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Chemoenzymatic synthesis of sialylated oligosaccharides for their evaluation in a polysialyltransferase assay

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Abstract—A series of sialylated β-D-Gal-(1→3)-α-D-GalNAc-octyl containing oligosaccharides representative of those found on mucin type complex *O*-glycans were synthesized by a chemoenzymatic approach for use in the kinetic characterization of recently cloned polysialyltransferases. Enzymatic incorporation of *N*-acetylneuraminic acid (sialic acid) into the synthetic acceptors was accomplished by 2,3-(*N*) and (*O*)-sialyltransferases to give the target compounds **6**–**10** in a practical yield.

Oligosaccharides on cell surfaces play an important role in many biological recognition events including cell–cell adhesion, bacterial attachment and viral infection.[1] The availability of oligosaccharides and their analogues as inhibitors can provide insight into their biological roles and might lead to the discovery of novel carbohydrate-based therapeutics.[2] Despite many advances in the chemical synthesis of oligosaccharides, it is still time-consuming and often difficult to synthesize these highly asymmetric and densely functionalized molecules.[3] Progress in recombinant DNA technology has made it possible to express large quantities of enzymes, including several glycosyltransferases that biosynthesize oligosaccharides. Many glycosyltransferases have been isolated and cloned are now readily available in quantities sufficient for their use in synthesis.[4] The uses of glycosyltransferases to prepare oligosaccharides offer a number of advantages: high regio- and stereoselectivity, no requirement for chemical protection of functional groups, and very mild reaction conditions.[5]

Numerous examples have been reported in which glycosyltransferases tolerate structural changes on both donor and acceptor substrates, thus making enzymatic synthesis a promising alternative for the preparation of both natural and unnatural oligosaccharides.[6]

Polysialic acid is a unique carbohydrate of a linear homopolymer of α-2,8-linked sialic acid, which may contain as many as 55 sialic acid residues per polymer.[7] Neural Cell Adhesion Molecules (NCAM) are highly polysialylated in embryonic tissues, although the majority of NCAM in the adult olfactory bulb and the hippocampus of adult brain contains polysialic acid where neuronal regeneration persists.[8] There is increasing evidence that polysialylated NCAM promotes cell migration and enhances neurite outgrowth and branching during development and neural regeneration.[9] Polysialic acid is thought to modulate the functional properties of NCAM by rendering it less adhesive to itself (homophilic binding)[10] or to other cell surface molecules (heterophilic binding). Taken together, these results strongly suggest that polysialylated NCAM plays a critical role in neural development and plasticity. Polysialic acid is present in mucin type *O*-glycans as shown in trout eggs.[11] Trisialylated core 1 disaccharide (β-D-Gal-(1→3)-α-D-GalNac-Ser/Thr) was also found in the human erythrocyte membrane.[12] However, it has not been shown which core structure(s) is a preferable acceptor for polysialyltransferases.

Herein, we describe the chemoenzymatic syntheses of the sialylated oligosaccharides **6**–**10**, which represent biosynthetic intermediates in the synthesis of polysialylated mucin type *O*-glycans (Fig. 1). These compounds are for use in kinetic studies of recently cloned polysialyltransferase, ST8SiaIV.[13] Our synthetic approach involves enzymatic incorporation of sialic acid residues to the synthetic di-, tri- and tetrasaccharide acceptors by recombinant rat liver Galβ-1,4/3-GlcNAcα-2,3-(*N*)-sialyltransferase (EC 2.4.99.5) and recombinant rat liver

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Galβ-1,3-GalNAcα-2,3-(*O*)-sialyltransferase (EC 2.4.99.4) (purchased from Calbiochem, USA) using cytidine-5′-monophospho-*N*-acetylneuraminic acid sodium salt (CMP-NeuAc) as sialic acid donor. The construction of branched Galβ-(1→3)-[NeuAcα-(2→6)]-GalNAcα-octyl trisaccharide **5b** was achieved by a chemical approach.

