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

Regioselective Chemoenzymatic Synthesis of Ganglioside Disialyl Tetrasaccharide Epitopes

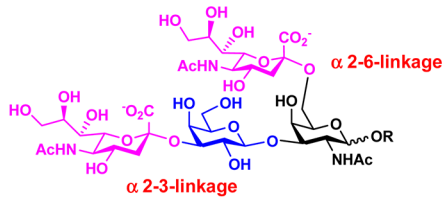
Xin Meng,[†] Wenlong Yao,[†] Jiansong Cheng,[‡] Xu Zhang,[†] Lan Jin,[†] Hai Yu,[§] Xi Chen,[§] Fengshan Wang,*[,†,∥] and Hongzhi Cao*[,†]

[†]National Glycoengineering Research Center, School of Pharmaceutical Science, Shandong University, Jinan 250012, China
[‡]College of Pharmacy, Nankai University, Tianjin 300071, China
[§]Department of Chemistry, University of California, Davis, California 95616, United States
[∥]Key Laboratory of Chemical Biology (Ministry of Education), Shandong University, Jinan 250012, China

Supporting Information

ABSTRACT: A novel chemoenzymatic approach for the synthesis of disialyl tetrasaccharide epitopes found as the terminal oligosaccharides of GD1α, GT1aα, and GQ1bα is described. It relies on chemical manipulation of enzymatically generated trisaccharides as conformationally constrained acceptors for regioselective enzymatic α2−6-sialylation. This strategy provides a new route for easy access to disialyl tetrasaccharide epitopes and their derivatives.

Sialic acids are common terminal residues on the glycan chains of various cell-surface glycoproteins and glycolipids. These unique nine-carbon monosaccharides on the outermost position of cell-surface glycoconjugates play important roles in many physiological and pathological processes.[1] For example, the disialyl tetrasaccharide **1α** (Figure 1) with an α



1α, Di-sialyl TF-antigen (α-isomer)
1β, Terminal tetrasaccharide epitope of GD1α, GT1aα, GQ1bα (β-isomer)

Figure 1. Naturally occurring disialyl tetrasaccharide epitopes.

configuration at the reducing end is found in the *O*-glycans of glycophorin (a major erythrocyte membrane glycoprotein),[2] mucin MUC II,[3] and erythropoietin (EPO).[4] The disialyl tetrasaccharide **1β** with a β configuration at the reducing end is an essential component of gangliosides GD1α, GT1aα, and GQ1bα[5] and the minimal binding epitope for high-affinity myelin-associated glycoprotein (MAG, Siglic-4) ligands[6] (Figure 1). Although the exact mechanism of the MAG−ganglioside interaction is not well-understood, the axon regeneration inhibited by MAG can be completely reversed by sialidase treatment, suggesting that sialic acid in gangliosides plays an essential role in high-affinity binding between MAG and gangliodes.[7] Structure−activity relationship (SAR) studies have revealed that the terminal disialyl tetrasaccharide **1β** of GQ1bα shows superior binding to MAG compared with the terminal trisaccharide epitope without the internal α2−6-linked sialic acid on GalNAc, which is present in GM1b, GD1a, and GT1b.[5,6d] Tetrasaccharide **1β** has been considered as a leading compound for the development of potent glycan inhibitors of MAG to enhance axon regeneration for the injured adult mammalian central nervous system.[7]

