Development of Synthetic Routes of Oligosaccharide Units Related to Molecular Recognition, Employing Glycosidase-mediated Transglycosylation

(グリコシダーゼの糖転移反応による
分子認識オリゴ糖鎖合成法の開発)

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ABBREVIATIONS

CD	cyclodextrin
DM-β-CD	2,6-di-*O*-methyl β-cyclodextrin
Me_2SO	dimethylsulfoxide
HP-β-CD	hydroxypropyl-β-cyclodextrin
endo-α-GalNAc-ase	endo-α-*N*-acetylgalactosaminidase
α-NAHase	*N*-acetyl-α-D-hexosaminidase
β-NAHase	*N*-acetyl-β-D-hexosaminidase
FAB-MS	fast atom bombardment-mass spectrometry
Hex	hexose
HexNAc	*N*-acetylhexosamine
HMBC	heteronuclear multiple bond correlation
HSQC	^{1}H-detected heteronuclear signal quantum coherence spectroscopy
HPLC	high pressure liquid chromatography
Lac	lactose
LacNAc	β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucose
MeCN	acetonitrile
MeOH	methanol
NMR	nuclear magnetic resonance
$(GlcNAc)_2$	*N,N'*-diacetylchitobiose
*p*NP	*p*-nitrophenol or *p*-nitrophenyl
*o*NP	*o*-nitrophenol or *o*-nitrophenyl

INTRODUCTION

Oligosaccharides play many important roles in biological processes such as cell-cell recognition, growth, differentiation, pathogenic infection, and cancers[1]. Cell surface oligosaccharides also change in characteristic fashion during normal cellular development[2], malignant alteration[3], and apoptosis[4]. Therefore, there is high current interest in investigation of the biological functions of oligosaccharides. Oligosaccharides that serve as competitive rigands represent potential drug targets for the treatment of infections diseases, inflammation, and cancer. However, it is difficult to get enough amounts of oligosaccharides from natural sources for biological research, because they are limited quantities in nature. For providing of large amounts of oligosaccharides, significant attention has been focused to synthesis of oligosaccharides and its analogs.

My purpose is to develop the efficient synthetic method to obtain oligosaccharide units in sufficient amounts to elucidate other function of oligosaccharide units. Carbohydrate molecules are recognized as particularly challenging targets for regioselective glycosylation by either chemical or enzymatic method because of their nature having multiple hydroxyl groups. Chemical methods for obtaining oligosaccharide units have been extensively developed[5-7], but they involves various elaborated procedures for protection, glycosylation and deprotection. Enzymatic approach has the advantages of the ability to provide efficient and stereospecific bond formation and the elimination of the need to use protecting groups on carbohydrate moieties. This method has been done in part with the glycosyltransferase and glycosidase[8, 9]. The former enzyme has been classified into two categories. The first is Leloir pathway enzyme (Fig. 1), which requires sugar nucleotides as donors, and the second is non-

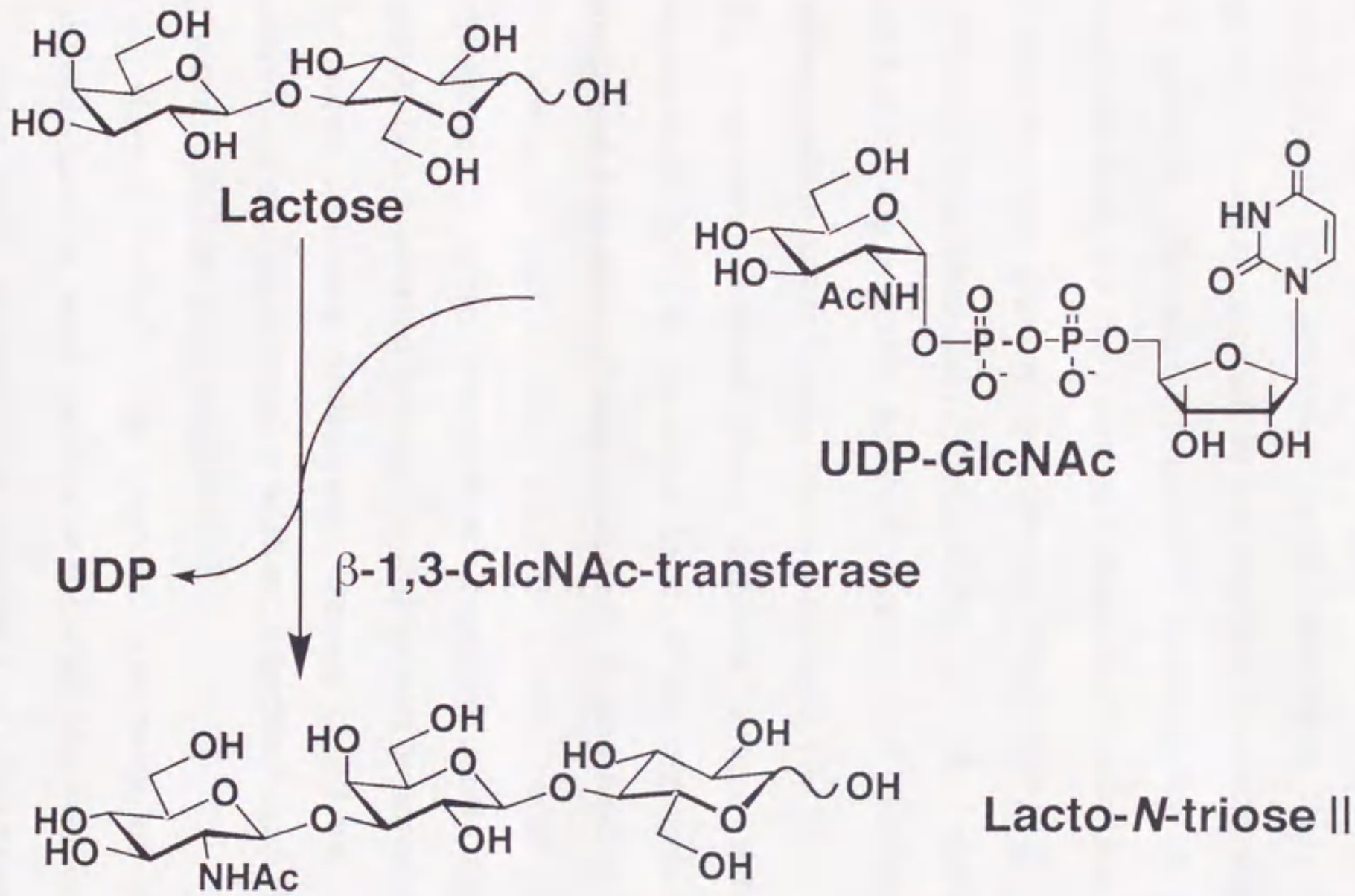


Fig. 1. Oligosaccharide synthesis of the Leloir pathway.

Enzymatic synthesis of lacto-*N*-triose II by β-1,3-GlcNAc-transferase.

Leloir pathway enzyme, which requires sugar-1-phosphates as donors (Fig. 2). The glycosidases usually hydrolyze glycosidic bonds, but they can be used for glycoside formation (Fig. 3). From a practical viewpoint, the use of glycosidases is attractive for oligosaccharide synthesis, because the glycosidases are generally more available, less expensive than the glycosyltransferases, and do not require expensive sugar nucleotide donors. In general, glycosidase-catalyzed transglycosylation do exhibit some regioselectivity for the hydroxyl linkage to the acceptor. For example, the β-D-galactosidase from *Kluyveromyces lactis* affords β-D-Gal-(1→6)-D-GlcNAc (*N*-acetyl-isolactosamine, IsoLacNAc) as the major transglycosylation product of the reaction between lactose and GlcNAc[10], whereas the β-D-galactosidase from bovine testes produced the β-(1→3)-linked isomer[11,12]. The enzymes isolated from different source reflect on the product regioselectivity. On the other hand, if the regioselectivity on glycosidase-mediated formation of oligosaccharide is modified by changing the reaction conditions, it will be applicable to a wide range of glycosidases in the synthesis. Some researchers reported that the regioselectivity of glycosidase-mediated transglycosylation can be manipulated to some extent by using following techniques: organic co-solvent system[13,14], inclusion complex of acceptor glycoside with cyclodextrin[15], and the configuration of the glycosidic linkage of acceptor[16-18].

Here I describe the practical synthesis of biologically important oligosaccharides, with particular focus on the regioselectivity. CHAPTER I deals with regioselective synthesis of disaccharides containing β-D-galactosyl residue. CHAPTER II deals with synthesis of 3'-*O*- and 6'-*O*-*N*-acetylglucosaminyl-*N*-acetyllactosaminide glycosides and manipulation of GlcNAc-transfer reaction by using inclusion complex of acceptor substrate with cyclodextrins. CHAPTER III deals with consecutive synthesis of β-D-

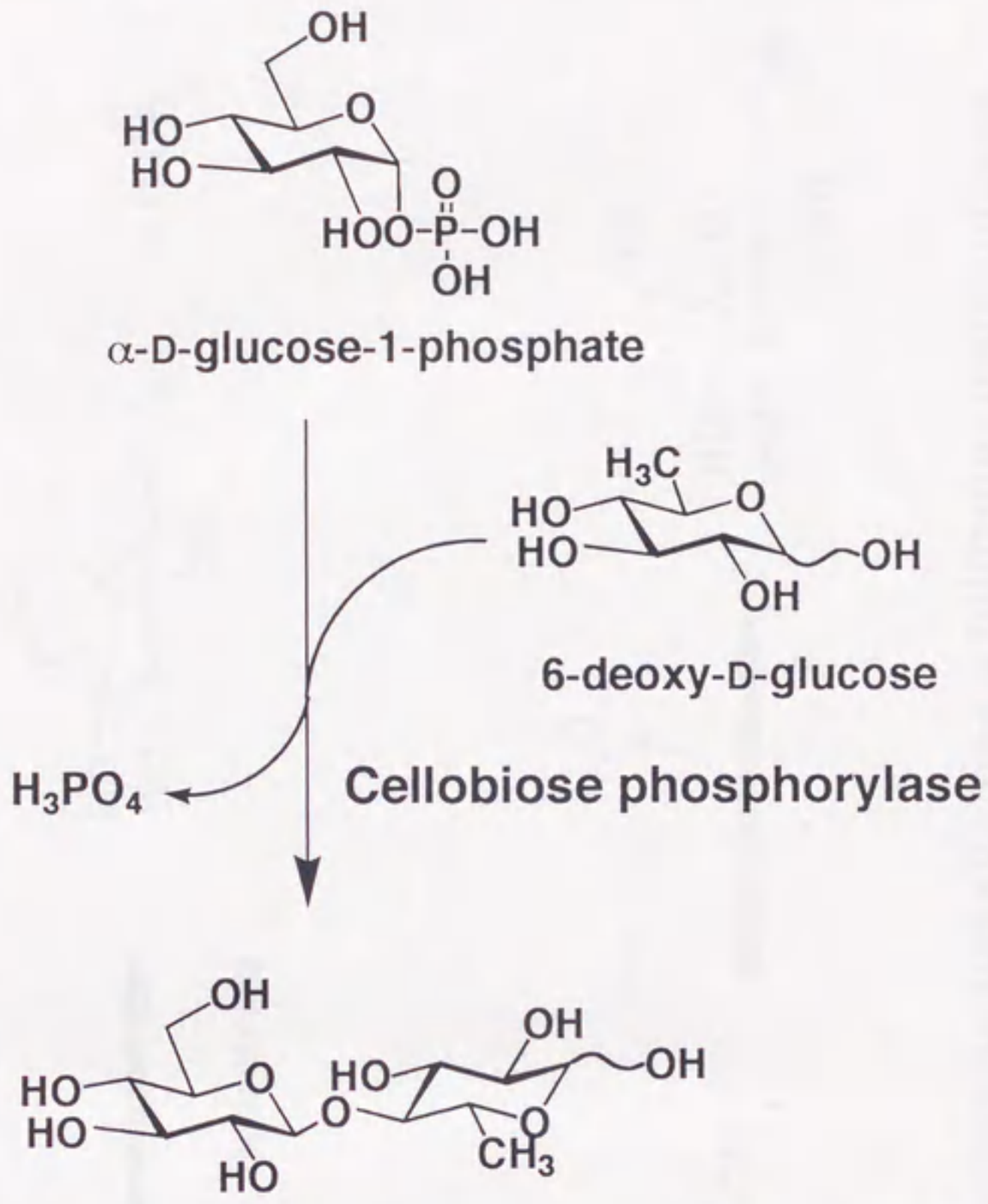
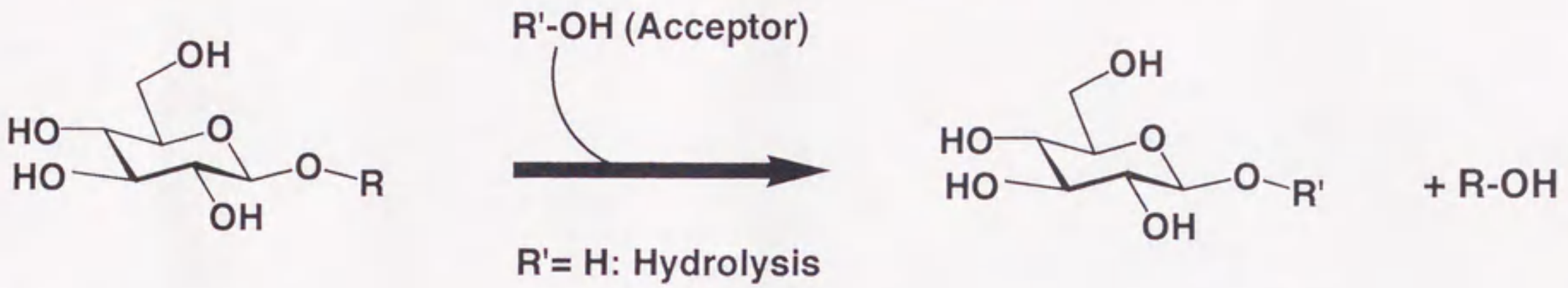


Fig. 2. Oligosaccharide synthesis of the non-Leloir pathway.

Enzymatic synthesis of 4-*O*-β-D-glucopyranosyl-6-deoxy-D-glucose by cellobiose phosphprylase.

(A) Transglycosylation reaction



(B) Reverse reaction

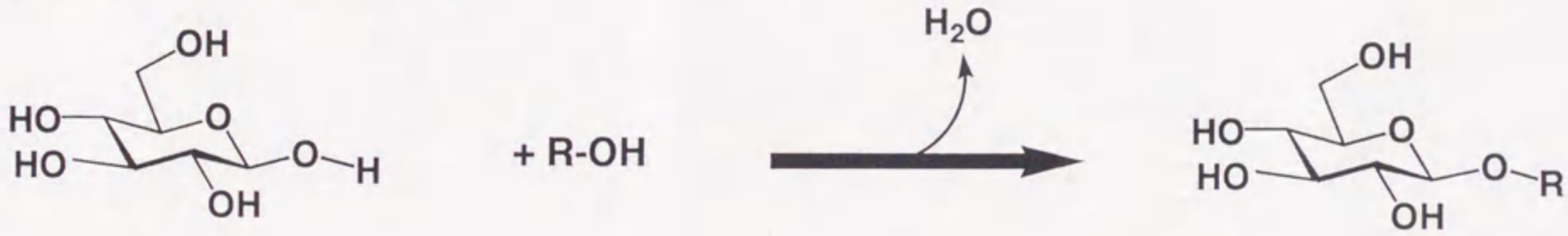


Fig. 3. Approaches to oligosaccharide synthesis utilizing glycosidases.

Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* and β-D-Gal-(1→3)-[β-D-GlcNAc-(1→6)]- α-D-GalNAc-$OC_6H_4NO_2$-*p* as carbohydrate units of mucin-types 1 and 2 core.

CHAPTER I

Enzymatic synthesis of galactosyl residue-containing disaccharide glycosides

1. Introduction

Galactosyl residue-containing disaccharide units were found in many glycoproteins and glycolipids, and play numerous biological roles. β-D-Gal-(1→3)-β-D-GalNAc sequence is a component of G_{M1} ganglioside, which is a precursor compound of ganglioside a-series glycolipids[19], and of asialo G_{M1}, which is a marker glycolipid of mouse NK cells[20,21]. G_{M1} and its pentasaccharide component induced differentiation and proliferation of Neuro 2a. Lacto-*N*-biosidic [β-D-Gal-(1→3)-β-D-GlcNAc] linkage (Type 1 structure) identified as a core structure of blood group ABH, Le^a, Le^b and sialyl-Le^a antigens, and also found a terminal sequence of asialo *N*-linked oligosaccharides[22-24]. *p*-Nitrophenyl lacto-*N*-bioside is a suitable substrate for lacto-*N*-biosidase[25,26]. There is, therefore, a high current interest in developing synthetic routes to oligosaccharides involved in glycoconjugates and its analogues. An organic chemical method for obtaining β-D-Gal-(1→3)-β-D-GalNAc-$OC_6H_4NO_2$-*p* and β-D-Gal-(1→3)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* has been developed[27,28], but it involves various elaborate procedures for protection, glycosylation and deprotection. On the other hand, reducing disaccharides β-D-Gal-(1→3)-D-GalNAc and β-D-Gal-(1→3)-D-GlcNAc were also synthesized by transglycosylation utilizing β-D-galactosidase from several sources[11, 12, 29, 30]. My interest was directed to develop a system for selective transfer of a galactosyl residue to the C-3 position of β-glycoside acceptors.

This chapter details a convenient synthesis of *p*-nitrophenyl galactosyl-glycosides, including β-D-Gal-(1→3)-β-D-GalNAc-$OC_6H_4NO_2$-*p*, β-D-Gal-(1→3)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* and β-D-Gal-(1→3)-β-D-Glc-$OC_6H_4NO_2$-*p*, by using porcine testes and *Bacillus circulans* β-D-galactosidase-catalyzed

transfer from lactose to *p*-nitrophenyl β-glycosides, and the regioselectivity of their transglycosylation reactions.

2. Materials and Methods

2.1. Materials

Commercially available β-D-galactosidase (EC 3. 2. 1. 23), Biolacta (Daiwa Kasei Co., Ltd., Osaka, Japan) from *Bacillus circulans* was directly used for the enzymatic synthesis. The crude enzyme was further purified by the previously developed method to analyze exactly the regioselectivity of the enzyme on the galactosyl transfer reaction[13]. Crude porcine testes β-D-galactosidase was prepared by 20-75% saturated ammonium sulfate precipitation. Nagstatin, an inhibitor of *N*-acetyl-β-D-hexosaminidase (β-NAHase) [31], was obtained from Meiji Seika Co., Ltd. (Tokyo, Japan). All other chemicals were obtained from commercial sources.

2.2. Enzyme assay

β-D-Galactosidase activity was assayed as follows. A mixture containing 2 mM *o*-nitrophenyl β-D-galactopyranoside in 0.9 ml of 50 mM sodium phosphate buffer (pH 6.0) and an appropriate amount of enzyme in a total volume of 0.1 ml was incubated for 10 min at 30℃. The reaction was stopped by adding 2 ml of 0.1 M Na_2CO_3, and then the liberated *o*-nitrophenol was measured spectrophotometrically at 420 nm. One unit of enzyme was defined as the amount hydrolyzing 1 μ mole of *o*-nitrophenyl β-D-galactopyranoside per min.

2.3. Analytical Method

HPLC was done with a YMC-packed column type AQ-312 (ODS) (ϕ 6×

150 mm) in a Hitachi 6000-series liquid chromatograph with an L-4000 ultraviolet detector. Elution of the column was done with H_2O-MeOH of 88 : 12. The flow rate was 0.8 ml/min at 40℃. ^{13}C- and ^{1}H-NMR spectra were recorded on a JEOL JNM-EX 270 or JNM-LA 500 spectrometer. Chemical shifts are expressed in δ relative to sodium 3-(trimethylsilyl)-propionate as an external standard. FAB-MS analyses were done in the positive ion mode using a JEOL JMS DX-303HF mass spectrometer, coupled to a JEOL DA-5000 mass data system. An accelerating voltage of 10 kV and a mass resolution of 1000 was used. A sample of 1 μl in distilled water was put onto a probe tip and mixed with 1 μl of glycerol as a matrix. Mass calibration was done using Ultramark. Specific rotation was measured with a digital polarimeter DIP-1000 apparatus (JASCO Corp. Ltd., Tokyo, Japan).

2.4. Hydrolytic reaction of β-D-galactosidase on p-nitrophenyl galactosyl residue-containing disaccharide glycosides

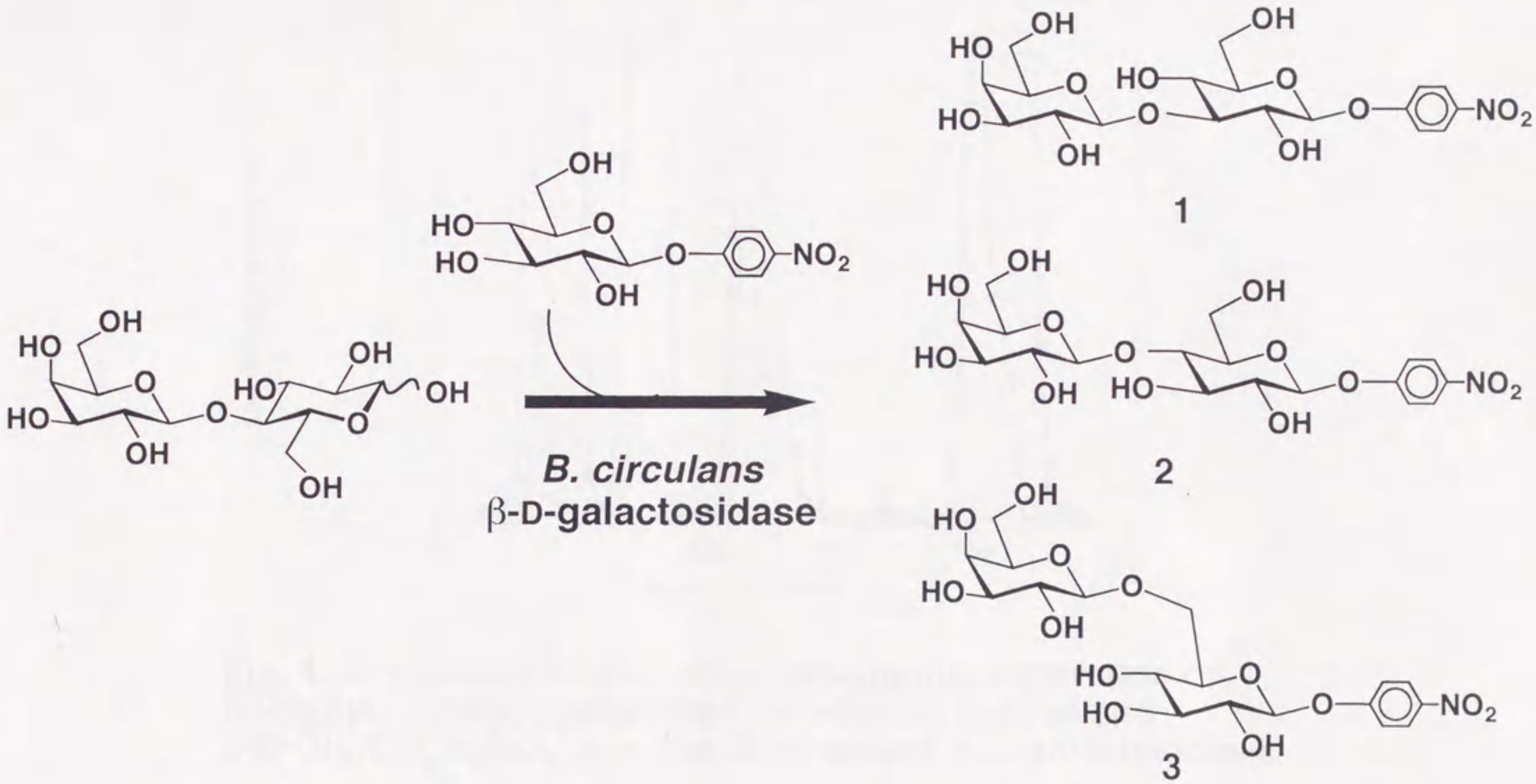
The relative rates of attack of β-D-galactosidase on *p*-nitrophenyl galactosyl-glucosides were measured by incubating a mixture (1 ml) containing 0.4 mM of substrates in 50 mM sodium phosphate buffer (pH 6.0) with 0.5 U of the enzyme at 40℃. Samples (100 μl) were taken out at 5-min intervals during the reaction (0, 5, 10, and 15 min). After inactivation of each sample by adding 200 μl of 1 M acetic acid, the liberated *p*-nitrophenyl glycosides were measured by HPLC and spectrophotometry at 300 nm. The reaction was linear from 5 to 15 min. The rate of attack on **1** (or **4**) was arbitrarily set at 100.

3. Results and Discussion

3.1. Enzymatic synthesis of β-D-Gal-(1→3)-β-D-Glc-$OC_6H_4NO_2$-p

3.1.1. Preparation of β-D-Gal-(1 → 3)-β-D-Glc-$OC_6H_4NO_2$-p (1) and its isomers

Commercially available crude β-D-galactosidase was used directly for the preparation of galactosyl-glucosides. Thus, following the previously developed method[15], compounds **1**, β-D-Gal-(1→4)-β-D-Glc-$OC_6H_4NO_2$-*p* (**2**), and β-D-Gal-(1→6)-β-D-Glc-$OC_6H_4NO_2$-*p* (**3**) were prepared by the galactosyl transfer reaction of *B. circulans* β-D-galactosidase (Scheme 1). These compounds were readily synthesized in a mmol scale. To a solution (12 ml) of lactose (2.4 g) and *p*-nitrophenyl β-D-glucoside (2.0 g) in 20 mM phosphate buffer (pH 7.0) containing 20% MeCN was added β-D-galactosidase from *B. circulans* (20 U). The molar ratio of the donor and acceptor was 1 : 1 and the total substrate concentration was 36.7%. After being incubated for 6 h at 40℃, the reaction mixture was heated for 10 min at 95℃ and centrifuged. The supernatant was directly put onto a Toyopearl HW-40S column (ϕ 5× 100 cm) as in Fig. 4. The eluate was monitored by measuring the absorbance at 300 nm (*p*-nitrophenyl group) and at 485 nm (phenol-sulfuric acid method). The chromatogram showed four peaks (F-1, tubes 117-122; F-2, tubes 133-142; F-3, tubes 152-160; F-4, tubes 161-168) as transglycosylation products displaying coincident absorbance at 300 nm and 485 nm. Fractions F-1, F-3, and F-4, after concentration to dryness followed by crystallization from MeOH, gave compounds **3** (75.7 mg), **1** (119.5 mg), **2** (270.4 mg) and respectively. Compound **1** and its isomers **2** and **3** were obtained in a total yield of 15.1% based on the *p*-nitrophenyl β-D-galactoside



Scheme 1. Formation of disaccharide glucosides by *B. circulans* β-D-galactosidase-catalyzed transglycosylation.

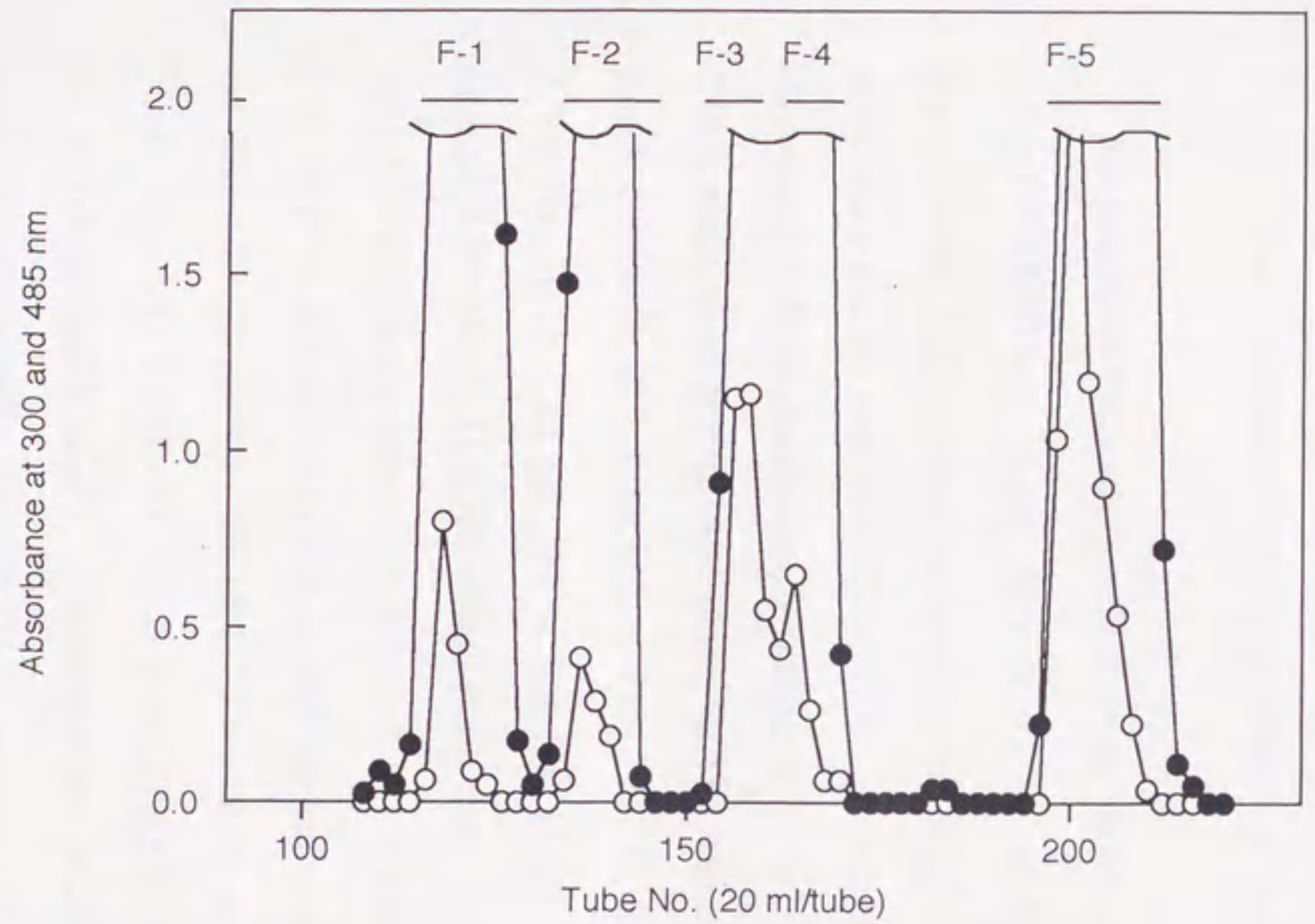


Fig. 4. Toyopearl HW-40S chromatographic separation of transglycosylation products formed from lactose and β-D-Glc-$OC_6H_4NO_2$-*p* by the *B. circulans* β-D-galactosidase.

