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New derivatives of reducing oligosaccharides and their use in enzymatic reactions: efficient synthesis of sialyl Lewis a and sialyl dimeric Lewis x glycoconjugates

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Abstract

The reducing oligosaccharides lactose and lacto-*N*-tetraose were reductively aminated with benzyloxycarbonylaminoaniline and sodium cyanoborohydride, followed by treatment of the resulting secondary amines with acetic anhydride. The resulting *N*-acetyl-*N*-(4-benzyloxycarbonylaminophenyl)-1-amino-1-deoxyalditol oligosaccharide derivatives were subjected to stepwise enzymatic elongation with various glycosyltransferases/nucleotide sugars. Purification of the products after each enzymatic step was conveniently performed by solid-phase extraction on silica gel C-18 cartridges. Two oligosaccharide derivatives (with sialyl Lewis a and sialyl dimeric Lewis x structures) were prepared. Conversion of the obtained derivatives into neoglycoproteins by the sequence hydrogenolysis, thiophosgene treatment, and protein coupling was carried out.

Keywords: Derivatives; Oligosaccharides; Enzymatic reactions

1. Introduction

The use of glycosyltransferases in preparative oligosaccharide synthesis is an alternative to traditional chemical synthesis [1]. Increased availability of nucleotide sugars and glycosyltransferases from commercial sources has today made enzymatic synthesis possible in laboratories that do not have cloning or enzyme purification capabilities. Some practical difficulties in the preparative use of glycosyltransferase enzymes remain, however. For example, purification of the oligosaccharide product after each enzymatic steps is not trivial. Enzymatic reactions are carried out in aqueous solutions containing considerable amounts of buffers and metal salts, and it is from this mixture that the oligosaccharide product (usually a minor component compared with the salts) has to be isolated. One approach to the product purification problem has been to attach the oligosaccharide acceptor to a solid phase and conduct the enzymatic steps in a solid-phase synthesis fashion [2]. Another approach has been to attach the oligosaccharide acceptor to a lipophilic moiety before the enzymatic steps. This enables facile product isolation from the reaction mixture by solid-phase extraction using C-18 silica cartridges (Sep-Pak, Bond-Elut or similar). The lipophilic moiety most commonly used for this purpose has been the methoxycarbonyloctyl glycosidic group [3,4]. However, this group has to be attached to the oligosaccharide acceptor by a multi-step organic synthetic se-

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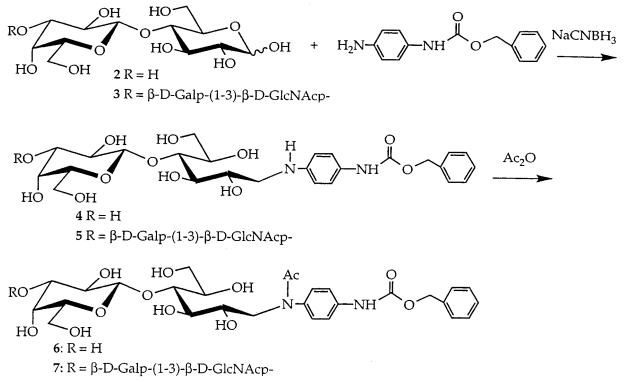
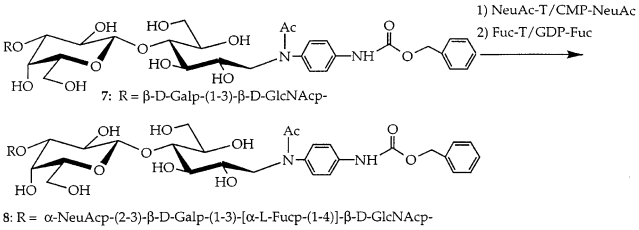


Scheme 1.

quence, and it might be difficult to realize this attachment onto larger oligosaccharides. Therefore, alternative oligosaccharide derivatization procedures are interesting, especially for work with complex reducing oligosaccharides of natural origin. We have previously reported [5] the use of glycosylamine derivatization/squaric acid coupling to long-chain aliphatic amines for this purpose.

