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Convenient enzymatic synthesis of a *p*-nitrophenyl oligosaccharide series of sialyl *N*-acetyllactosamine, sialyl Le^x and relevant compounds

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Abstract—From the β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**1**) prepared by the transglycosylation of β-galactosidase from *Bacillus circulans*, α-D-Neu5Ac-(2→3)-β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**9**) and α-D-Neu5Ac-(2→6)-β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**10**) were effectively synthesized with an equimolar ratio of CMP-Neu5Ac by recombinant rat α-(2→3)-*N*-sialyltransferase and rat liver α-(2→6)-*N*-sialyltransferase, respectively. The former enzyme also transferred effectively the Neu5Ac residue from CMP-Neu5Ac to the location of OH-3 in the non-reducing terminal of β-D-Gal-(1→4)-β-D-Gal-$OC_6H_4NO_2$-*p* or β-D-Gal-(1→4)-β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p*, while the latter enzyme did not. In the case of equimolar ratio of GDP-Fuc/acceptor, **1** and **9** were further fucosylated quantitatively to form β-D-Gal-(1→4)-β-D-(α-L-Fuc-(1→3)-)-GlcNAc-$OC_6H_4NO_2$-*p* (**14**) and α-D-Neu5Ac-(2→3)-β-D-Gal-(1→4)-β-D-(α-L-Fuc-(1→3)-)-GlcNAc-$OC_6H_4NO_2$-*p* (**13**) by recombinant human α-(1→3)-fucosyltransferase VII, respectively.

Keywords: Enzymatic synthesis; Sialyl Lewis X; Lewis X

1. Introduction

The oligosaccharides found in glycoconjugates and glycoproteins play crucial roles in a variety of biological events, for example, immunological responses, cellular recognition and host–toxin interactions.[1,2] Sialyl Lewis^x (sLe^x) tetrasaccharide is considered to serve as a common ligand of the three members of selectins, namely, E-, P- and L-selectins, which are involved in the biological events of inflammation and metastasis.[3,4] Sialyloligosaccharides and sialylglycoproteins also serve as the cell-surface receptor determinants of pathogenic bacteria, viruses, bacterial toxins and carbohydrate-recognizing proteins like lectins.[5–7] As the understanding of these biological functions increases, the need for practical synthetic procedures of oligosaccharides and their analogs in large quantities has become a major subject. Practical syntheses potentially contribute to studies on the molecular mechanisms of carbohydrate-mediated biological processes. In the literature, several synthetic approaches have been proposed to sialyl-*N*-acetyllactosamine, sLe^x and their related derivatives.[8–12] Among them, enzymatic and chemo-enzymatic methods seem to have received increasing attention from the viewpoint of green chemistry.[13] They are advantageous over their chemical counterparts also in terms of efficiency in obtaining the desired oligosaccharides without using protecting groups. Moreover, the problem of controlling the anomeric configuration is eliminated because the enzymatic glycosylation is usually stereospecific to provide a well-defined glycosidic linkage.

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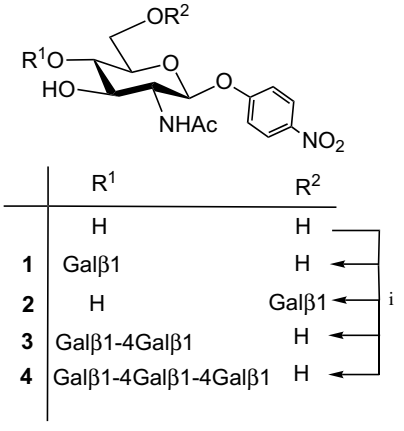
Recently, we reported the chemo-enzymatic synthesis of sialyl-α-(2→3)-LacNAc by using α-(2→3)-(*N*)-sialyltransferase.[14] In our chemo-enzymatic synthesis, a 3-aminopropyl group is introduced persistently at the reducing terminal sugar to give the corresponding β-glycosides. These functional groups are valuable for immobilization onto sensor chip surfaces and fabrication of glycomaterials. For instance, the *p*-aminophenyl group can be converted to thiolated compounds for this purpose.[15]

In this paper, we describe a convenient synthesis of *p*NP sLe^x and Le^x, which utilizes *p*NP monosaccharides as the starting sugars and the multiple enzymes of β-galactosidase, sialyltransferases and fucosyltransferase for their assembly. Obviously, these *p*NP-based oligosaccharides are designed for the study on specific carbohydrate–carbohydrate and carbohydrate–protein interactions with a quartz crystal microbalance or surface plasmon resonance methods.[16–19]

