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Note

Effective chemoenzymatic synthesis of *p*-aminophenyl glycosides of sialyl *N*-acetyllactosaminide and analysis of their interactions with lectins

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Abstract—A convenient chemoenzymatic procedure for the synthesis of *p*-aminophenyl glycosides of sialyl *N*-acetyllactosaminide has been developed from *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide as starting material through three steps: synthesis of *p*-nitrophenyl *N*-acetyllactosaminide with β-D-galactosidase, chemical reduction of the *p*-nitrophenyl group, and sialylation with sialyltransferase. The *p*-aminophenyl glycosides were then successfully biotin-labeled through the coupling with *N*-(+)-biotinyl-6-aminohexanoic acid to afford biotinylated oligosaccharides with an aminohexanosyl group and phenyl group as the spacers between the biotin and glycan. Furthermore, the biotin-labeled sugars were shown to be useful for immobilization and assay of the carbohydrate–lectin interactions by an optical biosensor based on surface plasmon resonance.

Keywords: Aminophenyl glycoside; Biotinylation; Carbohydrate–protein interaction; Enzyme catalysis; Surface plasmon resonance

Specific interactions between carbohydrates and proteins are involved in a number of important biological processes, such as cell–cell communication, cell adhesion, inflammation, bacterial and viral infections, and tumor cell metastasis.[1] Understanding of the molecular basis for carbohydrate–protein interactions in detail, therefore, not only provides valuable information on biological processes but also aids the development of potent biomedical agents.[2] In order to evaluate the specific interactions, many biophysical and biochemical methods have been developed, including affinity chromatography, microdialysis, electrophoresis, microcalorimetry, NMR spectroscopy, and an optical biosensor based on surface plasmon resonance (SPR).[3] The SPR method is particularly attractive because it enables the interaction be monitored kinetically in real-time without fluorescence or radioisotope labeling.[4] However, a frequent problem that must be overcome is the immobilization of oligosaccharides onto the surfaces of a sensor chip to facilitate kinetic measurements. With this objective, several procedures including introducing a biotinyl group for an oligosaccharide have already been reported.[5] It is well known that introducing a biotinyl group to the reducing end of an oligosaccharide makes it useful for immobilization onto a solid surface and for preparation of neoglycoconjugates.[6] In our previous study, we synthesized a number of *p*-nitrophenyl glycosides of galactosyl, fucosyl, and sialyl oligosaccharides, and used these for the preparation of glycopolymers.[7] In fact, our other purpose to synthesize those glycosides is for SPR analysis through immobilization onto a sensor chip after convenient reduction of nitrophenyl group

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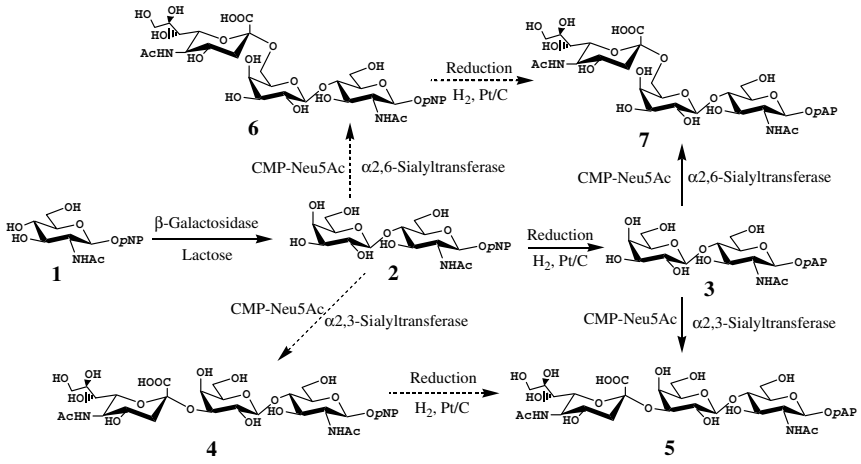


