Chemoenzymatic synthesis of GM_3 and GM_2 gangliosides containing a truncated ceramide functionalized for glycoconjugate synthesis and solid phase applications†

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Analogues of GM_3 and GM_2 gangliosides were chemoenzymatically synthesized on a multifunctional ceramide-type tether designed to facilitate diverse strategies for glycoconjugate synthesis. The truncated ceramide aglycon maintains the stereogenic centres of natural ceramide while avoiding extensive hydrophobicity that can hamper synthesis and purification of the glycolipids. Tetanus toxoid and BSA glycoconjugates of these two gangliosides were prepared for immunization of mice, and for solid phase assays to screen for ganglioside-specific antibodies. Inhibition experiments showed that antibodies generated by tetanus toxoid conjugates of GM_3 and GM_2 exhibited specificity for the carbohydrate epitope and the stereogenic centres of the ceramide.

Introduction

Oligosaccharides at the surface of mammalian cells act as key elements in recognition processes occurring during embryogenesis, metastasis, inflammation and pathogen recognition. Among other glycoconjugates at the cell surface, gangliosides, a group of complex sialylated glycosphingolipids, play a significant role in a variety of cell functions. They have been proposed to control growth and differentiation of cells, mediate cell–cell interactions, regulate cell signalling by acting as receptors for growth factors, and act as specific receptors for various microbial toxins.[1] Furthermore, these glycolipids have found wide applications in oligosaccharide microarrays,[2] investigations of sialic acid binding proteins,[3] immunotherapy,[4] and in the study of cell membrane events. Their importance in biological recognition requires efficient methods for their preparation.

The synthesis of gangliosides is particularly challenging due to their amphiphilic nature and the difficulties associated with stereoselective coupling of *N*-acetyl neuraminic acid.[5] Enzymatic methods have proved to be very useful in the synthesis of sialylated structures,[6] avoiding many of the problems associated with chemical approaches. Due to their hydrophobic lipid chain and their hydrophilic head group, gangliosides exhibit surfactant-like properties such as low solubility in organic solvents and aqueous solutions, as well as aggregation, resulting in low reaction yields and purification problems.[7] Several lines of evidence in the literature relating to ganglioside recognition by proteins, implicate the ceramide portion of glycolipids either directly or *via* its influence on the orientation of the polar head group when the ganglioside is located in the mammalian cell membrane.[8] In order to build a capability to include tandem recognition of the ceramide and the carbohydrate, we developed a truncated version of sphingosine.[9] This moiety confers increased aqueous solubility and allows for the elaboration with different lipid chains at a late stage of the synthesis, thereby facilitating manipulation of the intermediates involved.

Gangliosides, in particular GM_3, GM_2, GD_3 and GD_2 (Fig. 1) are highly overexpressed in melanoma and other tumors of neuroectodermal origin, making these structures interesting targets for active cancer immunotherapy.[4a,c,10] Unfortunately, gangliosides are poorly immunogenic, making their use as immunogens impractical and the preparation of anti-ganglioside antibodies for passive immunization difficult. Conjugation of an oligosaccharide epitope to an immunogenic carrier protein has been widely used as a strategy to enhance the immunogenicity of otherwise T-cell independent carbohydrate antigens, and has been applied to augment the immunogenicity of gangliosides.[11] Thus far, vaccines based on naturally occurring gangliosides or semi-synthetic gangliosides conjugated to keyhole limpet hemocyanin (KLH) have displayed only limited success in clinical trials.[4b,c,12] These results have motivated new strategies for the design of ganglioside-based vaccines in order to improve their immunogenicity.[4b,c,12,13] In this context, ganglioside analogues containing functional groups that facilitate flexible conjugation strategies, constitute a valuable tool for the design and development of cancer vaccines as well as for the study and modulation of ganglioside functions at membrane interfaces.

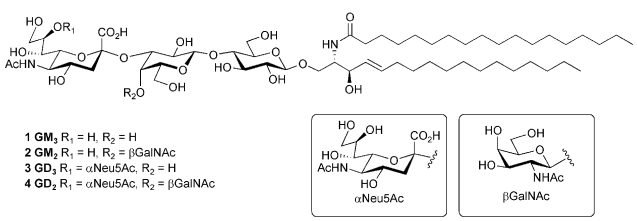

Fig. 1 Structure of gangliosides GM_3 (**1**), GM_2 (**2**), GD_3 (**3**), and GD_2 (**4**).

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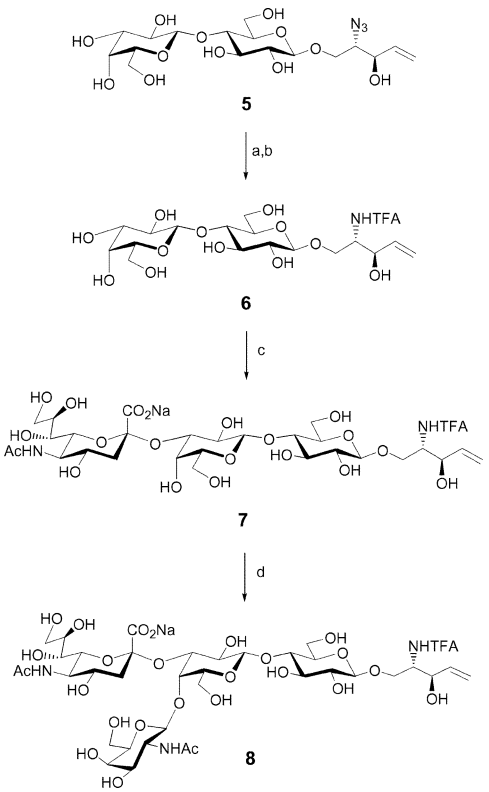
Here we address some of these issues and report the chemoenzymatic synthesis of versatile GM_3 and GM_2 ganglioside analogues designed for conjugation *via* their ceramide moiety. These compounds contain a truncated ceramide aglycon[9] which enhances water solubility of the molecule while conserving the important stereochemical and structural features of naturally occurring ceramides. The inherent flexibility to functionalize the ganglioside analogues with different linkers for covalent attachment to proteins, surfaces, or fluorescent probes, is demonstrated by the synthesis of tetanus toxoid (TT) and bovine serum albumin (BSA) glycoconjugates of GM_3 and GM_2. The immune response to both conjugates shows a good IgG response in mice, directed against the sialylated portion of the molecule *and* the ceramide moiety.

Results and discussion

Our chemoenzymatic approach was centred on a versatile lactosyl ceramide analogue **6**, which could be elaborated to gangliosides of higher complexity or functionalized with different acyl chains at the sphingosine amine. Intermediate **6** was synthesized as outlined in Scheme 1. The azide **5** was reduced in the presence of hydrogen sulfide in a mixture of pyridine, water and triethylamine.[9,14] The use of the free amine in the next enzymatic step has proven not to be very practical in our hands. Therefore, we envisioned that protection of the amine group would ease purification of the compound in the subsequent enzymatic reactions. Due to the acid lability of the *N*-acetyl neuraminic acid residue,[15] an amine protecting group such as trifluoroacetamide, which could be removed under mild basic conditions, was preferred. The free amine was acylated by reaction with methyl trifluoroacetate to yield **6**.

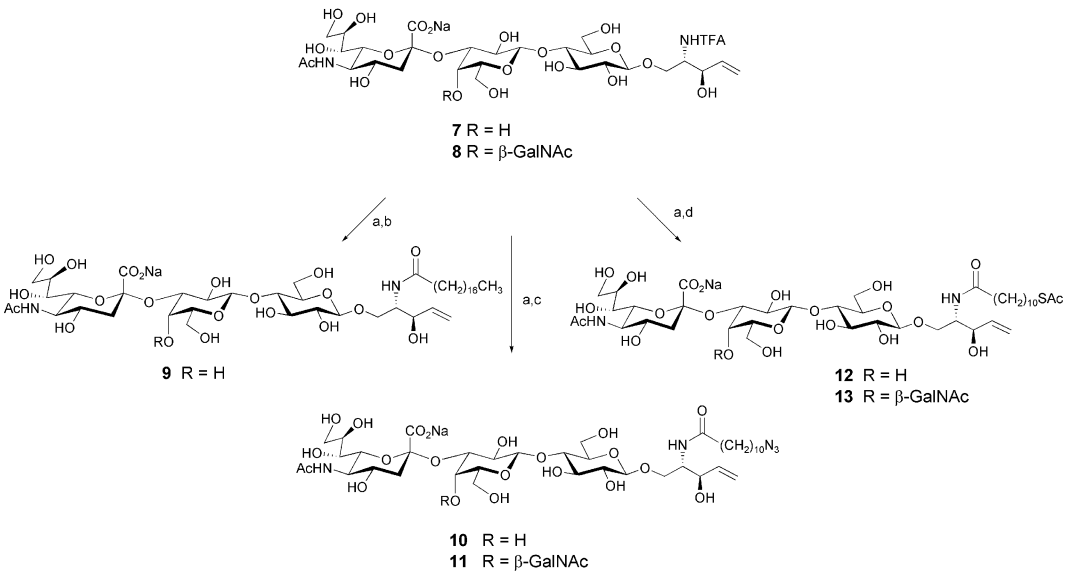


Scheme 1 *Reagents and conditions*: (a) H_2S, pyridine–H_2O–Et_3N (10 : 1 : 0.3), 87%; (b) methyl trifluoroacetate, MeOH, 75%; (c) α-(2,3)-Neu5Ac transferase, CMP-Neu5Ac, 88%; (d) β-(1,4)-GalNAc transferase, UDP-GlcNAc 4-epimerase, UDP-GlcNAc, 95%.

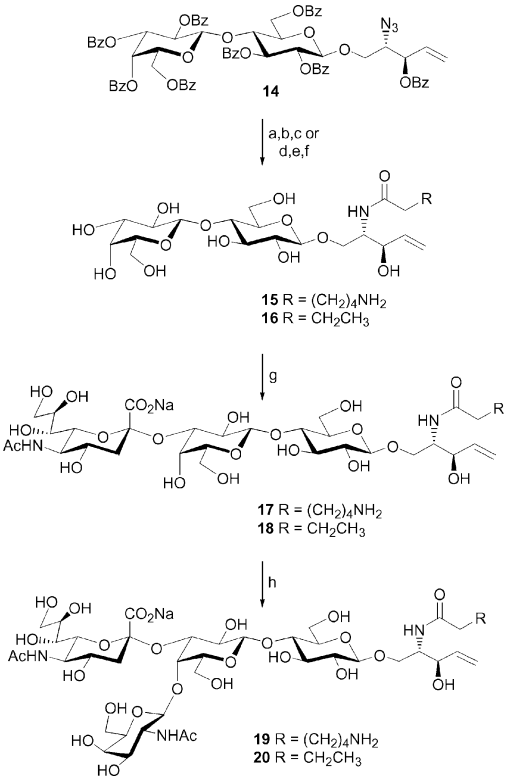
Enzymatic synthesis of oligosaccharides obviates many of the problems associated with chemical synthesis, including laborious protecting group manipulations and separation of numerous diastereomeric glycosylation products. We envisioned elaborating our ganglioside analogues from a lactose core using sialyltransferase- and *N*-acetyl-galactosaminyl transferase-catalyzed reactions. A major drawback of glycosylation reactions catalyzed by glycosyltransferases is their requirement for expensive nucleotide sugar donors. However, we have employed recombinant CMP-Neu5Ac synthetase and UDP-GlcNAc 4-epimerase in the production of glycosyl donors. The crude product obtained was subsequently used to effect sialylation of the acceptor **6** catalyzed by a recombinant α-(2,3)-sialyltransferase from *Campylobacter jejuni* to obtain **7** in good yield.[16] The carbohydrate portion of intermediate **7** was further elaborated *via* an enzymatic glycosylation yielding the GM_2 analogue **8** (Scheme 1). Using a recombinant β-(1,4)-*N*-acetylgalactosaminyl transferase[6] and UDP-GlcNAc 4-epimerase[17] from *Campylobacter jejuni*, GM_2 analogue **8** was produced from acceptor **7** in excellent yield.

The immobilization of oligosaccharides on solid surfaces and particles as well as their conjugation to proteins, are important tools in glycobiology for the study and modulation of carbohydrate functions. Oligosaccharide intermediates bearing a functional group designed for conjugation are valuable compounds for such undertakings. To this end, GM_3 and GM_2 analogues containing a functionalized *N*-acyl chain were synthesized from intermediates **7** and **8** (Scheme 2). Deprotection of the sphingosine amine followed by reaction with a succinimide-activated octadecanoate[18] yielded compound **9**. It can be envisioned that the long acyl chain could be used to non-covalently coat microtiter plates *via* hydrophobic interactions.[19] Furthermore, following protection, the truncated ceramide could be extended to a natural ceramide *via* olefin metathesis.[20] Alternatively, the alkene portion of the truncated ceramide could serve as a handle for protein conjugation *via* radical addition of cysteamine to the terminal olefin.[9,21] In an analogous fashion, compounds **7** and **8** were converted to GM_3 and GM_2 mimics **10** and **11**, which contain an azide group at the *N*-acyl chain terminus. These ganglioside analogues could be covalently attached to alkyne-functionalized surfaces *via* 1,4-dipolar cycloaddition or coupled to proteins or functionalized surfaces upon reduction of the azide to an amine. Similarly, compounds **7** and **8** were converted to **12** and **13**, which bear a terminal thioester. Upon deprotection of the thiol, these molecules would be suitable for attachment to gold surfaces or nanoparticles, and for conjugation to proteins.

The method described above for accessing GM_3 and GM_2 analogues from a lactose core, was particularly useful for the synthesis of compounds incorporating a long hydrophobic chain at the sphingosine nitrogen. However, compounds functionalized with a shorter acyl chain could be synthesized *via* an alternate pathway as shown in Scheme 3. The azide group of **14** was reduced under Staudinger conditions.[9] Acylation of the amine with *p*-nitrophenyl 6-trifluoroacetamidohexanoate followed by deprotection, afforded compound **15**. Alternatively, the benzoylated lactosyl azidosphingosine **14** could be deprotected and reduced in the presence of H_2S. Reaction of the amine with butyric anhydride afforded **16**. The lactosyl ceramides functionalized with



Scheme 2 *Reagents and conditions*: (a) NaOMe, MeOH, 40 °C; (b) *N*-(octadecanoyloxy)succinimide, 4-*N*,*N*-dimethylaminopyridine, pyridine–H_2O (9 : 1), 40 °C, 55%; (c) *N*-(11-azidoundecanoyloxy)succinimide, 4-*N*,*N*-dimethylaminopyridine, pyridine–H_2O (9 : 1), 40 °C, 78% for **10**, 76% for **11**; (d) *N*-(11-thioacetylundecanoyloxy)succinimide, 4-*N*,*N*-dimethylaminopyridine, pyridine–H_2O (9 : 1), 40 °C, 80% for **12**, 95% for **13**.