A few reports have appeared in the literature regarding the enzymatic synthesis of sialylated oligosaccharides.[14] Suitably protected TF-disaccharide acceptor **1** and deprotected mucin type core 2 tetrasaccharide acceptor **4** were prepared following the procedure reported earlier[15] (Scheme 1). Condensation of the sialic acid thioglycoside donor[16] **3** with the disaccharide diol acceptor **1** using dimethyl (methylthio)trifluoromethanesulfonate (DMTST) as glycosyl promoter in acetonitrile–dichloromethane (5:1) solvent at −10°C over 12 h gave the (2→6)-α-sialylated trisaccharide **5a** in 43% yield together with a minor amount of a (2→6)-β-sialylated trisaccharide (12%). On saponification of **5a** using wet sodium methoxide the deprotected (2→6)-α-sialylated TF-disaccharide **5b** was obtained in 62% yield.[17] The low yield in the glycosylation step may be due to the lower reactivity of the sialic acid donor as well as competition between glycosylation and elimination reactions. To increase the access of the donor to the acceptor site, a 4,6-diol acceptor was used instead of using only a 6-hydroxylated acceptor.

The enzymatic incorporations of sialic acid into compounds **2**, **4** and **5b** were achieved through the initial addition of the CMP-NeuAc donor along with rat liver Galβ-1,4-GlcNAc-α-2,3-(*N*)-sialyltransferase (EC 2.4.99.5), which selectively adds sialic acid to the 3-OH of the D-galactose residue linked to a (β-D-Gal-(1→4)-α-D-GlcNac) chain of the *N*-glycan structure,[18] or Galβ-1,3-GalNAc-α-2,3-(*O*)-sialyltransferase (EC 2.4.99.4), which selectively adds sialic acid to the 3-OH of the D-galactose residue linked to a core-1 chain (β-D-Gal-(1→3)-α-D-GalNac) of the *O*-glycan structure[19] at 37°C on a 1–2 μmol scale (Scheme 2). For the 2,3-(*O*)-sialyltransferase, the reaction was complete in 16–18 h,[20] while in the case of the 2,3-(*N*)-sialyltransferase, the reaction required from 30 min to 18 h depending upon the number of sialic acids incorpo-

