at the blue and red dotted lines annotated by their expected mass.

Figure S6



^{1}H-^{13}C HSQC-TOCSY experiments and assignments of $TT_{830-844}$

Figure S7



^{1}H-^{13}C HSQC-TOCSY assignments of activated Sia$_3$ using a 5:1 molar ratio of NaIO$_4$:TT$_{830\text{-}844}$

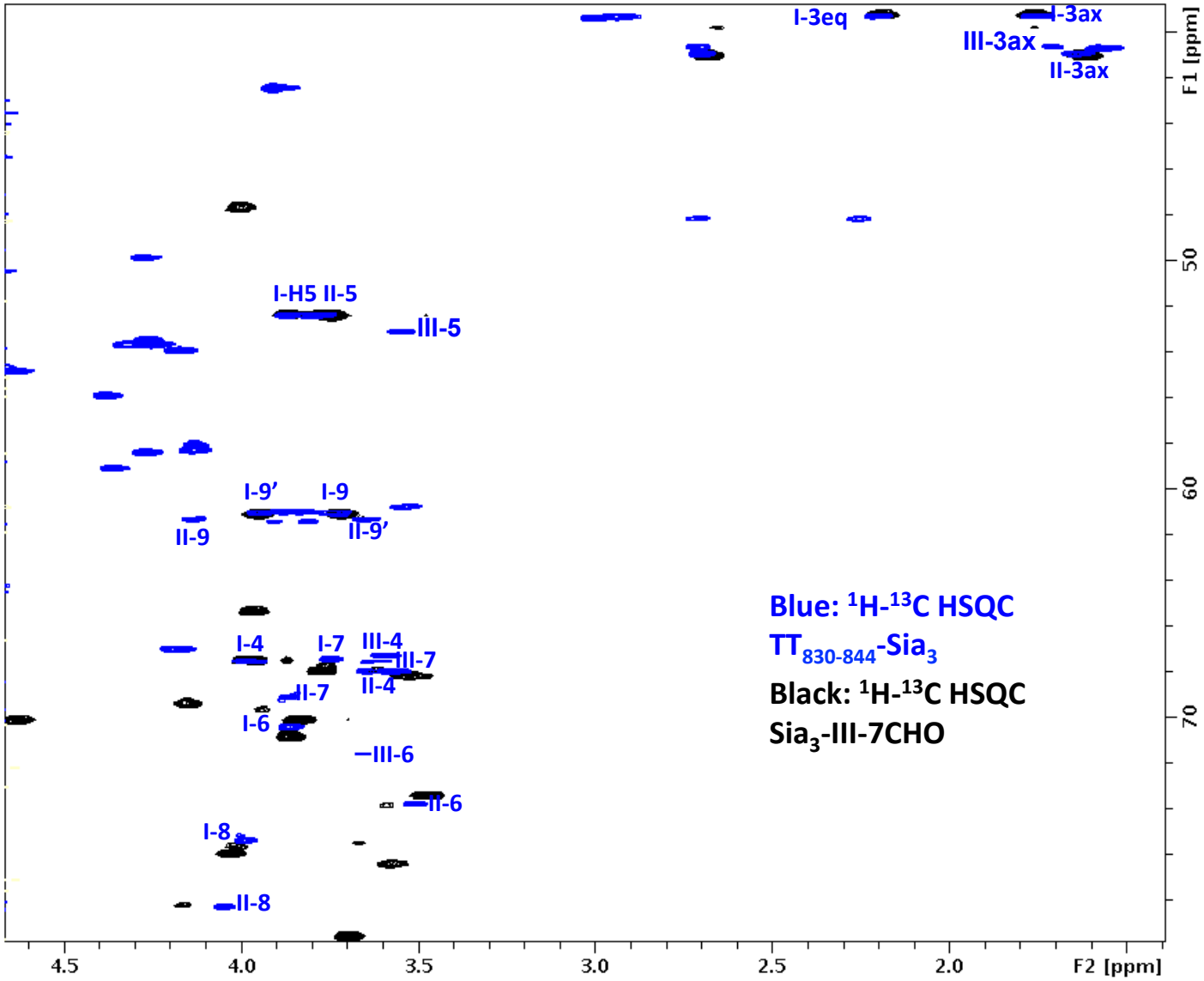


Figure S8. ^{1}H-^{13}C HSQC-TOCSY assignments of Sia$_3$-TT$_{830-844}$ (blue) overlaid with Sia$_3$ activated at C-7 of residue III.