(●), Absorbance at 300 nm; (○), Absorbance at 485 nm.

added and in a ratio of 26 : 58 : 16. Another fraction F-2 was combined, concentrated and lyophilized to yield 43.8 mg. It was assumed to be trisaccharide glycoside as transfer product, because its FAB-MS spectrum showed *m/z* 626 corresponding to the molecular ion (Hex-Hex-Hex-$OC_6H_4NO_2$-*p*). Fraction F-5 (tubes 195-213) contained *p*-nitrophenyl β-D-glucoside used acceptor substrate.

*3.1.2. Characterization of compounds **1**, **2**, and **3***

All physical data for **1** and **2** were identical to those of β-D-Gal-(1→3)-β-D-Glc-$OC_6H_4NO_2$-*p* and β-D-Gal-(1 → 4)-β-D-Glc-$OC_6H_4NO_2$-*p* reported previously[15]. The positive ion mode FAB-MS spectrum of **3** shows a molecular ion at *m/z* 464 ($[M+H]^+$). It indicated that compound **3** is a *p*-nitrophenyl disaccharide consisting of Hex-Hex-$OC_6H_4NO_2$-*p*. ^1H and ^{13}C-NMR were used for further study of the structure of compound **3**. ^1H-NMR (D_2O): δ 8.25 (d, 2H, *J* 9.2 Hz, *m*-Ph), 7.27 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.27 (d, 1H, *J* 7.6 Hz, H-1), 4.41 (d, 1H, *J* 6.9 Hz, H-1'). The carbon resonances were assigned by the C-H shift-correlation map and by comparing the spectrum with the data for compounds **1** and **2** [15]. ^{13}C-NMR (D_2O) data of **3** (Fig. 5): δ 164.45 (Ph carbon attached to the phenolic oxygen), 145.48 (*p*-Ph), 128.94 (*m*-Ph), 119.42 (*o*-Ph) 106.15 (C-1') 102.21 (C-1), 78.22, (C-5'), 78.14 (C-5), 77.91 (C-3), 75.51 (C-3'), 75.49 (C-2), 73.57 (C-2'), 71.99 (C-4), 71.42 (C-4'), 71.13 (C-6), 63.75 (C-6'). These results indicated that compound **3** is a *p*-nitrophenyl disaccharide β-D-Gal-(1→6)-β-D-Glc-$OC_6H_4NO_2$-*p*: $[\alpha]_D^{25}$-106.4° (*c* 1, H_20); m.p. 137-139° .

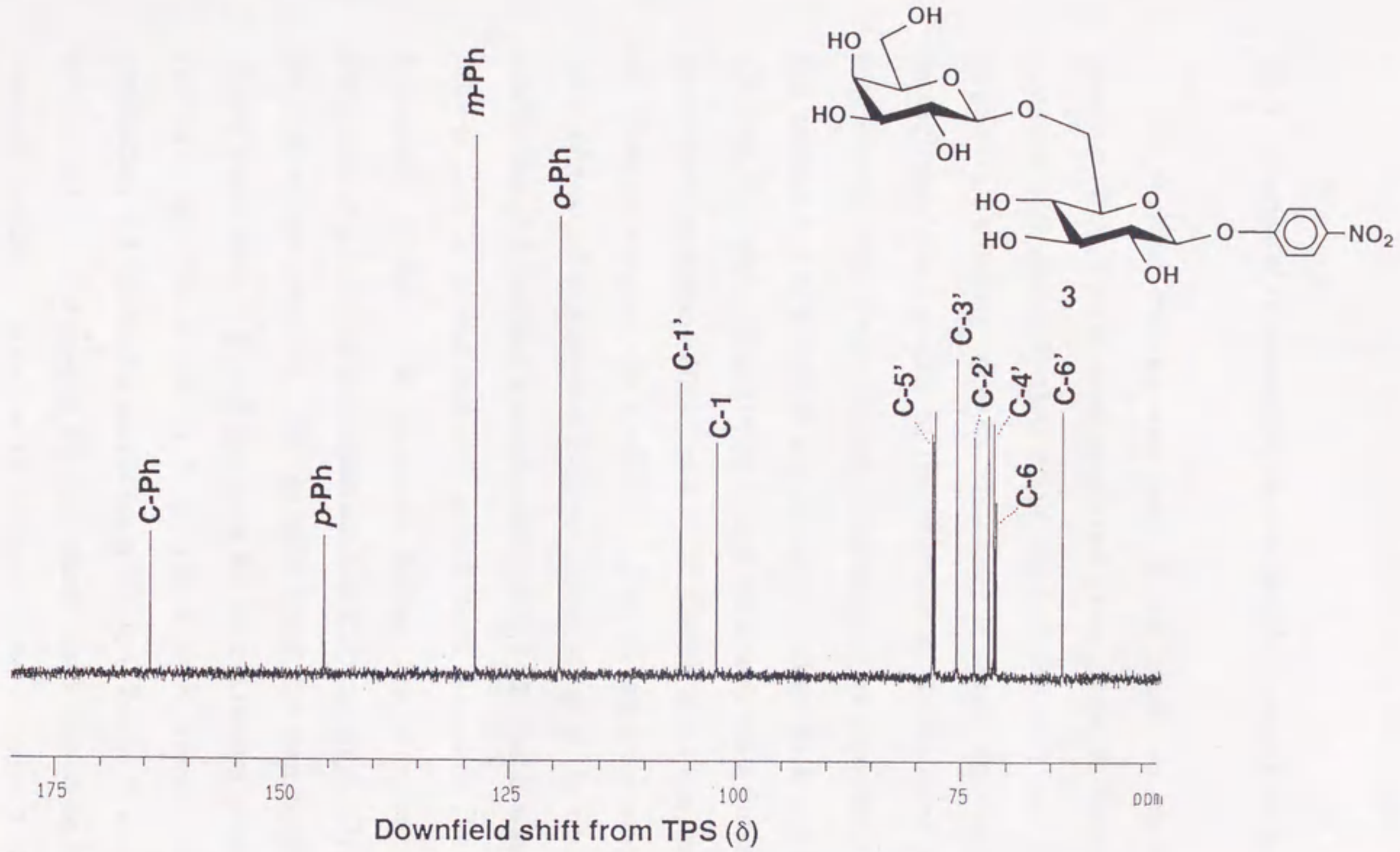


Fig. 5. ^{13}C-NMR analysis of the compound 3 in D_2O.

3.1.3. Course of B. circulans β-D-galactosidase-catalyzed transglycosylation

The purified enzyme was used in the study on regioselectivity of *B. circulans* β-D-galactosidase-catalyzed transglycosylation, since it can be assumed with certainly that all of the transfer reactions observed with the respective substrate were catalyzed by a single enzyme. Figure 6 is a transglycosylation profile of the reaction of *B. circulans* β-D-galactosidase with lactose and *p*-nitrophenyl β-D-glucoside in phosphate buffer (pH 7.0). The amounts of **1**, **2**, and **3** as a function of time were examined on the 0.3-ml scale. Samples (10 μ l) were taken out at intervals during the incubation, inactivated by adding 20 μ l of 1 M CH_3COOH and then were diluted with 4 vol. of water for analysis by HPLC. The transglycosylation reaction led to the preferential synthesis of **2** in the initial stage of the reaction. When the production of **2** reached a maximum at 4 h, **1**, **2**, and **3** were obtained in the ratio of 17 : 79 : 4 and in 21.6% overall yield (based on the donor). No (1→2)-transfer product was detected during the reaction. However, once formation of **2** reached its maximum, the amount markedly decreased during the subsequent reaction. On the other hand, the formation of **1** was a little slower than that of **2** and the time for its maximum production was 12 h. At that time, the ratios of **1**, **2**, and **3** were almost equal. When the production of **3** reached a maximum at 70 h, **1**, **2**, and **3** were obtained in the ratio of 12 : 9 : 79 and in 31.1% overall yield (based on the donor). This reaction makes it possible to prepare three isomers **1**, **2**, and **3** and to selectively synthesize **1** and its isomer **3** by controlling the reaction time.

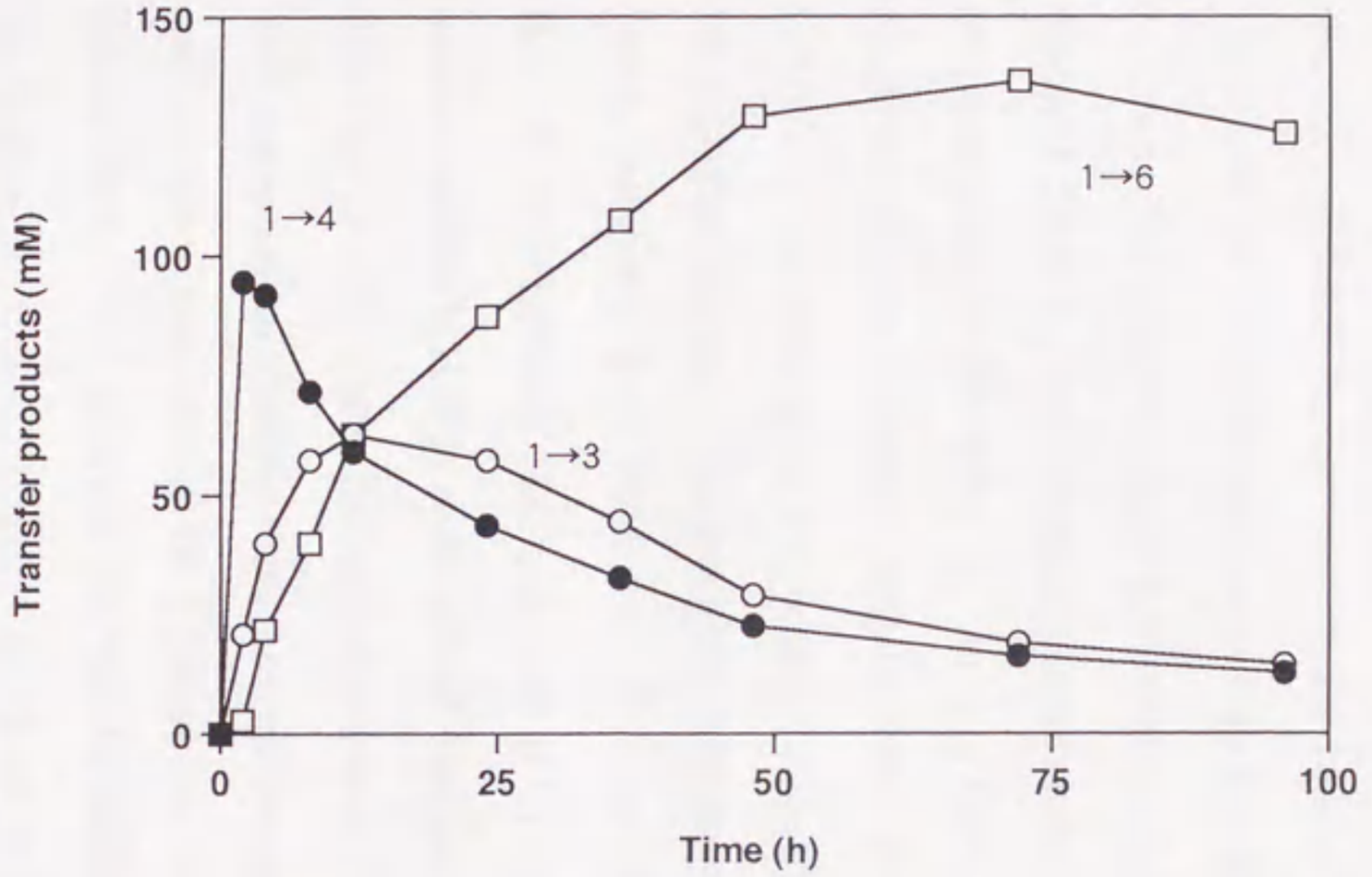


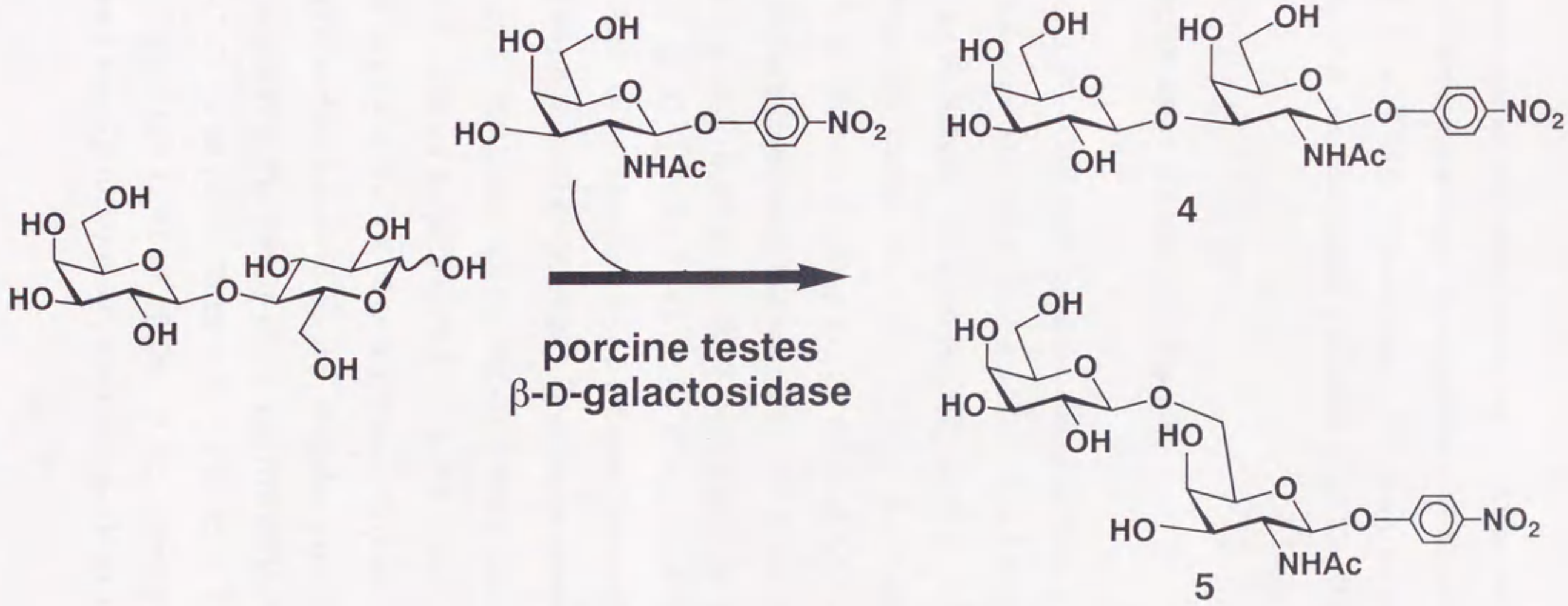
Fig. 6. Course of *B. circulans* β-D-galactosidase-mediated transglycosylation from lactose and β-D-Glc-$OC_6H_4NO_2$-*p*.

The amounts of **1** (●), **2** (○), and **3** (□) as a function of time were examined on the 0.3-ml scale as described in the Materials and Methods section, and samples were analyzed by HPLC during incubation.

3.2. Enzymatic synthesis of β-D-Gal-(1→3)-β-D-GalNAc-$OC_6H_4NO_2$-p

*3.2.1. Preparation of β-D-Gal-(1→3)-β-D-GalNAc-$OC_6H_4NO_2$-p (**4**) and β-D-Gal-(1→6)-β-D-GalNAc-$OC_6H_4NO_2$-p (**5**)*

In our previous study, *B. circulans* β-D-galactosidase predominantly catalyzed β-D-galactosyl transfer from lactose to the OH-4 positions of GlcNAc and GalNAc, but no transfer to the OH-3 positions of the acceptors[13]. Therefore, we selected porcine testes β-D-galactosidase for the synthesis of **4**. Crude β-D-galactosidase preparation from the extract of porcine testes followed by precipitation with ammonium sulfate was directly used for the preparation of **4** (Scheme 2). In this case, much attention was paid to the reaction system, because contaminant β-NAHase activity degrades β-D-GalNAc-$OC_6H_4NO_2$-*p* as an acceptor substrate, accompanied by a loss of the transglycosylation. This problem was solved by using Nagstatin, which is a strong inhibitor for β-NAHase[31]. To a solution of previously dissolved β-D-GalNAc-$OC_6H_4NO_2$-p (66 mg) and β-CD (219 mg) in 12.5 ml of 20 mM acetate buffer (pH 5.5) were added lactose (2.79 g) and Nagstatin (10 mM) followed by the crude β-D-galactosidase (1.0 U) prepared by precipitation with ammonium sulfate from porcine testes. The molar ratio of the donor and acceptor was about 10 : 1 and the total substrate concentration was about 8.8%. The mixture was incubated for 113 h at 40℃ and terminated by heating 95℃ for 10 min. The resulting insoluble material was removed by centrifugation and the supernatant was extracted with diethylether in order to remove the *p*-nitrophenol liberated during the reaction. The aqueous phase was concentrated to a low volume (20 ml) and loaded onto a Toyopearl HW-40S column mentioned above. The chromatogram showed two main peaks (F-1: tube numbers 67-75 and F-2: tube numbers 84-98) for



Scheme 2. The formation of disaccharide glycosides by porcine testes β-D-galactosidase-catalyzed transglycosylation.

which the absorbance at 300 nm coincides with that at 485 nm as in Fig. 7. F-1 and F-2 were each combined, concentrated and lyophilized to afford **5** (2.8 mg) and **4** (12.2 mg), respectively. F-3 peak corresponding to the absorbance at 300 nm contained β-D-GalNAc-$OC_6H_4NO_2$-*p* used as the acceptor.

*3.2.2. Characterization of compounds **4** and **5***

The ^{1}H- and ^{13}C-NMR were used for characterization of compounds **4** and **5**. ^{1}H-NMR data (D_2O) of **4**: ^{1}H, δ 8.28 (d, 2H, *J* 8.9 Hz, *m*-Ph), 7.23 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.33 (d, 1H, *J* 8.6 Hz, H-1), 4.51 (d, 1H, *J* 7.3 Hz, H-1'), 2.00 (s, 3H, NAc). The carbon resonanses of **4** were assigned by the HSQC (Fig. 8, 9). ^{13}C-NMR data (D_2O,): δ 177.90 (C=O of Ac), 164.61 (Ph carbon attached to the phenolic oxygen), 145.45 (*p*-Ph), 128.90 (*m*-Ph), 119.38 (*o*-Ph), 107.65 (C-1'), 101.66 (C-1), 82.17 (C-3), 78.16 (C-5'), 77.86 (C-5), 75.30 (C-3'), 71.44 (C-2'), 73.42 (C-4), 71.44 (C-4'), 63.84 (C-6'), 63.50 (C-6), 53.80 (C-2), 24.98 (Me of Ac). Other physical data for **4** were almost identical to those of β-D-Gal-(1→3)-β-D-GalNAc-$OC_6H_4NO_2$-*p* reported previously[29]. **5** had : $[\alpha]_D^{25}$ +7.66 (*c* 0.5 , H_20); and *m/z* 505 $(M+H)^+$. NMR data (D_2O): ^{1}H, δ 8.28 (d, 2H, *J* 9.2 Hz, *m*-Ph), 7.28 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.31 (d, 1H, *J* 8.6 Hz, H-1), 4.48 (d, 1H, *J* 7.2 Hz, H-1'), 2.05 (s, 3H, NAc); ^{13}C (D_2O,) δ 177.93 (C=O of Ac), 164.65 (Ph carbon attached to the phenolic oxygen), 145.44 (*p*-Ph), 128.93 (*m*-Ph), 119.47 (*o*-Ph), 106.16 (C-1'), 101.875 (C-1), 77.99 (C-5'), 77.32 (C-5), 75.51 (C-3'), 73.60 (C-2'), 73.22 (C-6), 71.84 (C-4), 71.42 (C-4'), 70.51 (C-3), 63.83 (C-6'), 54.91 (C-2), 24.96 (Me of Ac). These results indicated that compound **5** was a β-D-Gal-(1→6)-β-D-GalNAc-$OC_6H_4NO_2$-*p* .

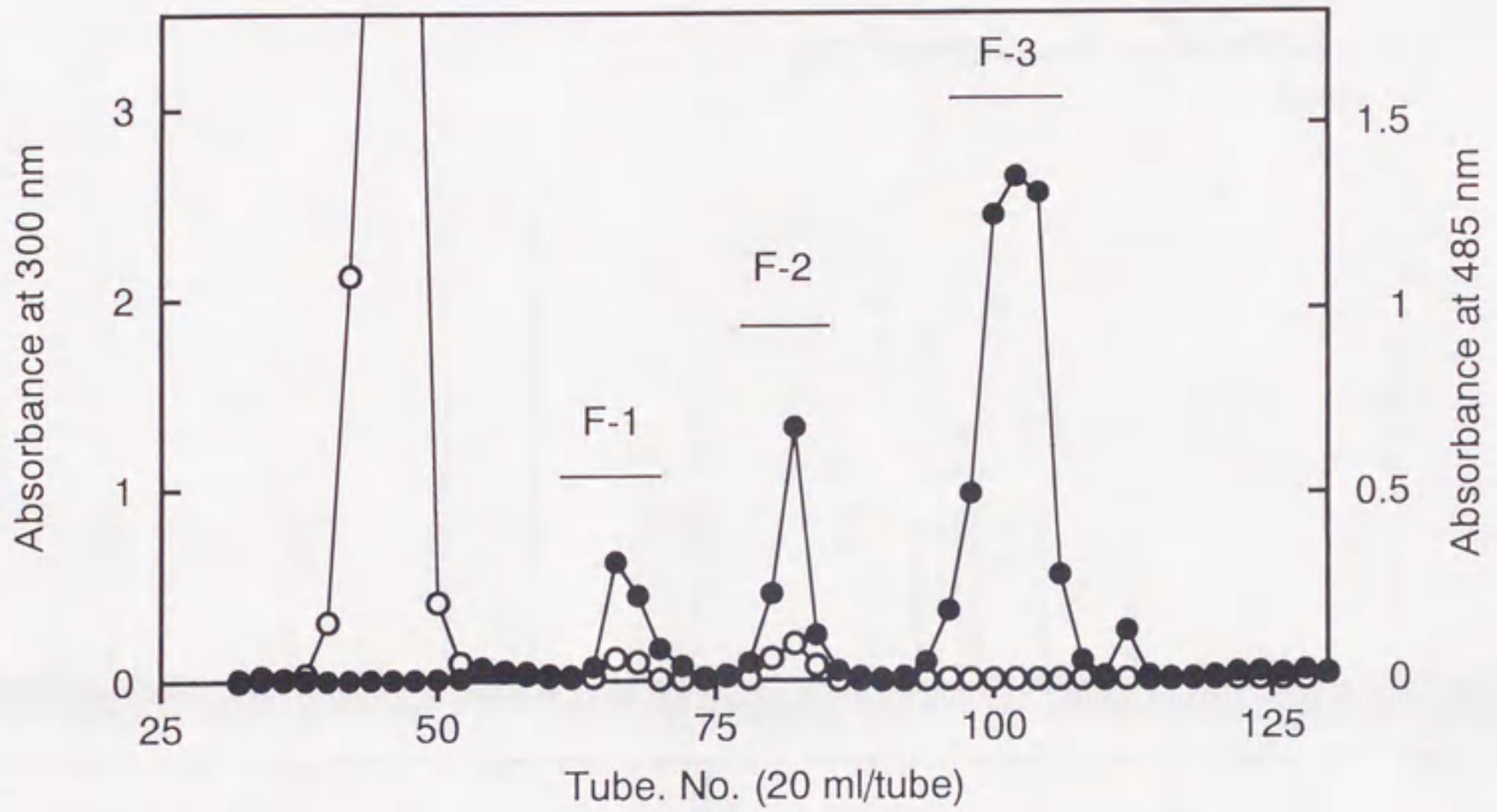


Fig. 7. Toyopearl HW-40S chromatographic separation of transglycosylation products formed from lactose and β-D-GalNAc-$OC_6H_4NO_2$-*p* by the porcine testes β-D-galactosidase.

(●), Absorbance at 300 nm; (○), Absorbance at 485 nm.

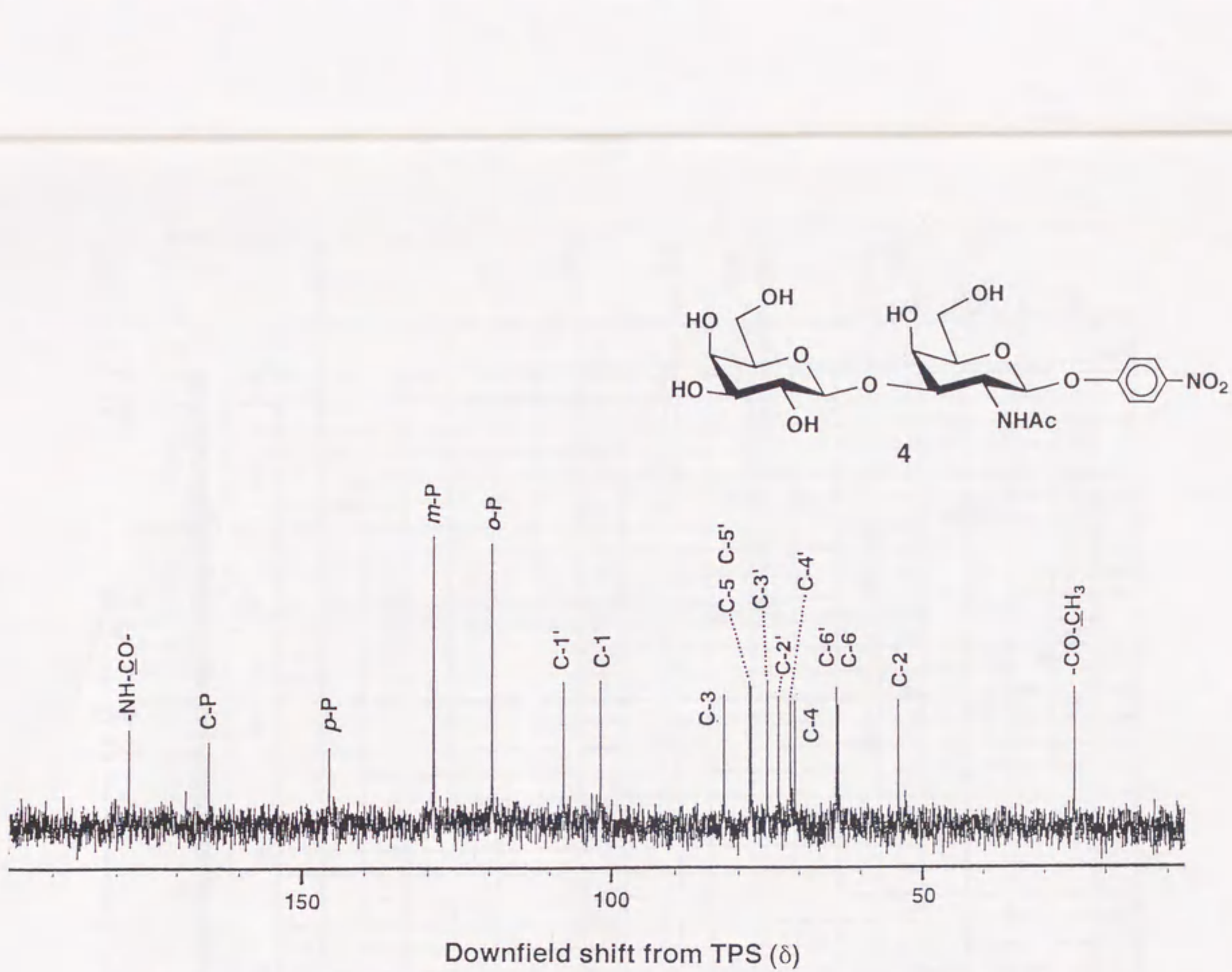


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Fig. 8. ^{13}C-NMR analysis of compound 4 in D_2O.

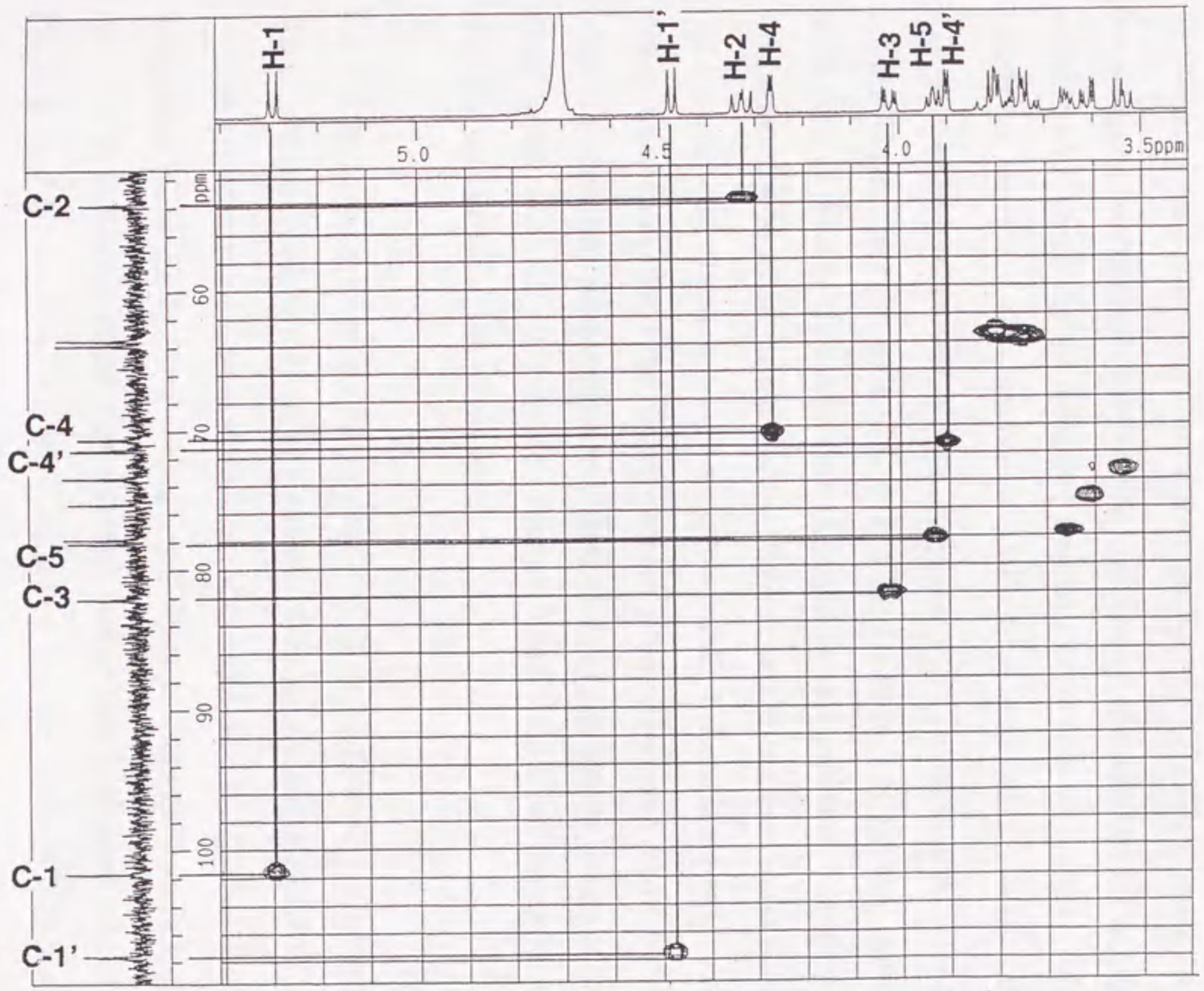


Fig. 9. HSQC spectrum of the compound 4 with ^{1}H and ^{13}C spectra printed on the sides of the 2D spectrum.

