CHEMISTRY
A European Journal

www.chemeurj.org

Reprint

A Journal of
ChemPubSoc Europe
ACES
Asian Chemical Editorial Society
WILEY-VCH

ChemPubSoc Europe

DOI: 10.1002/chem.201700506

CHEMISTRY A European Journal Full Paper

Carbohydrates

Synthesis of an S-Linked α(2→8) GD3 Antigen and Evaluation of the Immunogenicity of Its Glycoconjugate

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Abstract: Replacing the interglycosidic oxygen atom of oligosaccharides with a nonhydrolyzable sulfur atom has attracted significant interest because it provides opportunities for developing new glycoconjugate vaccines. Herein, a stereocontrolled and highly convergent method to synthesize a non-reducing-end inter-S-glycosidic variant of the GD3 antigen (S-linked α(2→8) GD3) that is resistant to enzymatic hydrolysis is reported. The key steps in the synthesis are a regio- and stereoselective α(2→3) sialylation of a lactoside acceptor with a C8-iodide-derivatized sialyl donor and an anomeric S-alkylation, which enable stereoselective construction of a terminal S-linked α(2→8) disialyl residue. The sulfhydryl-reactive maleimide group was used as the linker for the well-defined conjugation of these antigens to the immunogenic protein keyhole limpet hemocyanin (KLH). Groups of mice were immunized with the GD3–KLH and S-linked GD3–KLH glycoconjugates in the presence of complete Freund's adjuvant. Microarray analysis of the sera showed the promise of the S-linked GD3–KLH vaccine: it stimulated a high immunoglobulin G response against S-linked GD3 and cross-reactivity with the O-linked GD3 antigen was low. The activity of the S-linked GD3–KLH vaccine was comparable to that of the O-linked GD3–KLH vaccine, which highlighted the effectiveness of generating glycoconjugate vaccines and immunotherapies by relatively simple means.

Introduction

Gangliosides are unique cell-surface acidic glycolipids comprised of one or more sialic acid (*N*-acetylneuraminic acid, Neu5Ac) residues at the non-reducing terminus of oligosaccharide chains.[1] Primarily, these glycolipids are components of the outer leaflet of the plasma membranes of vertebrate cells and are essential mediators of diverse cellular functions.[2] Although detectable in normal healthy tissues at low levels, human tumors of neuroectodermal origin showed aberrant cell-surface expression patterns of gangliosides. For example, ganglioside GD3 (**1**; Figure 1), which is ubiquitous in all types of tissues and cells in higher vertebrates (including humans), is the most-abundant ganglioside in human melanoma and glioma cells—more abundant than GM2, GM3, GD2, and fucosyl-GM1 gangliosides.[3] Furthermore, the high surface expression of **1** on many types of malignant cell often provokes the immune system to recognize and target these tumor-associated carbohydrate antigens (TACAs).[4] Consequently, tumor-derived gangliosides have been extensively considered as immunotargets for the treatment of cancer.[5] However, **1** is known to have low antigenicity because of its self-antigen nature. Further studies of GD3-based conjugates showed that **1** can elicit a T-cell-dependent immune response that is characterized by immunoglobulin G (IgG) production when tethered to an immunocarrier protein such as keyhole limpet hemocyanin (KLH) and co-administered with an immunological adjuvant such as QS-21, a saponin natural product.[6]

Glycoconjugates composed of homogeneous synthetic and structurally well-defined carbohydrate haptens can enable better structure–function analysis for the design and optimization of oligosaccharide-based vaccines. Unlike vaccine constructs of glycoconjugate-derived glycans from natural sources, these glycotherapeutics possess well-defined and uniform glycan structures and conjugation sites and, more importantly, completely lack contaminating immunogens or residual microbes.[7–9]

Structural modification of cell-surface glycans that mimic native antigens can induce changes in the immunogenicity and antigenicity of the original carbohydrate antigen in cancer cells. One such class of mimics is primarily designed to minimize chemical degradation and enhance metabolic stability to

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*Supporting information for this article can be found under https://doi.org/10.1002/chem.201700506. It contains enzymatic hydrolysis, protein conjugation, immunization, and microarray assay protocols, synthetic methods, and characterization data (including ^{1}H and ^{13}C NMR spectra) for compounds **4**, **6**, **7**, **9**–**12**, **21**–**24**, **27**–**39**, **43**, and **44**.*

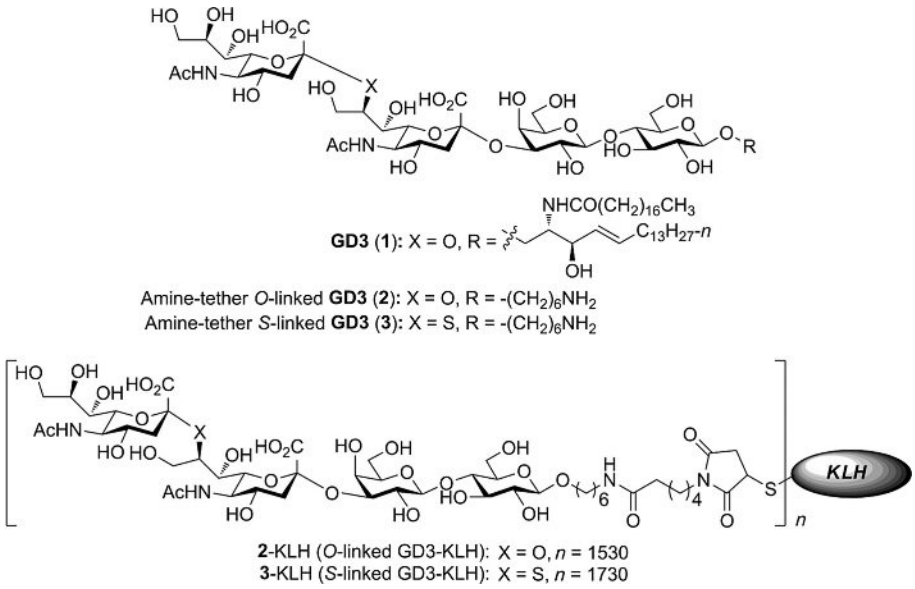


Figure 1. Structures of the ganglioside GD3 **1**, target GD3 antigens **2** and **3**, and the corresponding KLH–GD3 glycan conjugates.

endogenous glycosyl hydrolases through the introduction of hydrolysis-resistant glycosidic linkages such as C- or S-linked (oligo)saccharides.[10–12] In solution the latter often show similar conformations to the corresponding *O*-glycosides and similar or even more potent biological activities when bound to a protein.[13] The average O–C bond length in *O*-glycosides is typically 1.42 Å, whereas the anomeric S–C bond length in thioglycosides is relatively long at 1.78 Å, and therefore thioglycosides have significantly greater flexibility than natural *O*-glycosides.[14] Consequently, thioglycosides have been considered as inhibitors for glycoside hydrolases that facilitate crystallographic characterization of these enzymes.[15] Initial studies demonstrated that S-linked α(2→3) ganglioside GM3 trisaccharide antigens conjugated to tetanus toxoid produce antigen-specific, high-titer immunoglobulin M (IgM) and IgG antibodies in mice, which exceeds the response from native O-linked antigens.[16,17] These findings indicate the potential of S-linked α-sialosides as therapeutic cancer vaccines.

The stereoselective formation of the α-*S*-glycosidic bond in thioglycosides is a notoriously difficult task.[18] A survey of the literature revealed that α-*S*-sialosides are most commonly prepared by an S_N2 reaction of electrophilic glycosides (e.g., β-sialyl chloride) with either non-anomeric or anomeric metal thiolates.[19,20] Despite the almost universal use of β-sialyl chloride[12,21] for the synthesis of α-*S*-sialosides, use of this reagent is often complicated by the competing elimination reaction.[22] Among other reported approaches, anomeric S-alkylation has been investigated for the synthesis of α-*S*-sialosides via generation of a free sulfhydryl group at the anomeric position in situ.[21,23] It should be noted that the synthesis of 2-mercapto-Neu5Ac derivatives suffers from unsatisfactory yields because of competing reactions such as elimination and disulfide bond formation under the experimental conditions,[22] which further complicates the formation of the desired thioglycosides. Recently, a more-reactive cyclic *N*-acetyl-*N*5,*O*4-oxazolidinone-protected sialyl donor with a dibutyl phosphate anomeric leaving group has been introduced for the synthesis of α-*S*-sialosides, which eliminates some of these limitations.[24]

Previously, we reported the use of sialyl disulfides as nucleophilic donors for the exclusive formation of S-linked α(2→9) disialyl linkages.[25] These unsymmetrical disulfide donors undergo α-selective sialylation by nucleophilic substitution, and a major advantage is that the *tert*-butyl disulfide protecting groups can be readily reduced to thiol moieties, which enables reactivity tuning at any stage of the Neu5Ac chain elongation. Furthermore, we extended this novel anomeric S-alkylation strategy to masked thiosialyl donors that exhibit similar reactivity in an S_N2 displacement reaction with C8-iodide-activated electrophilic sialyl acceptors to construct S-linked α(2→8) disialyl linkages.[26] Notably, the anomeric configuration of the active intermediate—a 2-mercapto Neu5Ac derivative, generated in situ by unmasking *tert*-butyl disulfide-protected sialosides by treatment with sodium 2-mercaptoethanesulfonate (MESNa)—is stable and mutarotation does not occur instantaneously, unlike Neu5Ac hemiacetals[27] and other sugar hemiacetals, which are prone to mutarotation under most conditions. This approach was successfully exemplified in the highly stereoselective synthesis of S-linked α(2→9)- and α(2→8)-oligosialic acids. However, this versatile strategy has not yet been applied to the formation of a thioglycosidic bond in complex gangliosides such as **3** (Figure 2). Synthetic access to the isosteric S-linked ganglioside antigen is a prerequisite to further investigation of structure–function relationships and would be of significant value to determine the T-cell epitopes of these conjugates (Figure 1).