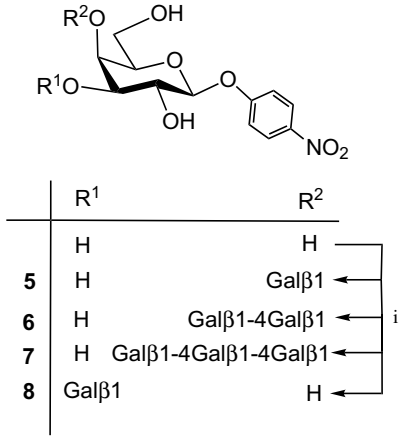
2. Results and discussion

2.1. Preparation of *p*NP galactosylated substrates for sialyltransferases

The glycosyl acceptors for sialylation were prepared by use of the transglycosylation of β-galactosidase from *Bacillus circulans* in a similar way to the previous reports.[20–22] As our initial approach, the commercially available β-galactosidase used here was partially purified because it contained high β-*N*-acetylhexosaminidase (NAHase) activity. When β-D-GlcNAc-$OC_6H_4NO_2$-*p* is applied as a glycosyl acceptor as shown in Scheme 1, the enzyme preparation should be free of the β-*N*-acetylhexosaminidase (NAHase) activity that might hydrolyze the acceptor used. Therefore, the crude enzyme was partially purified to remove the NAHase activity by hydrophobic interaction chromatography on a column of highly substituted Phenyl Sepharose 6 Fast Flow. This simple and convenient purification enabled us to synthesize a key substrate **1** in large scale. To the solution of β-D-GlcNAc-$OC_6H_4NO_2$-*p* (1.0 g) and lactose (4.0 g) dissolved in 35 mL of sodium phosphate buffer (20 mM, pH 6.8) containing 50% acetonitrile was added the partially purified β-galactosidase (3.2 U). The progress of the reaction at 30 °C was monitored by an HPLC fitted with an Amide-80 column and a UV detector, showing that four new peaks appeared. The reaction mixture was stopped by heating at 95 °C for 2 min and each product was purified with ODS (C-18), Toyopearl HW-40S and Bio-Gel P-2, successively, to yield transfer products **1**–**4** in the order of **1** > **3** > **4** ≈ **2**. Apparently, **3** and **4** are produced by further regioselective galactosylation from **1** formed initially. The structure of galactosyl trisaccharide **3** was easily confirmed by ^{1}H NMR spectroscopy [δ_H 5.30 ($J_{1,2}$ 7.8 Hz), 4.57 ($J_{1'',2''}$ 7.8 Hz), 4.51 ($J_{1',2'}$ 7.8 Hz); δ_C 79.9 (C-4) and 78.9 (C-4′)], which was accorded well with the data of the previous report.[20] Similarly, the structure of tetrasaccharide **4** was also confirmed by ^{1}H NMR [δ 5.31 ($J_{1,2}$ 8.4 Hz), 4.63 ($J_{1''',2'''}$ 7.8 Hz), 4.57 ($J_{1'',2''}$ 8.4 Hz), 4.52 ($J_{1',2'}$ 7.8 Hz)] and ^{13}C NMR spectra [δ 79.9 (C-4), 79.3 (C-4′) and 78.9 (C-4″)]. These ^{13}C NMR signals were shown to be clearly shifted downfield compared to other carbons (δ 56.6–76.8 ppm). FABMS analysis supports the structures. In a similar analysis, self-condensed galactosyl oligosaccharides (**6** and **7**) were formed by successive regioselective galactosylation through the initially formed major intermediate **5** from β-D-Gal-$OC_6H_4NO_2$-*p* which was applied as a common glycosyl donor and acceptor (Scheme 2). The structure of *p*NP galactosylated trisaccharide **6** was confirmed compared to the previous data.[23] The peaks appearing at δ 5.22



Scheme 1. Reagents and conditions: (i) Partially purified *B. circulans* β-galactosidase and lactose in 20 mM sodium phosphate buffer (pH 6.8) containing 50% acetonitrile.



Scheme 2. Reagents and conditions: (i) Partially purified *B. circulans* β-galactosidase and β-D-Gal-$OC_6H_4NO_2$-*p* in 20 mM sodium phosphate buffer (pH 6.8) containing 50% acetonitrile.

