Glycoconjugate Journal 15, 575–582 (1998)

Enzymatic synthesis of β-D-Gal-(1→3)-[β-D-GlcNAc-(1→6)]-α-D-GalNAc-$OC_6H_4NO_2$-*p* as a carbohydrate unit of mucin-type 2 core

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We have established a synthetic method for obtaining β-D-Gal-(1→3)-[β-D-GlcNAc-(1→6)]-α-D-GalNAc-$OC_6H_4NO_2$-*p* (1), which is a carbohydrate unit of mucin-type 2 core. A *N*-acetyl-D-hexosaminidase from *Nocardia orientalis* catalyzed the synthesis of the desired compound 1 with its isomers β-D-GalNAc-(1→6)-β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* (2) β-D-GlcNAc-(1→3)-β-D-Glc-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* (3) through *N*-acetylglucosaminyl transfer from *N*,*N*′-diacetylchitobiose and β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*. The enzyme formed the trisaccharides 1, 2, and 3 in 14% overall yield based on β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* as an acceptor substrate, and in the ratio of 44:32:24. In this way, *N*-acetylglucosaminyl transfer favored O-6 of the acceptor rather than O-6′, and occurred to a lesser extent at O-3′. This reaction was efficient enough to allow a one-pot preparation of the desired carbohydrate unit of mucin-type 2 core. When β-D-Gal-(1→3)-β-D-GalNAc-$OC_6H_4NO_2$-*p* was used as an acceptor, the enzyme also synthesized three kinds of trisaccharides in the same regioselectivity with respect to O-6 and O-6′ versus O-3′ of the acceptor.

***Keywords*: β-*N*-acetyl-D-hexosaminidase, β-D-galactosidase, β-D-Gal-(1→3)-[β-D-GlcNAc-(1→6)]-α-D-GalNAc-$OC_6H_4NO_2$-*p*, mucin-type 2 core, transglycosylation**

***Abbreviations*: endo-α-GalNAc-ase, endo-α-*N*-acetylgalactosaminidase; β-NAHase, β-*N*-acetyl-D-hexosaminidase; Lacβ-*p*NP, *p*-nitrophenyl β-lactoside; LacNAcβ-*p*NP, *p*-nitrophenyl β-*N*-acetyllactosaminide; $(GlcNAc)_2$, *N*,*N*′-diacetylchitobiose; CD, cyclodextrin**

Introduction

Mucin type oligosaccharides are found in serum, cell membrane glycoproteins and high molecular weight mucins. They can present multivalent carbohydrate antigenic or functional determinants for antibody recognition, mammalian cell adhesion and microorganism binding [1, 2]. The majority of the mucin-type carbohydrates of serum and membrane glycoproteins are of the core 1 which is β-D-Gal-(1→3)-α-D-GalNAc-Ser/Thr. However, human activated T-cells [3], leukemias [4], and immunodeficiencies [5] have all been associated with stimulation by β1-6GlcNAc-transferase (EC 2.4.1.102) giving oligosaccharides with predominantly core 2 rather than core 1. Thus, core 2 structure may have numerous roles in cell differentiation and transformation. There has been present a great interest in developing synthetic routes to core 2 oligosaccharide. An organic chemical method for obtaining β-D-Gal-(1→3)-[β-D-GlcNAc-(1→6)]-α-D-GalNAc-$OCH_2C_6H_5$ (**1**) has been developed [6], but it involves various elaborate procedures for protection, glycosylation and deprotection. From a practical viewpoint, the use of glycosidase is an attractive alternative for synthesis of such a trisaccharide glycoside [7, 8]. We have already established a preparative synthetic method for obtaining β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* [9]. It has made possible the use of large amounts of β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* as a starting substance for the synthesis of compound **1**. Therefore, our interest was directed to an enzymatic synthesis involving a β-D-Gal-(1→3)-[β-D-GlcNAc-(1→6)]-D-GalNAc unit α-glycosidically linked to *p*-nitrophenol as a core 2 oligosaccharide. This compound would be useful as an exogenous substrate for a new type of endo-α-*N*-acetylgalactosaminidase (EC 3.2.1.97) from *Streptomyces* sp. (endo-α-GalNAc-ase-S) [10, 11], and as a probe for carbohydrate antibodies and lectins. Thus, the object of the present investigation is to develop a system for selective transfer of the *N*-acetylglucosaminyl residue to the C-6 position of β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*.

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This paper describes a preparatives synthetic method for β-D-Gal-(1 → 3)-[β-D-GlcNAc-(1 → 6)]-α-D-GalNAc-$OC_6H_4NO_2$-*p*, which is a carbohydrate unit of mucin-type 2 core, and its analogs, by means of *Nocardia orientalis* β-*N*-acetyl-D-hexosaminidase (EC 3.2.1.52)-catalyzed transglycosylation.