We now report an alternative procedure for attachment, through reductive amination, of a lipophilic benzyloxycarbonylaminoaniline group to the reducing end of oligosaccharides. Oligosaccharides thus derivatized were enzymatically elongated and the products conveniently isolated from the reaction mixture after each step by solid-phase extraction with C-18 silica cartridges. The benzyloxycarbonylaminoaniline group of the synthesized oligosaccharides could be converted, by catalytic hydrogenation, followed by treatment with thiophosgene, into an isothiocyanate, which coupled well to proteins giving glycoconjugates. Two biologically interesting oligosaccharide derivatives, with sialyl Lewis a and sialyl dimeric Lewis x carbohydrate structures, were synthesized in good yield from lacto-*N*-tetraose and lactose, respectively, and were subsequently conjugated to human serum albumin (HSA).



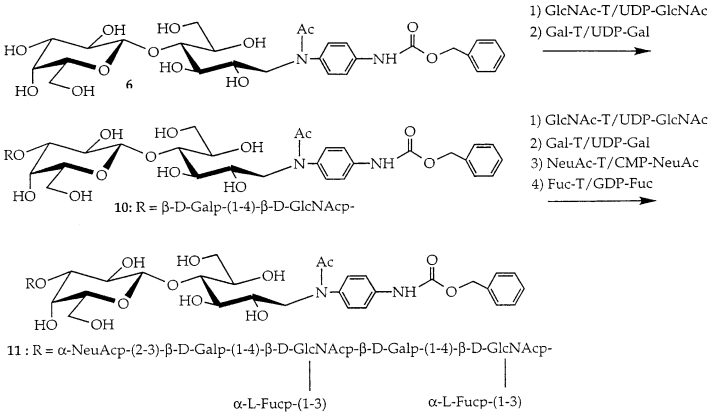
Scheme 2.

2. Results and discussion

Reaction of *p*-benzyloxycarbonylaminoaniline (**1**) [6,7] and sodium cyanoborohydride with lactose (**2**) or lacto-*N*-tetraose (**3**) gave the reductive amination products **4** and **5**, respectively. Treatment in situ of **4** or **5** with acetic anhydride produced the corresponding N-acetylated derivatives **6** and **7**, which were isolated (60 and 80% yields, respectively) and characterized. The N-acetylations were performed in order to stabilize the derivatives towards air oxidation, which is otherwise known [8] to be rather rapid with *p*-phenylenediamine derivatives like **4** or **5** (Scheme 1).

The lacto-*N*-tetraose derivative **7** was subjected to enzymatic elongation first using recombinant α-(2→3)-(*N*)-sialyltransferase/CMP-Neu5Ac, then human milk α-(1→3/4)-fucosyltransferase/GDP-fucose (Scheme 2). Purification by solid-phase extraction with C-18 silica cartridges after each step was performed, as well as a final gel filtration. The resulting sialyl Lewis a (sialyl LNF II) hexasaccharide derivative **8** was isolated in a 67% yield. The ^{1}H NMR data of **8** agreed well with those for a commercial sample of sialyl-Le^a hexasaccharide (which has the same oligosaccharide structure as **8** except for the reducing glucosyl unit).

It should be noted here that earlier attempts at enzymatic elongation of an oligosaccharide derivative carrying a *p*-trifluoroacetamidoaryl group instead of a benzyloxycarbonylaminoaryl group resulted [9] in partial loss of the trifluoroacetamido group. Other unpublished experiments have given the same result. Presumably, enzyme preparations often contain a de-trifluoroacetylating activity. Because of this sensitivity to deacylation, the use of *p*-trifluoroacetamidoaryl derivatized oligosaccharides in enzymatic synthesis is less practical, even though the *p*-trifluoroacetamidoaryl group is also lipophilic and the oligosaccharide is therefore easy to isolate by solid-phase extraction. No corresponding cleavage was observed with the present benzyloxycarbonylaminoaniline derivatives.

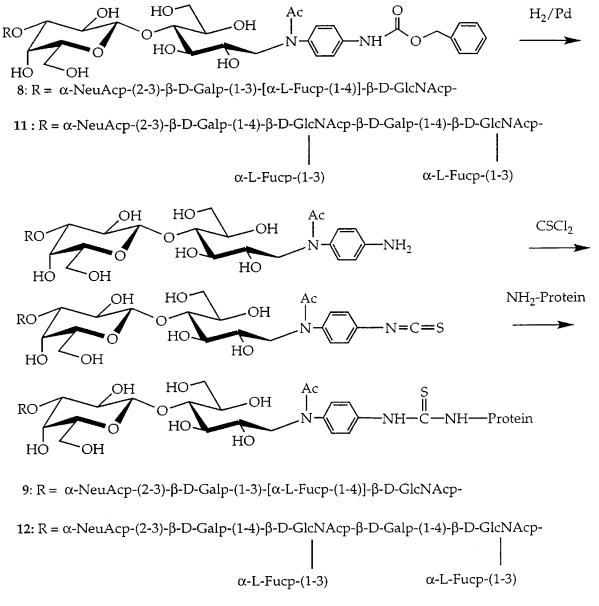


Scheme 3.

