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# Enzymatic syntheses of GlcNAc$\beta$1-2Man and Gal$\beta$1-4GlcNAc$\beta$1-2Man as components of complex type sugar chains

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**GlcNAc$\beta$1-2Man and GlcNAc$\beta$1-6Man were synthesized using the reverse hydrolysis activity of $\beta$-*N*-acetylglucosaminidase from both jack beans and *Bacillus circulans*. In turn, Gal$\beta$1-4GlcNAc$\beta$1-2Man and Gal$\beta$1-4GlcNAc$\beta$1-6Man were synthesized regioselectively using the transglycosylation activity of $\beta$-galactosidase from *Diplococcus pneumoniae* and *B. circulans*, respectively. These di- and trisaccharides are important components of complex type sugar chains and will be used as intermediates in our synthetic studies.**

***Keywords*: GlcNAc$\beta$1-2Man, Gal$\beta$1-4GlcNAc$\beta$1-2Man, Gal$\beta$1-4GlcNAc$\beta$1-6Man, components of complex type sugar chains**

***Abbreviations*: *p*Np-$\beta$-GlcNAc, *p*-nitrophenyl 2-acetamido-2-deoxy-$\beta$-D-glucopyranoside; *p*Np-$\beta$-Gal, *p*-nitrophenyl $\beta$-D-galactopyranoside**

## Introduction

The synthesis of carbohydrate chains is essential to the elucidation of their function in glycoproteins and glycolipids. Both organic chemical methods [1, 2] and enzymatic methods [3] have been employed in the synthesis of biologically important sugar chains.

There are in general two distinct classes of enzyme used for the synthesis of oligosaccharides: glycosidases and glycosyltransferases. In this study we used glycosidases, since most are commercially available and relatively inexpensive. Glycosidases can catalyse two types of reactions: reverse hydrolysis reactions and transglcosylation reactions. While the regioselectivity of reverse hydrolysis reactions is generally low, that of transglycosylation reactions can be quite high, assuming that the enzyme has been properly selected [4, 5].

We previously described the enzymatic synthesis of mannooligosaccharides for use as building blocks in the synthesis of high mannose type sugar chains [6]. In this communication, we would like to describe the enzymatic preparation of GlcNAc$\beta$1-2Man (1) and GlcNAc$\beta$1-6Man (2) using $\beta$-*N*-acetylglucosaminidase (EC 3.2.1.30), as well as the use of these products to in turn synthesize the trisaccharides, Gal$\beta$1-4GlcNAc$\beta$1-2Man (3) and Gal$\beta$1-4GlcNAc$\beta$1-6Man (4), using the transglycosylation activity of $\beta$-galactosidase (EC 3.2.1.23). These disaccharides and trisaccharides are important components of complex type sugar chains and can be used as intermediates for their total synthesis [7–9].

## Materials and methods

### Materials

$\beta$-Galactosidase from *Diplococcus pneumoniae* is a product of Boehringer Mannheim. Culture broth of *B. circulans* (commercial name of BIOLACTA N5) was purchased from Daiwa Kasei (Osaka, Japan). $\beta$-*N*-Acetylglucosaminidase from jack beans is a product of Sigma Chemical Co.

### Enzyme assays

Enzyme activities were measured as follows. A reaction mixture consisting of 0.2 ml of 5 mM *p*-nitrophenyl glycoside, 0.1 ml of 0.1 M potassium phosphate buffer (pH 6.0) and 0.2 ml of an enzyme solution was incubated at 37 °C for 15 min. The reaction was terminated by the addition of 1.5 ml of 0.2 M $Na_2CO_3$. The absorbance of *p*-nitrophenol was measured at 410 nm. One unit of enzyme was defined as

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the amount which liberated 1 μmole of *p*-nitrophenol per min under the above conditions. For the measurement of *β*-*N*-acetylglycosaminidase activity, 2.5 mM *p*Np-*β*-GlcNAc was used as the substrate.

### NMR measurement

The $^{1}H$ and $^{13}C$-NMR spectra were measured at 500 and 125 MHz, respectively, on a Varian Unity-500 spectrometer using $D_2O$ as solvent and a small amount of acetonitrile ($\delta$2.00 for $^{1}H$ spectra and 1.27 ppm for $^{13}C$ spectra) as the internal standard.

