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The synthesis of galactopyranosyl derivatives with β-galactosidases of different origins

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Abstract

β-Galactosidases from four different sources were used to catalyze the transfer of β-D-galactopyranosyl from 4-nitrophenyl-β-D-galactopyranoside to a hydroxyl group of 2-acetamido-2-deoxy-galactopyranose in the synthesis of Galβ(1–3)GalNAc (**1**), Galβ(1–4)GalNAc (**2**) and Galβ(1–6)GalNAc (**3**), in triethyl phosphate buffered solutions (20–60%). When β-galactosidases from *Penicillium multicolor* and *Aspergillus oryzae* were used as the catalysts, the β(1–6)-linked disaccharide was produced as the major product. However, with β-galactosidase from *Bifidobacterium bifidum*, the major products were the β(1–4) and β(1–6)-linked disaccharides. On the other hand, with β-galactosidase from *Streptococcus* 6646K, β(1–3)-linked disaccharide was predominant together with β(1–4)-linked isomer. Galβ(1–3)GlcNAc (**4**), Galβ(1–4)GlcNAc (**5**) and Galβ(1–6)GlcNAc (**6**) were also synthesized with β-galactosidase from *S.* 6646K and *B. bifidum* with 2-acetamido-2-deoxy-glucopyranose as the acceptor and PNPGal as the donor. In both cases, the β(1–4)-linked disaccharide was predominantly produced. In addition, a comparative study was carried out to determine the regioselectivity of the transglycosylation reaction as well as the hydrolytic specificity toward the same linked disaccharides. © 1996 Elsevier Science Ltd.

Keywords: Glycosidases; Chemoenzymatic synthesis; Bacterial galactosidases; Transglycosylation

1. Introduction

The glycopeptide, Galβ(1–3)GalNAc-Ser, is considered to be the representative unit of mucin type sugar chains and is also found in the core structure of many glycoproteins

Abbreviations: PNPGal, 4-nitrophenyl-β-D-galactopyranoside

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and glycolipids [1–4]. In the context of malignancy, the same glycopeptide unit, in this case called a T-antigen, is highly expressed in many carcinoma cells and is regarded as an important tumor marker [3,4]. The lactosamine unit, Galβ(1–4)GlcNAc, is a typical terminal sequence of *N*-linked oligosaccharides [5]. This sequence, and also Galβ(1–3)GlclNAc, are components of blood group determinants of the ABO system [6]. There is, therefore, a demand for the synthesis of such artificial antigens for immunological studies.

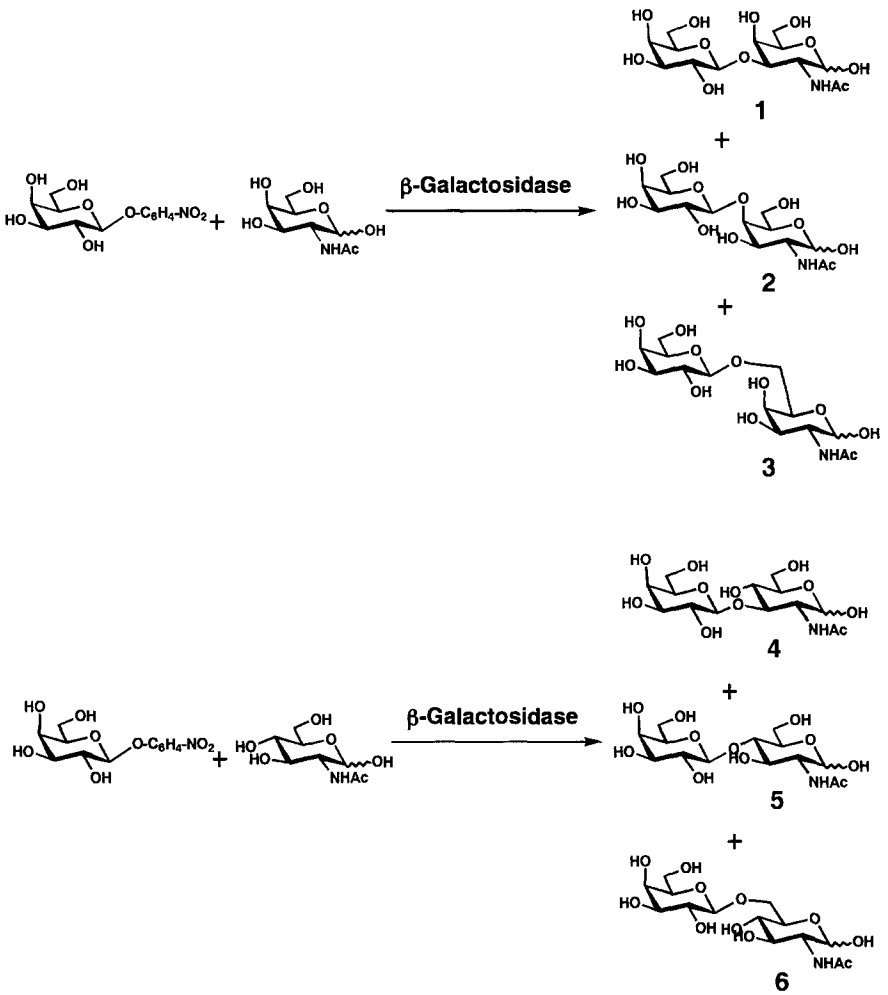
Many elegant methods for the synthesis of these disaccharides have been demonstrated using either chemical methods [7] or enzymic methods [8]. Since Hedbys et al. have reported the enzymic synthesis of β(1–3)-linked galactosyl derivatives with β-galactosidase from bovine testes, many syntheses have been performed with the same enzyme [6,8,9]. In the case of β(1–4)-linked disaccharides, β-galactosidase from either *Bacillus circulans* or *Diplococcus pneumoniae* is commonly used [10–12]. Our laboratory recently reported that a lactosamine unit can be synthesized regioselectively by choosing β-galactosidases according to their hydrolytic activity toward isomeric oligosaccharides [12].