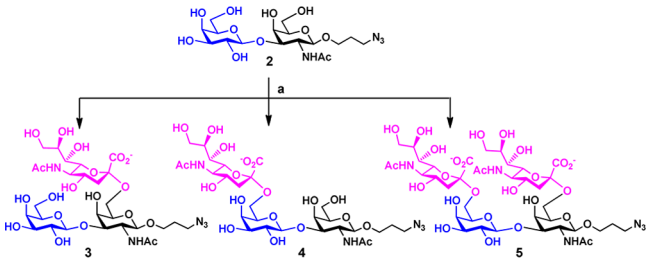
In order to study the functions of these widely distributed disialyl tetrasaccharides at the molecular level and evaluate their therapeutic potential, it is of great interest to develop an efficient and practical synthetic approach for these tetrasaccharides and their derivatives. Unfortunately, despite great progress over the last two decades, the chemical synthesis of sialic acid-containing complex structures is still challenging.[8] Several elegant chemical synthetic methods of these disialyl tetrasaccharides and related structures have been reported,[4,9] but they are quite lengthy and time-consuming and require tedious protecting-group manipulations. Alternatively, enzymatic synthesis using glycosyltransferases proceeds regio- and stereoselectively without protection. Two different sialyltransferases, α2−3-sialyltransferase and α2−6-sialyltransferase, are required to introduce two *N*-acetylneuraminic acid (Neu5Ac) groups at C6 and C3′ of the Galβ1−3GalNAc disaccharide core, respectively (Figure 1). To date, only a few recombinant *N*-acetylgalactosamine α2−6-sialyltransferases (ST6GalNAc) from mammalian sources have been employed in the synthesis of the Neu5Acα2−6GalNAc sequence.[10] A recombinant α2−6-sialyltransferase from chicken (chST6GalNAc I) and a recombinant α2−3-sialyltransferase from porcine (pST3Gal I) have been successfully utilized for enzymatic production of disialyl TF-antigen.[10a] However, a major problem encountered in these processes is the low expression level of mammalian sialyltransferases.[10,11] To circumvent this issue with the use of mammalian sialyltransferases, we herein report a chemoenzymatic approach for the synthesis of disialyl tetrasaccharide epitopes and their derivatives through regioselective sialylation of conformation-constrained trisaccharide acceptors by utilizing a bacterial α2−6-sialyltransferase from *Photobacterium damselae* (Pd2,6ST).[12]

Received: January 3, 2014
Published: March 20, 2014

   dx.doi.org/10.1021/ja5000609 | *J. Am. Chem. Soc.* 2014, 136, 5205−5208

In contrast to mammalian sialyltransferases, several bacterial sialyltransferases can be produced in sufficient amounts in convenient bacterial expression systems and have remarkable activities and promiscuous substrate specificities.[11a,13] Several bacterial sialyltransferases have been successfully employed in highly efficient one-pot multienzyme (OPME) sialylation systems for chemoenzymatic syntheses of various naturally occurring and non-natural α2−3-, α2−6-, and α2−8-linked sialosides.[12,14] Our previous work showed that both terminal Gal and GalNAc can be recognized by Pd2,6ST to form Neu5Acα2−6Gal and Neu5Acα2−6GalNAc, respectively.[14b] Structures containing both Gal and GalNAc such as Galβ1−3GalNAc, however, have not been tested as acceptor substrates for Pd2,6ST. When disaccharide Galβ1−3GalNAcβProAzide (**2**)[15] was used as an acceptor for Pd2,6ST and a varying amount of Neu5Ac was used as the donor precursor, Pd2,6ST was able to add Neu5Ac at both C6−OH of the internal GalNAc and C6′−OH of the terminal Gal. A mixture of monosialyl trisaccharides **3** and **4** and disialyl tetrasaccharide **5** was obtained, and the relative amount of **5** increased as the amount of Neu5Ac used increased. When 1.0 equiv of Neu5Ac was used, the yields of monosialyl trisaccharide **3** with Neu5Ac α2−6-linked to the internal GalNAc, monosialyl trisaccharide **4** with Neu5Ac α2−6-linked to the terminal Gal, and disialyl tetrasaccharide **5** were 34%, 32%, and 13%, respectively (Scheme 1). The products can be easily separated from each other by silica gel flash chromatography.

Scheme 1. One-Pot Two-Enzyme α2−6-Sialylation of Galacto-*N*-biose 2[a]

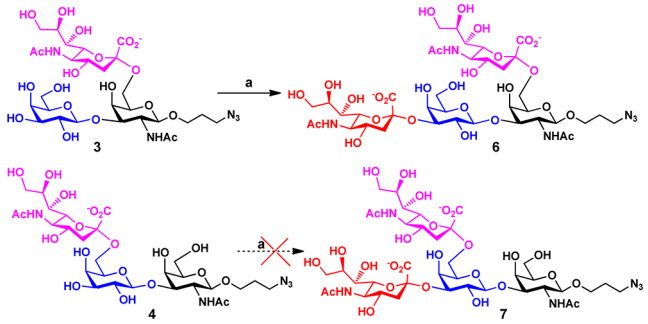


[a]Reagents and conditions: (a) Neu5Ac (1.0 equiv), CTP (1.0 equiv), Mg^{2+}, Tris-HCl buffer (pH 8.5), NmCSS, Pd2,6ST, 37 °C, 2 h. Yields: 34% for **3**; 32% for **4**; 13% for **5**.