Here, we report the first stereoselective synthesis of S-linked α(2→8) GD3 antigen **3** modified with an amine tether by the anomeric S-alkylation strategy. We show that the terminal S-linked α(2→8)-disialyl residue in **3** is completely resistant to bacterial sialidase hydrolysis. Once synthesized, S-linked GD3

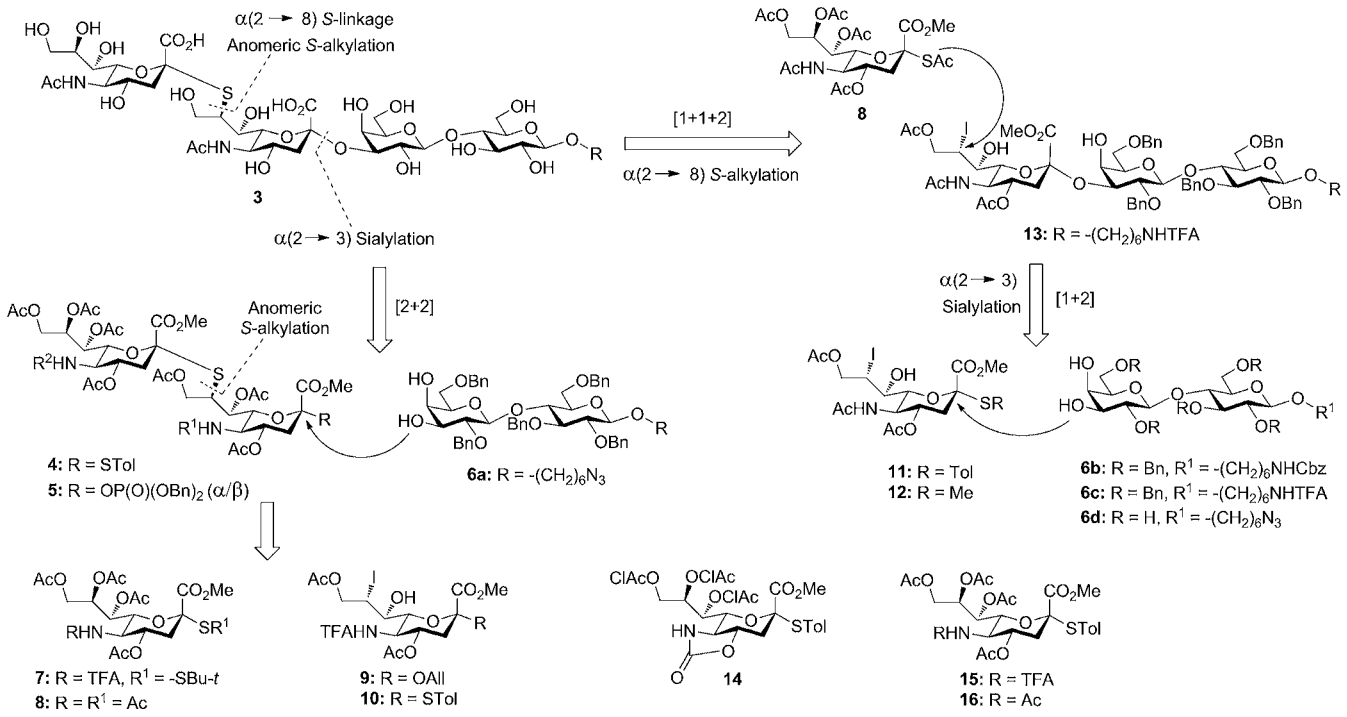


Figure 2. Retrosynthetic analysis of S-linked GD3 antigen **3** and building blocks for its chemical synthesis.

was conjugated with KLH, and the resulting vaccine (S-linked GD3–KLH) was used to immunize mice. A comparative microarray analysis of the sera of the immunized mice revealed that S-linked GD3–KLH induces antibody responses capable of recognizing the original S-linked GD3 **3** as well as the native O-linked GD3 antigen **2**.

Results and Discussion

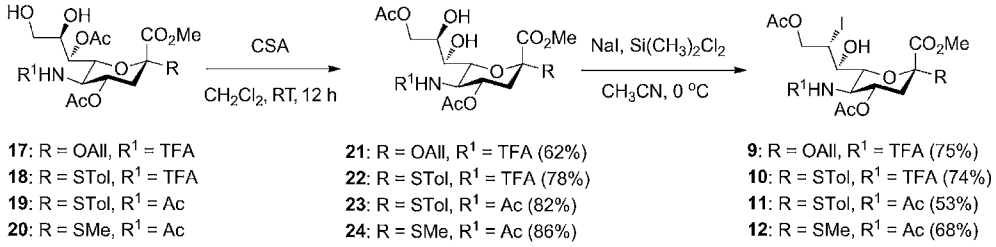
Despite continuing advances in sialic acid-containing glycan synthesis,[28–30] access to a non-natural S-glycosidic linkage such as that in S-linked GD3 **3** (Figure 2) represents a considerable synthetic challenge. Because the isosteric glycans, GD3 **2** and S-linked GD3 **3** contain Neu5Acα(2→3)Gal and Neu5Acα(2→8)Neu5Ac glycosidic linkages, respectively, the stereoselective introduction of either molecule is difficult to achieve by chemical synthesis.[31–34] In addition, thioether formation by nucleophilic substitution at the C8 position in **3** is particularly challenging because of the low reactivities of the tertiary thiol nucleophile and secondary leaving group. The reaction is also disfavored by the sterically crowded tertiary center of the anomeric Neu5Ac C2 thiolate moiety. Although chemoenzymatic methods that use glycosyltransferases are suitable for the large-scale production of GD3 and its derivatives,[35–38] this strategy is not feasible for the analogous S-linked GD3 tetrasaccharide. Previous studies of glycosyltransferase-catalyzed glycosidic bond formation with thiol-based acceptors, such as 3′-thiolactosides, showed promising results for the synthesis of thiooligosaccharides,[39] but the generation of inter-S-glycosidic linkages during S-linked GM3 synthesis was hampered[17] largely because of substrate intolerance. Thus, chemical synthesis is the most-promising route to prepare large quantities of S-linked carbohydrate antigens.

To probe and overcome this inefficiency, we considered two synthetic approaches to construct the critical α(2→8)-*S*-glycosidic linkage in **3** (Figure 2). In the first approach, we envisioned a direct and highly convergent [2+2] assembly of S-linked disialyl donor **4** and lactose-derived disaccharide acceptor **6a** reminiscent of an α(2→3) sialylation strategy. The S-linked α(2→8) disialosides **4** and **5** could be prepared by a highly stereoselective anomeric S-alkylation reaction[26] between a highly reactive thiol (generated in situ from **7** or **8**) and C8-iodide-activated acceptor **9** or **10**. The second approach involved stepwise union of a C8-iodide-activated thiosialyl donor (**11** or **12**) with lactoside acceptor **6c** to form trisaccharide **13**. This strategy would permit us to exploit a similar S-glycosidic bond-forming reaction with Neu5Ac 2-thioacetate **8**[21] to form the α(2→8)-*S*-glycosidic bond by a [1+1+2] glycosylation (Figure 2). To investigate stereoselective α-sialylation we used building blocks **9**–**15** modified by a set of carefully chosen *N*5-[40] and hydroxyl-protecting groups, such as trifluoroacetyl (TFA),[32,41] cyclic *N*5,*O*4-oxazolidinone,[42] and chloroacetyl (ClAc)[43] groups, along with traditional thiosialosides **8** and **16**. In addition, we also investigated sialyl donors with a different anomeric leaving group (e.g., phosphate **5**),[44,45] as well as the effect of the solvent on the α-selective sialylation reaction.[42,46] Furthermore, different masked amines in the hydrophobic C6 spacer appended at the reducing terminus of **6** were installed for further conjugation to a carrier protein to avoid undesired side reactions during the synthesis.