Scheme 3 *Reagents and conditions*: (a) PPh_3, pyridine–H_2O (9 : 1), 50 °C; (b) *p*-nitrophenyl 6-trifluoroacetamidohexanoate, pyridine–H_2O (9 : 1), 50 °C; (c) NaOMe, MeOH, 58%, three steps; (d) NaOMe, MeOH; (e) H_2S, pyridine–H_2O–Et_3N (10 : 1 : 0.3); (f) butyric anhydride, MeOH, 40 °C, 71%, three steps; (g) α-(2,3)-Neu5Ac transferase, CMP-Neu5Ac, 80% for **17**, 79% for **18**; (h) β-(1,4)-GalNAc transferase, UDP-GlcNAc 4-epimerase, UDP-GlcNAc, 79% for **19**, 83% for **20**.

shorter acyl chains were soluble in aqueous solutions and thus could be used as acceptors in enzymatic glycosylation reactions. Reaction in the presence of α-(2,3)-sialyltransferase yielded GM_3 analogues **17** and **18**, which could be further elaborated to GM_2 analogues **19** and **20** using β-(1,4)-*N*-acetylgalactosaminyl transferase.

Recent work from our group has focused on the incorporation of thiooligosaccharides in anti-cancer vaccines as a means to increase their immunogenicity.[22] We hypothesized that the metabolic stability of the *S*-linked glycans[23] could increase the immunogenicity of the conjugates, while producing antibodies cross-reactive with the corresponding *O*-linked antigen. In order to compare the immunogenicity, cross-reactivity, and metabolic stability of the *S*-linked GM_3 and GM_2 antigens, their *O*-linked counterparts were required. Evaluation of the response against the immunogen by ELISA necessitates the synthesis of a set of glycoconjugates, incorporating heterologous proteins and linkers as well as water-soluble oligosaccharides. The methods described herein were used to rapidly access GM_3 (**25** and **26**), and GM_2 (**27**) conjugates for immunization and solid phase assays (ELISA).

The use of tetanus toxoid in commercial carbohydrate conjugate vaccines and our recent successes in inducing high antibody titers against tetanus toxin glycoconjugates, motivated the use of this carrier protein for our studies.[24] Typical strategies for the generation of a conjugate vaccine involve the coupling of a carbohydrate antigen *via* a linker to reactive groups at the surface of a carrier protein.[25] Ideally, the coupling chemistry should allow for appropriate levels of substitution, while using a minimal amount of precious synthetic hapten. To this end, an adipate-based linker[26] was chosen to effect conjugation of haptens **17** and **19**, without introducing a potentially immunogenic tether. The generation of the GM_3 and GM_2 conjugates (Scheme 4) first involved the introduction of an amine-terminated spacer unit. Treatment of intermediates **7** and **8** with sodium methoxide in methanol yielded the corresponding amines. *N*-Acylation with 6-trifluoroacetamidohexanoic anhydride afforded intermediates **21** and **22**. Following deprotection of the primary amine, compounds **17** and **19** were functionalized for conjugation to proteins.

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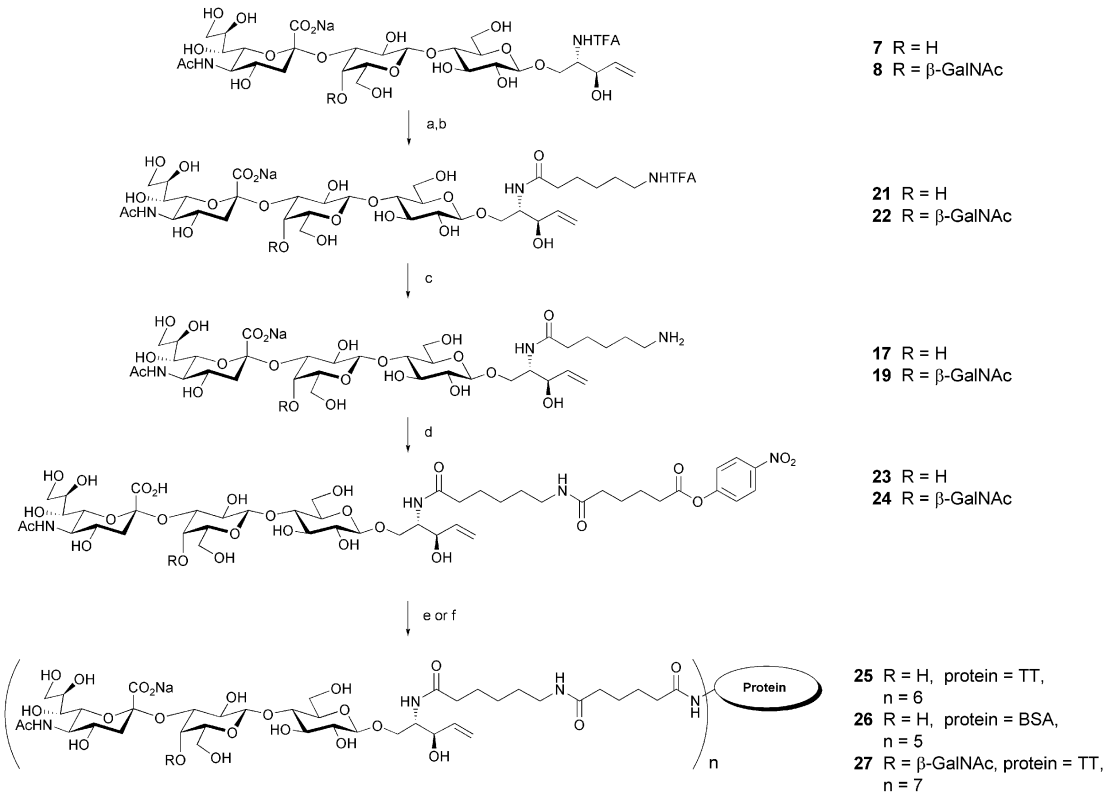

Scheme 4 *Reagents and conditions*: (a) NaOMe, MeOH, 40 °C; (b) 6-trifluoroacetamidohexanoic anhydride, MeOH, 78% for **21**, 78% for **22**, two steps; (c) NaOMe, MeOH, 40 °C, 72% for **17**, 73% for **19**; (d) *p*-nitrophenyl adipate diester, DMF–Et_3N; (e) tetanus toxoid, PBS, pH 7.2 (f) bovine serum albumin, water.

Reaction of **17** and **19** with *p*-nitrophenyl adipate diester in a mixture of *N*,*N*-dimethylformamide and triethylamine followed by reverse phase HPLC purification in the presence of 1% acetic acid afforded activated ganglioside analogues **23** and **24** for protein coupling. Incubation of tetanus toxoid with approximately 30 equivalents of the activated sugars yielded glycoconjugates **25** and **27** with hapten incorporation levels of 6 and 7 respectively.

The tetanus toxoid glycoconjugates **25** and **27** were used to immunize mice and their ability to raise an immune response was evaluated by ELISA. Synthetic glycoconjugates possessing a heterologous protein were required to detect carbohydrate-specific antibodies by solid phase assays (ELISA). For this purpose, ganglioside analogue **17** was coupled to bovine serum albumin using a similar approach to the one used to generate tetanus toxoid conjugates (Scheme 4). A hapten incorporation level of 5 was obtained for the GM_3-bovine serum albumin conjugate. In order to perform a more stringent assay for carbohydrate-specific antibodies and to exclude the possibility of tether-specific antibodies, we sought a glycoconjugate consisting of the same carbohydrate hapten conjugated to a heterologous protein *via* a tether that was distinct from that of the immunizing agent. Bovine serum albumin conjugates containing a squarate linker were prepared from intermediates **17** and **19** to address this requirement.[22b]

Preliminary experiments established that mice immunized with either GM_3-TT conjugate **25** or GM_2-TT conjugate **27** had a high titer of glycolipid-specific antibodies.[24c] Mice immunized with GM_3-TT conjugate **25** responded with an IgG titer of 10^4.[24c] In order to establish that the antibodies recognize sialoside-containing epitopes and not just the internal segment of the oligosaccharide hapten, inhibition of antibody binding to glycoconjugate-coated ELISA plates was performed with a panel of oligosaccharides (Fig. 2). These results showed that antibody binding had a strict requirement for the terminal sialic acid residue. GM_3 derivative **21** was at least 1000-fold more effective inhibitor than the lactosyl derivative **31** (IC_{50} of 0.39 μM *vs.* >1000 μM, data not shown). The ceramide portion of the molecule also has an effect on antibody

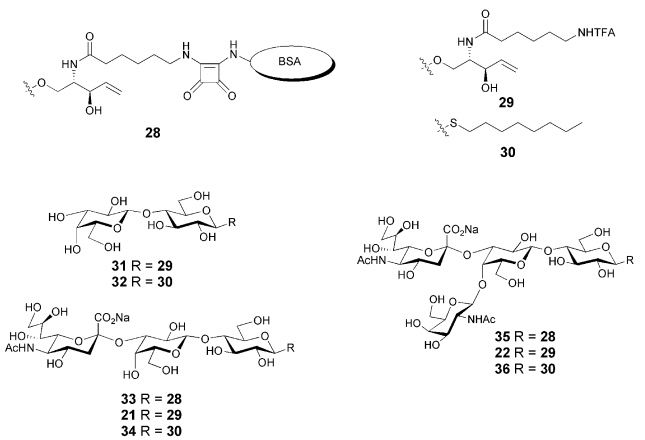

Fig. 2 Structure of immobilized glycoconjugates (**33** and **35**) and soluble inhibitors (**21**, **22**, **31**, **32**, **34** and **36**) used for inhibition ELISA experiments.

binding, since substitution of the truncated sphingosine aglycon by an *S*-octyl analogue resulted in a 100-fold decrease in the IC_{50} (Fig. 3).

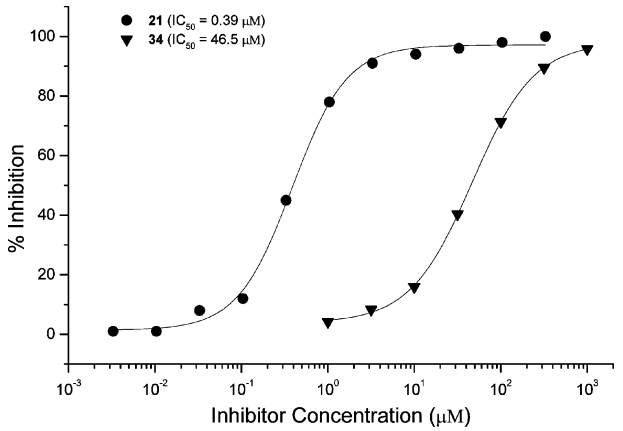

Fig. 3 ELISA inhibition experiments with serum obtained from mice immunized with GM_3-TT conjugate **25**. Synthetic oligosaccharides **21** and **34** were used to inhibit antibody binding to immobilized GM_3 conjugate **33**.

Similar results were observed for mice immunized with GM_2-TT conjugate **27**. Sera from immunized mice were titered against an immobilized GM_2-BSA conjugate (**35**). The mice responded to immunization with an IgG titer of $\sim 10^5$.[24c] Further study of the binding epitope by ELISA inhibition experiments indicated that both the terminal sialic acid residue and the *N*-acetylgalactosamine residue were required for antibody binding (Fig. 4). Comparison of IC_{50} values for compounds containing the truncated ceramide and for compounds containing the *S*-octyl aglycon again indicated that the ceramide portion of the molecule is also recognized by antibodies. Substitutions at the ceramide end of the molecule affect antibody binding to a smaller extent than either deletion of the sialic acid or the *N*-acetylgalactosamine residues.

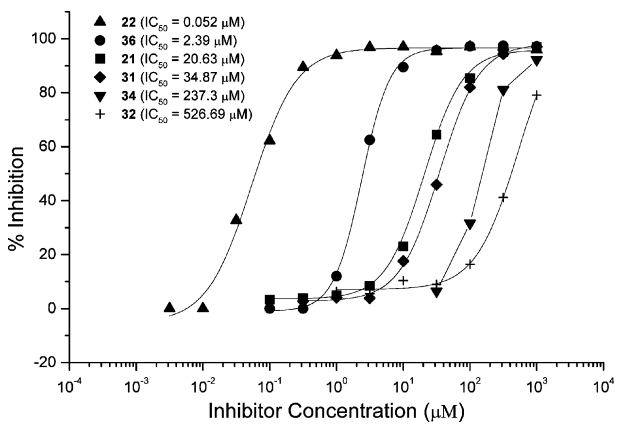

Fig. 4 ELISA inhibition experiments with serum obtained from mice immunized with GM_2-TT conjugate **27**. Synthetic oligosaccharides **21**, **22**, **31**, **32**, **34**, and **36** were used to inhibit antibody binding to immobilized GM_2 conjugate **35**.

Conclusions

We have demonstrated a versatile approach for the chemoenzymatic synthesis of ganglioside analogues in a form suitable for conjugation. The truncated ceramide aglycon allows coupling of the molecule *via* a wide variety of conjugation strategies, while maintaining the relevant stereochemical and structural features of natural ceramide. Mice immunized with GM_3-TT and GM_2-TT conjugates gave a high IgG hapten-specific response. The preponderance of the IgG subtype is indicative of an antibody class switch and activation of T_H cells. Immunochemical data obtained with the synthetic antigens created by this approach indicate that ceramide elements are recognized by antibodies induced by glycoconjugates **25** and **27**. This suggests that it can be important to include at least the stereogenic centres of the ceramide portion of gangliosides in conjugate vaccine design. Although the main thrust of the current work was directed toward ganglioside analogues in the context of tumor immunotherapy, the design of the tether is not limited to vaccine applications. Through the introduction of different terminal functionalities, these ganglioside mimics may be tailored for applications that present glycolipids in ordered arrays and model membranes.

Experimental

General methods

All chemical reagents were of reagent grade and used as supplied from Sigma-Aldrich, without further purification unless otherwise indicated. Organic solvents used in reactions were distilled under an inert atmosphere, except *N*,*N*-dimethylformamide, which was stored over 3 Å molecular sieves and subjected to reduced pressure for several minutes (<0.5mm Hg) prior to use. Pyridine was dried over solid KOH and used without further purification. Analytical thin layer chromatography (TLC) was conducted on silica gel 60-F_{254} (Merck). Plates were visualized under UV light, and/or by treatment with either acidic cerium ammonium molybdate or 5% ethanolic sulfuric acid, followed by heating. HPLC purification was conducted using a UV absorbance detector. Separations were performed on Beckman C_{18}-silica, Kromasil C_{18}-silica or Hamilton PRP-1 reverse-phase semi-preparative columns with combinations of water and methanol as eluent (flow rate 1.5–2.0 mL min^{-1}). Size exclusion chromatography purification was performed using Biorad P-4 Biogel resin and 5% methanol–water as eluent (flow rate 0.4 mL min^{-1}).

^1H NMR spectra were recorded at 500 or 600 MHz and ^{13}C NMR spectra were recorded at 125 MHz. Chemical shifts are reported in δ (ppm) units using ^{13}C and residual ^1H signals from deuterated solvents as references. ^1H and ^{13}C NMR spectra were assigned with the assistance of COSY, HMQC, HMBC, and TOCSY spectra. NMR data for compounds containing sphingosine/ceramide and derivatives such as the aglycon were labelled as follows. Hydrogen atoms of sphingosine are denoted $H–X_{sphing}$, where numbering begins with H-1 at the glycosyl linkage. The *cis* and *trans* olefinic hydrogen atoms of the truncated lipid are denoted $H–Xc_{sphing}$ and $H–Xt_{sphing}$ respectively. Similarly, the axial and equatorial protons at the 3-position of the neuraminic acid unit are denoted H-3a and H-3e respectively. For some compounds, the coupling pattern of the H-2 of the glucopyranosyl unit is reported as a multiplet, due to high order coupling.

