Supporting Information

^{13}C-Sialic Acid Labeling of Glycans on Glycoproteins Using ST6Gal-I

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Synthesis of ^{13}C-[1,2,3,10,11]-CMP-β-NeuAc

^{13}C-CMP-NeuAc was made in a four-step synthesis process following published procedures. *N*-hydroxysuccinimide and ^{13}C-acetyl chloride were combined to produce *N*-^{13}C-[1,2]-acetoxy-succinimide, which was further reacted with D-mannosamine to make *N*-^{13}C[1,2]-acetyl-mannosamine (^{13}C-ManNAc).[1] The crude ^{13}C-ManNAc was purified by ion exchange column (Dowex 50, H^+ form) and flash chromatography (Iatrobeads, $CHCl_3$, CH_3OH and H_2O 65:35:5). The enzyme, NeuAc aldolase, was then used to react ^{13}C-ManNAc with ^{13}C-pyruvate to produce ^{13}C-[1,2,3,10,11]-NeuAc.[2] ^{13}C-[1,2,3,10,11]-CMP-β-NeuAc was then synthesized using the enzyme, CMP-NeuAc synthetase, and cytidine-5'-triphosphophate to produce the final product.[3] ^{13}C-CMP-NeuAc was further purified by size exclusion chromatography on Bio-Gel P2 column (extra fine, water, 4°C) before use. The product was characterized by ^{1}H NMR spectroscopy. ^{13}C-CMP-NeuAc was lyophilized and stored at -20 °C and has been observed to be stable under these conditions for up to two years.

^{13}C-CMP-NeuAc: ^{1}H NMR (500 MHz, D_2O): δ 7.96 (d, 1 H, J = 6.5 Hz, H6 cytosine), 6.12 (d, 1 H, J = 6.0 Hz, H5 cytosine), 5.99 (d, 1 H, J = 4.0 Hz, H1 ribose), 4.34 – 4.16 (m, 5 H, H3 ribose, H2 ribose, H5 ribose, H5 ribose, H4 ribose), 4.13 (d, 1H, J = 11.5 Hz, H6 NeuAc), 4.07 (m, 1H, H4 NeuAc), 3.96 – 3.88 (m, 2 H, H5 NeuAc, H8 NeuAc), 3.86 (dd, 1 H, J = 11.5 Hz, J = 4.0 Hz, H9 NeuAc), 3.62 (dd, 1 H, J = 11.5 Hz, J = 6.5 Hz, H9 NeuAc), 3.44 (d, 1 H, J = 10.0 Hz, H7 NeuAc), 2.62 (d, broad, 1H, J = 132.5 Hz, $H3_{eq}$ NeuAc), 2.15 (dd, 3H, J = 128.5 Hz, J = 6.0 Hz, NHCH_3), 1.74 (d, broad, 1H, J = 125.5 Hz, $H3_{ax}$ NeuAc).

Structural characterization of ST6Gal-1 glycans

Chemicals and Enzymes: Peptide *N*-glycosidase F and α-1,6-Mannosidase (Xanthomonas manihotis) were purchased from New England BioLabs. Trypsin, α-2,3-Sialidase (Jack beans) and β-*N*-acetylhexosaminidase (Jack beans) were obtained from Sigma. Other fine chemicals were from standard sources.

Composition analysis by GC-MS: The neutral and amino sugar composition of ST6Gal-I was analyzed by GC-MS. Methyl glycosides were prepared from a dried sample by methanolysis (mild acid treatment) in 1 M HCl in methanol at 80°C (18 h), followed by *N*-acetylation with pyridine and acetic anhydride in methanol for detection of amino sugars. The sample then was *O*-per-trimethylsilylated (TMS) with Tri-Sil (Pierce) at 80°C (0.5 h). Inositol (~5 μg) was used as an internal standard. These procedures were carried out as described previously.[4]

Release of N-linked glycans: An aliquot of the sample was dried in a Speed Vac (Savant SC 110) and re-dissolved in ammonium bicarbonate buffer (50 mM, pH 8.4) and heated at 100°C for 5 min to denature the glycoprotein prior to trypsin digestion (37°C, overnight). A second enzyme, peptide *N*-glycosidase F (New England BioLabs) was added to the tryptic digest and incubated at 37°C overnight to release the *N*-linked glycans. After enzymatic digestions, the sample was passed

through a C18 reversed phase cartridge. The carbohydrate fraction was eluted with 5% acetic acid and then was dried by lyophilization. A portion of *N*-linked glycans was permethylated and analyzed by MALDI-TOF-MS.