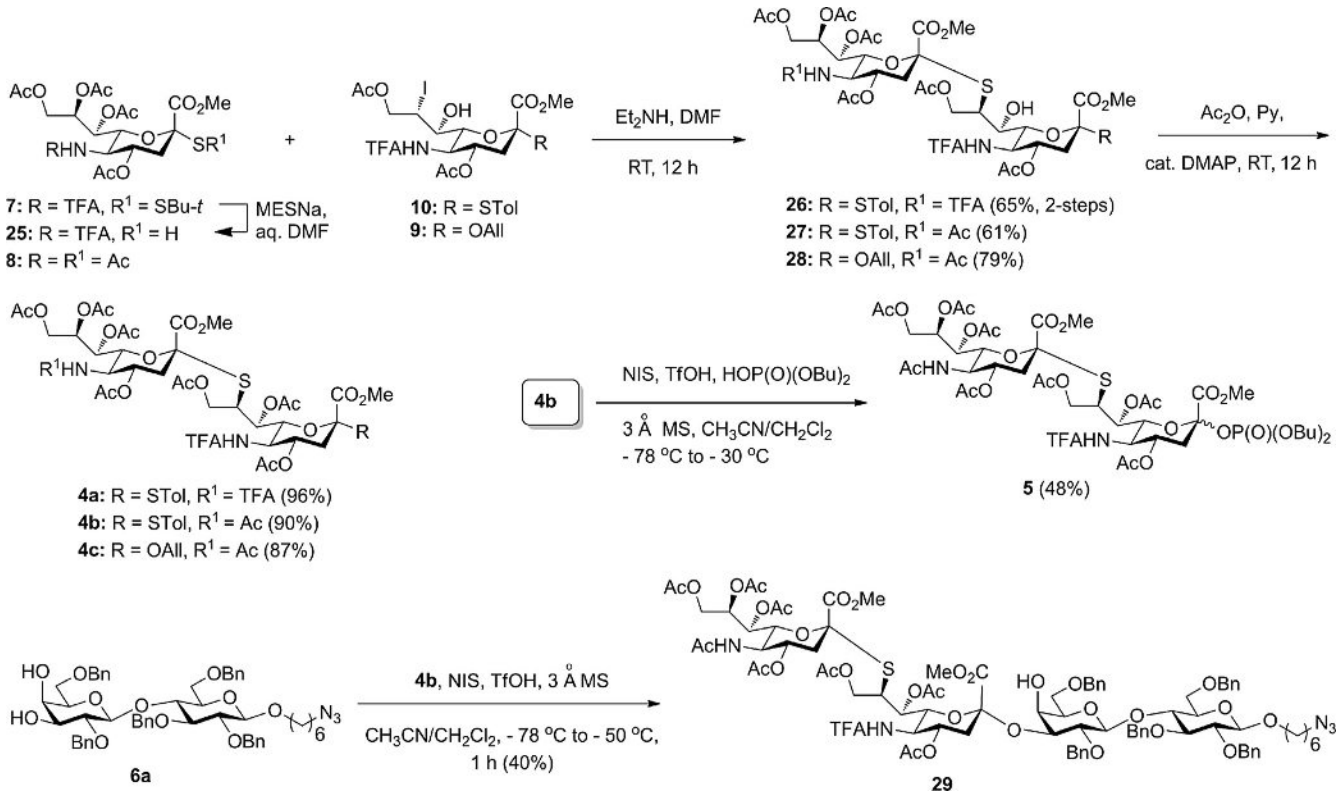
Synthesis of S-linked GD3 3 by a [2+2] strategy

The synthesis of S-linked α(2→8) disialosides **4** commenced with preparation of the two key C8-iodide-activated sialosides **9** and **10**, which were crucial electrophiles for the S_N2-type displacement reaction. Given the various regioselective methods available for intramolecular migration of acetyl groups in C9-OAc derivatives,[26] we developed an efficient synthesis to access **21**, **22**, and related derivatives **23** and **24** from the corresponding C7-OAc compounds **17**–**20** (Scheme 1). Attempts to transfer the acetate group in **17** from the C7 to C9 carbon atom by using previously reported conditions (basic resin Dowex 550 OH^-)[47] proved sluggish and provided C9-OAc compound **21** in disappointing yields (<10%; Table S1 in the Supporting Information). Additional reaction optimization—including neutral (MeOH), basic (Et_3N), acidic [(+)-10-camphorsulfonic acid (CSA)], or Lewis acid ($VO(OTf)_2$; Tf = triflyl) conditions—in different solvents and at different temperatures, revealed that this transformation could be accomplished in 62% yield in the presence of CSA in CH_2Cl_2 at room temperature (Scheme 1; Table S1 in the Supporting Information). When these conditions were applied to **18**,[41] **19**,[48] and **20**[49] the regioselective intramolecular acetyl group migration efficiently produced the C9-OAc derivatives **22**–**24** (78–86%). The C8-OH group in **21**–**24** was replaced by an iodide group (**9**–**12**, 53–75%, Scheme 1) with inversion of configuration upon treatment with dichlorodimethylsilane and sodium iodide in CH_3CN, by using our previously reported method.[26]

The α(2→8)-*S*-linkage of key disialosides **4a**–**c** (Scheme 2) was constructed by base-promoted (Et_2NH in DMF)[25,26] anomeric S-alkylation of masked thiols **7** and **8** with C8-iodide electrophiles **9** and **10**, respectively. Thus, thiol nucleophile **25** was formed by cleaving the *tert*-butyl disulfide **7** with MESNa (see the Supporting Information for details), and the revealed



Scheme 1. Synthesis of C8-iodosialosides **9**–**12**.



Scheme 2. Synthesis of α(2→8) S-linked disialosides **4** by α-stereoselective anomeric S-alkylation and partially protected S-linked GD3 tetrasaccharide **29** by a [2+2] strategy.

thiol moiety reacted with **10** to yield S-linked α(2→8) disialoside **26** (65% yield over two steps) with inversion of the C8 chiral center.

Neu5Ac 2-thioacetate **8** was also a competent nucleophile for reaction with **10** under basic conditions (Et_2NH in DMF); S-linked Neu5Acα(2→8)Neu5Ac **27** was obtained in 61% yield. Reacting nucleophile **27** with **9** did not alter the outcome of the S-alkylation reaction, and the corresponding allyl disialoside **28** was formed in good yield (79%). Subsequently, the C7-OH group in disialosides **26**–**28** was acylated to provide the fully protected S-linked α(2→8) disialosides **4a–c**. The S-linked thiodisialosides **4a** and **4b** were suitable sialylation donors, whereas **4b** was transformed into highly activated donor **5** (see below). The newly formed α-glycosidic bond of **4a** was unambiguously confirmed by the coupling constants of the two H3 axial protons ($H3_{ax}$) with the two methyl ester carbonyl carbon atoms (C1)[26,50] (^3J(C1,$H3_{ax}$) = 7.2 and 7.5 Hz, determined by selective proton-decoupled ^{13}C NMR spectroscopy).[50,51] Importantly, no β-anomer was detected by NMR spectroscopy. Thiosialoside **4b** was further transformed into phosphate donor **5** by treatment with dibutyl phosphate ($HOP(O)(OBu)_2$) in the presence of *N*-iodosuccinimide (NIS), catalytic triflic acid (TfOH), and 3 Å molecular sieves (MS) at −78 °C in a mixture of CH_2Cl_2 and CH_3CN to give an inseparable α/β-mixture (1:0.86) in 48% yield.

Our initial efforts towards the direct [2+2] α(2→3) sialylation of dibutyl phosphate donor **5** were conducted in 1:1 CH_3CN/CH_2Cl_2 with lactoside **6a**[52] and trimethylsilyl trifluoromethanesulfonate (TMSOTf) as the promoter at −78 °C, and afforded an inseparable anomeric mixture of **29** (Table S2 in the Supporting Information). A similar result was obtained when *N*5 mono-TFA-protected 4-thiotolyl donor **4b** was subjected to sialylation with **6a** (activation with NIS in the presence of catalytic TfOH in CH_3CN at −35 °C). Notably, when the same sialylation was performed in 1:1 CH_3CN/CH_2Cl_2 at −78 °C tetrasaccharide **29** was obtained in 40% yield, with the α-anomer as the major product (Scheme 2). Disappointingly, the more-reactive *N*5,*N*5′-di-TFA-protected donor **4a** was cleanly activated under the NIS/TfOH conditions, but acceptor **6a** remained unreacted and hydrolysis of **4a** was observed. However, an attempt to completely deprotect **29** in a five-step sequence (Scheme S1 in the Supporting Information) gave complex product mixtures that were difficult to purify, and therefore were not advanced further. This result highlighted the need for an alternative [1+1+2] strategy and possibly a more-effective glycosylation approach (see below).

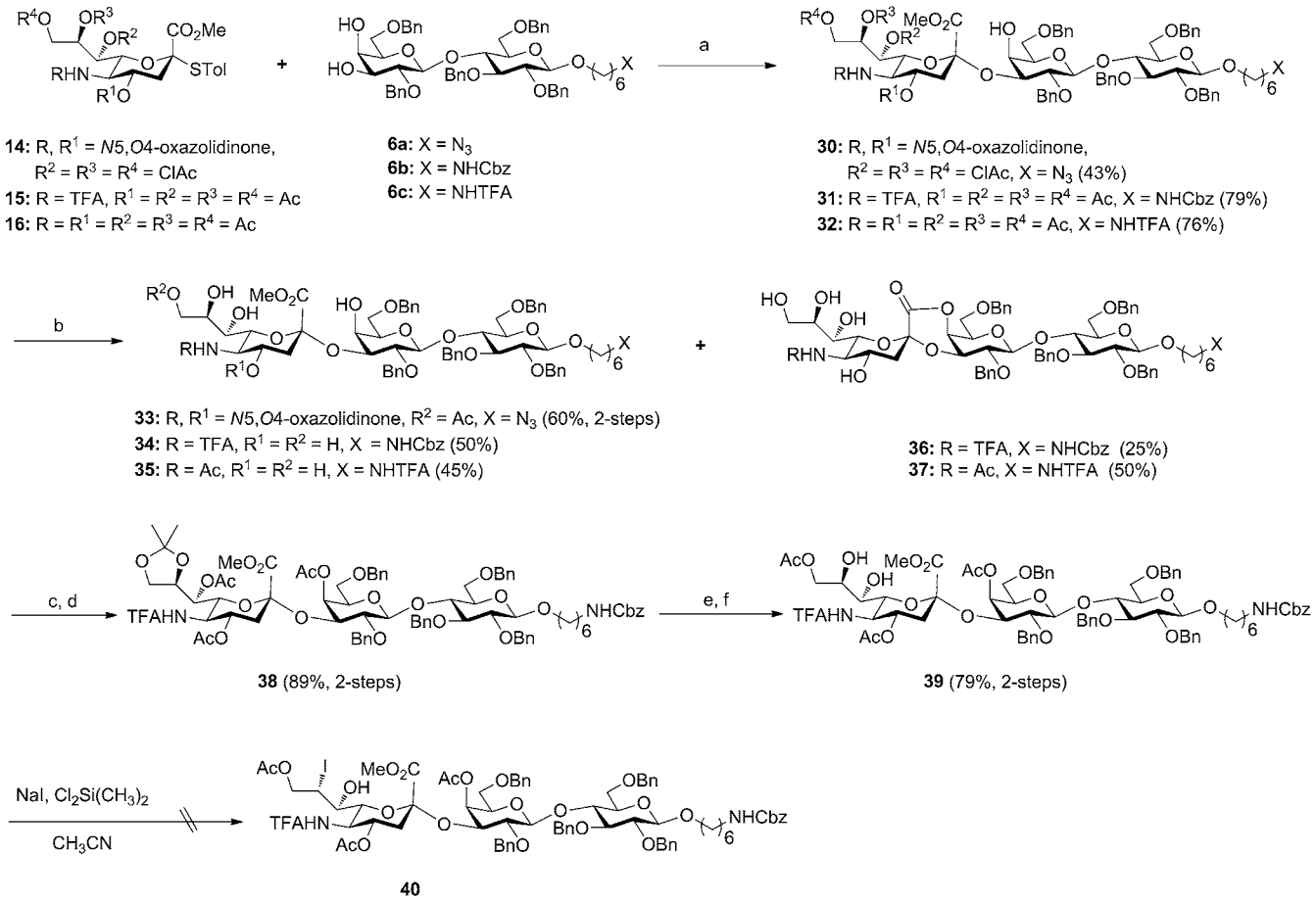
Synthesis of S-linked GD3 3 by a [1+1+2] strategy