Materials and methods

Materials

β-D-Galactosidase (EC 3.2.1.23) from porcine testes was purified according to our method [9]. β-NAHase (EC 3.2.1.52) prepared by 20–70% saturated ammonium sulfate precipitation from culture broth of *N. orientalis* was directly used for the enzymatic synthesis without further purification [12]. Endo-α-GalNAc-ase-A (EC 3.2.1.97) from *Alcaligenes* sp. and endo-α-GalNAc-ase-D from *Diplococcus pneumonia* were purchased from Seikagaku Corp. (Tokyo, Japan) and from Takara Shuzo Co. Ltd (Kyoto Japan), respectively. β-D-Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* and β-D-Gal-(1 → 3)-β-D-GalNAc-$OC_6H_4NO_2$-*p* were synthesized by our method [9]. All other chemicals were obtained from commerical sources.

Enzyme assay

β-NAHase activity was assayed as follows. A mixture containing 2 mM *p*-nitrophenyl *N*-acetyl-β-D-glucoaminide in 0.98 ml of 0.1 M sodium acetate buffer (pH 5.0) and 20 µl of enzyme was incubated for 10 min at 40 °C. The reaction was stopped by adding 2 ml of 1 M Na_2CO_3, and then the liberated *p*-nitrophenol was determined spectrophotometrically at 405 nm. One unit of enzyme was defined as the amount hydrolyzing 1 µmol of *p*-nitrophenol per min.

Analytical method

HPLC was performed with a YMC-packed column type AQ-312 (ODS) (ϕ 6 × 150 mm) and a TSK-GEL G-Oligo-PW (ϕ 7.8 × 300 mm) in a Hitachi 6000-series liquid chromatograph equipped with an L-4000 ultraviolet detector. Elution of the former column was effected with H_2O-CH_3OH in the ratio of 88:12, and that of the latter with H_2O. The flow rate are 0.8 ml min^{-1} at 40 °C. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL JNM-LA 500 spectrometer at 30 °C. Chemical shifts are expressed in δ relative to sodium 3-(trimethylsialyl)-propionate as an external standard. FAB-MS analyses were carried out in the positive ion mode using JEOL JMS DX-303HF mass spectrometer, coupled to a JEOL DA-800 mass data system. An accelerating voltage of 10 kV and a mass resolution of 1000 were employed. A sample of 1 µl in distilled water was loaded onto a probe tip and mixed with 1 µl of glycerol as a matrix. Mass calibration was performed using Ultramark. Specific rotation was determined with a digital polarimeter DIP-1000 apparatus (JASCO Corp., Ltd.).

Preparation of β-D-Gal-(1 → 3)-[β-D-GlcNAc-(1 → 6)]-α-D-GalNAc-$OC_6H_4NO_2$-*p* (**1**) and its positional analogs

β-D-Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* (210 mg) and $(GlcNAc)_2$ (1827 mg) were dissolved in 18 ml of 20 mM sodium acetate buffer (pH 5.0), followed by β-NAHase from *N. orientalis* (20 U). The molar ratio of the acceptor to donor was about 1:10, and the total substrate concentration was about 12%. The mixture was incubated for 12 h at 40 °C and the reaction was terminated by heating for 5 min at 95 °C. To the reaction mixture was added 6 ml of methanol (MeOH), and it was then loaded onto a Toyopearl HW-40S column (ϕ 4.0 × 95 cm) equilibrated with 25% MeOH in aqueous solution, and the effluent solution was monitored by measuring the absorbances at 300 nm (*p*-nitrophenyl group), 485 nm (neutral sugar content, phenol-sulfuric acid method) and 210 nm (*N*-acetyl group). As shown in Figure 1, the chromatogram showed four peaks (F-1, tubes 75–94; F-2, tubes 100–108; F-3, tubes 110–118; and F-4, tubes 133–147) displaying coincident absorbances at 300 nm and 210 nm. The former three peaks were presumed to contain transfer products. Fractions F-1 and F-2 were further purified by preparative HPLC using an ODS column (YMC-pack ODS SH-345-5, ϕ 20 × 500 mm). The flow ratio was 3.0 ml min^{-1}. Elution of the column was effected with H_2O–MeOH in the ratio of 75:25 and monitored by measuring the absorbance at 300 nm. The eluates corresponding to F-1′ (63–68 min) and F-2′ (81–87 min) were each combined, concentrated, and lyophilized to afford compounds **1** (13.9 mg) and **2** (8.9 mg), respectively. Fraction F-3 was concentrated and lyophilized to afford compound **3** (6.0 mg). Fraction F-4 contained β-D-Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* used as the acceptor substrate.

