Supporting Information

Chemical Structure and Composition of Major Glycans Covalently Linked to Therapeutic Monoclonal Antibodies by Middle-down NMR

Jiangnan Peng[#], Sharadrao M. Patil[#], David A. Keire[§*] and Kang Chen[#*]

Division of Pharmaceutical Analysis, Office of Testing and Research, Office of Pharmaceutical Quality, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, [#]Silver Spring, MD 20993 and [§]St. Louis, MO 63110, United States.

Corresponding Author

* Kang Chen, kang.chen@fda.hhs.gov

* David Keire, david.keire@fda.hhs.gov

Table of Contents:

Table S1 Information on mAb drug products studied

mAb	Drug product	Concentration	Manufacture
Rituximab	Rituxan®	10 mg/mL	Genentech
	Ristova®		Roche
	Reditux®		Dr. Reddy's
Infliximab	Remicade®	100 mg/vial	Janssen
Bevacizumab	Avastin®	10 mg/mL	Genentech.
Etanercept	Enbrel®	25 mg/vial	Amgen
Adalimumab	Humira®	40 mg/0.8 mL	AbbVie

Table S2 ^{1}H and ^{13}C NMR chemical shifts assignment (ppm) for glycan standard G2F[a]

Nucleus	GN1α[b]	GN1β[b]	GN2	Man3	Fucα[c]	Fucβ[c]	Man4	Man4′	GN5/5′	Gal6/6′
C1	93.20	97.67	103.8	103.2	102.3	102.1	102.2	99.73	102.2	105.7
C2	56.40	58.89	57.67	72.91	70.94	70.93	79.10	79.03	57.58	73.69
C3	71.89	75.11	74.77	83.16	72.16	72.18	72.12	72.15	74.71/ 74.80[d]	75.23
C4	81.70	81.31	82.31	68.40	74.58	74.58	70.01	70.04	81.22	71.26
C5	71.91	76.21	77.10	77.07	69.56	69.55	76.27	75.57	77.44	78.08
C6	69.73	69.29	62.67	68.54	18.07	18.04	64.41	64.37	62.69	63.75
H1	5.189	4.703	4.675	4.772	4.897	4.903	5.126	4.933	4.590	4.478
H2	3.908	3.718	3.762	4.261	3.798	3.798	4.200	4.118	3.710	3.550
H3	4.018	3.685	3.754	3.775	3.907	3.915	3.910	3.907	3.739/3 .703[d]	3.678
H4	3.796	3.781	3.739	3.796	3.812	3.814	3.513	3.505	3.741	3.936
H5	3.881	3.651	3.607	3.632	4.142	4.107	3.754	3.625	3.582/ 3.574 [d]	3.737
H6a	3.852	3.910	3.883	3.969	1.222	1.222	3.933	3.920	3.995	3.779
H6b	3.722	3.676	3.799	3.792	-	-	3.627	3.624	3.856	3.741

[a] ^{1}H and ^{13}C NMR chemical shifts were obtained from the average of multiple cross peaks from HSQC, HSQC-TOCSY, and Long-range HSQC-TOCSY spectra; The errors of chemical shifts were less than 0.005 ppm for all ^{1}H and less than 0.020 ppm for all ^{13}C.

[b] GN1α and GN1β represent the two equilibrium anomeric configurations of GN1.

[c] Fucα and Fucβ were the two sets of data observed for Fuc due to the α/β equilibrium of GN1.

[d] Two sets of close signals were likely originated from the difference in 1,3- (GN5) or 1,6- (GN5′) linked branches.

