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Chemoenzymatic synthesis of GD3 oligosaccharides and other disialyl glycans containing natural and non-natural sialic acids

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Abstract

In order to understand the biological importance of naturally occurring sialic acid variations on disialyl structures in nature, we developed an efficient two-step multi-enzyme approach for the synthesis of a series of GD3 ganglioside oligosaccharides and other disialyl glycans containing a terminal Siaα2–8Sia component with different natural and non-natural sialic acids. In the first step, α2–3- or α2–6-linked monosialylated oligosaccharides were obtained using a one-pot three-enzyme approach. These compounds were then used as acceptors for the α2–8-sialyltransferase activity of a recombinant truncated multi-functional *Campylobacter jejuni* sialyltransferase CstII mutant, CstIIΔ32^{I53S}, to produce disialyl oligosaccharides. The α2–8-sialyltransferase activity of CstIIΔ32^{I53S} has promiscuous donor substrate specificity and can tolerate various substitutions at C-5 or C-9 of the sialic acid in CMP-sialic acid, while its acceptor substrate specificity is relatively restricted. The terminal sialic acid residues in the acceptable monosialylated oligosaccharide acceptors are restricted to Neu5Ac, Neu5Gc, KDN, and some of their C-9 modified forms but not their C-5 derivatives. The disialyl oligosaccharides obtained are valuable probes for their biological studies.

Keywords

carbohydrate; chemoenzymatic synthesis; enzyme; GD3 oligosaccharides; sialic acid; sialosides

Introduction

Sialic acids (Sia) are a diverse family of naturally occurring polyhydroxy keto aldonic acids that are broadly distributed in animals and are involved in a wide range of biological processes.[1] In most cases, *N*-acetylneuraminic acid (Neu5Ac), the most abundant sialic acid form, and other common forms such as *N*-glycolylneuraminic acid (Neu5Gc), keto-deoxy-nonulosonic acid (KDN), and their naturally occurring derivatives, are frequently located on cell surface as the terminal monosialyl residues α2–3- or α2–6-linked to galactosides or 2-acetamino-2-deoxy-galactosides in biologically active glycoconjugates.[2] Disialyl structures Siaα2–8Sia containing diverse sialic acid forms (Figure 1) have also been found as constituents of glycans

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Supporting Information Available: NMR spectra of monosialylated and disialylated oligosaccharide products. This material is available free of charge via the internet at http://pubs.acs.org.

in many glycoproteins and glycolipids including gangliosides which are sialylated glycosphingolipids that are presented on the outer leaflets of plasma membranes.[1c]

α2–8-Linked disialyl moiety Neu5Acα2–8Neu5Ac is a common structural unit of GD1c, b-series gangliosides (e.g. GD3, GD2, GD1b, GT1b, GQ1b, and GQ1bα), and of GT1a and GP1c belonging to aand c- series gangliosides respectively.[3] The simplest member of this group is ganglioside GD3 which has been shown to be a human melanoma associated antigen.4 Disialyl structures containing other sialic acid forms have also been reported. For example, Neu5Acα2–8Neu5Gc has been found on gangliosides in mouse thymoma and thymocytes,5 cat and sheep erythrocytes,6 and bovine brain.[7] Neu5Gcα2–8Neu5Gc has been found on gangliosides in mouse thymoma and thymocytes,5 human gastrointestinal adenocarcinoma, and gastric cancer cell MKN74.8 Neu5Gcα2–8Neu5Ac has been found on gangliosides in mouse5 and rabbit thymus.[9] 9OAc-Neu5Acα2–8Neu5Ac has been found on GD3 (9OAc-GD3) gangliosides in human melanoma,[10] GD1b in bovine brain,11 GT3 in chicken and rat brain,12 and GT2 in Alaskan pollack brain13 and cod brain.[14] 9OAc-GD3 has been shown to be an important regulatory molecule involved in signal transduction, regulation of cell growth and differentiation, apoptosis, and inflammation, etc.[15] Sulfated disialyl structure 8OSO_3^--Neu5Acα2–8Neu5Ac has been observed in ganglioside GD3 in bovine gastric mucosa16 and sea urchin sperm.[17] Although most disialyl sequences are α2–3-linked to a galactose (Gal) moiety in gangliosides, a few Neu5Acα2–8Neu5Ac sequences have also been found to link to *N*-acetylgalactosamine (GalNAc) or glucose (Glc) through an α2–6-sialyl linkage.[17]

The α2–8-linked disialyl glycans have also been discovered in glycoproteins.[18] For example, Siaα2–8Sia units have been found in both *O*-linked and *N*-linked polysialylglycoproteins from trout egg,[19] vertebrates/embryonic brain,[20] eel/rat brain,[21] human tumor,[22] fruit fly (*Drosophila*),[23] cicada,[24] and rainbow trout ovarian fluid.[25] More specifically, Neu5Acα2–8Neu5Ac has been found in *O*-linked glycoproteins from bovine adrenal medulla[26] and human erythrocyte glycophorin,[27] as well as *N*-linked glycoproteins from umbilical cord erythrocyte Band 3[28] and ovarian fluid of rainbow trout.[29] Neu5Gcα2–8Neu5Gc has been found in both *O*-linked and *N*-linked glycans in the proteins from bovine adrenal medulla,[26] pig spleen,[30] rat thymus,[31] and recently in mouse serum.[32] Although not existing in glycolipids, KDNα2–8KDN has been found in *O*-linked glycoproteins from rat kidney[33] and various rat organs.[34] Moreover, polysialic acids with Neu5Acα2–8Neu5Ac repeating units are the major components of capsular polysaccharides of group B *Neisseria meningitidis*, *Escherichia coli* K1, *Moraxella nonliquifaciens*, and *Pasteurella haemolytica* A2.[35]

Disialyl structures are believed to play important roles in numerous biological events.[15,36] For example, Siglec-7, an inhibitory receptor expressed on natural killer (NK) cells, shows a significant preference for α2–8-linked disialyl ligands[37] such as GD3 whose expression on the target cells can suppress NK cell-mediated cytolytic activity.38

Nevertheless, the low availability of pure disialyl glycans and glycoconjugates from natural sources make it difficult to elucidate their biological functions. On the other hand, chemical formation of Siaα2–8Sia-linkage is one of the most challenging tasks in chemical glycosylation due to the sterically hindered tertiary anomeric center, lack of a stereo-directing group adjacent to the anomeric position, the presence of an electron-withdrawing carboxyl group in sialyl donors, and the low reactivity of the C8 hydroxyl group caused by C1 carboxyl and/or the C5 acetamide group in the sialic acid of sialyl acceptors.[39] Recently, the introduction of *N*,*N*-diacetyl,[40] azido,[41] *N*-trifluoroacetyl (*N*-TFA),[42] *N*-Troc,[43] *N*Fmoc,[43b,43c] *N*-trichloroacetyl,[43b,43c] and *N*-phthalimido group[44] at the C5 position in sialyl donors have been reported to exhibit improved donor reactivity towards sialylation. Some of these sialyl donors have been applied in the synthesis of α2–8-linked disialylated oligosaccharides in moderate yields.[40b,42b,44] The 1,5-lactam derivative of sialic acids[45] and 5-*N*,4-*O*-carbonyl protected

oxazolidinone sialyl donor[46] have also been developed for the synthesis of α2–8-linked disialosides. Despite the advance, current chemical synthesis of sialosides remains to be a time-consuming process and requires skillful expertise.

In comparison, sialyltransferase-catalyzed sialylation with intrinsic high regio- and stereoselectivity, as well as mild and environment-friendly reaction conditions, offers great advantages and is considered an attractive and a practical approach for the synthesis of sialosides including those containing disialyl motifs. Recent identification and cloning of a bi-functional bacterial sialyltransferase CstII from *Campylobacter jejuni* OH4384 that can catalyze the formation of both α2–8- and α2–3- sialyl linkages[47] provide a unique catalyst for efficient synthesis of ganglioside oligosaccharides and their derivatives.[48] Nevertheless, both chemical and enzymatic syntheses of α2–8-linked sialosides have been so far limited to Neu5Ac[40b, 42b, 44-49] and some Neu5Gc[50]-containing structures. In order to understand the importance of variations of naturally existing sialic acid forms in α2–8-linked sialosides, herein we report a facile two-step multi-enzyme approach for preparative chemoenzymatic synthesis of α2–8-linked disialyl oligosaccharides containing Neu5Ac, Neu5Gc, KDN, and their derivatives. The success of this method is demonstrated by the production of a series of GD3 ganglioside oligosaccharides and other disialyl glycans containing natural and non-natural sialic acids.

Results and Discussion

Two-step multi-enzyme approach for the synthesis of disialyl oligosaccharides

As shown in Figure 2, we used a two-step process to produce disialyl oligosaccharides. In the first step, α2–3- or α2–6-linked monosialyl oligosaccharides containing different sialic acid forms were prepared using the one-pot three-enzyme method and purified as described previously.[51] They were then used in the second step as acceptors for the α2–8-sialyltransferase activity of CstIIΔ32^{I53S} for the synthesis of Siaα2–8Siaα2–3/6Gal-terminated disialyl oligosaccharides using a one-pot multi-enzyme approach containing two (with a CMP-sialic acid synthetase and an α2–8-sialyltransferase) or three enzymes (with an additional sialic acid aldolase compared to the two-enzyme approach). CstIIΔ32^{I53S} is a recombinant truncated form of CstII with a single amino acid mutation (I53S mutation was introduced to enhance the α2–8-sialyltransferase activity and to stabilize the enzyme47b) compared to CstII from *C. jejuni* OH4384.[47a] It was cloned from a synthetic gene whose codons were optimized for an *E. coli* expression system.[47c] Although CstIIΔ32^{I53S} is multifunctional and can catalyze the formation of both Siaα2–3Gal and Siaα2– 8Sia linkages,[47,48] its α2–3-sialyltransferase activity is lower than its α2–8-sialyltransferase activity. Therefore, CstIIΔ32^{I53S} was used only for its α2–8-sialyltransferase activity in the two-step process for the synthesis of Siaα2–8Siaα2–3Gal terminated disialyl oligosaccharides to provide a higher efficiency and a better control over the sialylation process.

The preparation of α2–3- or α2–6-linked monosialylated oligosaccharides was carried out using an efficient one-pot three-enzyme chemoenzymatic approach[52] developed in our lab. In this system, *N*-acetylmannosamine (ManNAc), mannose (Man), or their derivatives obtained by chemical or enzymatic modification, was coupled with pyruvate to form sialic acid derivatives by a sialic acid aldolasecatalyzed reaction. The sialic acid derivatives formed were then activated by a CMP-sialic acid synthetase and transferred to a suitable sialyltransferase acceptor for the formation of sialosides. Depending on the specificity of the sialyltransferase, α2–3- or α2–6-linked sialosides could be produced efficiently in a single pot without the purification of intermediates. Sialic acid aldolases from *E. coli* K12[53] and *Pasteurella multocida*,[54] CMP-sialic acid synthetase from *N. meningitidis* (NmCSS),[53] a multifunctional sialyltransferase from *Pasteurella multocida* (PmST1) for the formation of α2–3-linked sialosides,[51a] and a sialyltransferase from *Photobacterium damsela* (Pd2,6ST) for the

formation of α2–6-linked sialosides,[51b] were shown to be excellent catalysts for the synthesis of monosialylated glycans because they were able to be expressed in *E. coli* in large amounts with high activity and promiscuous substrate specificity.

With α2–3- and α2–6-linked monosialylated oligosaccharides in hands, α2–8-linked disialyl oligosaccharides were synthesized in the second step using the one-pot multi-enzyme process with CstIIΔ32^{I53S} as the α2–8-sialyltransferase and NmCSS with or without *E. coli* K12 sialic acid aldolase.[47c] The application of the method in the synthesis of the targeted GD3-type disialyl glycans was explored for two major groups: one group contained a penultimate α2–3-linked Neu5Ac with different terminal α2–8-linked sialic acid forms (**Sia**α2–8Neu5Acα2–3LacβProN$_3$) and the other contained a terminal α2–8-linked Neu5Ac with different α2–3-linked penultimate sialic acid forms (Neu5Acα2–8**Sia**α2–3GalβOR). In addition, the synthesis of GD3-type disialyl glycans (Neu5Gc/KDNα2–8Neu5Gc/KDNα2–3LacβProN$_3$) containing the combination of two other common sialic acid forms such as Neu5Gc and KDN was also carried out. The synthesis of Sia2–8Siaα2–6GalβOR-type disialyl glycans was investigated for the compounds containing a terminal α2–8-linked Neu5Ac with different α2–6-linked penultimate sialic acid forms (Neu5Acα2–8**Sia**α2–6GalβOR) using a one-pot two-enzyme system.

