JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Two-Step Chemoenzymatic Detection of *N*-Acetylneuraminic Acid–α(2-3)-Galactose Glycans

Liuqing Wen,[†,‡] Yuan Zheng,[†,‡] Kuan Jiang,[†,§,‡] Mingzhen Zhang,[#] Shukkoor Muhammed Kondengaden,[†] Shanshan Li,[†] Kenneth Huang,[†] Jing Li,[†,§] Jing Song,[†] and Peng George Wang*[,†,§]

[§]State Key Laboratory of Medicinal Chemical Biology and College of Pharmacy, Nankai University, Tianjin 300071, China
[†]Department of Chemistry and [#]Institute for Biomedical Sciences, Georgia State University, Atlanta, Georgia 30303, United States

Supporting Information

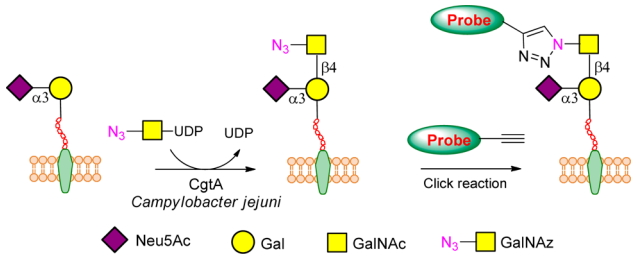
ABSTRACT: Sialic acids are typically linked α(2-3) or α(2-6) to the galactose that located at the non-reducing terminal end of glycans, playing important but distinct roles in a variety of biological and pathological processes. However, details about their respective roles are still largely unknown due to the lack of an effective analytical technique. Herein, a two-step chemoenzymatic approach for the rapid and sensitive detection of *N*-acetylneuraminic acid–α(2-3)-galactose glycans is described.

N-Acetylneuraminic acid (Neu5Ac) is the most widespread form of sialic acid and almost the only form found in humans.[1] *N*-Glycolylneuraminic acid (Neu5Gc) and ketodeoxynonulosonic acid (Kdn) are common in other vertebrates but rarely present in humans.[1] Neu5Ac is present in essentially all human tissues and always attaches to the galactose residue at the non-reducing terminal end of glycans through α(2-3) or α(2-6) linkage.[2] It is well established that Neu5Acα(2-3)Gal and Neu5Acα(2-6)Gal glycans play crucial but distinctive roles in diverse biological and pathological processes, including immune responses and cell–cell and cell–pathogen interactions.[3] However, studies are hindered due to the lack of an effective method to analyze such glycans or glycoproteins.

Lectin binding has been the primary method to analyze sialylated glycans,[4] but lectins often suffer from weak binding affinity, limited specificity, and cross-reactivity. In recent years, the development of bioorthogonal chemistry has provided a powerful tool for probing glycans, proteins, and lipids.[5] Bioorthogonal functional groups (azide and alkyne) carried by *N*-acetylmannosamine, or Neu5Ac analogues, were metabolically incorporated into glycans, allowing covalent conjugation by click chemistry reaction with either fluorescent tags for visualization or affinity probes for enrichment of sialylated glycans and glycoproteins.[6] A chemical approach to tag sialylated glycans has also been suggested.[7] Nevertheless, these methods suffer from low detection sensitivity and efficiency, toxicity of labeling regents, and the inability to detect complicated glycan structures.

As a complementary strategy to remodel glycans with non-natural functionalities, chemoenzymatic labeling of glycans relies on the substrate-specific glycosyltransferases, which transfer non-natural sugars that contain bioorthogonal functional groups onto target glycan *in vitro*.[8] Chemoenzymatic method does not rely on a cell's biosynthetic machinery and therefore can be employed in any desired biological contexts where feeding cells with non-natural sugar analogues is not possible, such as human tissue extracts.[8b] As the glycosyltransferase-mediated reaction and click chemistry reaction both proceed with high specificity and efficiency, chemoenzymatic labeling provides a higher sensitivity and selectivity compared to other analytical methods using, for instance, antibodies, lectins, and metabolic labeling. In this Communication, we report a two-step chemoenzymatic method that takes advantage of the substrate promiscuity of a β-(1,4)-*N*-acetylgalactosaminyltransferase from *Campylobacter jejuni* (CgtA) and click chemistry reaction to rapidly and sensitively detect Neu5Acα(2-3)Gal glycans (Scheme 1).