Cleavage of N-linked glycans by Exoglycosidases: A portion of the *N*-linked glycans was dissolved in 0.05 M sodium citrate with 0.1 M NaCl buffer at pH 6.0 and treated with α-2,3-Sialidase from Jack beans (Sigma) overnight at 37°C. The sample was dried in the speed vacuum centrifuge to change enzyme buffer. β-*N*-acetylhexosaminidase (pH 5.0) from Jack beans (Sigma) was added to the portion of sialidase digested sample and incubated for 5 days at 37°C in 0.1 M citrate phosphate buffer at pH 5.0. After enzyme digestion, the sample was dried and dissolved in 0.05 M sodium citrate buffer (pH 4.5). Another exoglycosidase, α-1,6-mannosidase from *Xanthomonas manihotis* (New England BioLabs) was added to the sialidase and β-*N*-acetylhexosaminidase digested glycans. After the enzymatic digestions, each portion of the samples was lyophilized and permethylated. The carbohydrates were analyzed by MALDI-TOF-MS to monitor the enzyme digestions.

Preparation of the O-permethylated carbohydrates: The lyophilized carbohydrate fraction was dissolved in dimethylsulfoxide and then methylated with NaOH and methyl iodide as described by K. Aumula, *et al*.[5]

Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI/TOF-MS): Profiling of *N*-linked glycans was performed initially using MALDI/TOF-MS (4700 Proteomics analyzer, Applied Biosystems). Permethylated glycans were crystallized on a MALDI plate with 2, 3-dihydroxybenzoic acid (DHBA, 20mg/mL solution in 50% methanol: water) as a matrix. All spectra were acquired in the reflector positive ion mode and averaged spectra of 50 laser shots with a 337 nm nitrogen laser**.**

Electrospray ionization – linear ion trap mass spectrometry (ESI-LCQ/MSn): The structure of the oligosaccharides detected by MALDI-TOF MS was confirmed by LCQ-ESI MS (Thermo Finnigan) spectrometry, in the positive ion mode. The permethylated glycans were dried, re-dissolved in 50% methanol containing 1 mM NaOH, and infused directly into the LCQ-MS instrument at a constant flow rate of 1μL/min via a syringe pump (Harvard Apparatus) and sprayed at 3.5 kV. The capillary temperature was set to 200^{o}C. A normalized collision energy of 35 and an isolation mass window of 2 Da were applied to obtain MSn described in detail by A. Lapadula, *et al*.[6]

Glycosyl composition Analysis: Glycosyl composition analysis of the released *N*-glycans from purified ST6Gal-1 glycoprotein by GC-MS showed fucose, galactose, mannose, *N*-acetylglucosamine, *N*-acetylneuraminic acid, and *N*-acetylgalactosamine. The glycosyl composition was performed by GC-MS in order to unambiguously determine if *N*-acetylgalactosamine was present in addition to the *N*-acetylglucosamine that was expected on the N-glycans.

MALDI-MS and ESI-MS/MS analysis of permethylated released oligosaccharides: The released and permethylated *N*-glycans from ST6Gal-1 were also analyzed by MALDI-MS (Figure 1A). More than ten oligosaccharides were detected in the spectrum, which consisted of various fucosylated and/or sialylated complex type biantennary and minor triantennary *N*-glycan structures. The structure of major glycans after sialidase digestion was confirmed by performing tandem mass spectrometry using ESI-MS/MS on the molecular ions and a series of exoglycosidase digestions. The main ions observed were m/z 3007.6 and 2792.5, which corresponded to compositions of biantennary structures $Hex_4HexNAc_5NeuAc_2Fuc_1$ and $Hex_5HexNAc_4NeuAc_2$, respectively. The structure of the ion m/z 2792.5 was confirmed to be a non-fucosylated disialylated biantennary glycan by exoglycosidase digestions and subsequent ESI-MS/MS analysis of exoglycosidase products (see Table 1 and Figure 1). For the ion m/z 3007.6, the presence of *N*-acetylgalactosamine in the glycosyl composition data by GC-MS and the $Hex_4HexNAc_5NeuAc_2Fuc_1$ composition with five hexosamine residues in the MALDI-MS suggested a possible sialyl LacdiNAc sequence (Sia -GalNAcß1-4GlcNAc-R) at the non-reducing end. Figure 2 shows the ESI-MS/MS data of the parent ion, m/z 1154 (D.C. of m/z 2285) from the released *N*-glycans after α 2-3 Sialidase digestion. The presence of the fragment ion m/z 527.2 showing a $HexNAc_2^+$ ion and the y and b fragment ions from the m/z 1154 ion confirmed the presence of the $HexNAc_2$ residue at one of the biantennary arms. The presence of sialyl LacdiNac (Sia2-6GalNAcβ1-4GlcNAcβ1-R) has previously been described by T. Ohkura *et al*.[7]