3.2.3. Course of porcine testes β-D-galactosidase-catalyzed transglycosylation from lactose and β-D-GalNAc-$OC_6H_4NO_2$-p

When an inclusion complex of β-D-GalNAc-$OC_6H_4NO_2$-*p* with β-CD was the acceptor, two transfer products **4** and **5** were observed by HPLC in 15.3% overall yield (based on the acceptor) and in a ratio of 5 : 1. These values are based on the time for maximum yield of **4** after 4 h. Fig. 10 is an transglycosylation profile of the reaction with lactose and β-D-GalNAc-$OC_6H_4NO_2$-*p*. In this case, the transfer reaction led to the preferential formation of **4** over **5** in the initial stage of the reaction. However, once formation of **4** reached its maximum, the amount decreased markedly during the subsequent reaction. On the other hand, **5** formation was extremely slower and, thus, the time for maximum production was ~240 h. At that time, its concentration surpassed that of **4** in the latter stage of reaction. Thus, much more (1→3) transfer product than the (1→6) transfer one was found in the initial stage of reaction.

3.3. Enzymatic synthesis of β-D-Gal-(1→3)-β-D-GlcNAc-$OC_6H_4NO_2$-p

3.3.1. Preparation of β-D-Gal-(1→3)-β-D-GlcNAc-$OC_6H_4NO_2$-p (6)

The crude enzyme preparation was also used for the synthesis of **6** with Nagstatin (Scheme 3). To a solution β-D-GlcNAc-$OC_6H_4NO_2$-*p* (1.0 g) and β-CD (3.3 g) in 50 ml of 20 mM acetate buffer (pH 5.5) were added lactose (21.0 g) and Nagstatin (0.1 mM), followed by the crude β-D-galactosidase from porcine testes (5 U). The molar ratio of the donor to acceptor was about 20 : 1, and the total substrate concentration was about 44%. The mixture was incubated for 50 h at 40℃ and the reaction was terminated by

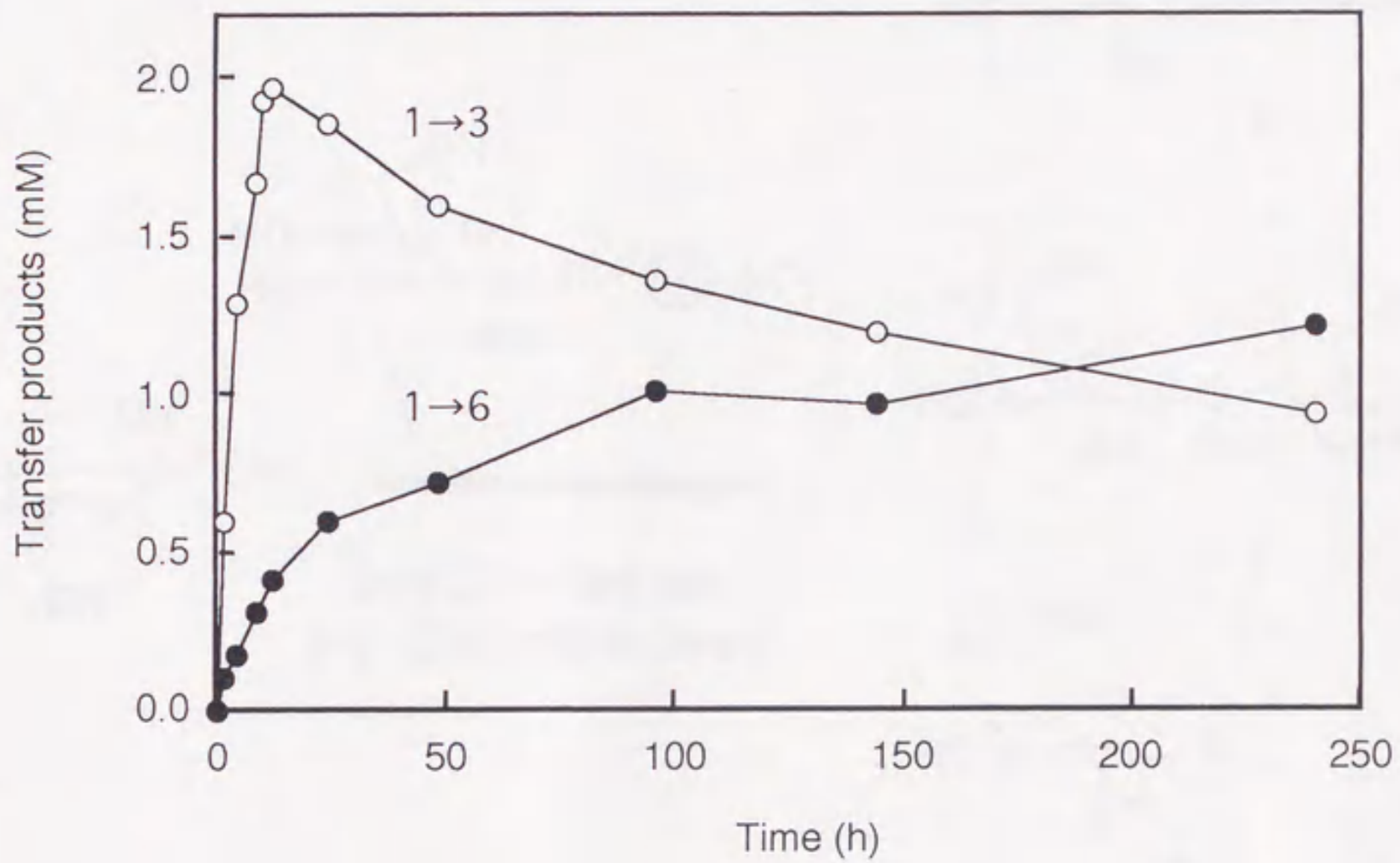
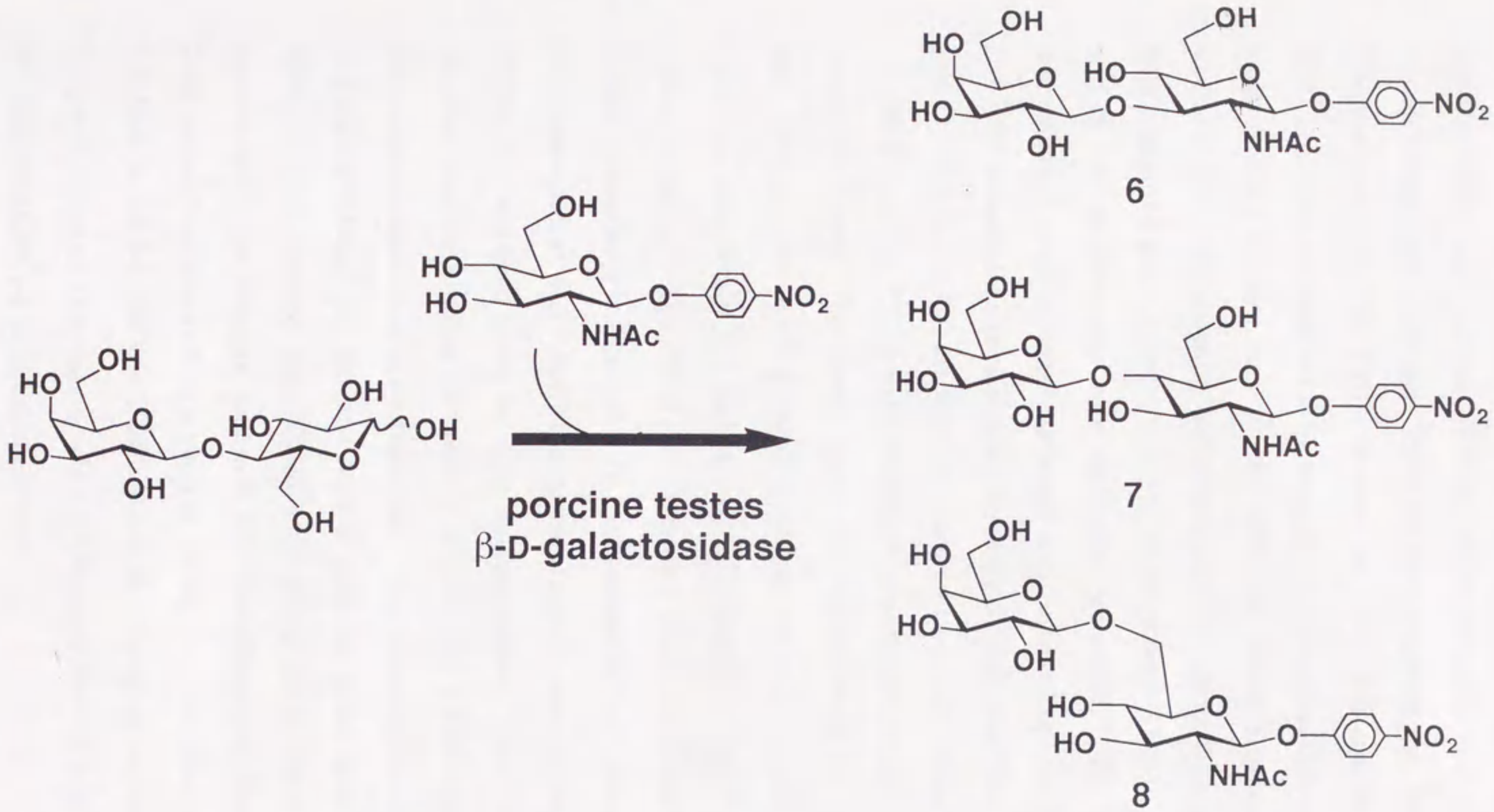


Fig. 10. Time course of porcine testes β-D-galactosidase-mediated isomer formation of 4 and 5 in the presence of β-CD.

The amounts of **4** (β1→3, ○), and **5** (β1→6, ●) products as a function of time were examined on the 0.5-ml scale as described in the Materials and Methods section, and samples were analyzed by HPLC during incubation.



Scheme 3. Formation of disaccharide glycosides by porcine testes β-D-galactosidase-catalyzed transglycosylation.

heating at 95℃ for 10 min. The resulting insoluble material was removed by centrifugation, and one half of the supernatant was loaded onto Chromatorex-ODS DM 1020T column (ϕ 3×50 cm) equilibrated with 20% MeOH in aqueous solution to remove the *p*-nitrophenol liberated during the reaction. The column was eluted with the same solution. The eluate fractions (600 ml) showing coincident absorbance at 300 nm and 485 nm were combined and concentrated to a small volume (15 ml). The remaining half of the supernatant was similarly processed. The two batches of concentrated solution were combined and further applied to the Toyopearl HW-40S column described above. The elution conditions were the same as in Fig. 11. The eluate (20 ml fraction) showed two main peaks (F-1, tubes 50-57; and F-2, tubes 67-79) with coincident absorbance at 485 nm and 300 nm (data not shown). The latter peak was concentrated and lyophilized (133 mg), then treated with β-D-galactosidase from *B. circulans* in order to hydrolyze selectively β-D-Gal-(1 → 4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**7**) in the product mixture. Thus, F-2 (133 mg) was dissolved in 66.5 ml of 20 mM sodium phosphate buffer (pH 7.0) containing the β-D-galactosidase (40 U) and incubated at 40℃ until the contaminant **7** was no longer detected by HPLC; the required time for this reaction was 4 h on the average. The reaction was terminated by heating at 95℃ for 10 min and the resulting insoluble material was centrifuged off. The supernatant was concentrated to a small volume (20 ml) and loaded onto the same column of Toyopearl HW-40S. F-2' fraction was eluted as a sharp peak (tubes 63-75). After concentration to dryness followed by crystallization from ethanol, this fraction gave compound **6** in a yield of 75 mg. F-1 was concentrated to afford β-D-Gal-(1→6)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**8**, 40 mg) as reported previously[14]. F-3 peak, showing absorbance only at 300 nm (tubes 85-100), contained β-D-GlcNAc-$OC_6H_4NO_2$-*p* used as an acceptor.

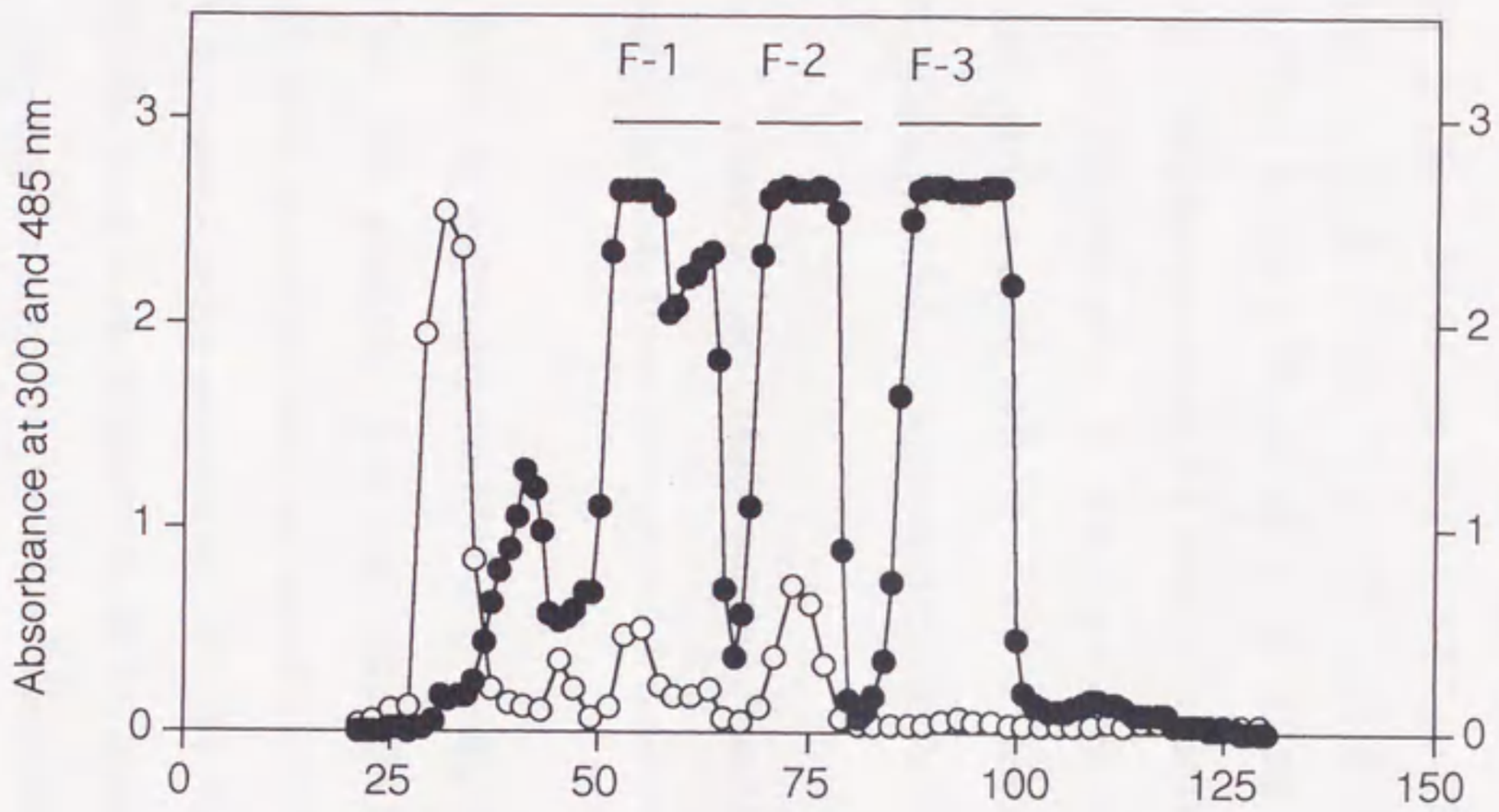


Fig. 11. Toyopearl HW-40S chromatographic separation of transglycosylation products formed from lactose and β-D-GlcNAc-$OC_6H_4NO_2$-*p* by porcine testes β-D-galactosidase.

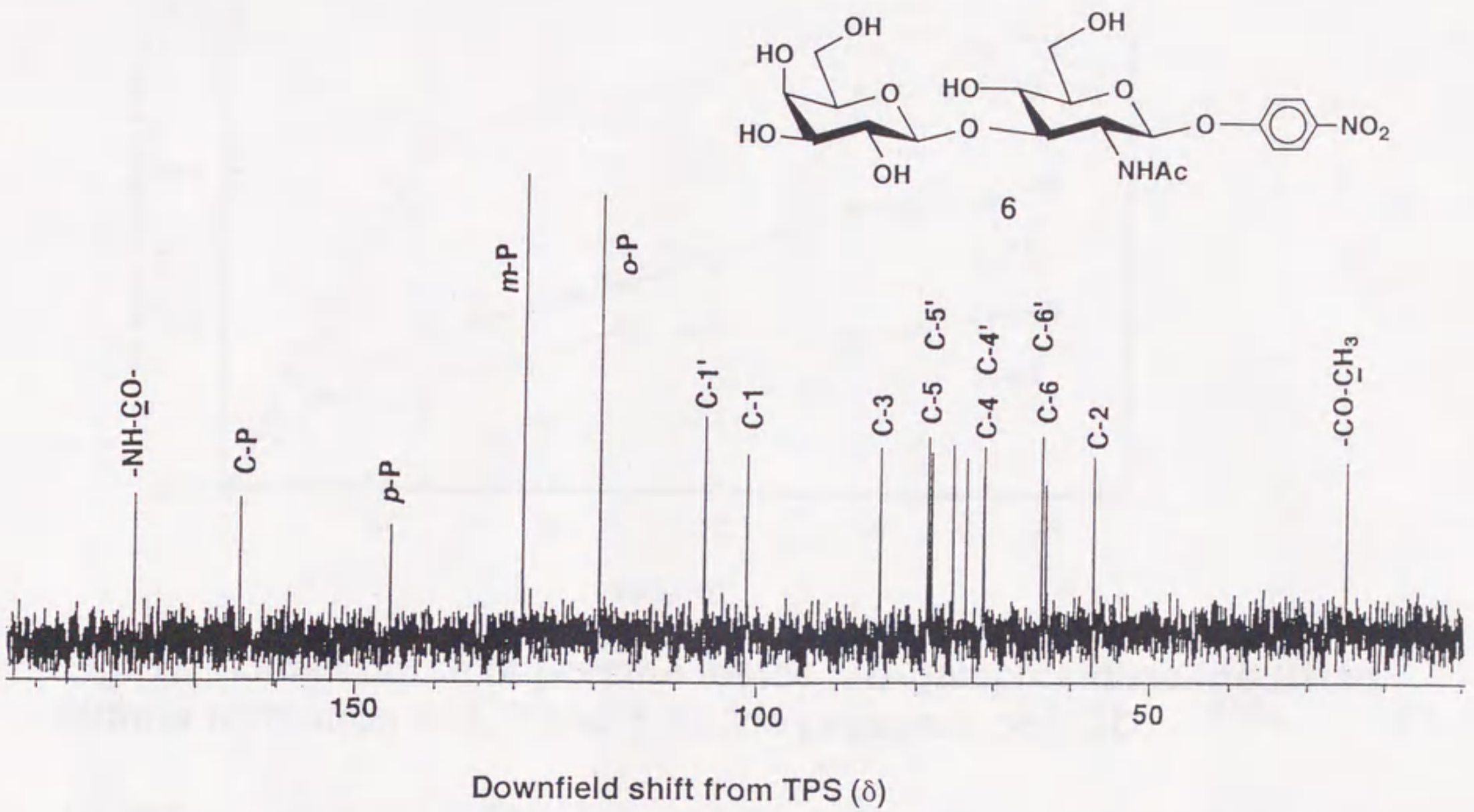
(●), Absorbance at 300 nm; (○), Absorbance at 485 nm.

*3.3.2. Characterization of compounds **6**, **7**, and **8***

NMR data (D_2O) of **6**: ^1H, δ 8.22 (d, 2H, *J* 9.2 Hz, *m*-Ph), 7.18 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.34 (d, 1H, *J* 8.6 Hz, H-1), 4.47 (d, 1H, *J* 7.6 Hz, H-1'); ^{13}C (Fig. 12), δ 177.79 (C=O of Ac), 164.47 (Ph carbon attached to the phenolic oxygen), 145.51 (*p*-Ph), 128.91 (*m*-Ph), 119.37 (*o*-Ph), 106.34 (C-1'), 101.18 (C-1), 84.64 (C-3), 78.63 (C-5'), 78.15 (C-5), 75.33 (C-3'), 73.51 (C-2'), 71.36 (C-4), 71.23 (C-4'), 63.86 (C-6'), 63.29 (C-6), 57.16 (C-2), 24.98 (Me of Ac). Other physical data for compound **6** were identical to those of β-D-Gal-(1→3)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* [28]. In the same way, the NMR and FAB-MS analyses revealed that compounds **7** and **8** were β-D-Gal-(1 → 4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* and β-D-Gal-(1→6)-β-D-GlcNAc-$OC_6H_4NO_2$-*p*.

3.3.3. Course of the porcine testes β-D-galactosidase-catalyzed transglycosylation from lactose and β-D-GlcNAc-$OC_6H_4NO_2$-p

When an inclusion complex of β-D-GlcNAc-$OC_6H_4NO_2$-*p* with β-CD was used as the acceptor, β-(1 → 3)- (**6**), β-(1 → 4)- (**7**), and β-(1 → 6)- (**8**) disaccharide glycosides were observed by HPLC, in 20.9% total yield (based on the acceptor) in the ratio of 48 : 20 : 32. These values are based on a time of 50 h for maximum production of the desired β-(1→3) compound (**6**) (Fig. 13). The ratio of the three transfer compounds varied little during the entire course of reaction: the (1→4)-linked transfer product was formed in significant amounts along with the (1→3)-linked and (1→6)-linked products. The regioselectivity is much lower than when the *N*-acetyl galactosaminide was used as acceptor. These transfer products were first separated into two



6

Fig. 12. ^{13}C-NMR analysis of compound 6 in D_2O.

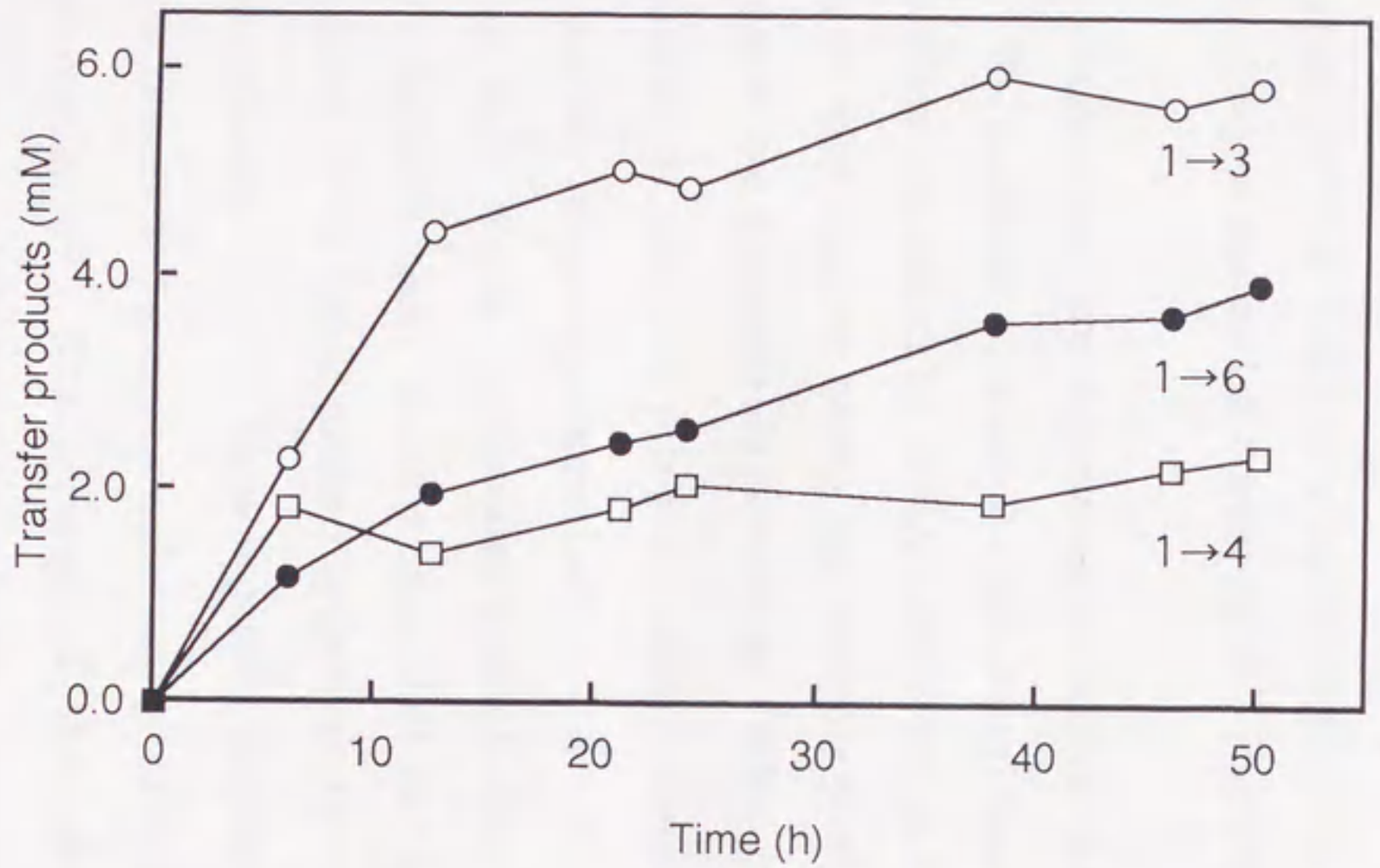


Fig. 13. Time course of porcine testes β-D-galactosidase-mediated isomer formation of 6, 7 and 8 in the presence of β-CD.

The amounts of **6** (β1→3, ○), **7**(β1→4,□) and **8** (β1→6, ●) products as a function of time were examined on the 0.5-ml scale as described in the Materials and Methods section, and samples were analyzed by HPLC during incubation.

fractions (F-1 and F-2) by chromatography on a Toyopearl HW-40S column. F-1 contained only compound **8**. The purification of F-2 into the desired compound **6** was somewhat cumbersome, because this fraction was heavily contaminated by **7**. The compound **7** was therefore selectively removed by hydrolytic treatment with *B. circulans* β-D-galactosidase, which does not hydrolyze **6**. When the lyophilized fraction was incubated at a 0.2% concentration with the enzyme, **7** was hydrolyzed completely after 4 h. The hydrolyzate was then rechromatographed on Toyopearl HW-40S, enabling the selective removal of **7** from the F-2 fraction.

3.4. Regioselectivity of galactosyl-disaccharide formation

The positions of enzymatic galactosylation in all galactosyl-disaccharide products, identified by HPLC, are depicted by arrows in Fig. 14. In four cases, each number shows the percentage of galactosylation in the initial stage of the disaccharide formation. When *p*-nitrophenyl β-D-glucoside was acceptor, *B. circulans* β-D-galactosidase transferred a galactosyl residue to O-4 of the acceptor preferentially to O-3 and reacted only weakly at the O-6 (Fig. 14A). No (1→2)-transfer product was detected during the reaction. The regioselectivity is lower than that of GlcNAc-$OC_6H_4NO_2$-*p* acceptor [14], because (1→3)-linked product is formed other than (1→4)- and (1→6)-linked ones. Yanahira *et al.* have reported the formation of β-D-Gal-(1→2)-D-Glc from lactose and glucose employing by transglycosylation of *B. circulans* β-D-galactosidase[32]. The presence of the aglycon moiety in the glycoside acceptor may hinder galactosylation to its neighboring hydroxyl group O-2. In the case that *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide is used as acceptor, galactosylation occurs overwhelmingly at O-4 and reacts only weakly at the OH-6. No (1→3)-transfer product was detected during the reaction (Fig. 14B). We have recently reported that, when 3-acetamido-3-deoxy-D-glucose

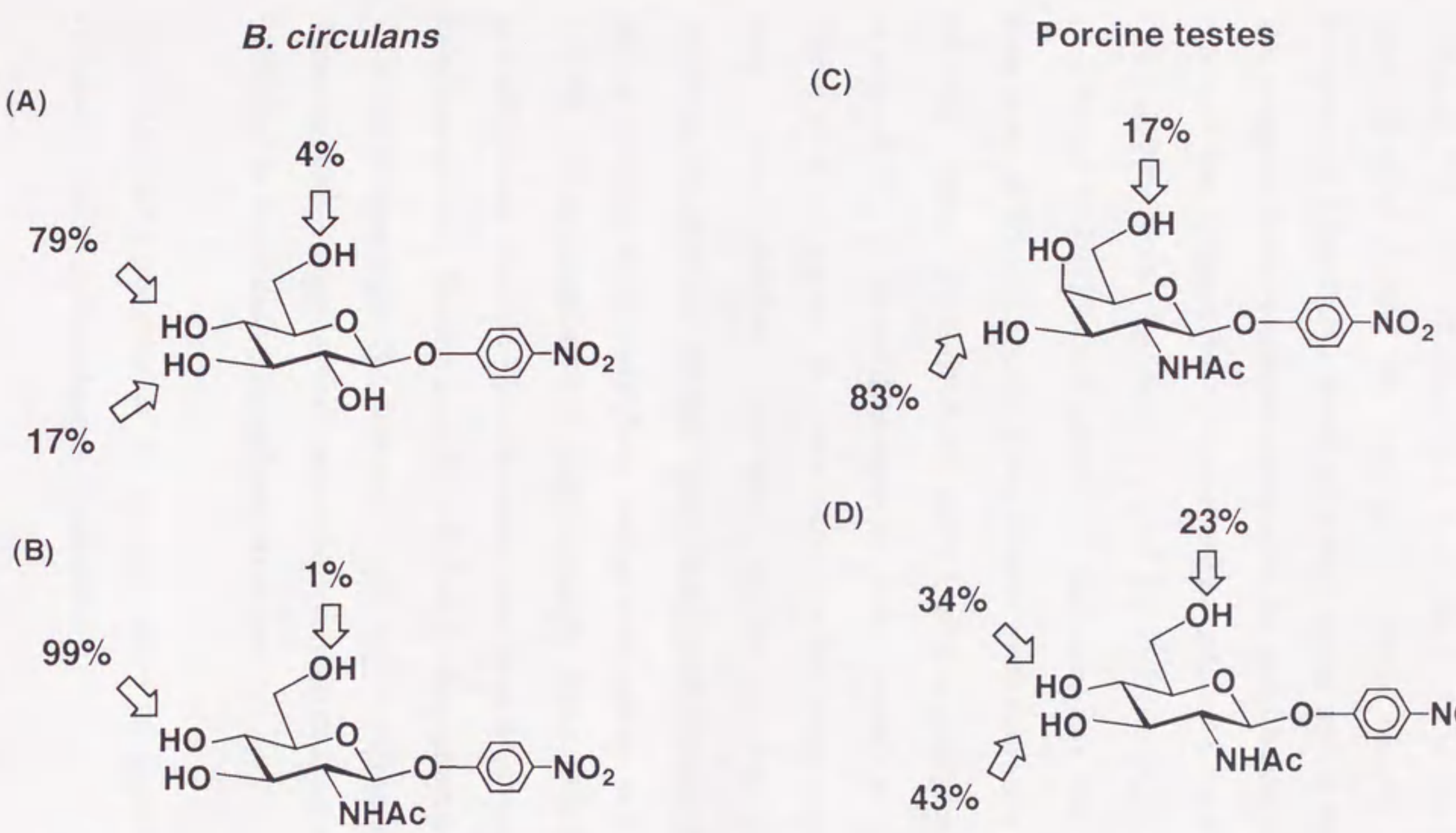


Fig. 14. Structurs of acceptor substrates for β-D-galactosidase-catalyzed galactosylation.