The inability to obtain pure material from the direct assembly of disaccharide building blocks prompted us to test a stepwise glycosylation approach for the preparation of sialyllactose derivatives. We focused first on α(2→3) sialylation (Scheme 3). We anticipated that post-glycosylation modification was anticipated would lead to incorporation of a C8-iodide leaving group on the Neu5Ac residue of GM3 derivatives **13**. Treatment of cyclic *N*5,*O*4-oxazolidinone-protected donor **14**[43] and **6a** with NIS and TfOH (cat.) in 1:1 CH_3CN/CH_2Cl_2 provided α-sialoside **30** in moderate yield (43%). However, sialylation of *N*-TFA-protected thiosialyl donor **15**[41] with **6b** (see the Supporting Information for details) in acetonitrile at −35 °C significantly increased the yield of trisaccharide **31** (79%). For comparison, traditional *N*-acetyl *p*-thiotolylsialyl donor **16**[53] was also coupled to acceptor **6c**.[43] To our delight, (2→3) sialylation under the same reaction conditions proceeded very cleanly to produce trisaccharide **32** in 76% isolated yield with complete anomeric stereoselectivity. The α-stereochemistry of the newly formed glycosidic bonds was assigned based on the chemical shift of the Neu5Ac H3 proton (**31**: $\delta = 2.48$ ppm, dd, $J = 13.1$, 4.8 Hz, **32**: $\delta = 2.52$ ppm, dd, $J = 13.1$, 4.7 Hz) and the coupling constant between the H3 axial proton and the C1 carbonyl carbon atom of the methyl ester group (d, ^3J(C1, $H3_{ax}$) = 6.5 and 5.7 Hz for **31** and **32**, respectively, determined by selective $\{^1H\}^{13}C$ NMR spectroscopy).

Installing an iodide leaving group at the C8 position of the sialic acid moiety in GM3 derivatives **30**–**32** requires selective protection of the most-reactive primary hydroxyl group at C9. Accordingly, removal of the *O*-ClAc protecting groups in **30** under basic conditions (Et_3N, MeOH) followed by a temperature-controlled regioselective O-acetylation (AcCl, collidine, CH_2Cl_2, −40 °C to RT) gave the C9 *O*-Ac derivative **33** in 60% yield over two steps. De-O-acetylation of **31** under Zemplén conditions (NaOMe, MeOH) produced building block **34** (50%), as well as lactone **36** (25%), which is a product of lactonization between the CO_2Me moiety of the non-reducing-end Neu5Ac moiety and the neighboring C4 hydroxyl group of galactose. By contrast, Zemplén deprotection of **32** produced a modest yield (45%) of the desired trisaccharide **35** and showed a notable shift toward lactone **37** (50%). As a representative example of the proposed C7-to-C9 acetyl group migration in trisaccharide-based substrates, 8,9-*O*-isopropylidenation of **34** (2,2-dimethoxypropane (DMP), CSA, acetone) was performed, and was followed by acetylation [Ac_2O, pyridine, 4-(dimethylamino)pyridine (DMAP) (cat.)] to provide fully protected trisaccharide **38** (89% over two steps). Finally, the 8,9-*O*-isopropylidene acetal in **38** was cleaved by trifluoroacetic acid in wet CH_2Cl_2, and the primary hydroxyl group of the resulting diol was selectively acetate protected by a CSA-catalyzed acetyl group migration from C7 to the C9 in CH_2Cl_2 to afford **39** (79% over two steps).

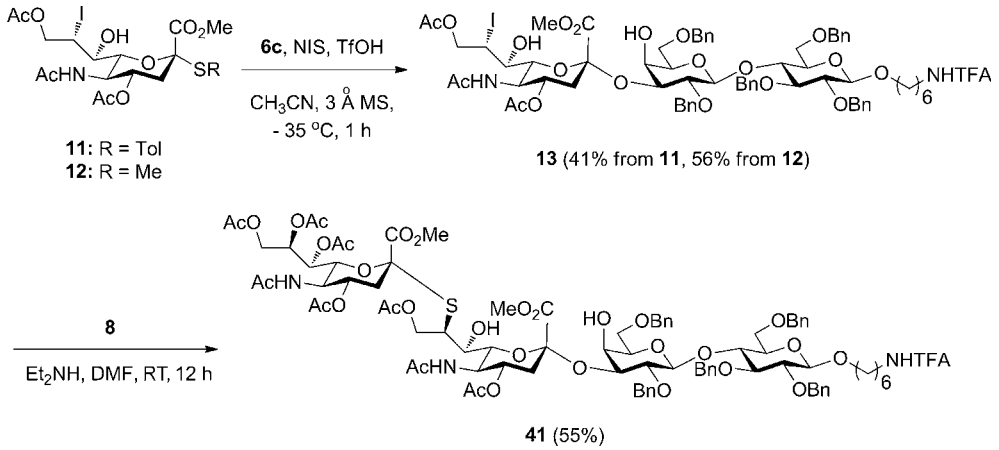
At this point, the C8-hydroxyl group in these representative sialosides (**33** and **39**) needed to be converted to the key C8-iodide-activated GM3-based trisaccharide acceptors required for the α(2→8) S-alkylation discussed above (Figure 2). We envisioned that this transformation could be accomplished by an iodotrimethylsilane (generated in situ) catalyzed transposition. However, treatment of the sterically encumbered C8 alcohols **33** and **39** with dichlorodimethylsilane and sodium iodide did not form the desired iodide compounds and produced capricious and intractable product mixtures.

Having demonstrated the effective regio- and stereoselective assembly of GM3 trisaccharide **32**, we next evaluated whether C8-iodide-activated thiosialoside donors **11** and **12** displayed similar reactivity in an analogous α(2→3) glycosylation



Scheme 3. Reagents and conditions: a) NIS, TfOH, CH_3CN/CH_2Cl_2 (1:1 v/v), 3 Å MS, −78 °C to −50 °C for **30**; NIS, TfOH, CH_3CN, 3 Å MS, −35 °C for **31** and **32**; b) MeOH, Et_3N; then AcCl, collidine, CH_2Cl_2, −40 °C to RT for **33**; NaOMe, MeOH for **34** and **35**; c) 2,2-dimethoxypropane, CSA, acetone; d) Ac_2O, pyridine, DMAP (cat.), RT, 12 h; e) trifluoroacetic acid, H_2O/CH_2Cl_2, 0 °C, 10 min; f) CSA, CH_2Cl_2, RT, 12 h. Cbz = carbobenzyloxy, Bn = benzyl.

(Scheme 4). Use of an *N*5-acetyl protecting group in **11** and **12** eliminated the requirement for additional reinstallation steps, which further simplified the final-stage modification. More importantly, the C8-iodide leaving group on these donors makes them competent electrophiles for post-glycosylation nucleophilic substitution with 2-mercapto Neu5Ac derivatives. Donors **11** and **12** were prepared in a similar manner to that outlined above for the synthesis of **9** and **10** (Scheme 1). Gratifyingly, both donors were reactive to acceptor **6c** under NIS/TfOH-promoted conditions in CH_3CN at −35 °C, and **13** was obtained in satisfactory yields (41% from **11**; 56% from **12**). Again, full characterization confirmed the excellent regio-(2→3) and ste-

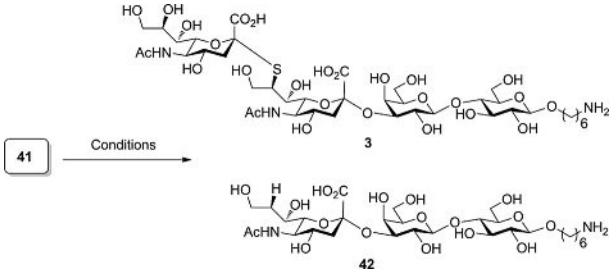


Scheme 4. Synthesis of partially protected S-linked α(2→8) GD3 **41**.

reoselectivity (α anomer, d, $^3J(C1'',H3''_{ax}) = 6.3$ Hz) of the sialylation reaction. Because the iodide leaving group is positioned at C8 of the non-reducing-end Neu5Ac residue in **13**, subsequent nucleophilic attack by the thiol generated in situ from **8** proceeded stereoselectively to produce protected S-linked α(2→8) GD3 tetrasaccharide **41** in moderate yield (55%).