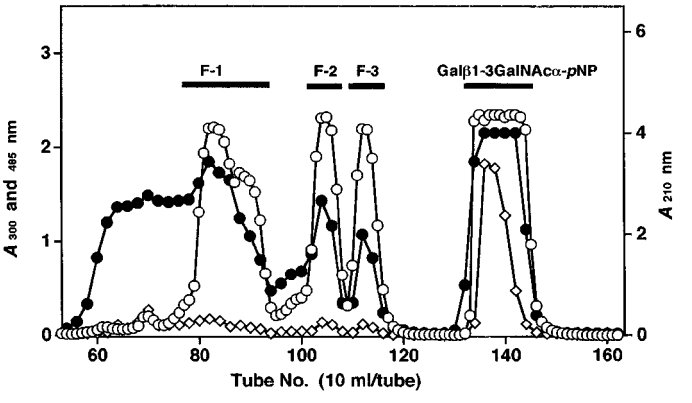


Figure 1. Toyopearl HW-40S chromatographic separation of transglycosylation products formed from $(GlcNAc)_2$ and β-D-Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* by *N. orientalis* β-NAHase (○), Absorbance at 300 nm (◇), Absorbance at 495 nm (●), Absorbance at 210 nm.

Preparation of β-D-Gal-(1 → 3)-[β-D-GlcNAc-(1 → 6)]-β-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* (**4**) and its positional analogs

β-D-Gal-(1 → 3)-β-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* (87.1 mg) and (GlcNAc)$_2$ (757.8 mg) were dissolved in 7.5 ml of 20 mM sodium acetate buffer (pH 5.0), followed by β-NAHase from *N. orientalis* (15 U). The molar ratio of the acceptor to donor was about 1:10, and the total substrate concentration was about 11%. The mixture was incubated for 12 h at 40 °C and the reaction was terminated by heating for 5 min at 95 °C. To the reaction mixture was added 2.5 ml of MeOH, and it was then loaded onto a Toyopearl HW-40S column (ϕ 2.2 × 90 cm) as described above. The chromatogram showed three peaks (F-5, tubes 36–41; F-6, tubes 44–51; and F-7, tubes 55–65) displaying coincident absorbances at 300 nm and 210 nm. The former two peaks were presumed to contain transfer products. Fractions F-5 and F-6 were further purified by preparative HPLC with the ODS column already mentioned. The elution of the column was effected with H$_2$O–MeOH in a ratio of 75:25, at a flow rate of 5.0 ml min^{-1}. The eluates correspondng to the F-5′ (66–70 min), F-6-a (72–75 min) and F-6-b (77–80 min) were each combined, concentrated, and lyophilized to afford compounds **4** (3.2 mg), **5** (2.2 mg) and **6** (1.2 mg), respectively. Fraction F-7 contained β-D-Gal-(1 → 3)-β-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* used as the acceptor substrate.

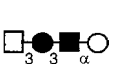
Results and discussion

Characterization of compound **1** and positional analogs

The positive ion mode FAB-MS/MS spectrum of compound **1** shows a molecular ion at *m/z* 708 ([M + H]$^+$) with a fragment ion at *m/z* 546 (fragment from HexNAc-HexNAc-OC$_6$H$_4$NO$_2$). It indicates that compound **1** is a branched trisaccharide Hex-(HexNAc-)HexNAc-OC$_6$H$_4$NO$_2$. The ^{1}H-NMR signals of the compound **1** were easily assigned by correlation with the reported spectrum for β-D-Gal-(1 → 3)-α-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* (Table 1). The additional signals at δ 4.44, 3.54, 3.42, 3.27, 3.36, 3.83 and 1.88 arise from proton signals of H-1, 2, 3, 4, 5, 6, and NAc due to a GlcNAc residue, respectively. The introduction of a GlcNAc residue to β-D-Gal-(1 → 3)-α-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* also resulted in 0.156 and 0.324 ppm downfield shifts of H-5 and H-6 of GalNAc residue on **1**, respectively (Table 1). It shows that the GalNAc residue on the trisaccharide is substituted at the 6-position. The ^{13}C-NMR spectrum of **1** using HSQC provided useful information on the composition and sugar sequence. All of the different carbon lines were resolved using carbon-proton shift correlation (Table 1). The spectrum was also correlated with that of β-D-Gal-(1 → 3)-α-D-GalNAc-OC$_6$H$_4$NO$_2$-*p*. Seven signals of the GlcNAc residue, which did not appear in the spectrum of β-D-Gal-(1 → 3)-α-D-GalNAc-OC$_6$H$_4$NO$_2$-*p*, were clearly differentiated from other signals. The most direct evidence that the GlcNAc residue is bound to the C-6 position of GalNAc, was obtained from the HMBC spectrum (Figure 2), due to long-range couplings from GlcNAc H-1 to GalNAc C-6 and GlcNAc C-1 to GalNAc H-6 and H-6′. The NMR and FAB-MS/MS analyses revealed that **1** is a β-D-Gal-(1 → 3)-[β-D-GlcNAc-(1 → 6)]-α-D-GalNAc-OC$_6$H$_4$NO$_2$-*p*: $[\alpha]_D^{25}$ + 116.9° (*c* 0.5, H$_2$O).

