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Regioselective transglycosylation in the synthesis of oligosaccharides: comparison of β-galactosidases and sialidases of various origins

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Abstract

N-Acetyl-lactosamine(β-D-Gal*p*-(1 → 4)-D-Glc*p*NAc) was synthesized regioselectively with the aid of the transglycosylation activity of β-galactosidase isolated from *Diplococcus pneumoniae* using *p*-nitrophenyl β-D-galactopyranoside as the donor. Also, transglycosylation of the sialyl group in an α-(2 → 8)-linked sialic acid dimer or *p*-nitrophenyl glycoside of sialic acid to *N*-acetyl-lactosamine was performed using sialidases of various origins. When sialidase from *Clostridium perfringens*, *Arthrobacter ureafaciens*, or *Vibrio cholerae* was used, α-(2 → 6)-linked sialyl *N*-acetyl-lactosamine was obtained regioselectively. In contrast, when sialidase from newcastle disease virus was used, the α-(2 → 3)-linked isomer was obtained regioselectively.

The regioselectivity of the transglycosylation reaction using β-galactosidase and sialidase was compared with hydrolysis specificity toward the same linkages.

Introduction

Recently, the demand for the synthesis of the oligosaccharides contained in glycoconjugates has increased dramatically, since it has been shown that carbohydrate chains in glycoproteins and glycolipids play important roles in the recognition of cell surface glycoproteins [1,2] and glycolipids [3]. Sialyl *N*-acetyl-lactosamine is found frequently in glycoproteins, glycolipids and the oligosaccharides derived from milk [4]. Moreover, α-(1 → 3)-fucosylated and α-(2 → 3)-sialylated *N*-acetyl-lactosamine, sialyl Lewis x, have received much attention as cellular

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adhesion molecules [5,6]. The establishment of a process for the large scale synthesis of sialyl oligosaccharides is important for the elucidation of the role played by sialyl groups in glycoconjugates.

Various transferases have been examined for the synthesis of oligosaccharides because of their high regioselectivities and yields [7,8]. However, the use of transferases causes various problems, which have recently been discussed [9].

In contrast, glycosidases can catalyze two different types of reactions: condensation reactions and transglycosylation reactions. Condensation reactions, the reverse of hydrolysis reactions, have been reported to give extremely poor yields and regioselectivities, especially in the synthesis of sialyl oligosaccharides [10]. Thiem et al. [11] and Nilsson [12] have reported transglycosylation reactions using glycosides of synthetic substrates as donors. Although they obtained oligosaccharides in fairly good yield, the product was often a mixture of various regioisomers.

We recently reported that α-Fuc-(1 → 2)-Gal or α-Fuc-(1 → 3)-GlcNAc could be synthesized regioselectively by choosing α-fucosidases according to their substrate specificity in the hydrolysis reaction [13]. Our current interest is to correlate the substrate specificity of glycosidases in hydrolysis reactions to the regioselectivity of their transglycosylation reactions.

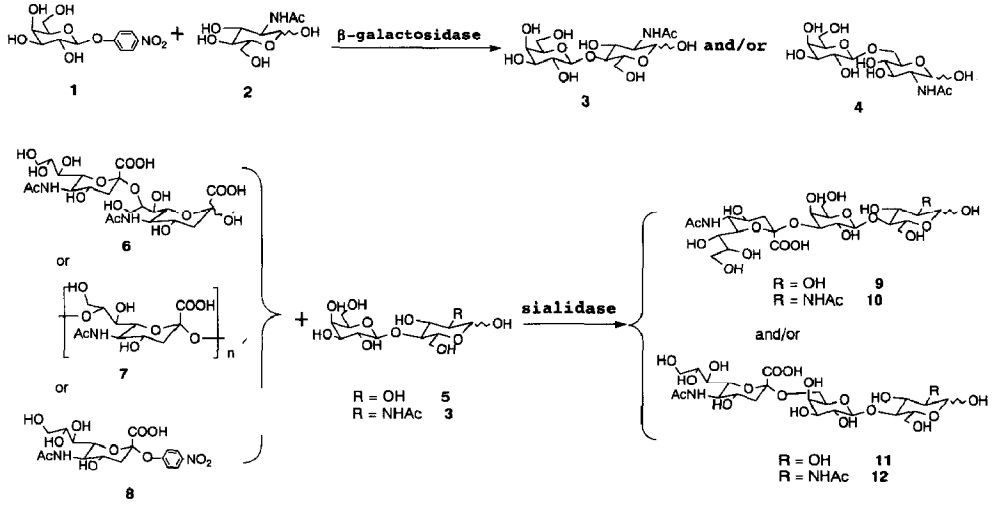
In the present study, we surveyed a number of β-galactosidases and sialidases for their abilities to regioselectively synthesize β-(1 → 4)-linked lactosamine and α-(2 → 3)-linked or α-(2 → 6)-linked sialyl oligosaccharides, respectively. As a result, we were able to synthesize α-(2 → 3)-linked sialyl *N*-acetyl-lactosamine by the combined use of β-galactosidase and sialidase.