Table S3. ^{1}H and ^{13}C NMR Chemical Shifts assignment for glycan G0F[a]

	GN1α[b]	GN1β[b]	GN2	Man3	Fuc3'α[c]	Fuc3'β[c]	Man4	Man4'	GN5/5′
C1	93.21	97.67	103.8	103.2	102.3	102.0	102.3	99.74	102.3
C2	56.39	58.86	57.65	72.90	70.92	ND[d]	79.13	79.04	58.05
C3	71.87	75.10	74.74	83.16	72.19	ND[d]	72.11	72.14	76.03/ 76.08
C4	81.68	81.33	82.38	68.41	74.56	ND[d]	70.00	70.03	72.61
C5	71.90	76.21	77.07	77.01	69.54	69.55	76.27	75.56	78.52
C6	69.70	69.27	62.65	68.62	18.00	18.05	64.39	64.34	63.33
H1	5.179	4.694	4.665	4.770	4.888	4.893	5.114	4.915	4.554
H2	3.895	3.712	3.761	4.250	3.791	3.802	4.187	4.107	3.701
H3	4.008	3.670	3.752	3.768	3.902	3.916	3.899	3.897	3.550/ 3.541
H4	3.786	3.775	3.730	3.774	3.803	ND[d]	3.506	3.495	3.459
H5	3.872	3.642	3.595	3.632	4.132	4.095	3.744	3.616	3.434/ 3.431
H6a	3.843	3.899	3.878	3.957	1.210	1.220	3.928	3.903	3.910
H6b	3.708	3.663	3.785	3.767	-	-	3.615	3.619	3.758

[a] ^{1}H and ^{13}C NMR chemical shifts were obtained from the average of multiple cross peaks from HSQC, HSQC-TOCSY, and Long-range HSQC-TOCSY spectra; The errors of chemical shifts are less than 0.005 ppm for all ^{1}H and less than 0.014 ppm for all ^{13}C.

[b] GN1α and GN1β represent the two equilibrium anomeric configurations of GN1.

[c] Fuc3′α and Fuc3′β were the two sets of data observed for Fuc3′ due to the equilibrium of GN1.

[d] ND indicated not determined.

Table S4. ^{1}H and ^{13}C NMR Chemical Shifts assignment for glycan G1[a]

	GN1α[b]	GN1β[b]	GN2	Man3	Man4	Man4'	GN5a[c]	GN5b[c]	Gal6
C1	93.17	97.53	104.1	103.2	102.3	99.76	102.2	102.3	105.7
C2	56.38	58.84	57.69	72.92	79.16	79.05	57.61	58.08	73.71
C3	72.73	75.24	74.76	83.18	72.13	72.17	74.69/ 74.83	76.03/ 76.13	75.25
C4	82.42	81.95	82.24	68.44	70.04	70.07	81.25	72.64	71.27
C5	71.97	77.32	77.12	77.10	76.30	75.58	77.45	78.54	78.09
C6	62.68	62.74	62.70	68.57	64.43	64.37	62.71	63.36	63.75
H1	5.206	4.710	4.625	4.781	5.132	4.938	4.599	4.572	4.487
H2	3.899	3.705	3.766	4.263	4.204	4.125	3.752	3.720	3.556
H3	3.899	3.692	3.781	3.776	3.912	3.907	3.739/ 3.709	3.563/ 3.551	3.688
H4	3.649	3.643	3.746	3.811	3.521	3.508	3.744	3.467	3.946
H5	3.892	3.527	3.626	3.636	3.762	3.629	3.587	3.453	3.745
H6a	3.895	ND[d]	3.814	3.974	3.925	3.920	4.001	3.925	3.785
H6b	3.761	3.679	ND[d]	3.793	3.635	3.638	3.867	3.774	3.767

[a] ^{1}H and ^{13}C NMR chemical shifts were obtained from the average of multiple cross peaks from HSQC, HSQC-TOCSY, and Long-range HSQC-TOCSY spectra; The errors of chemical shifts are less than 0.006 ppm for all ^{1}H and less than 0.015 ppm for all ^{13}C.

[b] GN1α and GN1β represented the two equilibrium anomeric configurations of GN1.

[c] GN5a indicates GN5/5' with galactosylation, while GN5b indicated GN5/5' without galactosylation;

[d] ND indicated not determined.