In this study, β-galactosidases from various origins were examined to determine their ability to specifically hydrolyze Gal-GalNAc and Gal-GlcNAc isomers. This was done in order to find new enzymes for use in our synthetic work. As a result, we have been able to find β-galactosidases capable of synthesizing β(1–3) and β(1–4)-linked galactosyl derivatives with high specificity.

2. Results

Synthesis of compounds **1**, **2** *and* **3** *by transglycosylation using* β*-galactosidase.*— Since the synthesis of Galβ(1–3)GalNAc and Galβ(1–6)GalNAc employing β-galactosidase from bovine testes has been reported [8], many researchers have used this enzyme for synthesis [8,9]. It may, however, be difficult to obtain sufficient amounts of this enzyme from an animal source for use in large scale preparations. We therefore screened β-galactosidases from a number of microbes for their ability to effect the same transformations. The formation of galactose derivatives in the presence of β-galactosidase is shown in Scheme 1. Fig. 1 shows the HPLC result of reaction mixtures with β-galactosidases from different origins. β-Galactosidases from *P. multicolor*, *A. oryzae*, and *B. bifidum* produced mixtures of β(1–3), β(1–4) and β(1–6)-linked isomers. However, the β-galactosidases from *P. multicolor* and *A. oryzae* produced mainly **3** along with **1** and **2** as minor by-products. In contrast, β-galactosidase from *B. bifidum* afforded **2** and **3** in ca. 3:2 ratio.

β-Galactosidase from *S.* 6646K gave **1** as a main product with smaller amounts of **2**. In this case 20% triethyl phosphate was used as cosolvent to improve the solubility of PNPGal. In general, the fact that solvents do not change regioselectivity shows that the structure of enzyme is not severely affected by organic solvent. Although this is the enzyme which preferentially produces the β(1–3)-linked, the amount of disaccharide is limited because this enzyme is also expensive. Further investigations of the nature of this enzyme are therefore needed.



Scheme 1. The formation of disaccharides with β-galactosidase.