Scheme 1. Two-Step Chemoenzymatic Detection of Neu5Acα(2-3)Gal Glycans by CgtA with UDP-GalNAz



The Neu5Acα(2-3)Gal epitope localized on a cell surface is well known to be the receptor of many infectious microbes, such as the influenza virus.[2] Abnormal Neu5Acα(2-3)Gal expression has frequently been observed in many carcinomas.[3b] Traditionally, lectin binding using *Maackia amurensis* leukoagglutinin (MAL I) and hemagglutinin (MAH or MAL II) is the main method for Neu5Acα(2-3)Gal detection. However, MAL I only binds terminal Neu5Acα(2-3)Galβ(1-4)GlcNAc trisaccharide in *N*-glycans.[9] MAH binds preferentially trisaccharide Neu5Acα(2-3)Galβ(1-3)GalNAc in *O*-glycans.[10] They also bind some non-sialylated structures such as SO_4^{3-}-Galβ(1-3)GalNAc.[11] Moreover, it was reported that *M. amurensis* lectins require a high minimum agglutinating concentration (up to 125–500 μg),[12] and therefore a long incubation time (typically overnight) is necessary for glycoprotein detection.[13]

Received: July 10, 2016
Published: August 23, 2016

DOI: 10.1021/jacs.6b07132
J. Am. Chem. Soc. 2016, 138, 11473–11476

Thus, a simple, rapid, and sensitive method for detecting Neu5Acα(2-3)Gal glycans remains an unmet need.

Neu5Acα(2-3)Gal disaccharide is the outer core component of many *C. jejuni* strains.[14] CgtA is responsible for the extension of Neu5Acα(2-3)Gal with GalNAc residue (Figure 1).[14] We

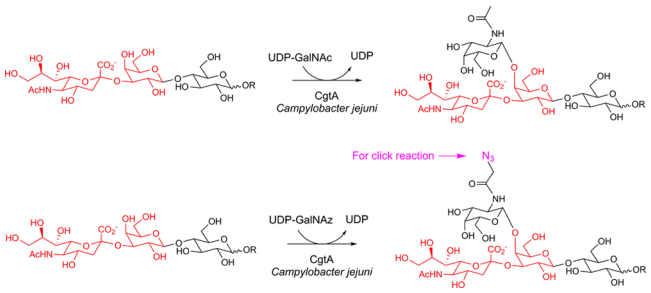
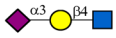
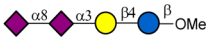
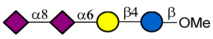
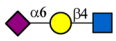
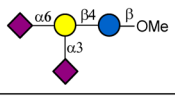


Figure 1. CgtA recognizes Neu5Acα(2-3)Gal with UDP-GalNAc or UDP-GalNAz.

reasoned that CgtA might tolerate substitution at the C-2 position of GalNAc, allowing for the introduction of an azide group for further click chemistry reaction (Figure 1). Substrate specificity study using Ganglio-oligosaccharide GM3 (Table 1, entry 1) shows that both UDP-GalNAz and UDP-GalNAc are efficient substrates of CgtA (Figure S1). Kinetic analysis revealed a k_{cat}/K_m value of 1.34 nM^{-1} min^{-1} for UDP-GalNAc, and 1.51 nM^{-1} min^{-1} for UDP-GalNAz. Indeed, treatment of GM3 with CgtA and UDP-GalNAz overnight led to a complete

Table 1. Substrate Specificity of CgtA with UDP-GalNAz toward Sialylated Oligosaccharides

Entry	Substrate	RA (%)[a]
1		100
2		104.3
3		100.6
4		113.4
5		96.5
6		0.08
7		0.28
8		0.31
9		0.41
10		0.25

[a]RA: relative activity. See SI for experimental details. Glc GlcNAc

conversion of GM3. The product was confirmed by MALDI-TOF-MS and NMR (see Supporting Information (SI)).