2. Results and discussion

Regioselective synthesis of β-D-galactopyranosyl-(1 → 4)-O-2-acetamido-2-deoxy-D-glucose by a transglycosylation reaction using β-galactosidase from different origins.—Since the first report by Kuhn et al. [14] on the enzymatic synthesis of **4** by a transglycosylation reaction using phenyl β-D-galactopyranoside as the donor, many investigators have attempted to synthesize **3** regioselectively [15]. Most β-galactosidases, however, afforded **4** preferentially to **3** [16]. Recently, Sakai et al. reported that β-galactosidase from *Bacillus circulans* gave **3** by a transglycosylation reaction using **5** as the substrate [17]. However, even in the reaction using β-galactosidase from *B. circulans*, **4** was also formed in ~ 10% yield.

We investigated alternative enzymes for the synthesis of **3** in complete regioselectivity (Fig. 1). *E. coli* β-galactosidase afforded **4** exclusively, as reported by Kuhn and co-workers [14]. Other enzymes from *Aspergillus oryzae* and *Penicillium multicolor* also gave **4** in preference to **3**. Although β-galactosidase from *B. circulans* afforded **3** preferentially, as reported by Sakai et al. [17], a small amount of **4** was obtained as a by-product.

In contrast, β-galactosidase from *Diplococcus pneumoniae* displayed complete regioselectivity toward the synthesis of **3**, according to analyses by HPLC and ^{1}H NMR spectroscopy (the differentiation limit of relative peak areas was ~ 1%).



Scheme 1.

The β-galactosidase from *D. pneumoniae* is the first enzyme to have shown such regioselectivity. The disaccharide **3** was isolated in 11.6% yield and the structure was confirmed by ^{1}H NMR spectroscopy.

Regioselective synthesis of α-(2 → 6)-linked sialyl oligosaccharides by a transglycosylation reaction using sialidases of different origins.—α-(2 → 8)-Linked sialic acid dimer **6** was used as the sialyl donor for transglycosylation reactions. Initially, **5** was used as an acceptor because the transglycosylation products **9** and **11** could be easily discriminated by comparison of their HPLC retention times or ^{1}H NMR spectra with those of authentic samples. Sialidases of various origins were employed as catalysts for the reaction. The reaction was monitored by HPLC using a CarboPac PA-1 column (Dionex) and a pulsed amperometric detector. A 0.1 M sodium hydroxide solution containing 0.1 M sodium acetate was used as the elution solvent. Elution patterns are shown in Fig. 2. As this column was able to separate **9** and **11**, the composition of the reaction products could easily be established. The sialyl oligosaccharide was isolated by HPLC using a Mono Q column (Pharmacia); a representative HPLC chromatogram is shown in Fig. 3.

The structure of the isolated sialyl oligosaccharide was confirmed by comparing its ^{1}H NMR spectrum with those of authentic **9** and **11**. The isomeric ratios of α-(2 → 3)- and α-(2 → 6)-linkages were estimated from HPLC peak areas (Table 1). It was revealed that sialidases from *Clostridium perfringens*, *Arthrobacter ureafaciens*, and *Vibrio cholerae* afforded **11** regioselectively in the reaction of **5** and **6**. One reason for the significantly reduced isolated yield, as compared with the transfer ratio (Table 1), is absorption of the sialyl oligosaccharides on the Hiload Q column. A recovery test conducted using a pure sample of **9** revealed that ~ 65% of the applied sample remained on the column following elution. We are now investigating alternate isolation conditions to avoid this loss of product.