Synthesis of compounds **4**, **5** *and* **6**.—Fig. 2 shows the HPLC analysis of the reaction mixtures in the presence of β-galactosidase from *B. bifidum* and *S.* 6646K. When β-galactosidase from *B. bifidum* was used in the synthesis of disaccharides, the β(1–4)-linked isomer **5** was formed almost exclusively, with a minor amount of the β(1–6)-linked **6**. This result was expected because the same linkages are preferred in the hydrolysis of the substrates. N-Acetyllactosamine was synthesized with *B. circulans* enzyme from lactose and GlcNAc in a yield of 23.2% [11]. The synthesis of the same compound was reported with the enzyme from *D. pneumoniae* in a yield of 11% [12]. In our case the enzymes from both *B. bifidum* and *S.* 6646K produced the desired product in over 30% yield. Especially, with β-galactosidase from *B. bifidum*, **5** was obtained in 35% yield, which was much higher than that reported by Usui et al. [13] using

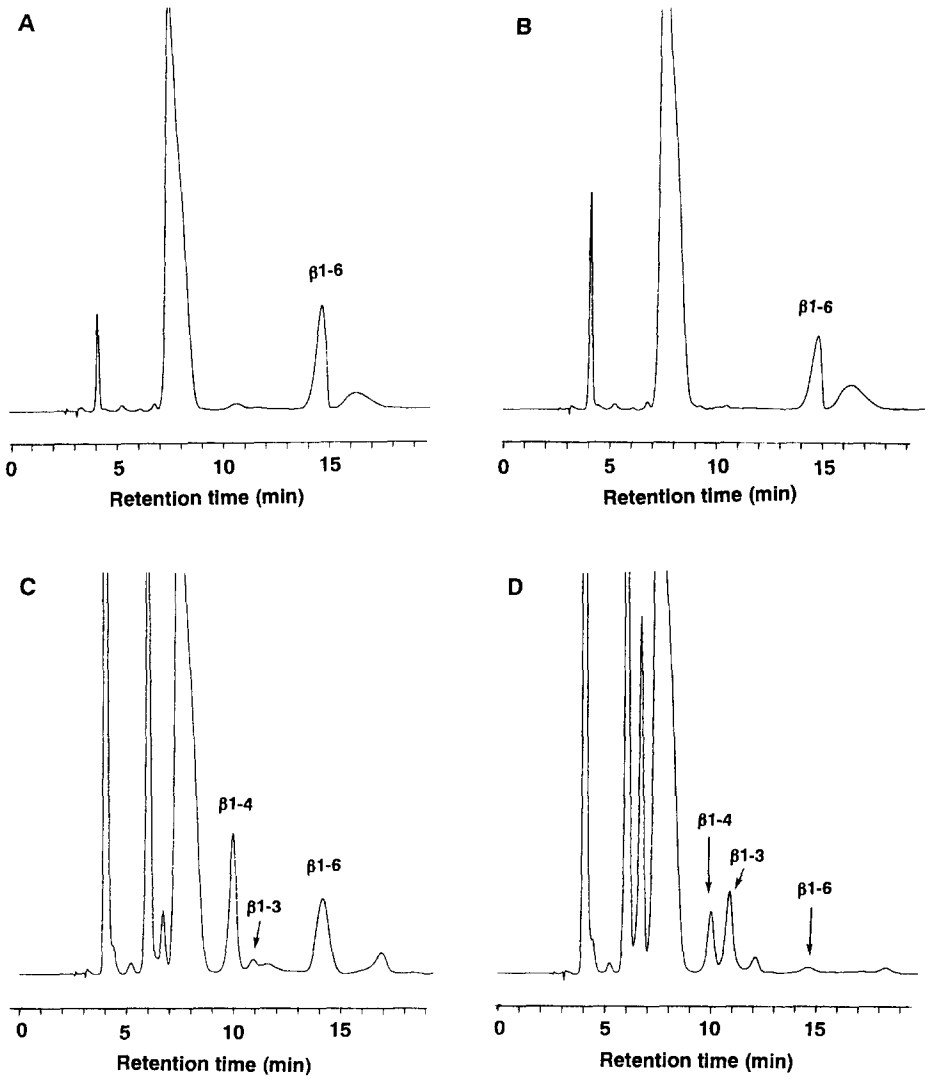


Fig. 1. HPLC chromatograms of the reaction mixtures in which **1**, **2** and **3** have been formed using β-galactosidases from *A. oryzae* (**A**), *P. multicolor* (**B**), *B. bifidum* (**C**), *S.* 6646K (**D**). CAPCEL PAK NH_2 column was used with 80% acetonitrile.

β-galactosidase from *B. circulans*. As *B. bifidum* is relatively easy to culture, it serves as a good enzyme source for preparation of β(1–4) disaccharides. With β-galactosidase from *S.* 6646K, **5** was also the major product with ten-times less of **4**. In both cases the results of syntheses were in agreement with those of the substrate specificity during hydrolysis.