In the same way, the structures of compounds **2** and **3** were similarly characterized. The each positive ion mode FAB-MS/MS spectra of **2** and **3** show a molecular ion at *m/z* 708 ([M + H]$^+$) with a fragment ion at *m/z* 505

Table 1. ^{1}H chemical shifts of the constituent monosaccharides for β-D-Gal-(1 → 3)-α-D-GalNAc-OC$_6$H$_4$NO$_2$-*p*, compounds **1**, **2**, and **3** in D$_2$O solution. *p*-nitrophenyl, (○); α-D-GalNAc, (■); β-D-Gal, (●); β-D-GlcNAc, (□). The superscripts by the sugar indicate the linkage positions of the subsequent monosaccharides in the sequence.

Residue	*Reporter group*	*Chemical shifts in compounds (δ)*			
			 **1**	 **2**	 **3**[a]
GalNAc	H-1	5.791	5.767	5.770	5.790
		(3.66)[b]	(3.36)	(3.66)	(3.36)
	H-2	4.560	4.547	4.551	4.568
	H-3	4.277	4.275	4.226	4.282
	H-4	4.300	4.287	4.269	4.296
	H-5	3.999	4.155	3.978	4.000
	H-6	3.675	3.999	3.666	ND[c]
	H-6′	3.728	3.735	3.736	ND
	NAc	1.998	1.989	1.995	2.007
Gal	H-1	4.534	4.526	4.538	4.531
		(7.63)	(7.63)	(7.63)	(7.63)
	H-2	3.543	3.537	3.541[d]	3.599
	H-3	3.639	3.636	3.620	ND
	H-4	3.909	3.915	3.895	4.144
	H-5	3.675	3.673	3.792	ND
GlcNAc	H-1		4.439	4.565	4.703
			(8.55)	(8.25)	(8.55)
	H-2		3.537	3.662	ND
	H-3		3.419	3.530[d]	3.563
	H-4		3.267	3.395	3.465
	H-5		3.355	3.438	ND
	H-6′		3.827	3.921	3.893
	NAc		1.880	2.011	2.028
pNP	*o*-	7.242	7.231	7.238	7.241
		(9.46)	(9.46)	(9.16)	(9.16)
	m-	8.216	8.246	8.197	8.215
		(9.16)	(9.16)	(9.16)	(9.16)

[a] Spectrum recorded at 25 °C.
[b] Coupling constant in Hz.
[c] ND, Not determined.
[d] These assignments may be changed.

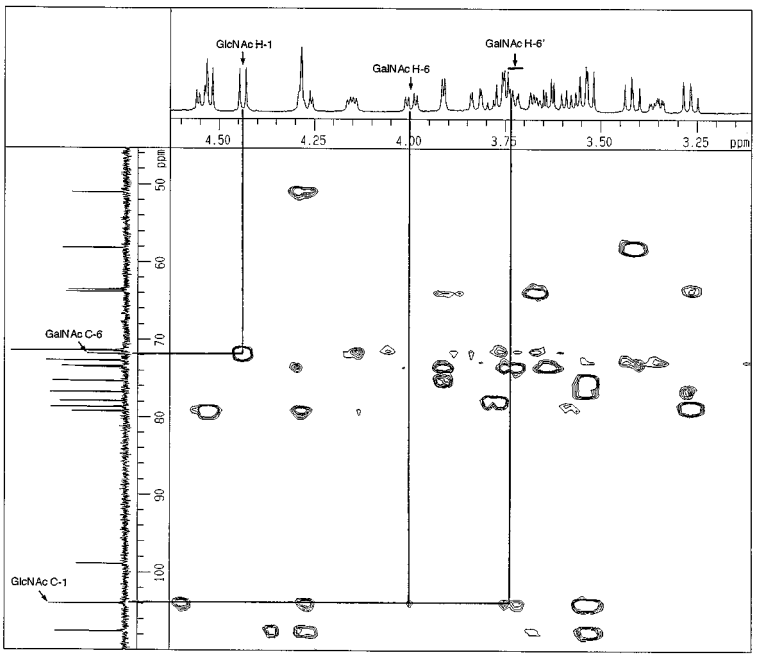


Figure 2. HMBC spectrum of the compound **1** with ^{1}H and ^{13}C spectra printed on the sides of the 2D spectrum.