Arrows indicate the positions of galactosylation. Parcentages above the arrows are of the fomation of a given transglycosylation compared with the total.

(Glc3NAc) was the acceptor, the enzyme transferred Gal not only to *O*-6 of Glc3NAc, but also to the β-anomeric position (*O*-1). No galactosylation to *O*-2 and *O*-4 of the acceptor (which are adjacent to the 3-acetamido group) was observed during the reaction[13]. These results indicate that the existence of substituent group (*N*-acetyl group and *p*-nitrophenyl group) in the acceptor hinders galactosylation to its neighboring hydroxyl group. In contrast, the enzyme from porcine testes induced β-D-galactosyl transfer to OH-3 of β-D-GlcNAc-$OC_6H_4NO_2$-*p* and β-D-GalNAc-$OC_6H_4NO_2$-*p*[10] (Fig. 14C, D). When β-D-GalNAc-$OC_6H_4NO_2$-*p* was acceptor, the enzyme catalyzed formation of β(1→3) linked disaccharide predominantly to β(1→6) and no β(1→4). When β-D-GalNAc-$OC_6H_4NO_2$-*p* was acceptor, the enzyme also catalyzed β(1→3) linked disaccharide predominantly to β(1→6) and β(1→4). These results suggests that existence of substituent group in the acceptors does not hinder porcine testes β-D-galactosidase-catalyzed transgalactosylation. Bovine testes β-D-galactosidase also shows similar regioselectivity on its transglycosylation with lactose as a donor and GalNAc and GlcNAc as acceptors[30, 31]. Interestingly, when *O*-(2-acetamide-2-deoxy-α-D-galactosyl)-L-serine and -threonine was used as acceptors, bovine testes β-D-galactosidase formed only β(1→3)-linked disaccharide, β-D-Gal-(1→3)-β-D-GalNAc-O-Ser and -Thr, but no (1→4)- and (1→6)-linked disaccharides[33]. These results suggests that regioselectivity on transglycosylation may be controlled to some extent by aglycon structure.

3.4. Hydrolytic reaction of β-D-galactosidase on p-nitrophenyl galactosyl residue-containing disaccharide glycosides

Comparison of hydrolytic specificity to regioselectivity during

transglycosylation was carried out with β-D-galactosidase from *B. circulans* and porcine testes. As shown in Table 1A, the relative rate of hydrolysis for *B. circulans* β-D-galactosidase of **1** and **3** compared with **2** (100) was 12 and 3, 8 ~ 33-fold differences. Compound **2** should be a much better substrate than **3** under the hydrolytic conditions. Thus, this order of the hydrolytic rate of $(1 \rightarrow 4) \gg (1 \rightarrow 3) > (1 \rightarrow 6)$ corresponded to that of their transglycosylation. It was reported that the rate of the formation of β-D-Gal-(1 → 4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**7**) through the enzymatic transglycosylation from lactose to *p*-nitrophenyl *N*-acetyl-β-lactosaminide is much faster than that of β-D-Gal-(1→6)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**8**), but no $(1 \rightarrow 3)$-transfer product, β-D-Gal-$(1 \rightarrow 3)$-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**6**) was detected during the reaction[14]. The same relationship among these disaccharides was also observed for the hydrolytic rate. Namely, the relative rate of hydrolysis of **8** compared with **7** (100) was 4, a 25-fold difference, and the (1→3)-linked isomer **6** was not hydrolyzed by this enzyme under the present condition. It indicates that the order of formation of transfer product closely corresponds to that of the hydrolytic rate.

This is also the case for the comparison of hydrolytic rates on β-D-Gal-(1 →3)-β-D-GlcNAc, β-D-Gal-(1→4)-β-D-GlcNAc and β-D-Gal-(1→6)-β-D-GlcNAc to regioselectivity on their isomers formation with porcine testes enzyme. Thus, the rate of hydrolysis are in the order (1→3) > (1→4) > (1→6) as shown in Table 1B. The same relationship was also observed for the formation of **6**, **7**, and **8** (Fig. 14D). Thus, the hydrolytic specificity of isomeric oligosaccharides by galctosidases can be useful for predicting the regioselectivity of transglycosylation.

Table.1 The hydrolytic rates of (A)*B. circulans* and (B)porcine testes β-D-galactosidases on disaccarides containing galactosyl residue.

(A)

Compounds	Relative rate (%)
β-D-Gal-(1→4)-β-D-Glc-$OC_6H_4NO_2$-*p* (**1**)	100*
β-D-Gal-(1→3)-β-D-Glc-$OC_6H_4NO_2$-*p* (**2**)	12
β-D-Gal-(1→6)-β-D-Glc-$OC_6H_4NO_2$-*p* (**3**)	3
β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**7**)	100*
β-D-Gal-(1→3)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**6**)	0
β-D-Gal-(1→6)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**8**)	4

* The hydrolytic rate on **1** (or **4**) was arbitrarily set at 100.

(B)

Compounds	Relative rate (%)
β-D-Gal-(1→4)-D-GlcNAc	60
β-D-Gal-(1→3)-D-GlcNAc	100*
β-D-Gal-(1→6)-D-GlcNAc	22

* The hydrolytic rate on β-D-Gal-(1→3)-D-GlcNAc was arbitrarily set at 100.

CHAPTER II

Enzymatic synthesis of 3'-*O*- and 6'-*O*-*N*-acetylglucosaminyl-*N*-acetyllactosaminide glycosides catalyzed by β-*N*-acetyl-D-hexosaminidase from *Nocardia orientalis*

1. Introduction

β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-GlcNAc and β-D-GlcNAc-(1→6)-β-D-Gal-(1→4)-GlcNAc sequences have been found in human milk[34,35] and red blood cell glycosylceramides[36]. These substances have been known to carry blood group ABH, Lewis and Ii antigens[36-44]. β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-GlcNAc has been also known to be the common trisaccharide unit of polylactosaminoglycans present in *N*-glycans[40], *O*-glycans[44] and glycolipids[44-46]. There is at present a great interest in developing synthetic routes to such oligosaccharide units. Two groups have been reported that human serum contains β(1→3) *N*-acetylglucosaminyltransferase [EC 2.4.1.149] which catalyzes the transfer of *N*-acetylglucosamine from UDP-GlcNAc to β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-D-glucose (LacNAc)[45,46]. Although this method is certainly elegant, the wide use of the above synthetase has been restricted. Because the most difficulty for the enzymatic method stays not only in the enzyme purification and maintenance of the active form, but also the donor substrate is very expensive. From a practical viewpoint, the use of glycosidases is very attractive in the synthesis of oligosaccharide involved in glycoconjugates utilizing the transglycosylation reaction. Several GlcNAc-containing disaccharides have been prepared conveniently by the *N*-acetylglucosaminyl transfer reaction utilizing β-*N*-acetyl-D-hexosaminidase (EC 3.2.1.52, β-NAHase) and lysozyme (EC 3.2.1.17)[47-49]. My interest was directed to an enzymatic approach to the synthesis of *N*-acetylglucosaminyl-trisaccharide glycosides from enough amounts of LacNAcβ-*p*NP as a starting material glycoside. Thus, the object of the present investigation is to develop a system for selective transfer of *N*-acetylglucosaminyl residue onto C-3 and C-6 of the Gal residue of LacNAcβ-*p*NP acceptor, based on this approach.

This chapter describes a preparative synthetic method for β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-GlcNAc and β-D-GlcNAc-(1→6)-β-D-Gal-(1→4)-GlcNAc glycosides, utilizing the transglycosylation reaction catalyzed by β-NAHase from *N. orientalis* and the manipulation of the regioselectivity of the glycosidase by the use of an inclusion complex of LacNAcβ-*p*NP acceptor with CD.

2. Materials and Methods

2.1. Materials

β-D-Galactosidase (EC 3. 2. 1. 23) from *Bacillus circulans* was obtained from Daiwa Kasei Co., Ltd. (Osaka, Japan). β-NAHase prepared by 20-70% saturated ammonium sulfate precipitation from culture broth of *N. orientalis* was directly used for the enzymatic synthesis without further purification[46]. LacNAcβ-*p*NP was synthesized according to the method of Usui *et al*[14]. Hydroxypropyl (HP)-β-CD (degree of substituent: 11.8) was a gift from Nihon Shokuhin Kako (Shizuoka, Japan). All other chemicals were obtained from commercial sources.

2.2. Enzyme assay

β-NAHase activity was assayed as follows. A mixture containing 2 mM *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide in 0.98 ml of 0.1 M sodium acetate buffer (pH 5.0) and 20 μ l of enzyme was incubated for 10 min at 40℃. 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 μ mol of *p*-nitrophenol per min.

2.3. Analytical method

HPLC was performed with a YMC-packed column type AQ-312 (ODS) (ϕ 6×150 mm) and TSK-GEL G-Oligo-PW (ϕ 7.8×300 mm) in a Hitachi 6000-

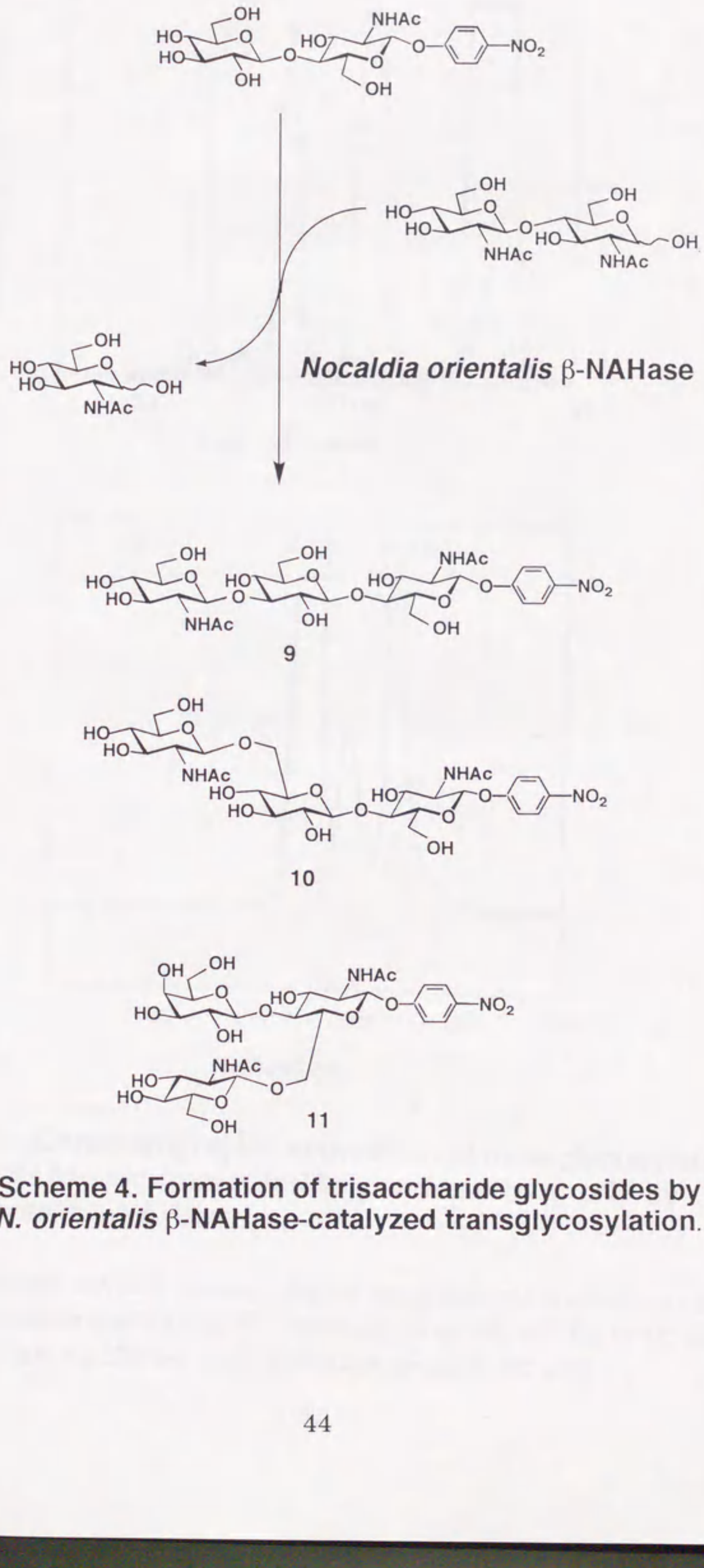
series liquid chromatograph equipped with an L-4000 ultraviolet detector. Elution of the former column was effected with 80 : 20 H_2O-MeOH at a flow rate of 1.0 ml/min at 40℃, and that of the latter with H_2O at a flow rate of 0.7 ml/min at 50℃. NMR, FAB-MS, and specific rotation were carried out as described in CHAPTER Ⅰ.

3. Results and Discussion

*3.1. Enzymatic synthesis of β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-p (**9**), β-D-GlcNAc-(1→6)-β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-p (**10**), and β-D-Gal-(1→4)-[β-D-GlcNAc-(1→6)]-β-D-GlcNAc-$OC_6H_4NO_2$-p (**11**)*

*3.1.1. Preparation of compounds **9** and **10** (Scheme 4)*

The enzyme used in this study was the crude β-NAHase from *Nocaldia orientalis*, which was prepared as a 20-70% saturated ammonium sulfate precipitation from the culture broth. The enzyme preparation was completely devoid of β-D-galactosidase, which degrades the acceptor substrate LacNAcβ-*p*NP (vide infra). LacNAcβ-*p*NP (625 mg) and α-CD (1247.5 mg) were first dissolved in 8 ml of 20 mM sodium acetate buffer (pH 5.0), and then 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucose[$(GlcNAc)_2$] (2.48 g) was added, followed by β-NAHase from *N. orientalis* (25 U). The molar ratio of the donor to acceptor was about 2 : 1, and the total substrate concentration was about 37%. The mixture was incubated for 12 h at 40℃ and the reaction was terminated by heating at 95℃ for 10 min. To the reaction mixture was added 32 ml of 40% methanol in aqueous solution. When allowed to stand at 4℃, α-CD partially crystallized out. The crystalline material was filtrated by a glass filter. The filtrate was concentrated to a small volume (20 ml) and loaded onto a Toyopearl HW-40S column (ϕ 5×100 cm) equilibrated with 25% MeOH in aqueous solution, and the eluate was monitored by measuring the absorbance at 300 nm (*p*-nitrophenyl group) and 485 nm (carbohydrate content, determined by phenol-sulfuric acid method). As shown in Fig. 15A, the chromatogram contained



Scheme 4. Formation of trisaccharide glycosides by *N. orientalis* β-NAHase-catalyzed transglycosylation.

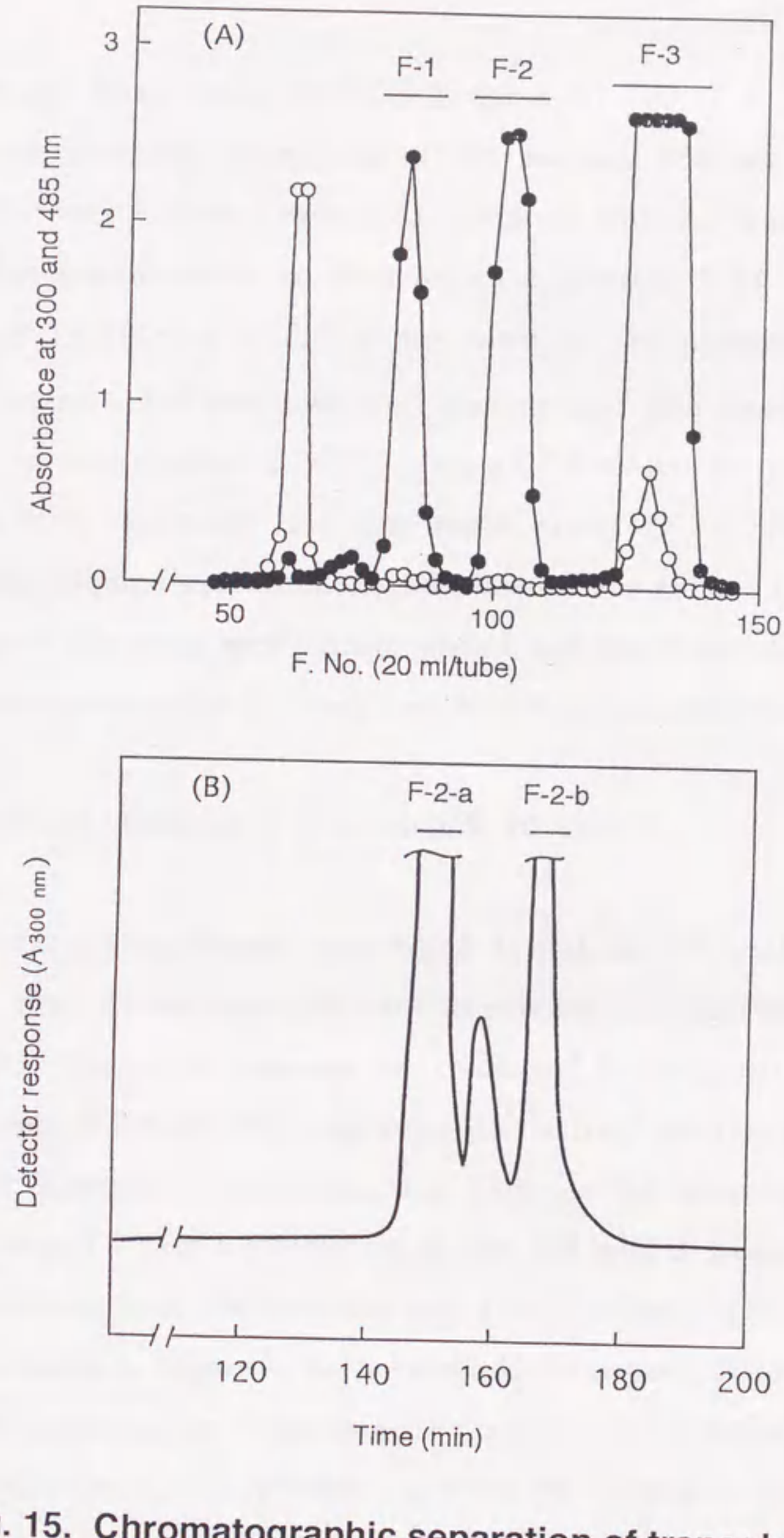


Fig. 15. Chromatographic separation of transglycosylation products formed from $(GlcNAc)_2$ and LacNAcβ-*p*NP by *N. orientalis* β-NAHase.

(A) Toyoperal HW-40S chromatographic separation of transfer products. (●), Absorbance at 300 nm. (○), Absorbance at 485 nm. (B) HPLC with a YMC-pack AQ-323 S-5 120A (ODS) column (ϕ 2×50 cm).

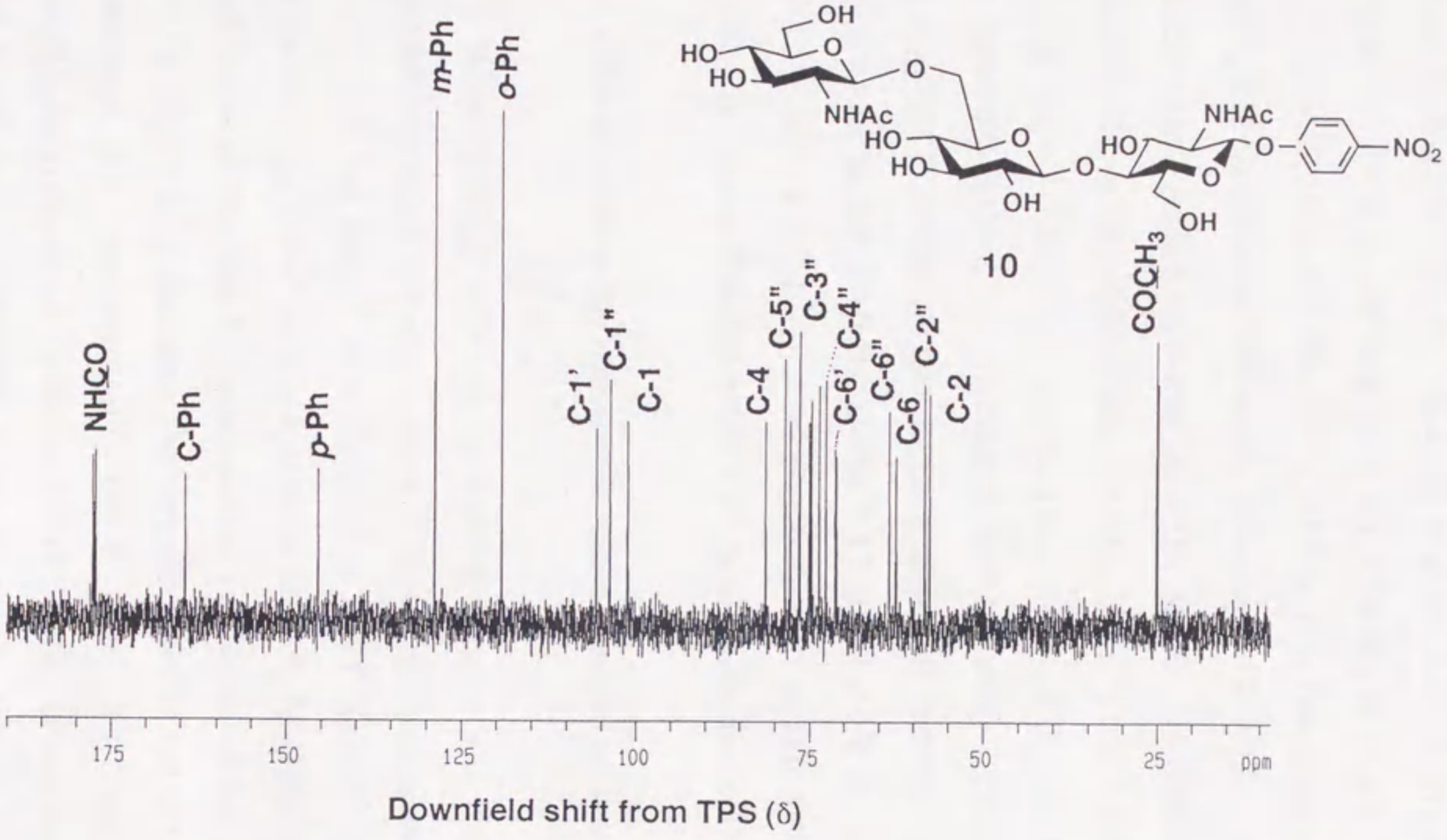
three peaks (F-1, tubes 79-89; F-2, tubes 97-109; F-3, tubes 124-139) displaying coincident absorbance at 300 nm and 485 nm. The first two peaks (F-1 and F-2) were presumed to contain transfer products. Fraction F-1, after lyophilization to dryness, gave compound **11** (25.8 mg). F-3 contained LacNAcβ-*p*NP (345.6 mg) used as the acceptor. The eluates corresponding to F-2 were combined, concentrated to a small volume (2 ml) and 1/4 vol. was applied to HPLC using a ODS column as in Fig. 15B. The fraction was separated into two main peaks (F-2-a and F-2-b). The remaining aliquots were similarly processed. The eluates corresponding to F-2-a and F-2-b peaks were each combined, and concentrated and lyophilized to afford compounds **10** (23.8 mg) and **9** (8.9 mg), respectively.

*3.1.2. Characterization of compounds **9**, **10**, and **11***

Structural assignments were made as follows. FAB-MS/MS indicated that **1** and **2** are trisaccharides consisting of HexNAc-Hex-HexNAc-$OC_6H_4NO_2$. The sugar sequence was confirmed in the positive ion mode by the presence of 708 ($[M+H]^+$) together with the fragment ions at *m/z* 204, 366 and 569 (HexNAc, HexNAc-Hex and HexNAc-Gal-HexNAc, respectively). The compound **3** gave a $[M+H]^+$ ion at *m/z* 708 with a fragment ion at *m/z* 545 (fragment from the reducing end HexNAc-HexNAc-$OC_6H_4NO_2$). The sugar sequence is shown to be a branched structure. Based on the sugar sequence, the structure of the transfer products were elucidated by their ^{1}H- and ^{13}C-NMR spectra as in Table 2 and Fig. 16. These results indicate that compounds **9**, **10**, and **11** are β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p*, β-D-GlcNAc-(1→6)-β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p*, and β-D-Gal-(1→4)-[β-D-GlcNAc-(1→6)]-β-D-GlcNAc-$OC_6H_4NO_2$-*p*, respectively. Compound **9**: $[\alpha]^{25}_D$ -13.8° (*c* 0.1, H_20 /MeCN=1/1); FABMS

Table 2. ^{13}C chemical shifts of compounds **9**, **10** and **11** in D_2O solution.

GlcNAcβ1-3Galβ1-4GlcNAcβ-*p*NP(**9**), III II I													
GlcNAcβ1-6Galβ1-4GlcNAcβ-*p*NP (**10**), III II I													
Galβ1-4 (GlcNAcβ1-6)GlcNAcβ-*p*NP (**11**) III II I													
Compound		C-1	C-2	C-3	C-4	C-5	C-6	CH_3	C=O	o-Ar	m-Ar	c-Ar	c-Ar
9	I	101.35	57.63	77.95	80.88	76.41	62.64	24.89	177.73	119.35	128.93	145.53	164.51
	II	105.68	72.83	84.81	71.16	77.74	63.79						
	III	105.68	58.49	74.82	72.52	78.49	63.31	25.00	177.79				
10	I	101.29	57.65	76.55	81.33	77.88	62.53	25.18	177.70	119.37	128.91	145.53	164.49
	II	105.86	73.66	74.84	71.16	76.55	71.39						
	III	103.92	58.35	75.15	72.72	78.67	63.50	24.92	177.37				
11	I	101.10	57.47	76.73	81.72	75.25	70.15	24.91	176.96	119.26	129.02	145.51	164.45
	II	103.70	58.17	76.62	72.67	78.63	63.52	24.91	177.68				
	III	106.09	73.69	74.75	71.27	78.24	63.77						



10

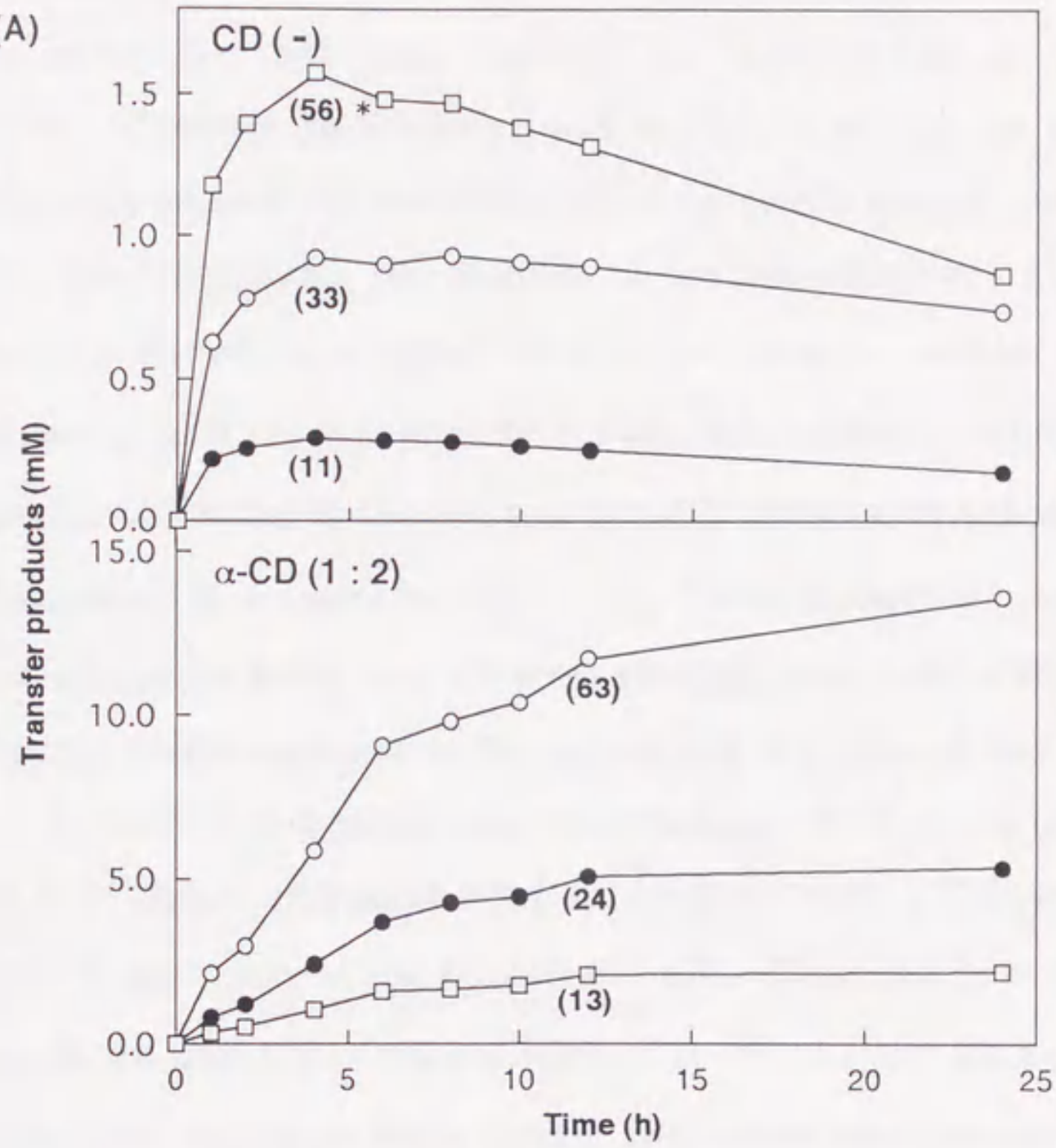
Fig. 16. ^{13}C-NMR analysis of compound 10 in D_2O.