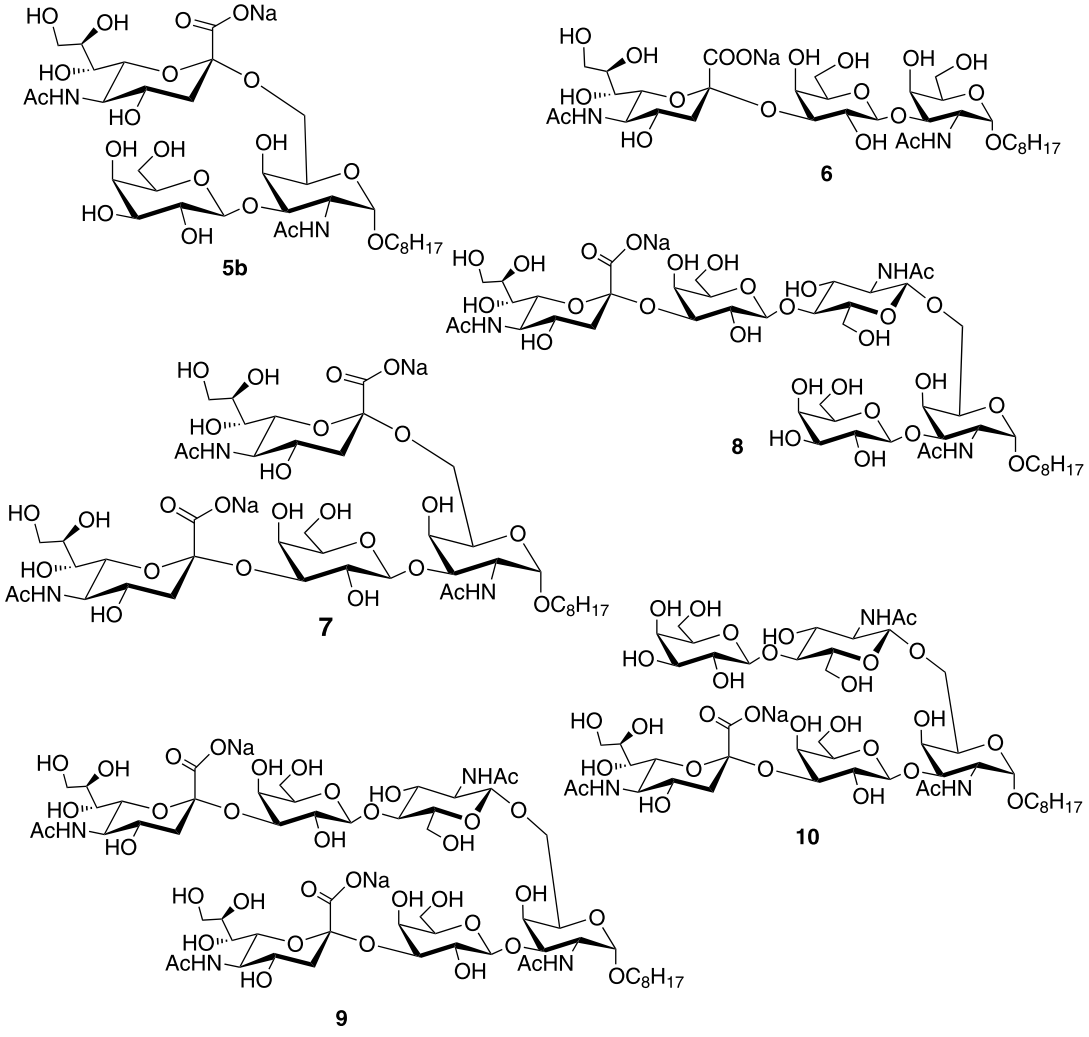
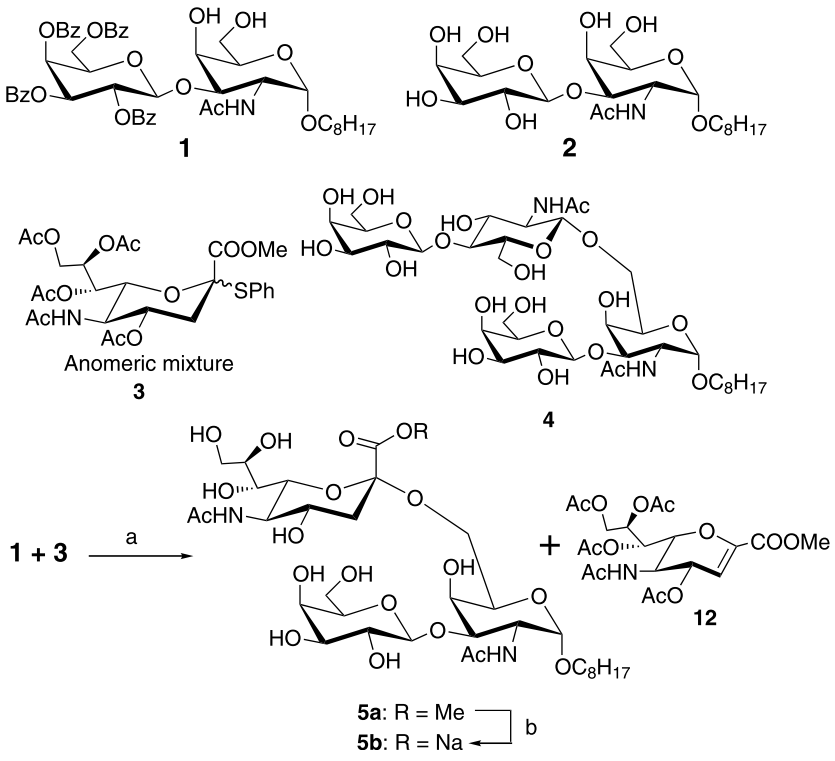
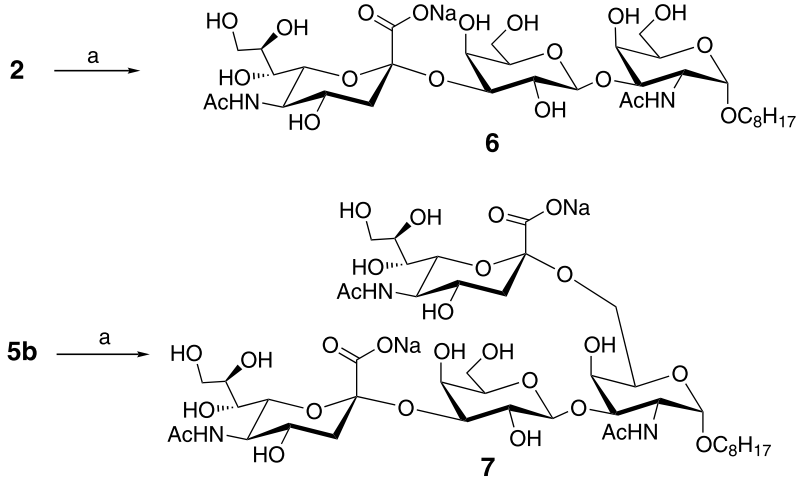