The derivative **8** could be converted into an isothiocyanate by catalytic hydrogenation, followed by in situ treatment of the product amine with thiophosgene. Coupling of the isothiocyanate to HSA gave the sialyl Lewis a neoglycoconjugate **9** (Scheme 4). The spacer in this conjugate is the same [8] as has been published previously.

To further demonstrate the usefulness of oligosaccharide benzyloxycarbonylaminoaniline derivatives in enzymatic synthesis, the lactose derivative **6** was subjected to a six-step enzymatic elongation sequence. The product nonasaccharide derivative **11** has an oligosaccharide part ('sialyl dimeric Lewis x') that is implicated as a high-affinity selectin ligand [10–12] or as a tumor marker [13]. The following steps were carried out (Scheme 3). The lactose derivative **6** was treated with first recombinant *Neisseria meningitidis* β-(1→3)-*N*-acetylglucosaminyltransferase/UDP - GlcNAc, then recombinant bovine milk β-(1→4)-galactosyltransferase/UDP-Gal. Purification after each step by solid-phase extraction with C-18 silica cartridges was performed here as well as in all subsequent steps. After gel filtration, the lacto-*N*-neotetraose derivative **10** was obtained in 53% yield from **6**. Further treatment of **10** first with recombinant *Neisseria meningitidis* β-(1→3)-*N*-acetylglucosaminyltransferase/UDP-GlcNAc, then recombinant bovine milk β-(1→4)-galactosyltransferase/UDP-Gal gave a hexasaccharide major product (approximately 60% yield), contaminated with residual tetrasaccharide **10** (approximately 40%). The reason for the incomplete conversion to hexasaccharide is the sluggish reaction with the first enzyme. Extended oligosaccharides like **10** are known [18] to be poor acceptors for *N. meningitidis* β - (1→3) - *N* - acetylglucosaminyltransferase. The hexasaccharide was purified by gel filtration prior to treatment first with recombinant α - (2→3) - (*N*) - sialyltransferase/CMP-NeuAc, then recombinant human α-(1→3)-fucosyltransferase V/GDP-Fuc. A final gel filtration purification gave **11** in 21% yield, calculated from **10**. The ^{1}H NMR data of **11** agreed well with data [15,16] for similar structures (Scheme 3). Conversion of **11** into the neoglycoconjugate **12** was carried out as for **8** (Scheme 4).

It should be noted that nonasaccharide derivatives containing the same sugar sequence as **11** have been prepared previously



Scheme 4.

[14–17], in these cases by multi-step chemical synthesis. The total yields (from component protected monosaccharides) were in the 1% range. The total yield (from the lactose derivative **2**) in the present enzymatic synthesis of **11** was 11%. This illustrates that preparative enzymatic synthesis of oligosaccharides is in certain cases an attractive alternative to chemical synthesis, provided that enzymes and nucleotide sugars are available and that purification after each step can be carried out in a simple way.

3. Experimental

General methods.—Concentrations were performed at reduced pressure at < 40 °C bath temperature. Proton NMR spectra were recorded at 25, 30 or 60 °C in D_2O using Bruker 400 and 600 MHz instruments. The benzyl methylene signal (at δ 5.244 against external TMS in compound **10** at 30 °C) present in all conjugates was used as internal ^{1}H NMR reference signal. For ^{13}C NMR spectra, dioxane (δ 67.4) was used as reference. The FABMS spectra were recorded with a JEOL JMS-SX/SX-102A instrument. Ions were produced by a beam of Xe atoms (6 keV), using a glycerol matrix. MALDI MS spectra were recorded in the positive ion mode with a Bruker reflex 3 instrument, using a 2,5-dihydroxybenzoic acid matrix. Thin-layer chromatography (TLC) was performed on Kieselgel 60 F_{254} Fertigplatten (E. Merck, Darmstadt, Germany). The plates were eluted with X:3:3:2 EtOAc–MeOH–AcOH–water mixtures, where X was varied between 4 and 12, depending on the polarity of the analytes. After elution, spots were visualized by UV light and by dipping in 5% H_2SO_4, followed by charring. For solid-phase extractions, Isolute C-18 EC silica cartridges (International Sorbent Technology, Mid-Glamorgan, UK) were used. The cartridges were wetted thoroughly with several void volumes of MeOH and then washed with at least 20 void volumes of water before use. Water for all solutions was from a MilliQ water purification system (Millipore Corp., Bedford, MA, USA), and was degassed by vacuum treatment before use. Gel filtrations were performed on Bio-Gel P2 (fine) columns using MilliQ-purified water with added *n*-butanol (5%, to prevent microorganism contamination) as eluant.

