Organic &
Biomolecular
Chemistry

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 5809

www.rsc.org/obc

PAPER

Chemoenzymatic synthesis of biotin-appended analogues of gangliosides GM2, GM1, GD1a and GalNAc-GD1a for solid-phase applications and improved ELISA tests

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Received 4th January 2011, Accepted 18th May 2011
DOI: 10.1039/c1ob00009h

Biotinylated analogues of gangliosides GM2, GM1, GD1a and GalNAc-GD1a were synthesized in high yields using glycosyltransferases from *Campylobacter jejuni*. The presence of a biotin moiety in the aglycone part of these mimics allows for attachment of these materials onto various streptavidin-coated surfaces. Analysis of the interaction of biotin-appended GM1 with the B subunit of *Escherichia coli* heat-labile enterotoxin performed in a modified ELISA procedure shows the potential of this compound to replace the natural GM1 in toxin detection.

Introduction

In recent years, the immobilization of oligosaccharides on solid supports and particles, along with their conjugation to proteins, has received increasing attention as a valuable tool to gain insight into the function of carbohydrates and improvement of their functionality.[1] Consequently, a need has risen for oligosaccharides that are suitably equipped with functional groups designed for such studies and applications.[2] Among the various immobilization techniques,[1a,3] those based on the (strept-)avidin-biotin couple[4] are especially useful for two reasons: 1) the high and specific affinity of (strept-)avidin to biotin,[5] and 2) the possibility to avoid complicated chemical treatments that would limit the scope of applicable sugars and (or) alter their primary structure during the immobilization. Accordingly, biotin-appended carbohydrates are of interest, and several procedures for the incorporation of a biotin moiety in the aglycone part of mono- and oligosaccharides have been reported,[6] along with subsequent immobilization on a solid surface or preparation of glycoconjugates[4,6a,7] and glyconanoparticles.[8]

However, not the method of biotinylation, but rather the synthetic availability of complex carbohydrates is often the limiting factor in such (and other) studies. This is particularly relevant in the case of gangliosides, a subclass of glycosphingolipids that all contain one or more sialic acid residues but vary in other parts, such as the number of ring structures and overall composition. Gangliosides are present in all mammalian cells, and are particularly abundant in neuronal tissues. They are key signaling molecules of major cellular processes, including cellular recognition, adhesion, receptor signal transduction, growth regulation, differentiation and apoptosis.[9] Apart from that, gangliosides act as receptors for bacterial toxins including *Vibrio cholerae* toxin and *Escherichia coli* heat-labile enterotoxin that bind with high affinity to GM1 ganglioside.[10] In addition, gangliosides are primary targets for auto-antibodies that cause immune-mediated forms of polyneuropathy including Guillain-Barré Syndrome,[11] in which antibodies against GM1 and several other related gangliosides are relevant.[11–12] The detection of toxins[13] and antibodies[14] is based on the recognition thereof in ELISA assays by bovine brain-derived gangliosides. These natural extracts have several drawbacks, as they are limited in scope, difficult to purify, expensive, potentially infected and usually contaminated with other glycolipids. Apart from that, active surfaces obtained *via* a direct adsorption of gangliosides are not always optimal. Thus, a benefit was reported of using an ordered GM1 monolayer on an air–water interface rather than the conventional GM1-adsorbed solid support for the selection of GM1-binding peptides.[15] Given the availability and wide use of streptavidin-coated ELISA plates, the synthesis of structurally analogous mimics of gangliosides containing a biotin-appended aglycone would thus bring advantages to the field of detection and diagnostics, as well as to other (bio)medical applications that involve biotin-streptavidin interactions.

The difficulties associated with the introduction of the sialic acid to an oligosaccharide core structure[16] make the task of an efficient preparation of gangliosides and their analogues particularly challenging,[17] despite several successful chemical approaches to their synthesis.[18] Chemoenzymatic strategies, however, are able to considerably facilitate the synthesis of sialylated compounds. Thus, a very efficient method[19] for the synthesis of gangliosides GD3, GT3, GM2, GD2, GT2, GM1 and GD1a was developed based on glycoengineering by selected recombinant

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glycosyltransferases from *Campylobacter jejuni*.[20] With this strategy, ganglioside analogues were prepared in high yields that contain a 2-azidoethyl aglycone,[19] a truncated functionalized ceramide unit,[21] S-linked ganglioside analogues,[22] or a long hydrophobic chain that also contains ω-functionalization for applications in biosensing.[23]

In the present study, we aim to combine the strength and specificity of the biotin-(strept)avidin interaction with the flexibility and efficiency of the chemoenzymatic synthesis, in the synthesis of biotin-appended analogues of gangliosides GM2, GM1, GD1a and GalNAc-GD1a *via* glycosyltransferases from *C. jejuni*. Authentic GalNAc-GD1a (with a ceramide tail) was very recently synthetized to reveal the power of a novel glycosylation strategy;[24] our synthesis targets the first analogue containing the oligosaccharide structure of GalNAc-GD1a, and involves the first chemoenzymatic route towards this oligosaccharide. Subsequently, as a first result of the exploration of the usefulness of synthetic gangliosides, we demonstrate here the advantage of the biotinylated GM1 mimic over the natural bovine brain-derived GM1 for the detection of *Escherichia coli* heat-labile enterotoxin in an ELISA-based procedure.