Electro-spray ionization (ESI) and MALDI mass spectra were recorded by the mass spectrometry laboratory service at the University of Alberta. For high resolution mass determination,

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spectra were obtained by voltage scan over a narrow range at a resolution of approximately 10 000. Optical rotations were determined in a 10 cm cell at 22 ± 2 °C. $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}.

Enzymatic synthesis

1. CMP-Neu5Ac synthetase from *Neisseria meningitidis*[27]. The enzyme was expressed in *Escherichia coli* AD202. After a 24 h incubation, the cells were harvested by centrifugation at 8000 rpm, 4 °C for 20 min. The cells were resuspended in 20 mM Tris, pH 8.0 and lysed using a French press. The enzyme was isolated by centrifugation of the supernatant at 6000 rpm, 4 °C for 30 min followed by ultracentrifugation of the supernatant at 40 000 rpm, 4 °C for 60 min. The supernatant, containing CMP-Neu5Ac synthetase, was used to produce the CMP-Neu5Ac needed for enzymatic sialylation reactions.

2. α-(2,3)-Sialyltransferase from *Campylobacter jejuni* OH43-84[17]. The enzyme was expressed in *Escherichia coli* AD202. After a 24 h culture, the cells were harvested by centrifugation at 8000 rpm, 4 °C for 20 min. The cells were resuspended in 50 mM HEPES, pH 7.5 containing 10% (v/v) glycerol. The enzyme was isolated by centrifugation at 13 000 rpm at 4 °C for 30 min followed by ultracentrifugation of the supernatant at 40 000 rpm at 4 °C for 60 min. The enzyme was recovered in the pellet, which was resuspended in 50 mM HEPES, pH 7.5 containing 10% (v/v) glycerol. The suspension was used without any further purification in subsequent sialylation reactions.

3. β-(1,4)-*N*-Acetylgalactosaminyl transferase from *Campylobacter jejuni* O:36[6]. The enzyme was expressed in *Escherichia coli* AD202. After a 24 h culture, the cells were harvested by centrifugation at 8000 rpm, 4 °C for 20 min. The cells were resuspended in 50 mM HEPES, pH 7.5 containing 10% (v/v) glycerol and disrupted using a French press. The enzyme was isolated by centrifugation of the supernatant at 6000 rpm, 4 °C for 30 min followed by ultracentrifugation of the supernatant at 40 000 rpm, 4 °C for 60 min. The supernatant, containing the enzyme, was used in subsequent reactions without any further purification.

4. Bifunctional UDP-GlcNAc/Glc 4-epimerase from *Campylobacter jejuni* NCTC 11168[28]. The enzyme was expressed in *Escherichia coli* AD202. After a 24 h culture, the cells were harvested by centrifugation at 8000 rpm, 4 °C for 20 min. The cells were resuspended in a solution of 20 mM HEPES, pH 7.0, 200 mM NaCl, 5 mM β-mercaptoethanol and 1 mM EDTA. Pop culture reagent (Novagen) (200 μL) followed by lysonase (Novagen) (2 μL) were added. The content was vortexed and subsequently incubated for 15 min at room temperature. The mixture was centrifuged at 14 000 rpm, 4 °C for 20 min. The supernatant, containing the enzyme, was collected and used in subsequent reactions without any further purification.

5. Radiochemical assays[29]. The activity of the α-(2,3)-sialyltransferase and the β-(1,4)-*N*-acetylgalactosaminyl transferase was determined by radiochemical assays using 8-(methoxycarbonyl)octyl *O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-β-D-glucopyranoside and 8-(methoxycarbonyloctyl) *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-*O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-β-D-glucopyranoside respectively as substrates.

The activity of the bifunctional UDP-GlcNAc/Glc 4-epimerase was determined by a radiochemical assay in which the formation of labeled UDP-GalNAc is monitored through a glycosyltransferase reaction using octyl *O*-(α-D-fucopyranosyl)-(1 → 2)-*O*-β-D-galactopyranoside as an acceptor.

One unit of enzyme activity was defined as the amount that catalyzes the formation of 1 μmol of product per minute at 37 °C using the corresponding acceptor.

6. Synthesis of CMP-Neu5Ac[27]. *N*-Acetyl neuraminic acid (25.0 mg; 75.5 μmol) and cytidine triphosphate disodium salt (4.77 mg; 90.6 μmol; 1.2 eq.) were suspended in 0.5 M $MgCl_2$ (2.0 mL; 50 mM in reaction) and 0.04 M dithiothreitol (100 μL; 0.2 mM in reaction). The pH was adjusted to 8.0 with 1 M NaOH solution and CMP-Neu5Ac synthetase enzyme preparation (17.9 mL) was added. The mixture was gently tumbled overnight at room temperature. The reaction mixture was then centrifuged at 14 000 rpm for 20 min, the supernatant was then collected and lyophilized. The crude solid obtained was used in sialylation reactions without any further purification assuming 90% conversion of starting material.

Immunization protocol

BALB/c mice were immunized by interperitoneal injection of tetanus toxoid conjugates **25** and **27** (50 μg in 100 μL PBS and 100 μL Freund's complete adjuvant, homogenized). Subsequent injections (50 μg in 100 μL PBS and 100 μL Freund's incomplete adjuvant, homogenized) were made subcutaneously on days 21 and 28. Mice were bled on day 38 and the sera were isolated by centrifugation and stored at −20 °C.

Enzyme-linked immunosorbent assay (ELISA)

BSA-carbohydrate-protein conjugates (10 μg mL^{-1} in PBS) were used to coat 96-well microtiter plates (MaxiSorp, Nunc) overnight at 4 °C. The plate was washed 5 times with PBST (PBS containing 0.05% (v/v) Tween 20). Sera were diluted with PBST containing 0.1% BSA. The solutions were distributed on the coated microtiter plate and incubated at room temperature for 2 hours. The plate was washed with PBST (5 times) and goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories; 1 : 2000 dilution in 0.1% BSA–PBST; 100 μL per well) was added. The mixture was then incubated for 1 hour. The plate was washed 5 times with PBST before addition of a 1 : 1 mixture of 3,3′,5,5′-tetramethylbenzidine (0.4 g L^{-1}) and 0.02% H_2O_2 solution (Kirkegaard & Perry Laboratories; 100 μL per well). After 2 minutes, the reaction was stopped by addition of 1 M phosphoric acid (100 μL per well). Absorbance was read at 450 nm.

Oligosaccharide inhibition of enzyme-linked immunosorbent assay (ELISA)

Carbohydrate-protein conjugates (10 μg mL^{-1} in PBS) were used to coat 96-well microtiter plates (MaxiSorp, Nunc) overnight at 4 °C. The plate was washed 5 times with PBST (PBS containing 0.05% (v/v) Tween 20). The serum solution (1 : 2000 dilution

in 0.1% BSA–PBST) was mixed with inhibitor dissolved in 0.1% BSA–PBST at concentrations ranging from 0.001 μM to 1 mM. The resulting solutions were added to the coated microtiter plate in triplicates, and incubated at room temperature for 2 hours. The plate was washed with PBST (5 times) and goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories; 1 : 2000 dilution in 0.1% BSA–PBST; 100 μL per well) was added. The mixture was then incubated for 1 hour. The plate was washed 5 times with PBST before addition of a 1 : 1 mixture of 3,3′,5,5′-tetramethylbenzidine (0.4 g L^{-1}) and 0.02% H_2O_2 solution (Kirkegaard & Perry Laboratories; 100 μL per well). After 2 minutes, the reaction was stopped by addition of 1 M phosphoric acid (100 μL per well). Absorbance was read at 450 nm and percent inhibition was calculated relative to wells containing sera without inhibitor.

***O*-(β-D-Galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-(trifluoroacetamido)-pent-4-ene-1,3-diol (6).** The lactosyl azidosphingosine **5** (151.5 mg; 324 μmol) was dissolved in a solution of pyridine–water–triethylamine (10 : 1 : 0.3; 11.3 mL). The solution was saturated with H_2S by bubbling the gas into the solution for 1 h at 0 °C, followed by stirring for 36 h. The solvent was evaporated and the residue was dissolved in water. After addition of acetic acid (200 μL), the mixture was applied to a Sep-Pak (C18; 5 g). The salt obtained was then eluted through Amberlite IRA-400 (Cl^-) resin preconditioned with 2 M NaOH yielding the free amine (124.7 mg; 282 μL; 87%). A portion of the material (76.8 mg; 174 μmol) was dissolved in dry degassed methanol (5.0 mL) and methyl trifluoroacetate (334.0 mg; 2.61 mmol; 15 eq.) was added. The reaction mixture was stirred at room temperature overnight. The solution was then concentrated. The residue was redissolved in water and applied to a Sep-Pak (C18; 5 g). Gradient elution (MeOH–H_2O) afforded **6**. A sample (110.0 mg; 249.0 μmol) was further purified by HPLC (100.0 mg; 18.6 μmol; 75%): $[\alpha]_D$ +9.1 (*c* 0.55, H_2O). ^{1}H NMR (500 MHz, D_2O): δ_H 5.85 (ddd, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.0 Hz, H-4_{sphing}), 5.34 (ddd, 1H, $J_{5t,4}$ = 17.3 Hz, $J_{5t,3}$ = 1.1 Hz, $J_{5t,5c}$ = 1.1 Hz, H-$5t_{sphing}$), 5.29 (ddd, 1H, $J_{5c,4}$ = 10.4 Hz, $J_{5c,5t}$ = 1.1 Hz, $J_{5c,3}$ = 1.1 Hz, H-$5c_{sphing}$), 4.48 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1_{Glc}), 4.44 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1_{Gal}), 4.31 (dd, 1H, $J_{3,2}$ = 7.0 Hz, $J_{3,4}$ = 7.0 Hz, H-3_{sphing}), 4.30 (dd, 1H, $J_{1a,1b}$ = 7.0 Hz, $J_{1a,2}$ = 7.0 Hz, H-$1a_{sphing}$), 4.22 (ddd, 1H, $J_{2,3}$ = 7.0 Hz, $J_{2,1a}$ = 7.0 Hz, H-2_{sphing}), 3.98 (dd, 1H, $J_{6a,6b}$ = 12.3 Hz, $J_{6a,5}$ = 2.1 Hz, H-$6a_{Glc}$), 3.92–3.89 (m, 2H, H-4_{Gal}, H-$1b_{sphing}$), 3.81–3.70 (m, 4H, H-$6b_{Glc}$, H-5_{Gal}, H-$6a_{Gal}$, H-$6b_{Gal}$), 3.67–3.57 (m, 4H, H-3_{Gal}, H-3_{Glc}, H-4_{Glc}, H-5_{Glc}), 3.53 (dd, 1H, $J_{2,3}$ = 9.9 Hz, $J_{2,1}$ = 7.9 Hz, H-2_{Gal}), 3.32 (m, 1H, H-2_{Glc}). ^{13}C NMR (125 MHz, D_2O): δ_C 160.0 (q, $J_{C,F}$ = 37 Hz), 136.4, 119.4, 116.7 (q, $J_{C,F}$ = 286 Hz), 103.8, 103.0, 79.3, 76.2, 75.7, 75.1, 73.6, 73.4, 72.4, 71.8, 69.4, 68.6, 61.9, 61.0, 54.8. ESI HRMS calcd for $C_{19}H_{30}F_3NO_{13}Na$: 560.15615. Found: 560.15593.

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-*O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-trifluoroacetamido-pent-4-ene-1,3-diol (7).** The acceptor **6** (13.6 mg; 25.2 μmol; 5.04 mM in reaction), crude CMP-Neu5Ac (~45.3 μmol; ~9.06 mM in reaction; ~1.8 eq.), $MnCl_2$ (100 μL of 0.5 M solution; 10 mM in reaction), $MgCl_2$ (100 μL of 0.5 M solution; 10 mM in reaction), dithiothreitol (125 μL of 0.1 M solution; 2.5 mM in reaction), water (600 μL), α-(2,3)-sialyltransferase (4.5 mL; ~4.5 U), and alkaline phosphatase (Roche Bioscience) (2.5 μL; ~2.5 U) in 0.05 M HEPES buffer, pH 7.5 containing 10% (v/v) glycerol were combined in a 15 mL Falcon tube. The mixture was tumbled gently at room temperature for 24 h after which time TLC ($CHCl_3$–MeOH–H_2O–AcOH; 10 : 7 : 1 : 2) indicated almost complete disappearance of the starting material **6**. The reaction mixture was centrifuged at 14 000 rpm for 30 min and the supernatant was applied to a Sep-Pak (C18; 10 g). Gradient elution (MeOH–H_2O) afforded **7**. Further purification by size exclusion chromatography (Biogel P-4 resin; 50 cm × 2 cm) using 5% MeOH–H_2O as an eluent yielded **7** (18.9 mg; 22.2 μmol; 88%): $[\alpha]_D$ +5.1 (*c* 1.08, H_2O). ^{1}H NMR (500 MHz, D_2O): δ_H 5.83 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.2 Hz, H-4_{sphing}), 5.33 (m, 1H, H-$5t_{sphing}$), 5.28 (m, 1H, H-$5c_{sphing}$), 4.51 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1_{Gal}), 4.47 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1_{Glc}), 4.30 (dd, 1H, $J_{3,2}$ = 7.2 Hz, $J_{3,4}$ = 7.2 Hz, H-3_{sphing}), 4.22 (ddd, 1H, $J_{2,3}$ = 7.1 Hz, $J_{2,1a}$ = 7.1 Hz, $J_{2,1b}$ = 3.6 Hz, H-2_{sphing}), 4.11–4.07 (m, 2H, H-$1a_{sphing}$, H-3_{Gal}), 3.98 (dd, 1H, $J_{6a,6b}$ = 12.2 Hz, $J_{6a,5}$ = 2.1 Hz, H-$6a_{Glc}$), 3.94 (d, 1H, $J_{4,3}$ = 3.05 Hz, H-4_{Gal}), 3.91–3.79 (m, 9H, H-$1b_{sphing}$, H-8_{Neu}, H-$9a_{Neu}$, H-5_{Gal}, H-$9b_{Neu}$, H-6_{Neu}, H-7_{Neu}, H-2_{Gal}, H-$6b_{Glc}$), 3.75–3.56 (m, 6H, H-4_{Neu}, H-$6a_{Gal}$, H-$6b_{Gal}$, H-5_{Neu}, H-3_{Glc}, H-4_{Glc}, H-5_{Glc}), 3.32 (m, 1H, H-2_{Glc}), 2.75 (dd, 1H, $J_{3e,3a}$ = 12.2 Hz, $J_{3e,4}$ = 4.7 Hz, H-$3e_{Neu}$), 2.02 (s, 3H, C(O)CH_3), 1.79 (dd, 1H, $J_{3a,3e}$ = 12.2 Hz, $J_{3a,4}$ = 12.2 Hz, H-$3a_{Neu}$). ^{13}C NMR (125 MHz, D_2O): δ_C 175.9, 174.7, 160.0, 136.4, 119.4, 116.7 (q, $J_{C,F}$ = 285 Hz), 103.6, 100.7, 79.2, 76.4, 76.1, 75.7, 75.1, 73.8, 73.6, 72.7, 72.4, 70.2, 69.2, 69.0, 68.6, 68.4, 63.5, 61.9, 60.9, 54.8, 52.6, 49.8, 40.5, 22.9. ESI HRMS calcd for $C_{30}H_{46}N_2O_{21}F_3$: 827.25448. Found: 827.25507.