(fragment from Hex-HexNAc-$OC_6H_4NO_2$). It suggests that both compounds are linear trisaccharides HexNAc-Hex-HexNAc-$OC_6H_4NO_2$. These ^{1}H- and ^{13}C-NMR data are shown in Tables 1 and 2, respectively. The NMR and FAB-MS/MS revealed that **2** and **3** are β-D-GalNAc-(1 → 6)-β-D-Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*: $[\alpha]_D^{25}$ + 129.8° (*c* 0.5, H_2O) and β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 3)]-α-D-GalNAc-$OC_6H_4NO_2$-*p*: $[\alpha]_D^{25}$ + 140.4° (*c* 0.5, H_2O), respectively.

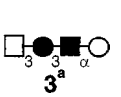
The structures of compounds **4**, **5**, and **6** were also characterized by ^{1}H- and ^{13}C-NMR (Tables 3 and 4). The NMR revealed that **4**, **5**, and **6** are β-D-Gal-(1 → 3)-[β-D-GlcNAc-(1 → 6)]-β-D-GalNAc-$OC_6H_4NO_2$-*p*, β-D-GlcNAc-(1 → 6)-β-D-Gal-(1 → 3)-β-D-GalNAc-$OC_6H_4NO_2$-*p* and β-D-Glc NAc-(1 → 3)-β-D-Gal-(1 → 3)-β-D-GalNAc-$OC_6H_4NO_2$-*p*, respectively.

Enzymatic synthesis of β-D-Gal-(1 → 3)-[β-D-GlcNAc-(1 → 6)]-α-D-GalNAc-$OC_6H_4NO_2$-*p* (**1**)

The enzyme used in the present study was the crude β-NAHase from *N. orientalis*, which was prepared as a 20–70% saturated ammonium sulfate fraction from the culture broth. The enzyme preparation was completely devoid of β-D-galactosidase acitvity, which degrades the acceptor substrate β-D-Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* (vide infra), and was used without further purification. The enzyme catalyzed the synthesis of the desired compound **1** with its isomers **2** and **3** through *N*-acetylglucosaminyl transfer from $(GlcNAc)_2$ to β-D-Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* substrates. The enzyme formed the trisaccharides **1**, **2**, and **3** in 14% overall yield based on the acceptor, and in a ratio of 44:32:24. These values were based on the time for the maximum production of desired compound **1** after 10 h. Figure 3 shows the transglycosylation profile with $(GlcNAc)_2$ and β-D-Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*. The time for maximum formation of **1**, **2**, and **3** was at 8 ∼ 12 h and their concentrations varied a little during the subsequent reaction. These were separated by chromatography on a Toyopearl HW-40S column followed by HPLC with an ODS column. Moreover, the unreacted acceptor could be recovered by straightforward chromatography and reutilized. The *N*-acetylglucosaminyl transfer favored O-6 of the acceptor rather than O-6′, and occurred to a lesser extent at O-3′.

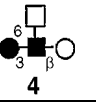
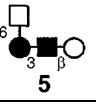
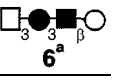
In this way, consecutive use of transgalactosylation [9] and trans-*N*-acetylglucosaminylation led to the formation of trisaccharide **1** as a carbohydrate unit of mucin-type 2 core, starting with α-D-GalNAc-$OC_6H_4NO_2$-*p* as shown in Figure 4. We have previously observed that increased solubility of *p*-nitrophenyl glycosides by using cyclodextrin (CD) which is thought to form an inclusion complex with the *p*-nitrophenyl group resulted in improved yields of transfer products [9, 13]. We also applied this concept to the present reaction system. As a result, the solubility of β-D-Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* was certainly increased, but the yields of three transfer products were not always increased (data not shown).

Table 2. ^{13}C chemical shifts of the constituent monosaccharides for *β*-D-Gal-(1 → 3)-*α*-D-GalNAc-OC$_6$H$_4$NO$_2$-*p*, compounds **1**, **2**, and **3** in D_2O solution. Abbreviations and superscripts as in legend to Table 1.

Residue		*Chemical shifts in compounds (δ)*			
			 **1**	 **2**	 **3**[a]
GalNAc	C-1	98.68	98.93	98.81	98.59
	C-2	50.02	51.02	51.04	50.92
	C-3	79.55	79.25	79.64	79.39
	C-4	71.37	71.44	71.18	71.36
	C-5	74.79	73.55	74.84	74.71
	C-6	63.74	71.84	63.77	63.67
	NAc	24.76	24.87	25.02	24.71
	C=O	177.53	177.53	177.53	177.44
Gal	C-1	107.61	107.57	107.49	107.56
	C-2	73.44	73.44	73.36	72.46
	C-3	75.34	75.33	75.27	84.56
	C-4	71.43	71.44	71.25	71.17
	C-5	77.88	77.90	76.56	77.36
	C-6	63.85	63.87	71.21	63.71
GlcNAc	C-1		103.98	104.08	105.49
	C-2		58.16	58.34	58.44
	C-3		76.77	76.56	76.32
	C-4		72.71	72.77	72.46
	C-5		78.69	78.77	78.42
	C-6		63.58	63.60	63.26
	NAc		24.74	24.77	24.92
	C=O		177.01	177.27	177.68
pNP	*o*-	119.42	119.54	199.45	199.30
	m-	128.81	128.90	128.81	128.73
	p-	145.12	145.17	145.11	145.01
	C–O	164.09	164.29	164.13	163.99

[a] Spectrum recorded at 25 °C.