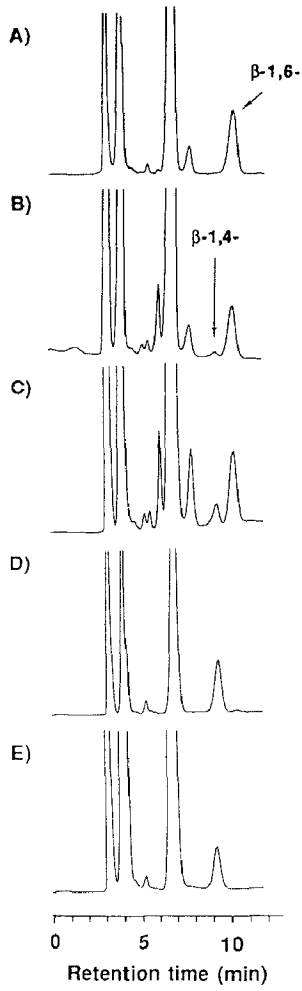


Fig. 1. HPLC of the mixtures of **1** and **2** in the presence of β-galactosidases from *E. coli* (A), *A. oryzae* (B), *P. multicolor* (C), *B. circulans* (D), and *D. pneumoniae* (E).

It should be noted that the reaction using sialidase from *V. cholerae* afforded **11** as the sole product, while Thiem et al. [11] reported that a mixture of α-(2 → 3)-linked and α-(2 → 6)-linked sialyl oligosaccharides was obtained in the reaction using **8** as the donor in the presence of immobilized sialidase from *V. cholerae*.

Regioselective synthesis of α-(2 → 3)-linked sialyl oligosaccharides by a transglycosylation reaction using sialidase from Newcastle disease virus.—In the reaction of **5** and **6** in the presence of sialidase from Newcastle disease virus (NDV), **9** was obtained as the sole product. In contrast, when sialidase from *V. cholerae* was used, only **11** was obtained (Fig. 2). Similarly, in the reaction of **3** with **6** in the presence of sialidase from NDV, **10** was obtained with high regioselectivity (Fig. 4), while **12** was the only product observed when sialidase from *C. perfringens* was

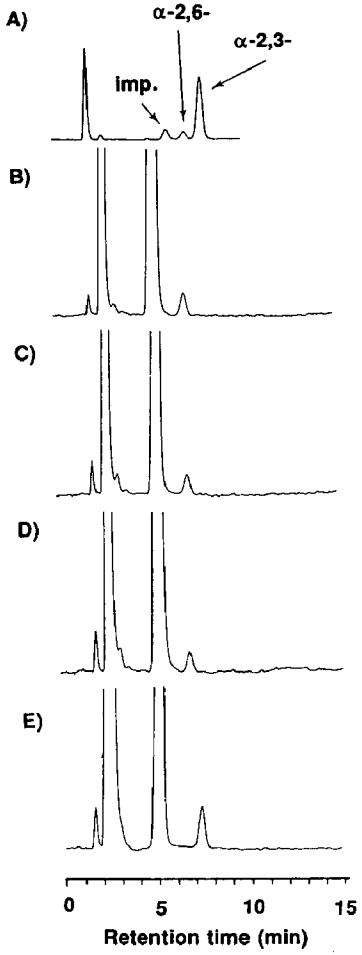


Fig. 2. Anion exchange HPLC of the mixtures of **5** and **6** in the presence of sialidases from *C. perfringens* (B), *A. ureafaciens* (C), *V. cholerae* (D), and NDV (E). (A) is a chromatogram of a mixed authentic standard of **9** and **11**.

used. The high purity and the regioselectivity of the product were confirmed by ^{1}H NMR spectroscopy as shown in Fig. 5. The α-(2 → 3)-linked structure was confirmed by the chemical shifts of the H-3 protons of the sialic acid residue, which resonated at 1.78 and 2.74 ppm. Moreover, the absence of **12** was demonstrated by the ^{1}H NMR spectrum, which lacked the characteristic signals at 1.71 and 2.67 ppm due to the H-3 protons of an α-(2 → 6)-linked sialic acid residue.

Transglycosylation reaction using **7** *as a sialyl donor.*—A similar transglycosylation reaction was performed using **7**, an α-(2 → 8)-linked polymer of sialic acid, as the donor for transferring the sialyl group. In order to monitor the profiles of the product and the degraded oligomers of **7**, an HPLC system attached to a Mono Q column was eluted with a 3-step gradient from 4 to 500 mM NaCl solution. A

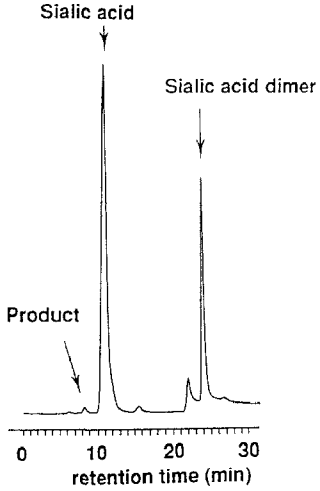


Fig. 3. HPLC of the mixture of **5** and **6** in the presence of β-galactosidase from *C. perfringens*. Column, Mono Q.

representative HPLC chromatogram is shown in Fig. 6. In the reaction of **5** and **7** with sialidase from *C. perfringens*, only **11** was isolated from the reaction mixture. When a similar reaction was performed using sialidase from NDV, a mixture of **9** and **11** was generated.