### HPLC

Our HPLC system employed an Asahipak NH2P-50 column (4.6 × 250 mm, Showa Denko, Tokyo, Japan) with 80% acetonitrile as the solvent. The Dionex Bio-LC system (CA, USA) with a pulsed amperometric detector and a CarboPac PA1 column (4 × 250 mm) was used with a 50 mM NaOH solution as the solvent.

### Partial purification of *β*-galactosidase and *β*-*N*-acetylglucosaminidase from *B. Circulans*

*Step 1. Partial purification of β-galactosidase*

Powdered culture broth of *B. circulans* (100 g) was dissolved in 300 ml of 10 mM sodium acetate buffer (pH 5.0). The precipitate was removed by centrifugation at 10 000 × **g** at 4 °C for 30 min. The supernatant was applied to a DEAE Sepharose FF column (2.6 × 30 cm) and the column run using a NaCl linear gradient (360 ml of 10 mM sodium phosphate buffer (pH 7.4) and 360 ml of the same buffer made to 0.5 M NaCl). The flow rate was maintained at 4 ml per min and 20 ml fractions were collected. The *β*-galactosidase activity appeared in tubes 27–30 (fraction F-1) while *β*-*N*-acetylglucosaminidase activity appeared in tubes 31–37 (fraction F-2). Fraction F-1 was used, after concentration and without further purification for the syntheses of *β*-galactosyl oligosaccharides.

*Step 2. Partial purification of β-N-acetylglucosaminidase*

A 5 ml aliquot of fraction F-2 was applied to a Sephacryl S-300 column (1.6 × 60 cm). The column was eluted with 10 mM sodium acetate buffer (pH 5.0) containing 0.2 M NaCl, at a flow rate of 0.5 ml per min and 2 ml fractions were collected. The *β*-*N*-acetylglucosaminidase activity appeared in tubes 38–44 (fraction F-3). Following concentration, fraction F-3 was used for the syntheses of GlcNAc-Man.

### Syntheses of GlcNAc*β*1-2Man (1) and GlcNAc*β*1-6Man (2)

A reaction mixture consisting of 15 g of *N*-acetylglucosamine (GlcNAc) and 51 g of mannose (Man) and *β*-*N*-acetylglucosaminidase from *B. circulans* (300 units) in 30 ml sodium acetate buffer (pH 5.0, 0.1 M) was incubated at 37 °C. After 15 days, the enzyme was denatured by heating in boiling water for 5 min. The reaction mixture was applied to an activated carbon column (10 × 130 cm). The products were eluted using a gradient from 0 to 30% aqueous ethanol solution (20 l each), at a flow rate of 20 ml per min. The eluent was collected as fractions (1 l each) and the amount of sugar was measured by the phenol-sulfuric acid method [10]. The oligosaccharide composition of each fraction was analysed by HPLC. The disaccharides were eluted in fractions no. 24–no. 26. The fractions no. 24 and no. 26 were separately concentrated to provide disaccharides **1** (73.1 mg) and **2** (527.9 mg), respectively. Fraction no. 25 (196.0 mg) contained a mixture of **1** and **2** in the ratio of ~1 : 2.

### Synthesis of Gal*β*1-4GlcNAc*β*1-2Man (3) by the transglycosylation reaction

A reaction mixture containing 210 mg of *p*Np-*β*-Gal, 267 mg of **1**, 420 μl of dimethyl sulfoxide (DMSO), and *β*-galactosidase from *D. pneumoniae* (1 unit) in 200 mM potassium phosphate buffer (pH 6.0, 2.1 ml) was incubated at 37° C. After 5 h, the enzyme was denatured by heating the mixture in boiling water for 5 min. The reaction mixture was then applied to an activated carbon column (1.6 × 40 cm). The transfer product was eluted using a gradient from zero to 30% aqueous ethanol solution (1 l each), at a flow rate of 2 ml per min. The eluent was collected in 20 ml fractions. After concentration of the trisaccharide fractions, 16.1 mg of **3** was obtained.

### Synthesis of Gal*β*1-4GlcNAc*β*1-6Man (4) by the transglycosylation reaction

A reaction mixture containing 120 mg of *p*Np-*β*-Gal, 150 mg of **2**, 540 μl of acetonitrile, and *β*-galactosidase from *B. circulans* (32 units) in 200 mM potassium phosphate buffer (pH 6.0, 1.23 ml) was incubated at 37 °C. After 2 h, the enzyme was denatured by heating the mixture in boiling water for 5 min. After purification using an activated carbon column, 36.9 mg of **4** was isolated.