Having demonstrated that CgtA accepts UDP-GalNAz as substrate, we next tested its substrate specificity toward sialylated oligosaccharides with UDP-GalNAz. Many sialylated oligosaccharides containing α(2-3)-, α(2-6)-, or α(2-8)-linked sialic acid (Table 1 and Table S1) were synthesized using the methods reported previously.[15] We found that CgtA requires only the linear disaccharide structure of Neu5Acα(2-3)Gal (Table 1, entries 1−5) or Neu5Gcα(2-3)Gal (Table S1, entry 2) when UDP-GalNAz is used as donor. Meanwhile, only very low relative activity was detected toward the structure containing α(2-6)-linked sialic acid or without sialic acid (Table 1, entries 6−10; Table S1, entries 3−5). Indeed, no observable product or byproduct (UDP) could be found by thin-layer chromatography after the incubation of these compounds with CgtA and UDP-GalNAz overnight. These findings indicate the potential of CgtA for application in selective labeling of Neu5Acα(2-3)Gal glycans.

To test the practicality of the described strategy for protein labeling, we used fetal bovine fetuin as an example. Fetal bovine fetuin is a well-studied model protein for sialylated glycans analysis and is commercially available. It contains three *N*-glycosylation and three *O*-glycosylation sites, on which Neu5Ac can attach to the terminal galactose residues through α(2-3) or α(2-6) linkage.[16] As a control, fetal bovine fetuin was treated with a sialidase (NanC), which specifically hydrolyzes α(2-3)-linked Neu5Ac.[17] After treatment with NanC, a slight migration change compare to native feutin was observed on SDS-PAGE gel (Figure 2A). The α(2-6)-linked Neu5Ac was confirmed by biotinylated *Sambucus nigra* agglutinin (B-SNA) (Figure 2A). Native fetuin or NanC-treated fetuin was labeled by CgtA with UDP-GalNAz at 37 °C for 1 h, while other control studies were performed in parallel. Following copper-free click reaction (DIBO-alkyne, 10 μM), the proteins were analyzed by Western blot using streptavidin-linked horseradish peroxidase (S-HRP). Strong fluorescence was observed in the labeling group, while all the control groups failed to be labeled (Figure 2A), demonstrating that the designed scheme could be used to specifically label Neu5Acα(2-3)Gal glycans on glycoprotein. The labeled fetuin was further treated with peptide *N*-glycosidase F (PNGF), which removes *N*-glycans from glycoproteins, and detected by S-HRP. Fluorescence labeling could still be observed in the PNGF-treated sample (Figure 2A, bottom graph), indicating that both *N*-glycans and *O*-glycans were labeled. Meanwhile, the probe of same amount of fetuin with biotinylated MAL II was unsuccessful. Thus, our chemoenzymatic approach provides a more credible detection strategy for Neu5Acα(2-3)Gal glycans and enables the highly sensitive detection of glycoproteins.

We next determined whether the approach could be used to track Neu5Acα(2-3)Gal glycoproteins in complex samples. Cell lysates from human embryonic kidney 293 (HEK293T) cells was incubated with CgtA and UDP-GalNAz at 37 °C for 1 h, while control groups were studied in parallel. Following the Cu(I)-catalyzed azide−alkyne cycloaddition (CuAAC) reaction with diazo-biotin-alkyne, biotinylated cell lysates were detected by Western blot using S-HRP (Figure 2B). Strong fluorescence labeling was observed in cell lysates, while only background level of nonspecific labeling was observed in the NanC-treated group and other control groups (Figure 2B), further highlighting the specificity of the designed strategy toward Neu5Acα(2-3)Gal glycans. Cell surface Neu5Acα(2-3)Gal