Figure 1. Structures of the compounds synthesized enzymatically.

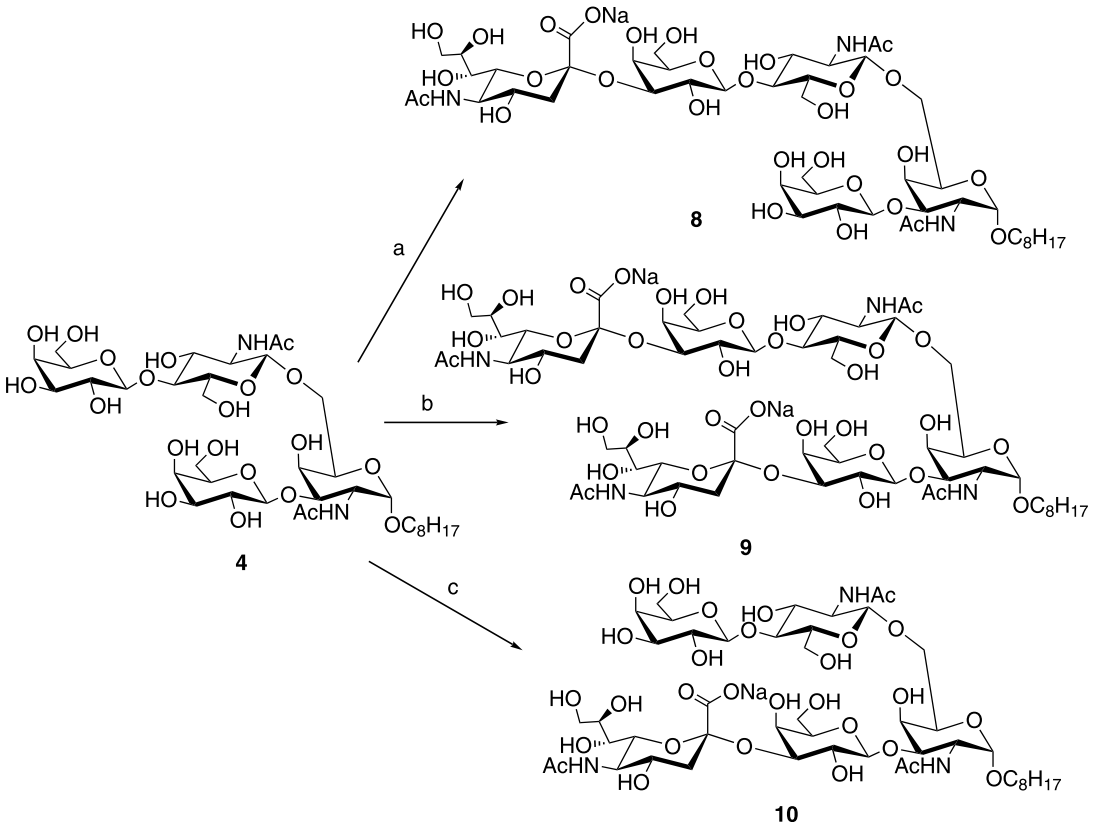


Scheme 1. *Reagents and conditions*: (a) DMTST, CH_3CN–CH_2Cl_2 (5:1), 3 Å MS, –10°C, 12 h, 43% together with 40% of **12**; (b) 0.5 M MeONa, MeOH, rt, 12 h, then a few drops of water, rt, 12 h, 62%.



