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Chemoenzymatic synthesis of 2-azidoethyl-ganglio-oligosaccharides GD3, GT3, GM2, GD2, GT2, GM1, and GD1a

Ola Blixt,[a,*] Daniela Vasiliu,[a] Kirk Allin,[a] Nathan Jacobsen,[a] Dawn Warnock,[a] Nahid Razi,[a] James C. Paulson,[a] Stéphane Bernatchez,[b] Michel Gilbert[b] and Warren Wakarchuk[b]

[a]*Carbohydrate Synthesis and Protein Expression Core D, Consortium for Functional Glycomics, The Scripps Research Institute, Department of Molecular Biology, CB-248A, 10550 North Torrey Pines Road, 92037 La Jolla, United States*
[b]*Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6*

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Abstract—We have synthesized several ganglio-oligosaccharide structures using glycosyltransferases from *Campylobacter jejuni*. The enzymes, α-(2→3/8)-sialyltransferase (Cst-II), β-(1→4)-*N*-acetylgalactosaminyltransferase (CgtA), and β-(1→3)-galactosyltransferase (CgtB), were produced in large-scale fermentation from *Escherichia coli* and further characterized based on their acceptor specificities. 2-Azidoethyl-glycosides corresponding to the oligosaccharides of GD3 (α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-), GT3 (α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-), GM2 (β-D-Gal*p*NAc-(1→4)-[α-D-Neu*p*5Ac-(2→3)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-), GD2 (β-D-Gal*p*NAc-(1→4)-[α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→3)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-), GT2 (β-D-Gal*p*NAc-(1→4)-[α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→3)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-), and GM1 (β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→4)-[α-D-Neu*p*5Ac-(2→3)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-) were synthesized in high yields (gram-scale). In addition, a mammalian α-(2→3)-sialyltransferase (ST3Gal I) was used to sialylate GM1 and generate GD1a (α-D-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→4)-[α-D-Neu*p*5Ac-(2→3)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-) oligosaccharide. We also cloned and expressed a rat UDP-*N*-acetylglucosamine-4′epimerase (GalNAcE) in *E. coli* AD202 cells for cost saving in situ conversion of less expensive UDP-GlcNAc to UDP-GalNAc.

Keywords: Ganglioside; Oligosaccharide; Chemoenzymatic; Synthesis; Glycosyltransferase

1. Introduction

Gangliosides are glycolipids that comprise a structurally diverse set of sialylated molecules that are found in most cells but are particularly abundant in neuronal tissues. They have been found to act as receptors for growth factors, toxins, viruses, and to facilitate the attachment of human melanoma and neuroblastoma cells. Specific gangliosides are also present in early stages of human neural development and affect major cellular processes including proliferation, differentiation, survival, and apoptosis. They are also well-known tumor associated antigens and active immunization using gangliosides may suppress melanoma growth.[1–6]

Despite the importance of the sialylated ganglioside structures, methods for their efficient preparation have been limited. The introduction of the sialic acid to a glycolipid core structure has shown to be a daunting task and complicated engineering with well executed synthetic strategies is needed. Several elegant chemical approaches have been reported over decades by various groups[7–10] as well as chemoenzymatic synthesis of GM3.[11–14] A recent report also demonstrated the in vivo production of the glycan chain of GM1 and GM2 oligosaccharides using metabolically engineered bacteria.[15,16] Until now, the synthesis of *N*-acetylneuraminic acid

* Corresponding author. Tel.: +1 858 784 7538; fax: +1 858 784 7542; e-mail: olablixt@scripps.edu

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(Neu*p*5Ac) containing ganglio-oligosaccharides and particularly the difficult formation of a α-(2→8)-linkage between two Neu*p*5Ac molecules has been the most tedious.

Due to recent advances in molecular biology and characterizations of various pathogens, several bacterial glycosyltransferases have been identified that accomplish transformations analogous to mammalian enzymes. Because they can be produced in simple bacterial expression systems, they are increasingly being explored as potential biochemical catalysts for glycosidic linkage formations in the preparation of synthetically defined carbohydrate structures.[17–21] Several glycosyltransferase genes from *Campylobacter jejuni* have been identified as coding genes responsible for the expression of various ganglioside related lipooligosaccharide (LOS) synthesized by this pathogenic bacterium.[22] Among these genes, Cst-II, coding for a bifunctional α-(2→3/8)-sialyltransferase (α3/8SiaT), CgtA, coding for a β-(1→4)-*N*-acetylgalactosaminyltransferase (β4GalNAcT), and CgtB, coding for a β-(1→3)-galactosyltransferase (β3GalT), have been cloned into *Escherichia coli* expression vectors and expressed as active glycosyltransferases.[23,24] We have now further explored the large-scale production of these enzymes and developed efficient synthetic procedures for ganglio-oligosaccharides GD3 (α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-), GT3 (α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-), GM2 (β-D-Gal*p*NAc-(1→4)-[α-D-Neu*p*5Ac-(2→3)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-), GD2 (β-D-Gal*p*NAc-(1→4)-[α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→3)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-), GT2 (β-D-Gal*p*NAc-(1→4)-[α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→3)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-), GM1 (β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→4)-[α-D-Neu*p*5Ac-(2→3)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-), and GD1a (α-D-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→4)-[α-D-Neu*p*5Ac-(2→3)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-).

2. Results and discussion

2.1. Large-scale production of recombinant enzymes

The genes encoding *cst*-II (α3/8SiaT), *cgt*A, (β4GalNAcT) and *cgt*B (β3Gal) were cloned into the expression vector pCWori+ and expressed in *E. coli* strain AD202 cell line.[22,25] Large-scale fermentation (100 L) gave high enzymatic activities from crude cell lysates after passing cells through a microfluidizer and removal of cell debris. The Cst-II was assayed based on α-(2→3)-activity using lactose (**7**) as acceptor substrate and cytidine-5′monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac) as donor substrate to give an enzyme activity of 50 U/L of the expression media. The CgtA was assayed under similar conditions to give 67 U/L using α-(2→3)-sialyllactose (**16**) and uridine-5′diphospho-*N*-acetylgalactosamine (UDP-GalNAc) as acceptor and donor substrates, respectively. The CgtB enzyme was assayed using GM2 (**24**) as the acceptor and uridine-5′diphosphogalactose (UDP-Gal) as the donor substrate to obtain an activity of 17 U/L of the expression media. All enzymes were stable as frozen cell lysate at −20 °C for at least 6 months of storage.