DOI: 10.1021/jacs.6b07132
J. Am. Chem. Soc. 2016, 138, 11473−11476

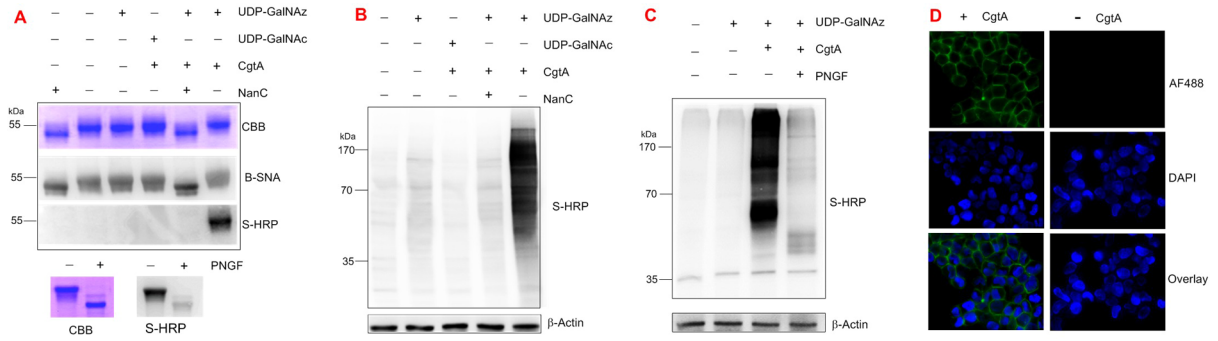


Figure 2. (A) Chemoenzymatic detection of Neu5Acα(2-3)Gal glycans on fetal bovine fetuin. Abbreviations: CBB, Coomassie brilliant blue staining; S-HRP, streptavidin-linked horseradish peroxidase; B-SNA, biotinylated *Sambucus nigra* agglutinin; NanC, sample was treated with NanC sialidase before labeling reaction; PNGF, the labeled fetuin was further treated with peptide *N*-glycosidase F (bottom graph). (B) Chemoenzymatic detection of Neu5Acα(2-3)Gal glycoproteins from HEK293T cell lysates. (C) Chemoenzymatic detection of Neu5Acα(2-3)Gal glycoproteins on cell surface of HEK 293T. (D) Imaging of cell surface Neu5Acα(2-3)Gal glycans on live HeLa cells (green) using fluorescence microscopy. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). See SI for experimental details.

glycans were selectively labeled by incubating a suspension of HEK293T living cells with CgtA and UDP-GalNAz at 37 °C for 30 min. Following biotinylation by the CuAAC reaction, strong fluorescence labeling was observed compared to control groups in Western blot (Figure 2C). Several other randomly selected human cancer cell lines, including A549, HeLa, and HepG2, were then chemoenzymatically labeled using the same strategy (see SI). The labeled samples were further treated with PNGF, resulting in significant fluorescence reduction, indicating that Neu5Acα(2-3)Gal mainly attached to *N*-glycans in these cell lines.

We next investigated the potential application of the described strategy for imaging and quantification of Neu5Acα(2-3)Gal glycans. The determination of the expression level of Neu5Acα(2-3)Gal glycans is very important to understand sialic-acid-related microbe infection and carcinogenesis.[18] Adherent HeLa cells were labeled by CgtA and UDP-GalNAz at 37 °C for 30 min. After copper-free click reaction (DIBO-biotin, 30 μM), a fluorescent reporter was subsequently installed by incubation with streptavidin-linked Alexa Fluor 488 (10 μg/mL). Membrane-associated fluorescence was observed for cells treated with both CgtA and UDP-GalNAz, whereas no fluorescence labeling was detected for control cells labeled in the absence of CgtA, confirming the specificity of the *in situ* chemoenzymatic reaction (Figure 2D). The fluorescence intensity, which reflects the expression level of Neu5Acα(2-3)Gal glycans proportionally, was determined by flow cytometry (Figure S9).