Materials.—Uridine 5′-diphosphogalactose (UDP-Gal), uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc), cytidine monophospho-*N*-acetylneuraminic acid (CMP-NeuAc), bovine serum albumin (BSA) and human serum albumin (HSA) were from Sigma Chemical Co., (St. Louis, IL, USA). Sialyl-Le^a hexasaccharide and lacto-*N*-tetraose were from IsoSep AB (Tullinge, Sweden). Guanosine 5′-(β-L-fucopyranosyl)-diphosphate (GDP-Fuc) was prepared by chemical synthesis as described previously [2]. Recombinant bovine milk β-(1→4)-galactosyltransferase (Gal-T), rat α-(2→3)-(*N*)-sialyltransferase (NeuAc-T), and human α-(1→3)-fucosyltransferase V (Fuc-T-V) were from Calbiochem/Novabiochem Corp. (La Jolla, CA, USA), the manufacturers unit definition was used. Recombinant *N. meningitidis* β-(1→3)-*N*-acetylglucosaminyltransferase (GlcNAc-T) was prepared as described [18], unit definition was based on *p*-nitrophenyl β-lactoside as substrate. Human milk α-(1→3/4)-fucosyltransferase (3/4-Fuc-T) was prepared from human milk as described [9], unit definition was based on compound **5** as the substrate.

β-D-Galactopyranosyl-(1→4)-D-[N-acetyl-N-(4-benzyloxycarbonylaminophenyl)-1-amino-1-deoxy]-glucitol (**6**).—Acetic acid (100 μL) was added to a solution of lactose (**2**, 33 mg, 0.1 mmol) in 2:1 EtOH–water (6 mL), then *p*-benzyloxycarbonylaminoaniline [6,7] (**1**, 100 mg, 0.41 mmol) was added. When all had dissolved, sodium cyanoborohydride (50 mg) was added, and the mixture was stirred for 48 h, after which TLC indicated no more change. Acetic anhydride (1.0 mL) was added, and the mixture was stirred for 5 h, then partitioned between EtOAc and water. The organic layer was washed with water, and the combined aqueous layers were concentrated to 2 mL and then applied onto an Isolute cartridge (1.0 g), which was then eluted first with water and then with MeOH. Appropriate fractions were concentrated and lyophilized to give **6** as a white powder (36 mg, 60%). ^{1}H

NME (25 °C): δ 4.459 (d, $J_{1,2}$ 7.8 Hz, H-1′), 3.541 (dd, H-2′), 1.893 (s, NAc); ^{13}C NMR (25 °C): δ 104.0 (C-1′), 81.4, 75.9, 73.4, 72.2, 71.9, 70.7, 70.1, 69.3 (C-2, 3, 4, 5, 2′, 3′, 4′, 5′), 68.0 (OCH_2Ph), 62.8, 61.5 (C-6, 6′), 52.2 (C-1), 22.8 (NAc). FABMS showed an [M + H] molecular ion at $m/e = 611$.