The final synthetic challenge was the deprotection of S-linked α(2→8) GD3 tetrasaccharide **41**. Birch reduction conditions have previously been effective for benzyl ether deprotection in S-linked GM3 derivatives.[17] The deprotection of nine base-sensitive protecting groups (acetyl esters, methyl esters, and trifluoroacetamide) also further complicated the sequence. Various conditions were tested for sequential and simultaneous removal of the benzyl ethers and base-labile protecting groups (Table 1). However, saponification (NaOH, MeOH) of **41** followed by a dissolving-metal reduction of the resulting intermediate was impractical and inferior for S-linked GD3-based substrates. The desired S-linked GD3 glycan **3** was obtained in only 48% yield over two steps (Table 1, entry 1). When catalytic hydrogenation (H_2, Pd/C) was performed instead of Birch reduction 8″-deoxy trisaccharide **42** (identified by HRMS (ESI); *m/z* calcd for $C_{29}H_{53}N_2O_{18}$ $[M+H]^+$: 717.3293; found: 717.3293) was the major product, obtained by hydrogenolytic cleavage of the terminal S-linked Neu5Ac moiety (Table 1, entry 2). The diminished yield of **3** reported in Table 1, entry 1 was probably due to competition from this undesired side reaction. Gratifyingly, catalytic hydrogenation (H_2, Pd/C, EtOH) followed by saponification afforded the target S-linked α(2→8) GD3 glycan **3** in good yield (77%, over two steps; Table 1, entry 3). Additionally, the linker was suitable for site-selective conjugation to a carrier protein (see below).

Table 1. Establishing the optimal sequential deprotection conditions for **41**.

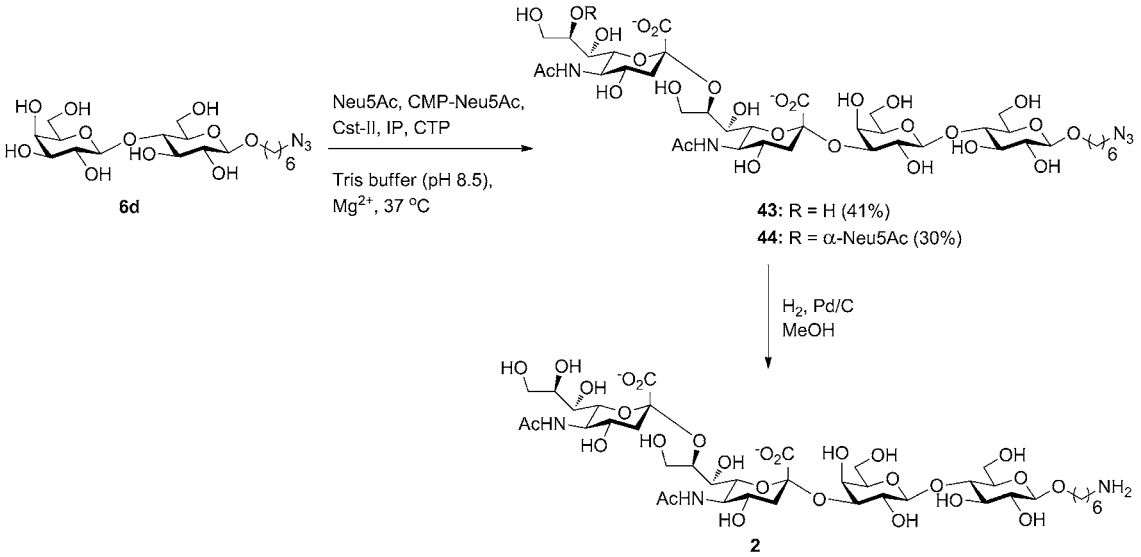


Entry	Conditions	42	Yield of 3 [%]
1	i) NaOH (aq.), MeOH ii) Na, NH_3 (l), −78 °C	–	48
2	i) NaOH (aq.), MeOH ii) H_2, Pd/C, MeOH, H_2O/AcOH	major	–
3	i) H_2, Pd/C, EtOH ii) NaOH (aq.), MeOH	–	77

Enzymatic synthesis of GD3 2

Chemical α-*O*-sialylations,[54,55] particularly the formation of Neu5Acα(2→8)Neu5Ac disialyl linkages, often pose significant synthetic challenges. Therefore, enzymatic synthesis with bacterial sialyltransferase (SiaT) Cst-II from *Campylobacter jejuni* was applied to prepare amine-tethered GD3 tetrasaccharide **2**. *C. jejuni* Cst-II is a bifunctional α(2→3/8) SiaT that transfers Neu5Ac to both 3-*O*-Gal and 8-*O*-Neu5Ac residues.[56] To simplify the synthesis, a sequential one-pot method was applied (Scheme 5).

First, cytidine-5′-monophosphosialic acid (CMP-Neu5Ac) was generated by using CMP-Neu5Ac synthetase (CSS, from *Neisseria meningitides*), cytidine-5′-triphosphate (CTP), and Neu5Ac (5 equiv), followed by addition of lactoside **6d**[57] and *C. jejuni* Cst-II to produce GD3 derivative **43** (41%) and trisialylated product **44** (30%). Traces of higher oligomers were detected but not isolated. Catalytic hydrogenation (H_2, Pd/C) of **43** in methanol produced amine-tethered GD3 **2** (in essentially quantitative yield), which was used for enzymatic stability studies



Scheme 5. One-pot enzymatic synthesis of GD3 **2**.

and converted to the sulfhydryl-selective maleimide derivative **45** (see below).

Stability of GD3 2 and S-linked GD3 3 against sialidase

With the key GD3 glycans **2** and **3** in hand, their stabilities during enzymatic hydrolysis by sialidase were examined.[12] Sialidases are hydrolytic enzymes that cleave the Neu5Ac residues on glycolipids or glycoproteins.[58] Bacterial *Vibrio cholerae* sialidase was chosen due to its accessibility and greater substrate promiscuity. The hydrolysis of compounds **2** and **3** (6 mM) was performed with sialidase (10 μL, 0.106 U of activity), and the reaction was monitored by following the equatorial H3 protons ($H3_{eq}$) of the Neu5Ac moieties of these glycans by ^{1}H NMR spectroscopy (Figure 3).

As expected, incubating GD3 glycan **2** with sialidase for 30 min led to a significant decrease of the $H3_{eq}$ proton signals ($\delta = 2.78$ and 2.68 ppm) and a new peak was observed centered at $\delta = 2.21$ ppm, which corresponds to the $H3_{eq}$ proton of β-Neu5Ac (Figure 3a versus b). As the enzymatic reaction progressed, complete hydrolysis of both the α(2→8)- and internal α(2→3)-linkages occurred within 120 min of incubation (Figure 3c). Due to the exoglycosidase activity of used sialidase, the hydrolysis is assumed to occur at the non-reducing-end Neu5Ac residues[58] to give α-Neu5Ac with concomitant mutarotation to thermodynamically favored β-Neu5Ac in solution. In contrast, no evidence was observed (within the NMR detection limit) for the cleavage of either the terminal S-linked α(2→8) or internal α(2→3) Neu5Ac moiety in **3** after 72 h incubation (Figure 3d versus f). These results indicate that S-linked GD3 antigen **3** is metabolically stable, and support the view that the inter-*S*-α(2→8) link is not enzymatically hydrolyzable, which prevents cleavage of the terminal Neu5Ac residue by sialidase.

Preparation of KLH–GD3 glycan conjugates

Glycans are typically weakly immunogenic and are often T-cell-independent antigens. However, a strong T-cell immune response[59] can be stimulated by conjugating glycan antigens with a highly immunogenic carrier protein. KLH was chosen as the foreign carrier protein due to its low cost, accessibility, and proven efficacy as a carrier protein for a clinical GD3 vaccine against melanoma.[60] The strong Michael-type reaction between sulfhydryl groups and maleimides was selected for the antigen–protein conjugation chemistry.[61–63] To obtain the conjugate, synthetic amine-tethered GD3 glycans **2** and **3** were reacted with a heterobifunctional cross-linker (6-maleimidocaproic acid *N*-succinimidyl ester) to produce maleimide derivatives **45** (79%) and **46** (70%), respectively (Scheme 6). Next, the lysine residues of KLH were derivatized by treatment with Traut's reagent (2-iminothiolane) to produce thiolated KLH.[64] On average, 2200 sulfhydryl groups were grafted onto KLH (estimated by using Ellman's reagent [5,5-dithio-bis(2-nitrobenzoic acid)]).