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-[2-acetamido-2-deoxy-*O*-β-D-galactopyranosyl-(1 → 4)]-*O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-trifluoroacetamido-pent-4-ene-1,3-diol (8).** The acceptor **7** (41.6 mg; 50.2 μmol) and UDP-*N*-acetylglucosamine disodium salt (49.0 mg; 75.2 μmol; 1.5 eq.) were combined in a Falcon tube. UDP-*N*-acetylglucosamine 4-epimerase (1.0 mL; ~1.0 U) in 0.2 M NaCl, 1.0 mM EDTA, 20 mM HEPES, 5 mM β-mercaptoethanol, pH 7.0 and *N*-acetylgalactosaminyl transferase (2.94 mL; ~2.5 U) were added to the mixture followed by 48 μL of 0.5 M $MgCl_2$ (6.0 mM in reaction) and alkaline phosphatase (10 μL; ~10 U). The reaction was gently tumbled for 16 h until the reaction was completed as observed by silica gel TLC (EtOAc–MeOH–H_2O–AcOH; 6 : 3 : 3 : 2). The mixture was centrifuged for 20 min at 14 000 rpm, the supernatant was collected and applied to a Sep-Pak (C18; 5g). Elution with a gradient of water–methanol afforded **8**. A portion (18.5 mg; 17.9 μmol) was further purified by size exclusion chromatography (Biogel P-4 resin; 50 cm × 2 cm) and eluted with 5% MeOH–H_2O to yield **8** (17.7 mg; 17.2 μmol; 95%): $[\alpha]_D$ +14.2 (*c* 0.32, H_2O). ^{1}H NMR (600 MHz, D_2O): δ_H 5.85 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.0 Hz, H-4_{sphing}), 5.33 (m, 1H, H-$5t_{sphing}$), 5.30 (d, 1H, H-$5c_{sphing}$), 4.74 (d, overlapped with HOD, 1H, H-1_{GalNAc}), 4.52 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1_{Gal}), 4.49 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1_{Glc}), 4.32 (dd, 1H, $J_{3,2}$ = 7.0 Hz, $J_{3,4}$ = 7.0 Hz, H-3_{sphing}), 4.23 (ddd, 1H, $J_{2,3}$ = 7.0 Hz, $J_{2,1a}$ = 7.0 Hz, $J_{2,1b}$ = 3.9 Hz, H-2_{sphing}), 4.14 (dd, 1H, $J_{3,2}$ = 9.8 Hz, $J_{3,4}$ = 2.6 Hz, H-3_{Gal}), 4.12–4.10 (m, 2H, H-$1a_{sphing}$, H-4_{Gal}), 3.99 (d, 1H, $J_{6a,6b}$ = 12.1 Hz, H-$6a_{Glc}$), 3.93–3.86 (m, 4H, H-4_{GalNAc}, H-2_{GalNAc}, H-$1b_{sphing}$,

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H-9a$_{Neu}$), 3.83–3.59 (m, 16H, H-5$_{Neu}$, H-6b$_{Glc}$, H-4$_{Neu}$, H-5$_{Gal}$, H-6a$_{Gal}$, H-6b$_{Gal}$, H-5$_{GalNAc}$, H-6a$_{GalNAc}$, H-6b$_{GalNAc}$, H-8$_{Neu}$, H-3$_{GalNAc}$, H-3$_{Glc}$, H-9b$_{Neu}$, H-4$_{Glc}$, H-7$_{Neu}$, H-5$_{Glc}$), 3.49 (d, 1H, $J_{6,5}$ = 10.1 Hz, H-6$_{Neu}$), 3.37–3.31 (m, 2H, H-2$_{Gal}$, H-2$_{Glc}$), 2.66 (dd, 1H, $J_{3e,3a}$ = 12.3 Hz, $J_{3e,4}$ = 4.5 Hz, H-3e$_{Neu}$), 2.03, 2.02 (2 × s, 2 × 3H, 2 × C(O)CH_3), 1.92 (dd, 1H, $J_{3a,3e}$ = 12.3 Hz, $J_{3a,4}$ = 11.8 Hz, H-3a$_{Neu}$). ^{13}C NMR (125 MHz, D_2O): δ_C 175.9, 175.7, 174.9, 159.8, 136.4, 119.4, 116.7 (q, $J_{C,F}$ = 285 Hz), 103.7, 103.5, 103.0, 102.5, 79.5, 78.0, 75.7, 75.6, 75.2, 75.1, 74.9, 74.0, 73.5, 73.1, 72.5, 72.2, 70.9, 69.6, 68.9, 65.1, 63.7, 62.1, 61.5, 61.0, 54.8, 53.2, 52.5, 49.8, 37.9, 23.6, 22.9. ESI HRMS calcd for $C_{38}H_{59}N_3O_{26}F_3$: 1030.33444. Found: 1030.33555.

Procedure (A) for deprotection and acylation of sphingosine amine using succinimide esters

Oligosaccharide **7** or **8** (5–40 μmol) was dissolved in dry methanol (2.0 mL). Sodium methoxide (~3.0 eq.) was added and the mixture, stirred for 3 days at room temperature until completion of the reaction. The pH of the reaction mixture was adjusted to 9.0 using 0.5 M HCl and the methanol was evaporated. The compound was purified by HPLC (Hamilton semi-preparative PRP-1 column) using a MeOH–H_2O gradient. A portion of the amine (5–15 μmol), the acylating agent (2.0–5.0 eq.) and 4-*N*,*N*-dimethylaminopyridine (1.0–3.0 eq.) were dissolved in a 9 : 1 pyridine–water mixture (1.0 mL). The reaction mixture was stirred overnight at 40 °C and concentrated. The residue was redissolved in a solution of 5% $NaHCO_3$–H_2O and applied to a C18 Sep-Pak cartridge. Gradient elution (MeOH–H_2O) afforded the target compound which was further purified by size exclusion chromatography (Biogel P-4 resin; 50 cm × 2 cm) using 5% MeOH–H_2O as an eluent.

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-*O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-octadecanamido-pent-4-ene-1,3-diol (9).** Compound **9** was prepared using procedure (A). Trisaccharide **7** (30.3 mg; 36.6 μmol) was deprotected and a portion of the amine (6.37 mg; 8.44 μmol) was acylated with *N*-(octadecanoyloxy)succinimide[18] (7.43 mg; 19.4 μmol; 2.3 eq.). Standard work-up and purification as described in procedure (A) afforded **9** (4.70 mg; 4.60 μmol; 55%): $[\alpha]_D$ +6.4 (*c* 0.34, H_2O). ^{1}H NMR (600 MHz, CD_3OD): δ_H 5.86 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 6.7 Hz, H-4$_{sphing}$), 5.26 (ddd, 1H, $J_{5t,4}$ = 17.2 Hz, $J_{5t,5c}$ = 1.5 Hz, $J_{5t,3}$ = 1.5 Hz, H-5t_{sphing}), 5.13 (ddd, 1H, $J_{5c,4}$ = 10.4 Hz, $J_{5c,4}$ = 1.5 Hz, $J_{5c,3}$ = 1.5 Hz, H-5c_{sphing}), 4.42 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1$_{Gal}$), 4.29 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1$_{Glc}$), 4.17–4.13 (m, 2H, H-1a$_{sphing}$, H-3$_{sphing}$), 4.04 (dd, 1H, $J_{3,2}$ = 9.7 Hz, $J_{3,4}$ = 3.2 Hz, H-3$_{Gal}$), 4.01 (m, 1H, H-2$_{sphing}$), 3.93–3.82 (m, 5H, H-4$_{Gal}$, H-6a$_{Glc}$, H-6a$_{Gal}$, H-6b$_{Gal}$, H-6$_{Neu}$), 3.76 (dd, 1H, $J_{9a,9b}$ = 11.7 Hz, $J_{9a,8}$ = 7.7 Hz, H-9a$_{Neu}$), 3.71–3.69 (m, 2H, H-4$_{Neu}$, H-5$_{Gal}$), 3.65 (dd, 1H, $J_{9b,9a}$ = 11.7 Hz, $J_{9a,8}$ = 4.2 Hz, H-9b$_{Neu}$), 3.61–3.52 (m, 7H, H-5$_{Neu}$, H-5$_{Glc}$, H-1b$_{sphing}$, H-8$_{Neu}$, H-2$_{Gal}$, H-3$_{Glc}$, H-4$_{Glc}$), 3.47 (dd, 1H, $J_{6b,6a}$ = 9.0 Hz, $J_{6b,5}$ = 1.8 Hz, H-6b$_{Glc}$), 3.42 (m, 1H, H-7$_{Neu}$), 3.27 (dd, 1H, $J_{2,3}$ = 7.8 Hz, $J_{2,1}$ = 7.8 Hz, H-2$_{Glc}$), 2.86 (m, 1H, H-3e$_{Neu}$), 2.18 (t, 2H, J = 7.5 Hz, CH_2C(O)), 2.00 (s, 3H, C(O)CH_3), 1.72 (m, 1H, H-3a$_{Neu}$), 1.59–1.56 (m, 2H, CH_2CH$_2$C(O)), 1.30–1.28 (m, 28H, alkane CH_2), 0.89 (t, J = 7.0 Hz, CH$_3$). ^{13}C NMR (125 MHz, CD_3OD): δ_C 176.2, 175.5, 174.9, 139.6, 116.9, 116.1, 105.1, 104.5, 101.1, 80.9, 77.7, 77.1, 76.5, 76.3, 75.0, 74.8, 73.3, 73.0, 70.9, 70.2, 69.71, 69.70, 69.4, 69.0, 64.7, 64.2, 62.8, 61.88, 61.86, 54.7, 54.00, 53.98, 49.0, 48.8, 42.1, 37.2, 33.1, 30.49, 30.46, 30.4, 27.1, 22.6, 14.4. ESI HRMS calcd for $C_{46}H_{81}N_2O_{21}Na_2$: 1043.51272. Found: 1043.51243.

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-*O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-(11-azidoundecanamido)-pent-4-ene-1,3-diol (10).** Compound **10** was synthesized according to procedure (A). Trisaccharide **7** (30.3 mg; 36.6 μmol) was deprotected and a portion of the amine (11.9 mg; 15.8 μmol) was acylated with *N*-(11-azidoundecanoyloxy)succinimide (see ESI†) (15.3 mg; 47.2 μmol; 3.0 eq.). Standard work-up and purification as described in procedure (A) yielded **10** (8.4 mg; 8.8 μmol; 78%): $[\alpha]_D$ +2.0 (*c* 0.45, H_2O). ^{1}H NMR (500 MHz, D_2O): δ_H 5.83 (ddd, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 6.9 Hz, H-4$_{sphing}$), 5.31 (ddd, 1H, $J_{5t,4}$ = 17.3 Hz, $J_{5t,5c}$ = 1.2 Hz, $J_{5t,3}$ = 1.2 Hz, H-5t_{sphing}), 5.26 (ddd, 1H, $J_{5c,4}$ = 10.4 Hz, $J_{5c,5t}$ = 1.2 Hz, $J_{5c,3}$ = 1.0 Hz, H-5b$_{sphing}$), 4.50 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1$_{Gal}$), 4.45 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1$_{Glc}$), 4.20 (dd, 1H, $J_{3,2}$ = 7.0 Hz, $J_{3,4}$ = 6.9 Hz, H-3$_{sphing}$), 4.11–4.04 (m, 3H, H-3$_{Gal}$, H-2$_{sphing}$, H-1a$_{sphing}$), 4.00 (dd, 1H, $J_{6a,6b}$ = 12.3 Hz, $J_{6a,5}$ = 2.2 Hz, H-6a$_{Glc}$), 3.95 (d, 1H, $J_{4,3}$ = 3.1 Hz, H-4$_{Gal}$), 3.90–3.78 (m, 4H, H-8$_{Neu}$, H-9$_{Neu}$, H-6b$_{Glc}$, H-5$_{Neu}$), 3.76 (m, 1H, H-1b$_{sphing}$), 3.73–3.66 (m, 2H, H-5$_{Gal}$, H-4$_{Neu}$), 3.65–3.61 (m, 5H, H-6a$_{Gal}$, H-6b$_{Gal}$, H-3$_{Glc}$, H-4$_{Glc}$, H-9b$_{Neu}$), 3.60–3.55 (m, 4H, H-5$_{Glc}$, H-6$_{Neu}$, H-7$_{Neu}$, H-2$_{Gal}$), 3.35–3.29 (m, 3H, H-2$_{Glc}$, CH_2N$_3$), 2.75 (dd, 1H, $J_{3e,3a}$ = 12.3 Hz, $J_{3e,4}$ = 4.7 Hz, H-3e$_{Neu}$), 2.25 (t, 2H, J = 7.3 Hz, CH_2C(O)), 2.03 (s, 3H, C(O)CH_3), 1.79 (dd, 1H, $J_{3a,3e}$ = 12.3 Hz, $J_{3a,4}$ = 12.3 Hz, H-3a$_{Neu}$), 1.61–1.56 (m, 4H, CH_2CH$_2$C(O), CH_2CH$_2$N$_3$), 1.38–1.29 (m, 12H, alkane CH_2). ^{13}C NMR (125 MHz, D_2O): δ_C 178.2, 175.9, 174.7, 137.2, 119.0, 103.6, 103.3, 100.7, 79.4, 76.4, 76.1, 75.7, 75.1, 73.8, 73.7, 72.7, 72.6, 70.3, 69.6, 69.2, 69.0, 68.4, 63.5, 61.9, 61.0, 53.9, 52.6, 52.2, 40.5, 36.8, 36.7, 29.4 (2C), 29.2, 29.1, 29.0, 28.8, 26.2, 22.9. ESI HRMS calcd for $C_{39}H_{67}N_5O_2Na$: 964.42263. Found: 964.42227.