β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-[N-acetyl-N-(4-benzyloxycarbonylaminophenyl)-1-amino-1-deoxy]-glucitol (**7**).—Acetic acid (50 μL) was added to a solution of lacto-*N*-tetraose (**3**, 30 mg, 0.042 mmol) in 2:1 EtOH–water (3.0 mL), then *p*-benzyloxycarbonylaminoaniline [6,7] (**1**, 60 mg, 0.25 mmol) was added. When all had dissolved, sodium cyanoborohydride (25 mg) was added, and the mixture was stirred for 48 h, after which TLC indicated no more change. Acetic anhydride (0.6 mL) was added, and the mixture was stirred for 3 h, then partitioned between EtOAc (20 mL) and water (20 mL). The organic layer was washed with 2:1 water–brine, and the combined aqueous layers were concentrated to 10 mL and then applied onto an Isolute cartridge (1.0 g), which was then eluted first with water and then with 20, 40, and 60% aq MeOH. Appropriate fractions were concentrated and lyophilized to give **7** as a white powder (33 mg, 80%). ^{1}H NMR (25 °C): δ 4.739 (d, $J_{1,2}$ 7.8 Hz, H-1″), 4.459 (d, $J_{1,2}$ 7.3 Hz, H-1′), 4.441 (d, $J_{1,2}$ 7.3 Hz, H-1‴), 2.038 (s, NAc), 1.885 (s, aromatic NAc); ^{13}C NMR (25 °C): δ 104.2 (C-1′), 103.7 (C-1‴), 103.3 (C-1″), 68.0 (OCH_2Ph), 62.8, 61.5 (C-6, 6′), 55.4 (C-2″), 52.2 (C-1), 23.0, 22.8 (NAc). FABMS showed an [M + H] molecular ion at $m/z = 976$.

O-*(Sodium 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulpyranosyluronate)-(2→3)-O-β-D-galactopyranosyl-(1→3)-O-[α-L-fucopyranosyl-(1→4)]-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→3)-O-β-D-galactopyranosyl-(1→4)-D-[N-acetyl-N-(4-benzyloxycarbonylaminophenyl)-1-amino-1-deoxy]-glucitol* (**8**).—A solution of **7** (6.0 mg, 6.15 μmol) and CMP-NeuAc (6.5 mg, 9.9 μmol) in buffer (0.05 M sodium cacodylate, 0.015 M $MnCl_2$, 0.2% Triton-X, pH 7.4, 1.0 mL) was mixed with NeuAc-T (100 mU, 0.1 mL of a 1.0 U/mL stock solution), and the mixture was kept at 37 °C for 24 h after which TLC indicated complete reaction. The mixture was diluted with water (1 mL), then slowly applied to an Isolute cartridge (1.0 g). Elution first with water and then with 20, 40, and 60% aq MeOH gave, after concentration and lyophilization of appropriate fractions, sialylated **7** (7.9 mg, homogenous on TLC), which was dissolved in buffer (0.05 M sodium cacodylate, 0.005 M $MnCl_2$, pH 7.2, 2.5 mL) containing GDP-Fuc (6.1 mg, 13 μmol) and human milk 3/4-Fuc-T (100 mU). After 24 h at 37 °C, TLC indicated complete reaction, and the mixture was purified on an Isolute cartridge as described above. The obtained lyophilized material (9.2 mg) was further purified by gel filtration to give pure **8** (5.8 mg, 67%); ^{1}H NMR (25 °C): δ 5.017 (d, $J_{1,2}$ 3.9 Hz, Fuc H-1), 4.700 (d, $J_{1,2}$ 8.6 Hz, GlcNAc H-1), 4.557 (d, $J_{1,2}$ 7.6 Hz, Gal H-1), 4.421 (d, $J_{1,2}$ 7.8 Hz, Gal H-1), 2.775 (dd, $J_{2a,3}$ 4.5, $J_{2a,2b}$ 12.3 Hz, NeuAc H-3a), 2.036, 2.033 (2 s, 2 NAc), 1.882 (s, aromatic NAc), 1.776 (t, NeuAc H-3b), 1.179 (d, $J_{5,6}$ 6.6 Hz, H-6 Fuc). FABMS showed an [M + Na] molecular ion at $m/z = 1436$.