Preparation of GD3-type disialyl oligosaccharides Siaα2–8Neu5Acα2–3LacβProN$_3$ containing a penultimate α2–3-linked Neu5Ac and different terminal α2–8-linked sialic acid forms

GM3 oligosaccharide with a propyl azide aglycon (Neu5Acα2–3Galβ1–4GlcβProN$_3$ or Neu5Acα2–3LacβProN$_3$) **3** was readily obtained in a quantitative yield by incubating 3-azidopropyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (LacβProN$_3$) **1** with *N*-acetylmannosamine (ManNAc) **2** in the one-pot three-enzyme system as described previously.[51a] The product Neu5Acα2–3LacβProN$_3$ **3** was then used as an acceptor for the α2–8-sialyltransferase activity of CstIIΔ32^{I53S} in the one-pot multiple-enzyme synthesis of GD3-type disialyl oligosaccharides containing different terminal sialic acid forms. As shown in Figure 3, CstIIΔ32^{I53S} has promiscuous donor substrate specificity and can catalyze the transfer of different sialic acids from CMP-sialic acid derivatives synthesized by NmCSS with or without *E. coli* K12 sialic acid aldolase to form GD3 oligosaccharides **4**, **6**, **8**, **10**, **12**, **14**, **16**, and **18** with different terminal sialic acid forms in good to excellent yields (51–92%).

The synthesis of GD3 oligosaccharides **4**, **6**, **8**, **10**, **12**, and **14** was carried out in the one-pot three-enzyme system at pH 8.5. We found that the use of 1.2-fold excess amount of sialic acid precursors was optimum to prevent the multiple α2–8-sialylation by CstIIΔ32^{I53S}.[47c, 48] Under these conditions, disialyl GD3 oligosaccharides Neu5Acα2–8Neu5Acα2–3LacβProN$_3$ **4** and Neu5Gcα2–8Neu5Acα2–3LacβProN$_3$ **6** were obtained in 76% and 73% yields, respectively, from ManNAc **2** and *N*-glycolyl mannosamine (ManNGc) **5** as sialic acid precursors. The yield (65%) for the formation of KDNα2–8Neu5Acα2–3LacβProN$_3$ **8** from mannose **7** was lower due to the formation of by-product with multiple α2–8-linked sialic acids. Interestingly, the synthesis of Neu5GcMeα2–8Neu5Acα2–3LacβProN$_3$ **10** containing a terminal modified Neu5Gc with a methyl group at the C5-OH was achieved in high efficiency with a 92% yield from *N*-methylglycolyl-mannose (ManNGcMe) **9**. This may be due to the prevention of additional α2–8-sialylation by the extra methyl group at the C5-OH in the terminal Neu5Gc in the disialyl product **10**. Non-natural GD3 oligosaccharides Neu5AcN$_3$α2–8Neu5Acα2–3LacβProN$_3$ **12** and Neu5Ac9N$_3$α2–8Neu5Acα2–3LacβProN$_3$ **14** containing an azido group at the C-5 or C-9 position of the terminal Neu5Ac were also obtained in good yields (87% and 78%, respectively) from C2- or C6-modified ManNAc derivatives *N*-azidoacetyl mannoseamine (ManNAz) **11** and *N*-acetyl-9-azido-mannosamine (9N$_3$ManNAc) **13**, respectively.

Gangliosides, including GD3, containing a terminal 9-*O*-acetyl modified Neu5Ac are common.[10,11,55] The biological functions of 9-*O*-acetylated GD3 are believed to be distinct from its non-acetylated counterpart. For example, it has been found as a marker for neural differentiation and malignant transformation[56] and has been suggested to protect tumor cells from apoptosis.[57] Both 9-*O*-acetylated Neu5Ac and Neu5Gc are readily available from their corresponding non-*O*-acetylated forms by a regioselective chemical acetylation.[58] Briefly, treatment of Neu5Ac or Neu5Gc with trimethyl orthoacetate in anhydrous DMSO in the presence of a catalytic amount of *p*-TsOH gave 9-*O*-acetyl-*N*-acetyl neuraminic acid (Neu5,9Ac$_2$) **15** or 9-*O*-acetyl-*N*-glycolyl neuraminic acid (Neu5Gc9Ac) **17** in excellent yields (over 90%). GD3 oligosaccharides Neu5,9Ac$_2$α2–8Neu5Acα2–3LacβProN$_3$ **16** and Neu5Gc9Acα2–8Neu5Acα2–3LacβProN$_3$ **18** were obtained in a one-pot two-enzyme reaction containing NmCSS and CstIIΔ32^{I53S} in 53% and 51% yields from **15** and **17**, respectively. A Tris-HCl buffer solution of pH 7.5 was used to prevent the de-acetylation under basic solutions with pH higher than 7.5.

The structures of all purified GD3 oligosaccharide products were confirmed by nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (HRMS). Compairing the ^{13}C NMR spectra of GD3 oligosaccharide Neu5Acα2–8Neu5Acα2–3LacβProN$_3$ **4** and GM3 oligosaccharide Neu5Acα2–3LacβProN$_3$ **3** indicated a downfield chemical shift of 6.39 ppm for the C-8 of the internal Neu5Ac in Neu5Acα2–8Neu5Acα2–3LacβProN$_3$ **4** (78.28 ppm) compared to that for the C-8 of Neu5Ac (71.89 ppm) in Neu5Acα2–3LacβProN$_3$ **3**. These data confirmed the formation of an α2–8-sialyl linkage by CstIIΔ32^{I53S}-catalyzed sialylation when Neu5Acα2–3LacβProN$_3$ **3** was used as an acceptor for CstIIΔ32^{I53S}. Among the GD3 oligosaccharides (**4**, **6**, **8**, **10**, **12**, and **14**) synthesized here, only the preparation of Neu5Acα2–8Neu5Ac terminated disialyl oligosaccharides using a similar CstII-catalyzed sialylation of Neu5Ac-containing GM3 oligosaccharides has been reported.[47c, 48, 49e–49i]

Preparation of GD3-type disialyl oligosaccharides Neu5Acα2–8Siaα2–3GalβOR containing terminalα2–8-linked Neu5Ac and different penultimate α2–3-linked sialic acid forms

Small scale one-pot three-enzyme reactions were performed first and analyzed by thin-layer chromatography (TLC) to study the acceptor specificity of the α2–8-sialyltransferase (or GD3 synthase) activity of CstIIΔ32^{I53S}. Preparative-scale syntheses were then carried out for suitable acceptors. As summarized in Figure 4, CstIIΔ32^{I53S} exhibited good activity towards monosialylated oligosaccharides **19** or **21** which posses a terminal Neu5Gc or KDN. GD3 oligosaccharides Neu5Acα2–8Neu5Gcα2–3LacβProN$_3$ **20** and Neu5Acα2–8KDNα2–3LacβProN$_3$ **22** were obtained in 72% and 71% yields, respectively, in the presence of *E. coli* K12 sialic acid aldolases, NmCSS, and CstIIΔ32^{I53S} using ManNAc **2** as the sialic acid precursor. The yields are comparable to that (76%) for the synthesis of Neu5Acα2–8Neu5Acα2–3LacβProN$_3$ **4** (Figure 3) from Neu5Acα2–3LacβProN$_3$ **3** (which contains the most abundant sialic acid form Neu5Ac) as an acceptor. Interestingly, substituting the C9-hydroxyl group on the terminal Neu5Ac in sialoside **23** with an azido did not block the α2–8-sialylation reaction catalyzed by CstIIΔ32^{I53S}. Preparative synthesis of disialyl oligosaccharide **24** was achieved in 63% yield. Quite surprisingly, further modification on the C-5 of the terminal Neu5Ac, Neu5Gc, and KDN in α2–3-linked monosialylated oligosaccharides Neu5AcN$_3$α2–3LacOMe **25**, Neu5GcMeα2–3LacβProN$_3$ **26**, and KDN5Meα2–3LacβProN$_3$ **27** with either a methyl or an azido group totally blocked the α2–8-sialylation reaction catalyzed by CstIIΔ32^{I53S}. Taken together, these results indicate that the α2–8-sialyltransferase activity of CstIIΔ32^{I53S} can tolerate a limited number of groups (*N*-acetyl, *N*-glycol, and hydroxyl) at C-5 and modifications on the C-9 of the terminal sialic acid residue in α2–3-linked monosialylated oligosaccharides as acceptor substrates.

Among the α2–3-monosialylated oligosaccharides used here (**19**, **21**, **23**, **25**, **26**, and **27**) as the acceptors for the α2–8-sialyltransferase activity of CstIIΔ32^{I53S}, the synthesis of **19**, **21**, **25**, and **26** has been reported previously.[51a] The synthesis of two new α2–3-linked monosialylated oligosaccharides **23** and **27** were carried out in a one-pot three-enzyme system containing an *E. coli* sialic acid aldolase, NmCSS, and PmST1 similar to that described previously for the synthesis of other α2–3-linked sialosides.[51a] Compound **23** was obtained from 6-azido-6-deoxy-*N*-acetyl-D-mannosamine (6-N_3-ManNAc) as the precursor of a sialic acid derivative and methyl β-D-galactopyranoside as a sialyltransferase acceptor. Compound **27** was obtained from 2-*O*-methyl-D-mannose as the precursor of a sialic acid derivative and azidopropyl β-D-lactoside as a sialyltransferase acceptor. The preparation of these starting materials for the one-pot three-enzyme synthesis of α2–3-linked sialosides has been reported previously.[51]

Preparation of GD3-type disialyl oligosaccharides Neu5Gc/KDNα2–8Neu5Gc/KDNα2–3LacβProN_3 containing the combination of Neu5Gc and KDN

GD3-type disialyl oligosaccharides containing naturally occurring Neu5Gc and KDN sialic acid forms including Neu5Gcα2–8Neu5Gc, KDNα2–8KDN, and hybrid KDNα2–8Neu5Gc and Neu5Gcα2–8KDN units were also synthesized. As shown in Figure 5, monosialylated oligosaccharides Neu5Gcα2–3LacβProN_3 **19** (96%) and KDNα2–3LacβProN_3 **21** (94%) were synthesized in the step 1 from LacβProN_3 **1** and ManNGc **5** or mannose **7** in the presence of *E. coli* aldolase, NmCSS, and PmST1. Using Neu5Gcα2–3LacβProN_3 **19** as the acceptor for the α2–8-activity of CstIIΔ32^{I53S}, disialyl oligosaccharides Neu5Gcα2–8Neu5Gcα2–3LacβProN_3 **28** and KDNα2–8Neu5Gcα2–3LacβProN_3 **29** were prepared from donor substrates **5** and **7** in the one-pot three-enzyme system in 75% and 57% yields, respectively. Similarly, sialylation of KDNα2–3LacβProN_3 **21** with the donor substrate **5** and **7** in the presence of *E. coli* K12 sialic acid aldolase, NmCSS, and CstIIΔ32^{I53S} produced Neu5Gcα2–8KDNα2–3LacβProN_3 **30** (68%) and KDNα2–8KDNα2–3LacβProN_3 **31** (55%) in comparable yields. Again, the lower yields (55-57%) for the formation of disialyl oligosaccharides containing a terminal KDN **29** and **31** compared to those (68-75%) for the formation of Neu5Gc-terminated disialyl oligosaccharides **28** and **30** were due to the formation of by-product with multiple α2–8-linked sialic acids for KDN-terminated glycans.