## Results and discussion

### Synthesis of **1** and **2** by a reverse hydrolysis reaction

We first tried to synthesize **1** by transglycosylation using various *β*-*N*-acetylglucosaminidases, but never observed any of the *β*1-2 linked isomer (data not shown). Nilsson has also reported encountering similar problems [11].

We therefore turned our attention to the preparation of **1** by reverse hydrolysis, and were successful when we used *β*-*N*-acetylglucosaminidase from jack beans (Fig. 1). A solution containing highly concentrated GlcNAc and Man was incubated in the presence of *β*-*N*-acetylglucosaminidase from jack beans. The disaccharides **1** and **2** were isolated in 0.23% and 1.7% yields, respectively, by activated carbon

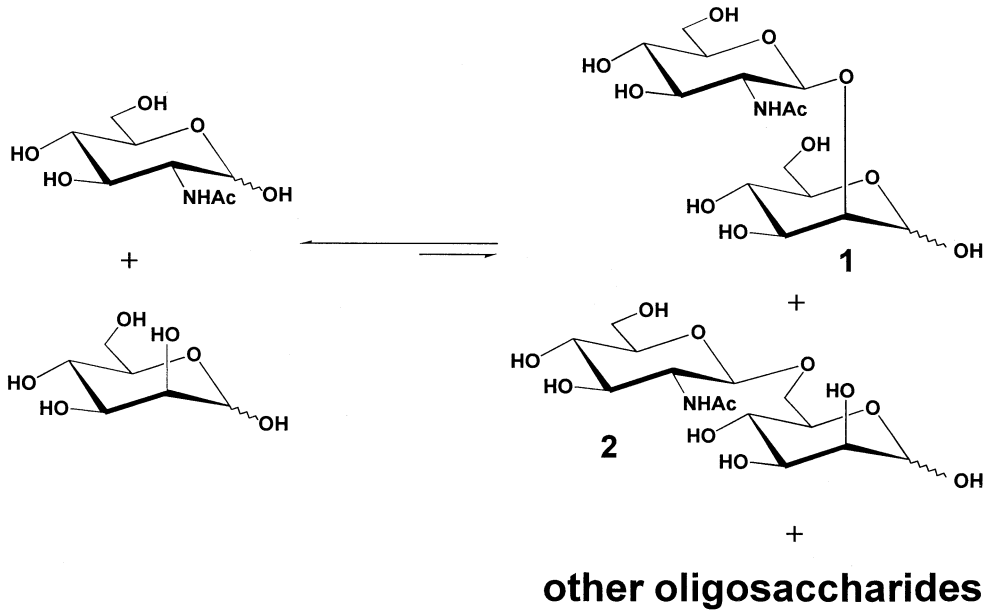


**Figure 1.** Scheme of the synthesis of **1** and **2**.

**Table 1.** $^{13}$C-NMR data of GlcNAc-Man (**1** and **2**) and Gal-GlcNAc-Man (**3** and **4**).

| | *1* | *2* | *3* | *4* |
|---|---|---|---|---|
| Gal | | | | |
| C-1 | | | 104.45 | 104.43 |
| C-2 | | | 72.49 | 72.50 |
| C-3 | | | 74.09 | 77.08 |
| C-4 | | | 70.07 | 70.09 |
| C-5 | | | 76.88 | 76.89 |
| C-6 | | | 62.55 | 62.52 |
| GlcNAc | | | | |
| C-1 | 100.21 | 101.45 | 101.05 | 102.97 |
| C-2 | 55.90 | 55.44 | 56.45 | 56.60 |
| C-3 | 73.97 | 73.68 | 74.04 | 73.91 |
| C-4 | 69.80 | 68.95 | 80.00 | 80.10 |
| C-5 | 76.32 | 75.76 | 76.25 | 76.29 |
| C-6 | 61.14 | 60.67 | 61.50 | 61.66 |
| Man | | | | |
| C-1 | 91.68 | 93.96 | 92.64 | 95.58 |
| C-2 | 77.86 | 70.57 | 78.74 | 71.84 |
| C-3 | 70.42 | 70.22 | 70.81 | 72.20 |
| C-4 | 67.91 | 69.84 | 68.91 | 70.63 |
| C-5 | 73.08 | 71.23 | 73.55 | 72.86 |
| C-6 | 62.03 | 66.74 | 63.08 | 68.37 |

column chromatography. Although these yields were somewhat low, the starting materials (GlcNAc and Man) are cheap and can be easily recovered from the reaction mixture. Therefore, we consider this reverse hydrolysis reaction to be a practical method for the synthesis of **1** and **2**. The