Transglycosylation reaction using **8** *as a sialyl donor*.—The reaction using **8** was performed using various sialidases, and the results are summarized in Table 1. It is

Table 1
Summary of the synthesis of sialyl oligosaccharides by a transglycosylation reaction

Enzyme source [a]	Donor	Accepter	α-(2 → 3):α-(2 → 6)	Transfer ratio [b]	Isolation yield (%)
A	6	5	0:100	8.5	2.5
	6	3	0:100	5.5	
	7	5	0:100	2.4	0.8
	8	5	30: 70	12.4	
B	6	5	0:100	5.6	
	8	5	0:100	7.4	
C	6	5	0:100	4.1	
	8	5	10: 90	10.2	
D	6	5	100: 0	9.9	1.8
	6	3	100: 0	8.0	
	7	5	75: 25	3.6	
	8	5	76: 24	10.2	
	8	3	100: 0	11.7	3.6

[a] Sialidases from (A) *C. perfringens*, (B) *A. ureafaciens*, (C) *V. cholerae*, and (D) NDV.
[b] Transfer ratio, [(product)/(product + sialic acid)] × 100 calculated from HPLC peak areas.

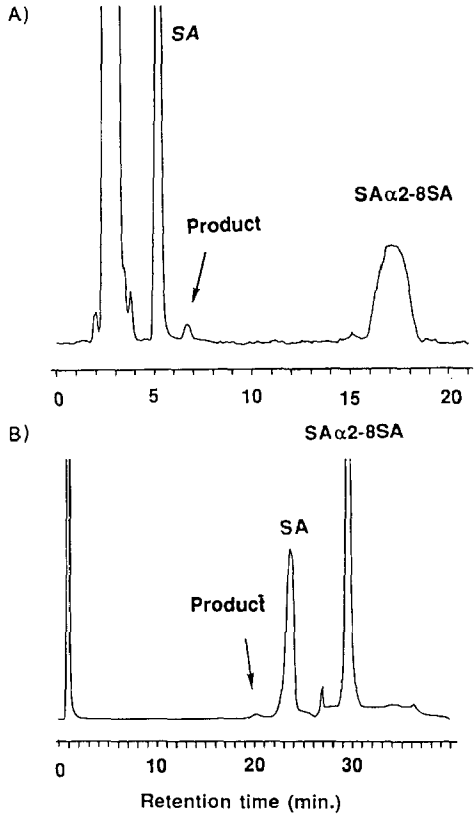


Fig. 4. HPLC of the mixture of **3** and **6** in the presence of sialidase from NDV. Column, (A) CarboPac PA-1 and (B) Mono Q.

remarkable that the yield of the reaction products is three to five times higher than the corresponding reaction using **6** or **7** as a sialyl donor. One reason for such high yields might be due to the addition of organic solvent to the acetate buffer in order to solubilize **8**. When the reaction of **8** and **5** was performed using sialidase from *C. perfringens* without organic solvent, the yield by HPLC was 8.3%. In contrast, the yield increased to 12.4% in 30% (v/v) acetonitrile as can be seen in Table 1. Another reason would be the difference in the K_m values between **8** and **6**, or **8** and **7**. The rate of hydrolysis of the products is the same irrespective of the donor of the transglycosylation reaction, therefore the faster the substrate (donor) changes to products, the greater the yields. In reactions using **6** or **7**, however, organic solvents could not be added due to the low solubility of these substrates in organic solvents.