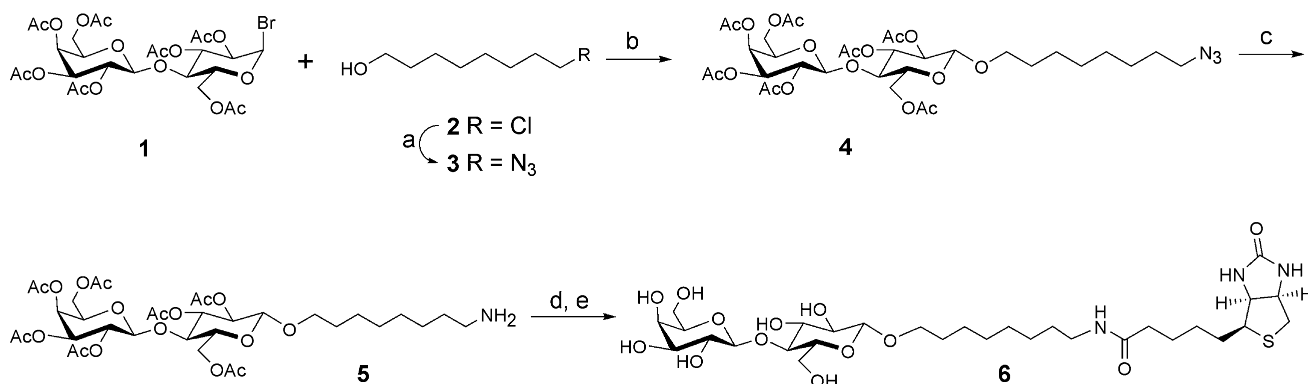
Results and discussion

Synthesis of biotin-appended ganglioside analogues

In order to accomplish enzymatic syntheses of ganglioside analogues with authentic oligosaccharide sequences, a lactose moiety is needed that is modified at the reducing end according to the envisioned application. For the use as a starting compound in the enzymatic modifications in this study, a biotin-appended lactoside **6** was synthesized (Scheme 1). Peracetylated lactosyl bromide **1** was glycosidated with 8-azidooctanol **3**, prepared by a substitution reaction from 8-chlorooctanol **2**, to give 8-azidooctyl heptaacetyl lactoside **4** in a moderate yield of 50%. Although ω-azidoalkyl lactosides were shown by others[19] and us[23b] to be excellent substrates for glycosyltransferases, it is logical to further modify the aglycone prior to enzymatic reactions with a view to both exploit the substrate acceptability of the glycosyltransferases and to avoid additional manipulations with the final (and expensive) ganglioside products. The azido group in **4** was reduced by hydrogenation, to give the amino-terminated lactoside **5** in a nearly quantitative yield. Coupling of **5** to biotin was accomplished with the use of EDC activation under inert atmosphere. The subsequent removal of acetyl groups by the classical Zemplén procedure[25] required 4 days and afforded the biotin-appended lactoside **6** in an excellent yield of 95% over two steps. The use of the unprotected amino group in the aglycone was shown not to be practical in the enzymatic reactions,[21] therefore we did not consider intermediate **5** as a suitable (after deacetylation) starting compound.

The conversion of lactoside **6** into biotinylated ganglioside analogues **8–11** (Scheme 2) was achieved in a series of enzymatic reactions that combine the use of recombinant glycosyltransferases from *Campilobacter jejuni* in well-described procedures[19,21] with our recently published modification[23b] at the early steps (syntheses of GM3 and GM2). Thus, sialylation of **6** was effected by CMP-NeuAc and catalyzed by the Cst-I α-(2–3)-sialyltransferase (construct CST-06) in a methanol/water (25/75 v/v) medium at 37 °C for 1 h. The resulting GM3 analogue **7** was not isolated, and after complete conversion of **6**, UDP-GlcNAc, UDP-GlcNAc 4′-epimerase (CPG-13) and the CgtA β-(1,4)-*N*-acetylgalactosaminyltransferase (construct CJL-30) from *C. jejuni* were added directly to the reaction mixture. The use of UDP-GlcNAc 4′-epimerase in one-pot mixtures with transferases effectively addresses a major drawback of glycosylation reactions, namely the limited availability and high cost of the necessary glycosyl donors. This allowed for the reaction of **7** with the *in situ* formed UDP-GalNAc, and after isolation on a Sep-Pak column, the GM2 analogue **8** was obtained in a yield of 90% (from the lactose-derivative **6**). Further elongation of GM2 analogue **8** to GM1 analogue **9** was performed in high yield (99%) using UDP-Gal, and the CgtB β-(1,3)-galactosyltransferase (construct CJL-20).

In the family of gangliosides that act as target epitopes for the immunological response in Guillain-Barré Syndrome, apart from GM1, an important role is also played by gangliosides GD1a[12c] and GalNAc-GD1a.[26] Given the possible usefulness of biotinylated analogues of these gangliosides in immunological studies, GD1a mimic **10** was synthesized by sialylation of **9** using the Cst-I α-(2–3)-sialyltransferase (construct CST-06). Compound **10** was obtained in 87% yield after isolation, and further elongated to GalNAc-GD1a analogue **11** using the CgtA β-(1,4)-*N*-acetylgalactosaminyltransferase (construct CJL-30). Compound **11** was obtained in 75% yield after isolation. To the best of our knowledge, this is the first report of a chemo-enzymatic synthesis



Scheme 1 Synthesis of biotin-appended lactose. *Reagents and conditions*: (a) NaN_3, DMSO, 102 °C, overnight, quant.; (b) AgOTf, toluene, –78 °C → 10 °C, 50%; (c) Pd/C (10%), MeOH, rt, quant.; (d) biotin (1 eq), EDC (1 eq), DMAP (1 eq), DMF, rt, N_2; (e) NaOMe, MeOH, rt, 95% over 2 steps.