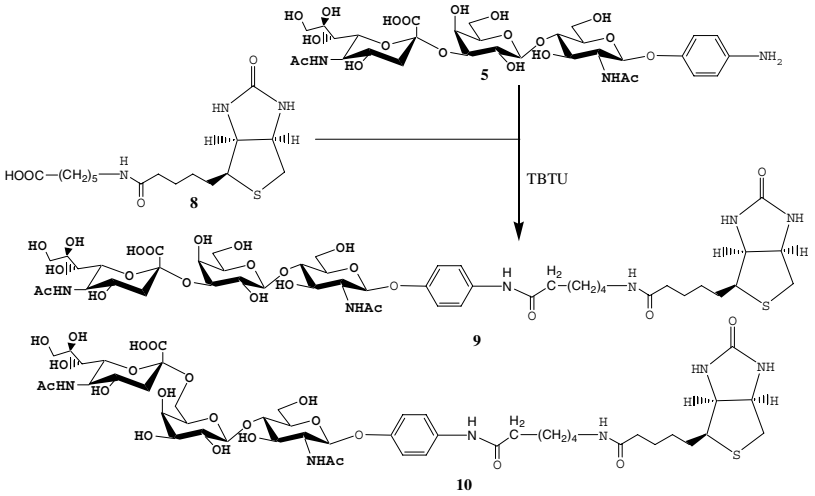
Scheme 1. Synthetic scheme for **5** and **7** from *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide by use of β-galactosidase, chemical reduction, and sialyl transferases.

and biotinylation. Here, we report a convenient chemoenzymatic procedure for the synthesis of *p*-aminophenyl glycosides of sialyl-*N*-acetyllactosaminide from *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (**1**) as starting material with β-D-galactosidase, chemical reduction, and sialyltransferases, and further introducing binotinyl group for immobilization and binding assay by SPR technology.[†]

We selected sialyl-*N*-acetyllactosaminides as model sugars because sialylated oligosaccharides are known to serve as the cell-surface receptor determinants for selectins, influenza virus and other viruses, bacterial toxins, and lectins.[8] According to our reported method, **2**, **4**, and **6** were synthesized from **1** as shown in Scheme 1 by using β-D-galactosidase from *Bacillus circulans*, recombinant rat α-(2→3)-sialyltransferase and rat liver α-(2→6)-sialyltransferase.[7a] After reduction of the *p*-nitrophenyl group,[9] the *p*-aminophenyl glycosides of sialyl-*N*-acetyllactosaminide **5** and **7** could be obtained from **4** and **6**, respectively. However, this synthetic procedure led to quite low yields (about 15% by HPLC) of **5** and **7** that may be due to the presence of Neu5Ac residues, comparing with that of the reduction of **2**. Therefore, we turned to try another procedure for the synthesis of **5** and **7** shown as solid arrow in Scheme 1. Firstly, **2** was easily converted to its *p*-aminophenyl derivative (**3**, obtained in 76% yield) through reduction reaction and purification with an ODS column. Then, **3** was sialylated by recombinant rat α-(2→3)-sialyltransferase with CMP-Neu5Ac sodium salt as the donor.[10] This enzyme reaction was monitored by an HPLC fitted with diode-array detector (DAD). After 26 h of incubation, at which time the peak for **3** almost disappeared in the HPLC chromatogram, the reaction was stopped by heating in a boiling-water bath for 5 min. The reaction mixture was then passed through a column of BioGel P-2. The transfer product **5** was obtained in 87% yield based on the donor used. The structure of **5** was confirmed by NMR and electrospray-ionization mass spectrometry (ESIMS). In the ^{1}H NMR spectrum of **5**, characteristic signals at 2.75 ppm (dd, 1H, *J* 4.8 and 12.0 Hz, H-3$''_{eq}$) and 1.81 (t, 1H, *J* 12.0 Hz, H-3$''_{ax}$) due to the H-3 of Neu5Ac were assigned. Negative-mode ESIMS gave a major molecular signal at *m*/*z* 764 due to $[M-H]^-$ as expected. In a similar manner, **7** was obtained in 85% yield based on the amount of donor (CMP-Neu5Ac sodium salt) added from **3** using rat liver α-(2→6)-sialyltransferase as biocatalyst. These results indicate that the chemoenzymatic procedure provides an effective way for the synthesis of the desired trisaccharides **5** and **7** with a *p*-aminophenyl group from simple monsaccharides in only three steps.