Comparison of hydrolytic specificity to regioselectivity during transglycosylation.—In many cases it has been noticed that the hydrolytic specificity of isomeric oligosaccha-

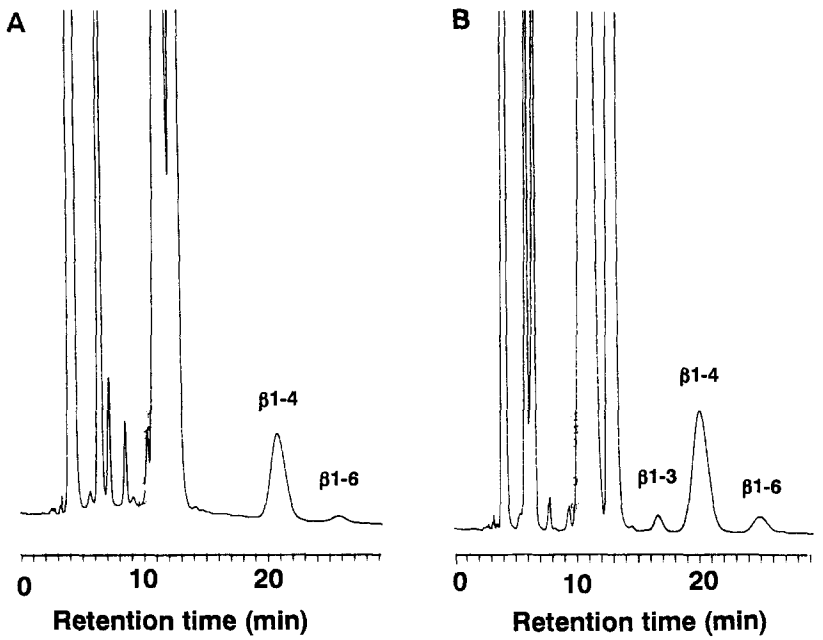


Fig. 2. HPLC chromatograms of the reaction mixtures in which **4**, **5** and **6** have been formed in the presence of β-galactosidases from *B. bifidum* (**A**) and *S.* 6646K (**B**). YMC-Polyamine II column was used with 80% acetonitrile.

rides by glycosidases can be useful for predicting the regioselectivity of transglycosylation. Several attempts have therefore been made to establish a correlation between hydrolytic specificity and regioselectivity during transglycosylation [6,12,16]. Similar experiments were carried out with β-galactosidase from *S.* 6646K and *B. bifidum* on Gal-GalNAc and Gal-GlcNAc isomers. Fig. 3 shows plots of residual amounts of substrates against time. The rates of hydrolysis for β-galactosidase from *S.* 6646K of Gal-GalNAc are in the order $\beta 1\text{–}3 > \beta 1\text{–}4 > \beta 1\text{–}6$, whereas the Gal-GlcNAc isomers are in a slightly different order $\beta 1\text{–}4 \gg \beta 1\text{–}3 > \beta 1\text{–}6$. With the *S.* 6646K enzyme the ratios of the rate constants for hydrolysis are $k_1/k_2 = 1.8$ and $k_5/k_4 = 3.9$, respectively. These results are in agreement with the preferential synthesis of **1** and **5**. β-Galactosidase from *B. bifidum* led to a faster hydrolysis of β(1–4)-linked isomers in agreement with the observed high yields of **2** and **5**. The ratios of the rates of hydrolysis were the values of $k_5/k_4 = 5.1$ and $k_5/k_6 = 23.8$. Thus the preferential production of β(1–4)-linked isomer seems plausible.