O-*(Sodium 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulpyranosyluronate)-(2→3)-O-β-D-galactopyranosyl-(1→3)-O-[α-L-fucopyranosyl-(1→4)]-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→3)-O-β-D-galactopyranosyl-(1→4)-D-[N-acetyl-N-(4-isothiocyanatophenyl)-1-amino-1-deoxy]-glucitol and its HSA conjugate* (**9**).—A solution of **8** (5.5 mg, 3.9 μmol) in water (0.50 mL) was stirred in a hydrogen atmosphere in the presence of 10% palladium on charcoal (5 mg) until TLC indicated complete conversion into a slower-migrating (and ninhydrin-positive) compound. Acetic acid (15 μL) and sodium acetate (20 mg) was then added, followed by EtOH (0.5 mL), then thiophosgene (5 μL) was added with stirring. Rapid (1 min) conversion into a faster-migrating compound was indicated by TLC, and the mixture was filtered (through glass wool), the filtrate was partitioned between water (1 mL) and diethyl ether (1 mL), the aqueous layer was, if necessary, pH-adjusted to 6–8, then concentrated to 0.3 mL and added to a solution of HSA (10 mg, 0.16 μmol, 1/24 equiv) in phosphate

buffer (1.5 mL, pH 9.0). The pH was adjusted to 9.5 with aq NaOH (0.5 M), then the mixture was gently stirred overnight at room temperature (rt). Low-molecular compounds were removed from the mixture by repeating (× 5) a water dilution–ultrafiltration cycle (10 K cut-off filter, 10 mL). The resulting solution was lyophilized to give conjugate (12.9 mg), which in MALDI showed a broad molecular ion centered around m/z 83743, which corresponds to an average sugar incorporation of 13 haptens/HSA molecule.

β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-[N-acetyl-N-(4-benzyloxycarbonylaminophenyl)-1-amino-1-deoxy]-glucitol (**10**).—A solution of **6** (44 mg, 72 μmol), UDP-GlcNAc (100 mg, 154 μmol, and BSA (60 mg) in buffer (0.5 M sodium cacodylate, 0.015 M $MnCl_2$, pH 7.3, 8.5 mL) was mixed with GlcNAc-T (1.4 U, 3.5 mL of a 0.41 U/mL stock solution), and the mixture was stirred for 3 days at rt. Then TLC analysis indicated 50% conversion to product (lower R_f). More UDP-GlcNAc (75 mg, 115 μmol) and GlcNAc-T (0.82 U, 2.0 mL) was added, and the mixture was stirred for another 48 h. The mixture was diluted with water (33 mL) and was then purified on an Isolute cartridge (4.0 g) as described for **3** and **8**, to give material (38 mg), which was taken up in buffer (0.5 M sodium cacodylate, 0.015 M $MnCl_2$, pH 7.3, 16 mL) and mixed with UDP-Gal (44 mg, 72 μmol) and Gal-T (1.5 U, 300 μL of a 5 U/mL stock solution). After 2 days at rt, the mixture was diluted with water and purified on an Isolute cartridge (4.0 g) as described for **3** and **8** to give material, which was further purified by gel filtration to give pure **10** (37 mg, 38 μmol, 53%); ^{1}H NMR (60 °C): δ 4.731 (d, $J_{1,2}$ 7.6 Hz, GlcNAc H-1), 4.475 (d, $J_{1,2}$ 7.6 Hz, Gal H-1), 4.433 (d, $J_{1,2}$ 7.6 Hz, Gal H-1), 4.097 (d, Gal H-4), 2.022 (s, NAc), 1.871 (s, aromatic NAc). FABMS showed an [M + H] molecular ion at $m/z = 976$.