Finally, Neu5Acα(2-3)Gal sialoglycoproteins that exist on the cell surface of HEK 293T were globally identified using the described strategy (Figure 3A). It is well established that cell surface glycoproteins containing Neu5Acα(2-3)Gal glycans play important roles in living cells, where these proteins are the potential therapeutic targets. However, there has been relatively little study profiling such sialoglycoproteins due to the lack of an effective enrichment method. Although lectin affinity chromatography has been explored to enrich sialoglycoproteins,[19] this method was limited by the weak binding affinity and limited specificity of lectins. Alternatively, avidin–biotin complex is the strongest known non-covalent interaction between a protein and a ligand (K_d = 10^{-14}–10^{-15} M), making avidin–biotin binding an ideal system for protein enrichment.[20] In this work, the biotinylated cell surface proteins of HEK293T were captured using streptavidin agarose resin. After digestion

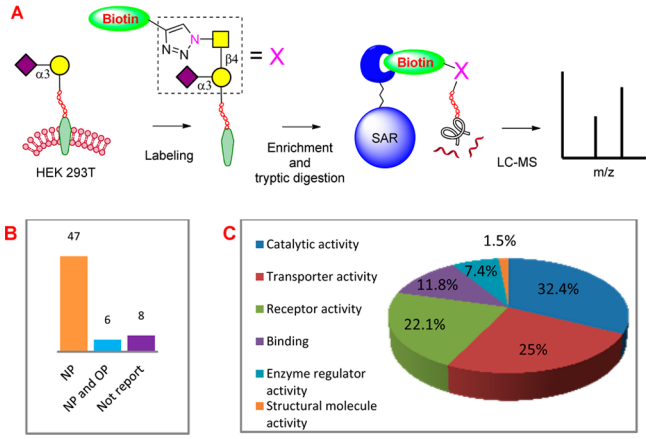


Figure 3. (A) Global identification of cell surface Neu5Acα(2-3)Gal glycoproteins from HEK293T. (B) In total, 61 proteins were probed. (C) Molecular function of the identified sialoglycoproteins. Abbreviations: SAR, streptavidin agarose resin; NP, *N*-glycosylation protein; OP, *O*-glycosylation protein.

with trypsin, the peptide fragments were analyzed by LS-MS. After the non-specific binding proteins in the control group were filtered, 61 proteins were identified (Table S2). A total of 53 of the probed proteins are the reported glycoproteins (Figure 3B), highlighting the feasibility of the described strategy. Among of the reported glycoproteins, 47 proteins contain *N*-glycans and 6 proteins contain both *N*- and *O*-glycans. These data are also well in accord with the Western blot observation (Figure 2C). Molecular function analysis using the Protein ANalysis THrough Evolutionary Relationships (PANTHER) Classification System[21] is displayed in Figure 3C. Four main categories, including catalytic activity (32.4%), transporter activity (25%), receptor activity (22.1%), and binding (11.8%), take up more than 90% of the total probed proteins. These are consistent with the well-known functions of cell surface sialoglycoproteins.[6f]

In conclusion, on the basis of a glycosyltransferase that could specifically recognize Neu5Acα(2-3)Gal with UDP-GalNAz and site-specific click chemistry reaction, we have developed the first strategy for the rapid and sensitive detection of Neu5Acα(2-3)Gal glycans. This method is far superior to the traditional lectin-based methods to detect Neu5Acα(2-3)Gal, which are limited by their inherent disadvantages. This method

DOI: 10.1021/jacs.6b07132
J. Am. Chem. Soc. 2016, 138, 11473–11476

also shows that the global analysis of Neu5Acα(2-3)Gal glycoproteins is achievable, providing a powerful tool for sialic-acid-related research. Moreover, substrate specificity study indicated that the described strategy can be also used to probe Neu5Gcα(2-3)Gal glycans, which are currently detected by polyclonal monospecific antibody.[2] Future studies will enable the exploration of new glycosyltransferases for use in further glycan detection.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b07132.