(glycerol): *m/z* 708 $(M+H)^+$; ^{1}H-NMR (D_2O): δ 8.27 (d, 2H, *J* 8.9 Hz, *m*-Ph), 7.20 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.35 (d, 1H, *J* 8.6 Hz, H-1), 4.51 (d, 1H, *J* 7.6 Hz, H-1'), 4.70 (d, 1H, *J* 8.6 Hz, H-1"), 2.06 (s, 3H, NAc due to GlcNAcβ-*p*NP), 2.02 (s, 3H, NAc due to GlcNAcβ3). Compound **10**: $[\alpha]_D^{25}$ -26.0° (*c* 0.2, H_2O /MeCN=1/1); FABMS (glycerol): *m/z* 708 $(M+H)^+$; ^{1}H-NMR (D_2O): δ 8.28 (d, 2H, *J* 9.2 Hz, *m*-Ph), 7.22 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.37 (d, 1H, *J* 8.3 Hz, H-1), 4.53 (d, 1H, *J* 7.6 Hz, H-1'), 4.63 (d, 1H, *J* 8.3 Hz, H-1"), 2.10 (s, 3H, NAc due to GlcNAcβ6), 2.05 (s, 3H, NAc due to GlcNAcβ-*p*NP). Compound **11**: $[\alpha]_D^{25}$ -41.7° (c 0.2, H_2O /MeCN=1/1); FABMS (glycerol): *m/z* 708 $(M+H)^+$. ^{1}H-NMR (D_2O): δ 8.25 (d, 2H, *J* 9.2 Hz, *m*-Ph), 7.17 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.33 (d, 1H, *J* 8.3 Hz, H-1), 4.53 (d, 1H, *J* 8.6 Hz, H-1'), 4.40 (d, 1H, *J* 7.6 Hz, H-1"), 1.99 (s, 3H, NAc due to GlcNAcβ-*p*NP), 1.82 (s, 3H, NAc due to GlcNAcβ6).

3.2. Effect of α-CD on the transglycosylation reaction system

When LacNAcβ-*p*NP was used as an acceptor and $(GlcNAc)_2$ as an *N*-acetylglucosaminyl donor, three *N*-acetylglucosaminyl trisaccharide glycosides **9**, **10**, and **11** were observed by HPLC, in 4.9% total yield (based on the acceptor added) and in a ratio of 11 : 33 : 56 (Fig.17). These values were based on the time for the maximum production of desired compounds **9** and **10** after 4 h. In this case, the transfer reaction led to the formation of compound **11** preferentially to **9** and **10**: one half of *N*-acetylglucosaminylation occurs at O-6 of GlcNAc moiety and the remaining at O-3' and O-6' of Gal. In this case, LacNAcβ-*p*NP shows a low solubility (2.8%) in aqueous medium. In general, the efficiency of the transglycosylation process is enhanced by the presence of a minimal amount of water and an excess of substrate[51]. Usui *et al.* previously reported that the

(A)



(B)

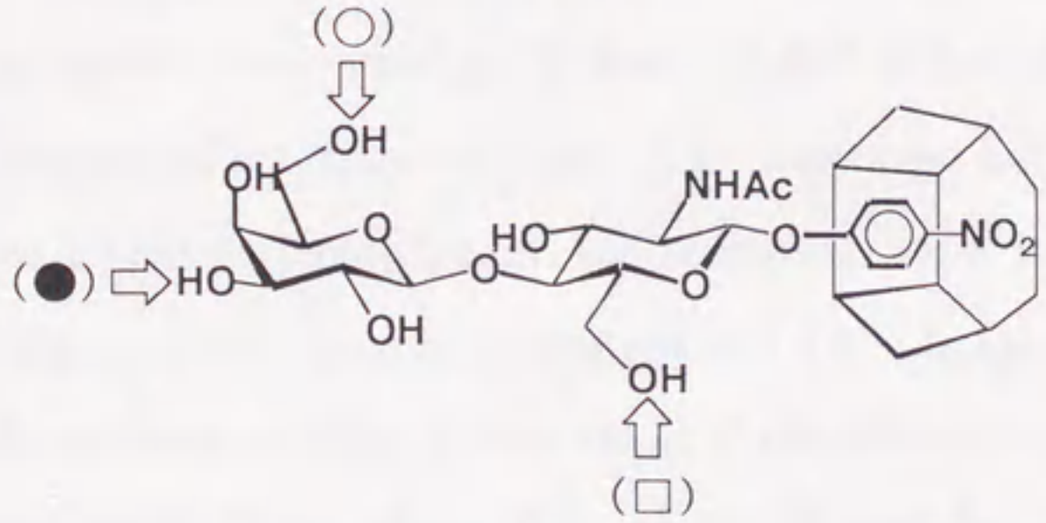


Fig. 17. Time course of *N. orientalis* β-NAHase-mediated transglycosylation from LacNAc β-pNP and $(GlcNAc)_2$.

* Values are parcentages of the formation of a given transglycosylation compared with the total.

use of an organic co-solvent (50-60% Me_2SO, acetonitrile etc.) in transfer reactions utilizing glycosidases not only ensured a sufficient solubility of *p*-nitrophenyl glycosides, but also resulted in high yields of desired compounds[52-54]. However, this concept was not applicable to the present reaction system because of the instability of the enzyme in organic co-solvent systems. On the other hand, the problem of low solubility was partially solved by using α-CD which is thought to form an inclusion complex with *p*-nitrophenyl group, as already reported[15]. Thus, the solubility of LacNAcβ-*p*NP was enhanced 5.9-fold by the presence of α-CD, when LacNAcβ-*p*NP and α-CD was dissolved in a molar ratio of 1 : 2. Three *N*-acetylglucosaminyl trisaccharide glycosides **9**, **10**, and **11** were similarly observed by HPLC, in 6.2 % total yield (based on the acceptor added) and in a ratio of 24 : 63 : 13 after 12 h. It resulted in a significant improvement of the yields in **9** and **10**. Figure 17 shows transglycosylation profiles with $(GlcNAc)_2$ and LacNAcβ-*p*NP in the presence and absence of α-CD. The time for maximum formation of **9**, **10** and **11** in the presence of α-CD was at ~12 h and its concentration then varied a little during the subsequent reaction. The maximum production in the presence of α-CD was 7.6-fold higher than in its absence in a given volume of reaction solution. The increased solubility by its formation of an inclusion complex of the acceptor with α-CD also facilitated the production of the desired products **9** and **10**. In this way, the regioselectivity of glycosidase-mediated formation of trisaccharide glycoside was substantially changed. Thus, about 70% of the *N*-acetylglucosaminy-lation occurs at the O-3' and O-6' on Gal residue and 30% at O-6 on GlcNAc residue, whereas, in absence of the α-CD, the most reactive OH is in O-6 position (56%). The existence of bulky α-CD region in an inclusion complex with the acceptor was effective for diminishing *N*-acetylglucosaminyl

transfer onto *O*-6 at the GlcNAc residue of the acceptor, which could be due to steric hindrance of α-CD. It resulted in improved yields of the desired compounds **9** and **10**.

3.3. Effect of molar ratio of α-CD to LacNAcβ-pNP acceptor on the β-NAHase-catalyzed transglycosylation

It is well established that α- and β-CDs can trap various compounds as guests in their cavities to form inclusion complex in solution. Rekharsky *et al.* have compared the thermodynamic quantities for the reaction of phenyl β-D-glucopyranoside and phenol with α-CD on the basis of ^{1}H-NMR studies[54]. It has been reported that only the phenol part of these substances includes in the α-CD cavity and the glucopyranose part of the phenyl β-D-glucopyranoside is excluded from the α-CD due to steric effect. The complexation reaction was reversible.

This concept was adopted in the present study that the LacNAc part of LacNAcβ-*p*NP would be also excluded from the α-CD cavity. Therefore, we investigated the regioselectivity of the β-NAHase-catalyzed formation of *N*-acetylglucosamininyl-trisaccharide products at different molar ratios of the acceptor to α-CD as depicted in Fig. 18A. The numbers on the ordinate show the percentage of three *N*-acetylglucosamininyl-trisaccharide products formed by transglycosylation, based on the time at which the desired **9** production reached its maximum. A change of regioselectivity could be to some extent achieved by using an inclusion complex of acceptor with α-CD. Thus, in the absence of α-CD, the enzyme transfers *O*-6 of the acceptor moiety preferentially to *O*-6' and reacts only weakly at *O*-3'. On the contrary, in the presence of α-CD, the enzyme transfers much less to *O*-6 of the

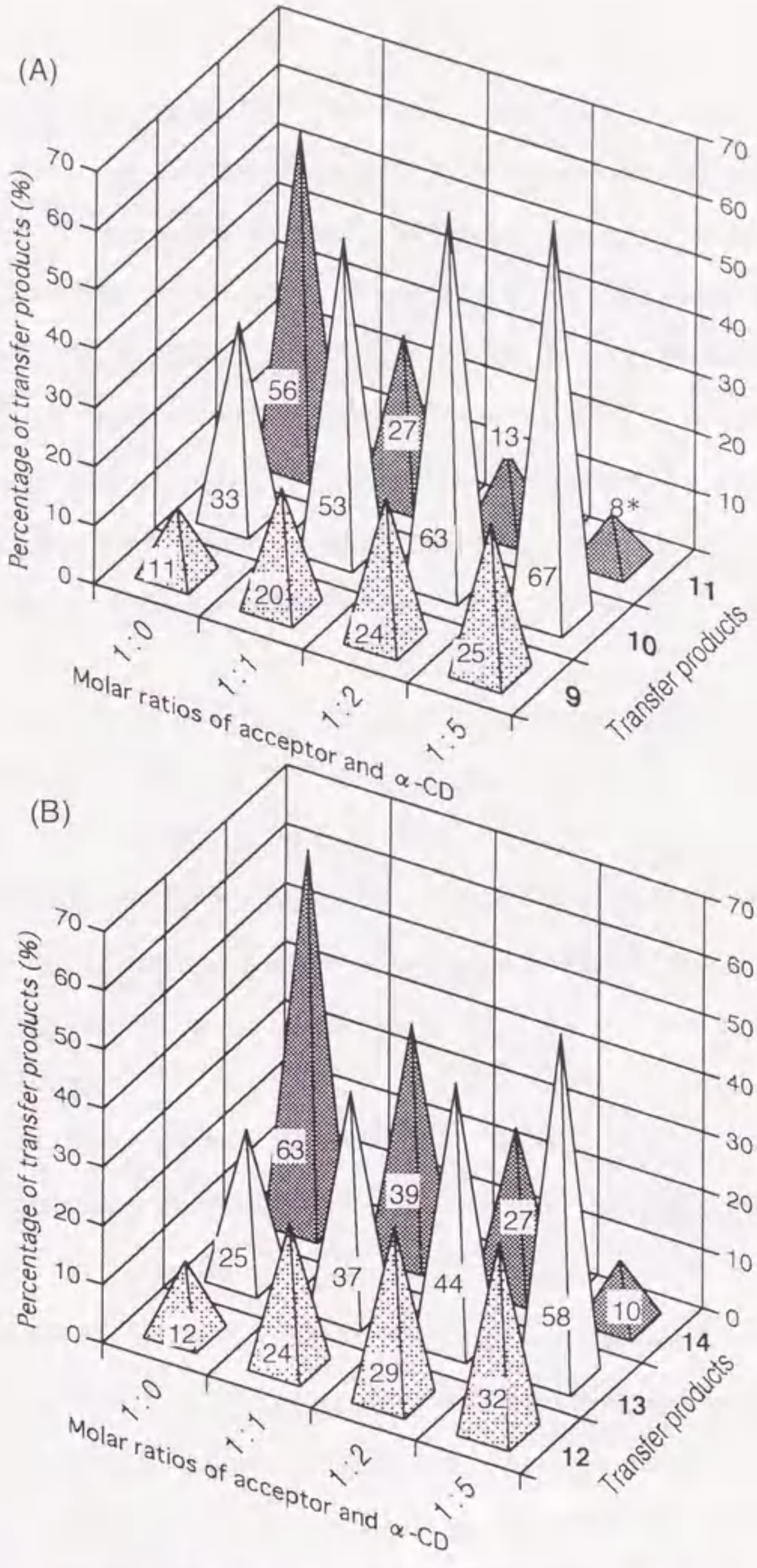


Fig. 18. Percentages of *p*-nitrophenyl *N*-acetylglucosaminyl-trisaccharide products formed by transglycosylation at different molar ratios of α-CD to acceptor. (A) LacNAcβ-pNP and (B) Lacβ-pNP were used as acceptor. * The numbers show the function of a given transglycosylation compared with the total.

acceptor, whereas more to *O*-6' and *O*-3'. Furthermore, with rising the molar ratio of α-CD to the acceptor, the proportion of **9** and **10** was increased, but that of **11** remarkably decreased. When the molar ratios of acceptor to α-CD were acceptor alone, 1 : 1, 1 : 2 and 1 : 5, the proportion of **11** production was 56, 27, 13 and 8%, respectively. It suggests that an excess of α-CD to the acceptor greatly enhances the probability of its complexation of α-CD with the acceptor. This effect is thought to contribute to diminishing the *N*-acetylglucosaminyl transfer onto *O*-6 at the GlcNAc residue of the acceptor, due to steric hindrance of the CD in an inclusion complex.

In this way, the regioselectivity of β-NAHase-catalyzed formation of trisaccharides was changed by utilizing the nature of hydrophobic *p*-nitrophenyl group in the glycosyl acceptor. Table 3 shows the effect of different molar ratios of the acceptor to α-CD on the transglycosylation. As a result, the molar ratio of 2 mole of α-CD to 1 mole of the acceptor was adopted, taking into account the efficiency of the desired compounds on the transglycosylation.

Usui *et al.* have already reported that, with Lacβ-*p*NP acceptor, the enzyme also produces β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-β-D-Glc-$OC_6H_4NO_2$-*p* (**12**) with its isomers β-D-GlcNAc-(1 → 6)-β-D-Gal-(1 → 4)-β-D-Glc-$OC_6H_4NO_2$-*p* (**13**) and β-D-Gal-(1 → 4)-[β-D-GlcNAc-(1 → 6)]-β-D-Glc-$OC_6H_4NO_2$-*p* (**14**) from Lacβ-*p*NP and $(GlcNAc)^{10}$ (Scheme 5). A similar change with respect to regioselectivity was observed in the absence and presence of α-CD as in Fig. 18B. With rising the molar ratio of α-CD to the acceptor, it gradually decreased the regioselectivity to *O*-6 of the glucose moiety of the acceptor, whereas increased to *O*-6' and *O*-3' of the galactose. The enzyme transfers more to *O*-3' of Gal of the acceptor in the presence of α-

Table 3. Effect of molar ratios of acceptor to α-CD on acceptor solubility and efficiency of transglycosylation.

Molar ratio (acceptor : α-CD)	Solubility [a] (%)	Yield [c] (%)	Production [d] (mM)
1 : 0	2.8 (1.0) [b]	4.9	2.8 (1.0) [e]
1 : 1	12.5 (4.5)	7.4	18.9 (6.8)
1 : 2	16.6 (5.9)	6.2	21.2 (7.6)
1 : 5	5.6 (2.0)	2.9	3.4 (1.2)

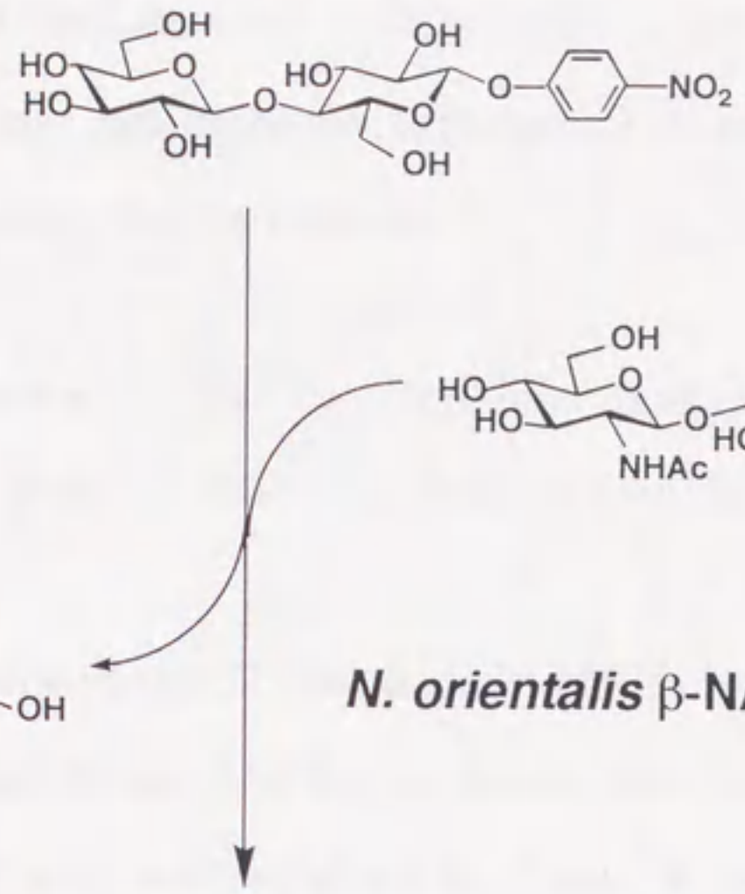
[a] The solubility of LacNAcβ-*p*NP in the absence and presence of α-CD.

[b] The solubility of LacNAcβ-*p*NP in the absence of α-CD was arbitrarily set at 1.0.

[c] Based on the acceptor added.

[d] A given volume of solution.

[e] The recovery of total transfer products in the absence of α-CD was arbitrarily set at 1.0.



12

13

14

Scheme 5. Formation of trisaccharide glycosides by *N. orientalis* β-NAHase-catalyzed transglycosylation.

CD, compared with the LacNAcβ-*p*NP acceptor. Replacement of Lacβ-*p*NP by LacNAcβ-*p*NP does not alter the direction of *N*-acetylglucosaminylation, but influences precisely the regioselectivity.

3.4. Effect of several CDs in inclusion complex with acceptor on regioselectivity of the β–NAHase-catalyzed N-acetylglucosaminylation

The regioselectivity of the *N. orientalis* β-NAHase-catalyzed formation of *N*-acetylglucosaminyl-trisaccharide products was further investigated by using three kinds of CDs instead of α-CD. When the molar ratio of LacNAcβ-*p*NP to respective β-CD, Hydroxypropyl (HP)-β-CD and 2, 6-di-*O*-methyl (DM)-β-CD was 1 : 2, the enzyme produced three transfer products **9**, **10**, and **11** as that observed for α-CD. Figure 19A shows the percentage of three transfer products **9**, **10**, and **11** in the absence and presence of respective CDs. The changes of regioselectivity were also observed in the presence of those CDs. In the absence of the CDs, *N*-acetylglucosaminyl transfer favored *O*-6 of the acceptor rather than *O*-6', and occurred to a lesser extent at *O*-3'. On the contrary, the use of respective β-CD, HP-β-CD and DM-β-CD greatly enhanced the regioselectivity at *O*-6' and weakened that at *O*-6. The use of these CDs certainly influenced the regioselectivity as that observed for α-CD.

In a similar manner, when Lacβ-*p*NP was acceptor, three transfer products **12**, **13** and **14** were formed as in Fig. 19B. In the presence of respective CDs, the regioselectivity with respect to *O*-3' and *O*-6' vs. *O*-6 of the acceptor was somewhat different from that of LacNAcβ-*p*NP. The enzyme tends to transfer GlcNAc more to *O*-3' of the acceptor and less to *O*-6. In the case of α-CD, β-CD and DM-β-CD, the effect for diminishing the

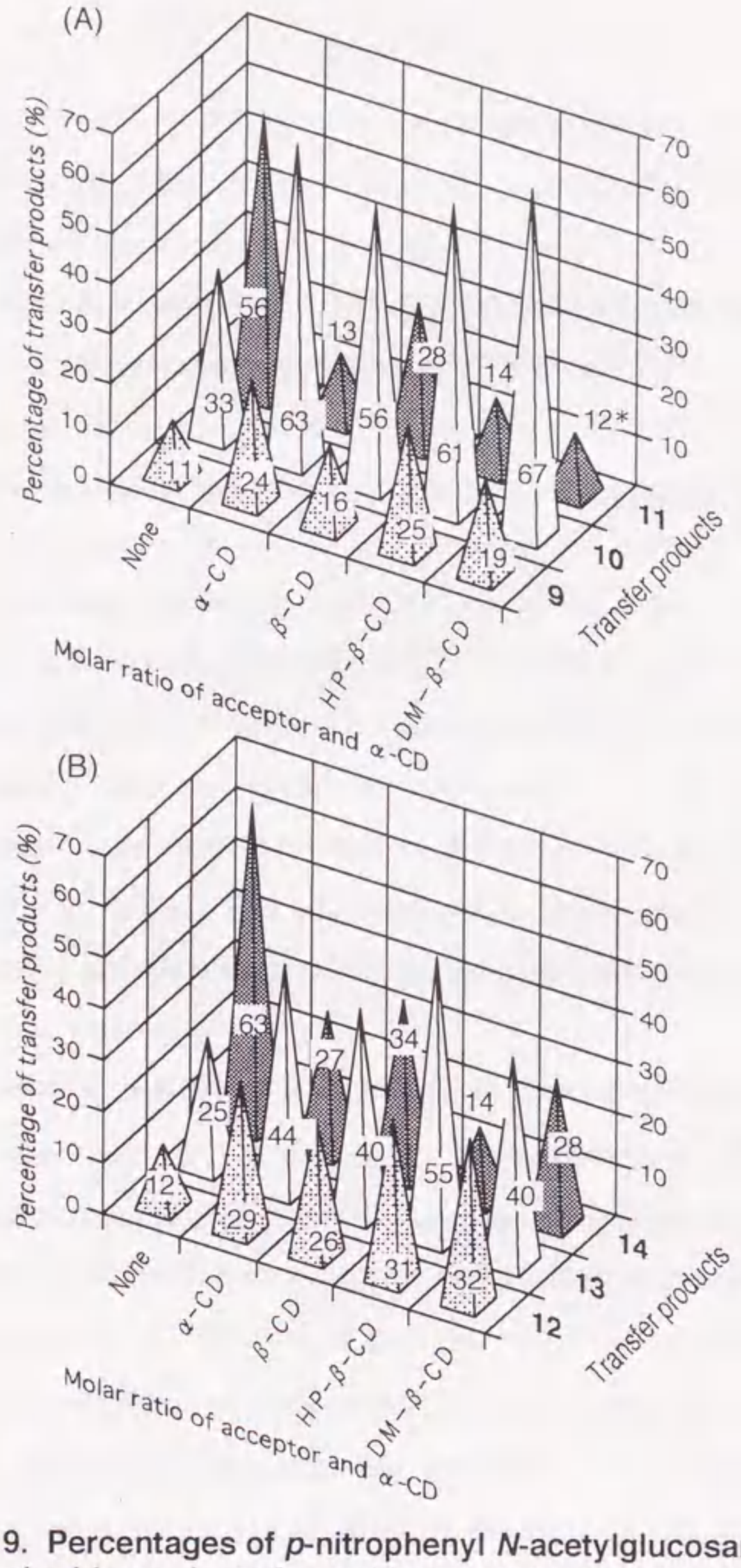


Fig. 19. Percentages of *p*-nitrophenyl *N*-acetylglucosaminyl-trisaccharide products formed by transglycosylation in the absence and presence of several CDs.

(A) LacNAcβ-pNP and (B) Lacβ-pNP were used as acceptor. * The numbers show the function of a given transglycosylation compared with the total.

regioselectivity at *O*-6 (27-34%) of the acceptor was not large as much as that (14%) observed for the HP-β-CD. In the presence of HP-β-CD, 86% of the *N*-acetylglucosaminylation occurs at the *O*-3' and *O*-6' of the acceptor and only 14% at *O*-6, whereas, in its absence, the most reactive OH is in the *O*-6 position. It suggests that the existence of the bulky HP-β-CD region in the complexation diminishes the regioselectivity at *O*-6 of the acceptor, due to unfavorable proximity between the hydroxypropyl group in the CD and the hydroxymethyl group at C-6 in the acceptor. In general, glycosidase do exhibit some regioselectivity, but this selectivity is less predictable and lower than that of such polysaccharide hydrolases as amylase[55], cellulase[57], β-D-mannanase[58] and lysozyme[59, 60]. Some researchers have reported that the regioselectivity can be manipulated to some extent. In the present studies, the observed regioselectivity of the β-NAHase-catalyzed formation of trisaccharide glycosides was also changed by manipulating the nature of hydrophobic *p*-nitrophenyl group in the disaccharide glycoside acceptor by using several kinds of CDs.

In conclusion, β-NAHase from *N. orientalis* catalyzed formation of the β-(1 → 3)-linked and β-(1 → 6)-linked *N*-acetylglucosaminyllactosaminides employing transglycosylation. In this case, the desired compounds **9** and **10** were obtained in improved yields by using inclusion complex of acceptor glycoside with α-CD. The use of α-CD in this reaction system made also possible to manipulate to some extent the regioselectivity of the β-NAHase-catalyzed formation of trisaccharide glycosides. Compounds **9** might be useful as a substrate for endo-β-galactosidase and as an acceptor substrate for β1,3 and 1,6-*N*-acetylglucosaminyltransferase. Nitrophenyl glycosides can be also reduced to aminated glycosides. The amino function may be derivatized for reactions with electrophiles[61]. The approach described above

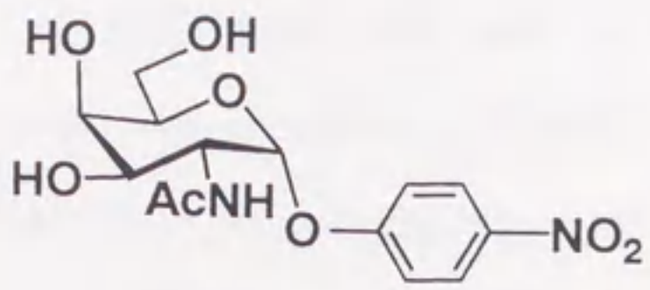
can be further extended to the synthesis of tetrasaccharide repeated LacNAc unit.