($J_{1,2}$ 7.2 Hz), 4.66 ($J_{1''',2'''}$ 8.4 Hz), 4.62 ($J_{1'',2''}$ 7.8 Hz) and 4.56 ($J_{1',2'}$ 7.8 Hz) in the ^{1}H NMR spectrum of tetrasaccharide **7** were shown to be all β-linkage. The ^{13}C NMR signals also revealed the structure **7** [δ 79.3 (C-4), 79.0 (C-4′) and 78.8 (C-4″)], which was downshifted compared to other carbons.

2.2. Substrate specificity in sialyltransferase reaction for galactosylated oligosaccharides

The synthetic galactosylated acceptors **1**–**3**, **5**, **8**, Galβ-(1→4)-Glcβ-$OC_6H_4NO_2$-*p*[24] and Galβ-(1→3)Glcβ-$OC_6H_4NO_2$-*p*[24] were then subjected to enzyme-catalyzed sialylation with two different enzymes, recombinant rat α-(2→3)-*N*-sialyltransferase (α-2,3-NST) and rat liver α-(2→6)-(*N*)-sialyltransferase (α-2,6-NST). The relative activities are summarized as shown in Table 1. The results are in good agreement with the specificity reported for both enzymes (entries 1, 2, 6 and 7).[25–28] α-2,6-NST shows limited acceptor specificity to type 2 structure (Galβ(1→4)GlcNAc) (entries 2 and 6), while α-2,3-NST shows a relatively broader specificity as compared to α-2,6-NST (entries 1, 2, 6 and 7). It has been reported that α-2,3-NST is capable of using terminal Galβ(1→4)Glc and both type 2 and type 1 (Galβ(1→3)GlcNAc) structures as acceptors. The present results demonstrated that even the GlcNAc residues in type 2 and type 1 could be replaced by the Gal residue (entries 4 and 5) although the replacement resulted in a decrease of activity. The relative rate for **3** was 29 when the rate of **1** was arbitrarily set at 100 for α-2,3-NST. It indicates that trisaccharide **3** further galactosylated at *O*-4 in **1** could be acceptable for α-2,3-NST (entry 3).

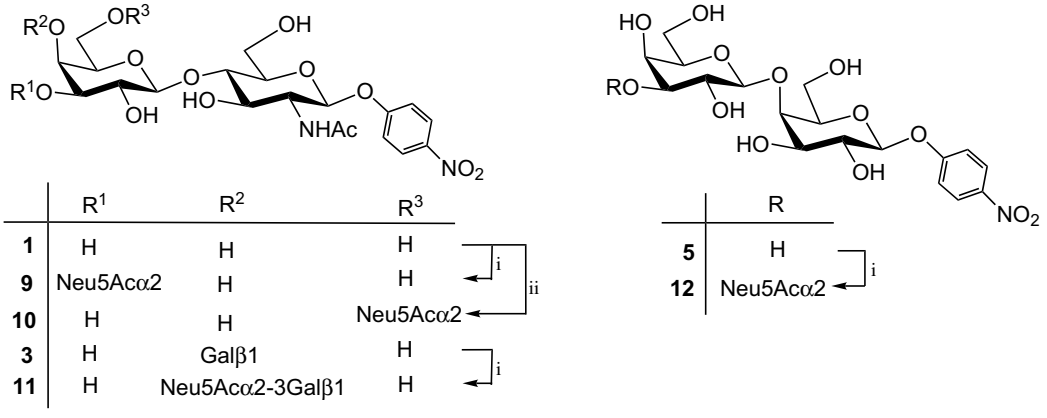
Sialylation of **1** was carried out with α-2,3-NST and α-2,6-NST to afford **9** and **10**, respectively (Scheme 3). Enzymatic synthesis was performed in the following way: a solution of **1** (8 mg, 15.8 μmol), CMP-Neu5Ac (10.5 mg, 16.0 μmol) in 40 mM MES (2-morpholinoethanesulfonic acid) buffer (pH 6.3, 0.8 mL) containing BSA (1.2 mg/mL) and 20 mM $MnCl_2$, 25 U alkaline phosphatase, and α-2,3-NST (20 mU) was incubated at 30 °C. The reaction was monitored by HPLC. After 48 h of incubation, HPLC analysis revealed that most of **1** was sialylated. The reaction mixture was purified by chromatography on ODS (15% acetonitrile containing 0.1% TFA as the eluent) and Bio-Gel P-2 columns to give **9** in 91% yield based on the donor. Similarly, **10** was obtained in excellent yield (93% based on donor) with α-2,6-NST as catalyst. In both cases, **1**