Table 3. ^{1}H chemical shifts of the constituent monosaccharides for *β*-D-Gal-(1 → 3)-*β*-D-GalNAc-OC$_6$H$_4$NO$_2$-*p*, compounds **4**, **5**, and **6**. Abbreviations and superscripts as in legend to Table 1

Residue	*Reporter group*	*Chemical shifts in compounds (δ)*			
			 **4**	 **5**	 **6**[a]
GalNAc	H-1	5.293 (8.55)[b]	5.287 (8.55)	5.297 (8.55)	5.304 (8.55)
	H-2	4.327	4.319	4.315	4.335
	H-3	4.020	4.013	4.005	4.022
	H-4	4.267	4.240	4.224	4.270
	H-5	3.928	4.076	ND[c]	3.938
	H-6	3.787	4.105	ND	ND
	H-6′	3.817	3.798	ND	ND
	NAc	1.968	1.969	1.975	1.989
Gal	H-1	4.474 (7.65)	4.467 (7.93)	4.471 (7.63)	4.475 (7.63)
	H-2	3.527	3.530	3.523	3.596
	H-3	3.611	3.609	3.591	ND
	H-4	3.900	3.901	3.882	4.131
	H-5	3.655	3.652	3.765	3.656
GlcNAc	H-1		4.526 (8.55)	4.556 (8.25)	4.688 (8.55)
	H-2		3.699	3.673	ND
	H-3		3.491	3.523	3.556
	H-4		3.435	3.420	3.458
	H-6′		3.915	ND	3.888
	NAc		1.733	2.034	2.026
pNP	*o*-	7.186 (9.46)	7.170 (9.16)	7.181 (9.16)	7.202 (9.46)
	m-	8.227 (9.45)	8.245 (9.46)	8.233 (9.15)	8.220 (9.46)

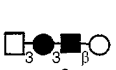
[a] Spectrum recorded at 25 °C.
[b] Coupling constant in Hz.
[c] ND, Not determined.

In a similar manner, when *β*-D-Gal-(1 → 3)-*β*-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* was used as the acceptor, three transfer products **4**, **5** and **6** were observed by HPLC in 8% total yield (based on the acceptor) and in a ratio of 54:31:15. The regioselectivity with respect to O-6 and O-6′ versus O-3 of the acceptor was not only similar to that of its anomer *β*-D-Gal-(1 → 3)-*α*-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* acceptor, but also of those of *p*-nitrophenyl *β*-lactoside (Lac*β*-*p*NP) and *β*-*N*-acetyllactosaminide (LacNAc*β*-*p*NP) ones as reported previously [12, 13]. However, the present yields based on the acceptor were somewhat different from previous data on Lac*β*-*p*NP and LacNAc*β*-*p*NP (Table 5). Especially, the transglycosylation with *β*-D-Gal-(1 → 3)-*α*-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* acceptor was much higher (4.7-fold) than that with Lac*β*-*p*NP acceptor. It seems to depend on the structure of acceptor disaccharides.

Effect of α-CD in the inclusion complex with acceptor substrate on regioselectivity of GlcNAc-transfer reaction

In our previous study [12, 13], when an inclusion complex of Lac*β*-*p*NP or LacNAc*β*-*p*NP with α-CD was used, the regioselectivity of *β*-NAHase catalysed formation of trisaccharide glycoside was substantially changed, due to steric hindrance of the CD in the inclusion complex. This was also applied to the reaction with *β*-D-Gal-(1 → 3)-*α*-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* and its anomer as acceptors. Figure 5 shows the percentages of three transfer products in the absence and presence of α-CD with four acceptors. With *β*-D-Gal-(1 → 3)-*α*-D-GalNAc-OC$_6$H$_4$NO$_2$-*p* acceptor, no matter how the molar ratio of α-CD to the acceptor was increased, it affected to a lesser extent the regioselectivity with respect

Table 4. ^{13}C chemical shifts of the constituent monosaccharides for β-D-Gal-(1 → 3)-β-D-GalNAc-$OC_6H_4NO_2$-*p*, compounds **4**, **5**, and **6**. Abbreviations and superscripts as in legend to Table 1.

