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Synthesis of 2-[(2-pyridyl)amino]ethyl β-D-lactosaminide and evaluation of its acceptor ability for sialyltransferase: a comparison with 4-methylumbelliferyl and dansyl β-D-lactosaminide

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Abstract—We report the synthesis of β-D-lactosaminide with a 2-aminopyridyl group that is linked to a glycosyl tether at the reducing end. This fluorescent disaccharide acts as an acceptor for both α-(2→6)- and α-(2→3)-sialyltransferases. In addition, the acceptor ability of this disaccharide was evaluated and compared with that of β-D-lactosaminide having a dansyl or a 4-methylumbelliferyl group.

Keywords: Sialyltransferase; Enzyme assay; 2-Aminopyridyl-tethered oligosaccharide; Fluorescence assay; *N*-Acetyl-D-lactosaminide

1. Introduction

Sialic acid residues at the nonreducing end of oligosaccharides are responsible for several biological events, such as binding with CD22 (Siglec), the lifetime of glycoproteins in plasma, and viral invasion.[1,2] Biosynthesis of the sialyl linkages are performed by the action of several sialyltransferases that catalyze transfer of a sialic acid residue to the nonreducing end of the oligosaccharide from cytidine-5′-monophospho-neuraminic acid (CMP-NeuAc). Increasing or decreasing the quantity of sialyltransferases in the cell may be related to pathogens, proliferation, and differentiation. Indeed, a deficiency of α-(2→6)-sialyltransferase causes a fatal decrease in IgM production due to a loss of ligand, NeuAc-α-(2→6)-Gal-β-(1→4)-GlcNAc, of the CD22 (Siglec) on the B-cell.[3] Therefore a convenient and sensitive diagnostic assay method for sialyltransferases is essential in order to estimate the quantity of sialyltransferase expressed in a target cell.

The most sensitive assay method is one using radiolabeled sugar nucleotides, such as CMP-[U-^{14}C]-sialic acid.[4] We have also examined fluorometric enzyme assay methods for sialyltransferases. However, although our methods are highly sensitive, these methods are time consuming[5] and the 4-methylumbelliferyl β-D-lactoside acceptor is only slightly soluble in buffer solution.[6] Palcic and co-workers have reported a fluorescence-labeled acceptor in order to assay several enzymes including sialyltransferases.[7,8] This superb substrate based on the LacNAc skeleton can be detected with as few as 100 molecules.

On the other hand, pyridyamination[9] is now widely used as a labeling method for oligosaccharides, and its high sensitivity makes it potentially useful for oligosaccharide analysis. This method, however, causes loss of the characteristic nature of the sugar ring at the reducing end, because pyridylamino labeling by reductive amination requires an aldehyde at the anomeric position.

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Although β-D-LacNAc can be easily prepared from lactose,[10] pyridylaminated LacNAc thus obtained by reductive amination does not act as an acceptor for α-(2→6)- and α-(2→3)-sialyltransferase due to its strict acceptor specificity requiring a complete LacNAc skeleton.[11] Although lactose having a pyridylamino group that is linked to the glycosyl tether derived from 2,3-dihydroxypropyl glycoside at the reducing end, has been synthesized,[12] the synthesis afforded several byproducts. In addition, the β-D-LacNAc derivative, which is a common acceptor for both α-(2→6)- and α-(2→3)-sialyltransferases, has not yet been synthesized. Therefore, we examined the synthesis of 2-aminopyridyl β-D-LacNAc (PA-LacNAc) as a new type of acceptor by a convenient synthetic route. In this paper, we describe the synthesis of the PA-LacNAc from lactose and the evaluation of its reactivity toward sialyltransferases in comparison with other acceptors.