Multi-gram-scale synthesis requires stoichiometric additives of generally expensive sugar nucleotide donor substrates. Since current prices of UDP-GalNAc are 20 times that of UDP-GlcNAc, we produced a rat UDP-*N*-acetylglucosamine-4′epimerase (GalNAcE) that converts less expensive UDP-GlcNAc to UDP-GalNAc in situ. The rat epimerase gene, kindly provided by Karl Johnson, Neose Technologies, was cloned into vector pCWori+ and expressed into *E. coli* strain AD202[25] with the standard procedure reported previously.[18] The cell lysates were used directly in the enzymatic reaction mixtures and no endogenous activity was seen in control reactions using cell lysate lacking the GalNAcE-producing plasmid (data not shown). Approximately 496 U/L media was produced as determined using the assay conditions described below. The GalNAcE enzyme also catalyzes the epimerization of UDP-Glc to UDP-Gal (174 U/L media). Therefore, the UDP-GalNAc epimerase worked exemplary well in combination with the CgtA and CgtB in preparative synthesis (see below).

2.2. α3/8SiaT and β4GalNAcT acceptor specificities

Bacterial glycosyltransferases typically have more relaxed substrate specificities than mammalian enzymes, which can be used to advantage for various synthetic applications.[20] To further explore the utility of the Cst-II and CgtA enzymes in preparative synthesis of various ganglio-oligosaccharides, we performed a detailed oligosaccharide acceptor substrate specificity study (Table 1). The Cst-II α-(2→3)-activity was measured using galactosides at high acceptor substrate concentrations (5 mM) and short incubation times (30 min) to minimize any α-(2→8)-sialylation on formed α-D-Neu*p*5Ac-(2→3)-products. The mono-sialylated assay products were isolated using Dowex-ion-exchange column as described previously.[17] Our data showed that neither terminal *N*-acetylglucosaminide nor *N*-acetylgalactosaminide could serve as acceptors. A spacer-derivatized galactoside (**2**) showed almost 2-fold higher activity than free galactose (**1**), suggesting the importance of beta configuration at the anomeric center of the terminal galactose moiety. Similar assumption can be made for lactose (**7**) and lactoside (**8**). No activity was observed with the detected thiogalactoside (**3**), *N*-acetylgalactosamine (**4** and **5**), or *N*-acetylglucosamine (**6**). As expected, lactosides (**7** and **8**) had the highest specificity of the compounds

Table 1. Relative acceptor substrate specificities of Cst-II and CgtA

No.	Acceptor	Relative specific activity (%)		
		Cst-II		CgtA
		α-(2→3)-	α-(2→8)-	
Dowex assays (Method A)				
1	D-Gal	21		
2	β-D-Gal*p*-sp[a]	38		
3	β-D-Gal*p*S*i*Pr[b]	<1		
4	D-Gal*p*NAc	0		
5	α-D-Gal*p*NAc-sp	0		
6	D-Glc*p*NAc	0		
7	β-D-Gal*p*-(1→4)-β-D-Glc*p*	**100**		0
8	β-D-Gal*p*-(1→4)-β-D-Glc*p*-sp	123		
9	β-D-Gal*p*-(1→4)-β-D-Glc*p*SCr[c]	8		
10	β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc	64		
11	β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-sp	88		
12	β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Lac[d]-sp	27		
13	[β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)]$_2$-sp	10		
14	[β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)]$_3$-sp	5		
Reversed phase (C18) assays (Method B)				
15	α-D-Neu*p*5Ac-(2→3)-β-D-Gal*p*R[e]		63	95
16	α-D-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)Glc*p*R		**100**	**100**
17	α-D-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)Glc*p*NAcR		199	84
18	α-D-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)Glc*p*R		116	8
19	α-D-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)Glc*p*NAcR		43	7
20	α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→3)-β-D-Lac-R		12	37
21	[α-D-Neu*p*5Ac-(2→8)]$_2$-α-D-Neu*p*5Ac-(2→3)-β-D-Lac-R		5	33
22	α-D-Neu*p*5Gc-(2→3)-β-D-Gal*p*-(1→4)Glc*p*NAcR		104	98
23	β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Lac-R	27		0
24	α-D-Neu*p*5Ac-(2→3)-[β-D-Gal*p*NAc-(1→4)]-β-D-Lac-R		10	
25	α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→3)-[β-D-Gal*p*NAc-(1→4)]-β-D-Lac-R		12	

Relative specific activity for each compound is calculated as a percentage of the reference compound (**7**) according to the formula: (value) = (acceptor/reference acceptor) × 100. Neutral acceptors were assayed by Method A and charged acceptors with Method B (see Experimental).

[a] sp, $OCH_2CH_2N_3$.

[b] S*i*Pr, thio-*iso*-propyl.

[c] *p*SCr, *para* thio-cresyl.

[d] Lac, lactose.

[e] R, OCH_2CH_2NH(Fmoc).

tested. *N*-Acetyllactosamines (LacNAc) (**10** and **11**) demonstrated excellent activity, but the extended dimeric and trimeric LacNAc (**13** and **14**) showed an approximate decrease of 9- and 18-fold, respectively, compared to monomeric LacNAc (**11**).