Table 1. Relative rates[a] of transfer Neu5Ac to synthetic acceptors with CMP-Neu5Ac as donor by recombinant rat α-2,3-NST and rat liver α-2,6-NST

Entry	Acceptor	Relative rate	
		α-2,3-NST	α-2,6-NST
1	**1**	100	100
2	**2**	0	0
3	**3**	29	0
4	**5**	48	0
5	**8**	10	0
6	Galβ(1→4)Glcβ-OR[b]	45	3
7	Galβ(1→3)Glcβ-OR[b]	97	0

[a] The activities were assayed with synthetic acceptors in the following way: a solution (200 μL) of 1 mM acceptor, 1 mM CMP-Neu5Ac, and α-2,3-NST (400 μU) or α-2,6-NST (400 μU) in 40 mM MES buffer (pH 6.3) containing 1.0 mg/mL BSA and 20 mM $MnCl_2$ was incubated at 30 °C. Samples (20 μL) were taken out at appropriate time intervals (0, 15, 30, 60 and 90 min) during the incubation, and were heated over water bath at 95 °C for 2 min to stop each enzyme reaction. Every sample was then analyzed by HPLC with a column of TSKgel ODS-80Ts (4.6 × 250 mm) eluted with 15% acetonitrile in 10 mM sodium phosphate buffer (pH 7.1) at a flow rate of 1.0 mL/min and with a UV detector monitored at 300 nm. The relative rates on **1** for both enzymes are arbitrarily set at 100.

[b] R=$C_6H_4NO_2$-*p*. Galβ(1→4)Glc-OR and Galβ(1→3)Glc-OR were prepared by β-galactosidase from *B. circulans* according to the previously reported method.[24]



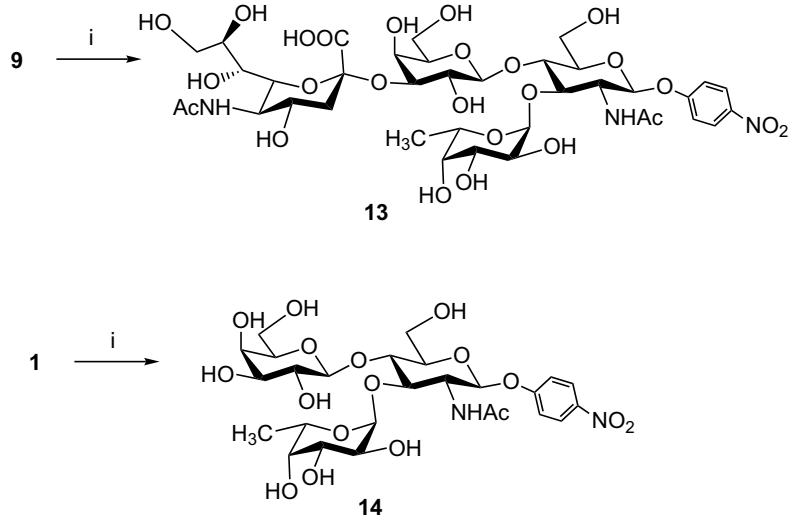
Scheme 3. Reagents and conditions: (i) Recombinant rat α-(2→3)-(*N*)-sialyltransferase, CMP-Neu5Ac, alkaline phosphatase in 40 mM MES buffer (pH 6.3) containing BSA and 20 mM $MnCl_2$ at 30 °C. (ii) Rat liver α-(2→6)-(*N*)-sialyltransferase, and other reagents and conditions are the same as (i).

was effectively sialylated although the donor CMP-Neu5Ac was used in an equimolar ratio to the acceptor. When **3** was used as the acceptor instead of **1**, HPLC chromatogram showed that one new peak with UV absorbance at 300 nm appeared in the reaction mixture with α-2,6-NST as catalyst. The new compound was purified and determined by NMR spectroscopy to be tetrasaccharide **11**. The downfield shift for galactose H-3″ at δ 4.09 ppm in the ^{1}H NMR spectrum and the characteristic peaks (δ 2.74 and 1.77 ppm for Neu5Ac H-3, δ 41.4 ppm for Neu5Ac C-3) of Neu5Ac residue in the ^{1}H and ^{13}C NMR spectra confirmed the structure of **11**. Similarly, **5** could be sialylated by α-2,3-NST to afford **12**. In the case of an equimolar ratio of acceptor/donor, **11** and **12** were obtained in yield of 72% and 76%, respectively. In contrast, none of the transfer products was detected when applying **3** and **5** with the α-2,6-NST as catalyst. These results indicate that the α-2,3-NST has broader specificity to **1**, **3**, **5** and **8**, while the α-2,6-NST could accept only **1**, not **2**–**5**.