2. Results and discussion

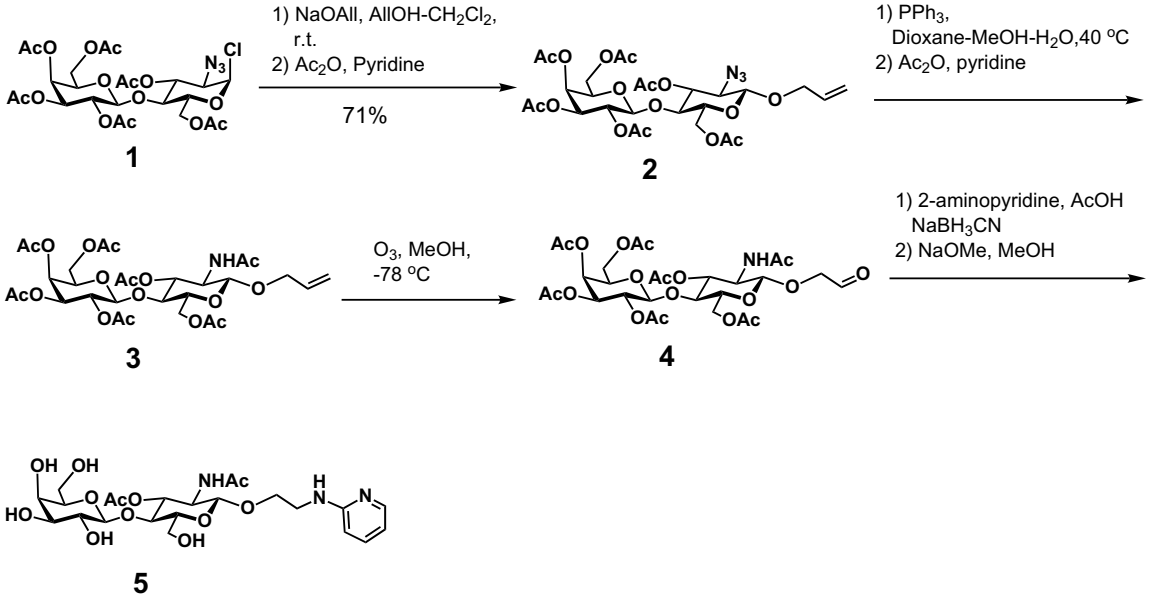
For the synthesis of allyl *O*-(LacN$_3$), LacNAc chloride (the glycosyl chloride)[13] was first prepared from lactose by use of azidonitration[10] and then conversion to the glycosyl chloride **1**. Sodium allyloxide was added to form the β-allyl glycoside **2**. This reaction proceeded smoothly and afforded the desired All β-D-LacN$_3$ (**3**), as the sole product.

In order to reduce the azide group to an amino group in the presence of the allyl glycosyl group, triphenylphosphine was used in dioxane–MeOH–H$_2$O. However, this reduction stopped after the formation of the iminophosphorane group, which did not hydrolyze at room temperature. Therefore, this reaction was performed at 40 °C to afford the 2-amino derivative (Scheme 1).

The 2-amino group was then acetylated using acetic anhydride, after which all the hydroxyl groups were acetylated for isolation as **3**. To attach the 2-aminopyridyl group by reductive amination, the allyl group at the anomeric position was converted into an aldehyde group by ozonolysis in 70% yield. This aldehyde was fluorescence labeled with 2-aminopyridine. After confirmation of imine formation between this aldehyde and the 2-aminopyridine by TLC, the imino group was reduced by acidic NaBH$_3$CN in quantitative yield. After O-deacetylation by NaOMe, the PA-LacNAc was analyzed by HPLC using a fluorescence detector. However, although fluorescence labeling was quantitatively performed, small amounts of impurities were detected due to the high sensitivity of detection. Therefore an aliquot of PA-LacNAc was isolated by HPLC and used for the sialyltransferase assays.

In order to evaluate the acceptor ability for the sialyltransferase reaction, other acceptors having 4-methylumbelliferyl or dansyl groups were also prepared and then compared for their acceptor ability toward PA-LacNAc.