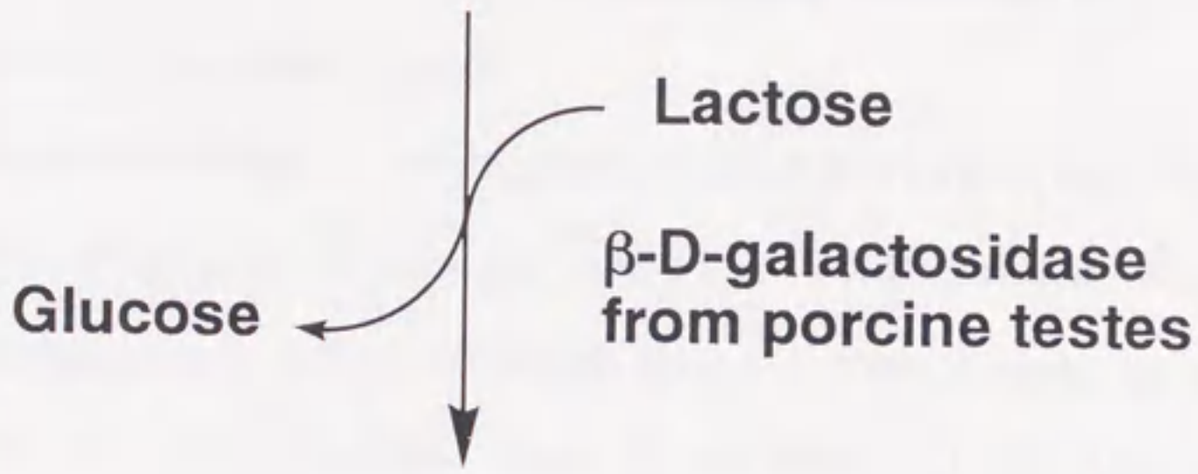
CHAPTER III

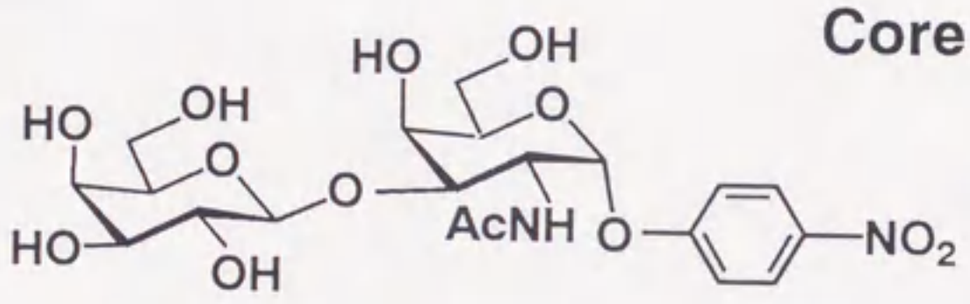
Consecutive synthesis of β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* and β-D-Gal-(1→3)-[β-D-GlcNAc-(1→6)]-α-D-GalNAc-$OC_6H_4NO_2$-*p* as carbohydrate units of mucin-types 1 and 2 core

1. Introduction

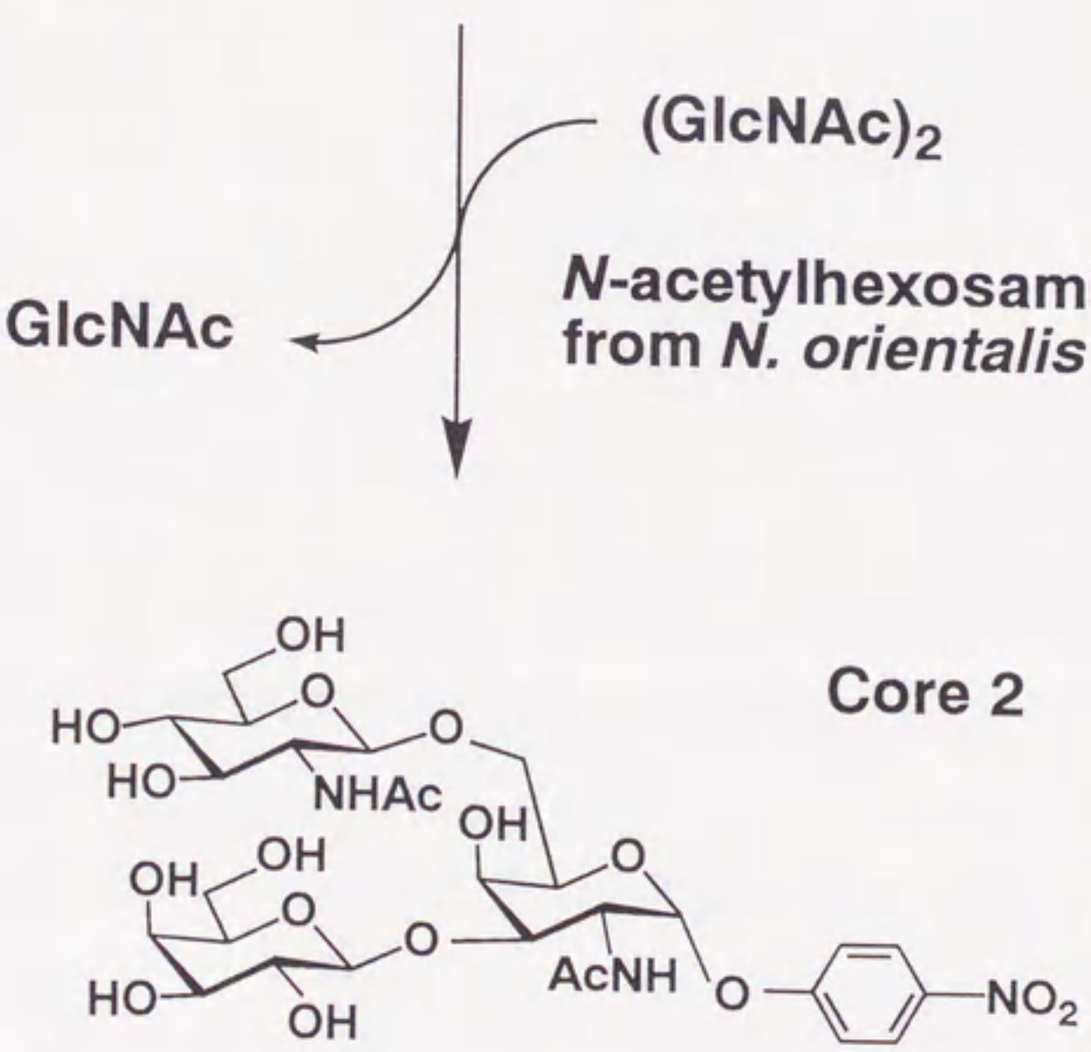
Mucin type oligosaccharides are found on serum, cell membrane glycoproteins and high molecular weight mucins. They can present multivalent carbohydrate antigenic or functional determinants for antibody recognition, mammalian cell adhesion and microorganism binding[62, 63]. The majority of the mucin-type carbohydrates of serum and membrane glycoproteins are of the core 1 which is β-D-Gal-(1→3)-α-D-GalNAc-Ser/Thr. On the other hand, human activated T-cell[64], leukemias[65], and immunodeficiencies[65] have all been associated with stimulation in β1-6GlcNAc-transferase (EC 2.4.1.102) giving oligosaccharides with predominantly core 2, β-D-Gal-(1 → 3)-[β-D-GlcNAc-(1 → 6)]-α-D-GalNAc-Ser/Thr, rather than core 1. Thus, core 2 structure may have numerous roles on cell differentiation and transformation. There has been at present a great interest in developing synthetic routes to core 2 oligosaccharide. Organic chemical methods for obtaining β-D-Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*[28] and β-D-Gal-(1 → 3)-[β-D-GlcNAc-(1 → 6)]-α-D-GalNAc-$OCH_2C_6H_5$[67] have been developed, but they involve 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 disaccharide glycoside[8, 9]. My interest was focused on a consecutive synthesis of β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* and β-D-Gal-(1→3)-[β-D-GlcNAc-(1→6)]-α-D-GalNAc-$OCH_2C_6H_5$ through transgalactosylation and trans-*N*-acetylglucosaminylation, starting with α-D-GalNAc-$OC_6H_4NO_2$-*p* as shown in Scheme 6. β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* and β-D-Gal-(1 → 3)-[β-D-GlcNAc-(1 → 6)]-α-D-GalNAc-$OCH_2C_6H_5$ would be useful as exogenous substrates for endo-α-*N*-acetylgalactosaminidase (endo-α-







Core 1



Core 2

Scheme 6. Consecutive synthesis of cores 1 and 2 through transgalactosylation and trans-*N*-acetyl-glucosaminylation.

GalNAc-ase, EC 3. 2. 1. 97)[68] and new type of endo-α-*N*-acetylgalactosaminidase from *Streptomyces* sp. (endo-α-GalNAc-ase-S) which released a tetrasaccharide β-D-Gal-(1 → 3)-[β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 6)]-D-GalNAc from asialofetuin [69, 70], respectively, as a probe for lectin and as a model compound of *O*-linked glycan.

This chapter describes a consecutive synthetic method for β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* and β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*, which are carbohydrate units of mucin-types 1 and 2 core, by means of porcine testes β-D-galactosidase- and *N. orientalis* β-NAHase-catalyzed transglycosylation.

2. Materials and Methods

2.1. Materials

β-D-Galactosidase (EC 3. 2. 1. 23) from porcine testes was purified as described previously. β-NAHase prepared by 20-70% saturated ammonium sulfate precipitation from culture broth of *N. orientalis* was directly used for the enzymatic synthesis without further purification[50]. 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 syuzo Co. Ltd. (Kyoto Japan). All other chemicals were obtained from commercial sources.

2.2. Enzyme assay

β-D-Galactosidase and β-NAHase activity were assayed as described in CHAPTERs Ⅰ and Ⅱ.

2.3. Analytical Method

HPLC was performed with a YMC-packed column type AQ-312 (ODS) (ϕ 6×150 mm) and 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 88 : 12 H_2O-MeOH, and that of the latter with H_2O. The flow rate was 0.8 ml/min at 40℃. NMR, FAB-MS, and specific rotation were carried out as described in CHAPTER Ⅰ.

2.4. Partial purification of β-D-galactosidase from porcine testes

A crude preparation of β-D-galactosidase was prepared by precipitation with 20-75% saturated ammonium sulfate from an extract of porcine testes by the same method as previously used with bovine testes[12]. For synthesis of β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*, the crude enzyme preparation was purified by removal of *N*-acetyl-α-D-hexosaminidase (α-NAHase) by column chromatography. The crude enzyme (1 g) was dissolved in 60 ml of 20 mM citrate phosphate buffer (pH 4.5) and charged onto a column (ϕ 3.2 × 26 cm) of CM-Sepharose Fast Flow equilibrated with the same buffer. The column was washed with 200 ml of the same buffer. The enzyme was eluted with a linear gradient of concentration of NaCl from 0 (500 ml) to 1.0 M (500 ml) and the eluate was collected in 20-ml fractions. The fractions containing β-D-galactosidase activity (tubes 34 - 42) were eluted at about 0.5 M NaCl. Eluates of the corresponding enzyme fractions were combined and concentrated to small volume (1.5 ml) using an Amicon Diaflo unit. The concentrated enzyme solution was loaded onto a column (ϕ 2.6 × 60 cm) of HiLoad 26/60 Sephacryl S-200 equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl and 0.02% NaN_3. Elution was performed with the same buffer at a flow rate of 1.5 ml/min, and fractions of 2.5 ml were collected. The elution pattern showed two β-D-galactosidase peaks (F-1: tubes 32-38 and F-2: 44-56) and one α-NAHase peak (tubes 52-62)(Fig. 20). The fractions consisting the F-1 peak were completely devoid of α-NAHase, and these was combined and used for synthesis of β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* (total activity: 0.12 U; specific activity: 0.044U/mg).

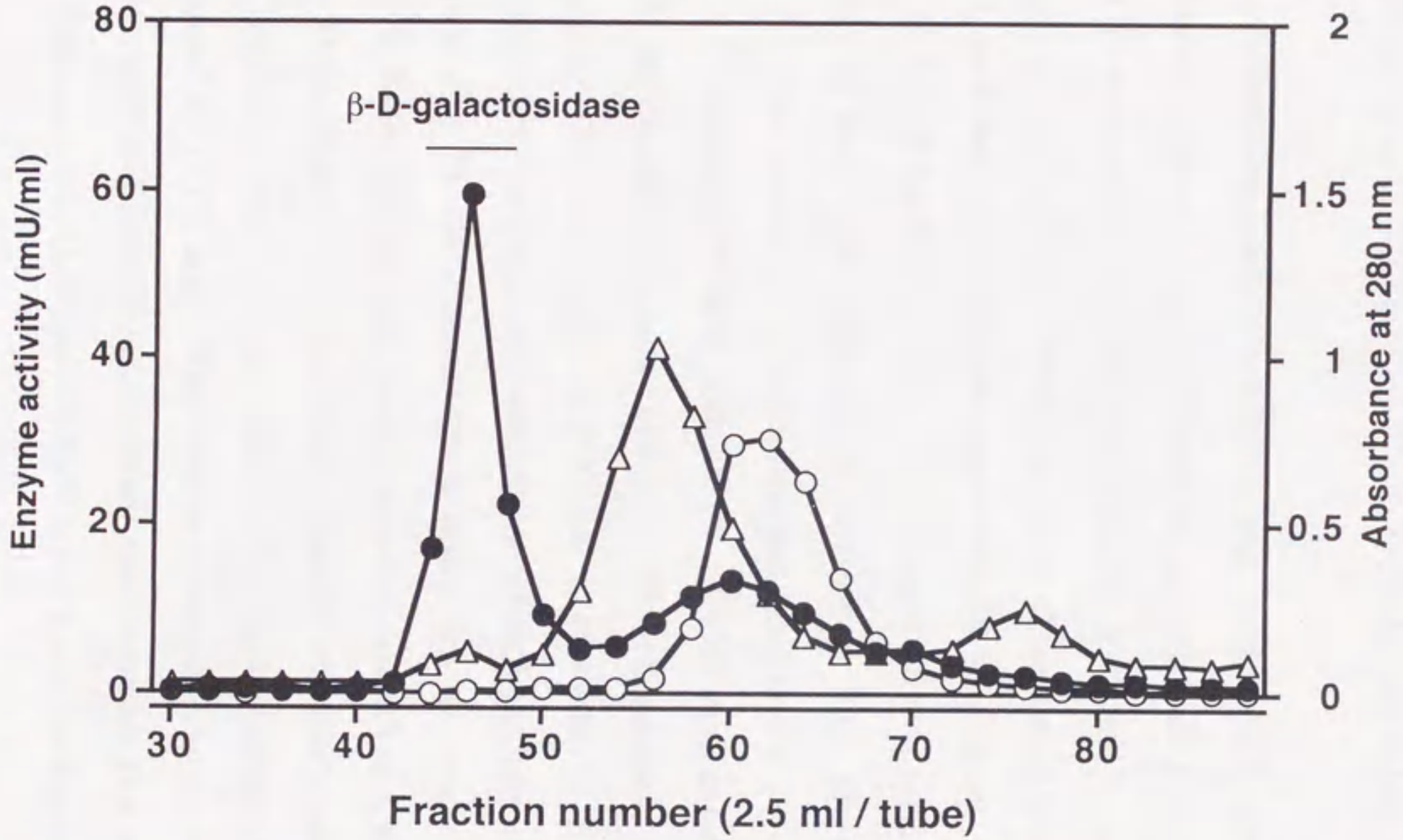


Fig. 20. Sephacryl S-200 HR gel filtration of a fraction containing β-D-galactosidase.

(●), β-D-galactosidase; (○), α-NAHase; (△), $A_{280\ nm}$. The separation was carried out as described in the Materials and Methods.

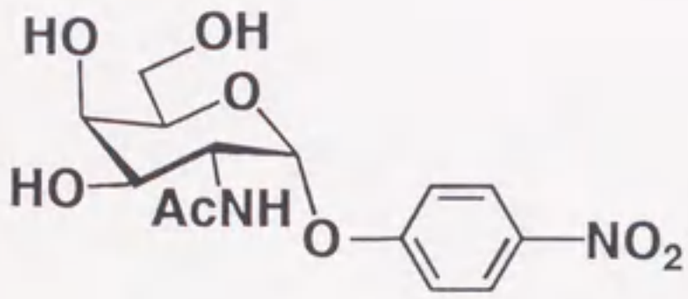
3. Results and Discussion

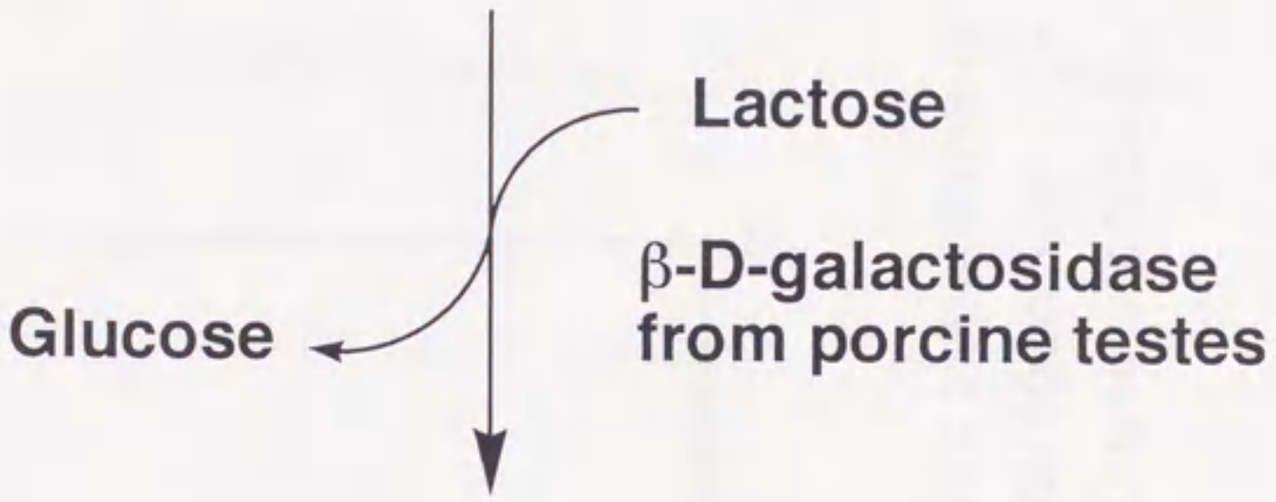
*3.1. Preparation of β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-p (**15**) and its positional analog β-D-Gal-(1→6)-α-D-GalNAc-$OC_6H_4NO_2$-p (**16**) (Scheme 7)*

α-D-GalNAc-$OC_6H_4NO_2$-*p* (120 mg) and β-CD (398 mg) were first dissolved in 18 ml of 20 mM sodium acetate buffer (pH 5.5), then lactose (2.48 g) was added, followed by partially purified β-D-galactosidase from porcine testes (0.12 U). The molar ratio of the donor to acceptor was about 20 : 1, and the total substrate concentration was about 14%. The mixture was incubated for 50 h at 40℃ and the reaction was terminated by adding 8 ml of 1 M acetic acid followed by heating at 95℃ for 10 min. Insoluble material was removed by centrifugation, and the supernatant was loaded onto a Toyopearl HW-40S column (ϕ 4.5×90 cm) equilibrated with 25% MeOH in aqueous solution, and the effluent solution was monitored by measuring the absorbance at 300 nm (*p*-nitrophenyl group) and 485 nm (carbohydrate content, determined by phenol-sulfuric acid method). As shown in Fig. 21, the chromatogram contained two peaks (F-1, tubes 56-61; and F-2, tubes 66-74) displaying coincident absorbance at 300 nm and 485 nm. These peaks were presumed to contain transfer products. Fraction F-2, after lyophilization to dryness followed by crystallization from ethanol, gave compound **15** (27.2 mg). The eluates corresponding to F-1 were combined, concentrated and lyophilized to afford compound **16** (8.8 mg). F-3 (tubes 93-104) contained α-D-GalNAc-$OC_6H_4NO_2$-*p* (64 mg) used as the acceptor.

*3.2. Characterization of **15** and **16***

The physical data for compound **15** were identical to those of β-D-Gal-(1





Core 1

15

16

Scheme 7. Enzymatic synthesis of core 1 disaccharide glycoside and its analog by porcine testes β-D-galactosidase-catalyzed transglycosylation.

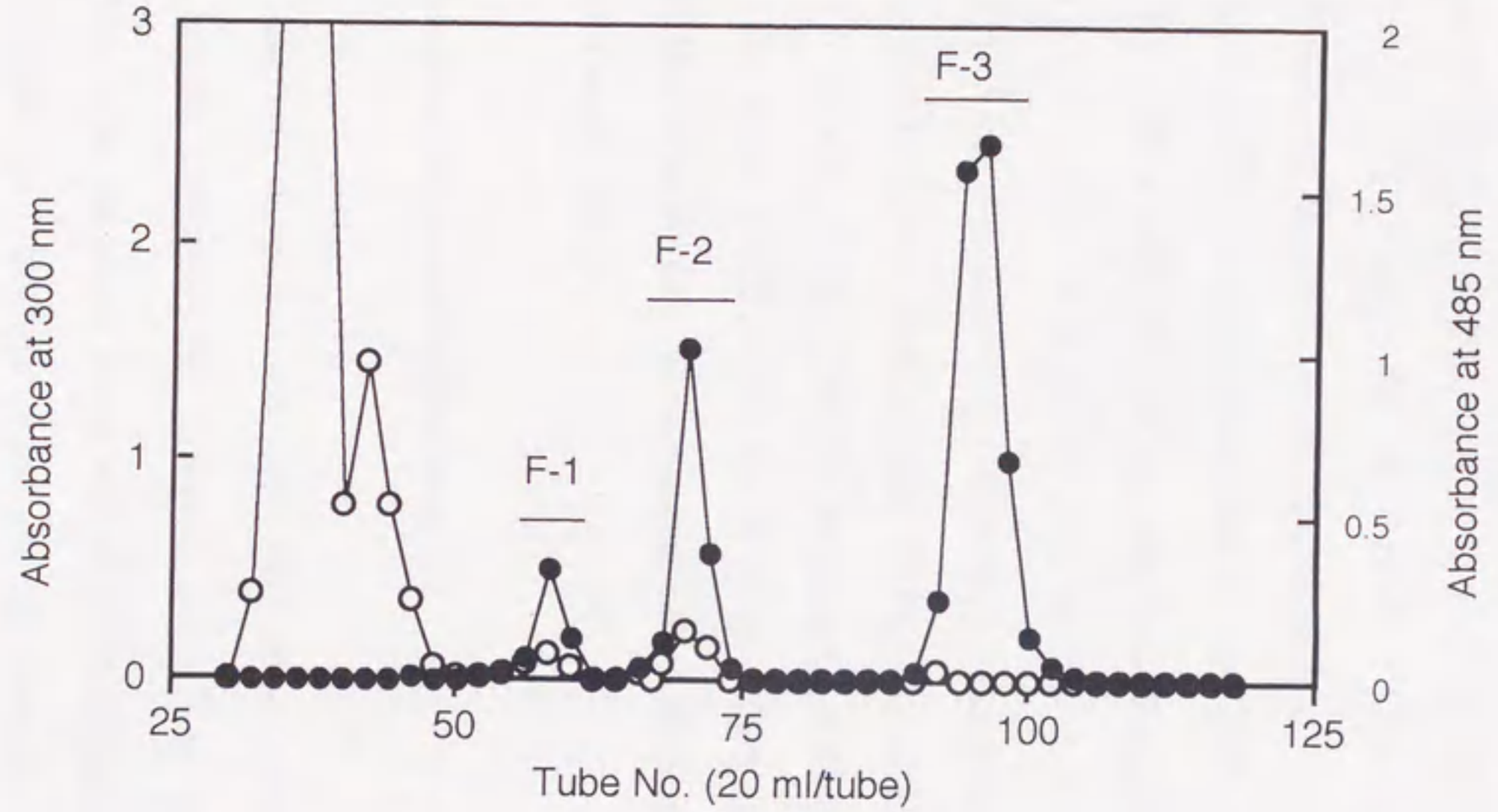


Fig. 21. Toyopearl HW-40S chromatographic separation of transglycosylation products formed from lactose and α-D-GalNAc-$OC_6H_4NO_2$-*p* by porcine testes β-D-galactosidase.

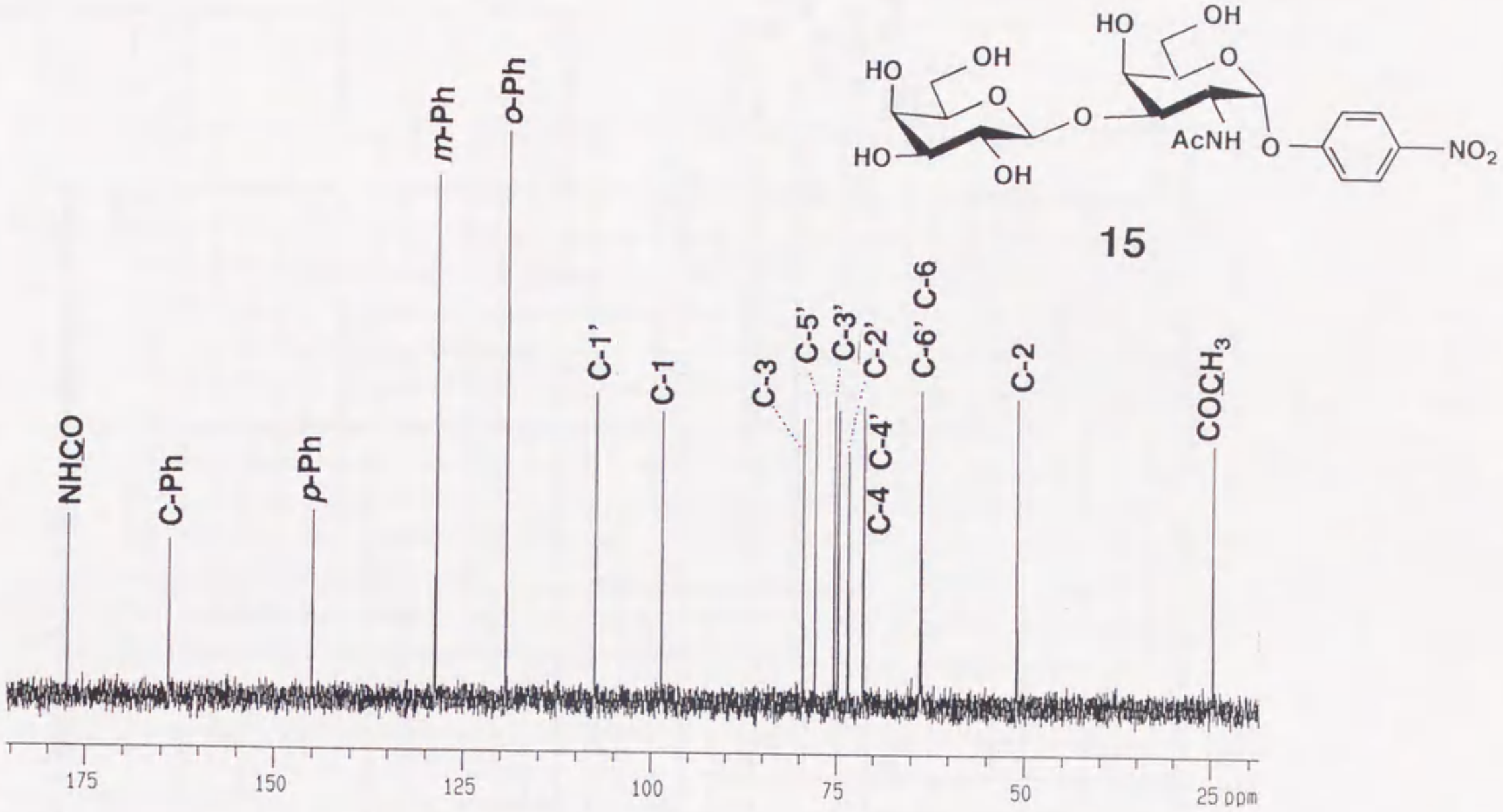
(●), Absorbance at 300 nm; (○), Absorbance at 485 nm.

→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* reported previously[28]. NMR data (D_2O) of **15** (Fig. 22, 23): ^1H, δ 8.25 (d, 2H, *J* 9.2 Hz, *m*-Ph), 7.26 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.80 (d, 1H, <*J* 1 Hz, H-1), 4.53 (d, 1H, *J* 7.6 Hz, H-1'), 1.92 (s, 3H, NAc); ^{13}C, δ 177.52 (C=O of Ac), 164.02 (Ph carbon attached to the phenolic oxygen), 145.12 (*p*-Ph), 128.79 (*m*-Ph), 119.39 (*o*-Ph), 107.64 (C-1'), 98.60 (C-1), 79.31 (C-3), 77.86 (C-5'), 75.29 (C-3'), 74.77 (C-5), 73.39 (C-2'), 71.37 (C-4,4'), 63.83 (C-6'), 63.74 (C-6), 50.10 (C-2), 24.73 (Me of Ac).

16 had : $[\alpha]_D^{25}$ +135.4° (*c* 0.5, H_20); m.p. 207° (from ethanol); and *m/z* 505 $(M+H)^+$. NMR data (Me_2SO-d_6): ^1H, δ 8.15 (d, 2H, *J* 9.2 Hz, *m*-Ph), 7.22 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.53 (d, 1H, *J* 3.5 Hz, H-1), 4.09 (d, 1H, *J* 7.6 Hz, H-1'), 1.84 (s, 3H, NAc); ^{13}C, δ 163.09 (Ph carbon attached to the phenolic oxygen), 143.31 (*p*-Ph), 127.06 (*m*-Ph), 118.51 (*o*-Ph), 104.30 (C-1'), 97.70 (C-1), 76.03 (C-5'), 74.00 (C-3'), 72.10 (C-5), 71.59 (C-2'), 69.37 (C-4'), 69.37 (C-4), 69.02 (C-6'), 67.91 (C-3), 61.73 (C-6), 49.76 (C-2), 22.94 (Me of Ac). These results indicated that compound **16** is *p*-nitrophenyl disaccharide β-D-Gal-(1→6)-α-D-GalNAc-$OC_6H_4NO_2$-*p*.

3.3. Effect of β-CD on the formation of ***15***

The enzyme used in this case was completely devoid of α-NAHase activity, which degrades the acceptor substrate α-D-GalNAc-$OC_6H_4NO_2$-*p* (vide infra). On the other hand, α-D-GalNAc-$OC_6H_4NO_2$-*p* is only spasingly soluble (0.1%) in aqueous medium. In general, the efficiency of the transglycosylation process is enhanced by the presence of a minimal amount of water and an excess of substrate[51]. The problem of low solubility was partially solved by using β-CD, which is thought to form an inclusion complex with a *p*-nitrophenyl group, as already reported[15]. Thus, the solubility of α-



15

Fig. 22. ^{13}C-NMR analysis of compound 15 in D_2O.

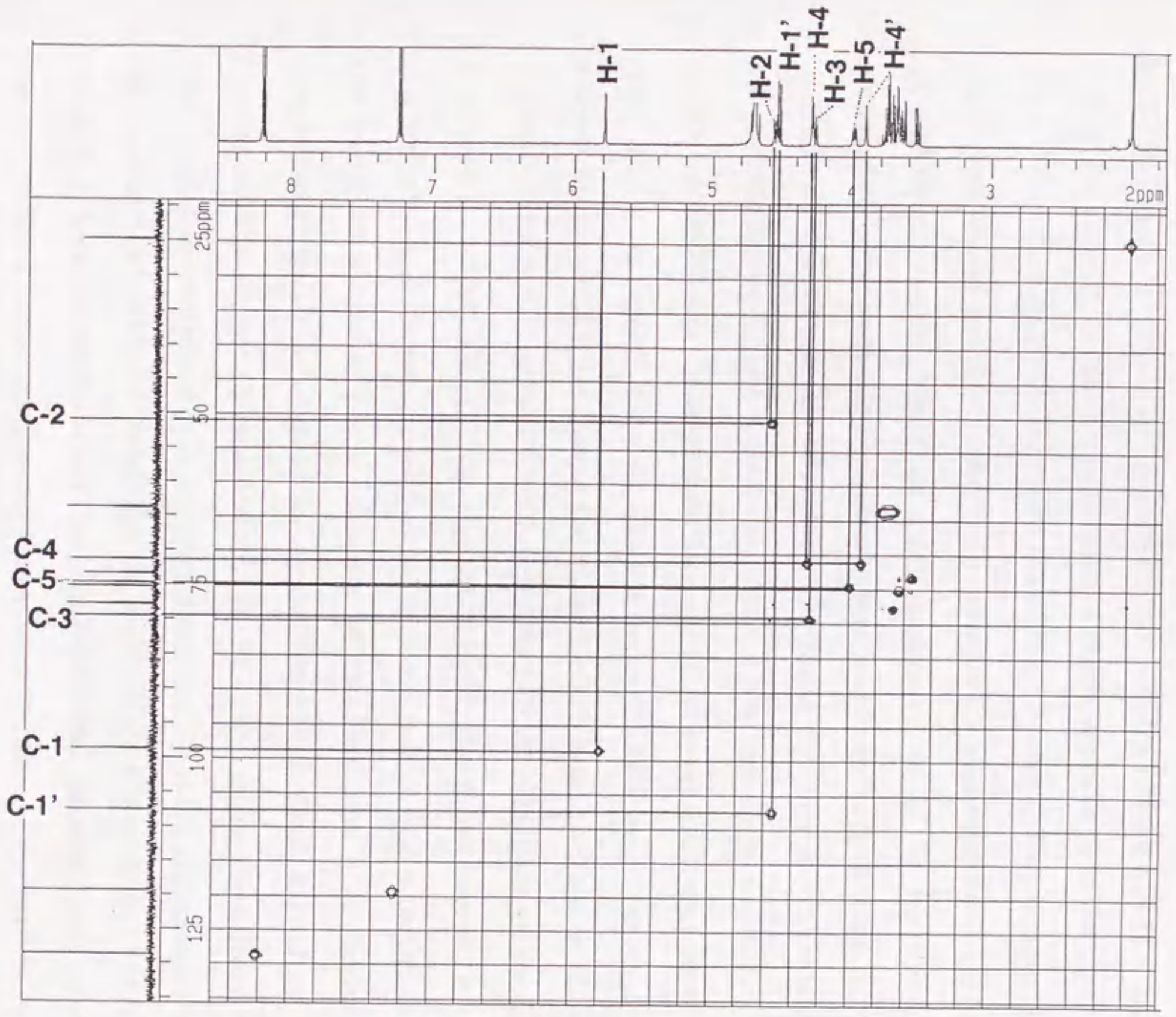


Fig. 23. HSQC spectrum of the compound 15 with ^{1}H and ^{13}C spectra printed on the sides of the 2D spectrum.