Scheme 2. *Reagents and conditions*: Rat recombinant 2,3-α-(*O*)-sialyltransferase (0.25 mU), CMP-NeuAc (2.25 equiv.), sodium cacodilate, Triton, calf intestine alkaline phosphatase, 37°C, 16 h, quantitative.

rated.[21] An interesting point to note here is that, while attempting to prepare **8** by using the 2,3-(*N*)-sialyltransferase, the reaction seemed to be completed in only 30 min. When we allowed the reaction to proceed for a longer time, disialylated product **9** was obtained in a quantitative yield, as confirmed by TLC (^{i}PrOH:H_2O:NH_4OH = 8:2:0.2 as running solvent, R_f values for compounds **8**: 0.32, **10**: 0.26 and **9**: 0.5), NMR and MS analyses. In order to confirm the formation of product **9**, two control experiments were performed. For one, the further incorporation of sialic acid to the compound **10** (obtained from the reaction of CMP-sialic acid and acceptor **4** in presence of 2,3-(*O*)-sialyltransferase) was done by using 2,3-(*N*)-sialyltransferase and CMP-sialic acid, while in the other case, the same reaction was performed using compound **8** (obtained from the reaction of CMP-sialic acid and acceptor **4** in the presence of 2,3-(*N*)-sialyltransferase), CMP-sialic acid and 2,3-(*O*)-sialyltransferase (Scheme 3). In both cases **9** was isolated as the sole product (TLC, MS and NMR analyses). This result can be explained based on the extremely high



Scheme 3. *Reagents and conditions*: (a) Rat recombinant 2,3-α-(*N*)-sialyltransferase (0.025 mU), CMP-NeuAc (1.4 equiv.), MOPS buffer, BSA, Triton, calf intestine alkaline phosphatase, 37°C, 30 min, 87%; (b) Rat recombinant 2,3-α-(*N*)-sialyltransferase (0.25 mU), CMP-NeuAc (2.25 equiv.), MOPS buffer, BSA, Triton, calf intestine alkaline phosphatase, 37°C, 16 h, quantitative; (c) Rat recombinant 2,3-α-(*O*)-sialyltransferase (0.25 mU), CMP-NeuAc (2.25 equiv.), sodium cacodilate, Triton, calf intestine alkaline phosphatase, 37°C, 18 h, quantitative.

reactivity of 2,3-(*N*)-sialyltransferase in comparison with that of 2,3-(*O*)-sialyltransferase. All the sialylated oligosaccharides **5b**–**10** were purified using C_{18}-SepPak cartridges and LH-20 sephadex gel filtration. Their NMR and MS[22] spectra supported the structures of all the products.

These synthetic oligosaccharides and NeuAcα-(2→3)-Galβ-(1→4)-GlcNAcβ-1→octyl **11**[23] were then incubated with a soluble form of ST8Sia IV and a donor substrate CMP-[^{3}H]-NeuAc.[24] After the reaction, the incubation mixture was directly applied to a column of Mono-Q anion exchange chromatography and eluted with a linear gradient of increasing concentrations of NaCl as described previously.[25,26] Surprisingly, compounds **5b** (49%) and **10** (65%) served as better acceptors than NeuAcα-(2→3)-Galβ-(1→4)-GlcNAcβ1-octyl **11** (both in the relative reaction rates). Interestingly, all these acceptors produced polysialic acid with 35–45 sialic acid residues, which is similar to that obtained on NCAM.[26] These results indicate that both α-(2,3)-linked and α-(2,6)-linked sialic acid on *O*-glycans serve as efficient precursors. Similarly, we have previously found that both α-(2,3)- and α-(2,6)-linked sialic acids on *N*-glycans serve as efficient precursors.[25] In the human erythrocyte membrane, the second sialic acid is added to both compounds **5b** and **10**, which is consistent with the results obtained in the previous study.[12] By contrast, in trout eggs, polysialic acid is added only to compound **5b**.[11] These results suggest either that polysialylation in trout eggs is unique or polysialic acid can be added to the α-(2,3)-sialylated core 1 if the core 2 branch is also present as seen in compound **10**.