In contrast to the high yields observed using **8** as a donor, the regioselectivity of these reactions was less than satisfactory (Table 1). These results coincided with the report of Thiem et al. concerning a mixture of α-(2 → 3)-linked and α-(2 → 6)-linked sialyl oligosaccharides obtained by the reaction of **8** and immobilized

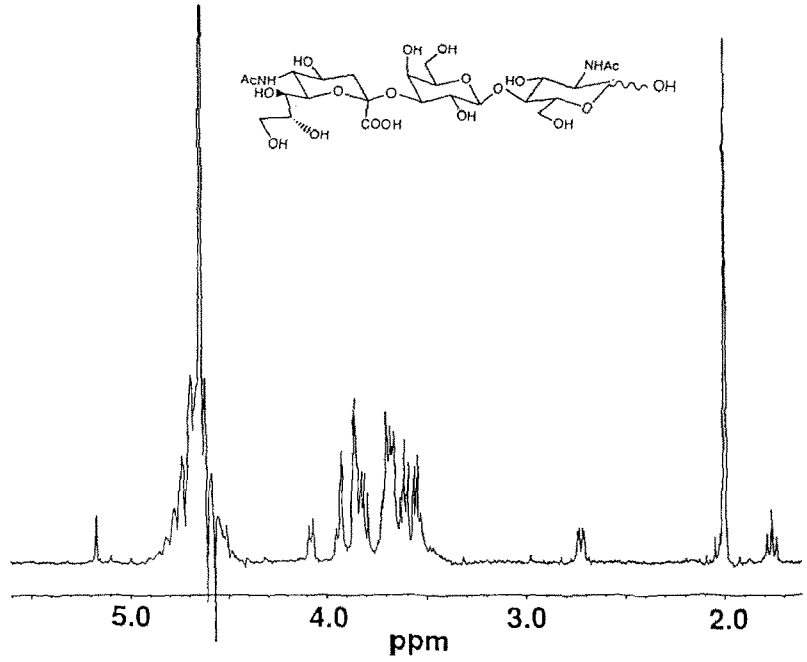


Fig. 5. 500-MHz ^{1}H NMR spectrum in D_2O of the isolated product from the mixture of **3** and **6** in the presence of NDV. The structure was determined to be **10**.

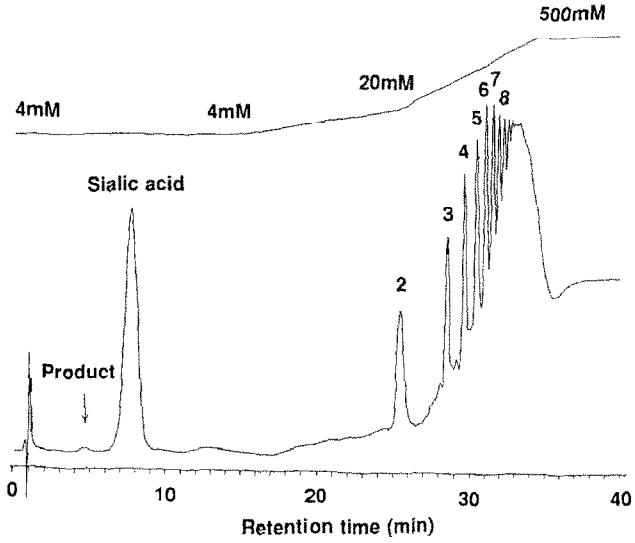


Fig. 6. Anion exchange HPLC of the mixture of **5** and **7** in the presence of sialidase from *C. perfringens* using a Mono Q column. Elution was performed with a linear gradient of sodium chloride solution as shown in the figure.