Subsequently, maleimide derivatives **45** and **46** were conjugated to iminothiolane-derivatized KLH in phosphate buffer (0.1 M, pH 7.2) at ambient temperature for 24 h. After dialysis, the glycan–KLH conjugates **2**–KLH and **3**–KLH were ready for immunization. Approximately 1530 (for **2**) and 1730 haptens (for **3**) were loaded per KLH molecule with a conjugation efficiency of 17 and 20%, respectively (determined by Warren's assay and Ellman's test). Compared with previous results for ganglioside GD3 conjugation to KLH by reductive amination,[65] the current protocol provides a higher degree of glycan load-

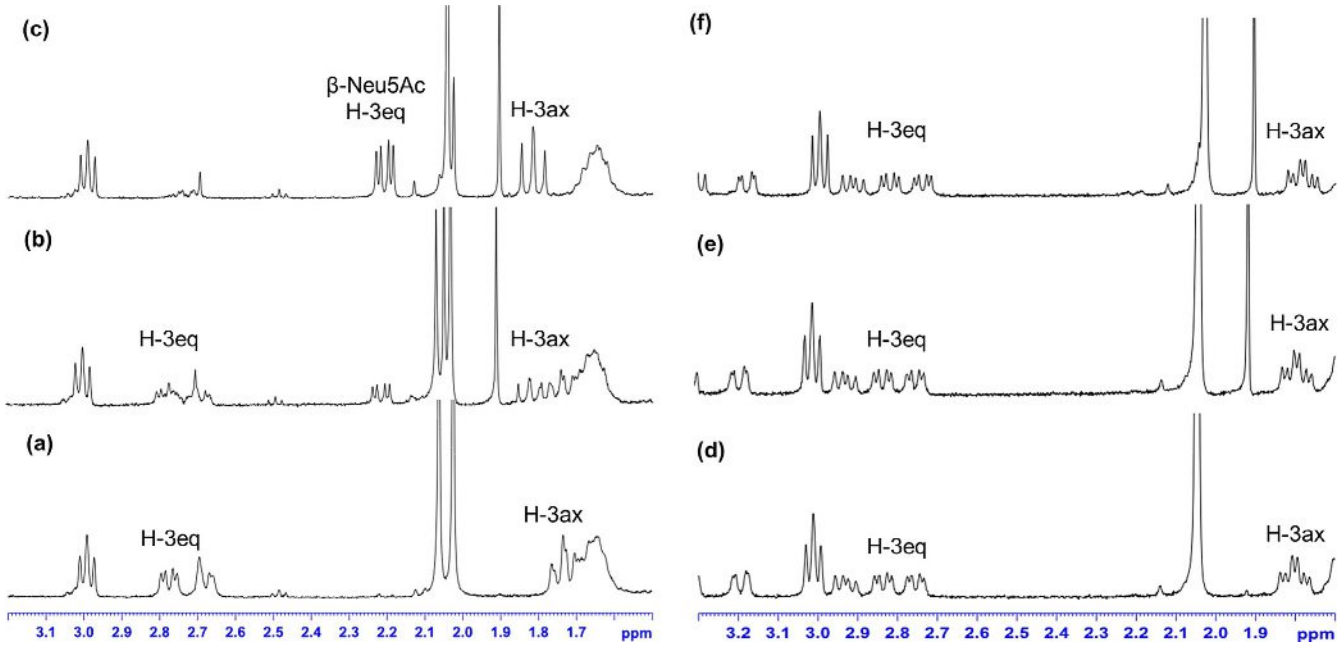
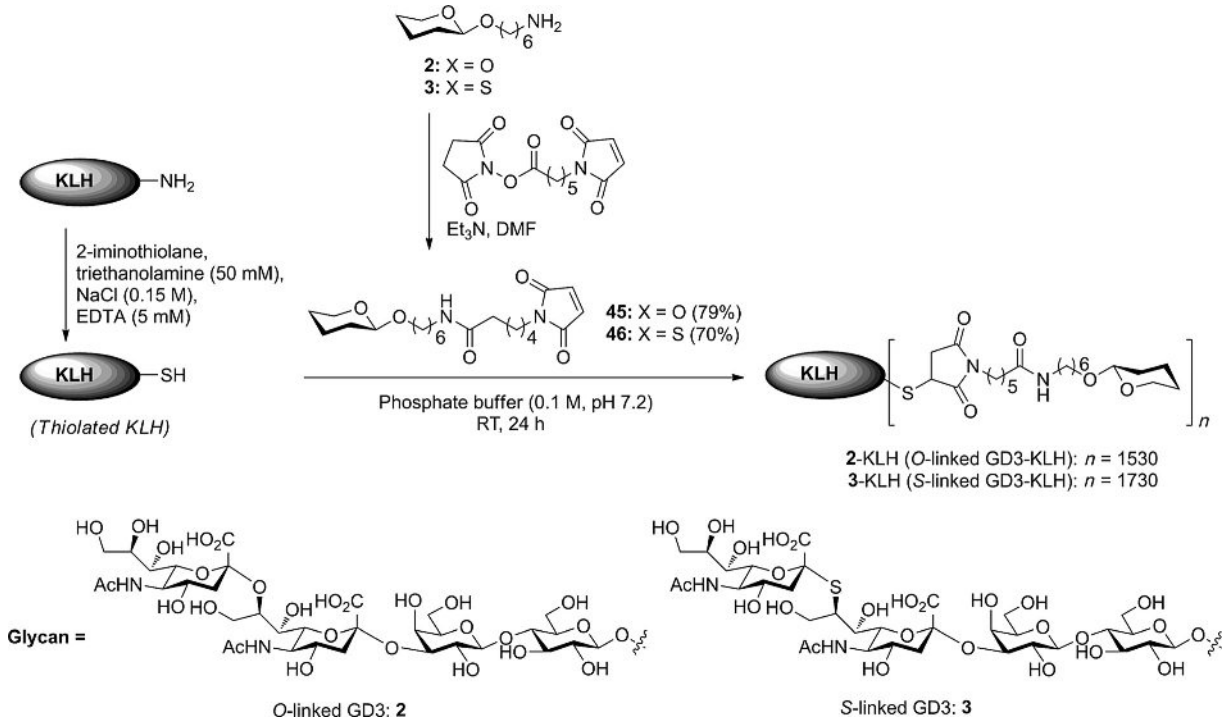


Figure 3. Comparing the hydrolytic stability of O-linked (**2**) and S-linked GD3 (**3**) glycans toward *V. cholerae* sialidase. Partial ^{1}H NMR (400 MHz, D_2O) spectra of **2** and **3** at different incubation times. Compound **2**: a) 0 min, b) 30 min, and c) upon complete hydrolysis. Compound **3**: d) 0 min, e) 30 min, and f) 72 h.



Scheme 6. Preparation of KLH–glycan conjugates **2**–KLH and **3**–KLH.

ing to the carrier protein. The key to achieving a superior epitope ratio is presumably the covalent attachment of the linkers to the preactivated biomolecules by the efficient Michael-type addition reaction.

Immunogenicity of 2–KLH and 3–KLH

The immunogenicity of both O-linked and S-linked GD3–KLH vaccines was evaluated in vivo. The vaccine was formulated by dissolving the immunogen in phosphate-buffered silane (PBS), which was then mixed (1:1 v/v) with Freund's complete adjuvant. BALB/c mice were immunized by subcutaneous injection of linker **47** (see Supporting Information, Figure S2), **2**, **3**, **2**–KLH, or **3**–KLH vaccine (antigen: 20 μg, $n=6$ per group) into the abdominal region on day 0, 14, and 28. Two weeks after the last immunization, sera from the immunized mice were collected to assess the level of antibody against O-linked GD3 glycan **2** and S-linked GD3 glycan **3** antigens by using a glycan microarray printed with **2**, **3**, and linker **47**. One-way analysis of variance and Tukey's post-hoc test were used to determine differences among the groups.

As shown in Figure 4, **3**–KLH stimulates a higher titer of IgG than **2**–KLH against the corresponding self-antigens (454.3 ± 24.88 versus 126.9 ± 118.8, $p<0.0001$), which indicated that the S-linked vaccine exhibited better immunogenicity than O-linked **2**–KLH for self-antigens. The relatively low antibody response of **2**–KLH relative to **3**–KLH might be due to the flexibility of the terminal S-glycosidic linkage in **3**,[16] as well as the nature of S-linked GD3 as a foreign antigen, which is in contrast to the endogenous nature of O-linked GD3. Furthermore, the results of the cross-reactivity test showed that the IgG

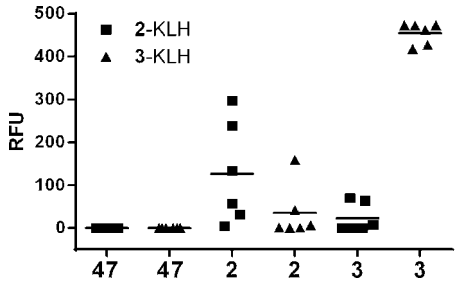


Figure 4. Evaluation of the immunogenicity responses of the **2**–KLH (■) and **3**–KLH (▲) vaccines in vivo. Sera were collected two weeks after the third immunization with linker–KLH, **2**–KLH, and **3**–KLH, and the binding of IgG to S-linked GD3 **3**, O-linked GD3 **2** and linker **47** was determined by glycan microarray. Each point represents the result from one mouse and the horizontal black line (–) is the average of the indicated group.

titers to the modified antigen (**2**–KLH: **2** versus **3**, $p=0.042$; **3**–KLH: **2** versus **3**, $p<0.0001$) stimulated by both vaccines are low. Notably, no statistical difference was observed for the levels of IgG against the *O*-GD3 antigen between mice treated with **2**–KLH (126.9 ± 118.8, and **3**–KLH (35.03 ± 62.91, $p=0.09$). It should be noted that the immune response of a carbohydrate-based vaccine also depends on the carrier protein and the adjuvant, which remains to be explored further.[60] In addition, no apparent binding of IgG to the linker was observed, which suggests that the linker does not stimulate an immune response.[61,63] These results indicate that an S-linked carbohydrate antigen may serve as a stable mimic of its O-linked counterpart.