2.3. Synthesis of sLe^{x}-OC$_6$H$_4$NO$_2$-*p* (13) and Le^{x}-OC$_6$H$_4$NO$_2$-*p* (14) oligosaccharides

Recombinant human α-(1→3)-fucosyltransferase VII (Fuc-T VII) has an extremely limited acceptor specificity restricted to 3′-sialylated type 2 oligosaccharides.[29,30] We examined the substrate specificity of this enzyme towards sialyl lactosamine **9** and lactosamine **1** (Scheme 4). The trisaccharide **9** was successfully fucosylated by incubation with GDP-Fuc, the α-(1→3)-fucosyltransferase and alkaline phosphatase in HEPES buffer (50 mM, pH 7.4) containing BSA (1.5 mg/mL) and $MnCl_2$ (15 mM) at 30 °C, producing **13** in a quite good yield. When using an equimolar ratio of acceptor and donor, **9** was fucosylated at O-3 of the GlcNAc residue almost quantitatively. The structure of **13** was determined by NMR spectroscopy. The characteristic peaks at δ 5.12 ppm (J 4.2 Hz) for the fucose H-1, 4.82 ppm for the fucose H-5, and 1.16 ppm (J 6.6 Hz) for the fucose H-6 in the ^{1}H NMR spectrum and peaks at δ 100.4 ppm for the fucose C-1 and 17.0 ppm for the fucose C-6 in the ^{13}C NMR spectrum evidenced the presence of the fucose residue to **9** through an α linkage. In the ^{13}C NMR spectrum, the signal of GlcNAc C-3 appearing at 76.6 ppm shifted down-field from 73.5 ppm in that of **9**, while the signal of GlcNAc C-4 appearing at 74.6 ppm shifted up-field from 79.5 ppm in that of **9**. These results indicate that the fucose has been transferred regioselectively onto GlcNAc OH-3 of **9**. Surprisingly, **1** was also fucosylated by human recombinant Fuc-T VII to give **14** in an excellent yield (91%), while **14** is obtained in a yield of 20% based on **1** (14% based on donor GDP-Fuc) by partially purified chicken serum α-(1→3)-fucosyltransferase.[31] Apparently, this Fuc-T VII in the present study is a valuable enzyme for the efficient synthesis of both sLe^{x}-OC$_6$H$_4$NO$_2$-*p* and Le^{x}-OC$_6$H$_4$NO$_2$-*p*.

In conclusion, a series of *p*NP sialyl oligosaccharides (**9**–**14**) were synthesized efficiently by combination with β-galactosidase, sialyltransferases and/or fucosyltransferase. These synthetic compounds will be very useful for enzyme kinetic analysis or activity assay, synthesis of artificial glycopolymers and investigation of carbohydrate-mediated biological functions with a surface plasmon resonance technique, a quartz crystal microbalance and other cell-free assay systems.



Scheme 4. Reagents and conditions: (i) Recombinant human α-(1→3)-fucosyltransferase VII, GDP-Fuc, and alkaline phosphatase in HEPES buffer (50 mM, pH 7.4) containing BSA and 15 mM $MnCl_2$ at 30 °C.

3. Experimental

3.1. General methods

Recombinant rat α-(2→3)-*N*-sialyltransferase and recombinant human α(1→3)-fucosyltransferase VII were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA) and used without further purification. Rat liver α-(2→6)-*N*-sialyltransferase was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). CMP-Neu5Ac sodium salt and GDP-Fuc were obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). β-Galactosidase from *B. circulans* (Biolacta) was a gift from Daiwa Kasei Co., Ltd. (Osaka, Japan). Optical rotations were measured with a JASCO DIP-1000 digital polarimeter at ambient temperature, using a 10-cm micro cell. The ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) spectra were recorded on a JEOL LA-600 spectrometer for solutions in D_2O. Chemical shifts are given in ppm and referenced to internal *tert*-butyl alcohol (δ 1.23 in D_2O or δ 31.2 in D_2O). All data are assumed to be first order with apparent doublet and triplets reported as d and t, respectively. Resonances that appear broad are designated b. FAB mass spectra (FABMS) were recorded using a JEOL DX 303 mass spectrometer.