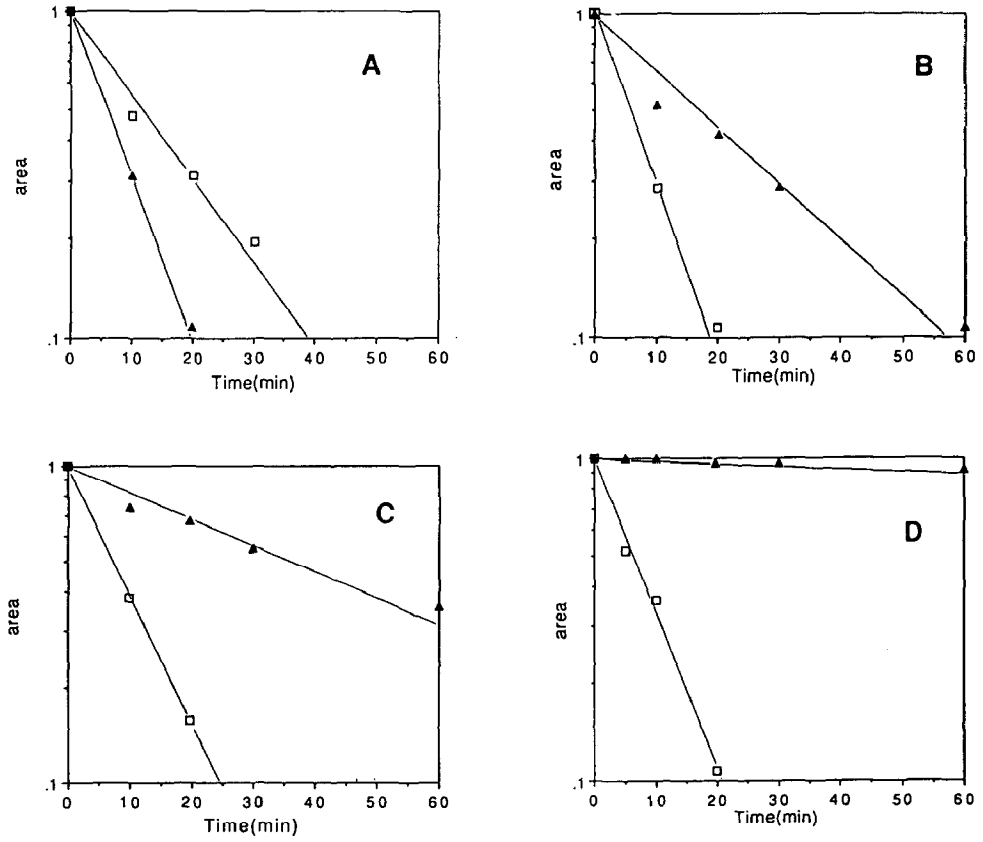


Fig. 7. The relative hydrolysis rates of **9** and **11**. The peak areas of **9** (□) and **11** (▲) in anion exchange HPLC were plotted according to the progress of the hydrolysis reaction using sialidases from *A. ureafaciens* (A), *V. cholerae* (B), *C. Perfringens* (C), and NDV (D).

sialidase from *V. cholerae* [11]. The reason for the poor regioselectivity in the experiment of Thiem et al. might have been due to the use of an immobilized sialidase. In our experiments, the immobilization of sialidase from *C. perfringens* or NDV severely decreased the substrate specificity of the hydrolysis reaction (data not shown).

Another interesting feature of the reaction using **8** as a donor is that the regioselectivity varies depending on the acceptor. Although a mixture of **9** and **11** was obtained when **5** was used as the acceptor, only **10** was obtained when **3** was used as the acceptor.

Comparison of the hydrolysis specificity with the regioselectivity in the transglycosylation reaction.—In order to survey the relation between the substrate specificity of the glycosidase in the hydrolysis reaction and the regioselectivity in the transglycosylation reaction, hydrolysis rates of **3** and **4** by various β-galactosidases and those of **9** and **11** by various sialidases were measured. The course of each hydrolysis

Table 2
Ratio of hydrolysis rates of **3** and **4** by β-galactosidases and that of **9** and **11** by sialidases

Origin of enzymes	k_4/k_3	k_9/k_{11}
β-Galactosidase		
E. coli	0.15	
A. oryzae	0.41	
P. Multicolor	0.67	
B. circulance	4.5	
D. pneumoniae	4.7	
Sialidase		
A. ureafaciens		0.51
V. cholerae		3.2
C. perfringens		4.8
NDV		28.1

reaction with time is shown in Fig. 7. The ratios of the hydrolysis rates, k_4/k_3 and k_9/k_{11}, are summarized in Table 2.

The order of k_4/k_3 values for each β-galactosidase coincides well with the formation ratio of **3** to **4**. For β-galactosidases from *B. circulans* and *D. pneumoniae*, k_4/k_3 values for both enzymes are extremely large in comparison with those of the other enzymes; thus, the preferential production of the β-(1 → 4)-linked isomer seems reasonable. In these cases, it may be concluded that the faster a specific disaccharide linkage is hydrolyzed, the faster the same disaccharide can be formed in a transglycosylation reaction. A similar relationship was observed in the transglycosylation reactions using α-fucosidases [13]. However, the difference of k_4/k_3 values between those of β-galactosidases from *B. circulans* and *D. pneumoniae* was not large enough in the present experiment, though the former enzyme gave a **3**/**4** product ratio of 10/1, while the latter enzyme did not form **4** at all.