3. Discussion

In general, glycosidases produce 1-6 linkages most easily during transglycosylation due to the high reactivity of the acceptor primary hydroxyl group. However, products

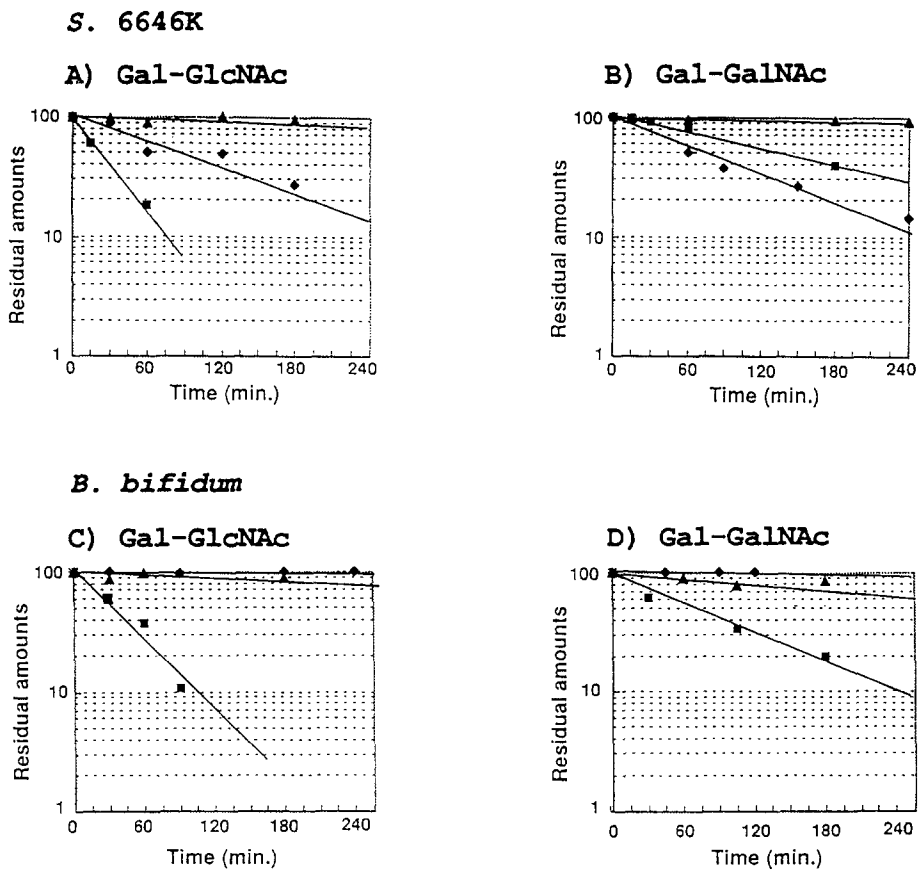


Fig. 3. Plots of % residual amounts of substrates versus time from hydrolysis in Gal-GlcNAc and Gal-GalNAc isomers catalyzed by β-galactosidases from *S.* 6646K or *B. bifidum*; —◆— β1–3, —■— β1–4, —▲— β1–6.

with other linkages (e.g. 1–3 or 1–4) are also formed, but their rates of hydrolysis may be rapid in comparison with those of 1–6 linked products. Since the accumulation of product depends on the ratio of the rate of transfer to the rate of hydrolysis, we used a high concentration of non-aqueous solvent, triethyl phosphate, in order to reduce the rate of hydrolysis. The β-galactosidase was reasonably stable in aq triethyl phosphate solution as previously reported [14].

In order to raise the yield of disaccharide, we stopped the reaction just before PNPGal disappear in HPLC, because prolonged incubation was found to decrease the production of desired compounds (data not shown). This can be interpreted as follows: as the donor glycoside is consumed and the transglycosylation product accumulates, the rate of hydrolysis of the glycoside product eventually becomes larger than its rate of formation; the secondary hydrolysis will then reduce the yield of glycoside by shifting it

toward the equilibrium concentration. A more rapidly formed product may thus be more susceptible to secondary hydrolysis than a more slowly formed product. We confirmed that high concentrations of organic solvent, large amounts of enzyme and short reaction times are favorable to the formation of desirable minor products. This result was also obtained by Usui et al. [13] who reported the formation of β(1–4) and β(1–6)-linked *o*-nitrophenyl galactosyl GalNAc by β-galactosidase from *B. circulans* in 20–50% acetonitrile: higher concentrations of acetonitrile and shorter reaction times gave a higher yield of β(1–4)-linked compound.

Early work with β-galactosidase from *B. bifidum* and lactose showed a preferential transfer to the 3 position linked to galactosyl derivatives [15]. However, in the present study, the enzyme gave β(1–4)-linked galactosyl derivatives in good yield (33%). The reason for the change in regioselectivity is attributed to the structure of the acceptor because the substitution of the 2-OH group with *N*-acetoamino group may lead to a greater affinity to an electrophilic center in the enzyme acceptor site and influences the conformation of the acceptor bound to the enzyme. The steric interaction between the acceptor substituent and the enzyme may be a relevant factor. Differences in the structure of the acceptor may influence its interaction with the enzyme and affect the bonding with the glycosyl moiety with unexpected results.