3.2. Purification of β-galactosidase from *B. circulans*

The crude β-galactosidase from *B. circulans* (Biolacta, 50 mg) was dissolved in sodium phosphate buffer (20 mM, pH 6.8) containing 0.4 M ammonium sulfate. The supernatant was loaded onto a phenyl-Sepharose 6FF column equilibrated with the same buffer as described above. The column was developed with a linear decrease of ammonium sulfate concentration (0.4–0 M) in sodium phosphate buffer. The β-galactosidase was eluted at the beginning of the gradient. The fractions containing β-galactosidase activity without NAHase activity were collected and concentrated to a small volume by Amico Diaflo Unit with a PM10 membrane.

3.3. β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (1), β-D-Gal-(1→6)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (2), β-D-Gal-(1→4)-β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (3) and β-D-Gal-(1→4)-β-D-Gal-(1→4)-β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (4)

A reaction mixture (35 mL) of β-D-GlcNAc-$OC_6H_4NO_2$-*p* (1.0 g), lactose (4.0 g) and partially purified β-galactosidase (3.2 U) in sodium phosphate buffer (20 mM, pH6.8) containing 50% acetonitrile was incubated at 30 °C. After 24 h incubation, the reaction mixture was stopped by heating in a boiling water bath for 5 min. The supernatant from the centrifugation was applied to a column (2.5 × 45 cm) of ODS equilibrated with 12% MeOH in water, and the column was eluted with the same solution. The fractions containing the transfer products were collected and concentrated to a small volume for further purification. The concentrated solution was loaded onto a column (4 × 50 cm) of Toyopearl HW-40S equilibrated with 25% methanol in water, and the column was eluted with the same solution. The fractions (tubes 46–50, 20 mL/tube; tubes 52–59) were collected, concentrated and dried to afford **3** (103.7 mg) and **1** (265.1 mg), respectively. Other fractions (tubes 41–44) were further purified, then the fractions were collected, concentrated and loaded in a column (2.0 × 100 cm) of Bio-Gel P-2, and the column was eluted with water at a flow rate of 0.8 mL/min. The transfer products **2** (11.2 mg) and **4** (24.5 mg) were obtained. The physical and NMR data for compounds **1**–**3** were identical to those of β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p*, β-D-Gal-(1→6)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* and β-D-Gal-(1→4)-β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p*, respectively.[20] Data for **4**: ^{1}H NMR, δ 8.21 (d, *J* 9.0 Hz, 2H, *o*-Ph), 7.16 (d, *J* 9.0 Hz, 2H, *m*-Ph), 5.31 (d, *J* 8.4 Hz, 1H, H-1), 4.63 (d, *J* 7.8 Hz, 1H, H‴-1), 4.57 (d, *J* 8.4 Hz, 1H, H″-1), 4.52 (d, *J* 7.8 Hz, 1H, H′-1) and 1.99 (s, 3H, NHAc); ^{13}C NMR, δ 79.9, 79.3 and 78.9 (C-4, C-4′ and C-4″); $[\alpha]_D$ −86.5 (0.53, H_2O); FABMS 829 *m*/*z* for $[M+H]^+$.

3.4. β-D-Gal-(1→4)-β-D-Gal-$OC_6H_4NO_2$-*p* (5), β-D-Gal-(1→4)-β-D-Gal-(1→4)-β-D-Gal-$OC_6H_4NO_2$-*p* (6), β-D-Gal-(1→4)-β-D-Gal-(1→4)-β-D-Gal-(1→4)-β-D-Gal-$OC_6H_4NO_2$-*p* (7) and β-D-Gal-(1→3)-β-D-Gal-$OC_6H_4NO_2$-*p* (8)