O-*(Sodium 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulpyranosyluronate)-(2→3)-O-β-D-galactopyranosyl-(1→4)-O-[α-L-fucopyranosyl-(1→3)]-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→3)-O-β-D-galactopyranosyl-(1→4)-O-[α-L-fucopyranosyl-(1→3)]-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→3)-O-β-D-galactopyranosyl-(1→4)-D-[N-acetyl-N-(4-benzyloxycarbonylaminophenyl)-1-amino-1-deoxy]-glucitol* (**11**).—A solution of **10** (26 mg, 26.6 μmol), UDP-GlcNAc (265 mg, 407 μmol, and BSA (26 mg) in buffer (0.5 M sodium cacodylate, 0.015 M $MnCl_2$, pH 7.3, 1.8 mL) was mixed with GlcNAc-T (1.5 U, 3.6 mL of a 0.41 U/mL stock solution), and the mixture was stirred for 5 days at rt. Then TLC analysis indicated 60% conversion to product (lower R_f). The mixture was diluted with water (20 mL) and was then purified on an Isolute cartridge (1.0 g) as described for **3** and **8**, to give material (33 mg, a mixture of **10** and pentasaccharide product), which was taken up in buffer (0.5 M sodium cacodylate, 0.02 M $MnCl_2$, pH 7.3, 8.0 mL) and mixed with UDP-Gal (33 mg, 54 μmol) and Gal-T (0.75 U, 150 μL of a 5 U/mL stock solution). After 2 days at rt, the mixture was diluted with water and purified on an Isolute cartridge (1.0 g) as described for **3** and **8**, to give material, which was purified by gel filtration to give a hexasaccharide material (14.7 mg); ^{1}H NMR (40 °C): δ 4.718 (d, $J_{1,2}$ 7.8 Hz, GlcNAc H-1), 4.710 (d, $J_{1,2}$ 7.8 Hz, GlcNAc H-1), 4.480 (d, $J_{1,2}$ 7.6 Hz, Gal H-1), 4.467 (d, $J_{1,2}$ 7.7 Hz, Gal H-1), 4.426 (d, $J_{1,2}$ 7.7 Hz, Gal H-1), 4.151 (d, Gal H-4), 4.102 (d, Gal H-4), 2.034, 2.020 (2 s, 2 NAc), 1.877 (s, aromatic NAc). Part of this material (9.7 mg) and CMP-NeuAc (8.9 mg, 14.4 μmol) in buffer (0.05 M sodium cacodylate, 0.015 M $MnCl_2$, 0.2% Triton-X, pH 7.0, 1.1 mL) was mixed with NeuAc-T (250 mU, 0.25 mL of a 1.0 U/mL stock solution), and the mixture was kept at 37 °C for 4 days. Dilution with water (5 mL) and purification on an Isolute cartridge (0.5 g) gave material (11.8 mg), which was taken up in buffer (0.10 M sodium cacodylate, 0.020 M $MnCl_2$, pH 7.3, 1.3 mL) and mixed with GDP-Fuc (19 mg, 23 μmol) and Fuc-T-V (0.21 U, 0.42 mL of a 0.5 U/mL stock solution). After 4 days at 37 °C, the mixture was diluted with water (8 mL) and purified on an Isolute cartridge (0.5 g) as described for **3** and **8**, to give material, which was further purified by gel filtration to give pure **11** (7.0 mg, 3.6 μmol, 21% from **10**); ^{1}H NMR (25 °C): δ 5.112, 5.108 (2 d, 2 H, $J_{1,2}$ 3.3 Hz, 2 H-1 Fuc), 4.809 (d, GlcNAc H-1), 4.797 (d, GlcNAc H-1), 4.524 (d, $J_{1,2}$ 7.7 Hz, Gal H-1), 4.439 (d, $J_{1,2}$ 7.7 Hz, Gal H-1),

4.418 (d, $J_{1,2}$ 7.7 Hz, Gal H-1), 2.763 (dd, $J_{2a,3}$ 4.0, $J_{2a,2b}$ 12.3 Hz, NeuAc H-3a), 2.029, 1.999 (2 s, 2 NAc), 1.878 (s, aromatic NAc), 1.788 (t, NeuAc H-3b), 1.166, 1.145 (2 d, $J_{5,6}$ 6.6 Hz, 2 H-6 Fuc). MALDI-MS showed an [M + Na] molecular ion at $m/z = 1946$.

O-*(Sodium 5-acetamido-3,5-dideoxy*-D-glycero-α-D-galacto-*2-nonulpyranosyluronate)-(2→3)*-O-β-D-*galactopyranosyl-(1→4)*-O-*[α*-L-*fucopyranosyl-(1→3)]*-O-*(2-acetamido-2-deoxy-β*-D-*glucopyranosyl)-(1→3)*-O-β-D-*galactopyranosyl-(1→4)*-O-*[α*-L-*fucopyranosyl-(1→3)]*-O-*(2-acetamido-2-deoxy-β*-D-*glucopyranosyl)-(1→3)*-O-β-D-*galactopyranosyl-(1→4)*-D-*[*N-*acetyl*-N-*(4-isothiocyanatophenyl)-1-amino-1-deoxy]-glucitol and its HSA conjugate* (**12**).—Compound **11** (4.8 mg, 2.5 μmol) was treated first with thiophosgene (5 μL) and then HSA (7.6 mg, 1/22 equiv), as described under the preparation of **9** to give conjugate **12** (10.2 mg), which in MALDI MS showed a broad molecular ion centered around m/z 86730, which corresponds to an average sugar incorporation of 11 haptens/HSA molecule.

Acknowledgements

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