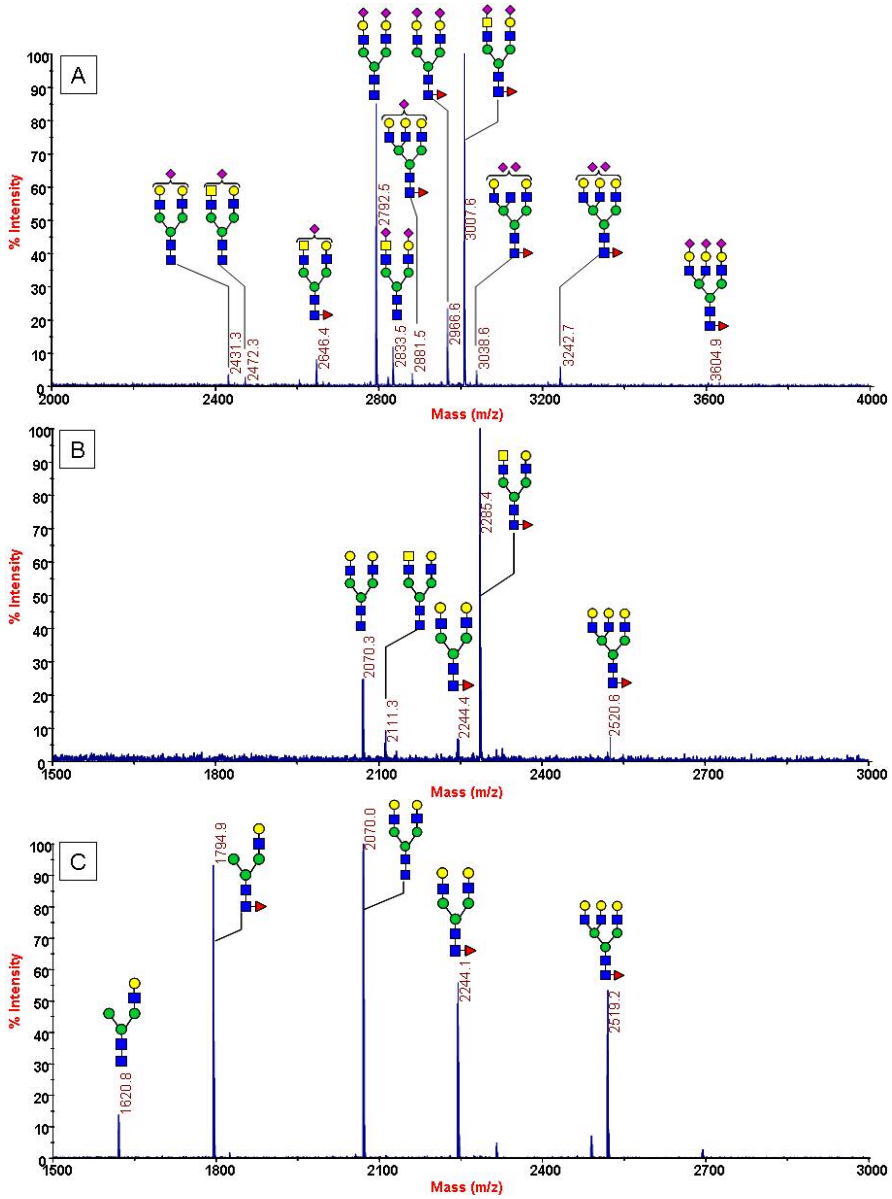
Exoglycosidase digestions: In order to confirm the glycan structures and determine the position of the LacdiNAc sequences (GalNAcß1-4GlcNAc) on the main glycan, the released *N*-glycans from ST6Gal-1 were subjected to a series of exoglycosidase digestions. Figure 1B shows the MALDI-MS spectrum of permethylated released *N*-glycans after α 2-3 sialidase treatment. The spectrum is greatly simplified compared to the intact glycan profile. The major ion m/z 2285 confirms loss of two sialic acids from the original ion m/z 3007 and corresponds to the $Hex_4HexNAc_5Fuc_1$ structure.