For the synthesis of 4-methylumbelliferyl β-D-LacNAc (UM-LacNAc),[14] a galactosyltransferase-mediated reaction with commercially available 4-methylumbelliferyl β-D-GlcNAc (UM-GlcNAc) was performed (Scheme 2). Although the solubility of UM-GlcNAc in the buffer solution was extremely poor, the galactosyltransferase reaction occurred in 90% yield. We assumed that the small amount of UM-GlcNAc dissolved was



Scheme 1.

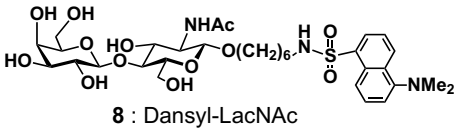


Figure 1.

galactosylated, after which UM-LacNAc (**7**) easily dissolved.

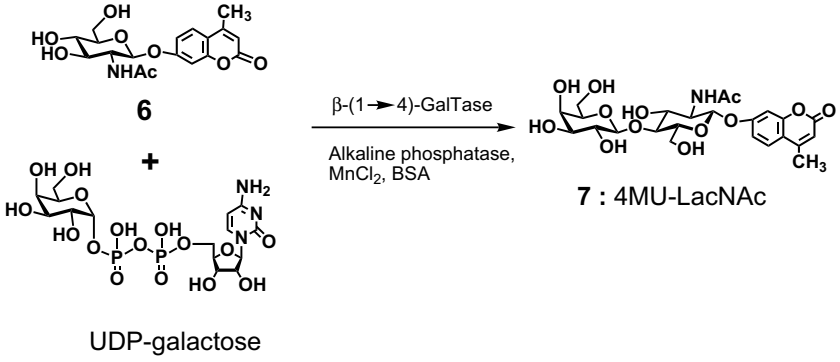
For the synthesis of the dansyl-β-D-LacNAc (**8**) (Fig. 1), reported procedure was used.[15]

In order to evaluate acceptor ability of these three fluorescence-labeled-LacNAcs, we measured their transfer rates by HPLC equipped with a fluorescence detector. For the estimation of sialyl-LacNAc formation, a new fluorescence peak by the sialyltransfer reaction on HPLC analysis was estimated by use of a calibration curve made from the corresponding fluorescence-labeled acceptor.

For the assay toward PA-LacNAc, we also optimized assay conditions by use of rat α-(2→6) and rat recombinant α-(2→3) sialyltransferases. As shown in Figure 2, in the case of sodium cacodylate buffer, a higher transfer velocity by α-(2→6)-sialyltransferase at pH 6 was observed, but only half the transfer velocities were observed at pH 5 and 7 compared to those in HEPES buffer. With the rat recombinant α-(2→3)-sialyltransferase, adequate transfer velocity was only observed at pH 5 in cacodylate buffer. Therefore, HEPES buffer (pH 7.0) and sodium cacodylate buffer (pH 5.0) were chosen for use of α-(2→6)- and α-(2→3)-sialyltransferase assays, respectively. In order to estimate K_m and V_{max} values, an assay solution containing CMP-NeuAc (300 μM), acceptor (various concentrations), sialyltransferase, and bovine serum albumin were used, and the formation of sialyl-LacNAc was estimated by HPLC.[6] Although a solution of PA-LacNAc can be prepared as a 100 mM solution, MU-LacNAc, and dansyl-LacNAc are troublesome to prepare over 20 and 2 mM, respectively.

Lineweaver–Burk plots of MU-, PA-, and dansyl-LacNAc toward α-(2→6)- and α-(2→3)-sialyltransferases are shown in Figure 3, and K_m and V_{max} values thus obtained are also summarized in Tables 1 and 2.