Both monosialyl trisaccharides **3** and **4** were then used as acceptors for enzymatic sialylation using a recombinant *Pasteurella multocida* α2−3-sialyltransferase (PmST1)[14b] to introduce another Neu5Ac at the C3′ position on the Gal (Scheme 2). PmST1-catalyzed α2−3-sialylation of monosialyl trisaccharide **3** formed the desired disialyl tetrasaccharide **6** in 95% yield. In contrast, trisaccharide **4** was not a suitable acceptor for PmST1, and no tetrasaccharide **7** was detected under the same conditions. These results are consistent with our previous findings[16] and the observations from a recent report by the Paulson group.[17]

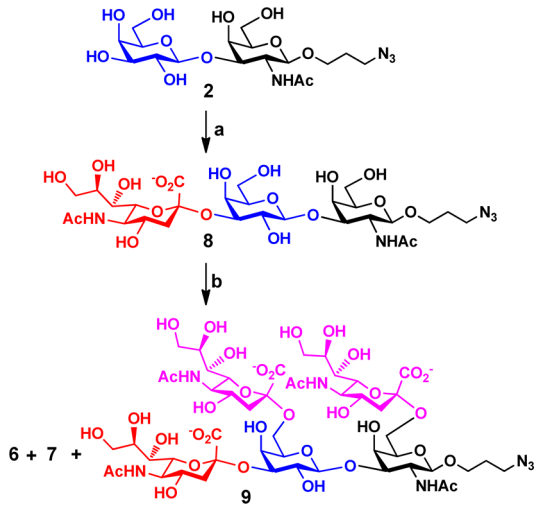
The desired disialyl tetrasaccharide **6** can also be prepared by an alternative two-step procedure with one-pot two-enzyme α2−3-sialylation of disaccharide **2** to form α2−3-sialoside **8**[14b] followed by one-pot two-enzyme α2−6-sialylation (Scheme 3). However, α2−6-sialylation of **8** by Pd2,6ST led to the production of a mixture of disialyl tetrasaccharides **6** and **7** and trisialyl pentasaccharide **9**. Compounds **6** and **7** were readily purified from the reaction mixture and from compound

Scheme 2. One-Pot Two-Enzyme α2−3-Sialylation of Trisaccharides 3 and 4[a]



[a]Reagents and conditions: (a) Neu5Ac (2.0 equiv), CTP (2.0 equiv), Mg^{2+}, Tris-HCl buffer (pH 8.5), NmCSS, PmST1, 37 °C, 1 h, 95% yield for **6**.

Scheme 3. One-Pot Two-Enzyme α2−3-Sialylation of Disaccharide 2 Followed by One-Pot Two-Enzyme α2−6-Sialylation of Trisaccharide 8[a]



[a]Reagents and conditions: (a) Neu5Ac (1.2 equiv), CTP (1.2 equiv), Mg^{2+}, Tris-HCl buffer (pH 8.5), NmCSS, PmST1, 37 °C, 1 h; (b) Neu5Ac (1.0 equiv), CTP (1.2 equiv), Mg^{2+}, Tris-HCl buffer (pH 8.5), NmCSS, Pd2,6ST, 37 °C, 2 h. Yields: 63% for **6** and **7** (**6**:**7** = 29:71 based on ^{1}H NMR analysis); 9% for **9**.

9 as a mixture, but further separation proved to be challenging. Quite interestingly, close examination of the NMR spectrum of the mixture of **6** and **7** in comparison with that of the reference pure tetrasaccharide **6** prepared by the previous two-step procedure (Scheme 2) indicated that Pd2,6ST preferred to add a Neu5Ac to the Gal instead of the GalNAc in monosialyl trisaccharide **8** to produce the non-natural structure **7**. A 29:71 ratio was observed for compound **6** to compound **7** as shown by ^{1}H NMR spectroscopy [see the Supporting Information (SI) for details].