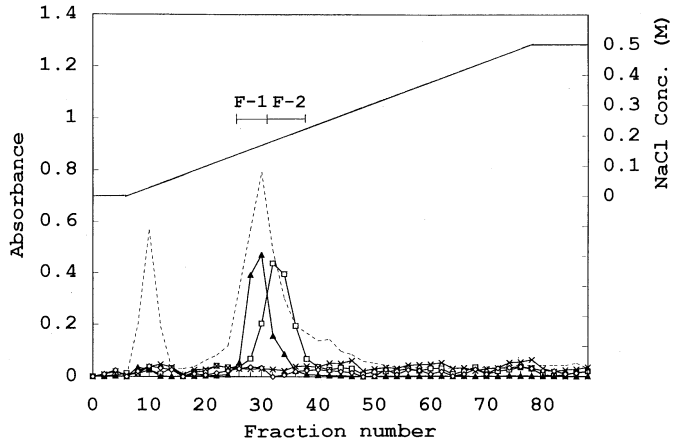


**Figure 2.** Elution pattern of *B. circulans* culture broth following a DEAE Sepharose FF column. -----, protein concentration measured at 280 nm; —□—, *β-N*-acetylglucosaminidase activity; —▲—, *β*-galactosidase activity; —×—, *α*-mannosidase activity; —◇—, *β*-mannosidase activity; ——, NaCl concentration.

$^{13}$C-NMR spectra data for **1** and **2** are summarized in Table 1.

From among the various *β-N*-acetylglucosaminidases which are commercially available, we decided to use *β-N*-acetylglucosaminidase from *B. circulans* for our large scale synthesis of **1**. There were two reasons for our choice of this enzyme source: (a) culture broth of *B. circulans* is commercially available in large amounts, and (b) *β-N*-acetylglucosaminidase from *B. circulans* can be easily isolated using the same chromatographic procedure we use for the isolation of *β*-galactosidase (*vide infra*). Figure 2 shows the

elution pattern following DEAE Sepharose FF column chromatography. The *β-N*-acetylglucosaminidase in fraction F-2 was further purified by gel-permeation chromatography using a Sephacryl S-300 column as shown in Fig. 3. The partially purified *β-N*-acetylglucosaminidase, fraction F-3 in Fig. 3, was free of both α-mannosidase (EC 3.2.1.24) and *β*-mannosidase (EC 3.2.1.25) activities. If this partially purified *β-N*-acetylglucosaminidase fraction had been contaminated with either of these enzymes, mannosyl oligosaccharides would also have been formed.

*The syntheses of **3** and **4** by transglycosylation*

As *β*-galactosidase from *D. pneumoniae* is known to cleave only *β*1-4 galactosyl linkages, we employed the transglycosylation activity of this enzyme for the synthesis of **3** (Fig. 4). The disaccharide **1** and *p*Np-*β*-Gal in aqueous DMSO were incubated in the presence of *β*-galactosidase from *D. pneumoniae*. Figure 5 shows the HPLC profile of this reaction mixture. The new peak B in Fig. 5 was isolated by activated carbon column chromatography and identified as the trisaccharide **3** (Fig. 6A). The *β*1-6 linked galactosyl trisaccharide isomer was not detected.

We used *β*-galactosidase from *B. circulans* instead of *β*-galactosidase from *D. pneumoniae* for the large scale synthesis of **4**, since it is much cheaper and provides nearly the same degree of selectivity. The fraction F-1 (Fig. 2) was used as our source of *β*-galactosidase. Using the transglycosylation activity of *β*-galactosidase, the galactosyl residue was transferred from *p*Np-*β*-Gal to the disaccharide **2** to provide a single product, the trisaccharide **4** (Fig. 6B). Figure 7 shows the activated carbon column elution pattern from which the transglycosylation products were isolated. Peaks A and B were identified as the disaccharide **2** and the trisaccharide **4**, respectively. Usui *et al.* [4] synthesized *β*1-4 linked galactosyl disaccharides using *β*-galactosidase from *B. circulans*, but approximately 10% of the product mixture consisted of a by-product, the *β*1-6 linked galactosyl disaccharide. In contrast, we detected no *β*1-6 linked trisaccharide in our product mixture. This might be due to the disaccharide acceptor being more tightly bound to the active site of the enzyme. The $^{13}$C-NMR chemical shift data for **3** and **4** are summarized in Table 1.