Materials, experimental methods, and supporting figures and tables (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*pwang11@gsu.edu

Author Contributions

‡These authors contribute equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to Dr. Xi Chen from University of California—Davis for generously providing us with CgtA plasmid. This work was supported by NIH grants (R01 GM085267 and R01 AI083754) and the National Natural Science Foundation of China (Grant 21372130).

■ REFERENCES

(1) Tangvoranuntakul, P.; Gagneux, P.; Diaz, S.; Bardor, M.; Varki, N.; Varki, A.; Muchmore, E. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 12045.

(2) Varki, N. M.; Varki, A. *Lab. Invest.* **2007**, *87*, 851.

(3) (a) Lou, Y. W.; Wang, P. Y.; Yeh, S. C.; Chuang, P. K.; Li, S. T.; Wu, C. Y.; Khoo, K. H.; Hsiao, M.; Hsu, T. L.; Wong, C. H. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 2482. (b) Varki, A. *Trends Mol. Med.* **2008**, *14*, 351. (c) Wang, B.; Brand-Miller, J. *Eur. J. Clin. Nutr.* **2003**, *57*, 1351. (d) Crocker, P. R.; Paulson, J. C.; Varki, A. *Nat. Rev. Immunol.* **2007**, *7*, 255.

(4) (a) Singh, H.; Sarathi, S. P. *Int. J. Sci. Eng. Res.* **2012**, *3*, 1. (b) Ghazarian, H.; Idoni, B.; Oppenheimer, S. B. *Acta Histochem.* **2011**, *113*, 236. (c) Blixt, O.; Han, S.; Liao, L.; Zeng, Y.; Hoffmann, J.; Futakawa, S.; Paulson, J. C. *J. Am. Chem. Soc.* **2008**, *130*, 6680.

(5) (a) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007. (b) Prescher, J. A.; Bertozzi, C. R. *Nat. Chem. Biol.* **2005**, *1*, 13. (c) Sletten, E. M.; Bertozzi, C. R. *Angew. Chem., Int. Ed.* **2009**, *48*, 6974. (d) Lu, L.; Gao, J.; Guo, Z. *Angew. Chem., Int. Ed.* **2015**, *54*, 9679.

(6) (a) Feng, L. S.; Hong, S. L.; Rong, J.; You, Q. C.; Dai, P.; Huang, R. B.; Tan, Y. H.; Hong, W. Y.; Xie, C.; Zhao, J.; Chen, X. *J. Am. Chem. Soc.* **2013**, *135*, 9244. (b) Chang, P. V.; Chen, X.; Smyrniotis, C.; Xenakis, A.; Hu, T. S.; Bertozzi, C. R.; Wu, P. *Angew. Chem., Int. Ed.* **2009**, *48*, 4030. (c) Hsu, T. L.; Hanson, S. R.; Kishikawa, K.; Wang, S. K.; Sawa, M.; Wong, C. H. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 2614. (d) Laughlin, S. T.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 12. (e) Xie, R.; Hong, S.; Feng, L.; Rong, J.; Chen, X. *J. Am. Chem. Soc.* **2012**, *134*, 9914. (f) Chen, W. X.; Smeekens, J. M.; Wu, R. H. *Chem. Sci.* **2015**, *6*, 4681.

(7) Zeng, Y.; Ramya, T. N.; Dirksen, A.; Dawson, P. E.; Paulson, J. C. *Nat. Methods* **2009**, *6*, 207.

(8) (a) Khidekel, N.; Arndt, S.; Lamarre-Vincent, N.; Lippert, A.; Poulin-Kerstien, K. G.; Ramakrishnan, B.; Qasba, P. K.; Hsieh-Wilson, L. C. *J. Am. Chem. Soc.* **2003**, *125*, 16162. (b) Chaubard, J. L.; Krishnamurthy, C.; Yi, W.; Smith, D. F.; Hsieh-Wilson, L. C. *J. Am. Chem. Soc.* **2012**, *134*, 4489. (c) Zheng, T. Q.; Jiang, H.; Gros, M.; del Amo, D. S.; Sundaram, S.; Lauvau, G.; Marlow, F.; Liu, Y.; Stanley, P.; Wu, P. *Angew. Chem., Int. Ed.* **2011**, *50*, 4113. (d) Li, Q.; Li, Z.; Duan, X.; Yi, W. *J. Am. Chem. Soc.* **2014**, *136*, 12536. (e) Mbua, N. E.; Li, X.; Flanagan-Steet, H. R.; Meng, L.; Aoki, K.; Moremen, K. W.; Wolfert, M. A.; Steet, R.; Boons, G. J. *Angew. Chem., Int. Ed.* **2013**, *52*, 13012.