The MALDI-MS spectrum of the released *N*-glycans after α 2-3 sialidase followed by β-*N*-hexosaminidase digestion is shown in Figure 1C. The β-*N*-hexosaminidase digestion, which should only affect the oligosaccharides containing the

GalNAc-GlcNAc structures, resulted in ions m/z 1621 and 1795 as products after removal of GalNAc-GlcNAc from their respective original ions, m/z 2111 and 2285. As expected, the ions m/z 2070, 2244, and 2519, which contained galactose at the non-reducing end, stayed unchanged after the β-*N*-hexosaminidase digestion.

In order to determine which arm the GalNAcß1-4GlcNAc moiety was attached in the biantennary structure, the product of the β-*N*-hexosaminidase digestion was subjected to α 1-6 mannosidase digestion. If the GalNAc-GlcNAc was originally attached through the 6-linked mannose then removal of $HexNAc_2$ after β-*N*-hexosaminidase and subsequent digestion with α 1-6 mannosidase should result in loss of one mannose residue and detection of the ions m/z 1417 and 1591. Figure 1D shows the MALDI-MS spectrum of the product of the above exoglycosidases after α 1-6 mannosidase digestion. The lack of the ions at m/z 1417 and 1591 suggests that the GalNAc-GlcNAc is probably not on the α 1,6 arm and is possibly attached through the α1, 3- mannose residue. However, more experiments with will be needed to confirm this.

Conclusion: In conclusion, the major *N*-glycan structure on the ST6Gal-1 glycoprotein was determined to be a fucosylated biantennary oligosaccharide with a Sia2-3/6GalNAcβ1-4GlcNAc attached through the α1, 3-mannose residue. The other main *N*-glycan structure was a disialylated biantennary glycan.



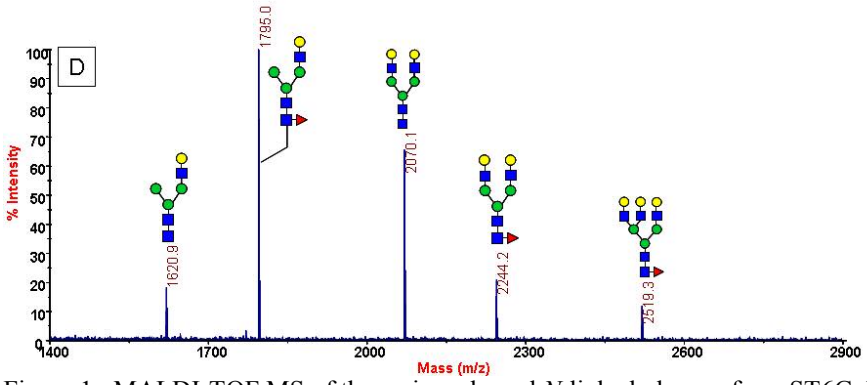


Figure 1. MALDI-TOF MS of the major released *N*-linked glycans from ST6Gal-l and of the products of exoglycosidase digests. (A) Intact glycans released from ST6Gal-l with PNGase F were permethylated and analyzed. Structural assignments are based on fragmentation (MS/MS) by ESI-LCQ (data are not presented). (B) Incubation with α 2-3 sialidase produced intact biantennary structures that were devoid of sialic acids. (C) The full MALDI-MS spectrum of ST6Gal-l permethylated *N*-liked glycans after α 2-3 sialidase and β-*N*-hexosaminidase digestions. (D) The full MALDI-MS spectrum of ST6Gal-l permethylated *N*-liked glycans after α 2-3 sialidase, β-*N*-hexosaminidase, and α 1-6 mannosidase digestions.