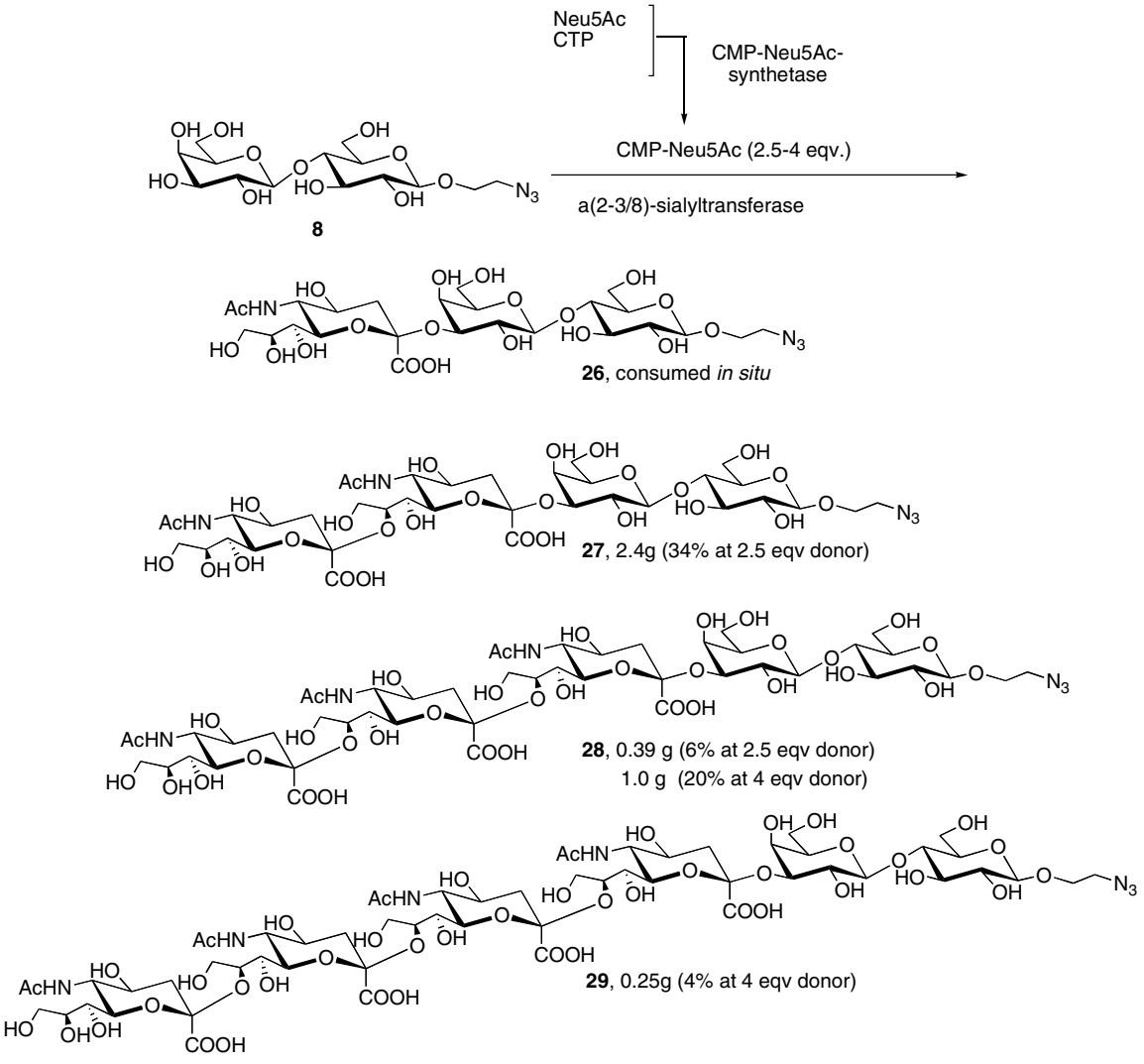
The Cst-II α-(2→8)-activity was measured with different sialosides (**15**–**25**) tagged with a hydrophobic 9-fluorenyl methoxycarbonyl (Fmoc) functional group for convenient solid-phase reversed phase C-18 SepPak extraction assays.[26] We found that products containing up to four sialic acid units could bind to the C18 column in our study (confirmed in parallel size exclusion chromatography assays, data not shown). The α-(2→8)-activity study showed that both α-(2→3)-sialosides (**15**–**17**) and α-(2→6)-sialosides (**18**, **19**) were excellent substrates for Cst-II. To confirm the α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→6)-product formation, **19** was synthesized in 100 mg quantities and verified by 1D and 2D-NMR-spectroscopy (data not shown). The larger oligosaccharides GD3 (**20**) and GT3 (**21**) showed a significant reduced activity (10–20-fold), compared to α-(2→3)-sialyllactose (**16**). A similar trend was also observed with the α-(2→3)-activity of the extended di- and trimeric-LacNAc structures above. Additionally, the *N*-glycolylneuraminic acid (Neu5Gc) oligosaccharide (**22**) was also a very good substrate and enabled the synthesis of various *N*-glycolylneuraminic acid structures commonly found in the murine species.

We also investigated the specificity of the β4GalNAcT (cgtA) with Fmoc acceptor substrates (**15**–**25**). The CgtA enzyme did not show activity on non-sialylated substrates as demonstrated with lactose (**7**)[24] and lacto-*N*-neotetraose (**23**) in contrast to the mammalian GM2 synthase that was reported capable in generating asialo-GM2 structures.[27] Sialic acid α-(2→3)-linked to terminal galactosides (**15**–**17**) showed 10-fold higher activity compared to α-(2→6)-sialosides (**18**, **19**) and approximately one third of the activity on the GD3

and GT3 oligosaccharides, compared to **16**. We speculate that **16** could be the preferred substrate for the enzyme in *C. jejuni* LOS biosynthesis. The CgtA glycosyltransferase could also utilize the sugar nucleotide donor UDP-Gal to the same extent as UDP-GalNAc (data not shown), and thus increase the synthetic diversity of oligosaccharide derivatives.[16]

2.3. Synthesis of GD3- (27), GT3- (28), and poly-sialylated-oligosaccharides

To synthesize ganglio-oligosaccharide structures for conjugation, the reducing end on lactose was equipped with the 2-azidoethyl-functionality prior to enzymatic modifications. These derivatives can be used *as is* in competitive inhibition experiments, conjugated to various supports or converted to other functional groups. With excess amounts of CMP-Neu5Ac, the Cst-II demonstrates extensive α-(2→8) multi-sialylation activities. Therefore, the synthesis of GD3 (**27**), GT3 (**28**), and tetra-sialyllactose (**29**) required carefully controlled conditions using restricted amounts of CMP-Neu5Ac. 2-Azidoethyllactoside (**8**) was elongated with Cst-II and 2.5 equiv of CMP-Neu5Ac[17] to produce 2.4 g (34%) of GD3 and 0.39 g (6%) of GT3 (in Scheme 1). GM3 (**26**) was completely consumed and converted to GD3 and GT3 using this molar ratio of CMP-Neu5Ac donor. By increasing the molar ratio of CMP-Neu5Ac to 4 equiv, the reaction was driven to >80% conversion of the newly formed GD3 to generate larger amounts of GT3 (1.0 g, 20%). Smaller amounts of the tetra-sialic acid α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→8)-α-D-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(**29**) (0.25 g, 4%) were also isolated and traces of higher sialylated fractions were also detected but not purified. Products were subject to ion-exchange and size-



Scheme 1.

exclusion chromatography to give pure material (>90%) per NMR and MS analysis.

2.4. Synthesis of GM2- (30), GD2- (31), GT2- (32), GM1- (33), and GD1a- (34) oligosaccharides

To extend the series of ganglioside oligosaccharide structures, GM3 (**26**),[18] GD3 (**27**), and GT3 (**28**) were further branched by using the one-pot mixture of CgtA (β4GalNAcT), UDP-GlcNAc, and GalNAcE to efficiently generate the GM2 (**30**), GD2 (**31**), and GT2 (**32**) derivatives in high yields (typical 80–90%) (Scheme 2). We have further elongated GM2 (**30**) to GM1 (**33**) by using the CgtB (β3GalT), UDP-glucose, and GalNAcE in high yields (0.5 g, 86%). Recently, chemical synthesis of similar aminoethyl glycoside was reported, in which total amounts of 38 mg, in a yield of 30%, were produced.[7] Starting from lactoside (**8**), we enzymatically generated gram-scale amounts of GM1 in >75% total yield within a week.