Compounds **5** and **7** were used for subsequent biotinylation as shown in Scheme 2. The coupling of the aminophenyl group in **5** or **7** to the carboxylic acid group in *N*-(+)-biotinyl-6-aminohexanoic acid (**8**) could be achieved using several well-known peptide coupling techniques. In the present study, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) was used because the use of uronium salts in a peptide coupling reaction has been proven to be fast and with limited side reactions.[11] The coupling reaction was detected by HPLC at 210, 254 and 300 nm [TSKgel ODS-80Ts column, 15% acetonitrile in phosphate sodium buffer (10 mM, pH 6.8) as mobile phase and at a flow rate of 0.5 mL/min]. The HPLC monitoring showed that the reaction was almost finished in 5 h. In a practical synthesis, the reaction was kept overnight,

[†] IUPAC chemical names are *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1**) and *p*-aminophenyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulosyl-2-onic acid-(2→3)-β-D-galactopyranoside-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (**5**), and *p*-aminophenyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulosyl-2-onic acid-(2→6)-β-D-galactopyranoside-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (**7**).



Scheme 2. Synthetic scheme for biotin-labeled sugars **9** and **10** from **5** and **7** through biotinylation with *N*-(+)-biotinyl-6-aminohexanoic acid (**8**).

which was long enough for the coupling reaction at room temperature. The biotin-labeled sugar was purified from the reaction mixture by sequential ODS column and BioGel P-2 column purification and characterized by NMR spectroscopy and ESIMS. The ^{1}H NMR spectra showed all expected signals from the two portions of the molecules, and the compounds were identified by comparison of their ^{1}H NMR spectra with literature data.[12] In the ^{1}H NMR spectrum of **9**, for example, the characteristic signals at 4.53 ppm (1H, dd, *J* 4.8 and 8.0 Hz, H-4) are due to H-4, and those at 4.30 ppm (1H, dd, *J* 4.4 and 8.0 Hz, H-3) are due to H-3 of the biotin moiety, while characteristic signals at 2.73 ppm (dd, 1H, *J* 5.2 and 13.2 Hz, H-3$''_{eq}$) and 1.82 (t, 1H, *J* 12.4 Hz, H-3$''_{ax}$) are due to the H-3 of the Neu5Ac residue. Negative-mode ESIMS for **9** gives a molecular ion signal at *m*/*z* 1104 for $[M-H]^-$. In a similar manner, **7** was coupled with **8** to afford biotin-labeled sugar **10**. The yields were 71% and 75% for **9** and **10**, respectively, in the present study. The results indicate that sialylated sugars were converted to their corresponding biotinyl sugars without any removal of sialic acid residues, which indicate the feasibility of this method for sialylated glycosides. The biotin-labeled oligosaccharides have both the ability to be immobilized due to the presence of the biotinyl portion and detected or purified with aid of the strong ultraviolet absorbing group.

A biosensor, BIAcore 3000, was used to measure the carbohydrate–lectin interaction according to our previous method.[13] The solution of biotin-labeled sugar at a concentration of 100 μg/mL was introduced onto the surfaces of sensor chip SA-5, resulting about 200 RU increase at the end of immobilization, which indicated the amount of immobilized ligand. To evaluate whether immobilized sugars interact with lectins or not, we monitored their interactions with appropriate lectins. As shown in Figure 1, the solutions of lectin from *Mackia*

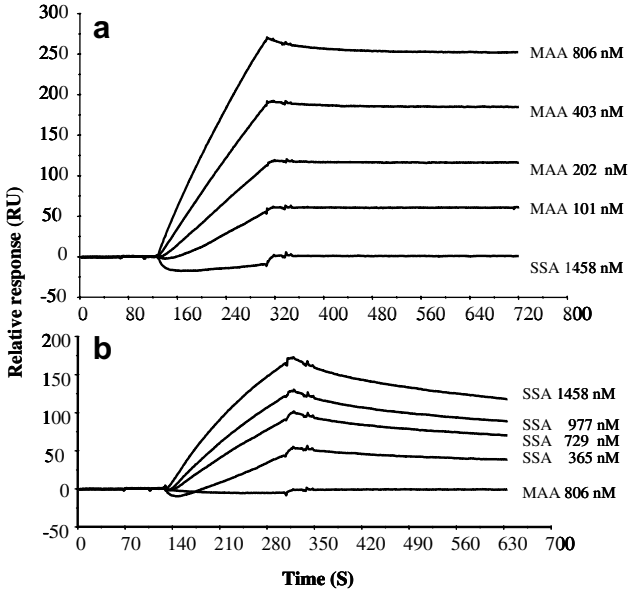


Figure 1. Sensorgrams show the interactions of surface-bound **9** or **10** with lectins: (a) sensor chip surface-bound **9**; (b) sensor chip surface-bound **10**.