Preparation of Neu5Ac2–8Siaα2–6GalβOR-type disialyl oligosaccharides containing a terminal α2–8-linked Neu5Ac and different penultimate α2–-6-linked sialic acid forms

Acceptor specificity of the α2–8-sialyltransferase activity of CstIIΔ32^{I53S} was also explored in a one-pot two-enzyme system with a panel of α2–6-linked monosialylated oligosaccharides containing natural sialic acid forms (Neu5Ac, Neu5Gc, and KDN) and non-natural sialic acids with various modifications at C-9 or C-5. To do this, α2–6-linked monosialylated oligosaccharides Siaα2–6GalβOR were synthesized from GalβOR using *Photobacterium damsela* α2–6-sialyltransferase (Pd2,6ST) in the one-pot three-enzyme system as described previously.[51b] Evaluation of Siaα2–6GalβOR as potential acceptors for the α2–8-sialyltransferase activity of CstIIΔ32^{I53S} was carried out in small-scale one-pot two-enzyme reactions from Neu5Ac the reactions and analyzed by thin-layer chromatography (TLC). Similar to human polysialyltransferases ST8SiaII (STX) and ST8SiaIV (PST) reported previously,[59] CstII exhibited acceptor specificity towards a list of α2–6-sialosides. α2–6-Linked sialyl lactosides Neu5Acα2–6LacβProN_3 **33**, Neu5Gcα2–6LacβProN_3 **35**, **and** KDNα2–6LacβProN_3 **37** containing naturally occurring sialic acid forms including Neu5Ac, Neu5Gc, and KDN served as good acceptor substrates for the α2–8-sialyltransferase activity of CstIIΔ32^{I53S}. As shown in Figure 6, preparative-scale sialylation of **33**, **35**, and **37** from Neu5Ac **32** as a donor precursor for CstIIΔ32^{I53S} successfully produced the disialylated products Neu5Acα2–8Neu5Acα2–6LacβProN_3 **34**, Neu5Acα2–8Neu5cα2–6LacβProN_3 **36**,and Neu5Acα2–8KDNα2–6LacβProN_3 **38** in 80%, 76%, and 64% yield, respectively. Similar to its α2–3-monosialylated counterpart Neu5Ac9N_3α2–3GalβOMe **23**,

α2–6-linked sialoside Neu5Ac9N_3α2–3GalβOMe **39** containing an azido substitution of the C9-OH of the terminal Neu5Ac was also a suitable acceptor for CstIIΔ32^{I53S}. Sialylation of **39** with Neu5Ac **32** in the one-pot two-enzyme system in preparative scale produced disialyl product Neu5Acα2–8Neu5Ac9N_3α2–6GalβOMe **40** in 56% yield. The α2–6-linked monosialylated oligosaccharides containing various substitutions at C-5 of Neu5Ac, Neu5Gc, or KDN, including Neu5AcN_3α2–6GalOMe **41**, Neu5NPgα2–6LacβProN_3 **42**, Neu5AcCbzα2–6LacβProN_3 **43**, Neu5GcMeα2–6LacβProN_3 **44**, KDN5Meα2–6LacβProN_3 **45**, and KDN5N_3α2–6GalOMe **46** did not serve as acceptor substrates for the α2–8-sialyltransferase activity of CstIIΔ32^{I53S}. These data, together with those obtained from the acceptor specificity studies using α2–3-linked monosialylated oligosaccharides, indicate the importance of the C-5 groups on the terminal sialic acid residues, instead of the sialyl linkages, in defining the acceptor specificity of the α2–8-sialyltransferase activity of CstIIΔ32^{I53S}.

Conclusions

In conclusion, we have developed an efficient two-step multi-enzyme approach for the synthesis of a series of GD3 ganglioside oligosaccharides and other disialyl glycans containing natural and non-natural sialic acids. The α2–8-sialyltransferase activity of a recombinant multi-functional CstIIΔ32^{I53S} has promiscuous donor substrate specificity and can tolerate various substitutions at C-5 or C-9 of sialic acid residues in the donor. In comparison, the α2–8-sialyltransferase activity of CstIIΔ32^{I53S} has relatively restricted acceptor substrate specificity. While both α2–3- and α2–6-linked monosialyl oligosaccharides are potential acceptors for CstIIΔ32^{I53S}, the terminal sialic acid residues in the acceptable monosialyl oligosaccharide acceptors are limited to Neu5Ac, Neu5Gc, KDN, and some of their C-9 modified forms. Additional modifications at the C-5 of the terminal sialic acid residues in the monosialyl oligosaccharides prevent them to be usable acceptors by the α2–8-sialyltransferase activity of CstIIΔ32^{I53S}. The disialyl oligosaccharides obtained in this work are valuable probes to study the biological importance of naturally occurring sialic acid modifications in disialyl structures in nature.

Experimental Section

Chemicals were purchased and used without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on Mercury-300, Varian Inova-400, or Varian VNMRS 600 MHz spectrometer. High resolution electrospray ionization (ESI) mass spectra were obtained at the Mass Spectrometry Facility in the University of California at Davis. Optical rotation was recorded on an Autopol IV Automatic Polarimeter at 589 nm wavelength. Silica gel 60 Å (40–63 m, Sorbent technologies) was used for flash chromatography. Analytical thin-layer chromatography was performed on silica gel plates 60 GF_{254} (Sorbent technologies) using *p*-anisaldehyde sugar stain for detection. Gel filtration chromatography was performed using a column (100 cm × 2.5 cm) packed with BioGel P-2 Fine resins (Bio-Rad, Hercules, CA).

Enzymatic Synthesis of Sialosides

Monosialosides

The synthesis of α2–3 and α2–6-linked monosialyl oligosaccharides **3**, **19**, **21**, **25**, **26**, **33**, **35**, **37**, **39**, **41**-**46** has been reported previously.[51] Monosialylated oligosaccharides **23** and **27** were prepared in a one-pot three-enzyme system containing an *E. coli* sialic acid aldolase, NmCSS, and PmST1 as described previously.[51a]

Methyl *O*-(5-acetamido-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranoside (Neu5Ac9N$_3$α2–3GalβOMe, 23)

Yield, 88%; white foam. ^{1}H NMR (600 MHz, D_2O) δ 4.36 (d, 1H, *J* = 7.8 Hz, H-1), 4.06 (dd, 1H, *J* = 10.2 and 3.0 Hz), 3.98 (m, 1H), 3.93–3.46 (m, 11H), 3.56 (s, 3H, OMe), 2.74 (dd, 1H, *J* = 12.6 and 4.8 Hz, H-3$_{eq}$'), 2.03 (s, 3H), 1.78 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$'); ^{13}C NMR (125 MHz, D_2O) δ 172.48, 171.32, 101.06, 97.31, 73.36, 72.42, 70.17, 67.91, 66.63, 65.80, 65.01, 58.47, 56.89, 54.59, 50.55, 49.20, 37.20, 19.63. HRMS (ESI) *m/z* calculated for $C_{18}H_{29}N_4Na_2O_{13}$ (M+Na), 532.1629, found 532.1637.

3-Azidopropyl *O*-(5-*O*-methyl-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-O-β-D-galactopyranosyl-(1→4)-D-glucopyranoside (KDN5Meα2–3LacβProN$_3$, 27)

Yield, 70%; white form. ^{1}H NMR (600 MHz, D_2O) δ: 4.54 (1 H, d, *J* = 7.8 Hz), 4.51 (1 H, d, *J* = 8.4 Hz), 4.11–3.59 (19 H, m), 3.59 (3H, s, OMe), 3.50 (2H, t, *J* = 7.20 Hz), 3.37–3.32 (2 H, m), 2.71 (1 H, dd, *J* = 12.6 and 4.2 Hz, H-3$_{eq}$'), 1.94 (2H, m), 1.80 (1H, t, *J* = 12.0 Hz, H-3$_{ax}$'); ^{13}C NMR (150 MHz, D_2O) δ: 174.17, 102.84, 102.33, 99.86, 79.88, 78.46, 75.63, 75.34, 74.96, 74.53, 73.22, 73.00, 72.35, 70.12, 69.54, 68.15, 67.56, 67.55, 62.82, 61.20, 60.28, 60.24, 48.08, 39.53, 28.42. HRMS (ESI) *m/z* calculated for $C_{25}H_{42}N_3Na_2O_{19}$ (M+Na), 711.2310, found 711.2318.

General procedures for one-pot multi-enzyme preparative synthesis ofα2–8-linked sialosides using CstIIΔ32^{I53S}

A monosialylated oligosaccharide as an acceptor for the α2–8-sialyltransferase activity of CstIIΔ32^{I53S} (2.5–3.0 mg), a sialic acid precursor (mannose, ManNAc, or their derivatives, 1.2 equiv.), sodium pyruvate (7.5 equiv.), and CTP (1.5 equiv.) were dissolved in H_2O. Stock solutions of Tris-HCl buffer (1 M, pH 8.5, 1 mL) and $MgCl_2$ (0.5 M, 0.4 mL) were added. After the addition of appropriate amounts of a recombinant *E. coli* K12 sialic acid aldolase, an *N. meningitidis* CMP-sialic acid synthetase, and CstIIΔ32^{I53S}, H_2O was added to bring the volume of the reaction mixture to 10 mL. The reaction was carried out by incubating the solution in an incubator shaker at 37 °C for 2 h (or at room temperature for overnight) with agitation at 140 rpm. The product formation was monitored by TLC developed with EtOAc:MeOH:H_2O:HOAc = 5:3:1.5:0.2 (by volume) and stained with *p*-anisaldehyde sugar stain. When an optimal yield was achieved, the reaction was quenched by adding the same volume (10 mL) of ice-cold EtOH and incubation at 4 °C for 30 min. The mixture was then centrifuged and the precipitates were removed. The supernatant was concentrated, passed through a BioGel P-2 gel filtration column, and eluted with water to obtain sialoside mixtures. Silica gel flash column was then used to obtain pure disalylated product.

3-Azidopropyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5Acα2–8Neu5Acα2–3LacβProN$_3$, 4)

Yield, 76%; white foam. $[\alpha]_D^{22}$ = -0.23° (*c* 2.15, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.53 (d, 1H, *J* = 8.4 Hz, Glc H-1), 4.50 (d, 1H, *J* = 8.4 Hz, Gal H-1), 4.19–3.99 (m, 6H), 3.94–3.55 (m, 21H), 3.47 (t, 2H, *J* = 6.6 Hz), 3.33 (t, 1H, *J* = 8.4 Hz), 2.80 (dd, 1H, *J* = 12.0 and 4.8 Hz, Neu5Ac H-3$_{eq}$"), 2.67 (dd, 1H, *J* = 12.6 and 4.2 Hz, Neu5Ac H-3$_{eq}$"), 2.08 (s, 3H), 2.05 (s, 3H), 1.93 (m, 2H), 1.77 (t, 1H, *J* = 12.6 Hz, Neu5Ac H-3$_{ax}$"), 1.75 (t, 1H, *J* = 12.0 Hz, Neu5Ac H-3$_{ax}$"); ^{13}C NMR (125 MHz, D_2O) δ 175.18 (2C), 173.87, 173.46, 102.86, 102.33, 100.74, 100.52, 78.28 (Neu5Ac C-8), 78.22, 75.59, 75.32, 74.98, 74.48, 74.25, 73.02, 72.81, 71.96, 69.49, 69.46, 68.64, 68.36, 68.13, 67.89, 67.55, 62.78, 61.73, 61.26, 60.20, 52.44, 51.93, 48.06, 40.68, 39.63, 28.41, 22.48, 22.22. HRMS (ESI) *m/z* calculated for $C_{37}H_{60}N_5O_{27}$ (M-2Na+H), 1006.3476, found 1006.3476.