GD1a is one of the several structures identified as target epitopes for immunological responses in Guillian–Barré syndrome.[24] We were also able to generate spacered GD1a (**34**) in a simple procedure using a α-(2→3)-sialyltransferase (ST3Gal-I). The ST3Gal-I is an enzyme known for its substrate specificity on *O*-glycans, where it creates sialylated T-antigen but it was also reported to have activity for ganglioside structures such as GM1 and GD1b.[28] Here, we used a recombinant porcine ST3Gal-I expressed in a baculovirus system[29] for sialylation of 2-azidoethyl GM1 (**33**) to synthesize GD1a (**34**) in high yield (Scheme 2).

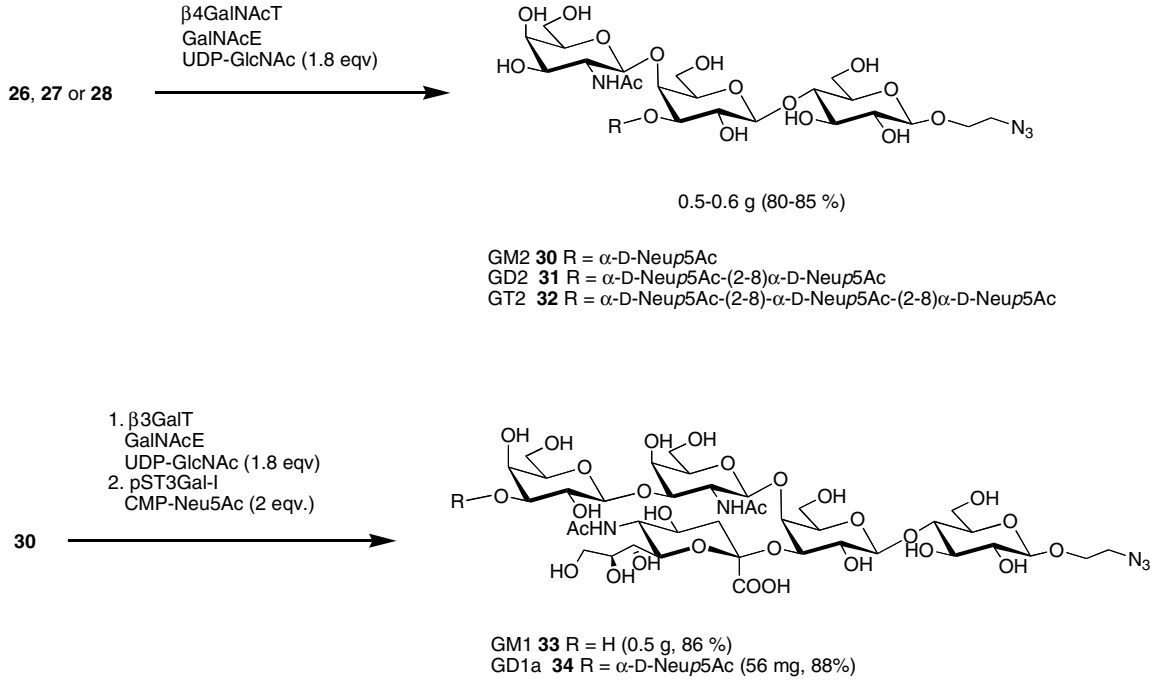
2.5. Conclusion

This work demonstrates that efficient synthesis of complex oligosaccharides representing the glycan chains of gangliosides can be achieved using microbial glycosyltransferases. Detailed acceptor specificities of Cst-II and CgtA revealed a relative broad substrate flexibility that enabled various enzymatic strategies. Large-scale fermentation in *E. coli* generated up to 5000 units of active enzymes that were incorporated into gram-scale synthesis of GD3, GT3, GM2, GD2, GT2, and GM1 ganglioside oligosaccharides. Expensive UDP-GalNAc was generated in situ from less expensive UDP-GlcNAc by the *E. coli* produced rat UDP-GalNAc-4′-epimerase. Furthermore, spacered GM1 was a substrate for a mammalian ST3Gal-I to generate GD1a, a structure that may have an important therapeutic value in neural disease. We are currently extending the ganglioside oligosaccharide series with the GD1b and GD1c using the approach described above. All structures have been linked to a convenient reducible azido-functional group that can be conjugated to proteins, solid-supports,[30] lipids, or other functional groups,[31] and they are available free of charge from CFG (http://www.functionalglycomics.org).

3. Experimental

3.1. General methods

Concentration of solvents was performed under reduced pressure at <40 °C bath temperature. NMR spectra



Scheme 2.

were recorded at 25 °C using a Bruker DRX-500 or 600 spectrometer. The following reference signals were used: acetone 2.225 ppm (^{1}H in D_2O), acetone 29.7 ppm (^{13}C in D_2O). ESI-TOF high-accuracy MS spectra were recorded with an LC MSD TOF (Agilent Technologies) using dihydroxybenzoic acid as matrix. Thin-layer chromatography was performed on Kieselgel 60 F_{254} Fertigplatten (Merck, Darmstadt, Germany). After development with appropriate eluants (ethyl acetate–MeOH–acetic acid–water by volume 10—3:3:3:2), spots were visualized by UV light and/or by dipping in 5% sulfuric acid in ethanol, followed by charring. Water was purified from a NanoPure Infinity Ultrapure water system (Barnstead/Thermolyne, Dubuque, Iowa, USA) and was degassed by vacuum treatment before use. Compounds **10**–**12** were synthesized by chemoenzymatic methods as described previously[18] and compounds **2** and **9** were prepared as reported.[32] Compounds **13** and **14** were synthesized by consecutive reaction of β-(1→3)-*N*-acetylglucoaminyltransferase[33] and β-(1→4)-galactosyltransferase[18] and will be described elsewhere. Radioactive labeled sugar nucleotides were from NEN Life Science Products Inc. (Boston, MA). The radioactive nucleotide-sugars were diluted with unlabeled UDP-sugars (Sigma) to the desired specific radioactivity. All other oligosaccharide substrates were purchased from Sigma Chemical Co. (St. Louis, MO).

3.2. Construction of UDP-GalNAc-epimerase expression plasmid

Plasmid pACGP67B-GalNAcE was amplified in *E. coli* DH5α and the GalNAcE gene was subcloned into the expression plasmid pCWori+ using the restriction sites *Nde*I and *Eco*RI of the vector. The 5′ primer (galNacE-5p) was designed with 30 nucleotides containing the *Nde*I restriction site. The start codon, ATG, is included within the *Nde*I restriction site (bold italics) (5′-GTGAGA***CATATG***GAGGAGAAGGTGCTCGTC-3′). The 3′ primer (gal NAcE-3p) was a 30 mer containing the *Eco*RI restriction site (bold) followed by the complement (TCA) of the stop codon, TGA, (bold italics); 5′-GAACCCCGCGTACCG***ACTCTTAAG***TTGATC-3′. The resulting vector, pCWori-GalNAcE, was first transformed into *E. coli* DH5α (cloning host) and subsequently to the bacterial cells AD202[25] for expression.