amurensis agglutinin (MAA) were injected across the surface of sensor chip bound with **9**, and increases in RU from the initial baseline were observed, while the solution of lectin from *Sambucus sieboldiana* agglutinin (SSA) did not show any binding. In contrast, SSA showed binding with the surface bound with **10**, while MAA did not. The kinetic parameters for the interactions were calculated by using BIAevalution 3.1 software as following: k_a ($M^{-1}\,s^{-1}$) values of 2.91×10^3 and 6.23×10^3, k_d (s^{-1}) values of 1.23×10^{-3} and 9.86×10^{-5}, and k_A (M^{-1}) values of 2.36×10^6 and 6.32×10^7 for the interactions of MAA with **9** and SSA with **10**, respectively. These binding properties correspond with the known carbohydrate-binding specificities of the two lectins.[14]

In conclusion, we have developed an efficient procedure to synthesize **5** and **7** from **1** by use of the β-D-galactosidase from *B. circulans*, chemical reduction, and sialyltransferases. Further, the synthesized glycosides were biotinylated to afford biotin-labeled sugars with an aminohexanosyl group and a phenyl group as the spacers between the biotin and glycan. The biotin-labeled sugars have been demonstrated to be useful for immobilization and assay of the carbohydrate–lectin interactions by SPR.

1. Experimental

1.1. General methods

Rat liver α-(2→6)-*N*-sialyltransferase, TBTU, **1**, and **8** were purchased from Sigma Chemical Co. Alkaline phosphatase from calf intestine, and recombinant rat α-(2→3)-*N*-sialyltransferase were purchased from Calbiochem–Novabiochem Corp. (La Jolla, CA). CMP-Neu5Ac sodium salt was obtained from Kyowa Hakko kogyo Co., Ltd. (Tokyo, Japan). MAA and SSA were got from Seikagaku Corp. (Tokyo, Japan). Sensor chip SA-5 was obtained from BIAcore International AB (Uppsala, Sweden). β-Galactosidase from *B. circulans* (Biolacta) was a gift from Daiwa Kasei Co., Ltd. (Osaka, Japan). HBS buffer was prepared from 10 mM 2-hydroxyethylpiperazine *N*′-2-ethanesulfonic acid (pH 7.4), 150 mM NaCl, and 0.05% BIAcore surfactant P20 in distilled water.

HPLC analysis was performed with an Agilent 1100 HPLC system equipped with a DAD. The conditions for analysis of galactosylation were as follows: TSKgel Amide-80 column (4.6 mm × 250 mm), 77% acetonitrile in water as mobile solution, and flow rate at 1.0 mL/min. The analytical conditions for sialylation were as follows: TSKgel ODS-80TsQA (4.6 mm × 250 mm), 15% acetonitrile in 0.2% formic acid as mobile phase at a flow rate of 0.5 mL/min. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Advance 400 or JEOL LA-600 spectrometer for solutions in D_2O. Chemical shifts are given in parts per million and referenced to internal *tert*-butanol (δ_H 1.23 in D_2O or δ_C 31.2 in D_2O).

1.2. Preparation of *p*-aminophenyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulosyl-2-onic acid-(2→3)-β-D-galactopyranoside-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (5) and *p*-aminophenyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulosyl-2-onic acid-(2→6)-β-D-galactopyranoside-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (7)

A solution of **3** (10.0 mg), CMP-Neu5Ac disodium salt (15.0 mg) in 40 mM MES (2-morpholinoethanesulfonic acid) buffer (pH 6.4, 2.0 mL) containing BSA (1.0 mg/mL) and 20 mM $MnCl_2$, 25 U alkaline phosphatase, and recombinant rat α-(2→3)-sialyltransferase (30 mU) was incubated at 30 °C for 26 h. After termination of the reaction, the transfer product was purified by gel chromatography on an column of BioGel P-2 to afford **5** (14.0 mg). Data for **5**: ^{1}H NMR, δ 7.22 (d, J 8.8 Hz, 2H, *o*-Ph), 7.11 (d, J 8.8 Hz, 2H, *m*-Ph), 5.16 (d, J 8.4 Hz, 1H, H-1), 4.59 (d, J 8.0 Hz, 1H, H′-1), 2.75 (dd, J 12.0, 4.8 Hz, 1H, H-3$''_e$), 2.04, 2.03 (2s, 6H, 2NHAc), and 1.81 (t, J 12.0 Hz, 1H, H-3$''_a$); ESIMS, m/z 764 for $[M-H]^-$.