In conclusion, chemoenzymatic syntheses of six mono- and disialylated mucin type *O*-glycans (**5b**–**10**) were performed by addition of sialic acid in a stepwise manner or in a single step using the regio- and stereoselectivity of two sialyltransferase enzymes (Fig. 2). The results obtained using these oligosaccharides indicate that ST8Sia IV acts on mucin-type *O*-glycans as efficiently as on *N*-glycans, and exhibits minimum discrimination towards different linkages of sialic acids on acceptors.

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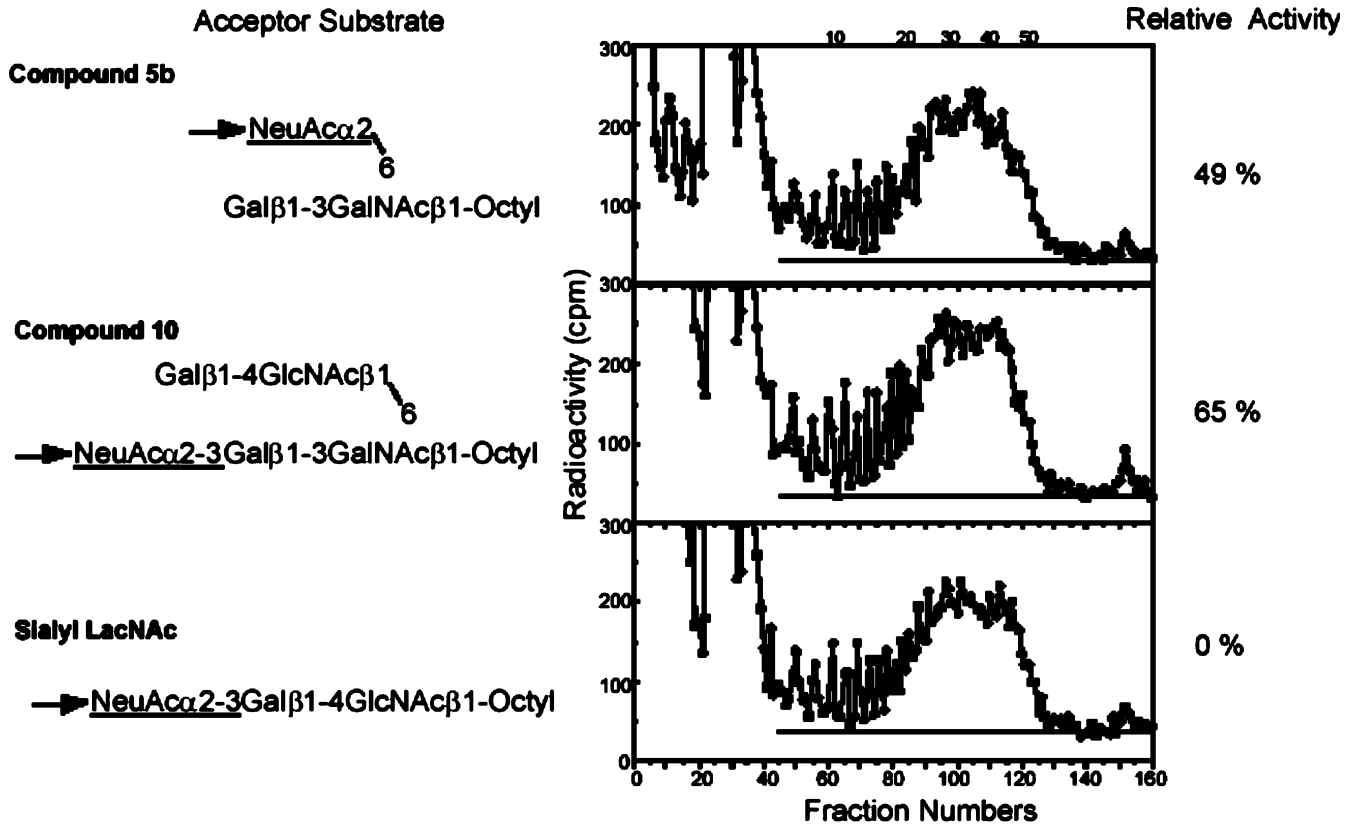