As shown in Figure 3, these three LacNAc derivatives act as acceptors for both α-(2→6) and α-(2→3)-sialyltransferases. In the case of α-(2→6)-sialyltransferase, the dansyl-LacNAc shows the smallest K_m value (56.0 μM) compared to MU-LacNAc (634 μM) and PA-LacNAc (442 μM). This phenomenon may be a cluster effect of the dansyl group or a hydrophobic interaction between the enzyme and the dansyl group. For MU- and PA-LacNAc, both acceptors show same K_m and V_{max}



Scheme 2.

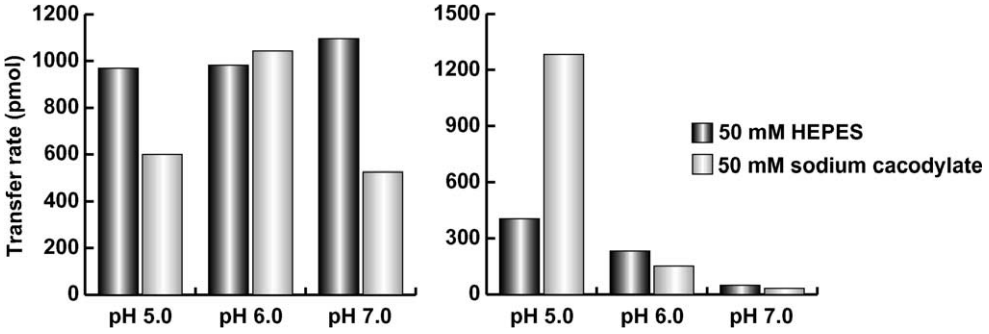


Figure 2. Transfer rate is dependent on buffer conditions. Rat-liver α-(2→6)-sialyltransferase (left), rat-recombinant α-(2→3)-sialyltransferase (right).

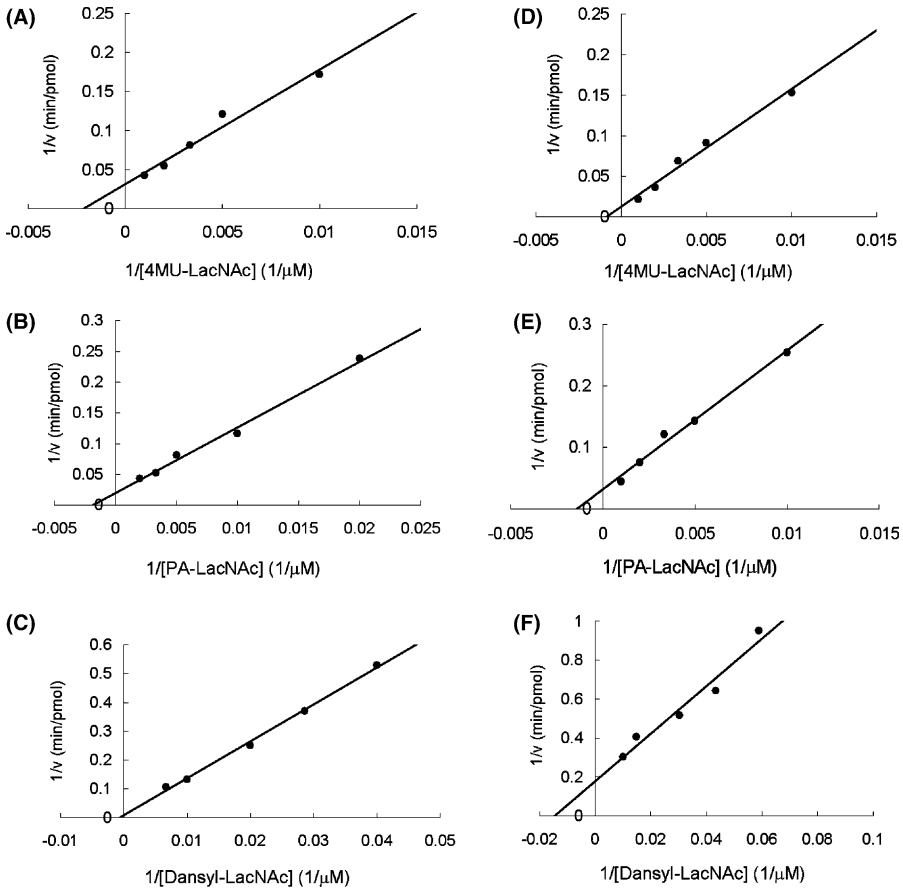


Figure 3. Kinetics of rat-liver α-(2→6)-(A), (B), and (C), and rat recombinant α-(2→3)-sialyltransferases (D), (E), and (F).