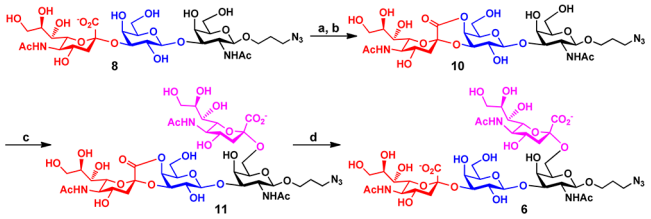
Previously, Boons and co-workers showed that conformation-constrained preorganized acceptor substrates can enhance the reaction efficiency of sialyltransferase-catalyzed reactions.[18] More recently, Withers and co-workers reported that the substrate promiscuity of a given glycosyltransferase can be expanded through substrate engineering.[19] It is unclear why Pd2,6ST regioselectively introduced Neu5Ac at the C6′ position of the Gal in trisaccharide acceptor **8** but showed no

dx.doi.org/10.1021/ja5000609 | J. Am. Chem. Soc. 2014, 136, 5205−5208

preference toward the Gal or the GalNAc in the disaccharide acceptor **2**. Nevertheless, these results indicate that the C6′ hydroxyl group is more accessible than the C6 hydroxyl group on trisaccharide acceptor **8** for Pd2,6ST and that the acceptor substrate modification (with or without Neu5Ac at the C3′ position of disaccharide **2**) can dramatically affect the reaction outcome. On the basis of these phenomena, we hypothesized that reversed regioselectivity could be achieved if the access to the C6′ hydroxyl group is hindered.

To test our hypothesis, trisaccharide **8** was converted to trisaccharide lactone **10** (Scheme 4) to constrain the

Scheme 4. Regioselective Sialylation of Trisaccharide Lactone 10[a]



[a]Reagents and conditions: (a) Ac_2O, pyridine, 0 °C, 12 h; (b) NaOMe, MeOH, 59% for two steps; (c) CMP-Neu5Ac (2.0 equiv), Tris-HCl buffer (pH 7.0), Pd2,6ST, 37 °C, 2 h, 86%; (d) 1 M NaOH, 3 h, 98%.

conformation of the Gal in the trisaccharide. The formation of lactone **10** was achieved by acetylation and simultaneous lactonization of trisaccharide **8** in the presence of acetic anhydride and pyridine at 0 °C for 12 h followed by removal of all of the *O*-acetyl groups from the resulting crude product under the Zemplén conditions to produce trisaccharide lactone **10**. Lactone formation is a common phenomenon in the chemical synthesis of Neu5Acα2−3Gal and oligosialic acid-containing structures under acidic conditions or during the peracetylation step.[20] The formation of the 1,4-lactone between the C1″ carboxyl group of Neu5Ac and the C4′ hydroxyl group of Gal during acetylation was confirmed by ^{1}H, ^{13}C, and 2D NMR spectroscopies, which showed long-range connectivity between the lactone C═O at 165.98 ppm and the downfield peak of Gal H4 at 5.24 ppm (see the SI for details). Trisaccharide lactone **10** was then used as an acceptor for one-pot two-enzyme α2−6-sialylation. The pH of the reaction was controlled at 7.0 to allow good enzymatic activity and to prevent spontaneous hydrolysis of the lactone at higher pH. To our delight, lactone **10** was stable under the reaction conditions used, and the reaction was completed in 2 h to produce disialyl tetrasaccharide lactone **11** in 86% yield. Saponification of **11** produced disialyl tetrasaccharide **6** as the only product in 98% yield (Scheme 4). The ^{13}C NMR spectrum (Figure 2c) was identical to that of the tetrasaccharide **6** prepared by the previous two-step procedure (Figure 2b). ^{13}C NMR analysis also revealed that the minor regioisomer in the unseparated mixture of **6** and **7** obtained from random sialylation of trisaccharide **8** (Figure 2a) was identical to tetrasaccharide **6**, as indicated by the three peaks at 173.9, 104.6, and 99.6 ppm.