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-[2-acetamido-2-deoxy-*O*-β-D-galactopyranosyl-(1 → 4)]-*O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-(11-azidoundecanamido)-pent-4-ene-1,3-diol (11).** Compound **11** was prepared using procedure (A). Tetrasaccharide **8** was deprotected and a portion of the amine (4.8 mg; 5.0 μmol) was acylated with *N*-(11-azidoundecanoyloxy)succinimide (see ESI†) (2.4 mg; 7.4 μmol; 1.5 eq.). Work-up and purification as described above afforded **11** (4.5 mg; 3.8 μmol; 76%): $[\alpha]_D$ +6.4 (*c* 0.45, H_2O). ^{1}H NMR (600 MHz, D_2O): δ_H 5.85 (ddd, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.0 Hz, H-4$_{sphing}$), 5.33 (m, 1H, H-5t_{sphing}), 5.27 (m, 1H, H-5c_{sphing}), 4.74 (d, 1H, J$_{1,2}$ = 7.3 Hz, H-1$_{GalNAc}$), 4.51 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{Gal}$), 4.47 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{Glc}$), 4.22 (dd, 1H, J$_{3,4}$ = 7.0 Hz, $J_{3,2}$ = 7.0 Hz, H-3$_{sphing}$), 4.15–4.06 (m, 4H, H-3$_{Gal}$, H-2$_{sphing}$, H-1a$_{sphing}$, H-4$_{Gal}$), 3.99 (d, 1H, $J_{6a,6b}$ = 12.1 Hz, H-6a$_{Glc}$), 3.92–3.90 (m, 2H, H-4$_{GalNAc}$, H-2$_{GalNAc}$), 3.87 (dd, 1H, $J_{9a,9b}$ = 11.0 Hz, $J_{9a,8}$ = 1.1 Hz, H-9a$_{Neu}$), 3.83–3.58 (m, 17H, H-5$_{Neu}$, H-6b$_{Glc}$, H-1b$_{sphing}$, H-4$_{Neu}$, H-5$_{Gal}$, H-6a$_{Gal}$, H-6b$_{Gal}$, H-5$_{GalNAc}$, H-6a$_{GalNAc}$, H-6b$_{GalNAc}$, H-8$_{Neu}$, H-3$_{GalNAc}$, H-3$_{Glc}$, H-9b$_{Neu}$, H-4$_{Glc}$, H-7$_{Neu}$, H-5$_{Glc}$), 3.48 (dd, 1H, $J_{6,5}$ = 9.9 Hz, $J_{6,7}$ = 1.9 Hz, H-6$_{Neu}$), 3.38–3.30 (m, 4H, H-2$_{Gal}$, H-2$_{Glc}$, CH_2N$_3$), 2.66 (dd, 1H, $J_{3e,3a}$ = 12.7 Hz, $J_{3e,4}$ = 4.5 Hz, H-3e$_{Neu}$), 2.25 (t, 2H, J = 7.3 Hz,

$CH_2C(O)N$), 2.03, 2.01 (2 × s, 2 × 3H, 2 × $C(O)CH_3$), 1.92 (dd, 1H, $J_{3a,3e}$ = 12.7 Hz, $J_{3a,4}$ = 11.4 Hz, H-3a$_{Neu}$), 1.59–1.56 (m, 4H, $CH_2CH_2N_3$, $CH_2CH_2C(O)$), 1.38–1.27 (m, 12H, alkene CH_2). ^{13}C NMR (125 MHz, D_2O): δ_C 178.2, 175.9, 175.7, 174.9, 137.2, 136.4, 119.0, 103.6, 103.3, 102.5, 79.6, 78.0, 75.6 (2C), 75.2, 75.1, 74.9, 74.0, 73.6, 73.1, 72.7, 72.2, 70.9, 69.6, 68.9, 68.7, 64.6, 63.7, 62.0, 61.4, 53.9, 53.2, 52.5, 52.1, 42.8, 37.8, 36.8, 29.4, 29.3, 29.2, 29.1, 29.0, 28.8, 26.2, 26.1, 23.5, 22.9. ESI HRMS calcd for $C_{47}H_{79}N_6O_{26}Na$: 1167.50200. Found: 1167.50205.

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-*O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-(11-thioacetylundecanamido)-pent-4-ene-1,3-diol (12).** Compound **12** was synthesized according to procedure (A). Trisaccharide **7** (30.3 mg; 36.6 μmol) was deprotected and a portion of the amine (4.0 mg; 5.3 μmol) was acylated with (11-thioacetylundecanoyloxy)succinimide[14] (3.8 mg; 10.6 μmol; 2.0 eq.). Work-up and purification as described above yielded **12** (4.2 mg; 4.2 μmol; 80%): $[\alpha]_D$ +2.3 (*c* 0.42, H_2O). ^{1}H NMR (500 MHz, D_2O): δ_H 5.84 (d, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.5 Hz, $J_{4,3}$ = 7.0 Hz, H-4$_{sphing}$), 5.32 (m, 1H, H-5t_{sphing}), 5.26 (m, 1H, H-5c_{sphing}), 4.51 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1$_{Gal}$), 4.46 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{Glc}$), 4.21 (dd, 1H, $J_{3,2}$ = 7.0 Hz, $J_{3,4}$ = 7.0 Hz, H-3$_{sphing}$), 4.11–4.05 (m, 3H, H-3$_{Gal}$, H-2$_{sphing}$, H-1a$_{sphing}$), 3.99 (dd, 1H, $J_{6a,6b}$ = 12.1 Hz, $J_{6a,5}$ = 1.8 Hz, H-6a$_{Glc}$), 3.95 (d, 1H, $J_{4,3}$ = 3.0 Hz, H-4$_{Gal}$), 3.89–3.55 (m, 16H, H-8$_{Neu}$, H-9a$_{Neu}$, H-6b$_{Glc}$, H-5$_{Neu}$, H-1b$_{sphing}$, H-5$_{Gal}$, H-4$_{Neu}$, H-6a$_{Gal}$, H-6b$_{Gal}$, H-3$_{Glc}$, H-4$_{Glc}$, H-9b$_{Neu}$, H-5$_{Glc}$, H-6$_{Neu}$, H-7$_{Neu}$, H-2$_{Gal}$), 3.33 (m, 1H, H-2$_{Glc}$), 2.89 (t, 2H, *J* = 7.2 Hz, CH_2SAc), 2.76 (dd, 1H, $J_{3e,3a}$ = 12.3 Hz, $J_{3e,4}$ = 4.6 Hz, H-3e$_{Neu}$), 2.36 (s, 3H, $SC(O)CH_3$), 2,25 (t, 2H, *J* = 7.2 Hz, $CH_2C(O)N$), 2.03 (s, 3H, $C(O)CH_3$), 1.80 (dd, 1H, $J_{3a,3e}$ = 12.3 Hz, $J_{3a,4}$ = 12.3 Hz, H-3a$_{Neu}$), 1.60–1.55 (m, 4H, $CH_2CH_2C(O)N$, $CH_2CH_2C(O)S$), 1.37–1.28 (m, 12H, alkane CH_2), ^{13}C NMR (125 MHz, D_2O): δ_C 203.4, 178.2, 175.9, 174.7, 137.2, 119.0, 103.6, 103.4, 100.7, 79.4, 76.4, 76.1, 75.7, 75.1, 73.8, 73.7, 72.7, 72.6, 70.3, 69.6, 69.2, 69.0, 68.4, 63.5, 61.9, 61.0, 53.9, 52.6, 40.5, 36.8, 31.0, 29.9, 29.41, 29.36, 29.3, 29.2, 29.1, 29.0, 28.7, 26.2, 22.9. ESI HRMS calcd for $C_{41}H_{69}N_2O_{22}NaS$: 1019.38581. Found: 1019.38566.

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-[2-acetamido-2-deoxy-*O*-β-D-galactopyranosyl-(1 → 4)]-*O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-(11-thioacetylundecanamido)-pent-4-ene-1,3-diol (13).** Compound **13** was prepared according to procedure (A). Tetrasaccharide **8** (14.7 mg; 14.0 μmol) was deprotected and a portion of the amine (7.0 mg; 7.3 μmol) was acylated with (11-thioacetylundecanoyloxy)-succinimide[14] (5.20 mg; 14.6 μmol; 2.0 eq.). Standard work-up and purification as described in procedure (A) afforded **13** (8.3 mg; 6.9 μmol; 95%): $[\alpha]_D$ +15.4 (*c* 0.31, H_2O). ^{1}H NMR (500 MHz, D_2O): δ_H 5.83 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.0 Hz, H-4$_{sphing}$), 5.31 (m, 1H, H-5t_{sphing}), 5.25 (m, 1H, H-5c_{sphing}), 4.73 (d, 1H, $J_{1,2}$ = 7.3 Hz, H-1$_{GalNAc}$), 4.50 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{Gal}$), 4.46 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{Glc}$), 4.20 (dd, 1H, $J_{3,4}$ = 7.0 Hz, $J_{3,2}$ = 7.0 Hz, H-3$_{sphing}$), 4.14–4.04 (m, 4H, H-3$_{Gal}$, H-2$_{sphing}$, H-1a$_{sphing}$, H-4$_{Gal}$), 3.98 (dd, 1H, $J_{6a,6b}$ = 11.7 Hz, $J_{6a,5}$ = 1.5 Hz, H-6a$_{Glc}$), 3.93–3.86 (m, 3H, H-4$_{GalNAc}$, H-2$_{GalNAc}$, H-9a$_{Neu}$), 3.80–3.57 (m, 17H, H-5$_{Neu}$, H-6b$_{Glc}$, H-1b$_{sphing}$, H-4$_{Neu}$, H-5$_{Gal}$, H-6a$_{Gal}$, H-6b$_{Gal}$, H-5$_{GalNAc}$, H-6a$_{GalNAc}$, H-6b$_{GalNAc}$, H-8$_{Neu}$, H-3$_{GalNAc}$, H-3$_{Glc}$, H-9b$_{Neu}$, H-4$_{Glc}$, H-7$_{Neu}$, H-5$_{Glc}$), 3.47 (dd, 1H, $J_{6,5}$ = 9.9 Hz, $J_{6,7}$ = 1.9 Hz, H-6$_{Neu}$), 3.37–3.31 (m, 2H, H-2$_{Gal}$, H-2$_{Glc}$), 2.88 (t, 2H, *J* = 7.2 Hz, CH_2SAc), 2.65 (dd, 1H, $J_{3e,3a}$ = 12.8 Hz, $J_{3e,4}$ = 4.4 Hz, H-3e$_{Neu}$), 2.35 (s, 3H, $SC(O)CH_3$), 2.24 (t, 2H, *J* = 7.3 Hz, $CH_2C(O)N$), 2.03, 2.01 (2 × s, 2 × 3H, 2 × $C(O)CH_3$), 1.92 (dd, 1H, $J_{3a,3e}$ = 12.8 Hz, $J_{3a,4}$ = 11.5 Hz, H-3a$_{Neu}$), 1.59–1.56 (m, 4H, CH_2CH_2SAc, $CH_2CH_2C(O)$), 1.30–1.28 (m, 12H, alkane CH_2). ^{13}C NMR (125 MHz, D_2O): δ_C 203.1, 178.2, 175.9, 175.7, 174.9, 137.5, 119.2, 103.6, 103.5, 102.7, 79.6, 78.0, 75.6 (2C), 75.2, 75.1, 74.9, 73.9, 73.6, 73.1, 72.7, 72.2, 70.9, 69.5 (2C), 68.9, 68.6, 63.7, 62.1, 61.4, 61.0, 59.8, 53.9, 53.2, 52.5, 37.9, 36.8, 31.0, 29.9, 29.4, 29.3, 29.2, 29.0, 28.9, 28.6, 26.2, 23.5, 22.9. ESI HRMS calcd for $C_{49}H_{82}N_3O_{27}SNa_2$: 1222.46522. Found: 1222.44480.

***O*-(β-D-Galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-(6-aminohexanamido)-pent-4-ene-1,3-diol (15).** The benzoylated lactosyl azidosphingosine **14**[9] (200 mg; 0.15 mmol) was dissolved in 90% aqueous pyridine (10.0 mL) and triphenylphosphine (100 mg; 0.38 mmol; 2.5 eq.) was added. After stirring for 8 h, *p*-nitrophenyl 6-trifluoroacetamidohexanoate (10 eq.) was added, and stirring was continued overnight. The mixture was concentrated, applied to a short plug of silica gel in 2 : 1 hexanes–ethyl acetate, and eluted with a gradient of the same mixture (3 : 2 → 1 : 1). The material obtained was deprotected by stirring with a catalytic amount of sodium methoxide in methanol (5.0 mL) at 45 °C overnight. After cooling to room temperature, the solution was neutralized by addition of acetic acid, concentrated, suspended in water–methanol (95 : 5) and filtered. The solution was then applied to a Sep-Pak (C18; 5 g) which had been preconditioned with methanol and water containing ~2% ammonium hydroxide, and eluted with a gradient of water and methanol containing the same. Concentration of the eluent afforded **15** (49.8 mg; 90.0 μmol; 58%). A portion of the material was purified further for analysis on a Hamilton PRP-1 semi-preparative HPLC column using a H_2O–MeOH gradient: $[\alpha]_D$ +4.9 (*c* 0.40, H_2O). ^{1}H NMR (600 MHz, D_2O): δ_H 5.85 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.0 Hz, H-4$_{sphing}$), 5.32 (m, 1H, H-5t_{sphing}), 5.27 (m, 1H, H-5c_{sphing}), 4.48 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{Glc}$), 4.44 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1$_{Gal}$), 4.22 (dd, 1H, $J_{2,3}$ = 7.0 Hz, $J_{2,1a}$ = 7.0 Hz, H-3$_{sphing}$), 4.11 (ddd, 1H, $J_{2,3}$ = 7.0 Hz, $J_{2,1a}$ = 6.4 Hz, $J_{2,1b}$ = 3.6 Hz, H-2$_{sphing}$), 4.06 (dd, 1H, $J_{1a,1b}$ = 10.6 Hz, $J_{1a,2}$ = 6.4 Hz, H-1a$_{sphing}$), 3.98 (dd, 1H, $J_{6a,6b}$ = 12.3 Hz, $J_{6a,5}$ = 2.2 Hz, H-6a$_{Glc}$), 3.93 (d, 1H, $J_{4,3}$ = 3.4 Hz, H-4$_{Gal}$), 3.86–3.69 (m, 5H, H-6b$_{Glc}$, H-1b$_{sphing}$, H-6a$_{Gal}$, H-6b$_{Gal}$, H-5$_{Gal}$), 3.67–3.62 (m, 3H, H-3$_{Gal}$, H-3$_{Glc}$, H-4$_{Glc}$), 3.60 (m, 1H, H-5$_{Glc}$), 3.54 (dd, 1H, $J_{2,3}$ = 9.9 Hz, $J_{2,1}$ = 7.9 Hz, H-2$_{Gal}$), 3.33 (m, 1H, H-2$_{Glc}$), 2.98–2.95 (m, 2H, CH_2NH_2), 2.36–2.30 (m, 2H, *J*, $CH_2C(O)N$), 1.68–1.58 (m, 4H, $CH_2CH_2NH_2$, $CH_2CH_2C(O)$), 1.40–1.34 (m, 2H, CH_2). ^{13}C NMR (125 MHz, D_2O): δ_C 177.6, 137.2, 119.0, 103.8, 103.2, 79.4, 76.3, 75.7, 75.1, 73.7, 73.4, 72.8, 71.8, 69.5, 61.9, 60.9, 53.8, 40.2, 36.4, 27.5, 26.0, 25.6, 24.2. ESI HRMS calcd for $C_{23}H_{43}N_2O_{13}$: 555.27597. Found: 555.27626.