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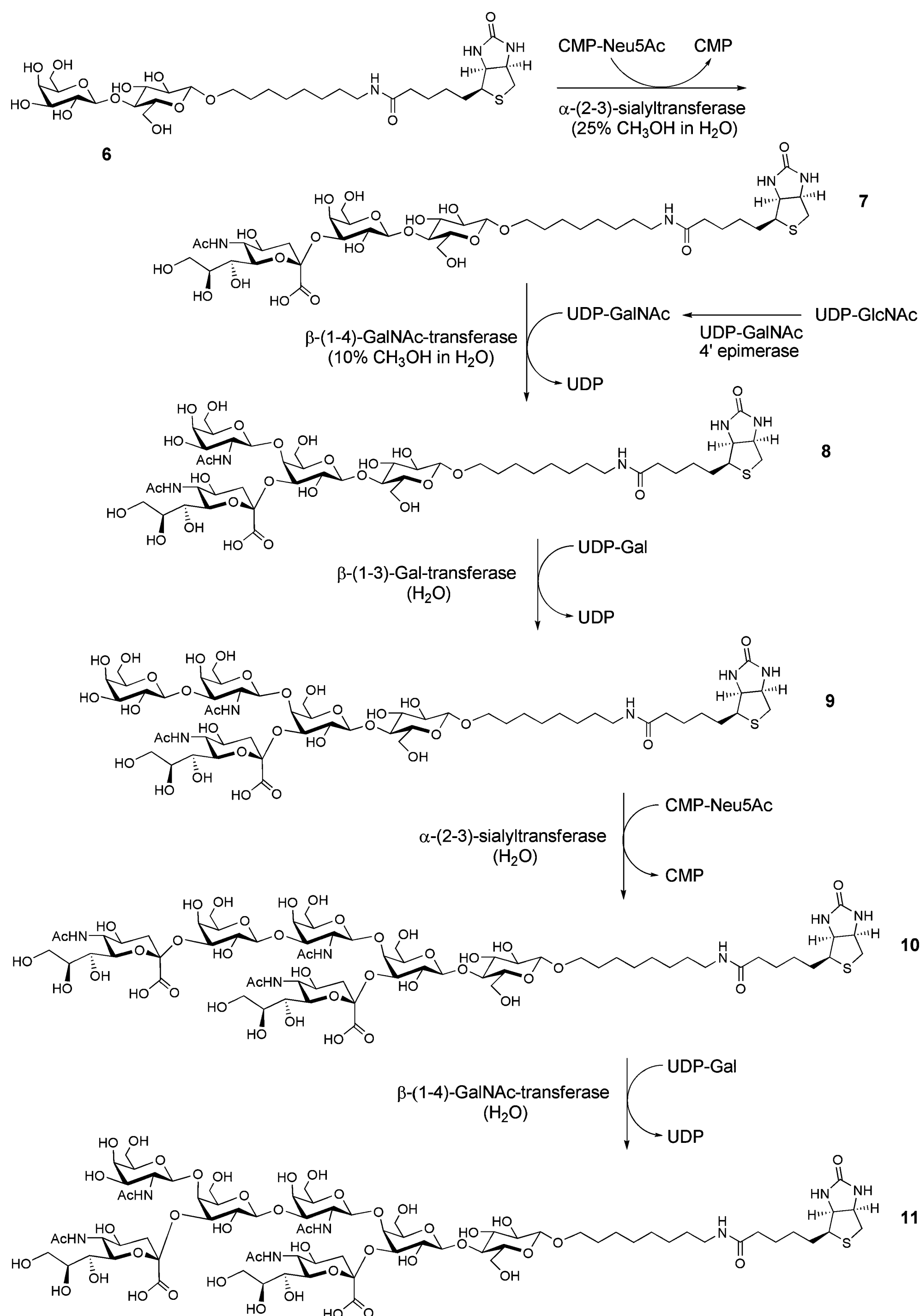

Scheme 2 Enzymatic syntheses of biotinylated ganglioside analogues. *Yields*: 90% for GM2 mimic **8** (for two steps), 99% for GM1 mimic **9**, 87% for GD1a mimic **10**, 75% for GalNAc-GD1a mimic **11**.

of an oligosaccharide that contains the GalNAc-GD1a sugar moiety.

Analysis of interaction of biotinylated GM1 with LT- B in a modified ELISA

It is of interest to investigate whether the conjugation of a biotin moiety to a ganglioside structure retains the recognition properties of both carbohydrate and biotin portions of the new molecule. In addition, it is worthwhile to test biotinylated mimics for their bioequivalence to the natural gangliosides and to evaluate the possibility of their use in bioapplications such as *e.g.* detection and diagnostics. Therefore a comparison was made of LT-B detection by GM1 analogue **9** on streptavidin-coated ELISA plates (indirect coating) to routinely used bovine brain-derived GM1 on non-streptavidin-coated plates. Binding assays were performed on

Nunc Maxisorp™ plates that – prior to the experiments with biotinylated GM1 **9** – were coated with streptavidin, and all incubations were done overnight at 4 °C. After incubation of streptavidin-coated and non-streptavidin-coated plates with GM1 mimic **9** and natural GM1 respectively, serial twofold dilutions of pure recombinant LT-B in coating buffer were added to the wells and binding was allowed for 16 h at 4 °C. For each LT-B concentration, experiments were done in triplicate. The results are shown in Fig. 1 and are given as mean specific optical densities at 415 nm.