Previous SAR studies of MAG and disialyl tetrasaccharide epitopes have demonstrated that the modification of Neu5Ac by introducing hydrophobic substituents at the C9 position in the Neu5Acα2−3GalNAc sequence can significantly increase the binding affinity of the glycan and MAG.[7] Encouraged by these results, we carried out the chemoenzymatic synthesis of

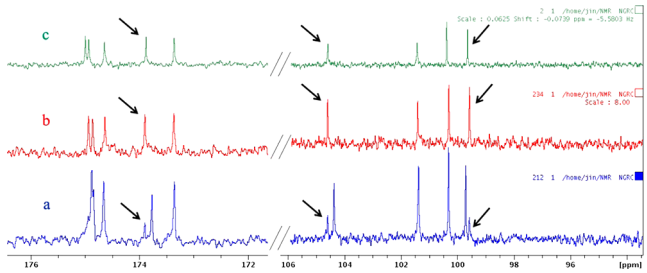
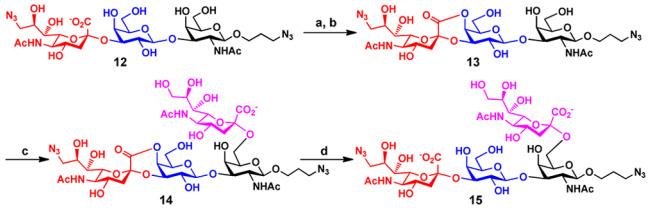


Figure 2. Overlay of a selected region of the ^{13}C NMR spectra of (a) a mixture of **6** and **7** obtained from α2−6-sialylation of trisaccharide **8**, (b) tetrasaccharide **6** obtained from α2−3-sialylation of trisaccharide **3**, and (c) tetrasaccharide **6** obtained from α2−6-sialylation of trisaccharide lactone **10** followed by saponification.

disialyl tetrasaccharide epitope **15** containing the non-natural sialic acid 9-N_3-Neu5Ac α2−3-linked to the Gal (Scheme 5)

Scheme 5. Regioselective Chemoenzymatic Synthesis of Tetrasaccharide 15 via Lactone Intermediates[a]

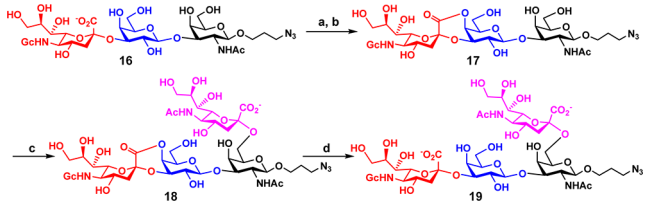


[a]Reagents and conditions: (a) Ac_2O, pyridine, 0 °C, 12 h; (b) NaOMe, MeOH, 55% for two steps. (c) CMP-Neu5Ac (1.5 equiv), Tris-HCl buffer (pH 7.0), Pd2,6ST, 2 h, 89%; (d) 1 M NaOH, 3 h, 96%.

using the efficient lactone method described above. To our delight, a similar high efficiency was achieved for the chemoenzymatic synthesis of disialyl tetrasaccharide **15** (Scheme 5). The 9-N_3 group in compound **15** can be used as a chemical handle for easy derivatization.[7]

The general applicability of the method was further explored by sialylation of trisaccharides containing a different sialic acid form or a different internal galactoside. As shown in Scheme 6, an α2−3-linked trisaccharide containing *N*-glycolylneuraminic acid (Neu5Gc), a nonhuman sialic acid form with an additional hydroxyl group at C5−NHAc, was also compatible with the regioselective sialylation approach, producing disialyl tetrasaccharide **19** as the only product via lactone intermediates. Furthermore, α2−3-linked sialyl galactoside Neu5Acα2−

Scheme 6. Regioselective Chemoenzymatic Synthesis of Tetrasaccharide 19 via Lactone Intermediates[a]

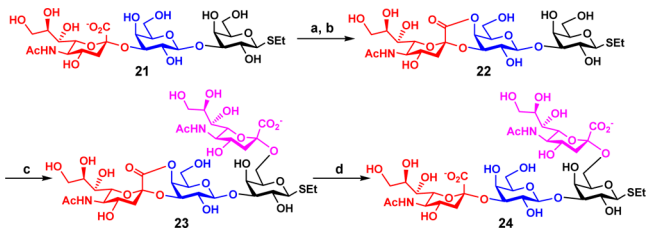


[a]Reagents and conditions: (a) Ac_2O, pyridine, 0 °C, 12 h; (b) NaOMe, MeOH, 45% for two steps. (c) CMP-Neu5Ac (2.0 equiv), Tris-HCl buffer (pH 7.0), Pd2,6ST, 2 h, 93%; (d) 1 M NaOH, 3 h, 98%.

dx.doi.org/10.1021/ja5000609 | J. Am. Chem. Soc. 2014, 136, 5205−5208

3Galβ1−3GalβSEt containing a different underlying disaccharide was also a suitable substrate for lactone-mediated regioselective sialylation, producing disialyl tetrasaccharide **24** as the only product (Scheme 7).