Although the hydrolysis results are well matched with the formation of disaccharides, it is interesting to note that in the case of *S.* 6646K the regioselectivity toward acceptors was different for Gal-GalNAc and Gal-GlcNAc. Another interesting phenomenon is that both enzymes have a different hydrolytic sensitivity for Gal-GalNAc and Gal-GlcNAc isomers. The hydrolysis of Gal-GlcNAc in both cases is more rapid than that of Gal-GalNAc. The rate of hydrolysis may depend on the structure of the acceptor and this selectivity is different from enzyme to enzyme. As shown previously [6,16], larger ratios for the rates of hydrolysis produced preferential formation of the same disaccharides. Thus, our results imply that the faster a specific disaccharide is hydrolyzed, the faster the same disaccharide can be formed in a transglycosylation reaction.

Therefore, the information on the hydrolytic specificity of glycosidases on isomeric oligosaccharides can be used to select enzymes for synthesis. Although enzyme sources are important, regioselectivity can be changed by the structure of the acceptor. In general, strategies to control the regioselectivity may be devised for the use of one glycosidase for the preferential formation of different linkages.

4. Experimental

General.—*N*-Acetyl galactosamine (Sigma), *N*-acetyl glucosamine (Tokyo Kasei Kogyo, Co., Tokyo, Japan) 4-nitrophenyl-β-D-galactopyranoside (Nacalai Tesque. Co., Kyoto, Japan) were commercial products. Chemicals were purchased from Wako (Osaka, Japan) and were used without further purification unless otherwise stated. β-Galactosidase of *S.* 6646K (Seikagaku Kogyo. Co., Tokyo, Japan), *A. oryzae* (Toyobo. Co., Osaka, Japan), *P. multicolor* (K.I.Chemicals., Shizuoka, Japan) were commercial products. Results are listed in Table 1

Table 1
Synthesis of D-galactosyl-containing disaccharides with various β-galactosidases

Acceptor	Origin of enzyme	Linkage	Yield %
D-GalNAc	*S.* 6646K	β-D-(1–3)	3.7
		β-D-(1–4)	1.2
	A. oryzae	β-D-(1–3)	1.4
		β-D-(1–4)	0.7
		β-D-(1–6)	25.5
	P. multicolor	β-D-(1–3)	trace
		β-D-(1–4)	1
		β-D-(1–6)	17
	B. bifidum	β-D-(1–3)	trace
		β-D-(1–4)	33.3
		β-D-(1–6)	19.1
D-GlcNAc	*S.* 6646K	β-D-(1–3)	4.4
		β-D-(1–4)	31.2
	B. bifidum	β-D-(1–4)	38.2
		β-D-(1–6)	2.5

One unit of activity is that amount of enzyme which will liberate one μmol of 4-nitrophenol from 4-nitrophenyl-β-D-galactopyranoside per min at 37 °C in 50 mM NaOAc/acetic acid buffer of pH 5.5 (buffer A) for *S.* 6646K and the same buffer of pH 4.5 (buffer B) for *B. bifidum*, *A. oryzae* and *P. multicolor*.

^{1}H, ^{13}C, HSQC and TOCSY NMR spectra were recorded in D_2O on a Varian unity-500 spectrometer (^{1}H, 499.82 MHz; ^{13}C, 125.68 MHz) with *tert*-butyl alcohol as the reference. Peak assignments were based on double resonance and TOCSY experiments. Optical rotations were measured on a Horiba SEPA-300 automatic polarimeter at 25 °C. An HPLC equipped with a JASCO PU-980 Intelligent HPLC pump and a UV monitor (215 nm) was performed using Asahipak NH2P-50 column (Showa-Denko Co., Ltd, Tokyo, Japan), CAPCEL PAK NH_2 (Shiseido Co., Ltd, Tokyo, Japan), or YMC-Pack Polyamine II (YMC Co., Ltd, Kyoto, Japan) for analytical scales or a LiChrospher 100 NH_2 column (10 mm × 250 mm, 10 μm; Merck) for preparative scales.