Figure 2. Addition of polysialic acid to compounds **5b**, **10** and NeuAc-α-(2-3)-Gal-β-(1-4)-GlcNAcβ-octyl **11**. Each compound (1.0 nmol each) was incubated with CMP-[^{14}C]-NeuAc and the soluble form of ST8SiaIV for 20 h at 37°C. The reaction products were directly subjected to Mono-Q anion exchange chromatography. The elution position for polysialic acid containing multiple numbers of sialic acids are shown as numbers above the chromatogram as described previously.[25,26] Sialic acid residues in acceptor molecules are underlined.

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17. Synthesis of **5b**: A solution of **1** (300 mg, 0.33 mmol), **3** (385 mg, 0.66 mmol) and freshly activated MS 3 Å (500 mg) in CH_3CN–CH_2Cl_2 (5:1; 6 mL) was stirred under Ar at room temperature for 1 h. The reaction mixture was cooled to −10°C and DMTST (682 mg, 2.64 mmol) was added. After stirring at −10°C for 12 h the reaction mixture was diluted with CH_2Cl_2 and filtered through Celite. The organic layer was washed with satd $NaHCO_3$, water and dried (Na_2SO_4) and concentrated. Column chromatography on SiO_2 using toluene–acetone (5:1)

gave pure 2,6-α-sialylated product **5a** (194 mg, 43%) along with some 2,6-β-sialylated product (12%). The protected trisaccharide (194 mg, 0.14 mmol) thus obtained was dissolved in 50 mM NaOMe in MeOH (5 ml) and stirred for 12 h at room temperature. A few drops of water was added to the reaction mixture which was then allowed to stir for another 12 h. The reaction mixture was neutralized with Amberlite IR-120 (H^+) resin, filtered and purified on LH-20 sephadex gel using MeOH as solvent to afford pure **5b** (70 mg, 62%).