Scheme 7. Regioselective chemoenzymatic synthesis of tetrasaccharide 24 via lactone intermediates[a]



[a]Reagents and conditions: (a) Ac_2O, pyridine, 0 °C, 12 h; (b) NaOMe, MeOH, 45% for two steps. (c) CMP-Neu5Ac (3.0 equiv), Tris-HCl buffer (pH 7.0), Pd2,6ST, 2 h, 92%; (d) 1 M NaOH, 3 h, 98%.

In summary, we have described a novel strategy for chemoenzymatic synthesis of disialyl tetrasaccharide epitopes containing natural and non-natural sialic acids using bacterial sialyltransferase-catalyzed regioselective sialylation of conformation-constrained acceptors. We have demonstrated that unwanted acceptor substrate promiscuity of a given sialyltransferase can be prevented through "substrate engineering".[19] Similar strategies can be explored for the synthesis of other carbohydrates using acceptor-substrate-promiscuous enzymes.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures and product characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

hzcao@sdu.edu.cn
fswang@sdu.edu.cn

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was financially supported by the National Basic Research Program of China (2012CB822102 to H.C. and F.W.), the National High Technology Research and Development Program of China (2012AA021504 to H.C. and F.W.), the National Natural Science Foundation of China (21172135 and 21372145 to H.C.), NIH (R01HD065122 to X.C.), and the Natural Science Foundation of Shandong Province, China (ZR2011CM038 to L.J.).

■ REFERENCES

(1) (a) Schauer, R. *Glycoconjugate J.* **2000**, *17*, 485. (b) Angata, T.; Varki, A. *Chem. Rev.* **2002**, *102*, 439. (c) Chen, X.; Varki, A. *ACS Chem. Biol.* **2010**, *5*, 163.

(2) Parkkinen, J.; Rogers, G. N.; Korhonen, T.; Dahr, W.; Finne, J. *Infect. Immun.* **1986**, *54*, 37.

(3) Thomsson, K. A.; Holmen-Larsson, J. M.; Angstrom, J.; Johansson, M. E. V.; Xia, L.; Hansson, G. C. *Glycobiology* **2012**, *22*, 1128.

(4) Schwarz, J. B.; Kuduk, S. D.; Chen, X. T.; Sames, D.; Glunz, P. W.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1999**, *121*, 2662.

(5) (a) Kolter, T.; Proia, R. L.; Sandhoff, K. *J. Biol. Chem.* **2002**, *277*, 25859. (b) Schnaar, R. L. *FEBS Lett.* **2010**, *584*, 1741.

(6) (a) Blixt, O.; Collins, B. E.; van den Nieuwenhof, I. M.; Crocker, P. R.; Paulson, J. C. *J. Biol. Chem.* **2003**, *278*, 31007. (b) Vyas, A. A.; Schnaar, R. L. *Biochimie* **2001**, *83*, 677. (c) Yang, L. J. S.; Zeller, C. B.; Shaper, N. L.; Kiso, M.; Hasegawa, A.; Shapiro, R. E.; Schnaar, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 814. (d) Collins, B. E.; Ito, H.; Sawada, N.; Ishida, H.; Kiso, M.; Schnaar, R. L. *J. Biol. Chem.* **1999**, *274*, 37637.

(7) For reviews, see: (a) Ernst, B.; Schwardt, O.; Mesch, S.; Wittwer, M.; Rossato, G.; Vedani, A. *Chimia* **2010**, *64*, 17. (b) Filbin, M. T. *Nat. Rev. Neurosci.* **2003**, *4*, 703. (c) Ernst, B.; Magnani, J. L. *Nat. Rev. Drug Discovery* **2009**, *8*, 661.