Residue		*Chemical shifts in compounds (δ)*			
					
			4	**5**	**6**[a]
GalNAc	C-1	101.66	101.53	101.84	101.72
	C-2	53.80	53.71	53.82	53.81
	C-3	82.17	81.85	82.12	82.01
	C-4	73.42	70.92	70.58	71.20
	C-5	77.86	76.81	78.24	78.14
	C-6	63.50	72.24	63.54	63.47
	NAc	24.98	24.97	25.00	24.98
	C=O	177.90	177.88	177.89	177.75
Gal	C-1	107.65	107.62	107.54	107.61
	C-2	73.42	73.41	73.39	72.55
	C-3	75.30	75.28	75.25	84.56
	C-4	71.44	71.40	71.10	70.64
	C-5	78.16	77.85	75.79	77.40
	C-6	63.84	63.83	71.10	63.79
GlcNAc	C-1		104.01	104.03	105.52
	C-2		58.30	58.31	58.51
	C-3		76.86	76.57	76.41
	C-4		72.77	72.72	72.55
	C-5		78.72	78.74	78.49
	C-6		63.59	63.54	63.33
	NAc		24.78	25.00	24.98
	C=O		177.11	177.32	177.88
pNP	*o*-	119.38	119.20	199.39	119.38
	m-	128.90	129.06	128.91	128.90
	p-	145.45	145.42	145.46	145.48
	C–O	164.61	164.66	164.61	164.60

[a] Spectrum recorded at 25 °C.

to O-6 and O-6′ versus O-3, compared with Lacβ-*p*NP and LacNAcβ-*p*NP. As a result, the regioselectivity of the β-NAHase-catalyzed formation of trisaccharides was changed only a little by utilizing the nature of the hydrophobic *p*-nitrophenyl group in the present acceptor. It suggests that the existence of a bulky CD region in the complexation has not much influence on the regioselectivity at O-6 of β-D-Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*, due to less steric hindrance between the CD and the hydroxymethyl group at C-6 in the acceptor.

Substrate specificity of endo-α-GalNAc-ases

Endo-α-GalNAc-ase which hydrolyzes the linkage between GalNAc and Ser/Thr glycoprotein has been isolated from a number of sources [14–17]. It has been reported that β-D-Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* is a suitable synthetic substrate for endo-α-GalNAc-ase [9, 18], which

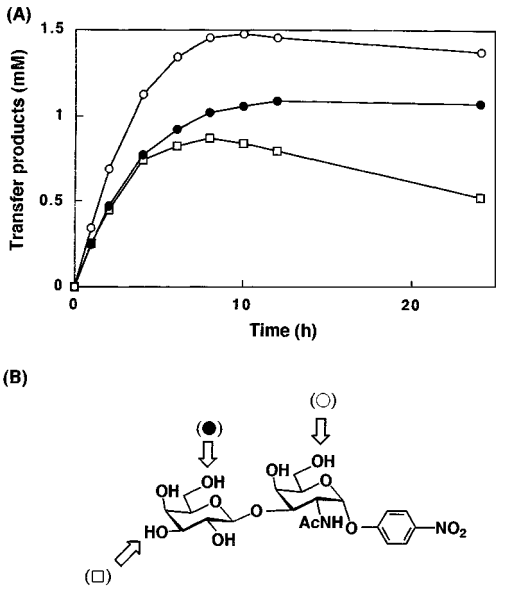


Figure 3. Time course of *N. orientalis* β-NAHase-mediated isomer formation of **1**, **2**, and **3**. (A) The amounts of 1 (○), 2 (●), and 3 (□) as a function of time were examined on the 0.25 ml scale as described in Materials and Methods, and samples were analyzed by HPLC during incubation. (B) The structure of β-D-Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* used as an acceptor substrate. Arrows show the position of *N*-acetylglucosaminylation.

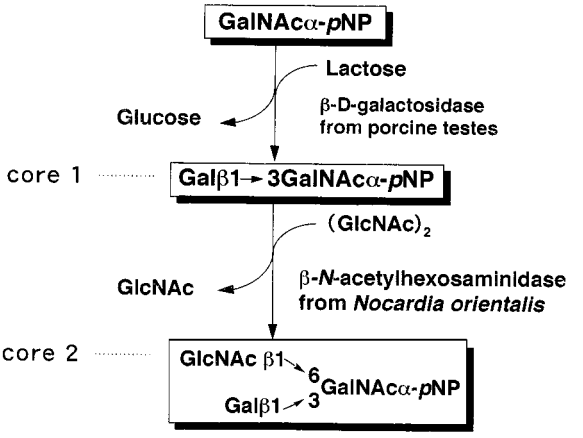


Figure 4. Synthetic scheme of carbohydrate region of cores 1 and 2 by consecutive use of transgalactosylation and trans-*N*-acetylglucosaminylation.