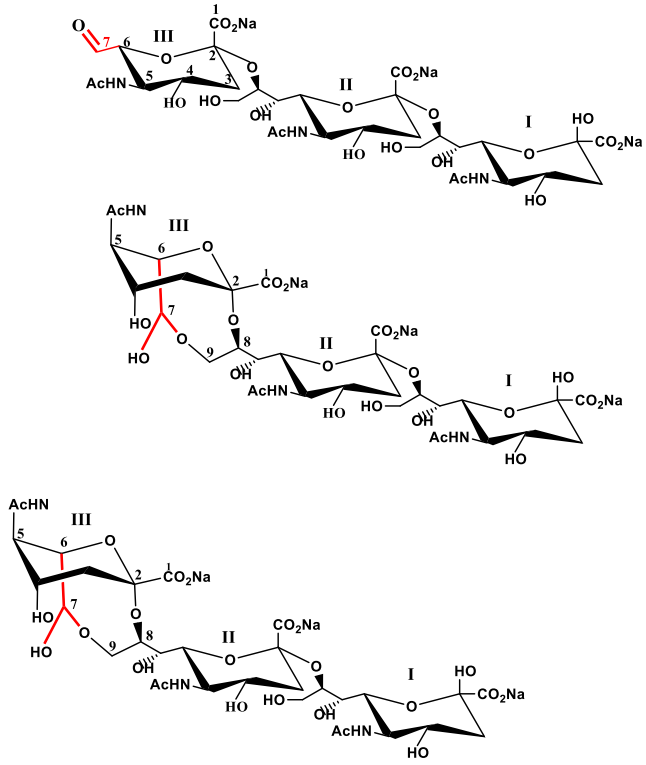


Figure S9. Hemiacetal structure of activated Sia_3.

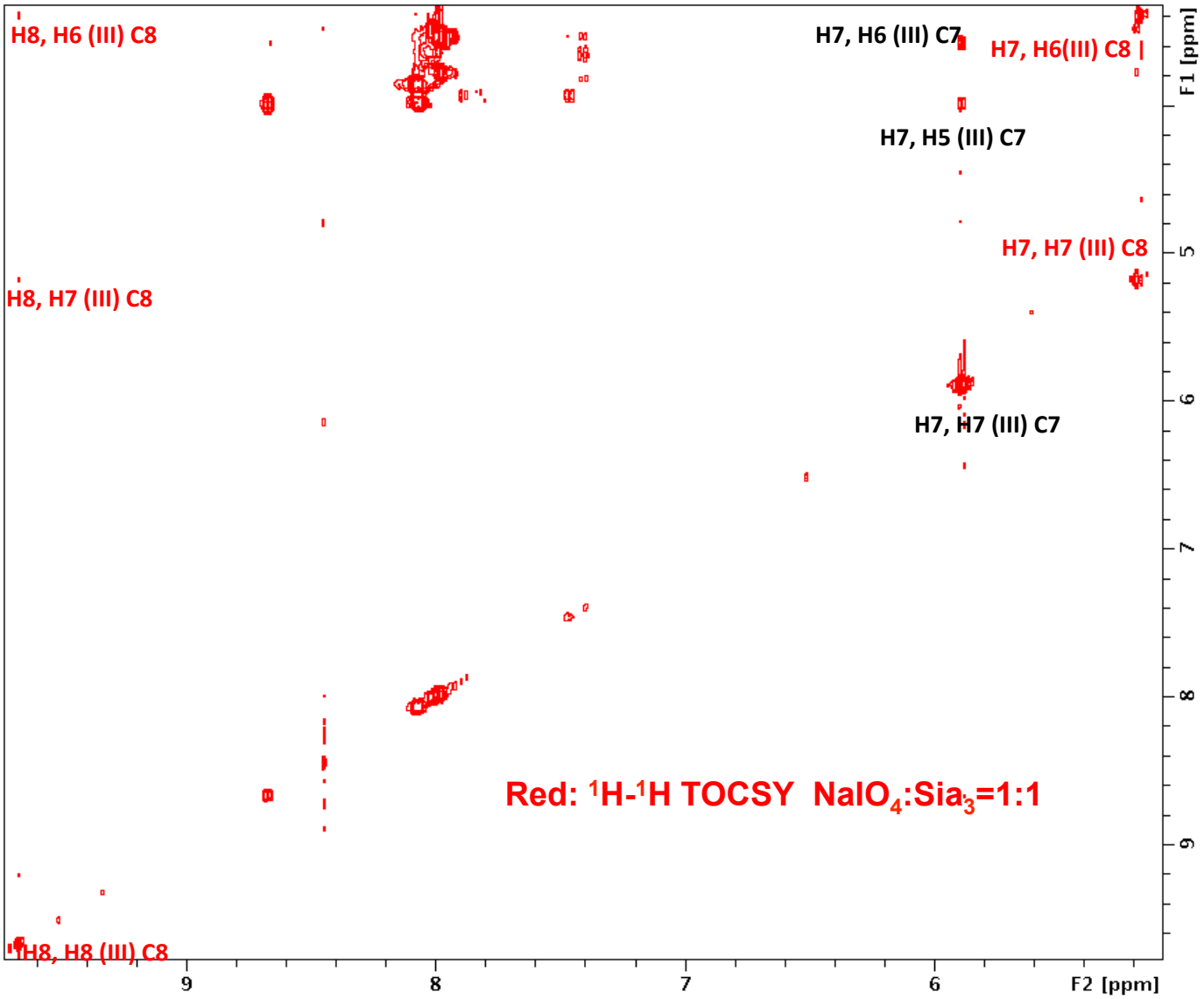


Figure S10. HSQC-TOCSY spectrum of Sia_3 activated in a 1:1 ratio with $NaIO_4$.