Table 1. Kinetic parameters toward rat-liver α-(2→6)-sialyltransferase

Substrate	K_m (μM)	V_{max} (pmol/min)	V_{max}/K_m (relative)
MU-LacNAc	634 ± 99	58.7 ± 5.0	22.3
PA-LacNAc	442 ± 104	43.9 ± 6.3	24.0
Dansyl-LacNAc	56.0 ± 12	23.2 ± 2.7	100

Table 2. Kinetic parameters toward rat recombinant α-(2→3)-sialyltransferase

Substrate	K_m (mM)	V_{max} (pmol/min)	V_{max}/K_m (relative)
MU-LacNAc	3.37 ± 0.93	1613 ± 406	84.8
PA-LacNAc	1.78 ± 0.34	62.05 ± 9.20	6.17
Dansyl-LacNAc	0.354 ± 0.091	200.1 ± 41.2	100

values. However, the V_{max}/K_m value of dansyl-LacNAc is four-fold higher than that of MU- and PA-LacNAc (Table 1).

For α-(2→3)-sialyltransferase, dansyl-LacNAc shows the smaller K_m (354 μM) value than that of the other acceptors. In contrast, although MU-LacNAc showed a large K_m value, a remarkable increase in the V_{max} value compared to that of the other acceptors was also observed. PA-LacNAc showed a moderate K_m value, but the lowest V_{max} value was observed (Table 2).

For the synthesis of these acceptors, MU-LacNAc was easily prepared by use of commercially available enzyme and substrates. However, the solubilities of both MU- and dansyl-LacNAc are remarkably poor compared to that of PA-LacNAc. Therefore preparation of relatively dilute solution by use of MU-LacNAc or dansyl-LacNAc in an assay mixture is troublesome. In addition, dansyl-LacNAc is the most insensitive during fluorescence detection, but PA-LacNAc is highly sensitive to fluorescence detection.

In summary, we synthesized PA-LacNAc and evaluated its ability to act as an acceptor. We found that solubility, sensitivity, and affinity toward both sialyltransferases make it suitable as a new probe for sialyltransferase assays.

3. Experimental

3.1. General procedure

^{1}H NMR spectra were recorded with Bruker Avance-400 instrument. Optical rotations were measured with a JASCO DIP-4 polarimeter. High-resolution mass spec-

tra were recorded using a Shimazu/Kratos concept-IIH under FAB conditions. The rat-liver α-(2→6)-sialyltransferases [EC 2.4.99.1] and rat-liver-recombinant α-(2→3)-sialyltransferases [EC 2.4.99.6] were purchased from Calbiochem.

3.2. Allyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-3,6-di-*O*-acetyl-2-azido-2-deoxy-β-D-glucopyranoside (2)

A solution of **1** (2.01 g, 3.15 mmol) and AllONa (8.0 mmol) in 1:2 CH_2Cl_2–AllOH (18 mL) was stirred at room temperature. After 10 min, the mixture was neutralized with AcOH and then concentrated. The residue was treated with pyridine–Ac_2O in the presence of 4-dimethylaminopyridine. After 12 h, the mixture was concentrated. Purification of the residue with a column of charcoal (3.5 i.d.×10 cm, water to 80% EtOH) afforded allyl glycoside **2** (908 mg, 71%), which was identical to the reported data.[16]