Conclusions

A straightforward method was developed for the stereoselective synthesis of a non-natural S-linked GD3 antigen. This synthetic strategy offers opportunities for the preparation of other biologically relevant oligosaccharides that feature non-natural inter-S-glycosidic linkages. The covalent attachment of maleimide-derivatized GD3 glycans to thiolated KLH permits the development of more-precisely defined vaccine candidates. The S-linked GD3–KLH conjugate elicited antibodies against not only the S-linked GD3 antigen but also the O-linked GD3 antigen. In addition to paving the way for the development of carbohydrate-based therapeutic vaccines, we anticipate that this approach will prove valuable for accessing other complex sialoconjugates of medical interest.

Experimental Section

Amine-tethered GD3 2

Compound **43** (12 mg, 11 μmol) was dissolved in methanol (4 mL) then Pd/C (2 mg) was added. The mixture was stirred at RT under an H_2 atmosphere for 6 h. The catalyst was removed by filtration, and the filtrate was concentrated under vacuum. The amine product **2** was used in the next step without further purification. $[\alpha]_D^{32} = -0.2$ ($c = 1.0$ in H_2O); ^{1}H NMR (400 MHz, D_2O): $\delta = 4.52$ (d, $J = 8.0$ Hz, 1H), 4.47 (d, $J = 8.0$ Hz, 1H), 4.21–4.11 (m, 2H), 4.08 (dd, $J = 9.6$, 1.3 Hz, 1H), 4.01–3.86 (m, 7H), 3.81 (dd, $J = 10.0$, 4.0 Hz, 2H), 3.76–3.53 (m, 16H), 3.35–3.27 (m, 1H), 2.99 (t, $J = 7.5$ Hz, 1H), 2.78 (dd, $J = 12.3$, 2.2 Hz, 1H), 2.68 (dd, $J = 12.0$, 2.0 Hz, 1H), 2.06 (s, 3H), 2.03 (s, 3H), 1.74 (t, $J = 12.3$ Hz, 1H), 1.73 (t, $J = 12.0$ Hz, 1H), 1.69–1.59 (m, 4H), 1.45–1.35 ppm (m, 4H); ^{13}C NMR (100 MHz, D_2O): $\delta =$ 174.89, 174.87, 173.4, 173.2, 102.6, 102.0, 100.5, 100.1, 78.1, 78.0, 75.4, 75.2, 74.7, 74.3, 73.9, 72.8, 72.6, 71.7, 70.3, 69.19, 69.17, 68.4, 68.0, 67.8, 67.4, 62.5, 61.5, 61.0, 60.0, 52.2, 51.7, 40.4, 39.7, 39.3, 28.3, 26.5, 25.1, 24.4, 22.2, 22.0 ppm; HRMS (ESI-TOF): *m/z* calcd for $C_{40}H_{69}N_3O_{27}Na$: 1046.4016 $[M+Na]^+$; found: 1046.4009.

S-linked GD3 3

Compound **41** (30 mg, 16 μmol) was dissolved in ethanol (5 mL) then Pd/C (60 mg) was added. The mixture was stirred at RT under an H_2 atmosphere for 18 h. The catalyst was removed by filtration, and the filtrate was concentrated under vacuum. The resulting residue was dissolved in MeOH (2.5 mL) then 1 N NaOH (aq.) (2.5 mL) was added. The reaction mixture was stirred for 4 h at RT then neutralized with 1 N HCl (aq.) and concentrated under reduced pressure. The crude product was eluted through a BioGel P-2 gel (packed in a BioRad column #737–1576, 1.5×75 cm) with 5% MeOH/H_2O to afford **3** (12.8 mg, 77% over two steps). $[\alpha]_D^{32} = +8.8$ ($c = 0.4$ in H_2O); ^{1}H NMR (600 MHz, D_2O): $\delta = 4.55$ (d, $J = 7.9$ Hz, 1H), 4.46 (d, $J = 8.0$ Hz, 1H), 4.06 (dd, $J = 9.9$, 2.9 Hz, 1H), 4.0–3.95 (m, 2H), 3.93–3.89 (m, 2H), 3.87–3.78 (m, 5H), 3.73–3.54 (m, 14H), 3.53–3.48 (m, 1H), 3.28 (t, $J = 8.6$ Hz, 1H), 3.15 (dd, $J = 12.9$, 2.9 Hz, 1H), 2.99–2.94 (m, 2H), 2.88 (dd, $J = 13.1$, 7.8 Hz, 1H), 2.79 (dd, $J = 12.6$, 4.6 Hz, 1H), 2.71 (dd, $J = 12.3$, 4.4 Hz, 1H), 2.02 (t, $J = 10.9$ Hz, 6H), 1.77 (t, $J = 12.6$ Hz, 1H), 1.75 (t, $J = 12.3$ Hz, 1H), 1.68–1.58 (m, 4H), 1.43–1.33 ppm (m, 4H); ^{13}C NMR (150 MHz, D_2O): $\delta = 175.9$, 175.8, 175.4, 174.8, 103.4, 103.0, 100.7, 85.6, 79.1, 76.5, 76.1, 75.8, 75.6, 75.4, 73.8, 73.6, 72.8, 71.4, 71.3, 71.2, 70.5, 69.6, 69.5, 69.2, 68.3, 63.5, 62.0, 61.1, 52.7, 41.9, 40.7, 40.4 (2C), 33.1, 29.4, 27.5, 26.2, 25.5, 23.1, 23.0 ppm; HRMS (ESI-TOF): *m/z* calcd for $C_{40}H_{69}N_3O_{26}NaS$: 1062.3788 $[M+Na]^+$; found: 1062.3739.

Compound 13

A representative procedure for the synthesis of 13: A mixture of donor **12** (72 mg, 0.13 mmol), acceptor **6c** (86.6 mg, 0.09 mmol), and activated molecular sieves (4 Å) in MeCN (2 mL) was stirred at RT for 30 min under an argon atmosphere. The mixture was then cooled to −35 °C. NIS (59 mg, 0.26 mmol) and TfOH (3 μL, 26 μmol) were added to the reaction mixture at −35 °C. When the reaction was complete (determined by TLC analysis), the reaction mixture was neutralized with NEt_3 then diluted with CH_2Cl_2. The solution was filtered through a Celite pad, and the filtrate was washed with a saturated aqueous solution of sodium thiosulfate then brine. The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (1:1 EtOAc/hexane, $R_f = 0.25$) to afford **13** (73.3 mg, 56%). $[\alpha]_D^{33} = +0.6$ ($c = 1.0$ in EtOAc); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.40$–7.13 (m, 25H), 6.21 (s, 1H; N*H*TFA), 5.88 (d, $J = 8.0$ Hz, 1H; N*H*Ac), 4.98 (d, $J = 10.7$ Hz, 1H), 4.92 (ddd, $J =$ 12.1, 10.7, 4.7 Hz, 1H; H-4″), 4.84 (d, $J = 11.1$ Hz, 1H), 4.73–4.65 (m, 6H), 4.51 (d, $J = 7.8$ Hz, 1H), 4.48–4.41 (m, 3H), 4.34 (d, $J = 12.1$ Hz, 1H), 4.32 (d, $J = 7.8$ Hz, 1H), 4.07 (dd, $J = 9.5$, 3.3 Hz, 1H), 3.98 (ddd, $J = 12.1$, 10.4, 8.2 Hz, 1H; H-5″), 3.93–3.85 (m, 2H), 3.82 (br s, 1H), 3.73 (s, 3H), 3.71–3.67 (m, 2H), 3.66–3.60 (m, 3H), 3.58 (dd, $J = 10.5$, 1.7 Hz, 1H), 3.55 (d, $J = 6.7$ Hz, 1H), 3.54–3.44 (m, 5H), 3.44–3.39 (m, 2H), 3.36 (dd, $J = 8.2$, 6.9 Hz, 1H), 3.33 (dd, $J = 4.4$, 2.2 Hz, 1H), 3.23 (dt, $J = 13.3$, 6.6 Hz, 2H), 2.62 (d, $J = 7.9$ Hz, 1H), 2.46 (dd, $J =$ 12.9, 4.6 Hz, 1H; H-3″$_{eq}$), 2.07 (s, 3H), 2.05 (s, 3H), 1.99 (s, 3H), 1.97 (t, $J = 12.9$ Hz, 1H; H-3″$_{ax}$), 1.62–1.56 (m, 2H), 1.50–1.43 (m, 2H), 1.42–1.34 (m, 2H), 1.32–1.26 ppm (m, 2H); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 172.8$, 172.1, 169.7, 168.5, 157.1 (q, J(C,F) = 36.6 Hz), 139.1, 138.9, 138.8, 138.4, 138.3, 128.24 (4C), 128.23, 128.19, 128.1 (4C), 128.0 (2C), 127.9 (2C), 127.8 (4C), 127.5, 127.44 (3C), 127.44, 127.35, 127.2, 115.8 (q, J(C,F) = 287.6 Hz), 103.51, 102.5, 98.0, 82.9, 81.8, 78.3, 76.1, 75.4, 75.2, 75.1, 74.8, 74.1, 73.2, 73.1, 72.3, 70.1, 69.5, 69.2, 68.6 (2C), 68.3, 67.9, 53.0, 51.7, 39.8, 36.6, 29.7, 29.4, 28.8, 26.3, 25.6, 23.1, 21.2, 21.0, 9.8 ppm; HRMS (ESI-TOF): *m/z* calcd for $C_{71}H_{86}N_2O_{21}F_3NaI$: 1509.4618 $[M+Na]^+$: found: 1509.4625.