3.3. Construction of CgtA, CgtB, and Cst-II expression plasmids

The *cgtA* gene was amplified from *C. jejuni* ATCC 43456 (GenBank accession # AF401528), the *cgtB* gene was amplified from *C. jejuni* NCTC 11168 (GenBank accession # AL139077, Cj1139c), and the *cst-II* gene was amplified from *C. jejuni* OH4384 (GenBank accession # AF130984). All three PCR products were digested with *Nde*I and *Sal*I and cloned in pCWori+ giving the constructs pCJL-30 (CgtA), pCJL-20 (CgtB), and pCST-68 (Cst-II). The *cst-II* gene in construct CST-68 also includes a point mutation (Ile53Ser) that enhances the production of sialyltransferase activity.[23]

3.4. Production of enzymes; Cst-II, CgtA, CgtB, and GalNAcE in *E. coli*

All the desired enzymes were overexpressed in *E. coli* AD202 and produced in a 100 L Fermentor (Braun Fermentor). Bacteria were cultured in 2xYT/Ampicillin (150 μg/mL) and induced with *iso*-propyl-thiogalactopyranoside (IPTG, 1 mM) with the conditions optimal for each enzyme. The constructs expressing the bacterial enzymes from *C. jejuni* (CgtA, CgtB, and Cst-II) demonstrated a slow growth and required incubation up to 24 h at 37 °C for CgtA, CgtB and 25 °C for Cst-II to reach the optimal OD of 0.3–0.6 at A_{600} for induction with IPTG. After induction, temperature decreased to 20 °C for CgtA and CgtB, whereas Cst-II continued to grow at 25 °C to reach the maximum growth of bacteria. The rat GalNAcE was incubated at 37 °C for 4 h to reach the optimal OD = 0.3–0.6 at A_{600} for induction. The incubation continued at 37 °C to complete the growth. Cells were harvested by spinning at 5000*g* for 30 min. The cell paste was weighed and re-suspended in the appropriate buffer (see assay conditions) in a 1 g/mL ratio of cell weight to the buffer. The cell paste was aliquoted to the desired volume and stored at −20 °C. To release the enzyme, cells were lysed with a microfluidizer procedure by adding 1/1 volume of HEPES buffer to the resuspended cell paste. The released enzymes were used as a crude suspension in the assays.

3.5. Enzyme activity assays

The assays were generally conducted in a final volume of 100 μL at 37 °C for 30 min with the desired enzyme (10 μL of crude mixture), $MnCl_2$ (100 mM), bovine serum albumin (1%), and the appropriate acceptor/donor substrate for each enzyme as follows: Cst-II assays were performed with lactose (100 mM) and [C^{14}]-CMP sialic acid (20 nmol, 2500 specific activity) in HEPES buffer (50 mM, pH 7.5). CgtA assays were carried out with sialyllactose (5 mM) and [H^3]-UDPGalNAc (50 nmol, 1000 specific activity) in HEPES (50 mM, pH 7.0). CgtB assays were performed with GM2 (20 mM) and [H^3]-UDPGal (50 nmol, 1000 specific activity) in MES (20 mM, pH 6.0). The enzyme activity of the GalNAcE was indirectly detected by GM2 formation using both epimerase and CgtA in the presence of UDP-GlcNAc (150 mM) as the donor and sialyllactose (5 mM) as an acceptor substrate. The CgtA and CgtB reactions were terminated by adding the ice-cold phosphate buffer (1 mL, 10 mM) to the reaction and were analyzed on

phosphate-Dowex-ion exchange column (method A). The Cst-II reaction was terminated with 1 mL of sodium formate (30 mM) and further analyzed on formate-Dowex-ion exchange.

3.6. Acceptor substrate specificity assays

3.6.1. Method A—neutral acceptors. Enzyme activity for different neutral acceptors was assayed at 25 °C for 30 min in a final volume of 100 μL containing HEPES (50 mM, pH 7.5), [H^3]-UDP-GalNAc (2 mM, 1.7 mCi/mol) (0.5 mM), or [C^{14}]-CMP-Neu5Ac acceptor (5 mM) final specific activity (0.70 Ci/mol) and diluted enzyme. Reactions were terminated by the addition of ice cooled EDTA (10 mM, 0.9 mL). In all cases, reactions were limited to transfer of less than 10% of the sugar nucleotide donor. The reaction mixtures were then passed through Pasteur pipet columns of Dowex resin (1-×8, 200–400 mesh, formate form, 0.5 × 5 cm, 1 mL). The columns were washed with sodium formate (20 mM, pH 7.0, 1.3 mL) and the effluents were directly collected in scintillation vials. The radioactivity was assayed as described above.

3.6.2. Method B—sialylated hydrophobic acceptors. The assays were conducted in a final volume of 100 μL at 25 °C for 30 min containing the acceptor (5 mM) and [H^3]-UDP-GalNAc (2 mM, 1.7 mCi/mol) (0.5 mM) or [^{14}C]-CMP-Neu5Ac in sodium HEPES buffer (50 mM, pH 7.5), $MnCl_2$ (10 mM), and bovine serum albumin (1%). Reactions were initiated by addition of the appropriate crude glycosyltransferase diluted in buffer containing bovine serum albumin (1%). Reactions were terminated by addition of ice-cooled EDTA buffer (10 mM, 0.9 mL). In all cases, reactions were limited to transfer of less than 10% of the sugar nucleotide donor. The mixture was applied to Sep-Pak C-18 reverse-phase cartridges (500 mg) (Isolute, International Sorbent Technology, Mid-Glamorgan, UK), which were washed with 5 mL of water. Radioactive products were eluted with MeOH (2 mL) and the eluate was evaporated to dryness and radioactivity was counted as described above.

3.7. General procedure for Fmoc-derivatization of 2-azidoethyl glycosides (15–25)

2-Azidoethyl glycoside (50 μmol) and Pd/C (10%, 50 mg) were suspended in water (5 mL) and ethanol (5 mL) and the mixture was vigorously stirred under a hydrogen atmosphere for 1 h at room temperature. When TLC (ethyl acetate–acetic acid–MeOH–water, 6:3:3:2 by volume) indicated complete reduction of azide to amine, sodium hydrogen carbonate was added (1 M, 2 mL) followed by 9-fluorenyl methylcarbonylchloroformate (2 equiv). After 30 min reaction at room temperature, the mixture was centrifuged to remove solid particles. The supernatant was diluted to 50 mL with water and loaded onto a SepPak cartridge (10 g). The column was washed with water and products were eluted with a gradient of MeOH (0–50%). Appropriate fractions were collected and lyophilized to dryness. Compounds were identified by MS and typical yields of 50–80% were obtained with a purity >90% according to TLC.