D-GalNAc-$OC_6H_4NO_2$-*p* was enhanced 6.5-fold by the presence of β-CD. When α-D-GalNAc-$OC_6H_4NO_2$-*p* was dissolved in amounts equimolar with β-CD, β-(1→3) and β-(1→6)-galactosyl disaccharide glycosides were observed by HPLC, in 22.1% total yield (based on the acceptor added) and in a molar ratio of 3 : 1. The maximum production of transfer products in the presence of β-CD was 2.5-fold higher (4.3 mM) than in its absence. This resulted in a significant improvement of the yield. Moreover, unreacted α-D-GalNAc-$OC_6H_4NO_2$-*p* acceptor, which is quite expensive, could be recovered by straightforward column chromatography and reutilized for synthesis.

Figure 24 shows a transglycosylation profile of the reaction with lactose and α-D-GalNAc-$OC_6H_4NO_2$-*p* in the absence and presence of β-CD. The maximum production of compound **15** in the presence of β-CD was observed after 24 h, at which time it predominated 3-fold over **16**, and its concentration varied little during the subsequent reaction. This result was a great contrast to that with the β-D-GalNAc-$OC_6H_4NO_2$-*p* as an acceptor described in CHAPTER Ⅰ. On the other hand, the formation of **16** was much slower, and maximum production was reached at ~50 h. In this way, the increased solubility of the acceptor by its formation of an inclusion complex with β-CD facilitated the production of the desired product **15**. Usui *et al.* have previously reported that the use of an organic co-solvent (50-60% Me_2SO, acetnitrile etc.) in transfer reactions utilizing glycosidases not only ensured a sufficient solubility of *p*-nitrophenyl glycosides, but also resulted in high yields of desired compounds[52,53,55,56]. However, this concept was not adopted in the present reaction system because of the instability of the enzyme in organic co-solvent systems.

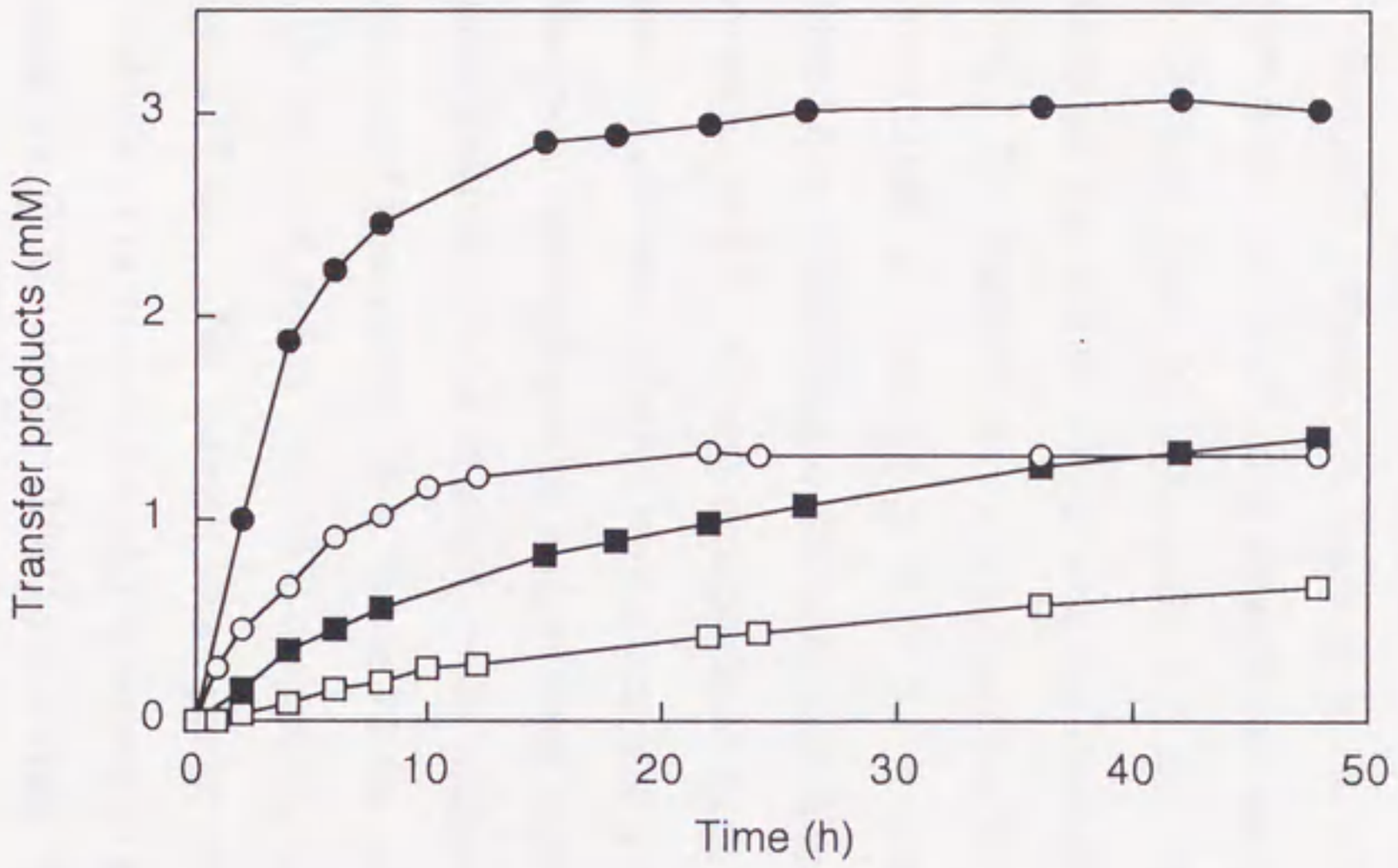
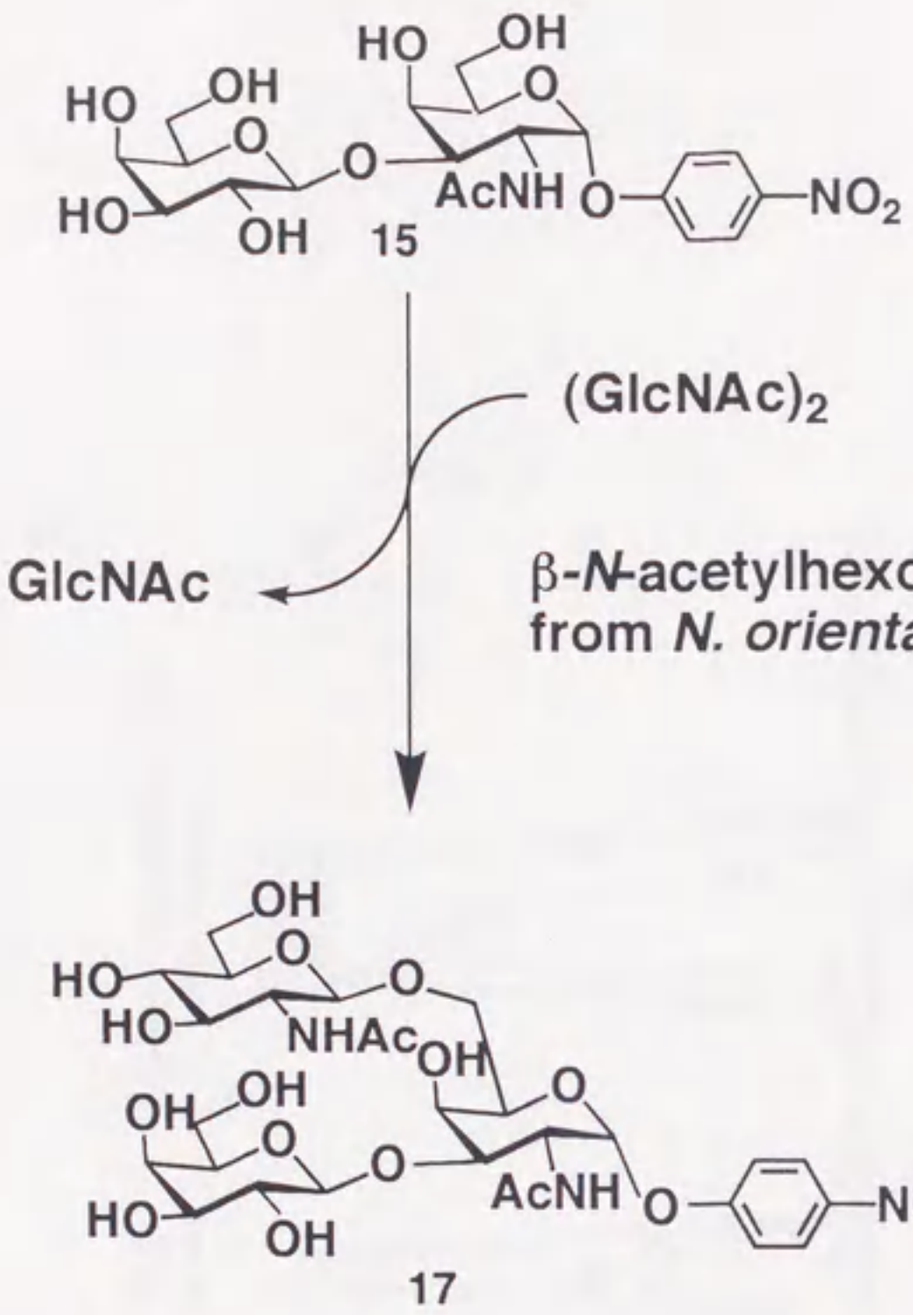


Fig. 24. Time course of porcine testes β-D-galactosidase-mediated isomer formation of 15 and 16.

The amounts of **15** (β1→3, ●, ○) and **16** (β1→6, ■, □) products as a function of time were examined on the 0.5-ml scale as described in the MATERIALS and METHODS section, and samples were analyzed by HPLC during incubation.

*3.4. Preparation of β-D-Gal-(1 → 3)-[β-D-GlcNAc-(1 → 6)]-α-D-GalNAc-$OC_6H_4NO_2$-p (**17**) and its positional analogs*

A preparative synthetic method for obtaining β-D-Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* was established as described above. It has made possible to use enough amounts of compound **15** as an acceptor substrate for obtaining β-D-Gal-(1→3)-[β-D-GlcNAc-(1→6)]-α-D-GalNAc-$OC_6H_4NO_2$-*p* (**17**) (Scheme 8). Thus, the compound **15** (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 donor to acceptor was about 10 : 1, and the total substrate concentration was about 12%. The mixture was incubated for 12 h at 40℃ and the reaction was terminated by heating at 95℃ for 5 min. To the reaction mixture was added 6 ml of methanol (MeOH), and 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 absorbance at 300 nm (*p*-nitrophenyl group) and 485 nm (carbohydrate content, determined by phenol-sulfuric acid method). As shown in Fig. 25, the chromatogram contained four peaks (F-1, tubes 75-94; and F-2, tubes 100-108; F-3, tubes 110-118; F-4, tubes 133-147) displaying coincident absorbances at 300 nm and 210 nm. The former three peaks were presumed to contain transfer products . Fraction 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 rate was 3.0 ml/min. Elution of the column was effected with H_2O-MeOH of 75 : 25 and monitored by measuring the absorbance at 300 nm. The eluates corresponding to the F-1' (63-68 min) and F-2' (81-87 min) were each combined, concentrated, and lyophilized to afford compound **17** (13.9 mg) and **18** (8.9 mg), respectively. Fraction F-3 was concentrated and



Core 1

Core 2

Scheme 8. Enzymatic synthesis of core 2 trisaccharide glycoside and its analogs by *N. olientalis* β-NAHase-catalyzes transglycosylation.

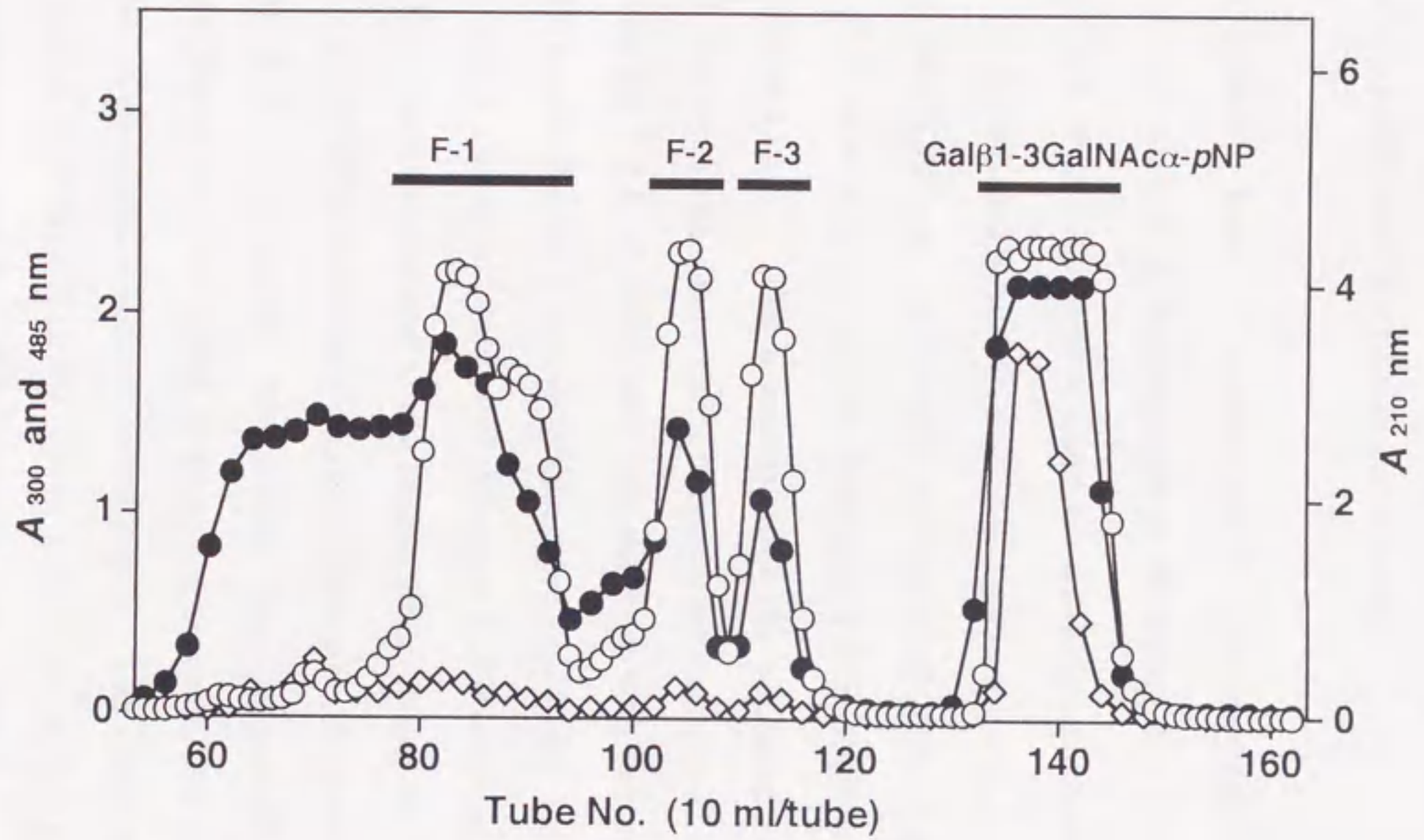


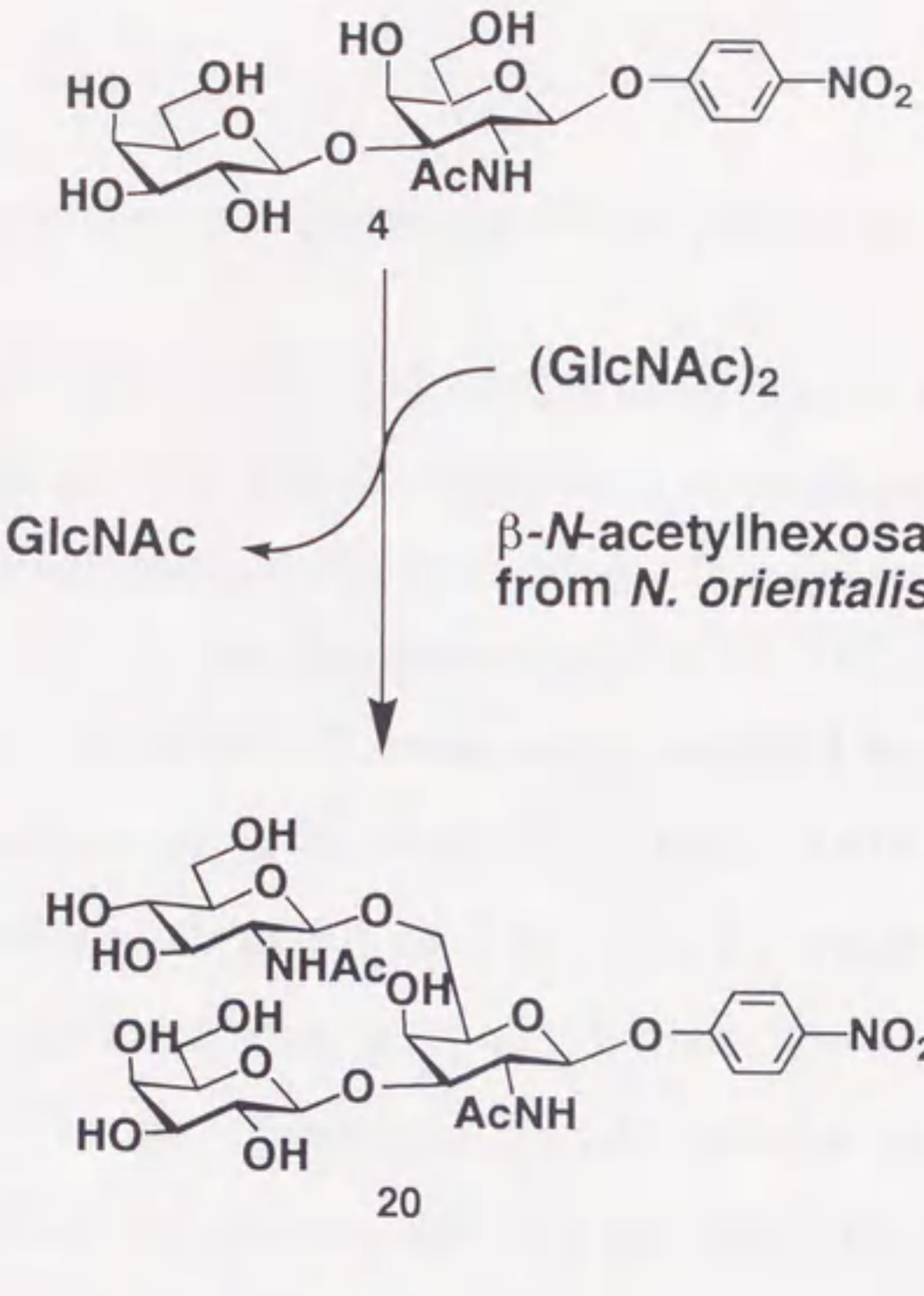
Fig. 25. Toyopearl HW-40S chromatographic separation of transglycosylation products formed from (GlcNAc) 2 and β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* by *N. orientalis* β-NAHase.

(○), Absorbance at 300 nm. (◇), Absorbance at 495 nm. (●), Absorbance at 210 nm.

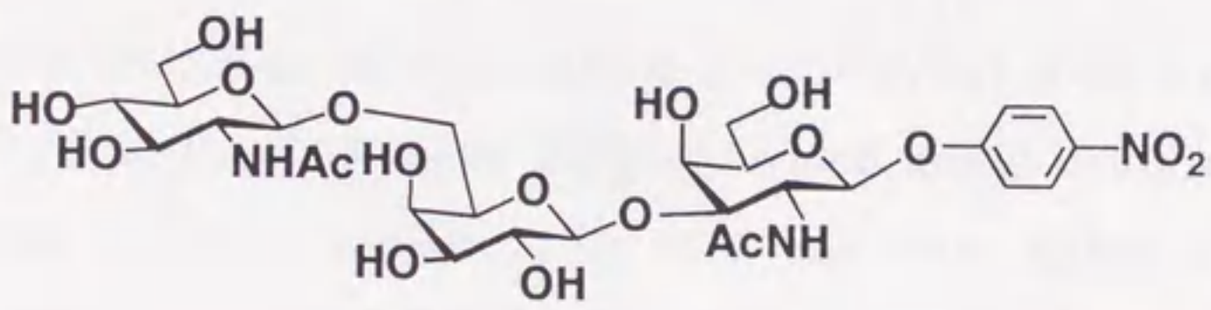
lyophilized to afford compound **19** (6.0 mg). Fraction F-4 contained β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* used as the acceptor substrate.

*3.5. Preparation of β-D-Gal-(1 → 3)-[β-D-GlcNAc-(1 → 6)]-β-D-GalNAc-$OC_6H_4NO_2$-p (**20**) and its positional analogs*

An isomeric form of mucin-type 2 core oligosaccharide, which are β-glycosidically linked to *p*-nitrophenol, utilizing β-D-Gal-(1→3)-β-D-GalNAc-$OC_6H_4NO_2$-*p* was also synthesized as a acceptor substrate instead of β-D-Gal-(1 → 3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* (Scheme 9). β-D-Gal-(1 → 3)-β-D-GalNAc-$OC_6H_4NO_2$-*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℃ and the reaction was terminated by heating at 95℃ for 5 min. To the reaction mixture was added 2.5 ml of MeOH, and 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 (Fig. 26). 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_2O-MeOH of 75 : 25 at a flow rate of 5.0 ml/min. The eluates corresponding 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 **20** (3.2 mg), **21** (2.2 mg) and **22** (1.2 mg), respectively. Fraction F-7 contained β-D-Gal-(1 → 3)-β-D-GalNAc-$OC_6H_4NO_2$-*p* used as the acceptor



20



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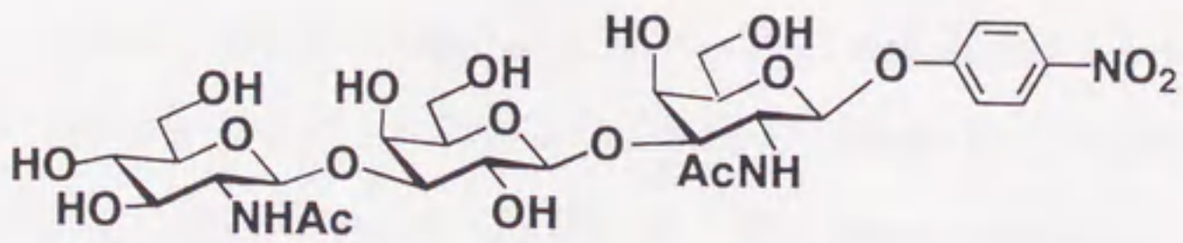


22

Scheme 9. Enzymatic synthesis of isomeric form of core 2 trisaccharide glycoside and its analogs by *N. olientalis* β-NAHase-catalyzes transglycosylation.

substrate.

3.6. Characterization of compound **17** *and positional analogs*

The positive ion mode FAB-MS/MS spectrum of compound **17** shows a molecular ion at *m/z* 708 ($[M+H]^+$) with a fragment ion at *m/z* 546 (a fragment of HexNAc-HexNAc-$OC_6H_4NO_2$). It indicates that compound **17** is a branched trisaccharide Hex-(HexNAc-)HexNAc-$OC_6H_4NO_2$. The ^{1}H-NMR signals of the compound **17** were easily assigned by correlation with the reported spectrum for β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* (Table 5). 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 GlcNAc residue, respectively. The introduction of GlcNAc residue to β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* also resulted in 0.156 and 0.324 ppm downfield shifts of H-5 and H-6 of GalNAc residue on **17**, respectively (Table 5). It shows that the GalNAc residue on the trisaccharide is substituted at the 6-position. The ^{13}C-NMR spectrum of **17** using HSQC provided useful information on the composition and sugar sequence (Fig. 27). All of the different carbon signals were resolved and assigned using carbon-proton shift correlation. The spectrum was also correlated with that of β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*. Seven signals of the GlcNAc residue, which did not appear in the spectrum of β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*, were clearly differentiated from other signals (Table 6). The most directed evidence, that GlcNAc residue is bound to C-6 position of GalNAc, was obtained from HMBC spectrum (Fig. 28), 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 **17** is a β-D-Gal-(1→3)-[β-D-GlcNAc-(1→6)]-α-D-GalNAc-$OC_6H_4NO_2$-*p*: $[\alpha]_D^{25}$ +116.9° (*c* 0.5, H_2O).

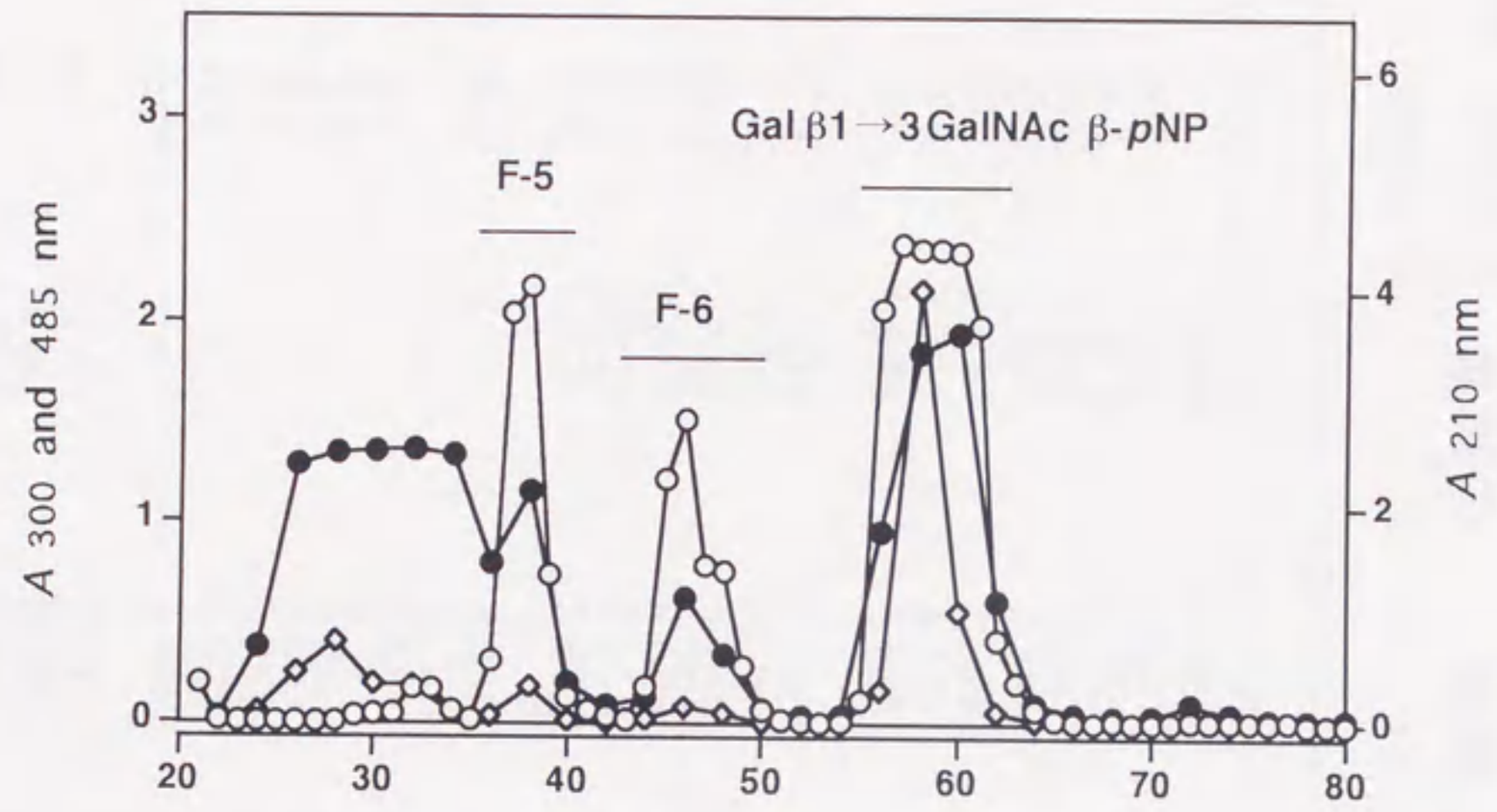
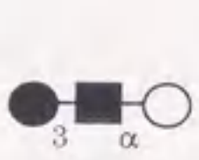
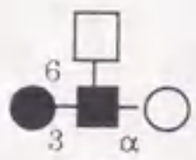
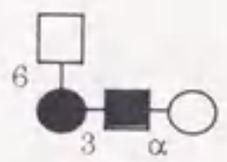
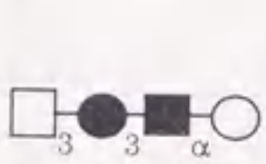


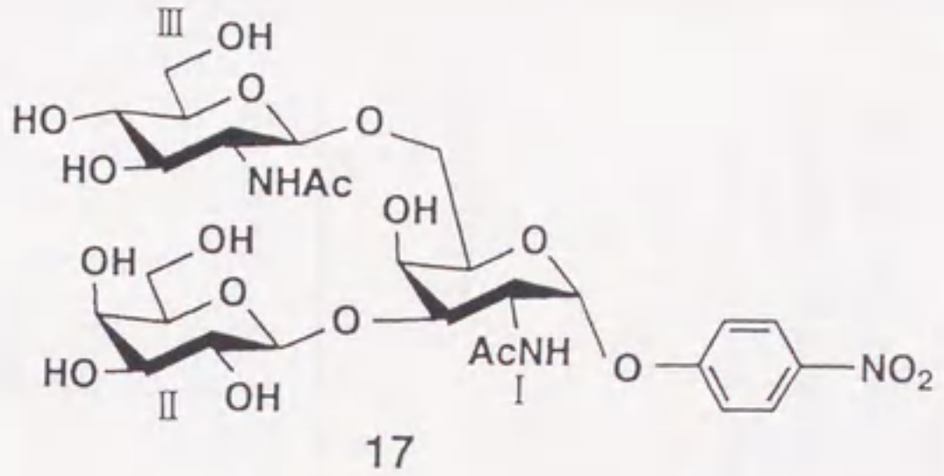
Fig. 26. Toyopearl HW-40S chromatographic separation of transglycosylation products formed from $(GlcNAc)_2$ and β-D-Gal-(1→3)-β-D-GalNAc-OC $_6H_4NO_2$-p by *N. orientalis* β-NAHase.

(○), Absorbance at 300 nm. (◇), Absorbance at 495 nm. (●), Absorbance at 210 nm.