***O*-(β-D-Galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-butanamidopent-4-ene-1,3-diol (16).** The benzoylated lactosyl azidosphingosine **14**[9] (3.6 g; 2.8 mmol) was dissolved in anhydrous methanol (40 mL) and treated with a catalytic amount of sodium methoxide. After stirring overnight, the mixture was neutralized with a Rexyn 101 ion exchange resin. Removal of the resin by filtration and evaporation of the solvent yielded

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a white solid. Purification of the compound by reverse-phase column chromatography using water as an eluent afforded the deprotected compound (1.1 g; 2.3 mmol; 83%). A portion of the lactosyl azidosphingosine (151.5 mg; 324 μmol) was dissolved in a solution of pyridine–water–triethylamine (10 : 1 : 0.3; 11.3 mL). The solution was saturated with H_2S by bubbling the gas in the solution for 1 h at 0 °C. The mixture was stirred for 36 h before the reaction was completed. The solvent was evaporated and the residue was dissolved in water. After addition of 200 μL of acetic acid, the mixture was applied to a Sep-Pak (C18; 5 g). The salt obtained was eluted through Amberlite IRA-400 (Cl^-) resin preconditioned with 2 M NaOH to yield the free amine (124.7 mg; 282 μmol; 87%). A portion of the material (20.0 mg; 45.0 μmol) was dissolved in methanol (2.0 mL) and butyric anhydride (19 μL; 113 μmol; 2.5 eq.) was added. After stirring for 2.5 hours the solution was concentrated and redissolved in water (1.5 mL). The mixture was applied to a C18 Sep-Pak cartridge and eluted with water to afford **16** (20.0 mg; 39.0 μmol; 86%): $[\alpha]_D$ +8.8 (*c* 0.15, H_2O). ^{1}H NMR (600 MHz, D_2O): δ_H 5.86 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.5 Hz, $J_{4,3}$ = 7.0 Hz, H-4$_{sphing}$), 5.33 (m, 1H, H-5t_{sphing}), 5.27 (m, 1H, H-5c_{sphing}), 4.48 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{Glc}$), 4.45 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1$_{Gal}$), 4.23 (dd, 1H, $J_{2,3}$ = 7.1 Hz, $J_{2,1a}$ = 7.1 Hz, H-3$_{sphing}$), 4.11 (ddd, 1H, $J_{2,3}$ = 7.1 Hz, $J_{2,1a}$ = 6.7 Hz, $J_{2,1b}$ = 3.7 Hz, H-2$_{sphing}$), 4.06 (dd, 1H, $J_{1a,1b}$ = 10.5 Hz, $J_{1a,2}$ = 6.4 Hz, H-1a$_{sphing}$), 3.98 (dd, 1H, $J_{6a,6b}$ = 12.3 Hz, $J_{6a,5}$ = 2.1 Hz, H-6a$_{Glc}$), 3.93 (d, 1H, $J_{4,3}$ = 3.3 Hz, H-4$_{Gal}$), 3.83–3.71 (m, 5H, H-6b$_{Glc}$, H-1b$_{sphing}$, H-6a$_{Gal}$, H-6b$_{Gal}$, H-5$_{Gal}$), 3.68–3.62 (m, 3H, H-3$_{Gal}$, H-3$_{Glc}$, H-4$_{Glc}$), 3.60 (m, 1H, H-5$_{Glc}$), 3.54 (dd, 1H, $J_{2,3}$ = 9.9 Hz, $J_{2,1}$ = 7.9 Hz, H-2$_{Gal}$), 3.35 (m, 1H, H-2$_{Glc}$), 2.23 (t, 2H, *J* = 7.4 Hz, C*H*$_2$C(O)N), 1.62–1.58 (sextet, 2H, *J* = 7.4 Hz, C*H*$_2$CH$_3$), 0.90 (t, 3H, *J* = 7.4 Hz, C*H*$_3$). ^{13}C NMR (from HMQC–HMBC spectra): δ_C 178.2, 137.2, 119.0, 103.8, 103.2, 79.5, 79.4, 76.4, 75.4, 75.2, 73.5, 73.3, 72.8, 71.8, 69.6, 69.4, 62.0, 53.9, 38.8, 19.9, 13.7. ESI HRMS calcd for $C_{21}H_{37}NO_{13}Na$: 534.21571. Found: 534.21514.

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-*O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-(6-aminohexanamido)-pent-4-ene-1,3-diol (17).** Compound **21** (15.3 mg, 15.9 μmol) was dissolved in dry degassed methanol (3.0 mL) and sodium methoxide (275 μL of 0.173 M solution; 47.6 μmol; 3.0 eq.) was added. The reaction mixture was stirred at 40 °C for 36 h. The pH of the reaction mixture was adjusted to 9.0 using 0.5 M HCl and the methanol was evaporated. The compound was purified by HPLC using a Hamilton PRP-1 semi-preparative column. Elution with a MeOH–H_2O gradient afforded **17** (10.0 mg; 11.5 μmol; 72%). Alternatively, the acceptor **15** (25 mg; 45 μmol; 9.0 mM in reaction) was combined with CMP-sialic acid (135 mg of crude mixture, estimated 1.3 eq.), 0.5 M $MnCl_2$ (100 μL; 10 mM in reaction), 0.5 M $MgCl_2$ (100 μL; 10 mM in reaction), 0.4 M dithiothreitol (37.5 μL; 3 mM in reaction), α-(2,3)-sialyltransferase (4.75 mL; ~4.75 U), and alkaline phosphatase (8 μL; 8 U). The mixture was tumbled gently overnight and then centrifuged at 14 000 rpm for 30 min to remove cellular debris. The supernatant was applied directly to a size exclusion column (Biogel P-4; 50 × 2 cm). Elution with 5% aqueous methanol afforded **17** (31 mg; 36 μmol; 80%): $[\alpha]_D$ +2.7 (*c* 0.24, H_2O). ^{1}H NMR (500 MHz, D_2O): δ_H 5.84 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.0 Hz, H-4$_{sphing}$), 5.31 (ddd, 1H, $J_{5t,4}$ = 17.2 Hz, $J_{5t,5c}$ = 1.2 Hz, $J_{5t,3}$ = 1.2 Hz, H-5t_{sphing}), 5.25 (ddd, 1H, $J_{5c,4}$ = 10.4 Hz, $J_{5c,5t}$ = 1.2 Hz, $J_{5c,3}$ = 1.2 Hz, H-5c_{sphing}), 4.51 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1$_{Gal}$), 4.46 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1$_{Glc}$), 4.21 (dd, 1H, $J_{3,4}$ = 7.0 Hz, $J_{3,2}$ = 6.6 Hz, H-3$_{sphing}$), 4.12–4.08 (m, 2H, H-2$_{sphing}$, H-3$_{Gal}$), 4.04 (dd, 1H, $J_{1a,1b}$ = 10.5 Hz, $J_{1a,2}$ = 6.4 Hz, H-1a$_{sphing}$), 3.98 (dd, 1H, $J_{6a,6b}$ = 12.3 Hz, $J_{6a,5}$ = 2.2 Hz, H-6a$_{Glc}$), 3.94 (d, 1H, $J_{4,3}$ = 3.1 Hz, H-4$_{Gal}$), 3.90–3.73 (m, 5H, H-8$_{Neu}$, H-5$_{Neu}$, H-9a$_{Neu}$, H-6b$_{Glc}$, H-1b$_{sphing}$), 3.71–3.67 (m, 3H, H-4$_{Neu}$, H-6a$_{Gal}$, H-6b$_{Gal}$), 3.66–3.61 (m, 5H, H-5$_{Gal}$, H-3$_{Glc}$, H-4$_{Glc}$, H-9b$_{Neu}$, H-6$_{Neu}$), 3.59–3.55 (m, 3H, H-5$_{Glc}$, H-7$_{Neu}$, H-2$_{Gal}$), 3.32 (m, 1H, H-2$_{Glc}$), 2.97 (t, 2H, *J* = 7.6 Hz, C*H*$_2$NH$_2$), 2.75 (dd, 1H, $J_{3e,3a}$ = 12.2 Hz, $J_{3e,4}$ = 4.6 Hz, H-3e$_{Neu}$), 2.28 (t, 2H, *J* = 7.4 Hz, C*H*$_2$C(O)), 2.03 (s, 3H, C(O)C*H*$_3$), 1.79 (dd, 1H, $J_{3a,3e}$ = 12.2 Hz, $J_{3a,4}$ = 12.1 Hz, H-3a$_{Neu}$), 1.69–1.59 (m, 4H, C*H*$_2$CH$_2$NH$_2$, C*H*$_2$CH$_2$C(O)), 1.41–1.34 (m, 2H, C*H*$_2$). ^{13}C NMR (125 MHz, D_2O): δ_C 177.6, 175.9, 174.7, 137.2, 118.9, 103.6, 103.2, 100.7, 79.2, 76.4, 76.1, 75.6, 75.1, 73.8, 73.7, 72.8, 72.7, 70.2, 69.5, 69.2, 69.0, 68.4, 63.5, 61.9, 60.9, 53.8, 52.6, 40.6, 40.2, 36.4, 27.4, 26.0, 25.6, 22.9. ESI HRMS calcd for $C_{34}H_{60}N_3O_{21}$: 846.37193. Found: 846.37177.

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-*O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-(butanamido)-pent-4-ene-1,3-diol (18).** Lactoside **16** (20.0 mg; 36.3 μmol) and crude CMP-Neu5Ac (~47.0 μmol; ~11.1 mM in reaction; ~1.2 eq.) were combined as solids. $MnCl_2$ (100 μL of a 0.5 M solution; 10 mM in reaction), $MgCl_2$ (100 μL of a 0.5 M solution; 10 mM in reaction), dithiothreitol (32 μL of a 0.4 M solution; 2.5 mM in reaction), α-(2,3)-sialyltransferase (4.0 mL; ~4.0 U) and alkaline phosphatase (5 μL; ~5 U) were then added sequentially and the mixture was tumbled gently overnight. When the starting material had been consumed as judged by TLC ($CHCl_3$–MeOH–H_2O–AcOH; 10 : 7 : 1 : 2), the mixture was centrifuged at 14 000 rpm for 30 min and the supernatant was applied to a Sep-Pak (C18; 5 g) which was eluted with water (30 mL), followed by a gradient of water–methanol. The product obtained was further purified by HPLC (Kromasil C_{18}-silica semi-preparative column) using a gradient of water and methanol as eluents to afford **18** (24.9 mg; 31.0 μmol; 79%): $[\alpha]_D$ +2.4 (*c* 0.30, H_2O). ^{1}H NMR (600 MHz, D_2O): δ_H 5.85 (dddd, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.5 Hz, $J_{4,3}$ = 7.1 Hz, $J_{4,2}$ = 1.1 Hz, H-4$_{sphing}$), 5.32 (m, 1H, H-5t_{sphing}), 5.27 (m, 1H, H-5c_{sphing}), 4.52 (dd, 1H, $J_{1,2}$ = 7.8 Hz, $J_{1,3}$ = 0.9 Hz, H-1$_{Gal}$), 4.47 (dd, 1H, $J_{1,2}$ = 7.9 Hz, $J_{1,3}$ = 0.9 Hz, H-1$_{Glc}$), 4.22 (ddd, 1H, $J_{3,4}$ = 7.1 Hz, $J_{3,2}$ = 7.1 Hz, $J_{3,1}$ = 0.9 Hz, H-3$_{sphing}$), 4.12–4.05 (m, 3H, H-3$_{Gal}$, H-2$_{sphing}$, H-1a$_{sphing}$), 3.99 (d, 1H, $J_{6a,6b}$ = 11.4 Hz, $J_{6a,5}$ = 1.8 Hz, H-6a$_{Glc}$), 3.95 (d, 1H, $J_{4,3}$ = 2.7 Hz, H-4$_{Gal}$), 3.90–3.73 (m, 5H, H-8$_{Neu}$, H-5$_{Neu}$, H-9a$_{Neu}$, H-6b$_{Glc}$, H-1b$_{sphing}$), 3.71–3.62 (m, 8H, H-4$_{Neu}$, H-6a$_{Gal}$, H-6b$_{Gal}$, H-5$_{Gal}$, H-3$_{Glc}$, H-4$_{Glc}$, H-9b$_{Neu}$, H-6$_{Neu}$), 3.60–3.55 (m, 3H, H-5$_{Glc}$, H-7$_{Neu}$, H-2$_{Gal}$), 3.33 (m, 1H, H-2$_{Glc}$), 2.75 (dd, 1H, $J_{3e,3a}$ = 12.3 Hz, $J_{3e,4}$ = 4.6 Hz, H-3e$_{Neu}$), 2.22 (t, 2H, *J* = 7.1 Hz, C*H*$_2$C(O)), 2.03 (s, 3H, C(O)C*H*$_3$), 1.79 (dd, 1H, $J_{3a,3e}$ = 12.3 Hz, $J_{3a,4}$ = 12.1 Hz, H-3a$_{Neu}$), 1.62–1.56 (m, 4H, C*H*$_2$CH$_2$C(O), C*H*$_2$CH$_2$CH$_2$C(O)), 0.89 (t, 3H, *J* = 9.3 Hz, C*H*$_3$). ^{13}C NMR (125 MHz, D_2O): δ_C 178.1, 175.9, 174.8, 137.1, 119.0, 103.6, 103.3, 100.7, 79.2, 76.4, 76.1, 75.6, 75.1, 73.8, 73.7, 72.74, 72.66, 70.3, 69.5, 69.2, 69.0, 68.4, 63.5, 61.9, 61.0, 53.9, 52.6, 40.7, 38.7, 22.9, 19.9, 13.7. ESI HRMS calcd for $C_{32}H_{53}N_2O_{21}$: 801.31336. Found: 801.31463.