3.8. Synthesis of 2-azidoethyl O-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (8)

O-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)1,2,4,6-tetra-*O*-acetyl-β-D-glucopyranose (90 g, 130 mmol) and 2-azidoethanol[34,†] (23 g, 260 mmol, 2 equiv) were dissolved in dry CH_2Cl_2 (100 mL) containing molecular sieves (4 Å) and cooled on ice. After 30 min stirring, boron trifluoride etherate (23 g, 160 mmol, 2 equiv) was added and the reaction was kept under a nitrogen atmosphere for 12 h while it was slowly reaching room temperature. The mixture was washed with water (1 L) and sodium hydrogen carbonate (1 M, 2 × 1 L). The organic layer was dried with magnesium sulfate, filtered, and evaporated to dryness. The crude mixture was dissolved in dry CH_2Cl_2 (100 mL), cooled in ice for 30 min, and then hydrazine acetate (6.0 g, 65 mol, 0.5 equiv) was added to hydrolyze un-reacted per-acetylated lactose. After 30 min, the mixture was washed with sodium hydrogen carbonate (1 M, 2 × 1 L). The organic layer was dried with magnesium sulfate, filtered, and evaporated to dryness. The crude mixture was loaded onto a silica gel column (10 × 40 cm) packed in toluene and the product was eluted with toluene–ethyl acetate (4:1 by volume, 1 vol), toluene–ethyl acetate (3:1 by volume, 1 vol), and toluene–ethyl acetate (2:1 by volume, 1 vol). Appropriate fractions containing **8** were collected and evaporated to dryness to give 38 g of 2-azidoethyl *O*-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside. The clear syrup was dissolved in dry MeOH (200 mL) and de-acetylated by adding sodium methoxide (20 mL, 0.5 M) at room temperature overnight. The mixture was neutralized with MeOH-washed Dowex-50-H^+, filtered, and evaporated to approximately 100 mL. Diethyl ether (100 mL) was added to initiate crystallization. After filtration and drying, white crystals of **8** were obtained (25 g, 63 mmol, 47%). Mp 145–147 °C; $[\alpha]_D^{20}$ +36.8 (*c* 0.025, water). Selected ^{1}H NMR (500 MHz, D_2O), δ (ppm): 4.53 (d, 1H, J = 8 Hz, Glc H-1), 4.45 (d, 1H, J = 8 Hz, Gal H-1), 4.06–4.04 (m, 1H, $OCH_2CH_2N_3$), 3.98 (dd, 1H, Gal H-4), 3.92 (d, 1H, Glc H-4), 3.36–3.33 (m, 1H, $OCH_2CH_2N_3$). Selected^{13}C NMR (600 MHz, D_2O), δ (ppm): 102.57, 101.81, 77.95, 75.00, 74.46, 73.98,

† 2-Azidoethanol is potentially explosive and adequate precaution should be taken when handling this reagent.

72.41, 72.16, 70.60, 68.19, 60.68, 59.69, 50.17. ESI-TOF high-accuracy MS *m/z* calculated for (M+H), 412.1567; found, 412.1533.

3.9. Synthesis of GD3 oligosaccharide (27)

Compound **8** (2 g, 5 mmol) and crude CMP-Neu5Ac[18] (25 g, 25 mmol, 46% by weight) were dissolved in cacodylate buffer (200 mM, 50 mL, pH 7.5) containing $MnCl_2$ (40 mM). The Cst-II enzyme (24 U) was added and the reaction was slowly stirred at room temperature for 48 h. When 95% of the starting material was converted to products (10% GM3 oligosaccharide, 60% GD3 oligosaccharide, and 25% GT3 oligosaccharide approximately as visualized by TLC), the mixture was centrifuged and loaded onto a column of Sephadex G15 (5 × 170 cm) equilibrated and eluted with water. Appropriate fractions, containing all products and nucleotide sugar, were collected (1.2 L) and loaded onto a Dowex 1 × 8-400 formate ion exchange resin (30 × 2.5 cm). Compound **8**, GM3, and free Neu5Ac were in the void fractions whereas GD3 oligosaccharide, GT3 oligosaccharide, and nucleotides absorbed to the resin. The column was washed with water (500 mL) followed by a gradient of aqueous sodium formate (20–80 mM, pH 7.5, 2.5 L). Fractions containing each product were concentrated by lyophilizing and each oligosaccharide was further purified by size exclusion chromatography (Sephadex G15, 5 × 170 cm and packed and eluted with 5% aqueous *n*-BuOH) to give **27** (2.42 g, 34%) and **28** (0.39 g, 6%). GD3 oligosaccharide **27** selected ^{1}H NMR (600 MHz, D_2O), δ (ppm): 4.53 (d, 2H, J = 8 Hz, Glc H-1, Gal H-1), 4.06–4.04 (2m, 2H, $OCH_2CH_2N_3$), 3.35–3.33 (m, 1H, $OCH_2CH_2N_3$), 2.78 (dd, 1H, J = 4 Hz, NeuAc H-3eq), 2.68 (dd, 1H, J = 4 Hz, NeuAc H-3eq), 2.07 (s, 3H, $NHCOCH_3$), 2.03 (s, 3H, $NHCOCH_3$), 1.75 (t, 2H, J = 12 Hz, NeuAc H-3ax). Selected ^{13}C NMR (600 MHz, D_2O), δ (ppm): 102.32, 101.85, 77.92, 77.55, 75.13, 74.89, 74.51, 73.89, 73.71, 72.45, 72.32, 74.45, 68.95, 68.19, 67.60, 67.11, 62.20, 62.10, 61.29, 61.26, 60.79, 59.61, 51.92, 51.40, 50.18, 40.19, 39.40, 21.99, 21.70. ESI-TOF high-accuracy MS *m/z* calculated for (M+H), 1038.3114; found, 1038.3098.