3-Azidopropyl *O*-(5-glycolylamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5Gcα2–8Neu5Acα2–3LacβProN$_3$, 6)

Yield, 73%; white foam. $[\alpha]_D^{22}$ = -0.95° (*c* 1.37, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.49 (d, 1H, *J* = 8.4 Hz, Glc H-1), 4.45 (d, 1H, *J* = 7.8 Hz, Gal H-1), 4.18–4.04 (m, 5H), 3.99–3.52 (m, 21H), 3.43 (t, 2H, *J* = 6.6 Hz), 3.28 (t, 1H, *J* = 8.4 Hz), 2.77 (dd, 1H, *J* = 12.0 and 4.2 Hz, H-3$_{eq}$"), 2.61 (dd, 1H, *J* = 12.0 and 3.6 Hz, H-3$_{eq}$"), 2.04 (s, 3H), 1.88 (m, 2H), 1.74 (t, 1H, *J* = 12.6 Hz, H-3$_{ax}$"), 1.72 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"); ^{13}C NMR (125 MHz, D_2O) δ 175.86 (2C), 175.15, 173.44, 102.81, 102.28, 100.70, 100.55, 78.21 (Neu5Ac C-8), 78.10, 75.50, 75.24, 74.91, 74.42, 74.26, 72.98, 72.44, 72.00, 69.43, 69.40, 68.31, 68.23, 68.07, 67.95, 67.50, 62.68, 61.70, 61.22, 61.11, 60.14, 52.36, 51.57, 48.00, 40.66, 39.46, 28.37, 22.44. HRMS (ESI) *m/z* calculated for $C_{37}H_{60}N_5O_{28}$ (M-2Na+H), 1022.3425, found 1022.3433.

3-Azidopropyl *O*-(3-deoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (KDNα2–8Neu5Acα2–3LacβProN$_3$, 8)

Yield, 65%; white foam. $[\alpha]_D^{22}$ = -10.0° (*c* 1.64, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.50 (d, 1H, *J* = 7.8 Hz, Glc H-1), 4.46 (d, 1H, *J* = 7.8 Hz, Gal H-1), 4.15–4.07 (m, 3H), 4.02–3.49 (m, 21H), 3.44 (t, 2H, *J* = 6.6 Hz), 3.29 (t, 1H, *J* = 8.4 Hz), 2.71 (dd, 1H, *J* = 12.0 and 4.2 Hz, H-3$_{eq}$"), 2.61 (dd, 1H, *J* = 12.0 and 4.2 Hz, H-3$_{eq}$"), 2.04 (s, 3H), 1.89 (m, 2H), 1.76 (t, 1H, *J* = 12.6 Hz, H-3$_{ax}$"), 1.67 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"); ^{13}C NMR (125 MHz, D_2O) δ 175.16 (2C), 174.00, 173.57, 102.80, 102.27, 100.72, 100.62, 78.24 (Neu5Ac C-8), 77.97, 75.48, 75.20, 74.90, 74.42, 74.31, 73.73, 72.96, 72.24, 70.52, 69.88, 69.44, 69.35, 68.09, 67.93, 67.49, 62.76, 61.67, 61.21, 60.15, 52.34, 47.99, 40.19, 39.31, 28.36, 22.42. HRMS (ESI) *m/z* calculated for $C_{35}H_{57}N_4O_{27}$ (M-2Na+H), 965.3210, found 965.3214.

3-Azidopropyl *O*-(5-methoxyacetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5GcMeα2–8Neu5Acα2–3LacβProN$_3$, 10)

Yield, 92%; white foam. $[\alpha]_D^{22}$ = -0.28° (*c* 3.2, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.49 (d, 1H, *J* = 8.4 Hz, Glc H-1), 4.45 (d, 1H, *J* = 7.8 Hz, Gal H-1), 4.15–4.10 (m, 2H), 4.06–4.01 (m, 3H), 3.98-3.52 (m, 24H), 3.43 (t, 2H, *J* = 7.2 Hz), 3.39 (s, 3H), 3.28 (t, 1H, *J* = 8.4 Hz), 2.75 (dd, 1H, *J* = 12.0 and 4.2 Hz, H-3$_{eq}$"), 2.64 (dd, 1H, *J* = 12.0 and 4.2 Hz, H-3$_{eq}$"), 2.04 (s, 3H), 1.88 (m, 2H), 1.73 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"), 1.71 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"); ^{13}C NMR (125 MHz, D_2O) δ 175.09 (2C), 173.65, 173.56, 173.54, 102.83, 102.29, 100.70, 100.34, 78.29 (Neu5Ac C-8), 78.16, 75.55, 75.32, 74.92, 74.39, 74.14, 72.99, 72.48, 72.00, 71.03 (OCH_3), 69.47, 69.40, 68.34, 68.25, 68.03, 67.60, 67.50, 62.70, 61.72, 61.23, 60.64, 59.17, 52.39, 51.55, 48.01, 40.69, 39.80, 28.38, 22.51. HRMS (ESI) *m/z* calculated for $C_{38}H_{62}N_5O_{28}$ (M-2Na+H), 1036.3581, found 1036.3579.

3-Azidopropyl *O*-(5-azidoacetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5AcN$_3$α2–8Neu5Acα2–3LacβProN$_3$, 12)

Yield, 87%; white foam. $[\alpha]_D^{22}$ = +1.37° (*c* 2.05, H_2O); H NMR (600 MHz, D_2O) δ 4.49 (d, 1H, *J* = 7.8 Hz, Glc H-1), 4.46 (d, 1H, *J* = 7.8 Hz, Gal H-1), 4.16–4.05 (m, 3H), 4.04 (s, 2H), 3.99–3.52 (m, 24H), 3.44 (t, 2H, *J* = 6.6 Hz), 3.29 (t, 1H, *J* = 9.0 Hz), 2.76 (dd, 1H, *J* = 12.6 and 4.8 Hz, H-3$_{eq}$"), 2.65 (dd, 1H, *J* = 12.6 and 4.8 Hz, H-3$_{eq}$"), 2.04 (s, 3H), 1.89 (m, 2H), 1.73 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"), 1.71 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"); ^{13}C NMR (125 MHz,

D_2O) δ 175.11, 173.65, 173.51, 171.27, 102.82, 102.29, 100.68, 100.34, 78.32 (Neu5Ac C-8), 78.15, 75.57, 75.34, 74.93, 74.39, 74.15, 72.99, 72.45, 71.99, 69.52, 69.41, 68.44, 68.21, 68.04, 67.60, 67.50, 62.70, 61.71, 61.23, 60.14, 52.40, 52.04, 51.97, 48.01, 40.62, 39.82, 28.38, 22.50. HRMS (ESI) *m/z* calculated for $C_{37}H_{59}N_8O_{27}$ (M-2Na+H), 1047.3490, found 1047.3486.

3-Azidopropyl *O*-(5-acetamido-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5Ac9N_3α2–8Neu5Acα2–3LacβProN_3, 14)

Yield, 78%; white foam. $[\alpha]_D^{22}$ = +5.11° (*c* 0.92, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.48 (d, 1H, *J* = 7.8 Hz, Glc H-1), 4.44 (d, 1H, *J* = 7.8 Hz, Gal H-1), 4.11 (dd, 1H, *J* = 12.0 and 3.0 Hz), 4.07-3.94 (m, 6H), 3.81–3.53 (m, 19H),), 4.47 (dd, 1H, *J* = 13.2 and 5.4 Hz), 3.42 (t, 2H, *J* = 6.6 Hz), 3.27 (t, 1H, *J* = 9.0 Hz), 2.74 (dd, 1H, *J* = 12.0 and 4.2 Hz, H-3_{eq}"), 2.60 (dd, 1H, *J* = 12.6 and 4.8 Hz, H-3_{eq}"), 2.02 (s, 3H), 1.99 (s, 3H), 1.87 (m, 2H), 1.72 (t, 1H, *J* = 12.0 Hz, H-3_{ax}"), 1.69 (t, 1H, *J* = 12.0 Hz, H-3_{ax}"); ^{13}C NMR (125 MHz, D_2O) δ 175.11, 175.06, 173.70, 173.30, 102.79, 102.27, 100.95, 100.39, 78.18 (Neu5Ac C-8), 78.14, 75.52, 75.26, 74.91, 74.41, 73.97, 72.97, 72.59, 72.58, 70.50, 69.40, 69.21, 68.78, 68.59, 68.05, 67.87, 61.56, 61.22, 60.13, 53.09, 52.37, 51.87, 47.99, 40.61, 39.62, 28.36, 22.43, 22.19. HRMS (ESI) *m/z* calculated for $C_{37}H_{59}N_8O_{26}$ (M-2Na+H), 1031.3540, found 1031.3552.

3-Azidopropyl *O*-(5-acetamido-9-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5,9Ac_2α2–8Neu5Acα2–3LacβProN_3, 16)

Yield, 53%; white foam. $[\alpha]_D^{22}$ = -2.36° (*c* 1.78, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.54 (d, 1H, *J* = 7.8 Hz, Glc H-1), 4.50 (d, 1H, *J* = 7.8 Hz, Gal H-1), 4.41 (d, 1H, *J* = 11.4 Hz), 4.24 (dd, 1H, *J* = 11.4 and 5.4 Hz), 4.19–4.08 (m, 3H), 4.03-3.57 (m, 22H), 3.48 (t, 2H, *J* = 6.0 Hz), 3.33 (t, 1H, *J* = 8.4 Hz), 2.80 (dd, 1H, *J* = 12.0 and 3.6 Hz, Neu5Ac H-3_{eq}"), 2.67 (dd, 1H, *J* = 13.2 and 4.8 Hz, Neu5Ac H-3_{eq}"), 2.16 (s, 3H), 2.08 (s, 3H), 2.05 (s, 3H), 1.92 (m, 2H), 1.76 (t, 2H, *J* = 12.0 Hz, Neu5Ac H-3_{ax}"); ^{13}C NMR (125 MHz, D_2O) δ 177.75 (2C), 176.44, 176.03, 105.44, 104.91, 103.33, 103.10, 80.87 (Neu5Ac C-8), 80.80, 78.17, 77.91, 77.56, 77.07, 76.82, 75.61, 75.39, 74.54, 72.06, 72.04, 71.22, 70.94, 70.71, 70.48, 70.12, 65.36, 64.31, 63.84, 62.79, 55.03, 54.51, 50.65, 43.26, 42.22, 30.99, 25.06, 24.80. HRMS (ESI) *m/z* calculated for $C_{39}H_{62}N_5O_{28}$ (M-2Na+H), 1048.3581, found 1048.3567.

3-Azidopropyl *O*-(9-*O*-acetyl-5-glycolylamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5Gc9Acα2–8Neu5Acα2–3LacβProN_3, 18)

Yield, 51%; white foam. $[\alpha]_D^{22}$ = +3.33° (*c* 0.63, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.51 (d, 1H, *J* = 7.8 Hz, Glc H-1), 4.47 (d, 1H, *J* = 8.4 Hz, Gal H-1), 4.38 (dd, 1H, *J* = 11.4 and 3.0 Hz), 4.20 (dd, 1H, *J* = 11.4 and 5.4 Hz), 4.16 (dd, 1H, *J* = 12.0 and 3.6 Hz), 4.11 (s, 2H), 4.08 (m, 3H), 4.02–3.53 (m, 21H), 3.44 (t, 2H, *J* = 6.6 Hz), 3.30 (t, 1H, *J* = 8.4 Hz), 2.78 (dd, 1H, *J* = 12.6 and 4.8 Hz, H-3_{eq}"), 2.65 (dd, 1H, *J* = 12.6 and 4.8 Hz, H-3_{eq}"), 2.12 (s, 3H), 2.05 (s, 3H), 1.89 (m, 2H), 1.75 (t, 1H, *J* = 12.6 Hz, H-3_{ax}"), 1.73 (t, 1H, *J* = 12.0 Hz, H-3_{ax}"); ^{13}C NMR (125 MHz, D_2O) δ 175.81, 175.08, 174.53, 173.62, 173.43, 102.77, 102.26, 100.75, 100.31, 78.32 (Neu5Ac C-8), 78.09, 75.56, 75.31, 74.92, 74.38, 74.01, 72.96, 72.95, 72.27, 69.46, 69.40, 69.38, 69.27, 68.39, 68.03, 67.66, 67.45, 62.59, 61.60, 61.21, 61.08, 52.39, 51.51, 47.96, 40.64, 39.75, 28.34, 22.41, 20.41. HRMS (ESI) *m/z* $C_{39}H_{62}N_5O_{29}$ (M-2Na+H), 1064.3530, found 1064.3521.