The trisaccharides **3** and **4** have previously been synthesized by organic chemical methods requiring numerous

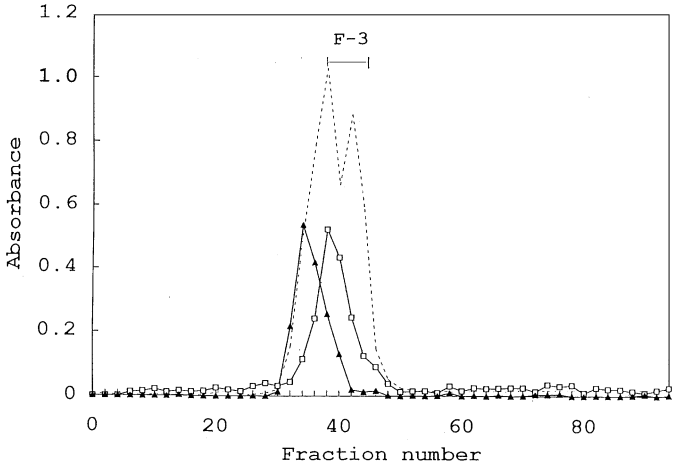


**Figure 3.** Elution pattern of the fraction F-2 in Fig. 2, following a Sephacryl S-300 column. -----, protein concentration measured at 280 nm; —□—, *β-N*-acetylglucosaminidase activity; —▲—, *β*-galactosidase activity.

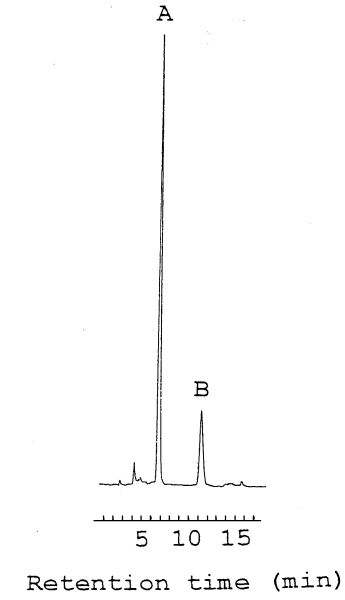


**Figure 5.** HPLC chart of the transglycosylation reaction products from disaccharide **1**. HPLC was measured using a UV detector. (A) disaccharide **1**; (B) trisaccharide **3**.