3.10. Synthesis of GT3 oligosaccharide (28) and tetra-sialyllactoside (29)

Compound **8** (1.5 g, 3.6 mmol) and crude CMP-Neu5Ac[18] (20 g, 46% by weight, 14.6 mmol) were dissolved in cacodylate buffer (200 mM, 50 mL, pH 7.5) containing $MnCl_2$ (40 mM), then Cst-II (20 U) was added, and the reaction was slowly stirred at room temperature for 48 h. After 4 h, 50% GD3 oligosaccharide and 10% GT3 oligosaccharide were formed and 30% of **8** remained. When <90% of the starting material was converted to products (15% tetra-sialyllactoside, 50% GT3 oligosaccharide, 20% GD3 oligosaccharide, and <10% lactoside as visualized by TLC), the mixture was centrifuged and loaded onto a column of Sephadex G15 (5 × 170 cm) equilibrated, packed, and eluted with water. Appropriate fractions, containing all products and nucleotide sugar, were collected (1.2 L) and loaded onto a Dowex 1 × 8-400 formate ion exchange resin (30 × 2.5 cm). Compound **8**, GM3, oligosaccharide, and free Neu5Ac were in the void fractions, whereas GD3 oligosaccharide, GT3 oligosaccharide, and nucleotides absorbed to the resin. The column was washed with water (500 mL) followed by elution with a gradient of aqueous sodium formate (20–80 mM, pH 7.5, 2.5 L). Fractions containing each product were concentrated by lyophilizing and further purified by size exclusion chromatography (Sephadex G15, 5 × 170 cm, packed and eluted with 5% aqueous *n*-BuOH) to give **28** (1.0 g, 20%) and tetra-sialo-lactoside **29** (0.25 g, 4%). GT3 oligosaccharide **28** selected ^{1}H NMR (600 MHz, D_2O), δ (ppm): 4.53 (d, 2H, J = 8 Hz, Glc H-1, Gal H-1), 4.07–4.05 (2m, 2H, $OCH_2CH_2N_3$), 3.37–3.34 (m, 1H, $OCH_2CH_2N_3$), 2.77 (dd, 2H, J = 4 Hz, NeuAc H-3eq), 2.69 (dd, 1H, J = 4 Hz, NeuAc H-3eq), 2.07 (s, 6H, $NHCOCH_3$), 2.03 (s, 3H, $NHCOCH_3$), 1.75 (t, 2H, J = 12 Hz, NeuAc H-3ax), 1.70 (t, 1H, J = 12 Hz, NeuAc H-3ax). Selected ^{13}C NMR (600 MHz, D_2O): 174.48, 102.12, 101.65, 77.89, 77.38, 74.99, 74.71, 74.33, 73.72, 73.22, 73.00, 72.27, 72.10, 71.20, 69.74, 68.82, 68.75, 68.55, 68.03, 68.03, 67.98, 67.88, 67.68, 67.62, 67.51, 67.45, 67.05, 66.93, 66.46, 62.03, 60.89, 60.57, 60.31, 59.47, 51.81, 51.21, 51.13, 49.99, 40.50, 39.89, 39.66, 39.14, 38.71, 21.91, 21.63, 21.50. ESI-TOF high-accuracy MS *m/z* calculated for (M+Na), 1351.3888; found, 1351.3858.

Tetra-sialyllactoside **29** selected ^{1}H NMR (600 MHz, D_2O), δ (ppm): 4.53 (d, 1H, J = 8 Hz, Glc H-1), 4.51 (d, 1H, J = 8 Hz, Gal H-1), 4.07–4.05 (2m, 2H, $OCH_2CH_2N_3$), 3.36–3.33 (m, 1H, $OCH_2CH_2N_3$), 2.76 (dd, 1H, J = 4 Hz, NeuAc H-3eq), 2.68 (dd, 3H, J = 4 Hz, NeuAc H-3eq), 2.08 (s, 6H, $NHCOCH_3$), 2.07 (s, 3H, $NHCOCH_3$), 2.03 (s, 3H, $NHCOCH_3$), 1.74 (q, 4H, J = 12 Hz, NeuAc H-3ax).

^{13}C NMR (600 MHz, D_2O), δ (ppm): 174.65, 174.58, 102.30, 101.83, 78.14, 77.55, 77.41, 75.17, 74.89, 74.52, 73.89, 73.42, 73.16, 72.86, 72.45, 72.27, 71.40, 69.24, 69.09, 68.94, 68.74, 68.55, 68.22, 68.17, 67.92, 67.87, 67.68, 67.12, 62.24, 61.17, 61.01, 60.86, 60.76, 59.64, 52.09, 52.02, 51.99, 51.39, 50.175, 40.08, 39.72, 39.30, 22.11, 21.99, 21.70. ESI-TOF high-accuracy MS *m/z* calculated for (M+Na), 1620.5015; found, 1620.5019.

3.11. General procedure for the synthesis of GM2 (30), GD2 (31), and GT2 (32) oligosaccharides

Sialylated acceptors **26**–**28** (0.70 mmol) and UDP-GalNAc (1.1 mmol, 1.8 equiv) were dissolved in Tris

(100 mM, 10 mL, pH 7.5) containing $MnCl_2$ (40 mM), then NAD^+ (0.3 mM), CgtA (30 U), and GalNAcE (800 U) were added, and the reaction was kept at 37 °C for 24 h. When >95% of the starting material was converted to product, the mixture was centrifuged and loaded onto a column of Sephadex G15 (2.5 × 170 cm) equilibrated and eluted with 5% *n*-BuOH in water. Appropriate fractions, containing the product, were collected by lyophilization and loaded onto a Sephadex G15 (1.6 × 170 cm) column and eluted with water to give **30**, **31**, or **32** (580–650 mg, 80–85%).

GM2 oligosaccharide **30**, Selected ^{1}H NMR (600 MHz, D_2O), δ (ppm): 4.74 (d, 1H, J = 8 Hz, GalNAc H-1), 4.54 (d, 1H, J = 8 Hz, Glc H-1), 4.53 (d, 1H, J = 8 Hz, Gal H-1), 4.06–4.04 (2m, 2H, $OCH_2CH_2N_3$), 3.36–3.33 (m, 1H, $OCH_2CH_2N_3$), 2.66 (dd, 1H, J = 4 Hz, NeuAc H-3eq), 2.03 (s, 3H, $NHCOCH_3$), 2.017 (s, 3H, $NHCOCH_3$ of GalNAc), 1.92 (t, 1H, J = 12 Hz, NeuAc H-3ax). Selected 13C NMR (600 MHz, D_2O) δ (ppm): 102.43, 102.26, 101.83, 78.20, 76.84, 74.57, 74.38, 74.12, 74.01, 73.68, 72.74, 72.36, 71.95, 70.94, 69.69, 68.38, 68.21, 67.67, 67.44, 74.57, 74.12, 62.50, 60.84, 60.22, 59.74, 56.10, 52.00, 51.25, 50.19, 36.58, 22.27, 21.71. ESI-TOF high-accuracy MS *m*/*z* calculated for (M+H), 928.3134; found, 929.3174.