β-D-Gal-$OC_6H_4NO_2$-*p* (500 mg) was incubated with partially purified β-galactosidase (5 U) in 1.8 mL of sodium phosphate buffer (20 mM, pH 6.8) containing 50% acetonitrile at 30 °C. After 5 h incubation, the reaction mixture was stopped and passed through columns of ODS, Toyopearl HW-40S and Bio-Gel P-2 as described above, affording **5** (162.7 mg), **6** (145.4 mg), **7** (45.7 mg) and **8** (3.9 mg) as transfer products. The physical and NMR data for compounds **5** and **6** were identical to those of β-D-Gal-(1→4)-β-D-Gal-$OC_6H_4NO_2$-*p* and β-D-Gal-(1→4)-β-D-Gal-(1→4)-β-D-Gal-$OC_6H_4NO_2$-*p*, respectively.[23] Data for **7**: ^{1}H NMR, δ 8.24 (d, *J* 9.6 Hz, 2H, *o*-Ph), 7.23 (d, *J* 9.6 Hz, 2H, *m*-Ph), 5.22 (d, *J* 7.2 Hz, 1H, H-1), 4.66 (d, *J* 8.4 Hz, 1H, H‴-1), 4.62 (d, *J* 7.8 Hz, 1H, H″-1) and 4.56 (d, *J* 7.8 Hz, 1H, H′-1); ^{13}C NMR, δ 79.3, 79.0 and 78.8 (C-4, C-4′ and C-4″); $[\alpha]_D$ −58.8 (0.96, H_2O); FABMS 788 *m*/*z* for $[M+H]^+$. Data for **8**: ^{1}H NMR, δ 8.25 (d, *J* 9.6 Hz, 2H, *o*-Ph), 7.24 (d, *J* 9.6 Hz, 2H, *m*-Ph), 5.25 (d, *J* 7.8 Hz, 1H, H-1), 4.64 (d, *J* 7.8 Hz, 1H, H′-1); ^{13}C NMR, δ 83.5 (C-3); FABMS 486 *m*/*z* for $[M+H]^+$.

3.5. α-D-Neu5Ac-(2→3)-β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (9), α-D-Neu5Ac-(2→6)-β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (10), α-D-Neu5Ac-(2→3)-β-D-Gal-(1→4)-β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (11) and α-D-Neu5Ac-(2→3)-β-D-Gal-(1→4)-β-D-Gal-$OC_6H_4NO_2$-*p* (12)

A reaction mixture of **1** (8 mg), CMP-Neu5Ac disodium salt (10.5 mg) in 0.8 mL MES (2-morpholinoethanesulfonic acid) buffer (40 mM, pH 6.3) containing BSA (1.2 mg/mL) and 20 mM $MnCl_2$, 25 U alkaline phosphatase, and α-2,3-NST (20 mU) was incubated at 30 °C. After 48 h incubation, the reaction solution was passed through columns of ODS (15% acetonitrile containing 0.1% TFA as eluent) and Bio-Gel P-2 to afford **9** (14.8 mg). Data for **9**: ^{1}H NMR, δ 8.18 (d, *J* 9.0 Hz, 2H, *o*-Ph), 7.14 (d, *J* 9.0 Hz, 2H, *m*-Ph), 5.31 (d, *J* 7.8 Hz, 1H, H-1), 4.57 (d, *J* 7.8 Hz, 1H, H′-1), 273 (dd, *J* 12.0, 4.8 Hz, 1H, H − 3″$_e$), 2.01, 1.99, (2s, 6H, 2 NHAc) and 1.78 (t, *J* 12.0 Hz, 1H, H − 3″$_a$); ^{13}C NMR, δ 77.1 (C-3); $[\alpha]_D$ −46.7 (1.20, H_2O); FABMS 796 *m*/*z* for $[M+H]^+$.