3.3. Allyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (3)

To a solution of allyl glycoside **2** (859 mg, 2.03 mmol) in 10:3:1 dioxane–MeOH–H_2O (26 mL) was added Ph_3P (705 mg, 2.69 mmol), and this mixture was stirred at 40 °C. After 20 h, Ac_2O (1.0 mL) was added, and the mixture was stirred for 1 h. The mixture was evaporated, and the residue was suspended into water. The water phase was washed with CH_2Cl_2, and then the water phase was concentrated in vacuo. The residue was dissolved in a solution of 1:1 Ac_2O–pyridine (42 mL) containing 4-dimethylaminopyridine (77 mg, 0.63 mmol), and this mixture was stirred for 12 h. After concentration of this mixture, purification of the residue by silica gel column chromatography (1:4 hexane–EtOAc) afforded lactosaminide **3** (687 mg, 50%), which was identical to that reported.[17]

3.4. 2-Oxoethyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (4)

Ozone was passed through a solution of lactosaminide **3** (130 mg, 0.19 mmol) in CH_2Cl_2 (9.6 mL) for 15 min at −78 °C until the solution became slightly blue. Excess ozone was displaced by an argon stream for 5 min, and then the ozonide was reduced by addition of an excess of Me_2S at −78 °C. The cooling bath was removed, and the mixture was concentrated in vacuo. Purification of the residue by silica gel column (20:1 EtOAc–MeOH) afforded aldehyde **4** (91 mg, 70%): $[\alpha]_D^{21}$ –10.0 (*c* 0.6, EtOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.65 (br s, 1H, aldehyde) 5.77 (d, 1H, $J_{NH,2}$ 9.3 Hz, NH) 5.36 (dd, 1H, $J_{4',3'}$ 3.4 Hz, $J_{4',5'}$ 1.0 Hz, H-4′) 5.12 (dd, 1H, $J_{2',1'}$ 7.8 Hz, $J_{2',3'}$ 10.5 Hz, H-2′) 5.08 (dd, 1H, $J_{3,2}$ 9.9 Hz, $J_{3,4}$ 8.4 Hz, H-3) 4.97 (dd, 1H, $J_{3',2'}$ 10.5 Hz, $J_{3',4'}$ 3.4 Hz, H-3′) 4.51 (d, 1H, $J_{1',2'}$ 7.9 Hz, H-1′) 4.50 (d, 1H, $J_{1,2}$ 7.9 Hz, H-1) 4.47 (dd, 1H, $J_{6a,5}$ 2.5 Hz, J_{gem} 11.9 Hz, H-6a) 4.31 (d, 1H, J_{gem} 17.9 Hz, –OCH_2CHO) 4.21 (dd, 1H, *J* 1.2 Hz, J_{gem} 17.9 Hz, –OCH_2CHO) 4.15 (ddd, 1H, $J_{2,1}$ 7.9 Hz, $J_{2,3}$ 9.9 Hz, $J_{2,NH}$ 9.3 Hz, H-2) 4.14 (dd, 1H, $J_{6'a,5'}$ 6.6 Hz, J_{gem} 11.2 Hz, H-6′a) 4.11 (dd, 1H, $J_{6b,5}$ 5.7 Hz, J_{gem} 11.9 Hz, H-6b) 4.08 (dd, 1H, $J_{6'b,5'}$ 7.2 Hz, J_{gem} 11.2 Hz, H-6′b) 3.89 (ddd, 1H, $J_{5',4'}$ 1.0 Hz, $J_{5',6'a}$ 6.6 Hz, $J_{5',6'b}$ 7.2 Hz, H-5′) 3.79 (dd, 1H, $J_{4,3} = J_{4,5}$ 8.4 Hz, H-4) 3.61 (ddd, 1H, $J_{5,4}$ 8.4 Hz, $J_{5,6a}$ 2.5 Hz, $J_{5,6b}$ 5.7 Hz, H-5) 2.15, 2.11, 2.09, 2.07, 2.05, 2.00, 1.97 (7s, 21H, Ac); HRFABMS: Calcd for $C_{28}H_{40}N_1O_{18}$ $(M+H)^+$ *m*/*z* 678.2245; found *m*/*z* 678.2182.