(8) For recent reviews, see: (a) Hsu, C. H.; Hung, S. C.; Wu, C. Y.; Wong, C. H. *Angew. Chem., Int. Ed.* **2011**, *50*, 11872. (b) Boltje, T. J.; Buskas, T.; Boons, G. J. *Nat. Chem.* **2009**, *1*, 611. (c) De Meo, C.; Priyadarshani, U. *Carbohydr. Res.* **2008**, *343*, 1540.

(9) Prabhanjan, H.; Aoyama, K.; Kiso, M.; Hasegawa, A. *Carbohydr. Res.* **1992**, *233*, 87.

(10) (a) Blixt, O.; Allin, K.; Pereira, L.; Datta, A.; Paulson, J. C. *J. Am. Chem. Soc.* **2002**, *124*, 5739. (b) George, S. K.; Schwientek, T.; Holm, B.; Reis, C. A.; Clausen, H.; Kihlberg, J. *J. Am. Chem. Soc.* **2001**, *123*, 11117.

(11) (a) Li, Y.; Chen, X. *Appl. Microbiol. Biotechnol.* **2012**, *94*, 887. (b) Takashima, S.; Tsuji, S. *Trends Glycosci. Glycotechnol.* **2011**, *23*, 178.

(12) Yu, H.; Huang, S.; Chokhawala, H.; Sun, M.; Zheng, H.; Chen, X. *Angew. Chem., Int. Ed.* **2006**, *45*, 3938.

(13) Schwardt, O.; Visekruna, T.; Rabbani, S.; Ernst, B. *Chimia* **2006**, *60*, 234.

(14) (a) Yu, H.; Cheng, J.; Ding, L.; Khedri, Z.; Chen, Y.; Chin, S.; Lau, K.; Tiwari, V. K.; Chen, X. *J. Am. Chem. Soc.* **2009**, *131*, 18467. (b) Yu, H.; Chokhawala, H.; Karpel, R.; Yu, H.; Wu, B. Y.; Zhang, J. B.; Zhang, Y. X.; Jia, Q.; Chen, X. *J. Am. Chem. Soc.* **2005**, *127*, 17618.

(15) Yu, H.; Thon, V.; Lau, K.; Cai, L.; Chen, Y.; Mu, S.; Li, Y.; Wang, P. G.; Chen, X. *Chem. Commun.* **2010**, *46*, 7507.

(16) Yu, H.; Chen, X. *Org. Lett.* **2006**, *8*, 2393.

(17) Nycholat, C. M.; Peng, W.; McBride, R.; Antonopoulos, A.; de Vries, R. P.; Polonskaya, Z.; Finn, M.G.; Dell, A.; Haslam, S. M.; Paulson, J. C. *J. Am. Chem. Soc.* **2013**, *135*, 18280.

(18) (a) Galan, M. C.; Dodson, C. S.; Venot, A. P.; Boons, G. J. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2205. (b) Galan, M. C.; Venot, A. P.; Boons, G. J. *Biochemistry* **2003**, *42*, 8522. (c) Galan, M. C.; Venot, A. P.; Glushka, J.; Imberty, A.; Boons, G. J. *J. Am. Chem. Soc.* **2002**, *124*, 5964. (d) Galan, M. C.; Venot, A. P.; Glushka, J.; Imberty, A.; Boons, G. J. *Org. Biomol. Chem.* **2003**, *1*, 3891.

(19) Lairson, L. L.; Watts, A. G.; Wakarchuk, W. W.; Withers, S. G. *Nat. Chem. Biol.* **2006**, *2*, 724.

(20) (a) Hayashi, M.; Tanaka, M.; Itoh, M.; Miyauchi, H. *J. Org. Chem.* **1996**, *61*, 2938. (b) Zhang, Y.; Lee, Y. C. *J. Biol. Chem.* **1999**, *274*, 6183. (c) Nakamura, T.; Bubb, W. A.; Saito, T.; Arai, I.; Urashima, T. *Carbohydr. Res.* **2000**, *329*, 471. (d) Pudelko, M.; Lindgren, A.; Tengel, T.; Reis, C. A.; Elofsson, M.; Kihlberg, J. *Org. Biomol. Chem.* **2006**, *4*, 713.