Preparation of β-galactosidase from Bifidobacterium bifidum.—β-galactosidase from *B. bifidum* (DSM 20456) was cultured in Briggs liver broth for 7 days in anaerobic condition: Components of Briggs liver broth; tomato juice 400 mL, neopeptone 15 g, yeast extract 6 g, liver extract 75 mL, glucose 20 g, soluble starch 0.5 g, tween 80 1 g, L-cysteine/HCl 0.2 g, distilled water 505 mL, pH 6.8. The culture broth was centrifuged at 6,000 rpm for 30 min at 4 °C. The obtained supernatant contained β-galactosidase activity of 101 units/mL and was used without further purification.

2-Acetamido-2-deoxy-3-O-β-D-galactopyranosyl-D-galactose (**1**): *(A) With β-galactosidase from* Streptococcus *6646K*.—To a solution of 4-nitrophenyl-β-D-galacto-

Table 2
^{13}C NMR data of D-galactose derivatives: GalNAc (I), GlcNAc (II), Gal (III)

Compound	Residue	C-1	C-2	C-3	C-4	C-5	C-6	C=O	CH_3
1	Iα	91.2	48.9	76.8	68.4	70.1	60.9	174.6	22.2
	Iβ	95.1	52.5	80.8	67.8	74.8	60.8	174.3	22.0
	III	104.3	70.8	72.5	68.2	74.9	60.8		
2	Iα	91.0	50.7	67.9	76.5	69.8	60.7	174.6	21.9
	Iβ	95.2	54.0	71.5	77.5	74.2	60.5	174.3	22.1
	III	104.3	71.3	72.7	68.5	75.0	60.9		
3	Iα	91.2	50.3	67.3	67.3	69.6	69.3	175.0	22.2
	Iβ	95.4	53.8	71.0	68.0	74.0	68.8	174.5	22.0
	III	103.3	70.8	72.8	68.8	75.2	61.0		
4	IIα	91.0	52.9	80.2	71.2	72.6	61.1	174.8	22.2
	IIβ	94.7	55.6	82.7	70.7	75.2	61.1	174.5	22.0
	III	103.4	72.8	74.9	68.7	75.4	60.6		
5	IIα	90.5	53.5	69.2	78.7	70.2	60.0	174.8	22.2
	IIβ	94.5	56.4	72.3	78.4	74.8	60.0	174.5	22.0
	III	104.1	70.8	72.4	68.4	75.3	61.0		
6	IIα	90.5	53.8	70.4	70.1	70.8	68.6	174.8	22.2
	IIβ	94.5	56.6	73.6	70.1	75.0	68.2	174.5	22.0
	III	104.1	71.2	72.6	68.8	75.0	60.8		

pyranoside (10 mg, 0.03 mmol) and 2-acetamido-2-deoxy-D-galactose (20 mg, 0.09 mmol) in 0.05 mL of buffer A incorporating 20% of triethyl phosphate was added β-galactosidase from *S.* 6646K (0.1 unit). The reaction mixture was shaken at 37 °C. Samples (5 μL) were taken at various time intervals. Each sample was heated at 100 °C for 2 min, and made up to 10 μL with deionized water. After centrifugation, 5 μL aliquots were examined by HPLC. In general, the reaction was stopped after 5 h then the sample was applied to HPLC and eluted with 80% acetonitrile at a flow rate of 1.0 mL/min to give **1** (0.9 mg, 3.7% based on PNPGal added); $[\alpha]_D$ +45.7 (*c* 1, H_2O): NMR data of galactose derivatives formed in the presence of β-galactosidase were listed in Table 2. Under the conditions described above, one other isomer was found, which was identified as **2** (0.3 mg, 1.2%) by ^{13}C NMR spectroscopy.