3.5. 2-[(2-Pyridyl)amino]ethyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (5)

To a solution of aldehyde **4** (255 mg, 0.376 mmol) in MeOH (4.0 mL) was added 2-aminopyridine (178 mg, 1.89 mmol) and HOAc (55 μL), and the mixture was stirred at room temperature. After 30 min $NaBH_3CN$ (48 mg, 0.76 mmol) was added, and the mixture was stirred for 18 h at room temperature. The mixture was diluted with EtOAc and washed with brine. The organic phase was dried ($MgSO_4$) and then concentrated in vacuo to give a residue that was dried in vacuo. To a solution of this residue in dry MeOH (5.0 mL) was added a catalytic amount of NaOMe. After 4 h the mixture was neutralized by addition of Dowex 50Wx8 (H^+ form) and then filtered. The filtrate was then concentrated in vacuo. Purification of this residue by HPLC (column: YMC-Pack ODS-A SH-343-5 S-5 120 A 2.0 i.d.×25 cm, 10:1 50 mM ammonium acetate–MeCN, monitoring by 210 nm or Ex 320 nm/Em 400 nm) and desalting by same HPLC system (H_2O for 30 min and then 1:1 H_2O–MeCN gradient system) afforded pure PA-lactosaminide **5** (55 mg, 29%): $[\alpha]_D^{21}$ –1.57 (*c* 0.57, H_2O); ^{1}H NMR (400 MHz, D_2O): δ 8.04, 7.67, 6.81, 6.76 (each m, each 1H, pyridylamino group) 4.64 (d, 1H, $J_{1',2'}$ 7.7 Hz, H-1′) 4.55 (d, 1H, $J_{1,2}$ 7.8 Hz, H-1) 4.01 (bd, 1H, $J_{4',3'}$ 3.3 Hz, H-4′) 3.75 (dd, 1H, $J_{3',2'}$ 10.0 Hz, $J_{3',4'}$ 3.3 Hz, H-3′) 3.62 (dd, 1H, $J_{2',1'}$ 7.8 Hz, $J_{2',3'}$ 10.0 Hz, H-2′) 1.96 (s, 3H, Ac); HRFABMS: Calcd for $C_{21}H_{34}N_3O_{11}$ $(M+H)^+$ *m*/*z* 504.2193; found *m*/*z* 504.2213.

3.6. 4-Methylumbelliferyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (7)

To a solution of 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside (10.0 mg, 0.026 mmol), BSA (5.0 mg), UDP-galactose (16.0 mg, 0.025 mmol), and alkaline phosphatase (50 U) in HEPES buffer (100 mM, pH 7.0 containing 30 mM $MgCl_2$) was added bovine

β-(1 → 4)-galactosyltransferase (12 mU), and the mixture was incubated at 37 °C. After 48 h, purification with an ODS column (1.5 i.d. × 22 cm, elution by H_2O (100 mL), then 9:1 H_2O–MeCN) afforded 4-methylumbelliferyl-LacNAc **7**[14] (10.8 mg, 0.019 mmol) in 74% yield. ^1H NMR (400 MHz, D_2O): δ 7.58 (d, 1H, J 8.8 Hz, MU group) 6.98 (dd, 1H, J 2.3 Hz, 8.8 Hz, MU group) 6.90 (d, 1H, J 2.3 Hz, MU group) 7.58 (s, 1H, MU group) 5.27 (d, 1H, $J_{1,2}$ 8.4 Hz, H-1) 4.54 (d, 1H, $J_{1',2'}$ 7.8 Hz, H-1′) 4.10 (dd, 1H, $J_{2,1}$ 8.4 Hz, $J_{2,3}$ 9.6 Hz, H-2) 3.95 (bd, 1H, $J_{4,3}$ 3.2 Hz, $J_{4',5'} \sim 0$ Hz, H-4′) 4.08–3.76 (m, 8H, H-3, H-4, H-5, H-6a, H-6b, H-5′, H-6′a, H-6′b) 3.70 (dd, 1H, $J_{3',2'}$ 9.9 Hz, $J_{3',4'}$ 3.2 Hz, H-3′) 3.59 (dd, 1H, $J_{2',1'}$ 7.8 Hz, $J_{2',3'}$ 9.9 Hz, H-2′) 2.35, 2.08 (2s, 6H, CH_3).