Compound 41

Diethylamine (12 μL, 0.11 mmol) was added to a solution of nucleophile **8** (25 mg, 45.4 μmol) and electrophile **7** (67.6 mg, 45 μmol) in DMF (1 mL). The mixture was stirred at RT for 12 h then the reaction mixture was concentrated under vacuum. The residue was dissolved in EtOAc then washed with water and brine. The organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (1:1 EtOAc/hexane + 10% MeOH, $R_f = 0.25$) to afford **41** (46 mg, 55%). $[\alpha]_D^{32} = -1.7$ ($c = 1.0$, EtOAc); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.40$–7.15 (m, 25H), 6.28 (s, 1H), 5.93 (d, $J = 8.8$ Hz, 1H), 5.34–5.31 (m, 1H), 5.09 (d, $J = 10.2$ Hz, 1H), 5.00 (d, $J = 10.8$ Hz, 1H), 4.91 (ddd, $J = 12.1$, 10.8, 4.5 Hz, 1H), 4.83 (d, $J = 11.1$ Hz, 1H), 4.80–4.75 (m, 1H), 4.72–4.66 (m, 4H), 4.49–4.43 (m, 4H), 4.35–4.30 (m, 3H), 4.18 (d, $J = 2.7$ Hz, 1H), 4.08 (dd, $J = 9.6$, 3.3 Hz, 1H), 4.06–4.00 (m, 2H), 3.92–3.85 (m, 3H), 3.78–3.66 (m, 14H), 3.57–3.44 (m, 6H), 3.36–3.32 (m, 2H), 3.25–3.20 (m, 2H), 3.05 (dd, $J = 13.7$, 2.8 Hz, 1H), 2.84 (s, 1H), 2.80 (dd, $J = 13.7$, 10.1 Hz, 1H), 2.59 (dd, $J = 12.7$, 4.6 Hz, 1H), 2.42 (dd, $J = 12.8$, 4.5 Hz, 1H), 2.13 (s, 3H), 2.09 (s, 3H), 2.07 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.99 (s, 3H), 1.98 (s, 3H), 1.97 (t, $J = 12.8$ Hz, 1H), 1.85 (t, $J = 12.7$ Hz, 1H), 1.84 (s, 3H), 1.63–1.56 (m, 2H), 1.50–1.42 (m, 2H), 1.41–1.34 (m, 2H), 1.32–1.26 ppm (m,

2H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 172.6, 171.5, 170.9, 170.7, 170.24, 170.16, 169.9 (2C), 168.7, 168.5, 157.1 (q, J(C,F) = 37.9 Hz), 139.1, 138.89, 138.85, 138.7, 138.4, 128.22 (2C), 128.21, 128.19, 128.16 (4C), 128.11 (2C), 128.0 (2C), 127.81 (4C), 127.8 (2C), 127.4 (4C), 127.3, 127.2, 127.1, 115.8 (q, J(C,F) = 288.6 Hz), 103.5, 102.43, 98.0, 82.9, 82.1, 81.8, 78.4, 76.6, 76.0, 75.4, 75.11, 75.07, 74.8, 74.0, 73.8, 73.2, 73.1, 72.8, 70.0, 69.7, 69.5, 69.0, 68.6, 68.5, 68.4, 68.0, 67.9, 67.6, 61.9, 53.0, 52.8, 50.9, 49.3, 39.8, 37.6, 36.5, 31.7, 29.4, 28.8, 26.3, 25.6, 23.2, 23.1, 21.3, 21.1, 20.9, 20.8, 20.78, 20.76 ppm; HRMS (ESI-TOF): m/z calcd for $C_{91}H_{114}N_3O_{33}F_3NaS$: 1888.6905 $[M+Na]^+$; found: 1888.6904.

Compound 45

N,*N*-Diisopropylethylamine (3.2 μL, 23 μmol) was added to a solution of GD3 **2** and commercially available 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester (7.2 mg, 23 μmol) in DMF (0.8 mL). The reaction mixture was stirred at RT for 2 h then concentrated under vacuum. The residue was eluted through a BioGel P-2 gel (packed in a BioRad column #737–1576, 1.5 × 75 cm) with water to afford **45** (9 mg, 67%). $[\alpha]_D^{32} = -2.2$ (c = 0.89 in H_2O); ^{1}H NMR (600 MHz, D_2O): δ = 6.79 (s, 2H), 4.48 (d, J = 7.8 Hz, 1H), 4.42 (d, J = 7.9 Hz, 1H), 4.18–4.08 (m, 2H), 4.04 (d, J = 10.0 Hz, 1H), 3.97–3.81 (m, 7H), 3.77 (d, J = 5.5 Hz, 1H), 3.75 (d, J = 5.7 Hz, 1H), 3.69–3.49 (m, 16H), 3.46 (t, J = 6.6 Hz, 2H), 3.25 (t, J = 8.6 Hz, 1H), 3.11 (t, J = 6.6 Hz, 1H), 2.73 (dd, J = 12.1, 4.0 Hz, 1H), 2.63 (dd, J = 11.7, 3.1 Hz, 1H), 2.17 (t, J = 7.4 Hz, 2H), 2.02 (s, 3H), 1.98 (s, 3H), 1.69 (t, J = 12.1 Hz, 2H), 1.59–1.48 (m, 6H), 1.48–1.40 (m, 2H), 1.36–1.24 (m, 4H), 1.24–1.15 ppm (m, 2H); ^{13}C NMR (150 MHz, D_2O): δ = 177.6, 175.9 (2C), 174.5, 174.35 (2C), 174.32, 135.32, 135.26, 103.7, 103.0, 101.5, 101.2, 79.2, 79.0, 76.4, 76.2, 75.8, 75.4, 75.0, 73.8, 73.6, 72.7, 71.5, 70.3, 69.5, 69.1, 68.9, 68.4, 63.5, 62.5, 62.1, 61.0, 60.3, 53.2, 52.7, 41.5, 40.7, 40.1, 38.5, 36.6, 29.6, 29.2, 28.3, 26.7, 26.3, 25.9, 25.6, 23.3, 23.0 ppm; HRMS (ESI-TOF): m/z calcd for $C_{50}H_{79}N_4O_{30}$: 1215.4779 $[M-H]^-$; found: 1215.4768.

Compound 46

Compound **46** (6 mg, 63%) was prepared from S-linked GD3 **3** (8 mg, 77 μmol) by following the same procedure described above for the synthesis of **45**. $[\alpha]_D^{32} = +10.8$ (c = 0.56 in H_2O); ^{1}H NMR (600 MHz, D_2O): δ = 6.80 (s, 2H), 4.55 (t, J = 6.7 Hz, 1H), 4.44 (d, J = 8.0 Hz, 1H), 4.06 (dd, J = 9.9, 3.1 Hz, 1H), 3.99 (dd, J = 8.1, 3.1 Hz, 1H), 3.96 (dd, J = 12.3, 2.3 Hz, 1H), 3.92–3.76 (m, 7H), 3.72–3.52 (m, 15H), 3.52–3.48 (m, 1H), 3.47 (t, J = 6.9 Hz, 1H), 3.28–3.24 (m, 1H), 3.15 (dd, J = 13.2, 3.2 Hz, 1H), 3.12 (t, J = 6.9 Hz, 2H), 2.87 (dd, J = 13.1, 7.9 Hz, 1H), 2.78 (dd, J = 12.4, 4.8 Hz, 1H), 2.70 (dd, J = 12.4, 4.6 Hz, 1H), 2.17 (t, J = 7.2 Hz, 2H), 2.00 (s, 3H), 1.99 (s, 3H), 1.75 (t, J = 12.4 Hz, 2H), 1.62–1.51 (m, 6H), 1.49–1.43 (m, 2H), 1.37–1.27 (m, 4H), 1.25–1.18 ppm (m, 2H); ^{13}C NMR (150 MHz, D_2O): δ = 176.6, 174.9, 174.8, 174.4, 173.9, 173.3 (2C), 134.2 (2C), 102.4, 102.0, 99.7, 84.5, 78.0, 75.4, 75.0, 74.7, 74.6, 74.4, 72.8, 72.6, 71.8, 70.5, 70.4, 70.2, 69.5, 68.6, 68.5, 68.2, 67.3, 62.5, 61.0, 60.1, 59.3, 51.6, 40.8, 39.6, 39.1, 37.4, 35.5, 32.1, 28.6, 28.1, 27.2, 25.7, 25.3, 24.9, 24.6, 22.1, 21.9 ppm; HRMS (ESI-TOF): m/z calcd for $C_{50}H_{79}N_4O_{29}S$: 1231.4551 $[M-H]^-$; found: 1231.4562.

The animal experiment protocol has been reviewed by the Institutional Animal Care and Use Committee of Chang Gung University and the Committee recognizes that the proposed animal experiment follows the guideline as shown in the Guide for Laboratory Animal Facilities and Care as promulgated by the Council of Agriculture.

Acknowledgements

This research was supported by Academia Sinica, the National Tsing Hua University, and the Ministry of Science and Technology of Taiwan. The authors are grateful to Dr. Wun-Shaing Wayne Chang and Ms. Chien-Yu Liao of the National Health Research Institutes for technical assistance.

Conflict of interest

The authors declare no conflict of interest.

Keywords: alpha-sialylation · carbohydrates · enzyme catalysis · glycosylation · sialic acids

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Manuscript received: February 2, 2017
Accepted Article published: March 12, 2017
Final Article published: April 24, 2017