Table S5. Demonstrated methods for glycan analysis

Method	Size of Analyte Molecule	Chemical reaction	Separation	Identification	Quantification	References
Intact	150 kDa	Denaturation	LC or CE or direct infusion	MS	MS	[1-3]
Middle-down MS	25 kDa	IdeS digestion and TCEP reduction	RP-HPLC	MS	MS	[3,4]
Middle-down-NMR	25 kDa	IdeS digestion, DTT reduction and Urea denaturation	SEC-FPLC	NMR	NMR	none
Glycopeptide or Multi-attribute method (MAM)	1 - 10 kDa	Alkylation, Trypsin or lysyl endopeptidase and urea/GuHCl denaturation	RP-HPLC	MS	MS	[1,3,5-7]
Free-glycan or glycan mapping method	1 – 2 kDa	Denaturation, PNGase-F and 2-AB labelling	CE or HILIC	MS and Retention time	Fluorescence or MS	[1,3,5,8]

CE: Capillary Electrophoresis
FPLC: Fast Protein Liquid Chromatography
HILIC: Hydrophilic Interaction Liquid Chromatography
HPLC: High Performance Liquid Chromatography
LC: Liquid Chromatography
RP: Reverse Phase
SEC: Size Exclusion Chromatography

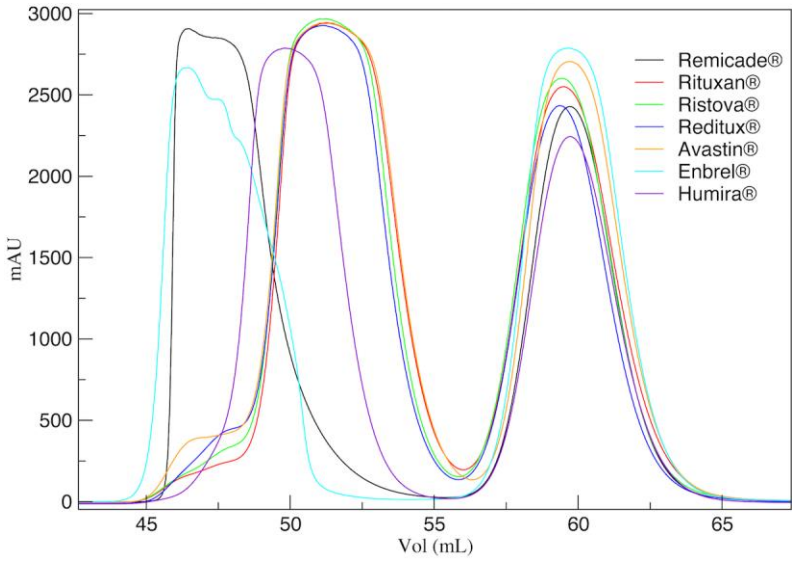

Figure S1. SEC-FPLC chromatograms of the digested 7 mAb drug products. A total of 17 samples from 7 mAb drug products were digested and the Fc of each mAb was readily separated from Fab using a Superdex 75g 16/600 FPLC column. The retention volume and peak elution shape for Fc domain of all seven mAbs are similarly at 59.5 mL, while the Fab portions of Infliximab and Adalimumab, and the TNF protein of Etanercept eluted earlier than the other four mAbs, with broadened and asymmetric peaks indicating aggregation.