3.7. Sialyltransferase assay with varying pH and buffer conditions

Assays[6] were performed with 50 mM HEPES or 50 mM cacodylate buffer solution (30 μL; pH was varied: pH 5.0, 6.0, and 7.0) containing CMP-β-D-NeuAc (300 μM), PA-β-D-LacNAc (1 mM), BSA (10 μg), and rat-liver-recombinant α-(2 → 3)- (1 mU) or rat-liver α-(2 → 6)-sialyltransferases (0.14 mU). The mixture were incubated at 37 °C for 30 min. The reaction was followed up to 15% consumption of donor substrate. After incubation, the mixture was diluted iced H_2O (170 μL), and then NeuAc transfer was estimated with HPLC (C_{18}-column: i.d. 0.46 × 25 cm, 15:1 25 mM ammonium acetate–MeCN) equipped with fluorescence detector (Ex 320 nm, Em 400 nm). The transfer rates were dependent on buffer conditions and are summarized in Figure 2.

3.8. Estimation of K_m and V_{max} values using three kinds of acceptors

Rat-liver α-(2 → 6)-sialyltransferase assays[6] were performed with 100 mM HEPES (30 μL; pH 7.0) containing CMP-β-D-NeuAc (300 μM), fluorescence labeled-β-D-LacNAc (varied concentrations), BSA (10 μg), rat-liver α-(2 → 6)-sialyltransferases (0.1–0.2 mU). The mixtures were incubated at 37 °C. The reaction was followed up to 15% consumption of donor substrate. After incubation, the mixture was diluted with iced H_2O (170 μL), and then NeuAc transfer was estimated by HPLC (C_{18}-column: i.d. 0.46 × 25 cm, 15:1 25 mM ammonium acetate–MeCN) equipped with fluorescence detector. K_m and V_{max} values were estimated using a method of Wilkinson[18] and by a Lineweaver–Burk plot. The data points are the averages of at least duplicate values. MU-β-D-LacNAc (100, 200, 300, 500, and 1000 μM), PA-β-D-LacNAc (50, 100, 200, 300, and 500 μM), and dansyl-β-D-LacNAc (17, 23, 33, 67, and 100 μM) were used for the assays. PA-LacNAc, MU-LacNAc, and dansyl-LacNAc were detected by following excitation and emission values: PA:Ex 320 nm, Em 400 nm; MU: Ex 325 nm, Em 372 nm; dansyl: Ex 340 nm, Em 540 nm.

Rat-liver-recombinant α-(2 → 3)-sialyltransferase assays were performed with 50 mM HEPES (30 μL; pH 5.0) containing CMP-β-D-NeuAc (300 μM), fluorescence labeled-β-D-LacNAc (varied concentrations), BSA (10 μg), rat-liver-recombinant α-(2 → 3)-sialyltransferases (0.1–0.2 mU). Estimation of K_m and V_{max} values were same as in the case of rat liver α-(2 → 6)-sialyltransferases. MU-β-D-LacNAc (100, 200, 300, 500, and 1000 μM), PA-β-D-LacNAc (100, 200, 300, 500, and 1000 μM), and dansyl-β-D-LacNAc (25, 35, 50, 100, and 150 μM) were used for the assays.

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