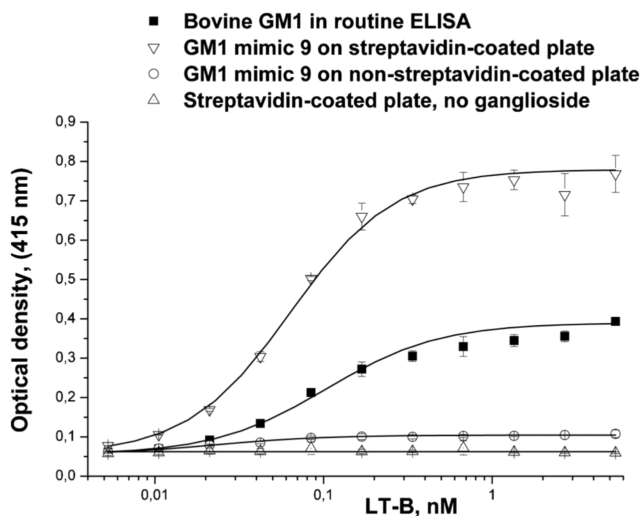

Fig. 1 Binding of B subunit of *E.coli* heat-labile enterotoxin (LT-B) to natural and synthetic gangliosides coated on ELISA plates, as visualized using a monoclonal antibody specific for LT-B-pentamer.

Fig. 1 clearly demonstrates that at all the applied toxin concentrations, assays based on biotinylated mimic **9** are consistently higher by at least a factor of two than conventional assays, indicating a higher affinity of the LT-B for the compound **9** in comparison with the natural GM1 in these experiments. The EC_{50} of LT-B in an assay based on **9** was determined to be 0.06 nM *versus* 0.10 nM determined in the bovine GM1-based ELISA.

The specificity of the LT-B binding to the biotin-appended GM1 **9** was confirmed in an experiment in which the toxin was added to the wells of a streptavidin-coated plate, to which no ganglioside was bound (Fig. 1). No positive reaction was obtained for any of the tested LT-B concentrations, thus demonstrating that it does not aspecifically bind to streptavidin under the applied conditions. Finally, the binding affinity of streptavidin for the biotin moiety of the compound **9** was unambiguously demonstrated in assays based on non-streptavidin-coated plates incubated with **9** and in assays based on streptavidin-coated plates incubated with bovine brain-derived GM1. The former assay shows negligible response to LT-B, while in the latter no positive reaction was obtained, equal to what was seen when using a blank streptavidin-coated plate. These results indicate that **9** is not adsorbed on a plate that is not coated with streptavidin, and also that streptavidin does not adsorb a ganglioside structure if that contains no biotin moiety. Thus, the specificity and increased sensitivity of the assay that involves GM1 analogue **9** and a streptavidin-coated plate is fully attributed to the specific binding of the biotin part of **9** to the surface-bound streptavidin. As a result of this interaction, a GM1-coated surface is formed, in which the natural conformation of GM1 is presumably presented better than on a surface derived from direct coating with bovine GM1. Analogous studies with a wider set of biotin-appended gangliosides and a wider variety of detrimental proteins are currently ongoing in our laboratories.

Conclusions

A series of biotin-appended ganglioside analogues was efficiently synthesized by a chemoenzymatic route that involves consecutive glycosylations of biotinylated lactose using recombinant glycosyltransferases from *C. jejuni*. In this manner, biotinylated glycoside analogues corresponding to gangliosides GM2, GM1, GD1a and GalNAc-GD1a were obtained in high yields. The synthesis of an oligosaccharide containing the GalNAc-GD1a sugar moiety in this manner is, in fact, the first reported chemo-enzymatic synthesis of this sugar moiety. Analysis of the interaction of GM1 analogue **9** with *Escherichia coli* heat-labile enterotoxin revealed that the biotinylated ganglioside surpasses the natural bovine GM1 in the detection of certain proteins involved in human diseases. This clearly displays the potential of such chemoenzymatically synthesized gangliosides in the detection of detrimental proteins (toxins/antibodies).

Experimental

General information

NMR spectra were recorded on a Bruker DPX 400, Bruker Avance 400 or Bruker Avance 900 (for the compound **11**) spectrometer. HRMS (ESI) data were collected on an AccuTOF-CS (JEOL) mass spectrometer. Solvents and chemicals were purchased from Sigma Chemical Co. Thin layer chromatography (TLC) was performed on Merck silica gel 60F254 aluminium backed plates, and detection was realized by either of the following methods: UV (254 nm), charring with a solution of $KMnO_4$(aq) or with 5% (v/v) sulfuric acid in ethanol and subsequent heating.