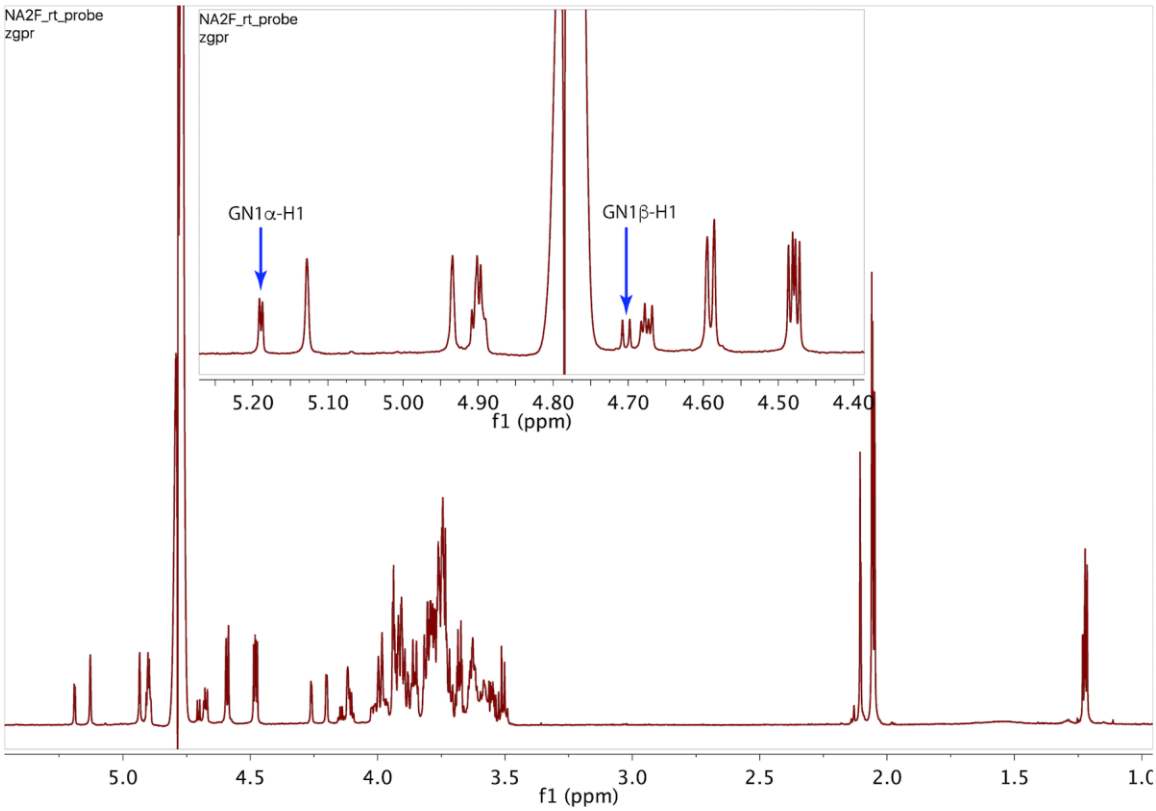

Figure S2. ^{1}H NMR spectrum of G2F in D_2O

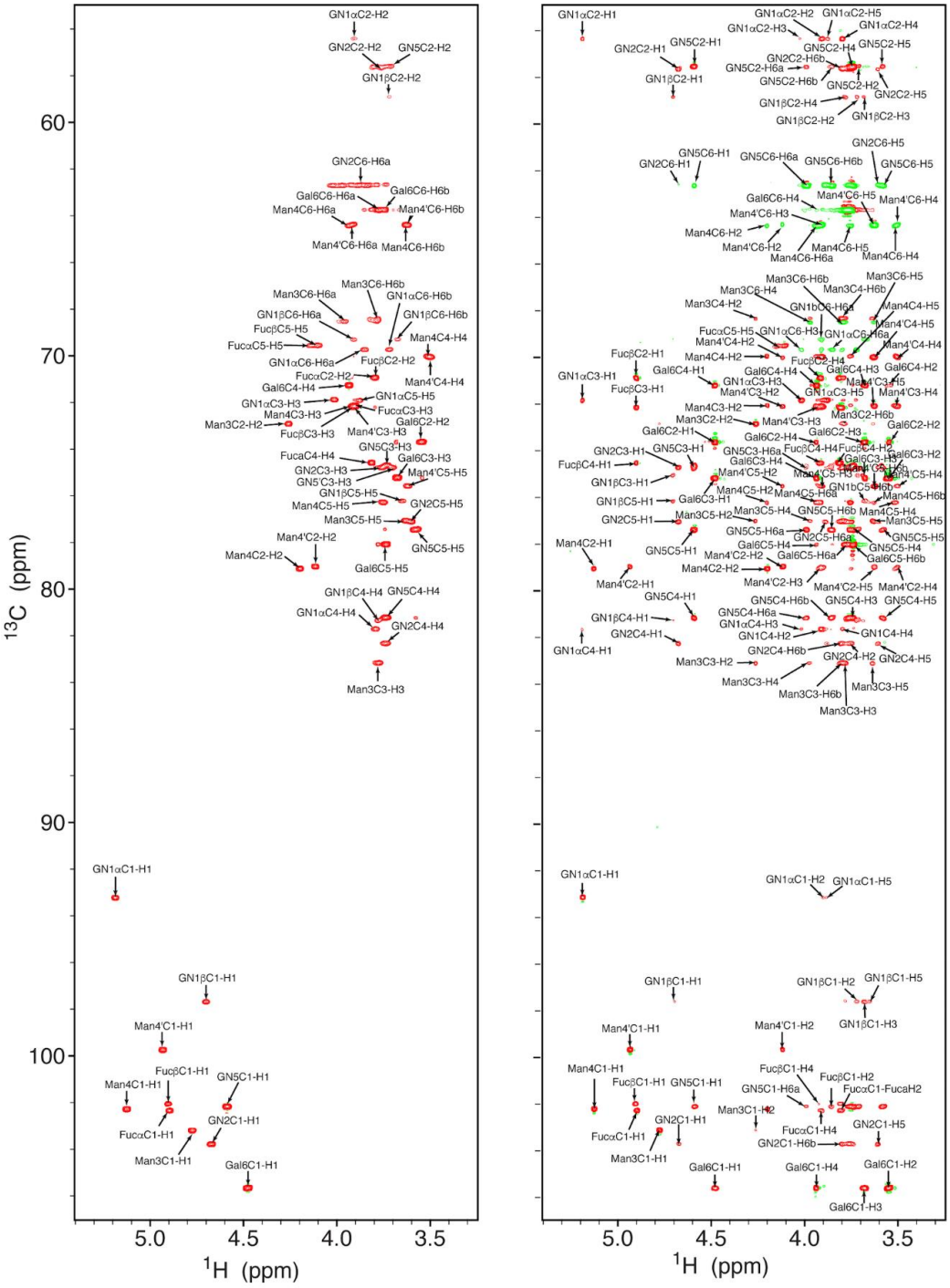

Figure S3. The 2D ^{1}H-^{13}C HSQC (left) and multiplicity-edited HSQC-TOCSY (right) spectra of standard glycan G2F. Peaks in green and red in the HSQC-TOCSY indicated the CH_2 and CH/CH_3 signals, respectively. GN1α and GN1β indicated the two anomeric isomers of GN1. Fucα and Fucβ indicated the two sets of signals for fucose because of the GN1 anomeric isomerization. The chemical shifts for GN5 and GN5' were labeled as GN5 in the annotation. The same unified labeling applied to GN6 and GN6'.