In a similar manner, **10** (15.2 mg) was obtained from compound **1** in the case with rat liver α-2,6-NST as catalyst. Also in a similar manner, **11** (8.6 mg) and **12** (9.9 mg) were obtained from **3** and **5**, respectively, with recombinant rat α-2,3-NST as catalyst. Data for **10**: ^{1}H NMR, δ 8.23 (d, *J* 9.6 Hz, 2H, *o*-Ph), 7.17 (d, *J* 9.6 Hz, 2H, *m*-Ph), 5.35 (d, *J* 8.4 Hz, 1H, H-1), 4.46 (d, *J* 7.2 Hz, 1H, H′-1), 2.67 (dd, *J* 12.0, 4.8 Hz, 1H, H − 3″$_e$), 2.01, 1.99, (2s, 6H, 2 NHAc) and 1.72 (t, *J* 12.0 Hz, 1H, H − 3″$_a$); ^{13}C NMR, δ 65.1 (C-6); $[\alpha]_D$ −132.7 (0.43, H_2O); FABMS 796 *m*/*z* for $[M+H]^+$. Data for **11**: ^{1}H NMR, δ 8.23 (d, *J* 9.0 Hz, 2H, *o*-Ph), 7.17 (d, *J* 9.0 Hz, 2H, *m*-Ph), 5.32 (d, *J* 9.0 Hz, 1H, H-1), 4.65 (d, *J* 8.4 Hz, 1H, H″-1), 4.51 (d, *J* 8.4 Hz, 1H, H′-1), 4.09 (dd, *J* 10.2 Hz, 3.0 Hz, 1H, H-3″), 2.74 (dd, *J* 12.0, 4.8 Hz, 1H, H − 3″$_e$), 2.00, 1.98 (2s, 6H, 2 NHAc) and 1.77 (t, *J* 12.0 Hz, 1H, H − 3″$_a$); ^{13}C NMR, δ 74.5 (C-3″) and 41.5 (C-3‴); FABMS 1002 *m*/*z* for $[M+H]^+$. Data for **12**: ^{1}H NMR δ 8.25 (d, *J* 9.0 Hz, 2H, *o*-Ph), 7.23 (d, *J* 9.0 Hz, 2H, *m*-Ph), 5.23 (d, *J* 7.8 Hz, 1H, H-1), 4.67 (d, *J* 7.8 Hz, 1H, H′-1), 4.11 (dd, *J* 3.0 Hz, 10.2 Hz, 1H, H-3′), 2.75 (dd, *J* 12.0, 4.8 Hz, 1H, H − 3″$_e$), 1.99 (s, 3H, NHAc) and 1.78 (t, *J* 12.0 Hz, 1H, H − 3″$_a$); FABMS 777 *m*/*z* for $[M+H]^+$.

3.6. α-D-Neu5Ac-(2→3)-β-D-Gal-(1→4)-β-D-(α-L-Fuc-(1→3)-)-GlcNAc-$OC_6H_4NO_2$-*p* (13) and β-D-Gal-(1→4)-β-D-(α-L-Fuc-(1→3)-)-GlcNAc-$OC_6H_4NO_2$-*p* (14)

In a reaction mixture of **9** (8 mg), GDP-Fuc disodium salt (6.3 mg), recombinant α-(1→3)-fucosyltransferase VII (20 mU), and alkaline phosphatase (20 U) in HEPES buffer (50 mM, pH 7.4) containing BSA (1.5 mg/mL) and $MnCl_2$ (15 mM) were incubated for 20 h at 30 °C. The reaction mixture was heated in boiling water for 5 min, and the reaction solution was passed through columns of ODS and Bio-Gel P-2 to afford **13** (8.9 mg). Data for **13**: ^{1}H NMR, δ 8.23 (d, *J* 9.0 Hz, 2H, *o*-Ph), 7.17 (d, *J* 9.0 Hz, 2H, *m*-Ph), 5.32 (d, *J* 8.4 Hz, 1H, H-1), 5.12 (d, *J* 4.2 Hz, 1H, H-1^{Fuc}), 4.82 (dd, 1H, H-5^{Fuc}), 4.54 (d, 1H, 8.4 Hz, H-1′), 4.22 (t, 1H, H-2), 2.74 (dd, *J* 12.0, 4.8 Hz, 1H, H − 3″$_e$), 2.00, 1.97 (2s, 6H, 2 NHAc), 1.77 (t, *J* 12.0 Hz, 1H, H − 3″$_a$) and 1.16 (d, 3H, 6.6 Hz, H-6^{Fuc}); ^{13}C NMR, δ 101.4 (C-1^{Fuc}), 76.7 (C-3), 74.6 (C-4) and 17.0 (C-6^{Fuc}). $[\alpha]_D$ −9.2 (0.86, H_2O); FABMS 942 *m*/*z* for $[M+H]^+$.

In a similar manner, **14** was prepared from **1** with recombinant α-(1→3)-fucosyltransferase VII as catalyst under the following conditions: **1** (8 mg), GDP-Fuc disodium salt (10.5 mg), recombinant α-(1→3)-fucosyltransferase VII (30 mU), and alkaline phosphatase (25 U) in HEPES buffer (50 mM, pH 7.4) containing BSA (1.5 mg/mL) and $MnCl_2$ (15 mM), incubation for 20 h at 30 °C. The reaction mixture was then processed in the same way as described for compound **13** to give **14** (9.9 mg, 91%). The NMR data for **14** were identical to that of β-D-Gal-(1→4)-β-D-(α-L-Fuc-(1→3)-)-GlcNAc-$OC_6H_4NO_2$-*p*.[31] Data for **14**: $[\alpha]_D$ −85.8 (0.46, H_2O); FABMS 651 *m*/*z* for $[M+H]^+$.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2005.08.019.

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