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-[2-acetamido-2-deoxy-*O*-β-D-galactopyranosyl-(1 → 4)]-*O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-(6-aminohexanamido)-pent-4-ene-1,3-diol (19).** Compound **22** (6.0 mg; 5.1 μmol) was dissolved in dry degassed methanol (1.5 mL) and sodium methoxide (89 μL of 0.17 M solution; 15.4 μmol; 3.0 eq.) was added. The reaction mixture was stirred at 35 °C for 36 h. The methanol was evaporated, redissolved in water and the pH of the mixture was adjusted to 9.0 with 1 N hydrochloric acid. The compound was purified by HPLC using a Hamilton PRP-1 semi-preparative column. Elution with a MeOH–H_2O gradient afforded **19** (4.0 mg; 3.7 μmol; 73%). Alternatively, UDP-*N*-acetylglucosamine disodium salt (15 mg; 34 μmol; 1.2 eq.) was added to a Falcon tube containing **17** (23.4 mg; 28.0 μmol; 1.0 eq.). *N*-Acetylgalactosaminyl transferase (1.5 mL, ~1.5 U) in 50 mM HEPES, pH 7.5 containing 10% glycerol (v/v) and UDP-*N*-acetylglucosamine 4-epimerase (1.5 mL, ~1.5 U) in 0.2 M NaCl, 1.0 mM EDTA, 20 mM HEPES, 5 mM β-mercaptoethanol, pH 7.0 were added to the mixture, followed by 75 μL of 0.5 M $MnCl_2$ and alkaline phosphatase (Roche Biosciences) (10 μL; 10 U). The mixture was tumbled overnight and then centrifuged for 30 min at 14 000 rpm. The supernatant was applied to a Sep-Pak (C18; 5 g), washed with water and eluted with a water–methanol gradient. The residue thus obtained was applied to a Biogel P-4 column (50 cm × 2 cm) and eluted with 5% MeOH–H_2O to yield **19** (23 mg; 22 μmol; 79%): $[\alpha]_D$ +27.1 (*c* 0.24, H_2O). ^{1}H NMR (600 MHz, D_2O): δ_H 5.85 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.0 Hz, H-4$_{sphing}$), 5.32 (m, 1H, H-5t_{sphing}), 5.27 (m, 1H, H-5c_{sphing}), 4.73 (d, 1H, $J_{1,2}$ = 7.3 Hz, H-1$_{GalNAc}$), 4.52 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1$_{Gal}$), 4.48 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{Glc}$), 4.23 (dd, 1H, $J_{3,4}$ = 7.0 Hz, $J_{3,2}$ = 7.0 Hz, H-3$_{sphing}$), 4.14 (dd, 1H, $J_{3,2}$ = 9.8 Hz, $J_{3,4}$ = 3.0 Hz, H-3$_{Gal}$), 4.12–4.09 (m, 2H, H-4$_{Gal}$, H-2$_{sphing}$), 4.06 (dd, 1H, $J_{1a,1b}$ = 10.4 Hz, $J_{1a,2}$ = 6.2 Hz, H-1a$_{sphing}$), 3.99 (dd, 1H, $J_{6a,6b}$ = 12.2 Hz, $J_{6a,5}$ = 1.9 Hz, H-6a$_{Glc}$), 3.93–3.90 (m, 2H, H-4$_{GalNAc}$, H-2$_{GalNAc}$), 3.87 (dd, 1H, $J_{9a,9b}$ = 12.0 Hz, $J_{9a,8}$ = 2.2 Hz, H-9a$_{Neu}$), 3.83–3.58 (m, 17H, H-5$_{Neu}$, H-6b$_{Glc}$, H-1b$_{sphing}$, H-4$_{Neu}$, H-5$_{Gal}$, H-6a$_{Gal}$, H-6b$_{Gal}$, H-5$_{GalNAc}$, H-6a$_{GalNAc}$, H-6b$_{GalNAc}$, H-8$_{Neu}$, H-3$_{GalNAc}$, H-3$_{Glc}$, H-9b$_{Neu}$, H-4$_{Glc}$, H-7$_{Neu}$, H-5$_{Glc}$), 3.49 (dd, 1H, $J_{6,5}$ = 10.1 Hz, $J_{6,7}$ = 2.1 Hz, H-6$_{Neu}$), 3.36 (dd, 1H, $J_{2,3}$ = 9.5 Hz, $J_{2,1}$ = 7.8 Hz, H-2$_{Gal}$), 3.32 (dd, 1H, $J_{2,3}$ = 9.2 Hz, $J_{2,1}$ = 8.1 Hz, H-2$_{Glc}$), 2.98 (t, 2H, *J* = 7.6 Hz, CH_2NH$_2$), 2.66 (dd, 1H, $J_{3e,3a}$ = 12.3 Hz, $J_{3e,4}$ = 4.3 Hz, H-3e$_{Neu}$), 2.28 (t, 2H, *J* = 7.3 Hz, CH_2C(O)), 2.03, 2.01 (2 × s, 2 × 3H, 2 × C(O)CH_3), 1.92 (dd, 1H, $J_{3a,3e}$ = 12.3 Hz, $J_{3a,4}$ = 12.3 Hz, H-3a$_{Neu}$), 1.69–1.60 (m, 4H, CH_2CH$_2$NH$_2$, CH_2CH$_2$C(O)), 1.40–1.36 (m, 2H, CH$_2$). ^{13}C NMR (125 MHz, D_2O): δ_C 177.6, 175.9, 175.7, 174.9, 137.2, 118.9, 103.6, 103.5, 103.2, 102.5, 79.5, 78.0, 75.6 (2C), 75.2, 75.1, 74.9, 74.0, 73.6, 73.1, 72.7, 72.1, 70.9, 69.6, 69.5, 68.9, 68.7, 63.7, 62.0, 61.5, 61.0, 53.8, 53.2, 52.5, 40.2, 37.9, 36.4, 27.4, 26.0, 25.6, 23.5, 22.9. ESI HRMS calcd for $C_{42}H_{73}N_4O_{26}$: 1049.45131. Found: 1049.45134.

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-[2-acetamido-2-deoxy-*O*-(β-D-galactopyranosyl)-(1 → 4)]-*O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-(butanamido)-pent-4-ene-1,3-diol (20).** UDP-*N*-acetylglucosamine disodium salt (15 mg; 18 μmol; 1.2 eq.) was added to a Falcon tube containing **18** (12 mg; 15 μmol; 1.0 eq.). Freshly prepared β-(1,4)-*N*-acetylgalactosaminyl transferase (2.0 mL; ~1.6 U) in 50 mM HEPES, pH 7.5 containing 10% glycerol (v/v) and UDP-*N*-acetylglucosamine 4-epimerase (2.0 mL; ~2.0 U) in 0.2 M NaCl, 1.0 mM EDTA, 20 mM HEPES, 5 mM β-mercaptoethanol, pH 7.0 were added to the mixture, followed by 4.0 μL of 0.5 M $MnCl_2$ (0.5 mM in reaction) and 8 μL (~8 U) of alkaline phosphatase (Roche Biosciences). The mixture was tumbled gently overnight and then centrifuged for 20 min at 14 000 rpm. The supernatant was applied to a C18 Sep-Pak cartridge (C18; 5 g), washed with water and eluted with a water–methanol gradient. The residue thus obtained was applied to a Biogel P-4 column in 5% aqueous methanol (50 cm × 2 cm) to yield **20** (13 mg; 13 μmol; 83%): $[\alpha]_D$ +14.8 (*c* 0.73, H_2O). ^{1}H NMR (600 MHz, D_2O): δ_H 5.85 (ddd, 1H, $J_{4,5t}$ = 17.3 Hz, $J_{4,5c}$ = 10.5 Hz, $J_{4,3}$ = 7.0 Hz, H-4$_{sphing}$), 5.32 (m, 1H, H-5t_{sphing}), 5.26 (m, 1H, H-5c_{sphing}), 4.73 (d, overlapped with HOD, 1H, H-1$_{GalNAc}$), 4.51 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{Gal}$), 4.47 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1$_{Glc}$), 4.22 (dd, 1H, $J_{3,4}$ = 7.0 Hz, $J_{3,2}$ = 7.0 Hz, H-3$_{sphing}$), 4.14 (dd, 1H, $J_{3,2}$ = 9.8 Hz, $J_{3,4}$ = 3.0 Hz, H-3$_{Gal}$), 4.11–4.05 (m, 3H, H-4$_{Gal}$, H-2$_{sphing}$, H-1a$_{sphing}$), 3.99 (dd, 1H, $J_{6a,6b}$ = 12.2 Hz, $J_{6a,5}$ = 1.9 Hz, H-6a$_{Glc}$), 3.92–3.89 (m, 2H, H-4$_{GalNAc}$, H-2$_{GalNAc}$), 3.87–3.58 (m, 18H, H-9a$_{Neu}$, H-5$_{Neu}$, H-6b$_{Glc}$, H-1b$_{sphing}$, H-4$_{Neu}$, H-5$_{Gal}$, H-6a$_{Gal}$, H-6b$_{Gal}$, H-5$_{GalNAc}$, H-6a$_{GalNAc}$, H-6b$_{GalNAc}$, H-8$_{Neu}$, H-3$_{GalNAc}$, H-3$_{Glc}$, H-9b$_{Neu}$, H-4$_{Glc}$, H-7$_{Neu}$, H-5$_{Glc}$), 3.48 (dd, 1H, $J_{6,5}$ = 10.1 Hz, $J_{6,7}$ = 1.8 Hz, H-6$_{Neu}$), 3.37–3.31 (m, 2H, H-2$_{Gal}$, H-2$_{Glc}$), 2.65 (dd, 1H, $J_{3e,3a}$ = 12.6 Hz, $J_{3e,4}$ = 4.5 Hz, H-3e$_{Neu}$), 2.22 (t, 2H, *J* = 7.3 Hz, CH_2C(O)), 2.02, 2.01 (2 × s, 2 × 3H, 2 × C(O)CH_3), 1.91 (dd, 1H, $J_{3a,3e}$ = 12.2 Hz, $J_{3a,4}$ = 12.2 Hz, H-3a$_{Neu}$), 1.61–1.55 (m, 4H, CH_2CH$_2$CH$_2$C(O), CH_2CH$_2$C(O)), 0.89 (t, 3H, *J* = 7.3 Hz, CH$_3$). ^{13}C NMR (125 MHz, D_2O): δ_C 178.0 (2C), 175.9, 174.9, 137.1, 119.0, 103.6, 103.5, 103.2, 102.5, 79.5, 78.0, 75.60, 75.59, 75.2, 75.1, 74.9, 74.0, 73.6, 73.1, 72.7, 72.2, 70.9, 69.6, 69.5, 68.9, 68.7, 63.7, 62.0, 61.4, 61.0, 53.9, 53.2, 52.5, 38.7, 37.9, 23.5, 22.9, 19.9, 13.7. ESI HRMS calcd for $C_{40}H_{66}N_3O_{26}$: 1004.39404 Found: 1004.39400.

Procedure (B) for deprotection and acylation of sphingosine amine using anhydride

Oligosaccharide **7** or **8** (17–40 μmol) was dissolved in dry methanol (2.0 mL). Sodium methoxide (~2 eq.) was added and the mixture was stirred for 3 days at room temperature until completion of the reaction. The pH of the reaction mixture was adjusted to 9.0 using 0.5 M HCl and the methanol was evaporated. The compound was purified by HPLC (Hamilton semi-preparative PRP-1 column) using a MeOH–H_2O gradient. A portion of the amine (15–18 μmol), 6-trifluoroacetamidohexanoic anhydride (~5.0 eq.), and triethylamine (2–4 eq.) were dissolved in dry degassed methanol (3.0 mL). The solution was stirred overnight at room temperature. The mixture was concentrated, redissolved in water, and applied to a C18 Sep-Pak cartridge. Gradient elution (MeOH–H_2O) afforded the target compound, which was further purified by HPLC (Hamilton semi-preparative PRP-1 column) using a MeOH–H_2O gradient.

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-*O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-(6-trifluoroacetamidohexanamido)-pent-4-ene-1,3-diol (21).** Compound **21** was prepared according to procedure (B). Trisaccharide **7** (33.5 mg;

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40.4 μmol) was deprotected and a portion of the amine (14.0 mg; 18.6 μmol) was acylated with 6-trifluoroacetamidohexanoic anhydride (40.5 mg; 92.8 μmol; 5.0 eq.). Work-up and purification as described in procedure (B) afforded **21** (13.9 mg; 14.4 μmol; 78%): $[\alpha]_D$ +16.8 (*c* 0.23, H_2O). ^1H NMR (500 MHz, D_2O): δ_H 5.82 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.5 Hz, $J_{4,3}$ = 7.0 Hz, H-4_{sphing}), 5.30 (ddd, 1H, $J_{5t,4}$ = 17.2 Hz, $J_{5t,5c}$ = 1.1 Hz, $J_{5t,3}$ = 1.1 Hz, H-$5t_{sphing}$), 5.24 (ddd, 1H, $J_{5c,4}$ = 10.5 Hz, $J_{5c,5t}$ = 1.1 Hz, $J_{5c,3}$ = 1.1 Hz, H-$5c_{sphing}$), 4.50 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1_{Gal}), 4.45 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1_{Glc}), 4.20 (dd, 1H, $J_{3,4}$ = 7.0 Hz, $J_{3,2}$ = 7.0 Hz, H-3_{sphing}), 4.10–4.03 (m, 3H, H-2_{sphing}, H-3_{Gal}, H-$1a_{sphing}$), 3.98 (dd, 1H, $J_{6a,6b}$ = 12.2 Hz, $J_{6a,5}$ = 2.2 Hz, H-$6a_{Glc}$), 3.94 (d, 1H, $J_{4,3}$ = 3.0 Hz, H-4_{Gal}), 3.89–3.61 (m, 13H, H-8_{Neu}, H-5_{Neu}, H-$9a_{Neu}$, H-$6b_{Glc}$, H-$1b_{sphing}$, H-4_{Neu}, H-$6a_{Gal}$, H-$6b_{Gal}$, H-5_{Gal}, H-3_{Glc}, H-4_{Glc}, H-$9b_{Neu}$, H-6_{Neu}), 3.59–3.54 (m, 3H, H-5_{Glc}, H-7_{Neu}, H-2_{Gal}), 3.32–3.29 (m, 3H, H-2_{Glc}, CH_2NHTFA), 2.75 (dd, 1H, $J_{3e,3a}$ = 12.3 Hz, $J_{3e,4}$ = 4.6 Hz, H-$3e_{Neu}$), 2.25 (t, 2H, *J* = 7.4 Hz, CH_2C(O)), 2.02 (s, 3H, C(O)CH_3), 1.79 (dd, 1H, $J_{3a,3e}$ = 12.3 Hz, $J_{3a,4}$ = 12.3 Hz, H-$3a_{Neu}$), 1.62–1.55 (m, 4H, CH_2CH$_2$NHTFA, CH_2CH$_2$C(O)), 1.35–1.30 (m, 2H, CH_2). ^{13}C NMR (125 MHz, D_2O): δ_C 177.8, 175.9, 174.7, 159.7 (q, $J_{C,F}$ = 37 Hz), 137.2, 119.0, 116.8 (q, $J_{C,F}$ = 285 Hz), 103.6, 103.3, 100.7, 79.3, 76.4, 76.1, 75.7, 75.1, 73.8, 73.7, 72.8, 72.7, 70.3, 69.5, 69.2, 69.0, 68.4, 63.5, 61.9, 61.0, 60.5, 53.8, 52.6, 40.5, 36.6, 28.3, 26.3, 25.8, 22.9. ESI HRMS calcd for $C_{36}H_{57}N_3O_{22}F_3$: 940.33913. Found: 940.33845.