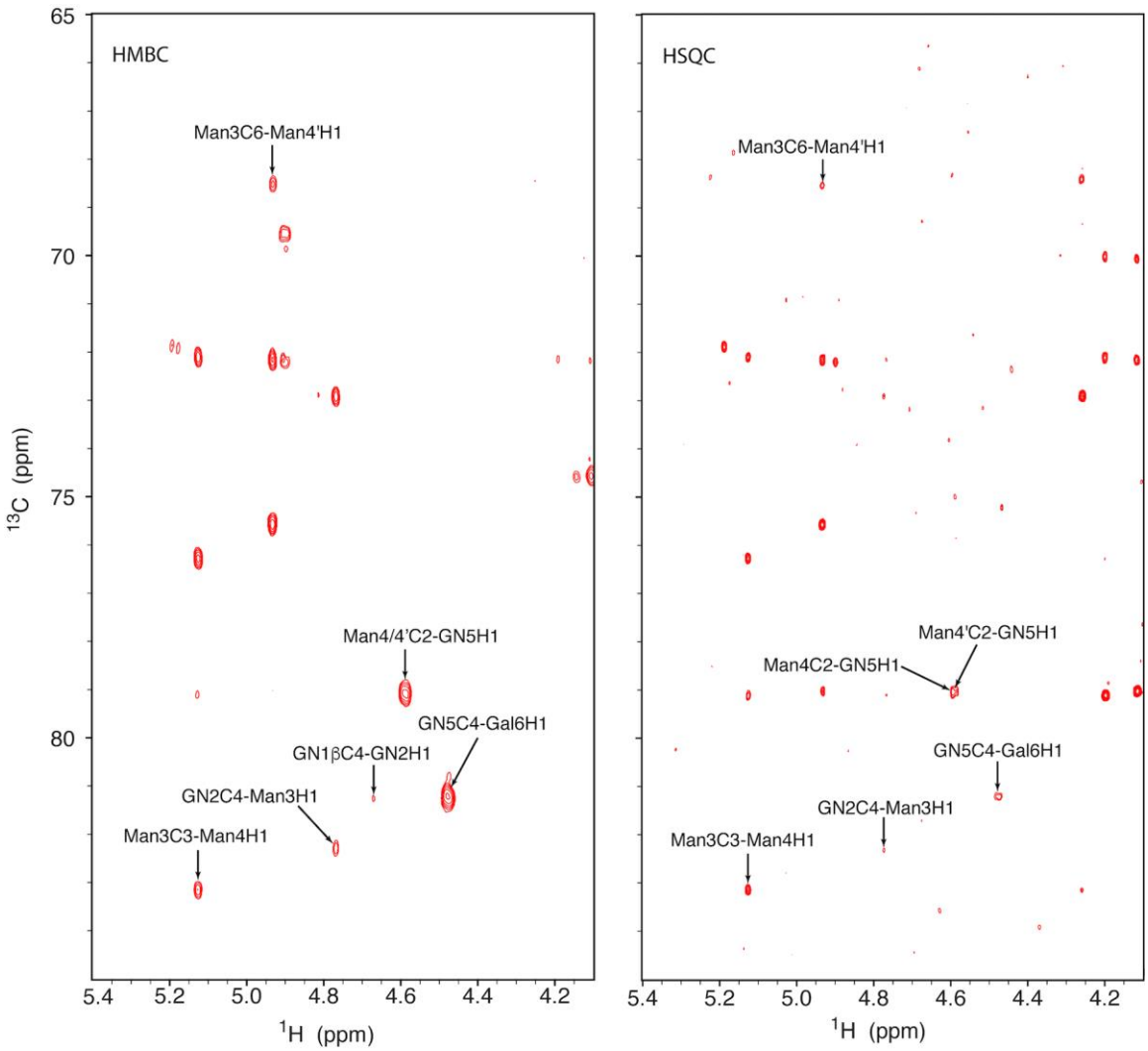

Figure S4. HMBC (left) and long-range HSQC (6 Hz) (right) spectra of G2F. Since the chemical shifts for GN5 and GN5' were mostly the same it is only labeled as GN5 in the annotation. The same labeling applies to GN6 and GN6'.