Sialidase from NDV afforded a k_9/k_{11} value of ~ 28, in agreement with the observed exclusive formation of **12**. On the other hand, although sialidase from *A. ureafaciens* gave a k_9/k_{11} value of 0.51, exclusive formation of **11** was observed. Moreover, irrespective of k_9/k_{11} values of 3.2 and 4.8 for sialidases from *V. cholerae* and *C. perfringens*, respectively, both afforded **11** selectively. If indeed the regioselectivity of the transglycosylation reaction is determined by the hydrolysis specificity of the glycosidase, **9** should be formed in preference to **11** by sialidases from either *V. cholerae* or *C. perfringens*. Since this is not the case, we conclude that some other factor is also at work influencing the regioselectivity of this transglycosylation reaction.

Although work remains to be done to fully quantify the relationship between hydrolysis specificity and regioselectivity of transglycosylation reactions, this relationship can now be used to rationalize the choice of enzymes for future reactions. For example, a sialidase with an extremely large k_9/k_{11} will most likely be required to obtain an α-(2 → 3)-linked transglycosylation product with complete regioselectivity.

3. Experimental

Materials.—β-Galactosidase from *A. oryzae* (Sigma), *P. multicolor* (K. I. Chemicals Co., Shizuoka, Japan), *B. circulans* (Daiwa Kasei Co., Osaka, Japan), and *D. pneumoniae* (Boehringer, Mannheim, Germany) were commercial products. Sialidases from *C. Perfringens*, *A. ureafaciens*, and *V. cholerae* were purchased from Sigma, and sialidase from NDV was obtained from Oxford GlycoSystems (UK). The *p*-nitrophenyl glycoside of sialic acid was purchased from Seikagaku Kogyo Co., Ltd., (Tokyo, Japan) and purified by silica gel chromatography and gel filtration using a Sephadex G-25 column. Sialic acid dimer and colomic acid were purchased from Nacalai Tesque Co. (Kyoto, Japan).

Analytical methods.—Galactosyl and sialyl oligosaccharides were analyzed with a Dionex Bio-LC system equipped with a CarboPac PA-1 column and pulsed amperometric detector. For galactosyl oligosaccharides, the column was eluted with 50 mM NaOH solution at a rate of 0.8 mL/min. For sialyl oligosaccharides, a mixture of 100 mM NaOH and 100 mM NaOAc was used as the eluant.

The second method for the analysis or isolation of sialyl oligosaccharides involved the use of an HPLC equipped with a Pharmacia P-3500 pump system, UV monitor (215 nm), and a Mono Q column, which was eluted with a gradient of 0 to 4 mM NaCl solution.

NMR spectra were measured on a Unity-500 system (Varian) in D_2O.

Typical reaction conditions were as follows:

Synthesis of **3** *using β-galactosidase from* D. pneumoniae.—To a mixed solvent of 2 mL of potassium phosphate buffer (pH 6.0) and 400 μL of Me_2SO, 200 mg of **1** and 734 mg of **2** were dissolved. A solution (400 μL, 1 unit) of β-galactosidase from *D. pneumoniae* was added. After incubation of the mixture at 37°C for 16 h, the mixture was heated in a boiling water bath for 5 min and was applied directly to an activated carbon column (2 × 50 cm). The disaccharide was eluted with a linear gradient of water (500 mL) and 20% aq EtOH (500 mL). Fractions containing disaccharide were detected by the phenol–H_2SO_4 method [18], and collected. After evaporation to dryness, 29.6 mg of **3** was obtained (yield, 11.6%).

Synthesis of **11** *using* **6** *as a donor.*—To a 1 mL solution of 100 mM NaOAc buffer (pH 5.0) containing 500 mg of **5** and 50 mg of **6**, 1 mg (0.34 unit) of sialidase from *C. perfringens* was added. After incubation of the solution at 37°C for 5 h, the reaction was stopped by heating the solution in a boiling water bath for 5 min. Portions (250 μL) of the solution were applied to an HPLC system equipped with a HiLoad Q column. The sialyl oligosaccharides were eluted with water at a rate of 4 mL/min for the first 30 min, then the NaCl concentration was increased with a linear gradient from 0 to 10 mM over the next 120 min. After 150 min, the residual material was eluted from the column using a 50 mM NaCl solution. Fractions containing sialyllactose were collected, concentrated, and applied to an HPLC system fitted with an Asahipack GS-220 column for gel-permeation chromatography. The desalted fractions of trisaccharide were collected and lyophilized, yielding 1.3 mg (2.5%) of **11**.