3-Azidopropyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-glycolylamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5Acα2–8Neu5Gcα2–3LacβProN$_3$, 20)

Yield, 72%; white foam. $[\alpha]_D^{22}$ = -0.78° (*c* 1.53, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.49 (d, 1H, *J* = 7.8 Hz, Glc H-1), 4.45 (d, 1H, *J* = 7.8 Hz, Gal H-1), 4.19–4.06 (m, 5H), 3.99–3.53 (m, 24H), 3.43 (t, 2H, *J* = 6.6 Hz), 3.29 (t, 1H, *J* = 8.4 Hz), 2.74 (dd, 1H, *J* = 12.6 and 4.2 Hz, H-3$_{eq}$"), 2.66 (dd, 1H, *J* = 12.6 and 4.2 Hz, H-3$_{eq}$"), 2.00 (s, 3H), 1.88 (m, 2H), 1.73 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"), 1.70 (t, 1H, *J* = 12.6 Hz, H-3$_{ax}$"); ^{13}C NMR (125 MHz, D_2O) δ 176.24, 175.18, 173.78 (2C), 102.89, 102.33, 100.42 (2C), 78.49 (Neu5Gc C-8), 78.28, 75.64, 75.36, 74.98, 74.47, 73.95, 73.03, 72.79, 71.97, 69.47, 69.25, 68.57, 68.37, 67.81, 67.66, 67.54, 62.79, 61.67, 61.31, 61.26, 60.22, 52.28, 51.93, 48.07, 40.77, 39.77, 28.41, 22.23. HRMS (ESI) *m/z* calculated for $C_{37}H_{60}N_5O_{28}$ (M-2Na+H), 1022.3425, found 1022.3456.

3-Azidopropyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(3-deoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5Acα2–8KDNα2–3LacβProN$_3$, 22)

Yield, 71%; white foam. $[\alpha]_D^{22}$ = -8.22° (*c* 3.2, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.48 (d, 1H, *J* = 7.8 Hz, Glc H-1), 4.45 (d, 1H, *J* = 7.8 Hz, Gal H-1), 4.16–4.12 (m, 3H), 4.04–3.79 (m, 5H), 3.75–3.51 (m, 18H), 3.43 (t, 2H, *J* = 6.6 Hz), 3.37 (t, 1H, *J* = 9.6 Hz), 3.28 (t, 1H, *J* = 9.0 Hz), 2.73 (dd, 1H, *J* = 12.6 and 4.8 Hz, H-3$_{eq}$"), 2.61 (dd, 1H, *J* = 12.6 and 4.8 Hz, H-3$_{eq}$"), 2.00 (s, 3H), 1.88 (m, 2H), 1.78 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"), 1.67 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"); ^{13}C NMR (125 MHz, D_2O) δ 175.13, 173.95, 173.81, 102.85, 102.30, 100.95, 100.29, 78.20 (KDN C-8), 78.08, 75.62, 75.38, 75.20, 74.95, 74.42, 73.00, 72.84, 71.87, 70.74, 69.65, 69.42, 68.67, 68.34, 67.60, 67.51, 62.74, 61.60, 61.22, 61.06, 60.17, 51.89, 48.03, 40.02, 39.43, 28.38, 22.21. HRMS (ESI) *m/z* calculated for $C_{35}H_{57}N_4O_{27}$ (M-2Na+H), 965.3210, found 965.3211.

Methyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranoside (Neu5Acα2–8Neu5Ac9N$_3$α2–3GalβOMe, 24)

Yield, 63%; white foam. $[\alpha]_D^{22}$ = +10.07° (*c* 1.47, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.36 (d, 1H, *J* = 9.6 Hz, Gal H-1), 4.34 (m, 1H), 4.05 (dd, 1H, *J* = 10.2 and 3.0 Hz), 3.99 (m, 1H), 3.94 (d, 1H, *J* = 3.0 Hz), 3.90–3.49 (m, 18H), 3.55 (s, 3H), 2.77 (dd, 1H, *J* = 12.6 and 4.8 Hz, H-3$_{eq}$"), 2.62 (dd, 1H, *J* = 12.0 and 4.2 Hz, H-3$_{eq}$"), 2.05 (s, 3H), 2.01 (s, 3H), 1.72 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"), 1.71 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"); ^{13}C NMR (125 MHz, D_2O) δ 175.17, 175.15, 174.13, 173.28, 103.66, 100.40, 100.23, 75.84, 75.72, 74.79, 74.30, 72.65, 71.80, 69.38, 69.23, 68.49, 68.10, 68.04, 62.74, 61.12, 60.68, 57.21, 52.25, 51.81, 51.59, 40.40, 39.42, 22.41, 22.23. HRMS (ESI) *m/z* calculated for $C_{30}H_{50}N_5O_{21}$(M-2Na+H), 816.2998, found 816.2986.

3-Azidopropyl *O*-(5-glycolylamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-glycolylamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5Gcα2–8Neu5Gcα2–3LacβProN$_3$, 28)

Yield, 75%; white foam. $[\alpha]_D^{22}$ = -6.90° (*c* 2.03, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.54 (d, 1H, *J* = 7.8 Hz, Glc H-1), 4.50 (d, 1H, *J* = 8.4 Hz, Gal H-1), 4.24–4.10 (m, 7H), 4.04–3.58 (m, 24H), 3.48 (t, 2H, *J* = 6.6 Hz), 3.33 (t, 1H, *J* = 9.0 Hz), 2.80 (dd, 1H, *J* = 12.6 and 4.82 Hz, H-3$_{eq}$"), 2.72 (dd, 1H, *J* = 12.0 and 4.2 Hz, H-3$_{eq}$"), 1.93 (m, 2H), 1.77(t, 2H, *J* = 12.0 Hz, H-3$_{ax}$"); ^{13}C NMR (100 MHz, D_2O) δ 176.24, 175.90, 173.85, 173.59, 102.86, 102.30, 100.34, 100.30, 78.56 (Neu5Gc C-8), 78.15, 75.59, 75.38, 74.95, 74.41, 73.88, 72.99, 72.48, 71.98,

69.41, 69.22, 68.29, 68.20, 67.60, 67.59, 67.50, 62.69, 61.63, 61.27, 61.12, 60.21, 60.15, 52.24, 52.59, 48.01, 40.79, 39.88, 28.37. HRMS (ESI) *m/z* calculated for $C_{37}H_{60}N_5O_{29}$ (M-2Na+H), 1038.3374, found 1038.3363.

3-Azidopropyl *O*-(3-deoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-glycolylamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (KDNα2–8Neu5Gcα2–3LacβProN$_3$, 29)

Yield, 57%; white foam. $[\alpha]_D^{22}$ = -7.72° (*c* 1.84, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.51 (d, 1H, *J* = 8.4 Hz, Glc H-1), 4.47 (d, 1H, *J* = 8.4 Hz, Gal H-1), 4.21–4.08 (m, 5H), 4.01–3.52 (m, 24H), 3.45 (t, 2H, *J* = 6.6 Hz), 3.31 (t, 1H, *J* = 8.4 Hz), 2.70 (dd, 1H, *J* = 12.0 and 4.2 Hz, H-3$_{eq}$"), 2.68 (dd, 1H, *J* = 12.0 and 4.2 Hz, H-3$_{eq}$"), 1.90 (m, 2H), 1.76 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"), 1.68 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"); ^{13}C NMR (125 MHz, D_2O) δ 178.84, 176.22, 176.20, 105.47, 104.89, 103.23, 102.99, 81.03 (Neu5Gc C-8), 80.85, 78.21, 77.95, 77.55, 77.03, 76.52, 76.38, 75.59, 74.84, 73.07, 72.55, 72.32, 72.02, 71.77, 70.56, 70.21, 70.11, 65.41, 64.19, 63.87, 62.78, 62.07, 54.86, 50.63, 42.96, 42.39, 30.98. HRMS (ESI) *m/z* calculated for $C_{35}H_{57}N_4O_{28}$ (M-2Na+H), 981.3159, found 981.3143.

3-Azidopropyl *O*-(5-glycolylamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(3-deoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5Gcα2–8KDNα2–3LacβProN$_3$, 30)

Yield, 68%; white foam. $[\alpha]_D^{22}$ = -9.90° (*c* 0.98, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.53 (d, 1H, *J* = 7.8 Hz, Glc H-1), 4.50 (d, 1H, *J* = 7.8 Hz, Gal H-1), 4.21–3.56 (m, 27H), 3.48 (t, 2H, *J* = 6.6 Hz), 3.42 (t, 1H, *J* = 9.6 Hz), 3.33 (t, 1H, *J* = 9.0 Hz), 2.80 (dd, 1H, *J* = 12.6 and 4.8 Hz, H-3$_{eq}$"), 2.66 (dd, 1H, *J* = 12.6 and 4.8 Hz, H-3$_{eq}$"), 1.93 (m, 2H), 1.84 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"), 1.73 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"); ^{13}C NMR (125 MHz, D_2O) δ 175.90, 173.93, 173.71, 102.88, 102.32, 100.99, 100.29, 78.27 (KDN C-8), 78.07, 75.65, 75.39, 75.27, 74.98, 74.47, 73.02, 72.59, 71.95, 70.76, 69.75, 69.67, 69.47, 68.44, 68.33, 67.71, 67.54, 62.74, 61.64, 61.25, 61.16, 60.22, 51.63, 48.06, 40.12, 39.37, 28.41. HRMS (ESI) *m/z* calculated for $C_{35}H_{57}N_4O_{28}$ (M-2Na+H), 981.3159, found 981.3097.

3-Azidopropyl *O*-(3-deoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(3-deoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (KDNα2–8KDNα2–3LacβProN$_3$, 31)

Yield, 55%; white foam. $[\alpha]_D^{22}$ = -20.0° (*c* 0.3, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.37 (d, 1H, *J* = 7.8 Hz, Glc H-1), 4.35 (d, 1H, *J* = 7.8 Hz, Gal H-1), 4.06–3.38 (m, 26H), 3.32 (t, 2H, *J* = 6.6 Hz), 3.26 (t, 1H, *J* = 9.6 Hz), 3.19 (t, 1H, *J* = 8.4 Hz), 2.55 (dd, 1H, *J* = 12.6 and 4.8 Hz, H-3$_{eq}$"), 2.50 (dd, 1H, *J* = 12.6 and 4.8 Hz, H-3$_{eq}$"), 1.77 (m, 2H), 1.64 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"), 1.57 (t, 1H, *J* = 12.0 Hz, H-3$_{ax}$"); ^{13}C NMR (125 MHz, D_2O) δ 173.36, 173.21, 105.46, 104.89, 103.49, 103.03, 80.86 (KDN C-8), 80.58, 78.33, 77.97, 77.85, 77.56, 77.03, 76.45, 75.59, 74.73, 73.31, 73.20, 72.54, 72.32, 72.29, 72.03, 70.62, 70.25, 70.11, 65.41, 64.17, 63.83, 62.79, 50.63, 41.97, 41.96, 30.98. HRMS (ESI) *m/z* calculated for $C_{33}H_{54}N_3O_{27}$ (M-2Na+H), 924.2945, found 924.2934.

3-Azidopropyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5Acα2–8Neu5Acα2–6LacβProN$_3$, 34)

Yield, 80%; white foam. $[\alpha]_D^{22}$ = -9.14° (*c* 1.04, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.51 (d, 1H, *J* = 7.8 Hz, Glc H-1), 4.45 (d, 1H, *J* = 7.8 Hz, Gal H-1), 4.41 (m, 1H), 4.14 (dd, 1H, *J* = 12.0 and 3.6 Hz), 4.03–3.54 (m, 25H), 3.48 (t, 2H, *J* = 6.6 Hz), 3.35 (t, 1H, *J* = 9.0 Hz), 2.79 (dd, 1H, *J* = 12.0 and 4.2 Hz, H-3$_{eq}$"), 2.64 (dd, 1H, *J* = 12.0 and 4.8 Hz, H-3$_{eq}$"), 2.08 (s, 3H),

2.05 (s, 3H), 1.94 (m, 2H), 1.76 (t, 1H, *J* = 12.6 Hz, H-3_{ax}"), 1.70 (t, 1H, *J* = 12.6 Hz, H-3_{ax}"); ^{13}C NMR (125 MHz, D_2O) δ 175.19, 175.13, 173.43, 173.30, 103.43, 102.19, 101.00, 100.72, 79.86 (Neu5Ac C-8), 78.49, 74.85, 74.83, 74.29, 73.92, 72.93, 72.88, 72.55, 71.92, 71.01, 69.67, 68.69, 68.60, 68.36, 67.99, 67.51, 63.89, 62.82, 61.70, 60.50, 52.43, 51.95, 48.09, 40.67, 40.12, 28.44, 22.50, 22.26. HRMS (ESI) *m/z* calculated for $C_{37}H_{60}N_5O_{27}$ (M-2Na+H), 1006.3476, found 1006.3489.