Enzymes

The enzymes were prepared as described by Pukin *et al.*[23b] The *C. jejuni* UDP-GlcNAc 4-epimerase (construct CPG-13) was expressed and purified as described by Bernatchez *et al.*[27] The *C. jejuni* Cst-I α-2,3-sialyltransferase (construct CST-06) was expressed as a fusion protein with the *E. coli* maltose-binding protein (without the leader peptide) and purified on amylose resin according to the manufacturer's instructions (New England Biolabs, Beverly, MA). The *C. jejuni* CgtB β-1,3-galactosyltransferase (construct CJL-20) was expressed as described by Linton *et al.*[28] and we used a cell extract that was clarified by centrifugation at 27 000 *g*. The *C. jejuni* CgtA β-1,4-*N*-acetylgalactosaminyltransferase (construct CJL-30) was expressed as described by Blixt *et al.*[19] and we used a cell extract that was clarified by centrifugation at 27 000 *g* and stored at −20 °C in 50 mM Hepes buffer (pH 7) with 40% glycerol.

Hepta-*O*-acetyl 8-azidooctyl lactoside (4)

A mixture of peracetylated lactosyl bromide (**1**, 3 g, 4.3 mmol), 8-azidooctanol (**3**, 1.1 g, 6.4 mmol) and molecular sieves (4 Å,

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1.5 g) in anhydrous toluene (50 ml) was cooled to – 78 °C and stirred for 1 h. Silver triflate (1.65 g, 6.4 mmol) was added and the mixture was allowed to warm up to 10 °C with stirring (~ 3 h). The precipitate was filtered off and washed with toluene. The combined filtrate was washed with a 15% aqueous NH_3, brine, and dried over Na_2SO_4. The solvent was evaporated *in vacuo* followed by silica gel chromatography of the residue (EtOAc–hexane, 1 : 3 to 1 : 1) which afforded the target **4** (1.7 g, 50.2%). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 5.27 (d, J = 2.5 Hz, 1 H), 5.12 (t, J = 9.5 Hz, 1 H), 5.03 (dd, J = 10.5, 7.5 Hz, 1 H), 4.89 (dd, J = 10.5, 3.5 Hz, 1 H), 4.80 (dd, J = 9.5, 8.0 Hz, 1 H), 4.46–4.36 (m, 3 H), 4.1–3.97 (m, 3 H), 3.82 (t, J = 7.5 Hz, 1 H), 3.79–3.69 (m, 2H), 3.53 (m, 1 H), 3.38 (m, 1 H), 3.18 (t, J = 7.0 Hz, 2 H), 2.07 (s, 3H), 2.04 (s, 3H), 1.99 (s, 3 H), 1.97 (s, 3H), 1.96 (s, 3H), 1.95 (s, 3H), 1.89 (s, 3H), 1.56–1.41 (m, 4 H), 1.35 –1.20 (m, 8 H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 170.2, 170.1, 169.9, 169.8, 169.6, 169.3, 168.9, 100.8, 100.4, 76.1, 72.6, 72.4, 71.5, 70.8, 70.4, 69.9, 68.9, 66.4, 61.8, 60.6, 51.2, 29.1, 28.9, 28.8, 28.6, 26.4, 25.5, 20.8, 20.63, 20.59, 20.5, 20.4 (2 × C), 20.3. **HRMS (ESI)** for $[M + NH_4]^+$ found 807.34696, calcd 807.35114 [Δ = 4.5 ppm].

Hepta-*O*-acetyl 8-aminooctyl lactoside (5)

Hepta-*O*-acetyl 8-azidooctyl lactoside **4** (0.8 g, 1.0 mmol) was dissolved in dry CH_3OH (10 ml). The solution was stirred with a catalytic amount of 10% palladium on carbon (80 mg) under hydrogen atmosphere (40 psi) for 6 h. The solids were filtered off, washed with methanol, and the filtrate concentrated under reduced pressure to give 0.75 g (98%) of **5** as a white solid. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 8.29 (bs, 2 H, NH_2), 5.34 (d, J = 3.2 Hz, 1 H), 5.22–5.15 (m, 1 H), 5.10 (dd, J = 10.5, 8.0 Hz, 1 H), 4.95 (dd, J = 10.5, 3.5 Hz, 1 H), 4.90–4.84 (m, 1 H), 4.52–4.42 (m, 3 H), 4.18–4.03 (m, 3 H), 3.92–3.85 (m, 1 H), 3.85–3.75 (m, 2 H), 3.65–3.58 (m, 1 H), 3.47–3.38 (m, 1 H), 3.00–2.92 (m, 2 H), 2.14 (s, 3H), 2.11 (s, 3H), 2.03 (s, 9 H), 2.02 (s, 3H), 1.96 (s, 3H), 1.80–1.70 (m, 2 H), 1.60–1.48 (m, 2 H), 1.42–1.23 (m, 8 H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 170.4, 170.3, 170.1, 170.0, 169.8, 169.6, 169.1, 101.0, 100.5, 76.3, 72.8, 72.5, 71.7, 71.0, 70.6, 70.1, 69.1, 66.6, 62.1, 60.8, 40.5, 29.5, 29.3, 29.2, 29.1, 26.8, 25.7, 21.0, 20.9, 20.8, 20.7, 20.6 (2xC), 20.5. **HRMS (ESI)** for $[M + H]^+$ found 764.32991, calcd 764.33409 [Δ = 4.8 ppm].