Synthesis of **10** *using* **8** *as a donor.*—To a mixed solvent of 600 μL of acetate

buffer (pH 5.0) and 180 μL of MeCN containing 75 mg of **8** and 300 mg of **3**, NDV sialidase (0.4 unit) was added. The solution was incubated at 37°C for 8 h, after which time the reaction was stopped by heating in a boiling water both for 5 min. Portions (50 μL) of the solution were applied to an HPLC fitted with a Mono Q column, and sialyl oligosaccharides were eluted using a linear gradient of 0 to 3 mM NaCl. Fractions containing **10** were collected and concentrated, and the mixture was desalted using Asahipack GS-220 column to give 3.9 mg (3.6%) of pure **10** after lyophilization. ^{1}H NMR data (D_2O): 1.78 (t, 1 H, *J* 12.0 Hz, H-3*a* of SA), 2.02 (s, 3 H × 2, –NAc of SA and GlcNAc), 2.74 (dd, *J* 4.7, 12.5 Hz, H-3*e* of SA), 4.53 (d, *J* 8 Hz, H-1α of GlcNAc), 5.18 (d, H-1β of GlcNAc), H-1 of Gal could not detected due to overlap with the HDO signal.

Measurement of hydrolysis rates.—Hydrolysis rates of various β-galactosidases were measured using the following general procedures. To 100 μL solutions of **3** and **4** (0.2 mg each) in 100 mM phosphate buffer (pH 6.0), 0.02 unit of β-galactosidase was added. The solution was incubated at 37°C, and at appropriate time intervals 5-μL portions of the solution were withdrawn for HPLC analysis using a CarboPac PA-1 column. The respective peak areas of the residual starting materials were measured by a Hitachi D-2500 chromato-integrator, and were plotted against the sampling time.

For the measurement of the hydrolysis rate of sialidases, similar experiments were performed using **9** and **11** in 100 mM acetate buffer (pH 5.0) instead of **3** and **4**.

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References

[1] A.D. Elbein, *Trends Biotechnol.*, 9 (1991) 346–352.
[2] H.J. Jennings, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 155–208.
[3] M. Takeuchi, S. Takasaki, M. Shimada, and A. Kobata, *J. Biol. Chem.*, 265 (1990) 12127–12130.
[4] J. Weinstein, V.S. Silva, and J.C. Paulson, *J. Biol. Chem.*, 257 (1982) 13845–13853.
[5] B.K. Brandley, S.J. Swiedler, and P.W. Robbins, *Cell*, 63 (1990) 861–863.
[6] M. Tiemeyer, S.J. Swiedler, M. Ishihara, M. Moreland, H. Schwiegruber, P. Hirtzer, and B.K. Brandley, *Proc. Natl. Acad. Sci. U.S.A.*, 88 (1991) 1138–1142.
[7] A. Lubineau, C. Auge, and P. Francois, *Carbohydr. Res.*, 228 (1992) 137–144.
[8] Y. Ichikawa, Y.C. Lin, D.P. Dumas, G.J. Shen, E.G. Junceda, M.A. Williams, R. Bayer, C. Ketcham, L.E. Walker, J.C. Paulson, and C.H. Wong, *J. Am. Chem. Soc.*, 114 (1992) 9283–9298.
[9] Y. Ichikawa, G.C. Look, and C.H. Wong, *Anal. Biochem.*, 202 (1992) 215–238.

[10] I. Maru, Y. Ohta, K. Okamoto, S. Suzuki, K. Kakehi, and Y. Tsukada, *Biosci. Biotech. Biochem.* 56 (1992) 1557–1561.
[11] J. Thiem and B. Sauerbrei, *Angew. Chem.*, 103 (1991) 1521–1523.
[12] K.G.I. Nilsson, *Carbohydr. Res.*, 167 (1987) 95–103.
[13] K. Ajisaka and M. Shirakabe, *Carbohydr. Res.*, 224 (1992) 291–299.
[14] R. Kuhn, H.H. Baer, A. Gauhe, *Chem. Ber.*, 88 (1955) 1713–1723.
[15] L. Hydbys, P.O. Larsson, K. Mosbach and S. Svensson, *Biochem. Biophys. Res. Commun.*, 123 (1984) 8–15.
[16] K. Ajisaka, H. Fujimoto, and H. Nishida, *Carbohydr. Res.*, 180 (1988) 35–42.
[17] (a) K. Sakai, R. Katsumi, H. Ohi, T. Usui, and Y. Ishido, *J Carbohydr. Chem.*, 11 (1992) 553–565; (b) T. Usui, S. Kubota, and H. Ohi, *Carbohydr. Res.*, 244 (1993) 315–323.
[18] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.