3-Azidopropyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-glycolylamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5Acα2–8Neu5Gcα2–6LacβProN$_3$, 36)

Yield, 76%; white foam. $[\alpha]_D^{22}$ = -5.53° (*c* 1.03, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.48 (d, 1H, *J* = 7.8 Hz, Glc H-1), 4.41 (d, 1H, *J* = 7.8 Hz, Gal H-1), 4.19–4.07 (m, 4H), 4.00–3.50 (m, 25H), 3.44 (t, 2H, *J* = 6.6 Hz), 3.31 (t, 1H, *J* = 9.0 Hz), 2.74 (dd, 1H, *J* = 12.6 and 4.2 Hz, H-3_{eq}"), 2.61 (dd, 1H, *J* = 12.0 and 4.8 Hz, H-3_{eq}"), 2.00 (s, 3H), 1.89 (m, 2H), 1.71 (t, 1H, *J* = 12.0 Hz, H-3_{ax}"), 1.68 (t, 1H, *J* = 12.0 Hz, H-3_{ax}"); ^{13}C NMR (125 MHz, D_2O) δ 178.20, 176.19, 175.18, 173.88, 103.45, 102.17, 101.16, 100.34, 79.88 (Neu5Gc C-8), 78.85, 74.84, 74.13, 73.92, 72.91, 72.79, 72.53, 71.95, 70.99, 69.80, 69.68, 68.70, 68.56, 68.39, 67.51, 63.92, 62.81, 61.73, 61.30, 60.47, 52.28, 51.94, 48.07, 40.76, 40.28, 28.42, 22.24. HRMS (ESI) *m/z* calculated for $C_{37}H_{60}N_5O_{28}$ (M-2Na+H), 1022.3425, found 1022.3431.

3-Azidopropyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(3-deoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5Acα2–8KDNα2–6LacβProN$_3$, 38)

Yield, 64%; white foam. $[\alpha]_D^{22}$ = -7.45° (*c* 0.98, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.47 (d, 1H, *J* = 7.8 Hz, Glc H-1), 4.39 (d, 1H, *J* = 7.8 Hz, Gal H-1), 4.20 (m, 1H), 4.13–4.08 (m, 2H), 3.99–3.47 (m, 23H), 3.43 (t, 2H, *J* = 6.6 Hz), 3.38 (t, 1H, *J* = 9.6 Hz), 3.29 (t, 1H, *J* = 9.0 Hz), 2.73 (dd, 1H, *J* = 12.6 and 4.8 Hz, H-3_{eq}"), 2.54 (dd, 1H, *J* = 12.6 and 4.8 Hz, H-3_{eq}"), 2.00 (s, 3H), 1.88 (m, 2H), 1.77 (t, 1H, *J* = 12.0 Hz, H-3_{ax}"), 1.62 (t, 1H, *J* = 12.0 Hz, H-3_{ax}"); ^{13}C NMR (125 MHz, D_2O) δ 175.14, 173.98, 173.70, 103.45, 102.17, 101.07, 100.88, 79.95 (KDN C-8), 78.40, 75.34, 74.81, 74.80, 73.94, 72.89, 72.86, 72.51, 71.86, 70.97, 70.75, 69.95, 69.59, 68.68, 68.67, 68.40, 67.50, 63.90, 62.77, 61.66, 60.47, 51.91, 48.06, 40.08, 39.84, 28.40, 22.22. HRMS (ESI) *m/z* calculated for $C_{35}H_{57}N_4O_{27}$ (M-2Na+H), 965.3210, found 965.3207.

Methyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→6)-*O*-β-D-galactopyranoside (Neu5Acα2–8Neu5Ac9N$_3$α2–6GalβOMe, 40)

Yield, 56%; white foam. $[\alpha]_D^{22}$ = -7.54° (*c* 1.14, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 4.37 (m, 1H), 4.27 (d, 1H, *J* = 7.8 Hz, Gal H-1), 3.97 (m, 1H), 3.89–3.44 (m, 18H), 3.53 (s, 3H), 2.75 (dd, 1H, *J* = 12.6 and 4.8 Hz, H-3_{eq}"), 2.57 (dd, 1H, *J* = 12.0 and 4.2 Hz, H-3_{eq}"), 2.03 (s, 3H), 1.99 (s, 3H), 1.72 (t, 1H, *J* = 12.0 Hz, H-3_{ax}"), 1.58 (t, 1H, *J* = 12.0 Hz, H-3_{ax}"); ^{13}C NMR (125 MHz, D_2O) δ 175.21, 175.13, 173.65, 173.56, 104.04, 101.03, 100.09, 76.29, 74.18, 73.58, 72.73, 72.69, 71.84, 70.77, 69.45, 68.72, 68.59, 68.39, 67.93, 63.65, 62.68, 60.51, 57.53, 52.51, 51.86, 51.37, 40.33, 22.45, 22.18. HRMS (ESI) *m/z* calculated for $C_{30}H_{50}N_5O_{21}$(M-2Na +H), 816.2998, found 816.2993.

Supplementary Material

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Acknowledgments

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REFERENCES

1. a Varki A. Glycobiology 1993;3:97–130. [PubMed: 8490246] b Schauer R. Glycoconj. J 2000;17:485–499. [PubMed: 11421344] c Angata T, Varki A. Chem. Rev 2002;102:439–469. [PubMed: 11841250]
2. a Kelm S, Schauer R, Crocker PR. Glycoconj. J 1996;13:913–926. [PubMed: 8981082] b Renkonen R. Adv. Exp. Med. Biol 1998;435:63–73. [PubMed: 9498066]
3. Varki, A.; Cummings, RD.; Esko, JD.; Freeze, HH.; Stanley, P.; Bertozzi, CR.; Hart, GW.; Etzler, ME., editors. Essentials of Glycobiology. 2nd Ed.. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, New York: 2008. p. 132
4. Nudelman E, Hakomori S, Kannagi R, Levery S, Yeh MY, Hellstrom KE, Hellstrom I. J. Biol. Chem 1982;257:12752–12756. [PubMed: 7130176]
5. Nakamura K, Suzuki M, Taya C, Inagaki F, Yamakawa T, Suzuki A. J. Biochem 1991;110:832–841. [PubMed: 1783616]
6. Furukawa K, Chait BT, Lloyd KO. J. Biol. Chem 1988;263:14939–14947. [PubMed: 3170570]
7. Nakao T, Kon K, Ando S, Hirabayashi Y. Biochim. Biophys. Acta 1991;1086:305–309. [PubMed: 1742322]
8. Nudelman ED, Levery SB, Stroud MR, Salyan ME, Abe K, Hakomori S. J. Biol. Chem 1988;263:13942–13951. [PubMed: 3417685]
9. Iwamori M, Nagai Y. J. Biol. Chem 1978;253:8328–8331. [PubMed: 711756]
10. Cheresh DA, Varki AP, Varki NM, Stallcup WB, Levine J, Reisfeld RA. J. Biol. Chem 1984;259:7453–7459. [PubMed: 6203895]
11. Hitoshi S, Kusunoki S, Kon K, Chiba A, Waki H, Ando S, Kanazawa I. J. Neuroimmunol 1996;66:95–101. [PubMed: 8964919]
12. Dubois C, Manuguerra JC, Hauttecoeur B, Maze J. J. Biol. Chem 1990;265:2797–2803. [PubMed: 2303428]
13. Farooqui AA, Horrocks LA. Neurochem. Pathol 1984;2:189–218. [PubMed: 6152665]
14. Waki H, Masuzawa A, Kon K, Ando S. J. Biochem 1993;114:459–462. [PubMed: 8276754]
15. Schauer R. Curr. Opin. Struct. Biol 2009;19:507–514. [PubMed: 19699080]
16. Slomiany A, Kojima K, Banas-Gruszka Z, Slomiany BL. Biochem. Biophys. Res. Commun 1981;100:778–784. [PubMed: 7271781]
17. Ijuin T, Kitajima K, Song Y, Kitazume S, Inoue S, Haslam SM, Morris HR, Dell A, Inoue Y. Glycoconj. J 1996;13:401–413. [PubMed: 8781971]
18. a Sato C, Kitajima K. Trends Glycosci. Glycotechnol 1999;11:371–390. a Sato C, Fukuoka H, Ohta K, Matsuda T, Koshino R, Kobayashi K, Troy FA 2nd, Kitajima K. J. Biol. Chem 2000;275:15422–15431. [PubMed: 10809778]
19. a Sato C, Kitajima K, Tazawa I, Inoue Y, Inoue S, Troy FA 2nd. J. Biol. Chem 1993;268:23675–23684. [PubMed: 8226894] b Inoue S, Iwasaki M. Biochem. Biophys. Res. Commun 1978;83:1018–1023. [PubMed: 708420]
20. Finne J. J. Biol. Chem 1982;257:11966–11970. [PubMed: 7118922]
21. a James WM, Agnew WS. Biochem. Biophys. Res. Commun 1987;148:817–826. [PubMed: 2446601] a Zuber C, Lackie PM, Catterall WA, Roth J. J. Biol. Chem 1992;267:9965–9971. [PubMed: 1315775]
22. a Roth J, Zuber C, Wagner P, Taatjes DJ, Weisgerber C, Heitz PU, Goridis C, Bitter-Suermann D. Proc. Natl. Acad. Sci. U.S.A 1988;85:2999–3003. [PubMed: 2834727] b Livingston BD, Jacobs JL, Glick MC, Troy FA. J. Biol. Chem 1988;263:9443–9448. [PubMed: 3288635] c Martersteck CM, Kedersha NL, Drapp DA, Tsui TG, Colley KJ. Glycobiology 1996;6:289–301. [PubMed: 8724137]
23. Roth J, Kempf A, Reuter G, Schauer R, Gehring WJ. Science 1992;256:673–675. [PubMed: 1585182]