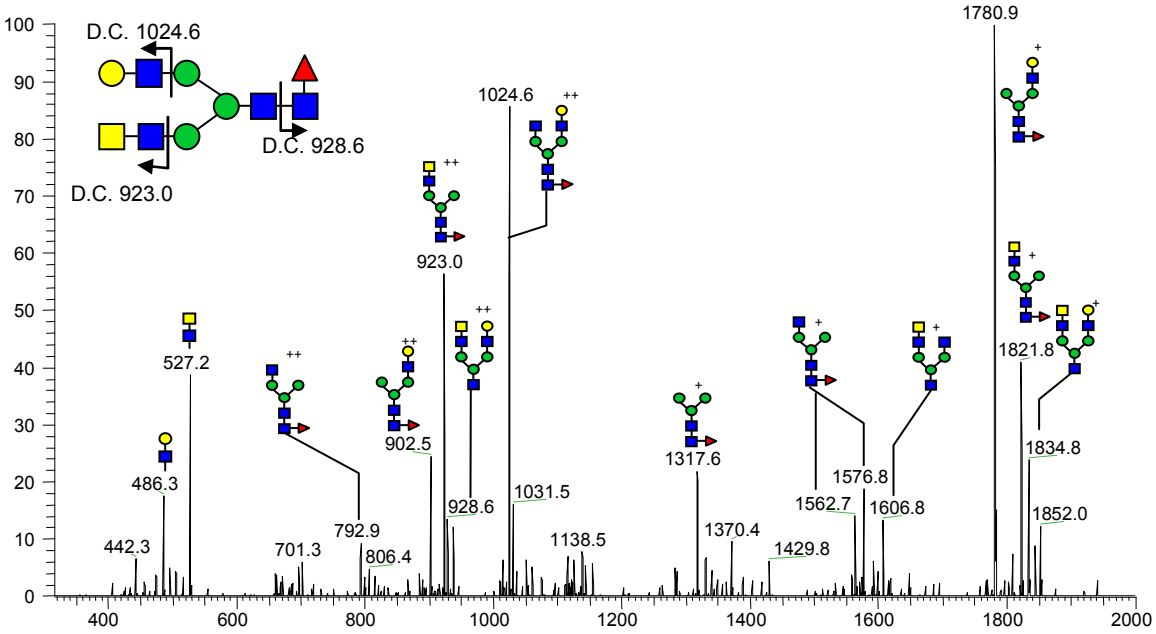
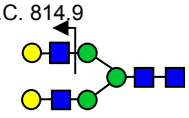
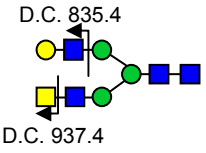
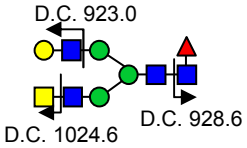
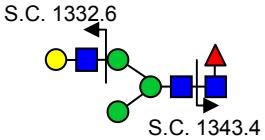
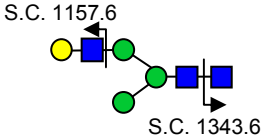


Figure 2. The ESI-MS/MS spectrum of permethylated *N*-glycans from ST6Gal-I parent ion, m/z 1154 (doubly charged of m/z 2285) after α 2-3 Sialidase treatment.

Table 1. The molecular ions observed by MALDI-MS of the released and permethylated *N*-glycans after α-2,3-sialidase digestions and the subsequent ESI-MS/MS fragments detected.

Observed Mass		Composition	Proposed structure
Single Charge	Double Charge		
2070.3	1046.7	$HEX_5HEXNAC_4$	D.C. 814.9 

2111.3	1067.5	$HEX_4HEXNAC_5$	
2285.4	1154.1	$HEX_4DHEX_1HEXNAC_5$	
1794.9	909.2	$HEX_4DHEX_1HEXNAC_3$	
1620.8	822.2	$HEX_4HEXNAC_3$	

Chemical shifts for observed NeuAc species

Compound	H_{3eq} (ppm)	H_{3ax} (ppm)	C_3 (ppm)
α-NeuAc	2.73	1.63	43.4
β-NeuAc	2.21	1.84	42.1
CMP-β-NeuAc	2.49	1.66	43.9
α-NeuAc bound to ST6Gal-I	2.68	1.72	42.8

Conditions: 10 mM potassium phosphate, 200 mM NaCl, pH 6.5, D_2O, 37ºC

References:

(1) Heidlas, J. E.; Lees, W. J.; Pale, P.; Whitesides, G. M. *J. Org. Chem*., **1992**, *57*, 146-151.
(2) Aubin Y. ; Prestegard J. H. *Biochem*. **1993**, *32*, 3422-3428.
(3) Weinstein, J.; Lee, E. U.; McEntee, K.; Lai, P. H.; Paulson, J. C. *J. Biol. Chem*. **1987**, *262*, 17735–17743.
(4) Merkle, R. K.; Poppe, I. *Methods in Enzymology* **1994**, *230*, 1-15.
(5) Anumula, K. R.; Taylor, P. B. *Anal. Biochem*., **1992**, *203*, 101-108.
(6) Lapadula, A. J.; Hatcher, P. J.; Hanneman, A. J.; Ashline D. J.; Zhang, H.; Reinhold V. N. *Anal. Chem*. **2005**, *77*, 6271-6279.
(7) Ohkura, T.; Seko, A.; Hara-Kuge, S.; Yamashita, K. *J. Biochem*. **2002**, *132*, 891-901.