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20. Enzymatic glycosylation using 2,3-(*O*)-sialyltransferase for the synthesis of **6**, **7** or **10**: To a solution of compound **2**, **4** or **5b** (2.0 μmol) in 50 mM sodium cacodylate buffer (pH 6.0; 100 μL) was added recombinant rat liver Galβ-1,3-GalNAcα-2,3-(*O*)-sialyltransferase (0.25 mU), CMP-NeuAc (3.0 mg, 4.50 μmol), Triton CF-54 (0.1%), calf intestinal alkaline phosphatase (5.0 U) and the solution was kept at 37°C for 16 h. The reaction mixture was passed through a QAE mini column to remove the side products formed and the product was isolated on a C_{18}-SepPak cartridge, which was pre-washed with water. The product was eluted with methanol. The methanolic fractions were concentrated and further purified on sephadex LH-20 to give pure **6**, **7** or **10**.
21. Enzymatic glycosylation using 2,3-(*N*)-sialyltransferase for the synthesis of **8** and **9**: To a solution of compound **4** (1.7 mg, 2.0 μmol) in 50 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS) buffer (pH 7.4; 100 μL) was added recombinant rat liver Galβ-1,4-GlcNAcα-2,3-(*N*)-sialyltransferase (0.025 mU for compound **8** and 0.25 mU for compound **9**), CMP-NeuAc (3.0 mg, 4.50 μmol for compound **9** and 1.8 mg, 2.70 μmol for compound **8**), Triton CF-54 (0.2%), calf intestinal alkaline phosphatase (5.0 U) and bovine serum albumin (1.0 mg) and the solution was kept at 37°C for 30 min to give **8** and for 16 h to give **9**. The reaction mixture was passed through a QAE mini column to remove the side products and the product was isolated on a C_{18}-SepPak cartridge, which was pre-washed with water. The product was eluted with methanol. The methanolic fractions were concentrated and further purified on sephadex LH-20 to give pure **8** or **9**.
22. Partial ^{1}H NMR (500 MHz, D_2O): The following common signals for the octyl aglycon were observed in D_2O soln: δ 1.60–1.40 (2H, m, OCH_2CH_2), 1.40–1.10 (10H, m, $OCH_2CH_2(CH_2)_5CH_3$), 0.85 (t, $J=6.9$ Hz, 3H, octyl CH_3). H-1 indicates the anomeric proton of the GalNAc residue, H-1′ the anomeric proton of the Gal residue linked to O-3 of the GalNAc residue and H-1″ the anomeric proton of the GlcNAc residue linked to O-6 of the GalNAc residue and onwards. Compound **5b**: δ 4.43 (d, $J=8.4$ Hz, 1H, H-1′), 4.28 (d, $J=3.6$ Hz, 1H, H-1), 2.72 (dd, $J=12.5$, 4.8 Hz, 1H, H-3$'_e$), 1.99 and 2.00 (2s, 6H, 2 NH*Ac*), 1.64 (t, $J=12.5$ Hz, 1H, H-3$'_a$); TOFMS m/z for $C_{33}H_{57}O_{19}N_2Na$ (found) 808.7 (calcd) 808.7. Compound **6**: δ 4.92 (d, $J=3.0$ Hz, 1H, H-1), 4.57 (d, $J=8.0$ Hz, 1H, H-1′), 2.77 (dd, $J=12.5$, 5.0 Hz, 1H, H-3$''_e$), 2.01–2.02 (2s, 6H, 2 NH*Ac*), 1.81 (t, $J=12.0$ Hz, 1H, H-3$'_a$); TOFMS m/z for $C_{33}H_{57}O_{19}N_2Na$ (found) 808.6, (calcd) 808.7. Compound **7**: δ 4.90 (d, $J=3.3$ Hz, 1H, H-1), 4.56 (d, $J=7.8$ Hz, 1H, H-1′), 2.78–2.82 (m, 2H, H-3$''_e$ and H-3$'''_e$), 2.01–2.04 (3s, 9H, 3 NH*Ac*), 1.68–1.82 (m, 2H, H-3$''_a$ and H-3$'''_a$); TOFMS m/z for $C_{44}H_{73}O_{27}N_3Na_2$ (found) 1122.0 (calcd) 1122.0. Compound **8**: δ 4.88 (d, $J=4.0$ Hz, 1H, H-1), 4.57 (d, $J=7.5$ Hz, 1H, H-1‴), 4.55 (d, $J=7.5$ Hz, 1H, H-1″), 4.48 (d, $J=7.5$ Hz, 1H, H-1′), 2.77 (dd, $J=12.0$, 4.5 Hz, 1H, H-3$'''_e$), 2.03–2.05 (3s, 9H, 3 NH*Ac*), 1.82 (t, $J=12.5$ Hz, 1H, H-3$'''_a$); TOFMS m/z for $C_{47}H_{80}O_{29}N_3Na$ (found) 1174.1 (calcd) 1174.1. Compound **9**: δ 4.86 (d, $J=3.9$ Hz, 1H, H-1), 4.53 (d, $J=7.5$ Hz, 1H, H-1‴), 4.52 (d, $J=7.5$ Hz, 1H, H-1″), 4.46 (d, $J=7.5$ Hz, 1H, H-1′), 2.73–2.75 (m, 2H, H-3$''''_e$ and H-3$''''_e$), 1.99–2.03 (4s, 12H, 4 NH*Ac*), 1.74–1.81 (m, 2H, H-3$''''_a$ and H-3$''''_a$); TOFMS m/z for $C_{58}H_{96}O_{37}N_4Na_2$ (found) 1487.4 (calcd) 1487.4. Compound **10**: δ 4.87 (d, $J=3.9$ Hz, 1H, H-1), 4.54 (d, $J=7.5$ Hz, 1H, H-1‴), 4.52 (d, $J=7.5$ Hz, 1H, H-1″), 4.45 (d, $J=7.5$ Hz, 1H, H-1′), 2.73 (dd, $J=12.5$, 5.0 Hz, 1H, H-3$'''_e$), 1.99–2.01 (3s, 9H, 3 NH*Ac*), 1.77 (t, $J=12.5$ Hz, 1H, H-3$'''_a$); TOFMS m/z for $C_{47}H_{80}O_{29}N_3Na$ (found) 1174.1 (calcd) 1174.1.
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