(9) (a) Knibbs, R. N.; Goldstein, I. J.; Ratcliffe, R. M.; Shibuya, N. *J. Biol. Chem.* **1991**, *266*, 83. (b) Wang, W. C.; Cummings, R. D. *J. Biol. Chem.* **1988**, *263*, 4576.

(10) Konami, Y.; Yamamoto, K.; Osawa, T.; Irimura, T. *FEBS Lett.* **1994**, *342*, 334.

(11) Sata, T.; Roth, J.; Zuber, C.; Stamm, B.; Heitz, P. U. *Am. J. Pathol.* **1991**, *139*, 1435.

(12) Wearne, K. A.; Winter, H. C.; O'Shea, K.; Goldstein, I. J. *Glycobiology* **2006**, *16*, 981.

(13) (a) Geisler, C.; Jarvis, D. L. *Glycobiology* **2011**, *21*, 988. (b) Machado, E.; Kandzia, S.; Carilho, R.; Altevogt, P.; Conradt, H. S.; Costa, J. *Glycobiology* **2011**, *21*, 376.

(14) Gilbert, M.; Karwaski, M. F.; Bernatchez, S.; Young, N. M.; Taboada, E.; Michniewicz, J.; Cunningham, A. M.; Wakarchuk, W. W. *J. Biol. Chem.* **2002**, *277*, 327.

(15) (a) Yu, H.; Chen, X. *Org. Biomol. Chem.* **2016**, *14*, 2809. (b) Wen, L.; Huang, K.; Liu, Y.; Wang, P. G. *ACS Catal.* **2016**, *6*, 1649. (c) Wen, L.; Huang, K.; Wei, M.; Meisner, J.; Liu, Y.; Garner, K.; Zang, L.; Wang, X.; Li, X.; Fang, J.; Zhang, H.; Wang, P. G. *Angew. Chem., Int. Ed.* **2015**, *54*, 12654. (d) Yu, H.; Chokhawala, H. A.; Huang, S.; Chen, X. *Nat. Protoc.* **2006**, *1*, 2485.

(16) Palmisano, G.; Larsen, M. R.; Packer, N. H.; Thaysen-Andersen, M. *RSC Adv.* **2013**, *3*, 22706.

(17) Xu, G.; Kiefel, M. J.; Wilson, J. C.; Andrew, P. W.; Oggioni, M. R.; Taylor, G. L. *J. Am. Chem. Soc.* **2011**, *133*, 1718.

(18) (a) Wu, Z.; Miller, E.; Agbandje-McKenna, M.; Samulski, R. J. *J. virol.* **2006**, *80*, 9093. (b) Nefkens, I.; Garcia, J. M.; Ling, C. S.; Lagarde, N.; Nicholls, J.; Tang, D. J.; Peiris, M.; Buchy, P.; Altmeyer, R. *J. Clin. Virol.* **2007**, *39*, 27.

(19) Zhao, J.; Simeone, D. M.; Heidt, D.; Anderson, M. A.; Lubman, D. M. *J. Proteome Res.* **2006**, *5*, 1792.

(20) Dundas, C. M.; Demonte, D.; Park, S. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 9343.

(21) Thomas, P. D.; Campbell, M. J.; Kejariwal, A.; Mi, H.; Karlak, B.; Daverman, R.; Diemer, K.; Muruganujan, A.; Narechania, A. *Genome Res.* **2003**, *13*, 2129.