8-biotinoylaminooctyl lactoside (6)

Hepta-*O*-acetyl 8-aminooctyl lactoside **5** (0.3 g, 0.4 mmol) was dissolved in dry DMF (5 ml). To this solution, biotin (0.1 g, 0.4 mmol), EDC (0.06 g, 0.4 mmol) and DMAP (0.05 g, 0.4 mmol) were added at room temperature, and the mixture was stirred for 4 h in the inert atmosphere. The reaction mixture was diluted with water and extracted (5 x) with ethyl acetate. The combined organic layer was washed with brine, and dried over Na_2SO_4. After evaporation of the solvent *in vacuo* the residue was dissolved in anhydrous methanol. A freshly prepared 1 M $NaOCH_3$ solution in anhydrous CH_3OH (100 µl) was added, and the mixture was stirred overnight at room temperature. More methanol was added to dissolve the formed precipitate, followed by addition of the acidic ion exchange resin Amberlite IR-120H, and the mixture was stirred for 10 min. The resin was filtered off, washed with methanol, and the filtrate concentrated under reduced pressure to give 0.25 g (95%) of **6** as a white powder. **^{1}H NMR** (400 MHz, DMSO-d_6): δ 7.75–7.7 (m, 1H), 6.51–6.20 (br s, 2H), 4.75–4.3 (br s, 7H), 4.31 (t, J = 4.4 Hz, 1 H), 4.20 (d, J = 7.2 Hz, 1 H), 4.17 (d, J = 8.0 Hz, 1 H), 4.13 (t, J = 4.4 Hz, 1 H), 3.8–3.32 (m, 13H), 3.15–2.95 (m, 4H), 2.82 (t, J = 5.2 Hz, 1 H), 2.58 (d, J = 12.4 Hz, 1 H), 2.09–2.00 (m, 2H), 1.67–1.20 (m, 18H). **^{13}C NMR** (101 MHz, DMSO-d_6): δ 172.6, 163.6, 104.7, 103.4, 81.7, 76.4, 75.9, 75.6, 74.1, 74.0, 71.4, 69.6, 69.0, 61.9, 61.4, 61.2, 60.1, 56.3, 39.2, 36.1, 30.1, 30.0, 29.8, 29.6, 29.0, 27.3, 26.4, 26.2. **HRMS (ESI)** for $[M + Na]^+$ found 718.31846, calcd 718.31968.

Synthesis of GM3-biotin 7

59 mg (85 µmol) of Lac-biotin was dissolved in 10 ml of methanol (needed to heat the sample at 60 °C to dissolve completely). The reaction was done in a final volume of 42 ml containing 50 mM Hepes pH 7.5, 10 mM $MgCl_2$, 155 µmol of CMP-NeuAc and 81.4 units of the α-2,3-sialyltransferase (construct CST-06). The reaction was complete after 1 h of incubation at 37 °C, as judged by TLC analysis, and the reaction mixture was directly used in the next step without isolation of the product.

Synthesis of GM2-biotin 8

40 ml of the GM3-biotin reaction (81 µmol) was used directly for the synthesis of GM2-biotin, after centrifugation for 10 min at 2 800 *g* to remove a precipitate. The reaction was done in a final volume of 80 ml containing 50 mM Hepes pH 7, 10 mM $MnCl_2$, 5 mM $MgCl_2$ (remaining from the GM3-biotin reaction), 200 µmol of UDP-GlcNAc, 36.8 units of the β-1,4-*N*-acetylgalactosaminyltransferase (construct CJL-30) and 68.1 units of the UDP-GlcNAc 4-epimerase (construct CPG-13). The reaction was complete after 3 h of incubation at 37 °C, as judged by TLC analysis. The reaction mixture was centrifuged 10 min at 2 800 *g*. The supernatant was diluted 2-fold with water and loaded on two Sep-Pak column (5 g) equilibrated with water. Hydrophilic material was washed off with water and the product was eluted with methanol. Appropriate fractions were collected and evaporated to give 87.4 mg of product (73 µmol, 90% yield). **^{1}H NMR** (400 MHz, D_2O, from HSQC): δ 4.74 (HNC**H**(CH)CH_2-biotin), 4.68, 4.66 (HNC**H**(CH)CH-biotin), 4.46, 4.41, 4.08, 4.05, 3.91, 3.86, 3.85 (C**H**-NAc), 3.84, 3.80 (C**H**$_2$OH-NeuAc), 3.745, 3.74 (C**H**-NAc), 3.735, 3.73 (2H), 3.71 (2H), 3.70, 3.68, 3.64, 3.62, 3.61, 3.58, 3.57 (C**H**$_2$-NH(C=O)), 3.56 (C**H**$_2$OH-NeuAc), 3.54, 3.53, 3.51, 3.42, 3.29, 3.27, 3.22, 3.16 (C**H**$_2$-NH(C=O)), 3.11 (2H), 2.60 (H_{NeuAc}-3eq), 2.21 (2H, HNC(O)C**H**$_2$$CH_2$-biotin), 1.97 (3H, Ac-N), 1.96 (3H, Ac-N), 1.86 (H_{NeuAc}-3ax), 1.80 (2H), 1.62 (2H), 1.55 (2H), 1.46 (2H), 1.43, 1.29–1.20 (5H), 1.26 (2H), 1.25 (2H). **^{13}C NMR** (100 MHz, D_2O): δ 104.5, 104.4, 103.8, 80.4, 78.9, 76.4, 76.4, 76.3, 76.1, 75.8, 74.8, 74.6, 74.0, 73.0, 72.5, 71.7, 71.5, 70.4, 69.8, 69.5, 64.6, 62.9, 62.3, 62.0, 59.0, 58.8, 56.0, 54.1, 53.3, 41.0, 38.7, 37.2, 30.4, 30.2, 30.0, 29.9, 27.9, 27.6, 26.8, 26.7, 26.5, 24.3, 23.8. **HRMS (ESI)** for $[M + Na]^+$ found 1212.49620, calcd 1212.49447 [Δ = 1.4 ppm].