24. Malykh YN, Krisch B, Gerardy-Schahn R, Lapina EB, Shaw L, Schauer R. Glycoconj. J 1999;16:731–739. [PubMed: 11003558]
25. Kanamori A, Inoue S, Iwasaki M, Kitajima K, Kawai G, Yokoyama S, Inoue Y. J. Biol. Chem 1990;265:21811–21819. [PubMed: 2254333]
26. Kiang WL, Krusius T, Finne J, Margolis RU, Margolis RK. J. Biol. Chem 1982;257:1651–1659. [PubMed: 7056734]
27. Fukuda M, Lauffenburger M, Sasaki H, Rogers ME, Dell A. J. Biol. Chem 1987;262:11952–11957. [PubMed: 3624241]
28. Fukuda M, Dell A, Fukuda MN. J. Biol. Chem 1984;259:4782–4791. [PubMed: 6715323]
29. Funakoshi Y, Taguchi T, Sato C, Kitajima K, Inoue S, Morris HR, Dell A, Inoue Y. Glycobiology 1997;7:195–205. [PubMed: 9134426]
30. Sato C, Kitajima K, Inoue S, Inoue Y. J. Biol. Chem 1998;273:2575–2582. [PubMed: 9446559]
31. Nohara K, Kunimoto M, Fujimaki H. J. Biochem 1998;124:194–199. [PubMed: 9644263]
32. Yasukawa Z, Sato C, Sano K, Ogawa H, Kitajima K. Glycobiology 2006;16:651–665. [PubMed: 16608914]
33. Ziak M, Kerjaschki D, Farquhar MG, Roth J. J. Am. Soc. Nephrol 1999;10:203–209. [PubMed: 10215318]
34. Ziak M, Meier M, Roth J. Glycoconj. J 1999;16:185–188. [PubMed: 10596892]
35. a Robbins JB, McCracken GH Jr. Gotschlich EC, Orskov F, Orskov I, Hanson LA. N. Engl. J. Med 1974;290:1216–1220. [PubMed: 4133095] b Rohr TE, Troy FA. J. Biol. Chem 1980;255:2332–2342. [PubMed: 6987220] c Aalto J, Pelkonen S, Kalimo H, Finne J. Glycoconj. J 2001;18:751–758. [PubMed: 12441664] d Adlam C, Knight JM, Mugridge A, Williams JM, Lindon JC. FEBS Microbiol. Lett 1987;42:23–25.
36. Varki A. Trends Mol. Med 2008;14:351–360. [PubMed: 18606570]
37. Yamaji T, Teranishi T, Alphey MS, Crocker PR, Hashimoto Y. J. Biol. Chem 2002;277:6324–6332. [PubMed: 11741958]
38. a Nicoll G, Avril T, Lock K, Furukawa K, Bovin N, Crocker PR. Eur. J. Immunol 2003;33:1642–1648. [PubMed: 12778482] b Attrill H, Imamura A, Sharma RS, Kiso M, Crocker PR, van Aalten DM. J. Biol. Chem 2006;281:32774–32783. [PubMed: 16895906]
39. Boons GJ, Demchenko AV. Chem. Rev 2000;100:4539–4566. [PubMed: 11749357]
40. a Demchenko AV, Boons GJ. Tetrahedron Lett 1998;39:3065–3068. b Demchenko AV, Boons GJ. Chem.-Eur. J 1999;5:1278–1283.
41. Yu CS, Niikura K, Lin CC, Wong CH. Angew. Chem. Int. Ed. Engl 2001;40:2900–2903. [PubMed: 11500903]
42. a Komba S, Galustian C, Ishida H, Feizi T, Kannagi R, Kiso M. Angew. Chem. Int. Ed. Engl 1999;38:1131–1133. b De Meo C, Demchenko AV, Boons GJ. J. Org. Chem 2001;66:5490–5497. [PubMed: 11485473]
43. a Ando H, Koike Y, Ishida H, Kiso M. Tetrahedron Lett 2003;44:6883–6886. b Adachi M, Tanaka H, Takahashi T. Synlett 2004:609–614. c Tanaka H, Adachi M, Takahashi T. Chem.-Eur. J 2005;11:849–862.
44. Tanaka H, Nishiura Y, Adachi M, Takahashi T. Heterocycles 2006;67:107–112.
45. a Ando H, Koike Y, Koizumi S, Ishida H, Kiso M. Angew. Chem. Int. Ed. Engl 2005;44:6759–6763. [PubMed: 16187391] b Tanaka H, Ando H, Ishida H, Kiso M, Ishihara H, Koketsu M. Tetrahedron Lett 2009;50:4478–4481.
46. a Tanaka H, Nishiura Y, Takahashi T. J. Am. Chem. Soc 2006;128:7124–7125. [PubMed: 16734441] b Tanaka H, Nishiura Y, Takahashi T. J. Am. Chem. Soc 2008;130:17244–17245. [PubMed: 19053458]
47. a Gilbert M, Brisson JR, Karwaski MF, Michniewicz J, Cunningham AM, Wu Y, Young NM, Wakarchuk WW. J. Biol. Chem 2000;275:3896–3906. [PubMed: 10660542] b Gilbert M, Karwaski MF, Bernatchez S, Young NM, Taboada E, Michniewicz J, Cunningham AM, Wakarchuk WW. J. Biol. Chem 2002;277:327–337. [PubMed: 11689567] c Cheng J, Yu H, Lau K, Huang S, Chokhawala HA, Li Y, Tiwari VK, Chen X. Glycobiology 2008;18:686–697. [PubMed: 18509108]

48. Blixt O, Vasiliu D, Allin K, Jacobsen N, Warnock D, Razi N, Paulson JC, Bernatchez S, Gilbert M, Wakarchuk W. Carbohydr. Res 2005;340:1963–1972. [PubMed: 16005859]

49. a Ito Y, Numata M, Sugimoto M, Ogawa T. J. Am. Chem. Soc 1989;111:8508–8510. b Ando H, Ishida H, Kiso M, Hasegawa A. Carbohydr. Res 1997;300:207–217. [PubMed: 9202407] c Ding Y, Fukuda M, Hindsgaul O. Bioorg. Med. Chem. Lett 1998;8:1903–1908. [PubMed: 9873456] d Castro-Palomino JC, Simon B, Speer O, Leist M, Schmidt RR. Chem.- Eur. J 2001;7:2178–2184. e Gilbert M, Karwaski M-F, Bernatchez S, Young NM, Taboada E, Michniewicz J, Cunningham A-M, Wakarchuk WW. J. Biol. Chem 2002;277:327–337. [PubMed: 11689567] f Antoine T, Heyraud A, Bosso C, Samain E. Angew. Chem. Int. Ed. Engl 2005;44:1350–1352. [PubMed: 15674992] g Schwardt O, Visekruna T, Zenhausern G, Rabbani S, Ernst B. J. Carbohydr. Chem 2006;25:543–556. h Rich JR, Wakarchuk WW, Bundle DR. Chem-Eur. J 2006;2:845–858. i Houliston RS, Yuki N, Hirama T, Khieu NH, Brisson J-R, Gilbert M, Jarrell HC. Biochemistry 2007;46:36–44. [PubMed: 17198373] j Zhang P, Zuccolo AJ, Li W, Zheng RB, Ling C-C. Chem. Commun 2009;28:4233–4235.

50. Roy R, Pon RA. Glycoconj. J 1990;7:3–12.

51. a Yu H, Chokhawala H, Karpel R, Yu H, Wu B, Zhang J, Zhang Y, Jia Q, Chen X. J. Am. Chem. Soc 2005;127:17618–17619. [PubMed: 16351087] b Yu H, Huang S, Chokhawala H, Sun M, Zheng H, Chen X. Angew. Chem. Int. Ed. Engl 2006;45:3938–3944. [PubMed: 16721893]

52. Yu H, Chokhawala HA, Huang S, Chen X. Nat. Protoc 2006;1:2485–2492. [PubMed: 17406495]

53. Yu H, Yu H, Karpel R, Chen X. Bioorg. Med. Chem 2004;12:6427–6435. [PubMed: 15556760]

54. Li Y, Yu H, Cao H, Lau K, Muthana S, Tiwari VK, Son B, Chen X. Appl. Microbiol. Biotechnol 2008;79:963–970. [PubMed: 18521592]

55. Schlosshauer B, Blum AS, Mendez-Otero R, Barnstable CJ, Constantine-Paton M. J. Neurosci 1988;8:580–592. [PubMed: 2448435]

56. Chen HY, Challa AK, Varki A. J. Biol. Chem 2006;281:7825–7833. [PubMed: 16434401]

57. Kniep B, Kniep E, Ozkucur N, Barz S, Bachmann M, Malisan F, Testi R, Rieber EP. Int. J. Cancer 2006;119:67–73. [PubMed: 16432837]

58. a Ogura H, Furuhata K, Sato S, Anazawa K, Itoh M, Shitori Y. Carbohydr. Res 1987;167:77–86. [PubMed: 3690578] b Rauvolfova J, Venot A, Boons GJ. Carbohydr. Res 2008;343:1605–1611. [PubMed: 18508039]

59. Angata K, Fukuda M. Biochimie 2003;85:195–206. [PubMed: 12765789]

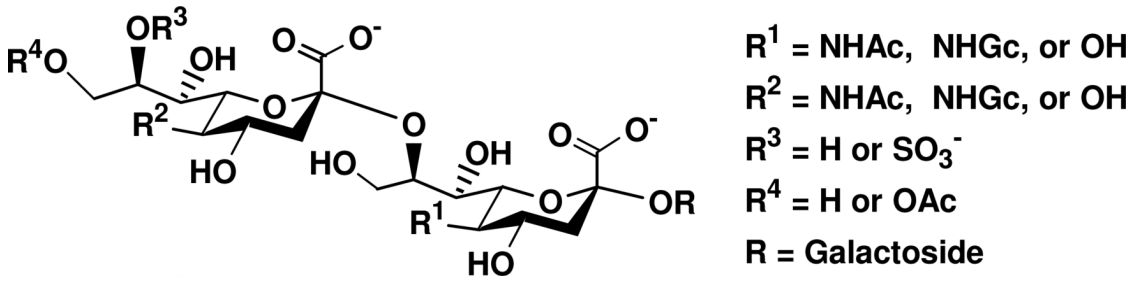

Figure 1.
Structures of common naturally occurring disialyl motifs in glycolipids and glycoproteins.

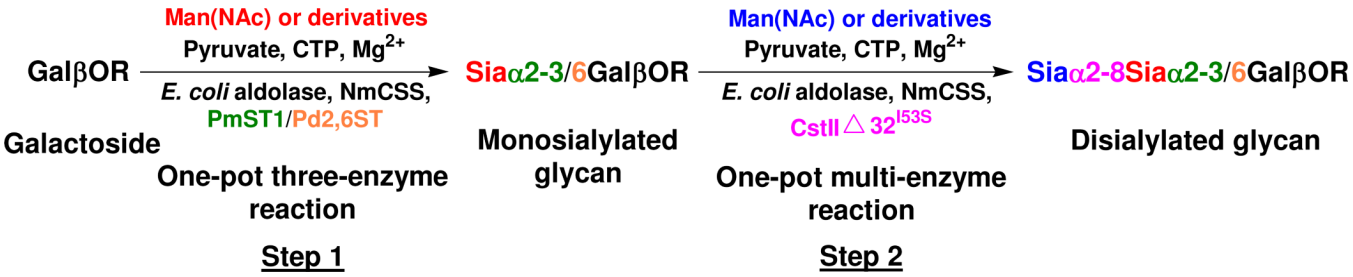

Figure 2.

Two-step multi-enzyme chemoenzymatic synthesis of diasialyl oligosaccharides containing different sialic acid forms and various sialyl linkages. Enzymes: *E. coli* aldolase, *Escherichia coli* K12 sialic acid aldolase; NmCSS, *Neisseria meningitidis* CMP-sialic acid synthetase; PmST1, *Pasteurella multocida* sialyltransferase for the formation of α2–3-linked sialosides; Pd2,6ST, *Photobacterium damsela* α2–6-sialyltransferase for the formation of α2–6-linked sialosides; CstII, *Campylobacter jejuni* sialyltransferase for the formation of α2–8-linked sialosides. Compounds: Man, mannose; ManNAc, *N*-acetylmannosamine; CTP, cytidine 5′-triphosphate; Sia, sialic acid.