2-Acetamido-2-deoxy-4-O-β-D-galactopyranosyl-D-galactose (**2**): *(A) Using β-galactosidase from* Bifidobacterium bifidum.—β-galactosidase from *B. bifidum* culture broth (0.2 mL, 20 units) was incubated with PNPGal (144 mg, 0.478 mmol) and GalNAc (320 mg, 1.447 mmol) in buffer B (400 μL) and triethyl phosphate (600 μL) for 2–4 h at 37 °C. The reaction procedures were the same as for *S.* 6646K. In this case, before HPLC was carried out the organic solvent was removed by a rotary evaporator and the reaction mixture in water was passed through an activated carbon column (2.0

cm × 50 cm). After washing the column with water for a few hours at a flow rate of 2 mL/min to remove monosaccharides, the disaccharides were eluted with the linear gradient of water (2 L) and 30% aq EtOH (2 L). Disaccharide fractions were detected by the phenol-sulfuric acid method and the desired fractions were collected. After the solution was concentrated it was applied to HPLC for further isolation of compound **2** (58 mg, 33.3%), $[\alpha]_D$ +43.2 (*c* 1, H_2O). Two other isomers were also isolated and identified as **1** (trace amount) and **3** (33.4 mg, 19.1%) by ^{13}C NMR spectroscopy.

2-Acetamido-2-deoxy-6-O-β-D-galactopyranosyl-D-galactose (**3**)*: (A) With β-galactosidase from* Aspergillus oryzae.—A solution of PNPGal (144 mg, 0.478 mmol) and GalNAc (320 mg, 1.477 mmol) in buffer B (400 μL) and triethyl phosphate (600 μL) was incubated with β-galactosidase from *A. oryzae* (80 units) at 37 °C. Samples were withdrawn for analysis after 45 min, the reaction mixture was then separated by preparative HPLC and gave, after freeze-drying **3** (43 mg, 25.5%), $[\alpha]_D$ +52 (*c* 1, H_2O). Under these conditions two other isomers were obtained: **1** (2.5 mg, 1.4%) and **2** (1.2 mg, 0.7%).

(B) With β-galactosidase from Penicillium multicolor.—β-galactosidase from *P. multicolor* (50 units) was used in the synthesis of disaccharides with the same donor and acceptor in buffer B at 55 °C for 2 h. Reaction conditions and procedures were the same as for *A. oryzae*. The yield of **3** was 17% (6.5 mg). Two other isomers were isolated: **1** (trace) and **2** (0.38 mg, 1%).

2-Acetamido-2-deoxy-4-O-β-D-galactopyranosyl-D-glucose (**5**)*: (A) With β-galactosidase from* Bifidobacterium bifidum.—A solution of PNPGal (144 mg, 0.478 mmol) and GlcNAc (320 mg, 1.477 mmol) in buffer B (total volume 1 mL) with 60% (v/v) triethyl phosphate was incubated with β-galactosidase from *B. bifidum* at 37 °C. Samples were withdrawn for analysis over a period of 4 h. In general, the reaction was stopped after 2 h then the solution was separated by preparative HPLC to give **5** (70 mg, 38.2%) $[\alpha]_D$ +27 (*c* 1, H_2O). One other isomer **6** was isolated (4.5 mg, 2.5%), $[\alpha]_D$ +22.3.

(B) With Streptococcus *6646K β-galactosidase.*—β-galactosidase from *S.* 6646K (0.1 unit) was used to synthesize disaccharides with GlcNAc (40 mg, 0.12 mmol) as the acceptor and PNPGal (20 mg, 0.06 mmol) as the donor in 20% (v/v) triethyl phosphate buffer A (total 0.12 mL) at 37 °C for 5 h. Reaction procedures were the same as those used with *B. bifidum*. The product was isolated by preparative HPLC to give **5** (7.8 mg, 31.2%) and **4** (1.1 mg, 4.4%), $[\alpha]_D$ +19.8 (*c* 1, H_2O).

Rates of hydrolysis.—The general procedure for measuring hydrolysis rates of β-galactosidase from *S.* 6646K and *B. bifidum* was as follows: 200 μL of solutions of A (**1** + **2** + **3**, 0.05 mg each) and B (**4** + **5** + **6**, 0.05 mg each) containing the same amounts of chitotriose as the standard were made up in a suitable buffer for each enzyme. The solution was incubated at 37 °C for 4 h. Aliquots (10 μL) of the solution were removed at various intervals for HPLC analysis. Asahipak NH2P-50 column was used for HPLC measurement with 80% aq acetonitrile. The peak areas of all components in the reaction mixtures were measured by a Hitachi D-2500 chromato-integrator and the residual areas were plotted against the sampling time. The peak area of the standard component was taken as 100% and those of residual components were expressed as a percentage of the standard component.

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