Synthesis of GM1a-biotin 9

The reaction was performed in a final volume of 32 ml and included: 38 mg (32 µmol) of GM2-biotin, 96 µmol of UDP-Gal, 50 mM Mes pH 6.0, 10 mM $MnCl_2$, 1 mM DTT and 14 units of

the β-1,3-galactosyltransferase (construct CJL-20). The reaction was complete after 3 h of incubation at 37 °C, as judged by TLC analysis (a sample had to be purified on a small Sep Pak column before doing the TLC analysis). The reaction mixture was diluted 6-fold with water before being applied to a Sep-Pak column (5 g) equilibrated with water. Hydrophilic material was washed off with water and the product was eluted with methanol. Appropriate fractions were collected and evaporated to give 43 mg of product (31.8 μmol, 99% yield).

Selected ^{1}**H NMR** (400 MHz, CD_3OD): δ 4.91 (d, 1H, J = 8.5 Hz, H_{GalNAc}-1), 4.49 (dd, 1H, J = 7.5 Hz, J = 4.5 Hz, HNC**H**(CH)CH_2-biotin), 4.44 (d, 1H, J = 7.5 Hz, $H_{Gal'}$-1), 4.42 (d, 1H, J = 8.0 Hz, H_{Gal}-1), 4.32–4.28 (m, 1H, HNC**H**(CH)CH-biotin), 4.27 (d, 1H, J = 8.0 Hz, H_{Gluc}-1), 2.71 (m, 1H, H_{NeuAc}-3eq), 2.19 (t, J = 7.1 Hz, 2H, HNC(O)C**H**$_2$$CH_2$-biotin), 2.01 (s, 3H, NHC(O)C**H**$_3$), 1.99 (s, 3H, NHC(O)C**H**$_3$), 1.93–1.87 (m, 1H, H_{NeuAc}-3ax). ^{13}**C NMR** (100 MHz, CD_3OD): δ 176.0, 175.7, 175.3, 174.9, 166.2, 106.7, 105.0, 104.3, 104.2, 103.5, 83.1, 81.6, 79.1, 76.5, 76.4, 76.0, 75.7, 75.2, 74.8, 74.6, 73.4, 72.6, 71.1, 70.9, 70.5, 70.3, 69.8, 65.5, 63.4, 63.0, 62.4, 62.1, 61.8, 61.7, 57.1, 53.8, 52.8, 41.1, 40.4, 38.6, 36.9, 30.8, 30.5, 30.4, 30.3, 29.8, 29.6, 28.0, 27.0, 23.8, 22.6. **HRMS (ESI)** for $[M + Na]^+$ found 1374.54558, calcd 1374.54729 [Δ = 1.2 ppm].

Synthesis of GD1a-biotin 10

The reaction was performed in a final volume of 7 ml and included: 34.6 mg (25.6 μmol) of GM1a-biotin, 42 μmol of CMP-NeuAc, 50 mM Hepes pH 7.5, 10 mM $MgCl_2$, and 2 units of the α-2,3-sialyltransferase CST-06. The reaction was complete after 2 h of incubation at 37 °C, as judged by TLC analysis. The reaction mixture was diluted 5-fold with water before being applied to a Sep-Pak column (5 g) equilibrated with water. Hydrophilic material was washed off with water and the product was eluted with 60% methanol. Appropriate fractions were collected and evaporated to give 36.6 mg of product (22.3 μmol, 87.1% yield). Selected ^{1}**H NMR** (400 MHz, D_2O): δ 4.53–4.50 (m, 1H, HNC**H**(CH)CH_2-biotin), 4.51 (d, J = 7.7 Hz, $H_{Gal'}$-1), 4.43 (d, 1H, J = 8.0 Hz, H_{Gal}-1), 4.38 (d, 1H, J = 8.0 Hz, H_{Gluc}-1), 4.35–4.30 (m, 1H, HNC**H**(CH)CH-biotin), 2.90 (dd, 1H, J = 13.2, J = 5.1, HCC**H**$_2$S-biotin), 2.69 (d, 1H, J = 13.2, HCC**H**$_2$S-biotin), 2.68–2.62 (m, 1H, $H_{NeuAc'}$-3eq), 2.61–2.55 (m, 1H, $H_{NeuAc'}$-3eq), 2.15 (t, J = 7.0 Hz, 2H, HNC(O)C**H**$_2$$CH_2$-biotin), 1.93 (2 × s, 6H, NHC(O)C**H**$_3$), 1.91 (s, 3H, NHC(O)C**H**$_3$), 1.86–1.78 (m, H_{NeuAc}-3ax), 1.74–1.66 (m, $H_{NeuAc'}$-3ax). ^{13}**C NMR** (100 MHz, CD_3OD): δ 176.0, 175.8, 175.5, 175.2, 174.9, 174.8, 165.6, 106.1, 104.8, 104.2, 104.1, 103.3, 101.0, 82.3, 81.2, 78.8, 77.3, 76.5, 76.4, 76.3, 75.9, 75.6, 75.1, 74.8, 74.7, 73.4, 72.9, 71.1, 71.0, 70.7, 70.4, 70.0, 69.7, 69.6, 69.5, 68.8, 65.2, 64.3, 63.6, 62.9, 62.7, 62.0, 61.8, 61.7, 57.0, 53.9, 53.8, 52.5, 42.3, 41.2, 40.5, 38.8, 34.9, 30.7, 30.6, 30.54, 30.51, 30.2, 30.1, 28.0, 27.0, 23.9, 22.7, 22.6. **HRMS (ESI)** for $[M + Na]^+$ found 1665.64236, calcd 1665.64270 [Δ = 0.2 ppm].