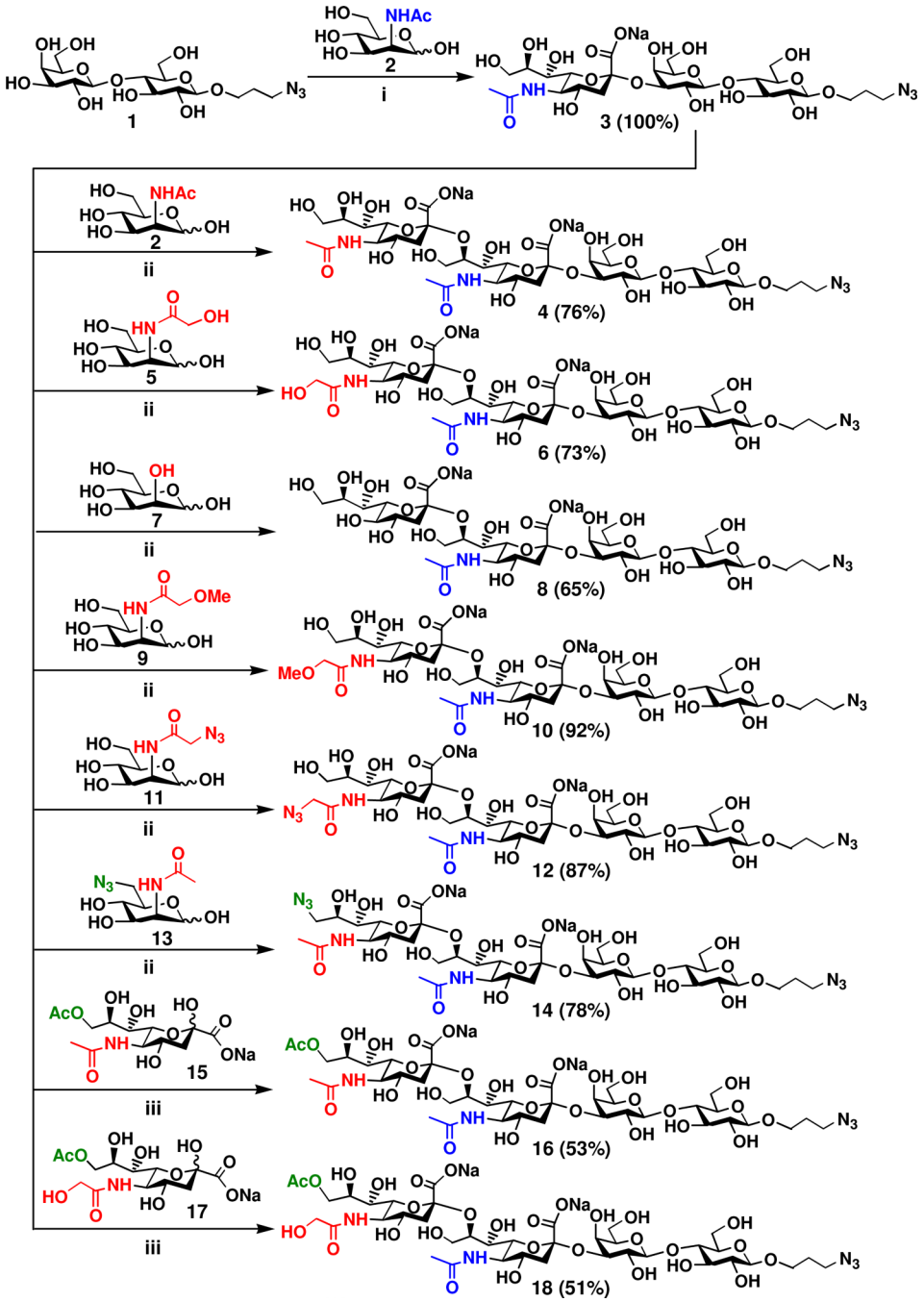

Figure 3.

Synthesis of disialyl GD3 oligosaccharides Siaα2–8Neu5Acα2–3LacβProN$_3$ containing a penultimate α2–3-linked Neu5Ac and different terminal α2–8-linked sialic acid forms. Reagents and conditions: (i) Pyruvate, CTP, Mg^{2+}, Tris-HCl buffer (pH 8.5), *E. coli* K12 sialic acid aldolase, NmCSS, and PmST1; (ii) Pyruvate, CTP, Mg^{2+}, Tris-HCl buffer (pH 8.5), *E. coli* K12 sialic acid aldolase, NmCSS, and CstIIΔ32^{I53S}; (iii) CTP, Mg^{2+}, Tris-HCl buffer (pH 7.5), NmCSS, and CstIIΔ32^{I53S}.

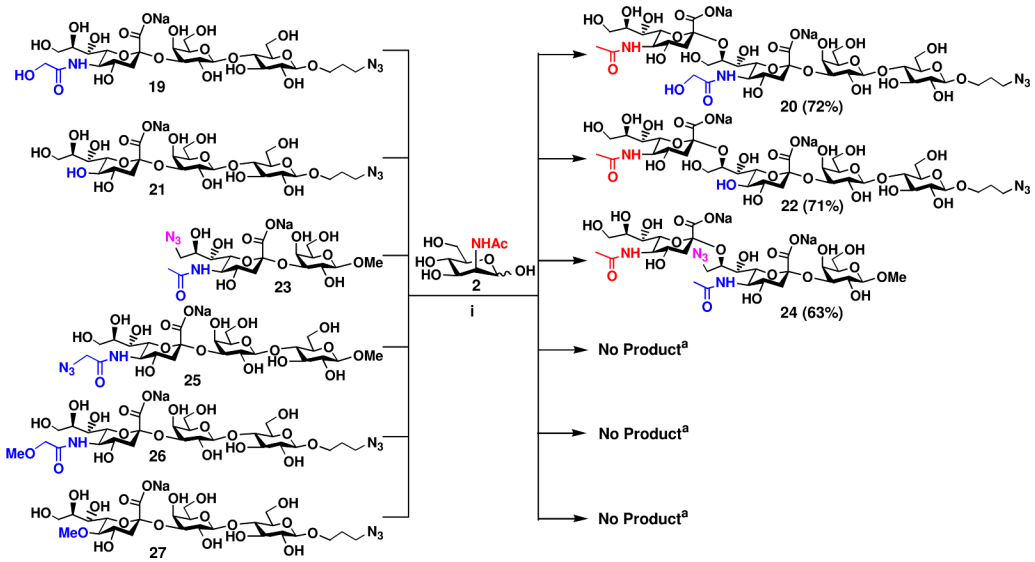

Figure 4.

Synthesis of disialyl GD3 oligosaccharides Neu5Acα2–8Siaα2–3GalβOR containing a terminal α2–8-linked Neu5Ac and different penultimate α2–3-linked sialic acid forms. Reagents and conditions: (i) Pyruvate, CTP, Mg^{2+}, Tris-HCl buffer (pH 8.5), *E. coli* K12 sialic acid aldolase, NmCSS, and $CstII\Delta32^{I53S}$. [a]Determined by small-scale reaction using TLC analysis.

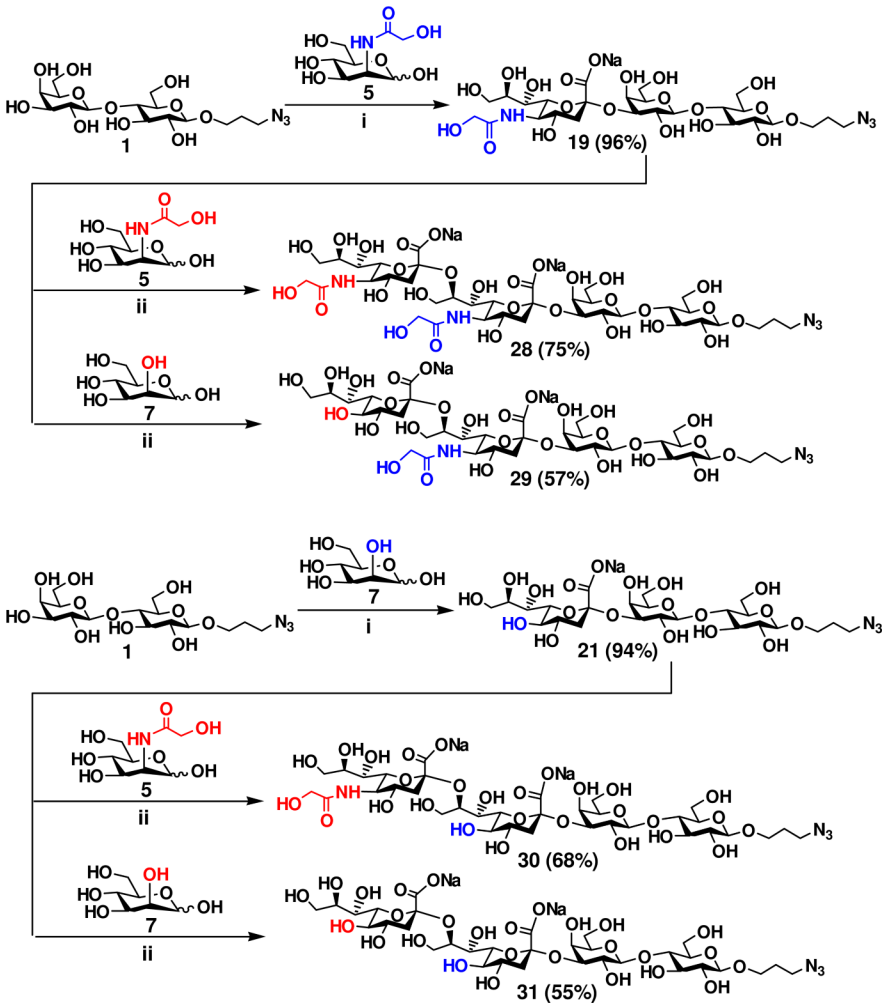

Figure 5.

Enzymatic preparation of GD3-type disialyl oligosaccharides Neu5Gc/KDNα2–8Neu5Gc/KDNα2–3LacβProN$_3$ containing the combination of Neu5Gc and KDN. Reagents and conditions: (i) Pyruvate, CTP, Mg^{2+}, Tris-HCl buffer (pH 8.5), *E. coli* K12 sialic acid aldolase, NmCSS, and PmST1; (ii) Pyruvate, CTP, Mg^{2+}, Tris-HCl buffer (pH 8.5), *E. coli* K12 sialic acid aldolase, NmCSS, and CstIIΔ32^{I53S}.

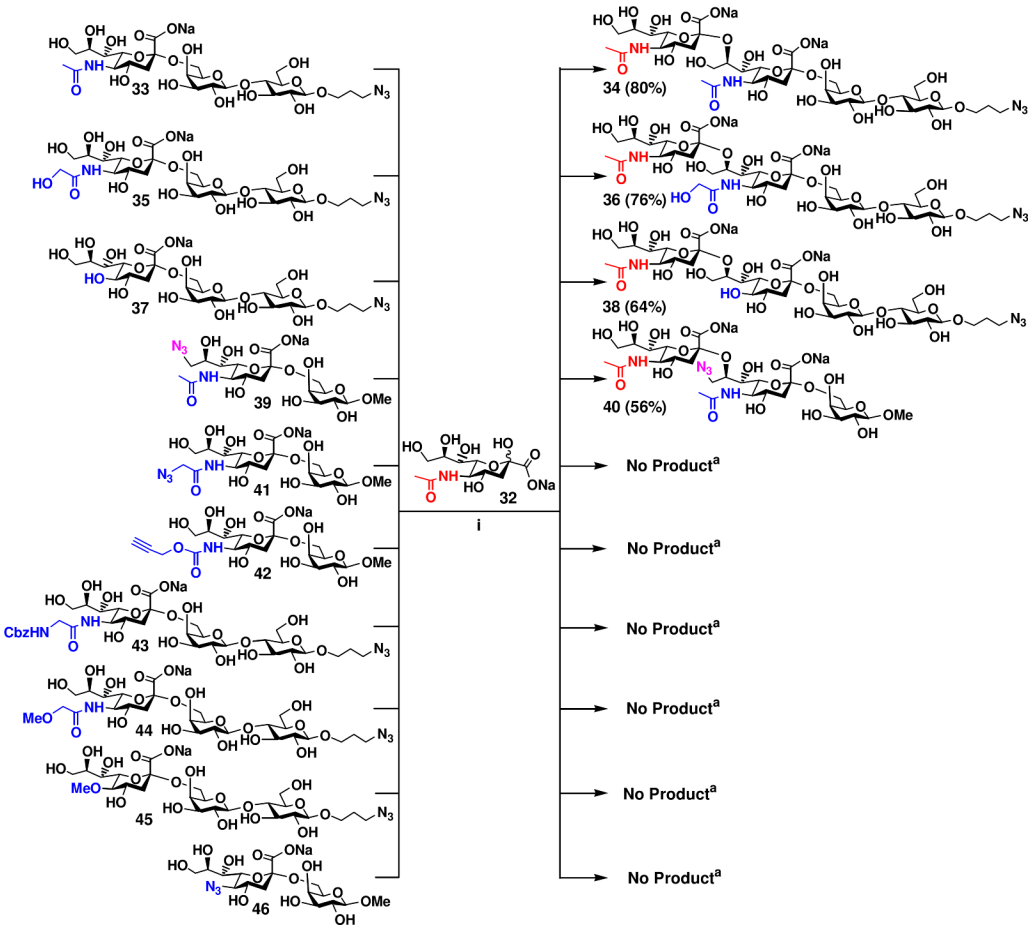

Figure 6.

Synthesis of Neu5Ac2–8Siaα2–6GalβOR-type disialyl oligosaccharides containing a terminal α2–8-linked Neu5Ac and different penultimate α2–6-linked sialic acid forms. Reagents and conditions: (i) CTP, Mg^{2+}, Tris-HCl buffer (pH 8.5), NmCSS, and CstIIΔ32^{I53S}. [a]Determined by small-scale reactions using TLC analysis.