GD2 oligosaccharide **31**, selected ^{1}H NMR (600 MHz, D_2O), δ (ppm): 4.70 (d, 1H, GalNAc H-1), 4.53 (d, 1H, J = 8 Hz, Glc H-1), 4.51(d, 1H, J = 8 Hz, Gal H-1), 4.06–4.04 (m, 1H, $OCH_2CH_2N_3$), 3.35–3.33 (m, 1H, $OCH_2CH_2N_3$), 2.77 (dd, 1H, J = 4 Hz, NeuAc H-3eq), 2.68 (dd, 1H, J = 4 Hz, NeuAc H-3eq), 2.07 (s, 3H, $NHCOCH_3$), 2.04 (s, 3H, $NHCOCH_3$), 2.03 (s, 3H, $NHCOCH_3$), 1.75 (m, 2H, J = 12 Hz, NeuAc H-3ax). Selected ^{13}C NMR (600 MHz, D_2O) δ (ppm): 102.40, 101.83, 81.20, 77.99, 77.87, 75.63, 74.56, 74.18, 73.91, 73.39, 72.72, 72.38, 72.31, 71.42, 71.04, 70.52, 69.37, 68.92, 68.87, 68.17, 67.78, 67.37, 61.13, 60.60, 60.30, 59.70, 59.64, 59.57, 52.02, 51.47, 51.31, 50.18, 49.81, 40.19, 40.15, 40.06, 38.89, 22.24, 22.01, 21.70. ESI-TOF high-accuracy MS *m*/*z* calculated for (M+H), 1241.3908; found, 1241.3892.

GT2 oligosaccharide **32**, selected ^{1}H NMR (600 MHz, D_2O), δ (ppm): 4.71 (d, 1H, J = 8 Hz, GalNAc H-1), 4.53 (d, 2H, J = 8 Hz, Glc H-1), 4.50 (d, 1H, J = 8 Hz, Gal H-1), 2.77 (dd, 2H, J = 4 Hz, NeuAc H-3eq), 2.68 (dd, 2H, J = 4 Hz, NeuAc H-3eq), 2.05 (m, 12H, $NHCOCH_3$), 1.74 (m, 3H, J = 12 Hz, NeuAc H-3ax). Selected ^{13}C NMR (600 MHz, D_2O) δ (ppm): 174.64, 174.59, 174.46, 102.41, 101.81, 77.92, 77.34, 76.82, 75.74, 74.78, 74.50, 74.42, 74.36, 74.14, 74.100, 73.97, 73.89, 73.66, 73.45, 72.99, 72.71, 72.47, 72.34, 72.30, 71.93, 71.77, 71.70, 71.40, 70.90, 70.50, 69.91, 60.81, 60.48, 60.26, 68.16, 68.04, 67.63, 67.41, 67.36, 67.19, 66.54, 62.21, 62.12, 60.58, 51.97, 51.32, 50.17, 40.67, 38.86, 22.21, 21.82, 21.70. ESI-TOF high-accuracy MS *m*/*z* calculated for (M+H), 1554.4682; found, 1554.4675.

3.12. Synthesis of GM1 oligosaccharide (33)

Compound **30** (500 mg, 0.552 mmol) and UDP-Glc (404 mg, 0.662 mmol) were dissolved in cacodylate buffer (200 mM, 1 mL, pH 7.5) containing $MnCl_2$ (40 mM), then CgtB (3U) and GalNAcE (30U) were added, and the reaction was slowly stirred at room temperature for 48 h. More than 95% of the starting material was converted to product and the mixture was centrifuged and loaded onto a column of Sephadex G15 (5 × 170 cm) equilibrated and eluted with water. The fractions containing product were concentrated by lyophilizing to give pure **33** (508 mg, 0.475 mmol, 86%). Selected ^{1}H NMR (600 MHz, D_2O), δ (ppm): 4.78 (d, 1H, J = 8 Hz, GalNAc H-1), 4.54 (d, 2H, J = 8 Hz, Gal H-1), 4.53 (d, 1H, J = 8 Hz, Glc H-1), 4.06–4.04 (m, 2H, $OCH_2CH_2N_3$), 3.36–3.33 (m, 1H, $OCH_2CH_2N_3$), 2.66 (dd, 1H, J = 4 Hz, NeuAc H-3eq), 2.03 (s, 3H, $NHCOCH_3$), 2.017 (s, 3H, $NHCOCH_3$ of GalNAc), 1.92 (t, 1H, J = 12 Hz, NeuAc H-3ax). Selected ^{13}C NMR (600 MHz, D_2O) δ (ppm): 174.63, 174.39, 173.75, 104.37, 102.20, 101.78, 101.27, 79.94, 78.15, 76.76, 74.50, 74.40, 73.97, 73.68, 72.70, 72.30, 72.10, 71.90, 70.29, 69.64, 68.34, 68.20, 68.16, 67.62, 67.52, 62.43, 60.72, 60.55, 60.22, 59.68, 51.21, 50.81, 50.15, 36.52, 29.85, 22.20, 21.66. ESI-TOF high-accuracy MS *m*/*z* calculated for (M+H), 1090.3663; found, 1090.3672.

3.13. Synthesis of GD1a oligosaccharide (34)

Compound **33** (50 mg, 46.8 μmol) and crude CMP-Neu5Ac[18] (108 mg, 93.6 μmol, 55% by weight) were dissolved in Tris buffer (100 mM, 2 mL, pH 7.5) containing $MnCl_2$ (20 mM), then ST3Gal I (2U) was added, and the reaction was slowly stirred at room temperature for 12 h. The conversion to product seemed complete by TLC. The mixture was centrifuged and loaded onto a column of Sephadex G15 (5 × 170 cm) equilibrated and eluted with water. Fractions containing product were lyophilized to give **34** (56 mg, 88%). Selected ^{1}H NMR (600 MHz, D_2O) δ (ppm): 4.61 (d, 1H, J = 8 Hz, GalNAc H-1), 4.54 (d, 2H, J = 8 Hz, Gal H-1), 4.52 (d, 1H, J = 8 Hz, Glc H-1), 2.75 (dd, 1H, J = 4 Hz, NeuAc H-3eq), 2.68 (dd, 1H, J = 4 Hz, NeuAc H-3eq), 2.03 (d, 6H, $NHCOCH_3$), 2.00 (s, 3H, $NHCOCH_3$ of GalNAc), 1.91 (t, 1H, J = 12 Hz, NeuAc H-3ax), 1.80 (t, 1H, J = 12 Hz, NeuAc H-3ax). Selected ^{13}C NMR (600 MHz, D_2O) δ (ppm): 174.65, 174.35, 173.70, 104.15, 102.25, 101.81, 101.08, 99.38, 80.14, 78.17, 76.51, 75.13, 74.45, 74.29, 73.99, 73.73, 72.70, 72.44, 72.34, 71.48, 69.62, 68.78, 68.36, 68.20, 68.15, 67.78, 67.69, 67.41, 67.06, 62.47, 62.15, 60.78, 60.60,

60.25, 59.71, 51.25, 50.70, 50.19, 39.31, 36.86, 22.26, 21.71. ESI-TOF high-accuracy MS *m/z* calcd for (M+H), 1403.4358; found, 1403.4382.

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