Table 5. Total yields of transfer products formed by β-NAHase-catalyzed transglycosylation with various disaccharide glycoside acceptors.

acceptor	*yield (%)**
Gal-(1 → 4)-β-D-Glc-$OC_6H_4NO_2$-*p*	3
Gal-(1 → 4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p*	5
Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*	14
Gal-(1 → 3)-β-DGalNAc-$OC_6H_4NO_2$-*p*	8

* Based on the acceptors added.

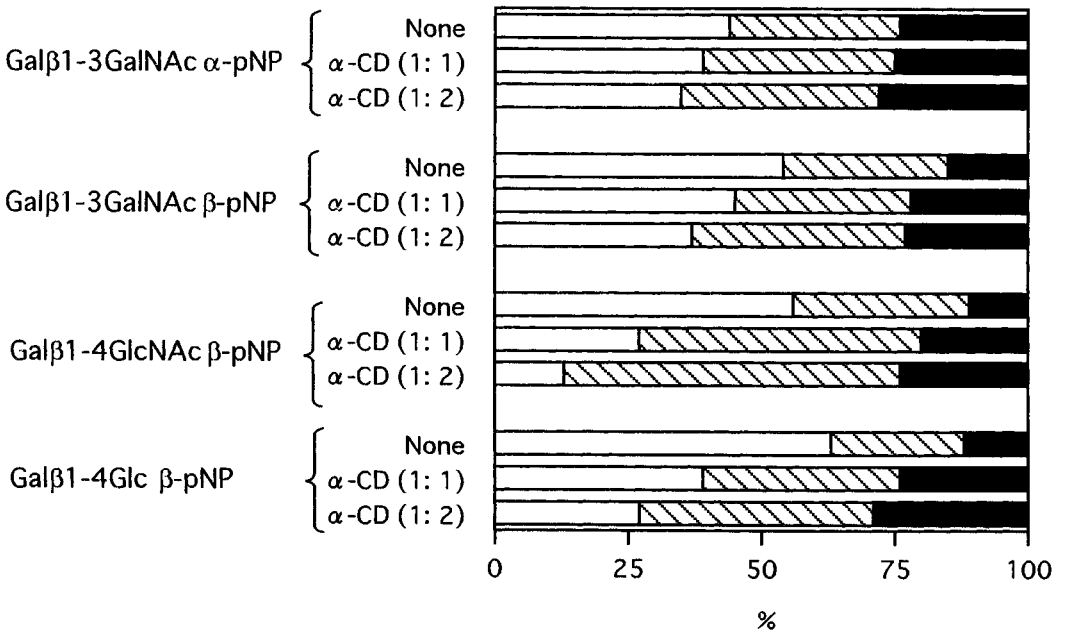


Figure 5. Effect of *α*-CD on regioselectivity of GlcNAc-transfer reaction. Abscissa presents percentages of transfer products formed by transglycosylation at different molar ratios of *α*-CD to acceptor. □, GlcNAc-transfer to O-6, ▨, GlcNAc-transfer to O-6′, ■, GlcNAc-transfer to O-3′.

can bypass a block of the disaccharide. However, the specificity of these enzymes with regard to core 2 oligosaccharide derivatives has not been reported. In this study, the substrate specificities of commercially available endo-α-GalNAc-ases-A and -D were further investigated using the synthetic compounds **1**, **2**, and **3**. *β*-D-Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* was used as a control to ascertain the effect of sugar modification. The enzyme hydrolyzates were detected by TLC and HPLC (data not shown). The enzyme was capable of liberating a reducing disaccharide only from the control sugar, but it did not show any activity on the compounds **1**, **2**, and **3**. This suggests that the O-substituted GlcNAc groups show some resistance to attack by these enzymes, and its specificity for the substrate is very high.

Conclusion

We have developed a preparative synthetic method for obtaining **1**, which is a carbohydrate unit of mucin-type 2 core, by using a *N. orientalis β*-NAHase-mediated GlcNAc-transfer reaction. Nitrophenyl glycoside cannot only serve as an enzyme substrate, but also it can be reduced to an aminated glycoside, whose amino function is derivatized for reactions with electrophiles [19, 20]. Such aminated mucin-type glycosides may be coupled with pendant carboxyl groups of polypeptides such as poly (L-glutamic acid) [21] and lead to a convenient synthetic route for artificial mucin glycoprotein.

Acknowledgments

This study was supported by grants-in-aid for scientific research from the Ministry of Education, Science, Sport and Culture of Japan, and a research grant for leading research utilizing potential of regional science and technology from the Science and Technology Agency of Japan, and a research grant (Glycotechnology program) from a Ministry of Agriculture, Forestry and Fishery of Japan.

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Received 26 September 1997, accepted 14 November 1997