Synthesis of GalNAc-GD1a-biotin 11

The reaction was performed in a final volume of 1 ml and included: 3.9 mg (2.4 μmol) of GD1a-biotin, 4 μmol of UDP-GalNAc, 50 mM Hepes pH 7, 10 mM $MnCl_2$ and 0.46 unit of the β-1,4-*N*-acetylgalactosaminyltransferase (construct CJL-30). The reaction was complete after 1 h of incubation at 37 °C, as judged by TLC analysis. The reaction mixture was diluted 5-fold with water before being applied to a Sep-Pak column (0.36 g) equilibrated with water. Hydrophilic material was washed off with water and the product was eluted with 60% methanol. Appropriate fractions were collected and evaporated to give 3.3 mg of product (1.8 μmol, 75% yield).). Selected ^{1}**H NMR** (400 MHz, D_2O, from HSQC): δ 4.74 (HNC**H**(CH)CH_2-biotin), 4.71, 4.68, 4.66 (HNC**H**(CH)CH-biotin), 4.47, 4.46, 4.41, 3.93 (C**H**-NAc), 3.81 (C**H**$_2$OH-NeuAc), 3.77 (C**H**$_2$OH-NeuAc′), 3.76 (2H, C**H**-NAc), 3.58 (C**H**$_2$-NH(C=O)), 3.57 (C**H**$_2$OH-NeuAc′), 3.56 (C**H**$_2$OH-NeuAc), 3.15 (C**H**$_2$-NH(C=O)), 2.61 (H_{NeuAc}-3eq), 2.49 ($H_{NeuAc'}$-3eq), 2.22 (2H, HNC(O)C**H**$_2$$CH_2$-biotin), 2.01 (3H, Ac-N), 2.00 (3H, Ac-N), 1.97 (3H, Ac-N), 1.96 (3H, Ac-N), 1.86 (H_{NeuAc}-3ax), 1.81 (2H), 1.62 (2H), 1.55 (2H), 1.46 (2H), 1.43 2H), 1.26 (2H), 1.30–1.22 (5H), 1.24 (2H). ^{13}**C NMR** (226 MHz, CD_3OD): δ 104.42, 102.78, 102.64, 102.55, 102.04, 100, 80.53, 78.75, 77.11, 77.01, 74.75, 74.73, 74.55, 74.4, 74.34, 74.29, 74.11, 73.54, 73.09, 73.05, 72.79, 72.3, 72.25, 71.34, 70.71, 69.99, 69.75, 68.77, 68.73, 68.12, 68.07, 67.89, 67.83, 62.87, 62.07, 62.52, 61.18, 60.26, 60.2, 60.69, 60.67, 60.62, 60.6, 60.42, 60.39, 55.41, 54.32, 52.37, 51.61, 51.06, 40.2, 39.8, 39.73, 39.31, 39.29, 35.55, 28.73, 28.37, 28.28, 28.26, 27.79, 27.68, 25.97, 25.22, 24.97, 22.07, 22.71, 22.67.

HRMS (ESI) for $[M - H]^-$ found 1844.73101, calcd 1844.72448 [Δ = 3.5 ppm].

ELISA

Nunc-Immuno Plates (Maxi Sorp Surface, Nalge Nunc International, Roskilde, Denmark) were coated with bovine brain monosialoganglioside GM1 (Sigma Aldrich, St. Louis, USA), synthetic GM1-biotin (REF) or Streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at 5 μg mL^{-1} in phosphate buffered saline (PBS) for 16 h at 4 °C. Plates were washed three times with deionized water and blocked for 1 h at room temperature with 2% skimmed milk, 0.1% bovine serum albumin and 0.1% Tween-20 in PBS under continuous shaking at 100 rpm. Next, plates coated with Streptavidin were incubated with 5 μg mL^{-1} monosialoganglioside GM1 or GM1-biotin in phosphate buffered saline (PBS; 100 μL/well) for 16 h at 4 °C. Subsequently, serial twofold dilutions of pure recombinant LT-B in coating buffer were added and binding was allowed for another 16 h at 4 °C. After washing plates three times with deionized water, plates were incubated with monoclonal antibody VD12 for 1 h at room temp in a 1 : 1000 dilution. After rinsing with deionized water three times, plates were further incubated with AP-labelled sheep-anti-mouse antibody and bound label was detected with 4-nitrophenylphosphate (disodium salt hexahydrate; Janssen Chimica). Detection was at 405 nm in a Bio-Rad Benchmark Microplate Reader (Bio Rad, Veenendaal, The Netherlands) using Microplate Manager/PC version 4.0 software for standard curve analysis, calculation of concentrations and standard deviation. Samples were analysed three times in independent experiments.

Acknowledgements

The authors thank the Human Science Frontier Program (RGP 38/2003) and Wageningen University for generous funding, and

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Peter van Galen (Radboud University, Nijmegen) for HRMS measurements.

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