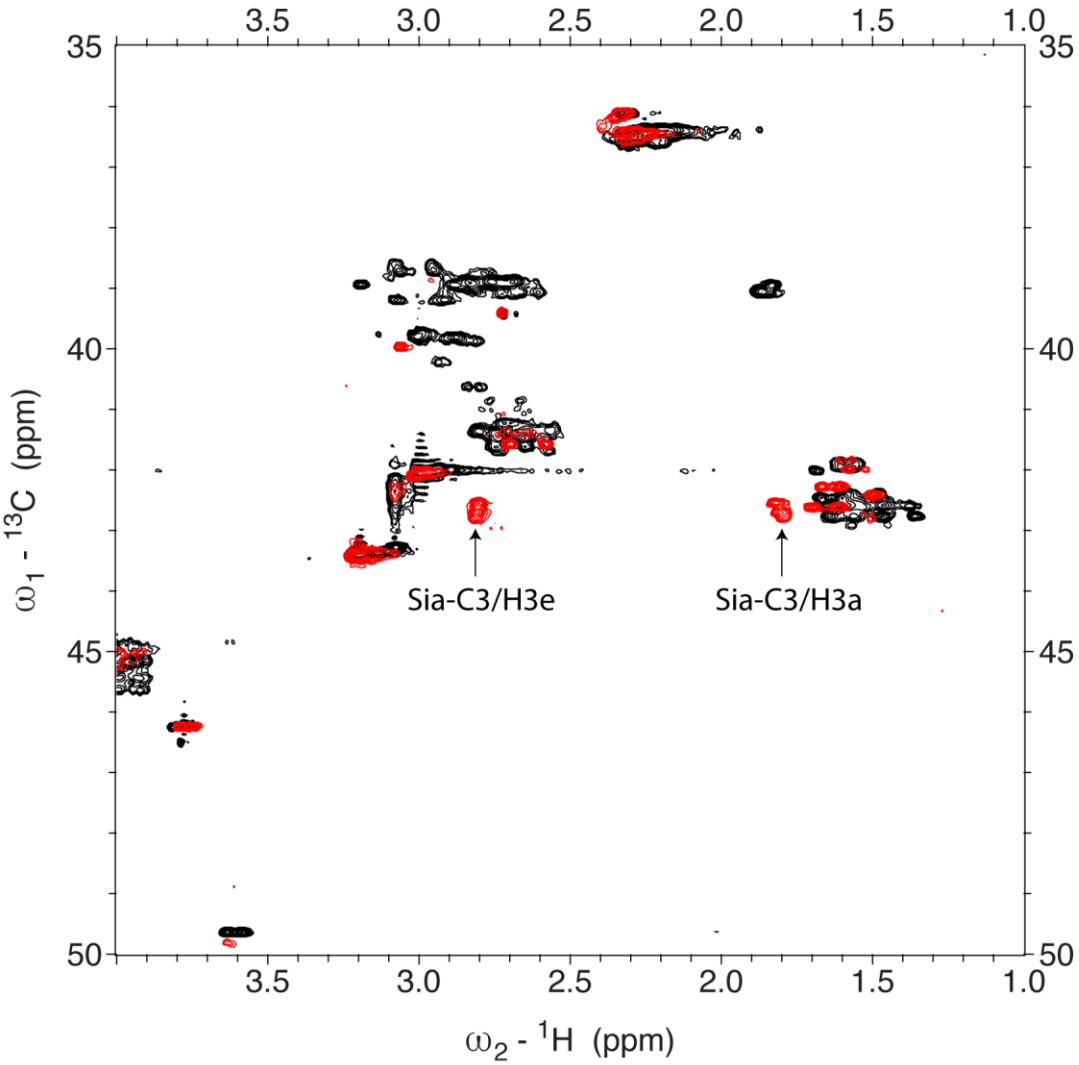

Figure S5. The overlay of ^{1}H-^{13}C HSQC spectra of Fc (black) and TNF receptor (red) domains of Etanercept. The signature sialic acid resonances C3/H3 in both axial (H3a) and equatorial (H3e) positions were identified in the TNF receptor domain spectrum only.

References:

(1) Xie, H.; Chakraborty, A.; Ahn, J.; Yu, Y. Q.; Dakshinamoorthy, D. P.; Gilar, M.; Chen, W.; Skilton, S. J.; Mazzeo, J. R. *MAbs* **2010**, *2*, 379-394.
(2) Chen, K.; Long, D. S.; Lute, S. C.; Levy, M. J.; Brorson, K. A.; Keire, D. A. *Journal of Pharmaceutical and Biomedical Analysis* **2016**, *128*, 398-407.
(3) Sinha, S.; Pipes, G.; Topp, E. M.; Bondarenko, P. V.; Treuheit, M. J.; Gadgil, H. S. *J Am Soc Mass Spectrom* **2008**, *19*, 1643-1654.
(4) Wang, B.; Gucinski, A. C.; Keire, D. A.; Buhse, L. F.; Boyne, M. T., 2nd. *Analyst* **2013**, *138*, 3058-3065.
(5) Melmer, M.; Stangler, T.; Schiefermeier, M.; Brunner, W.; Toll, H.; Rupprechter, A.; Lindner, W.; Premstaller, A. *Analytical and Bioanalytical Chemistry* **2010**, *398*, 905-914.
(6) Rogers, R. S.; Nightlinger, N. S.; Livingston, B.; Campbell, P.; Bailey, R.; Balland, A. *MAbs* **2015**, *7*, 881-890.
(7) Wang, T.; Chu, L.; Li, W. Z.; Lawson, K.; Apostol, I.; Eris, T. *Analytical Chemistry* **2017**, *89*, 3562-3567.
(8) Reusch, D.; Haberger, M.; Maier, B.; Maier, M.; Kloseck, R.; Zimmermann, B.; Hook, M.; Szabo, Z.; Tep, S.; Wegstein, J.; Alt, N.; Bulau, P.; Wuhrer, M. *Mabs* **2015**, *7*, 167-179.