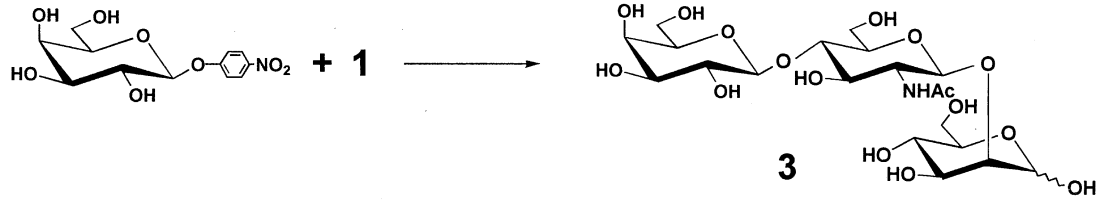


**Figure 4.** Scheme of the synthesis of **3**.

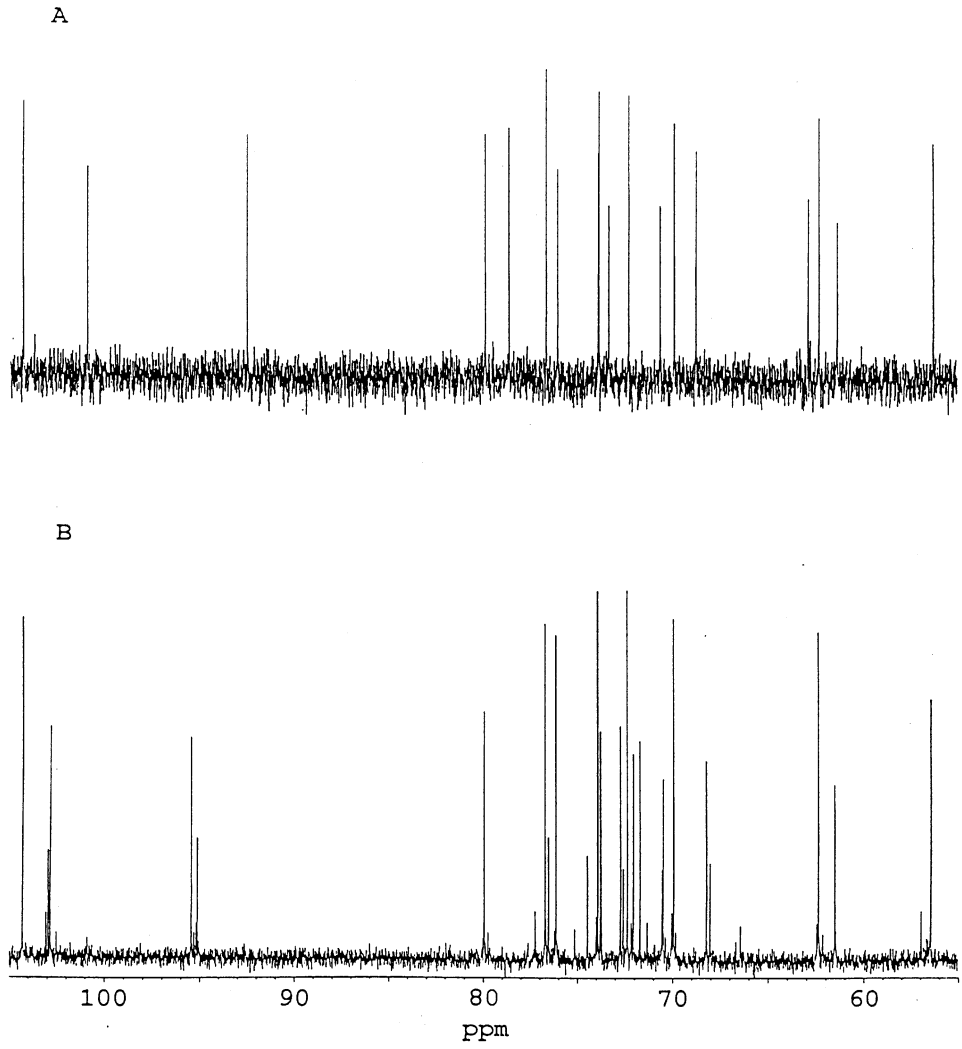


**Figure 6.** $^{13}$C-NMR of the transglycosylation reaction products. (A) trisaccharide **3**; (B) trisaccharide **4**.

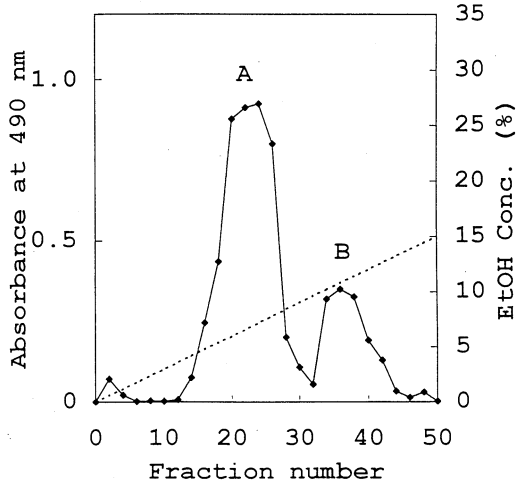


**Figure 7.** Activated carbon column chromatogram of the transglycosylation reaction products -----, ethanol concentration.
(A) disaccharide **2**; (B) trisaccharide **4**.

reaction steps [12–14]. In contrast, this enzymatic sequence afforded **3** and **4** in only two steps. Furthermore, both enzymes (*β-N*-acetylglucosaminidase and *β*-galactosidase) were obtained from commercially available *B. circulans* culture broth. Therefore, we consider this method to be well suited for the large scale synthesis of these oligosaccharides.

In general, reverse hydrolysis reactions using glycosidases produce mixtures. This makes the isolation process critical. In this study, although a number of GlcNAc-Man disaccharides were produced, the target disaccharide **1** could be easily isolated. In contrast, transglycosylation reactions are relatively regioselective if the enzyme is properly selected, as can be seen with our use of *β*-galactosidase. If their advantages and limitations are properly understood, both of these reactions can be effective synthetic tools for the synthesis of oligosaccharides. The disaccharides and trisaccharides synthesized in this study will be used in the construction of complex type sugar chains.

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