In a similar manner, **7** (13.7 mg) was synthesized from **3** (10 mg) with rat liver α-(2→6)-sialyltransferase as catalyst. Data for **7**: ^{1}H NMR, δ 7.19 (d, J 9.2 Hz, 2H, *o*-Ph), 7.10 (d, J 9.2 Hz, 2H, *m*-Ph), 5.18 (d, J 8.4 Hz, 1H, H-1), 4.48 (d, J 8.0 Hz, 1H, H′-1), 2.68 (dd, J 12.0, 4.8 Hz, 1H, H-3$''_e$), 2.06, 2.03 (2s, 6H, 2NHAc), and 1.73 (t, J 12.0 Hz, 1H, H-3$''_a$); ESIMS, m/z 764 for $[M-H]^-$.

1.3. Preparation of [(6-biotinylamido)hexanoylamido]-phenyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulosyl-2-onic acid-(2→3)-β-D-galactopyranoside-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (9) and [(6-biotinylamido)hexanoylamido]phenyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulosyl-2-onic acid-(2→6)-β-D-galactopyranoside-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (10)

A mixture of **5** (10 mg, 0.013 mmol), TBTU (5.2 mg, 0.016 mmol), and **8** (5.7 mg, 0.016 mmol) was dissolved in dry DMF (450 μL) under N_2 at room temperature. After stirring for 30 min, Et_3N (4 μL) was added, and the mixture was left to stir for overnight. After removal of solvents, the residue was passed through columns of ODS and BioGel P2 to afford **9** (9.8 mg). Data for **9**: ^{1}H NMR, δ 7.38 (d, J 9.2 Hz, 2H, *o*-Ph), 7.08 (d, J 9.2 Hz, 2H, *m*-Ph), 5.15 (d, J 8.4 Hz, 1H, H-1), 4.59 (d, J 8.0 Hz, 1H, H′-1), 4.53 (1H, dd, J 4.8 and 8.0 Hz, H-4 of biotinyl), 4.30 (1H, dd, J 4.4 and 8.0 Hz, H-3 of biotinyl), 2.73 (dd, J 13.2 and 5.2 Hz, 1H, H-3$''_e$), 2.04 (2s, 6H, 2NHAc), and 1.82 (t, J 12.0 Hz, 1H, H-3$''_a$); ESIMS, m/z 1104 for $[M-H]^-$.

In a similar manner as the preparation of **9** described above, **10** was prepared from **7** and **8**. Data for **10**: ^{1}H NMR, δ 7.36 (d, J 9.2 Hz, 2H, *o*-Ph), 7.08 (d, J 9.2 Hz, 2H, *m*-Ph), 5.18 (d, J 8.4 Hz, 1H, H-1), 4.53 (1H, dd, J 4.8 and 8.0 Hz, H-4 of biotinyl), 4.48 (d, J 8.0 Hz, 1H, H′-1), 4.29 (1H, dd, J 4.8 and 8.0 Hz, H-3 of biotinyl), 2.68 (dd, J 13.2 and 5.2 Hz, 1H, H-3$''_e$), 2.06, 2.03 (2s, 6H, 2NHAc), and 1.74 (t, J 12.0 Hz, 1H, H-3$''_a$); ESIMS, m/z 1104 for $[M-H]^-$.

1.4. Analysis of interaction between biotin-labeled oligosaccharide and lectin by a biosensor based on SPR

The immobilization of **9** or **10** was carried out according to the procedure reported.[3e,5e] Briefly, **9** or **10** at a con-

centration of 100 μg/mL was introduced onto a streptavidin-preimmobilized sensor surface of sensor chip SA-5, followed by injection of 10 μL 2 M NaCl solution. One flow cell of the sensor chip was injected with 10 μL solution of 2 M NaCl for a control. The lectin solution in HBS buffer was injected over the surfaces at a flow rate of 20 μL/min. The interaction was monitored at 25 °C as the change of in the SPR response. After 3 min of monitoring, the HBS buffer was introduced in place of the lectin solution to start the dissociation. Regeneration was accomplished by washing away the surface-bound lectin with 5 μL of 50 mM H_3PO_4, followed by extensive washing with HBS buffer. The data analyses were made with BIAevalution 3.1 software.

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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.2007.03.010.

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