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-[2-acetamido-2-deoxy-*O*-β-D-galactopyranosyl-(1 → 4)]-*O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-(6-trifluoroacetamidohexanamido)-pent-4-ene-1,3-diol (22).** Compound **22** was prepared according to procedure (B). Tetrasaccharide **8** (18.8 mg; 17.8 μmol) was deprotected and a portion of the amine (14.7 mg, 15.3 μmol) was acylated with 6-trifluoroacetamidohexanoic anhydride (30.4 mg; 69.7 μmol; 5.0 eq.). Work-up and purification as described in procedure (B) afforded **22** (13.9 mg; 11.9 μmol; 78%): $[\alpha]_D$ +26.4 (*c* 0.14, H_2O). ^1H NMR (500 MHz, D_2O): δ_H 5.82 (ddd, 1H, $J_{4,5t}$ = 17.2 Hz, $J_{4,5c}$ = 10.4 Hz, $J_{4,3}$ = 7.0 Hz, H-4_{sphing}), 5.28 (ddd, 1H, $J_{5t,4}$ = 17.2 Hz, $J_{5t,5c}$ = 1.0 Hz, $J_{5t,3}$ = 1.0 Hz, H-$5t_{sphing}$), 5.27 (ddd, 1H, $J_{5c,4}$ = 10.4 Hz, $J_{5c,5t}$ = 1.0 Hz, $J_{5c,3}$ = 1.0 Hz, H-$5c_{sphing}$), 4.72 (d, overlapped with HOD, 1H, H-1_{GalNAc}), 4.50 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1_{Gal}), 4.45 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1_{Glc}), 4.20 (dd, 1H, $J_{3,4}$ = 7.0 Hz, $J_{3,2}$ = 7.0 Hz, H-3_{sphing}), 4.13 (dd, 1H, $J_{3,2}$ = 9.8 Hz, $J_{3,4}$ = 3.1 Hz, H-3_{Gal}), 4.10–4.04 (m, 3H, H-4_{Gal}, H-2_{sphing}, H-$1a_{sphing}$), 3.97 (dd, 1H, $J_{6a,6b}$ = 12.1 Hz, $J_{6a,5}$ = 1.6 Hz, H-$6a_{Glc}$), 3.92–3.88 (m, 2H, H-4_{GalNAc}, H-2_{GalNAc}), 3.85 (dd, 1H, $J_{9a,9b}$ = 12.0 Hz, $J_{9a,8}$ = 2.2 Hz, H-$9a_{Neu}$), 3.82–3.57 (m, 17H, H-5_{Neu}, H-$6b_{Glc}$, H-$1b_{sphing}$, H-4_{Neu}, H-5_{Gal}, H-$6a_{Gal}$, H-$6b_{Gal}$, H-5_{GalNAc}, H-$6a_{GalNAc}$, H-$6b_{GalNAc}$, H-8_{Neu}, H-3_{GalNAc}, H-3_{Glc}, H-$9b_{Neu}$, H-4_{Glc}, H-7_{Neu}, H-5_{Glc}), 3.47 (dd, 1H, $J_{6,5}$ = 10.1 Hz, $J_{6,7}$ = 2.1 Hz, H-6_{Neu}), 3.36–3.29 (m, 4H, H-2_{Gal}, H-2_{Glc}, CH_2NHTFA), 2.65 (dd, 1H, $J_{3e,3a}$ = 12.1 Hz, $J_{3e,4}$ = 4.3 Hz, H-$3e_{Neu}$), 2.25 (t, 2H, *J* = 7.3 Hz, CH_2C(O)), 2.02, 2.01 (2 × s, 2 × 3H, 2 × C(O)CH_3), 1.91 (dd, 1H, $J_{3a,3e}$ = 12.1 Hz, $J_{3a,4}$ = 12.1 Hz, H-$3a_{Neu}$), 1.63–1.55 (m, 4H, CH_2CH$_2$NHTFA, CH_2CH$_2$C(O)), 1.35–1.29 (m, 2H, CH_2). ^{13}C NMR (125 MHz, D_2O): δ_C 177.8, 175.9, 175.7, 174.9, 159.7 (q, $J_{C,F}$ = 37 Hz), 137.2, 118.7, 116.8 (q, $J_{C,F}$ = 286 Hz), 103.6, 103.5, 103.3, 102.5, 79.6, 78.0, 75.6 (2C), 75.2, 75.1, 74.9, 74.0, 73.6, 73.1, 72.7, 72.2, 70.9, 69.6, 69.5, 69.0, 68.7, 63.7, 62.0, 61.4, 61.0, 53.8, 53.2, 52.5, 40.5, 37.9, 36.5, 28.3, 26.3, 25.8, 23.5, 22.9. ESI HRMS calcd for $C_{44}H_{70}N_4O_{27}F_3Na_2$: 1189.39749. Found: 1189.39720.

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-*O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-[6-(5-*p*-nitrophenyloxycarbonylpentanamido)hexanamido]-pent-4-ene-1,3-diol (23).** The amine **17** (4.5 mg; 5.2 μmol) was dissolved in dimethylformamide (1.5 mL), and triethylamine (2.2 μL; 15.6 μmol; 3.0 eq.) was added. *p*-Nitrophenyl adipate diester[26] (21.0 mg; 5.2 μmol; 10.0 eq.) was added. After 15 min, the reaction was complete as observed by TLC (CH_2Cl_2–MeOH–H_2O–AcOH; 4 : 5 : 1 : 0.5). The pH was adjusted to 5.0 with acetic acid and the reaction mixture was co-evaporated with toluene (3×). The residue was suspended in 1% acetic acid solution and filtered through cotton. The filtrate was concentrated and the residue obtained was purified by HPLC (Beckman C_{18}-silica semi-preparative column) using a gradient of MeOH–H_2O containing 1% acetic acid. The ^1H NMR spectrum was acquired in CD_3OD. Although visible, resonances were significantly broadened. ESI HRMS calcd for $C_{46}H_{69}N_4O_{26}$: 1093.41946. Found: 1093.41886.

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-[2-acetamido-2-deoxy-*O*-β-D-galactopyranosyl-(1 → 4)]-*O*-(β-D-galactopyranosyl)-(1 → 4)-*O*-(β-D-glucopyranosyl)-(1 → 1)-(2*S*,3*R*)-2-[6-(5-*p*-nitrophenyloxycarbonylpentanamido)hexanamido]-pent-4-ene-1,3-diol (24).** The amine **19** (3.4 mg; 3.2 μmol) was dissolved in dimethylformamide (1.0 mL) and triethylamine (2.0 μL; 14.2 μmol; 4.4 eq.) was added. *p*-Nitrophenyl adipate diester[26] (12.5 mg; 32.2 μmol; 10 eq.) was added. After 15 min, the reaction was complete as observed by TLC (CH_2Cl_2–MeOH–H_2O–AcOH; 4 : 5 : 1 : 0.5). The pH was adjusted to 5.0 with acetic acid and the reaction mixture was co-evaporated with toluene (3×). The residue was suspended in 1% acetic acid solution and filtered through cotton. The filtrate was concentrated and the residue obtained was purified by HPLC (Beckman C_{18}-silica semi-preparative column) using a gradient of MeOH–H_2O containing 1% acetic acid. The ^1H NMR spectrum was recorded in CD_3OD. Although visible, the resonances were significantly broadened. ESI HRMS calcd for $C_{54}H_{82}N_5O_{31}Na_2$: 1342.47892. Found: 1342.47920.

Preparation of tetanus toxoid conjugates 25 and 27

Purified monomeric tetanus toxoid (~6.6 mg mL^{-1}) in PBS, pH 7.2 (840 μL for **23**, 1.07 mL for **24**) was added to the activated sugars **23** or **24** (~30 eq.). The mixture was gently tumbled overnight before being dialyzed against PBS (5 × 2 L) at 4 °C. The incorporation level was determined by MALDI mass spectrometry. Results are presented in Scheme 4.

Preparation of bovine serum albumin conjugate 26

The activated sugar **23** (~30 eq.) was dissolved in water and bovine serum albumin (4.3 mg) was added. The mixture was gently tumbled overnight before being dialyzed against water (5 × 2 L) at 4 °C. The incorporation level was determined by MALDI mass spectrometry. Results are presented in Scheme 4.

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References

1 (*a*) S. I. Hakomori and Y. Igarashi, *Adv. Lipid Res.*, 1993, **25**, 147; (*b*) S. I. Hakomori, *Biochem. Soc. Trans.*, 1993, **21**, 583; (*c*) A. Varki, *Glycobiology*, 1993, **3**, 97; (*d*) C. Traving and R. Schauer, *Cell. Mol. Life Sci.*, 1998, **54**, 1330; (*e*) E. V. Dyatlovitskaya and A. G. Kandyba, *Russ. J. Bioorg. Chem.*, 2004, **30**, 201; (*f*) S. Hakomori and Y. M. Zhang, *Chem. Biol.*, 1997, **4**, 97.

2 (*a*) O. Blixt, S. Head, T. Mondala, C. Scanlan, M. E. Huflejt, R. Alvarez, M. C. Bryan, F. Fazio, D. Calarese, J. Stevens, N. Razi, D. J. Stevens, J. J. Skehel, I. van Die, D. R. Burton, I. A. Wilson, R. Cummings, N. Bovin, C. H. Wong and J. C. Paulson, *Proc. Natl. Acad. Sci. U.S.A.*, 2004, **101**, 17033; (*b*) Y. Fang, A. G. Frutos and J. Lahiri, *Langmuir*, 2003, **19**, 1500.

3 (*a*) S. A. Kalovidouris, O. Blixt, A. Nelson, S. Vidal, W. B. Turnbull, J. C. Paulson and J. F. Stoddart, *J. Org. Chem.*, 2003, **68**, 8485; (*b*) B. S. Bochner, R. A. Alvarez, P. Mehta, N. V. Bovin, O. Blixt, J. R. White and R. L. Schnaar, *J. Biol. Chem.*, 2005, **280**, 4307.

4 (*a*) S. J. Danishefsky and J. R. Allen, *Angew. Chem., Int. Ed.*, 2000, **39**, 836; (*b*) P. Fredman, K. Hedberg and T. Brezicka, *BioDrugs*, 2003, **17**, 155; (*c*) R. J. Bitton, M. D. Guthmann, M. R. Gabri, A. J. L. Carnero, D. F. Alonso, L. Fainboim and D. E. Gomez, *Oncol. Rep.*, 2002, **9**, 267; (*d*) S. M. Andersen, C.-C. Ling, P. Zhang, K. Townson, H. J. Willison and D. R. Bundle, *Org. Biomol. Chem.*, 2004, **2**, 1199; (*e*) H. J. Willison, K. Townson, J. Veitch, J. Boffey, N. Isaacs, S. M. Andersen, P. Zhang, C.-C. Ling and D. R. Bundle, *Brain*, 2004, **127**, 680.

5 G. J. Boons and A. V. Demchenko, *Chem. Rev.*, 2000, **100**, 4539.

6 O. Blixt, D. Vasiliu, K. Allin, N. Jacobsen, D. Warnock, N. Razi, J. C. Paulson, S. Bernatchez, M. Gilbert and W. Wakarchuk, *Carbohydr. Res.*, 2005, **340**, 1963.

7 A. S. Timen and P. Somfai, *J. Org. Chem.*, 2003, **68**, 9958.

8 (*a*) T. Kawano, J. Q. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki and M. Taniguchi, *Science*, 1997, **278**, 1626; (*b*) T. I. Prigozy, O. Naidenko, P. Qasba, D. Elewaut, L. Brossay, A. Khurana, T. Natori, Y. Koezuka, A. Kulkarni and M. Kronenberg, *Science*, 2001, **291**, 664; (*c*) D. B. Moody, B. B. Reinhold, M. R. Guy, E. M. Beckman, D. E. Frederique, S. T. Furlong, S. Ye, V. N. Reinhold, P. A. Sieling, R. L. Modlin, G. S. Besra and S. A. Porcelli, *Science*, 1997, **278**, 283; (*d*) K. Miyamoto, S. Miyake and T. Yamamura, *Nature*, 2001, **413**, 531; (*e*) C. A. Lingwood, *Glycoconjugate J.*, 1996, **13**, 495.

9 D. R. Bundle, C.-C. Ling and P. Zhang, *Chem. Abstr.*, 2003, **140**, 16928.

10 S. I. Hakomori, *Cancer Res.*, 1985, **45**, 2405.

11 G. Ada and D. Isaacs, *Clin. Microbiol. Infect.*, 2003, **9**, 79.

12 (*a*) P. Fredman, K. Hedberg and T. Brezicka, *BioDrugs*, 2003, **17**, 155; (*b*) P. O. Livingston, S. L. Zhang and K. O. Lloyd, *Cancer Immunol. Immunother.*, 1997, **45**, 1; (*c*) P. O. Livingston and G. Ragupathi, *Cancer Immunol. Immunother.*, 1997, **45**, 10.

13 (*a*) P. O. Livingston, *Semin. Cancer Biol.*, 1995, **6**, 357; (*b*) I. C. le Poole, M. A. T. Gerberi and W. M. Kast, *Curr. Opin. Oncol.*, 2002, **14**, 641; (*c*) G. Ragupathi, F. Koide, N. Sathyan, E. Kagan, M. Spassova, W. Bornmann, P. Gregor, C. Reis, H. Clausen, S. J. Danishefsky and P. Livingston, *Cancer Immunol. Immunother.*, 2003, **52**, 608.

14 J. Ohlsson and G. Magnusson, *Tetrahedron*, 2000, **56**, 9975.

15 J. L. Sonnenburg, H. van Halbeek and A. Varki, *J. Biol. Chem.*, 2002, **277**, 17502.

16 M. Gilbert, R. Bayer, A. M. Cunningham, S. Defrees, Y. H. Gao, D. C. Watson, N. M. Young and W. W. Wakarchuk, *Nat. Biotechnol.*, 1998, **16**, 769.

17 M. Gilbert, J. R. Brisson, M. F. Karwaski, J. Michniewicz, A. M. Cunningham, Y. Y. Wu, N. M. Young and W. W. Wakarchuk, *J. Biol. Chem.*, 2000, **275**, 3896.

18 Y. Lapidot, S. Rappoport and Y. Wolman, *J. Lipid Res.*, 1967, **8**, 142.

19 (*a*) M. C. Bryan, O. Plettenburg, P. Sears, D. Rabuka, S. Wacowich-Sgarbi and C.-H. Wong, *Chem. Biol.*, 2002, **9**, 713; (*b*) F. Fazio, M. C. Bryan, O. Blixt, J. C. Paulson and C. H. Wong, *J. Am. Chem. Soc.*, 2002, **124**, 14397; (*c*) F. Fazio, M. C. Bryan, H.-K. Lee, A. Chang and C.-H. Wong, *Tetrahedron Lett.*, 2004, **45**, 2689.

20 (*a*) A. N. Rai and A. Basu, *Org. Lett.*, 2004, **6**, 2861; (*b*) S. Torssell and P. Somfai, *Org. Biomol. Chem.*, 2004, **2**, 1643; (*c*) C.-C. Ling, Y. Cai and D. R. Bundle, unpublished work.

21 R. T. Lee and Y. C. Lee, *Carbohydr. Res.*, 1978, **37**, 193.

22 (*a*) J. R. Rich and D. R. Bundle, *Org. Lett.*, 2004, **6**, 897; (*b*) J. R. Rich, W. W. Wakarchuk and D. R. Bundle, *Chem.–Eur. J.*, DOI: 10.1002/chem.200500518.

23 (*a*) J. C. Wilson, M. J. Kiefel, D. I. Angus and M. von Itzstein, *Org. Lett.*, 1999, **1**, 443; (*b*) L. M. Mikkelsen, M. J. Hernaiz, M. Martin-Pastor, T. Skrydstrup and J. Jimenez-Barbero, *J. Am. Chem. Soc.*, 2002, **124**, 14940; (*c*) M. Qian, S. Spinelli, H. Driguez and F. Payan, *Protein Sci.*, 1997, **6**, 2285.

24 (*a*) M. Nitz, PhD Thesis, University of Alberta, 2001; (*b*) X. Wu and D. R. Bundle, *J. Org. Chem.*, 2005, **70**, 7381; (*c*) D. R. Bundle, J. R. Rich, S. Jacques, H. N. Yu, M. Nitz and C.-C. Ling, *Angew. Chem., Int. Ed.*, 2005, **44**, 7725.

25 G. T. Hermanson, *Bioconjugate Techniques*, Academic Press, San Diego, 1996.

26 X. Y. Wu, C.-C. Ling and D. R. Bundle, *Org. Lett.*, 2004, **6**, 4407.

27 M. F. Karwaski, W. W. Wakarchuk and M. Gilbert, *Protein Expression Purif.*, 2002, **25**, 237.

28 S. Bernatchez, C. M. Szymanski, N. Ishiyama, J. J. Li, H. C. Jarrell, P. C. Lau, A. M. Berghuis, N. M. Young and W. W. Wakarchuk, *J. Biol. Chem.*, 2005, **280**, 4792.

29 M. M. Palcic, L. D. Heerze, M. Pierce and O. Hindsgaul, *Glycoconjugate J.*, 1988, **5**, 49.

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