Table 5 . ^1H chemical shifts of the constituent monosaccharides for β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*, compounds **17**, **18**, and **19** in D_2O solution. *p*-nitrophenyl, (○); α-D-GalNAc, (■); β-D-Gal, (●); β-D-GlcNAc, (□). The superscripts at the name of the sugar indicate the linkage positions of the subsequent monosaccharides in the sequence.

Residues	Reporter groups	Chemical shifts in compounds (δ)			
			 **17**	 **18**	 **19**[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	n.d.[c]
	H-6'	3.728	3.735	3.736	n.d.
	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	3.531[d]	3.599
	H-3	3.639	3.636	3.620	n.d.
	H-4	3.909	3.915	3.895	4.144
	H-5	3.675	3.673	3.792	n.d.
GlcNAc	H-1		4.439	4.565	4.703
			(8.55)	(8.25)	(8.55)
	H-2		3.537	3.662	n.d.
	H-3		3.419	3.530[d]	3.563
	H-4		3.267	3.395	3.465
	H-5		3.355	3.438	n.d.
	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] Recorded at 25℃.
[b] Coupling constant in Hz.
[c] n.d., Not determined.
[d] These assignments may be changed.



17

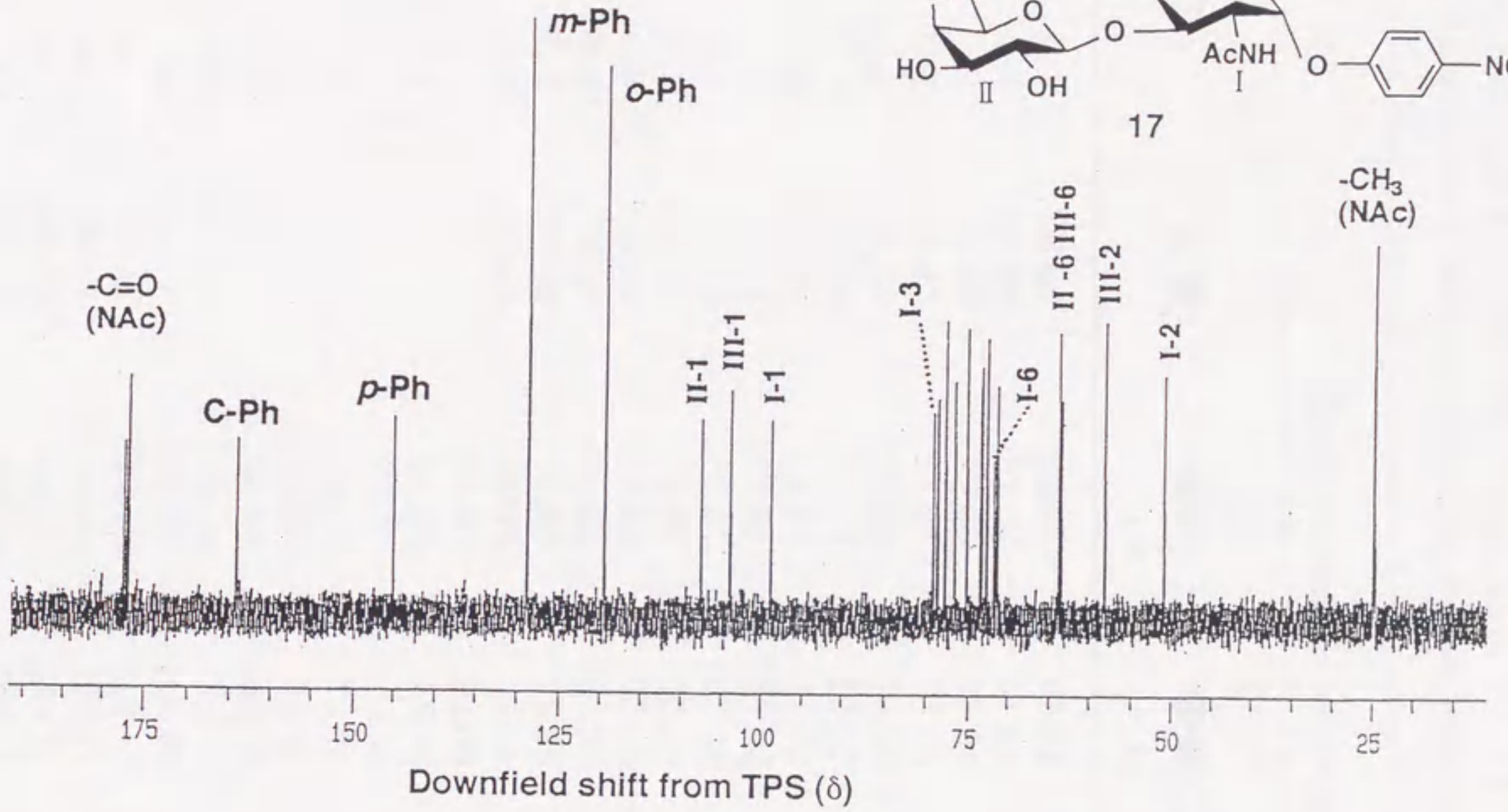
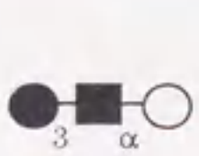
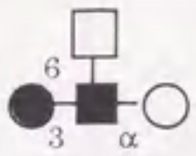
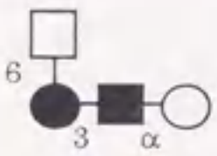
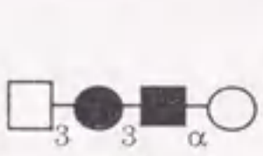


Fig. 27. ^{13}C-NMR analysis of the compound 17 in D_2O.

Table 6. ^{13}C chemical shifts of the constituent monosaccharides for β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*, compounds **17**, **18**, and **19** in D_2O solution. Abbreviations and superscripts as used in legend to Table 5.

Residues		Chemical shifts in compounds (δ)			
			 **17**	 **18**	 **19**[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	119.45	119.30
	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] Recorded at 25℃.

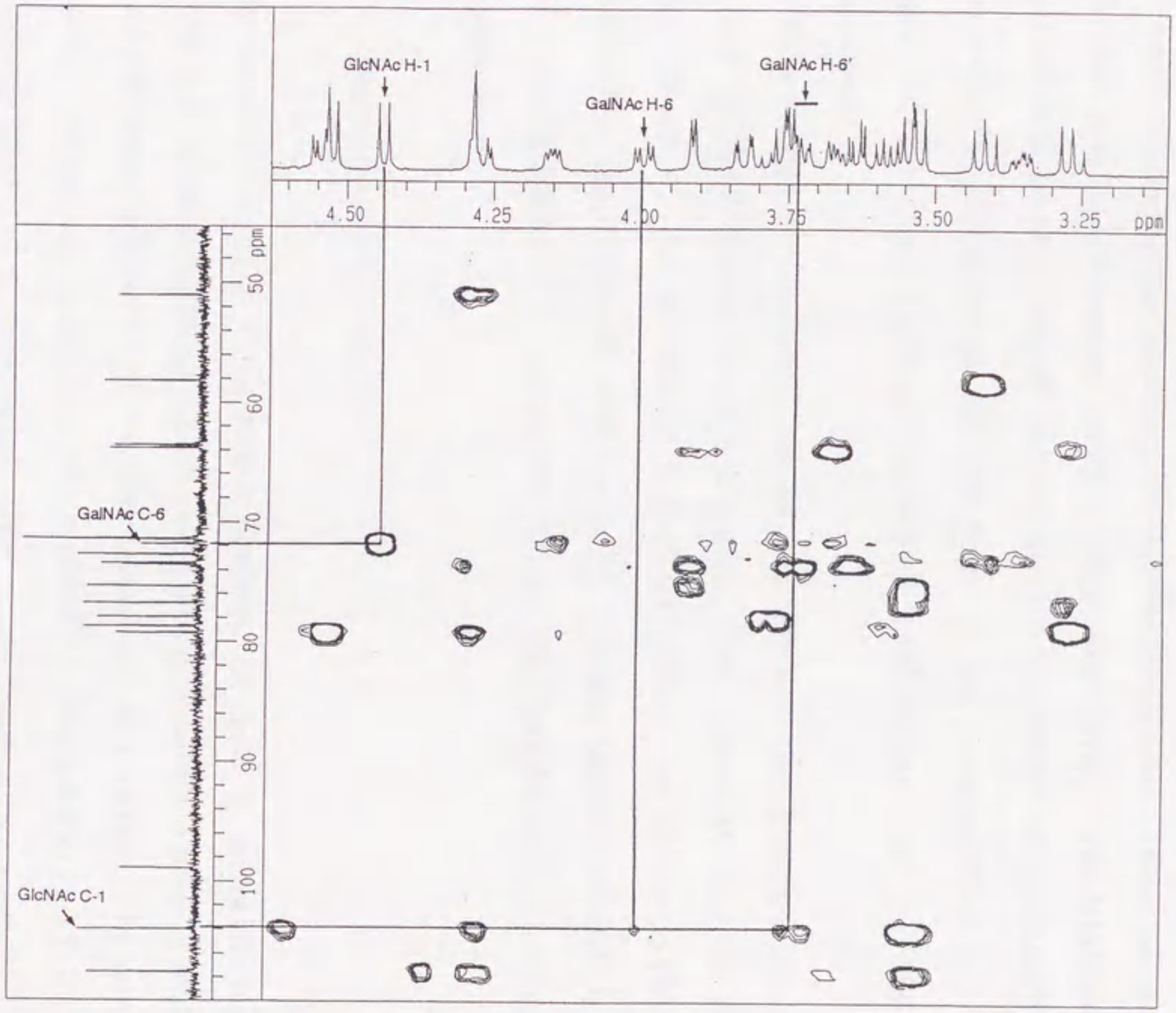


Fig. 28. HMBC spectrum of the compound 17 with ^{1}H and ^{13}C spectra printed on the sides of the 2D spectrum.

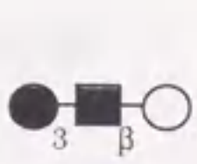
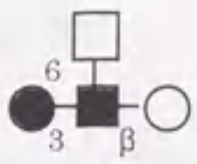
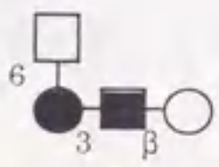
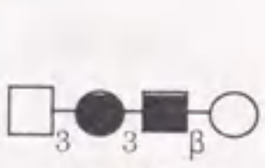
In the same way, the structures of compounds **18** and **19** were similarly characterized. The each positive ion mode FAB-MS/MS spectra of **18** and **19** show a molecular ion at *m/z* 708 ($[M+H]^+$) with a fragment ion at *m/z* 505 (fragment from Hex-HexNAc-$OC_6H_4NO_2$). It suggests that both compounds are linear trisaccharides HexNAc-Hex-HexNAc-$OC_6H_4NO_2$. These ^1H- and ^{13}C-NMR data were shown in Tables 5 and 6, respectively. The NMR and FAB-MS/MS revealed that **18** and **19** are β-D-GlcNAc-(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 **20**, **21**, and **22** were also characterized by ^1H and ^{13}C-NMR (Tables 7 and 8, Fig. 29). Compounds **20**, **21**, and **22** were shown to be 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-GlcNAc-(1 → 3)-β-D-Gal-(1 → 3)-β-D-GalNAc-$OC_6H_4NO_2$-*p*, respectively.

3.7. Enzymatic synthesis of **17**

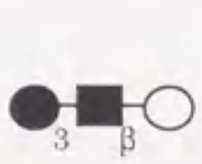
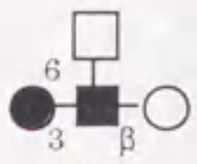
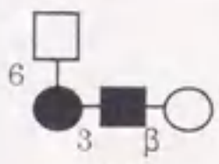
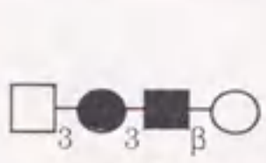
In my previous study in the former chapter, the from *N. orientalis* has proved to be efficient biocatalyst for transfer of *N*-acetylglucosaminyl residue onto 6-OH group of lactose and *N*-acetyllactosamine derivatives. The same procedure was applied to synthesis of compound **17** with β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* as an acceptor. The enzyme used in this synthesis is 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 contains chitinase activity, but is completely devoid of β-D-

Table 7. ^{1}H chemical shifts of the constituent monosaccharides for β-D-Gal-(1→3)-β-D-GalNAc-$OC_6H_4NO_2$-*p*, compounds **20**, **21**, and **22**. Abbreviations and superscripts as used in legend to Table 5.

Residues	Reporter groups	Chemical shifts in compounds (δ)			
			 **20**	 **21**	 **22**[a]
GalNAc	H-1	5.293	5.287	5.297	5.304
		(8.55)[b]	(8.55)	(8.55)	(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	n.d.[c]	3.938
	H-6	3.787	4.105	n.d.	n.d.
	H-6'	3.817	3.798	n.d.	n.d.
	NAc	1.968	1.969	1.975	1.989
Gal	H-1	4.474	4.467	4.471	4.475
		(7.65)	(7.93)	(7.63)	(7.63)
	H-2	3.527	3.530	3.523	3.596
	H-3	3.611	3.609	3.591	n.d.
	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	4.556	4.688
			(8.55)	(8.25)	(8.55)
	H-2		3.699	3.673	n.d.
	H-3		3.491	3.523	3.556
	H-4		3.435	3.420	3.458
	H-6'		3.915	n.d.	3.888
	NAc		1.733	2.034	2.026
pNP	*o*-	7.186	7.170	7.181	7.202
		(9.46)	(9.16)	(9.16)	(9.46)
	m-	8.227	8.245	8.233	8.220
		(9.45)	(9.46)	(9.15)	(9.46)

[a] Recorded at 25℃.
[b] Coupling constant in Hz.
[c] n.d., Not determined.

Table 8 . ^{13}C chemical shifts of the constituent monosaccharides for β-D-Gal-(1→3)-β-D-GalNAc-$OC_6H_4NO_2$-*p*, compounds **20**, **21**, and **22**. Abbreviations and superscripts as used in legend to Table 5.

Residues		Chemical shifts in compounds (δ)			
			 **20**	 **21**	 **22**[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	119.39	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] Recorded at 25℃.

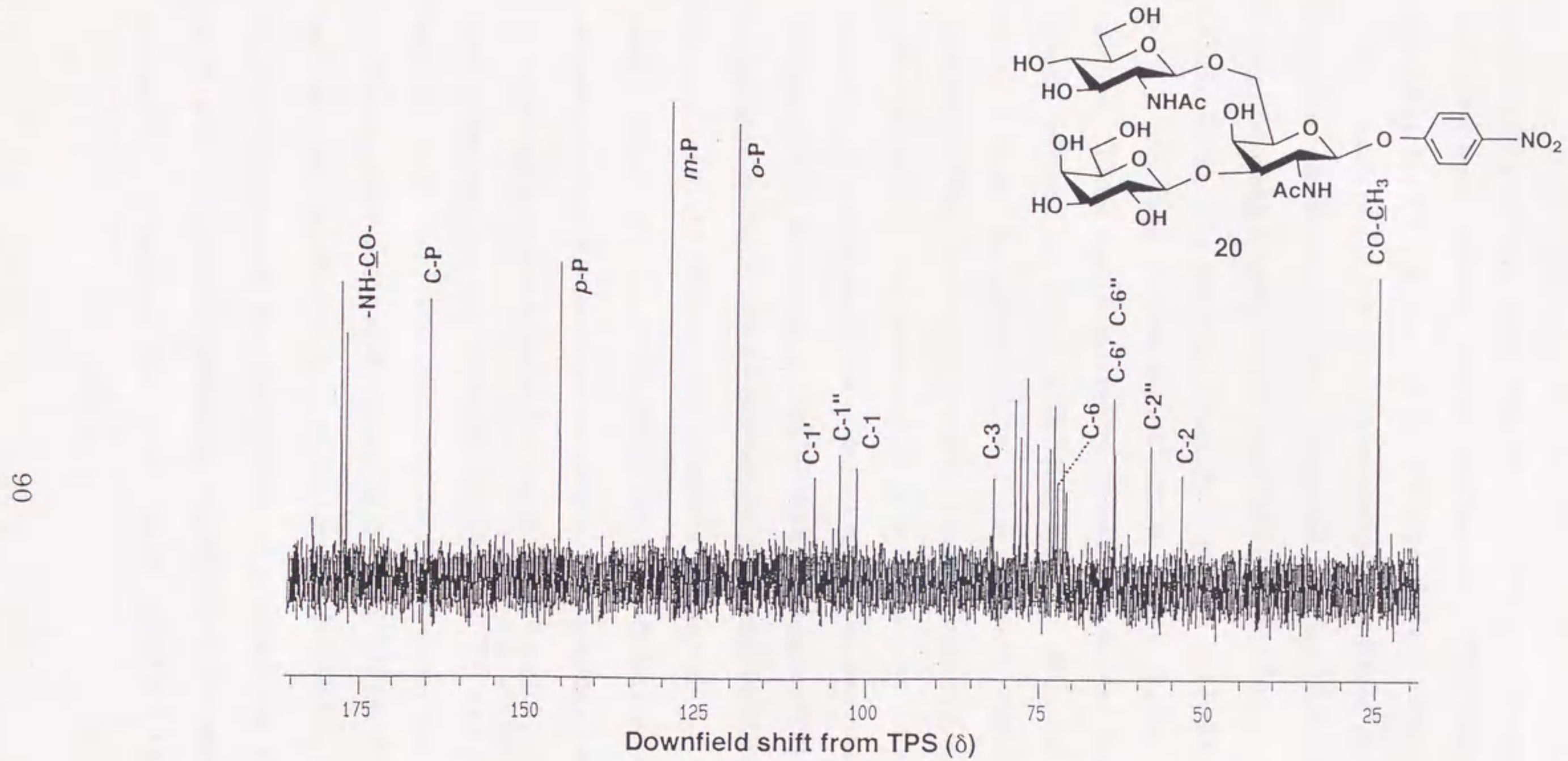
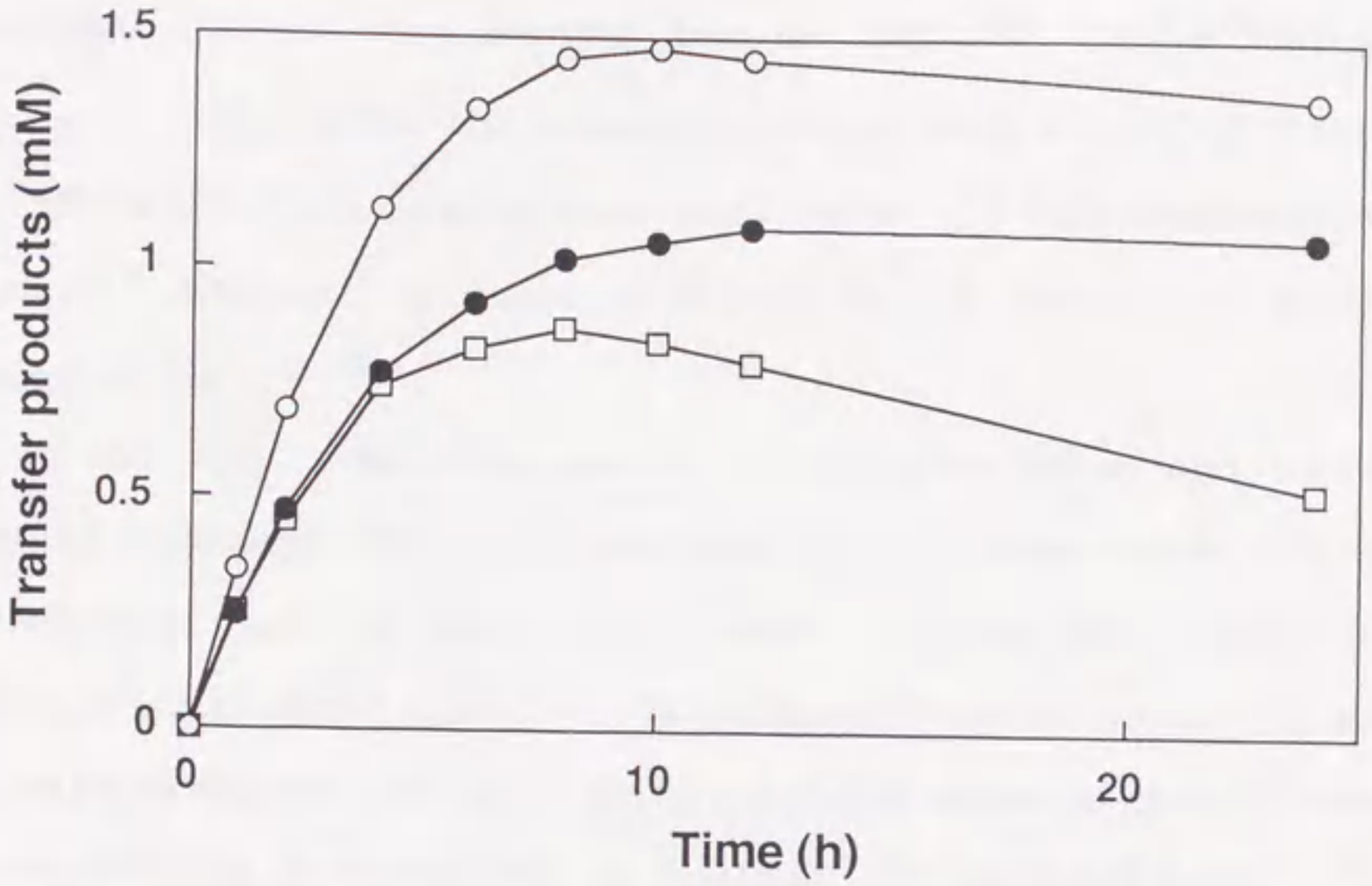


Fig. 29. ^{13}C-NMR analysis of the compound 20 in D_2O.

galactosidase activity, which degrades the acceptor substrate compound **15**, and was used without further purification. The enzyme formed the trisaccharides **17**, **18**, and **19** 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 **17** after 10 h. Figure 30 shows 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 **17**, **18**, and **19** 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 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'. Increased solubility of *p*-nitrophenyl glycosides by using cyclodextrins (CD), which are thought to form an inclusion complex with the *p*-nitrophenyl group, resulted in improved yields of transfer products as previously described. The concept was applied 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).

In a similar manner, when β-D-Gal-(1→3)-β-D-GalNAc-$OC_6H_4NO_2$-*p* was used as the acceptor, three transfer products **20**, **21**, and **22** 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' vs. O-3 of the acceptor was not only similar to that of its anomer β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* acceptor, but also to those of *p*-nitrophenyl β-lactoside (Lacβ-*p*NP) and β-*N*-acetyllactosaminide (LacNAcβ-*p*NP) ones as previously reported[15,17]. However, the present yields based on the acceptor were

(A)



(B)

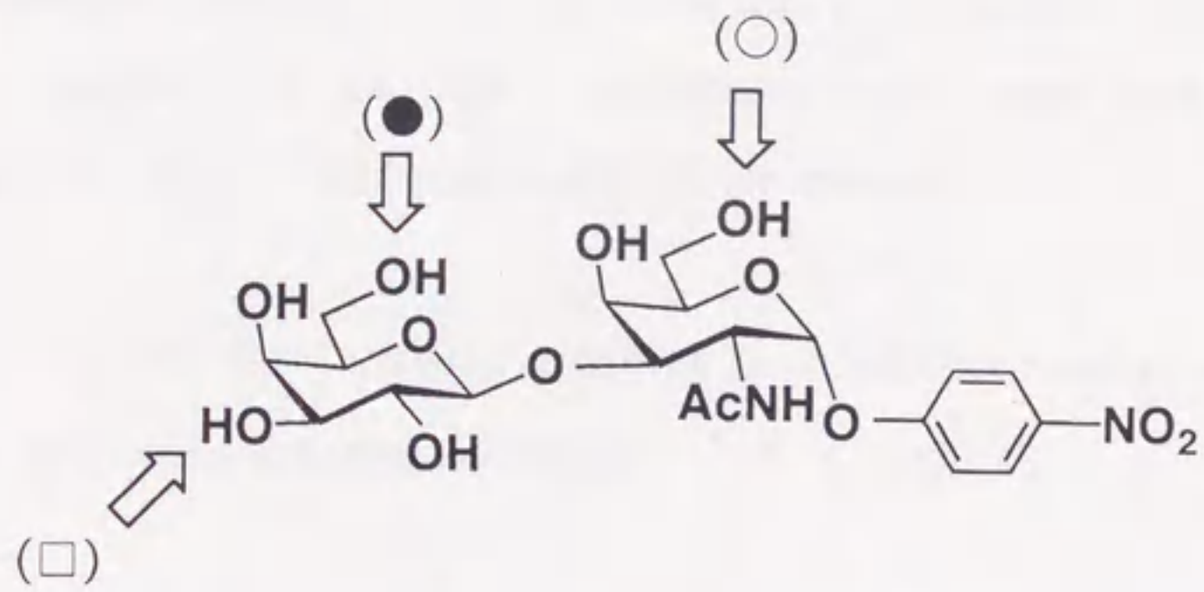


Fig. 30. Time course of *N. orientalis* β-NAHase-mediated isomer formation of 17, 18, and 19.

(A) The amounts of **17** (○), **18** (●), and **19** (□) 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.

somewhat different from previous data on Lacβ-*p*NP and LacNAcβ-*p*NP (Table 9). Especially, the transglycosylation with β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* acceptor was much higher (4.7-fold) than that with Lacβ-*p*NP acceptor. It seems to depend on the structure of acceptor disaccharides.

In this way, consecutive use of transgalactosylation and trans-*N*-acetylglucosaminylation led to the formation of trisaccharide **17** as a carbohydrate unit of mucin-type 2 core, starting with α-D-GalNAc-$OC_6H_4NO_2$-*p* as shown in Fig. 31. Nitrophenyl glycoside can not only serve as enzyme substrate, but also it can be reduced to aminated glycoside, whose amino function is derivatized for reactions with electrophiles[61,71]. Such aminated mucin-type glycosides may be coupled with pendant carboxyl groups of polypeptide such as poly (L-glutamic acid)[72] and lead to a convenient synthetic route of artificial mucin glycoprotein.

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

In the chapter Ⅱ, when an inclusion complex of Lacβ-*p*NP or LacNAcβ-*p*NP with α-CD was used, the regioselectivity of β-NAHase-catalyzed formation of trisaccharide glycoside was substantially changed, due to steric hindrance of the CD in an inclusion complex. This was also applied to the reaction with β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* and its anomer as acceptors. Figure 32 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-$OC_6H_4NO_2$-*p* acceptor, no matter how the molar ratio of α-CD to the acceptor was increased, it affected to a lesser extent the regioselectively

Table 9. Total yields of transfer products formed by β-NAHase-catalyzed transglycosylation with various disaccharide glycoside acceptors.

acceptors	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-D-GalNAc-$OC_6H_4NO_2$-*p*	8

*Based on the acceptors added.

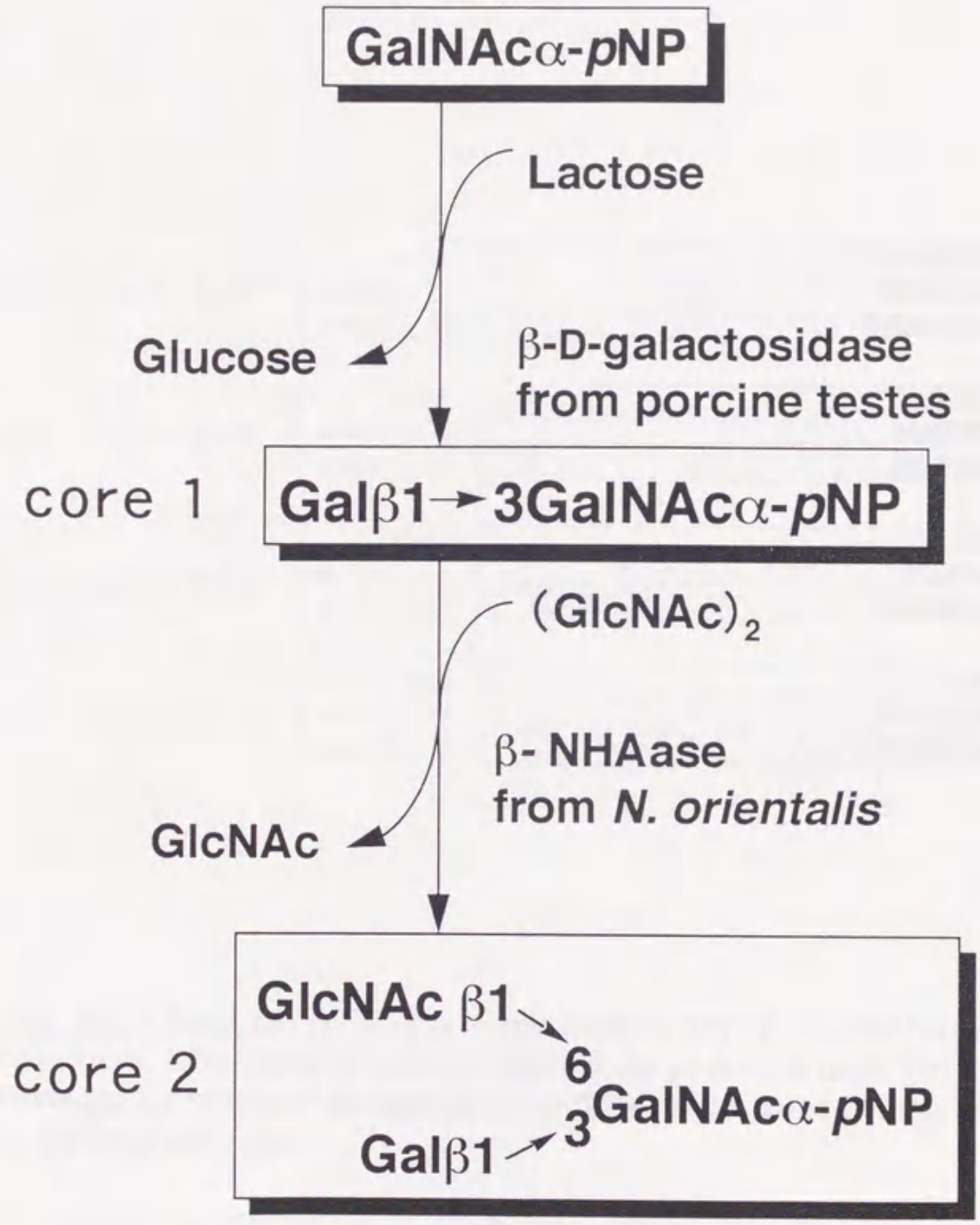


Fig. 31. Synthetic scheme of carbohydrate region of cores 1 and 2 by consecutive use of transgalactosylation and trams-*N*-acetylglucosaminylation.

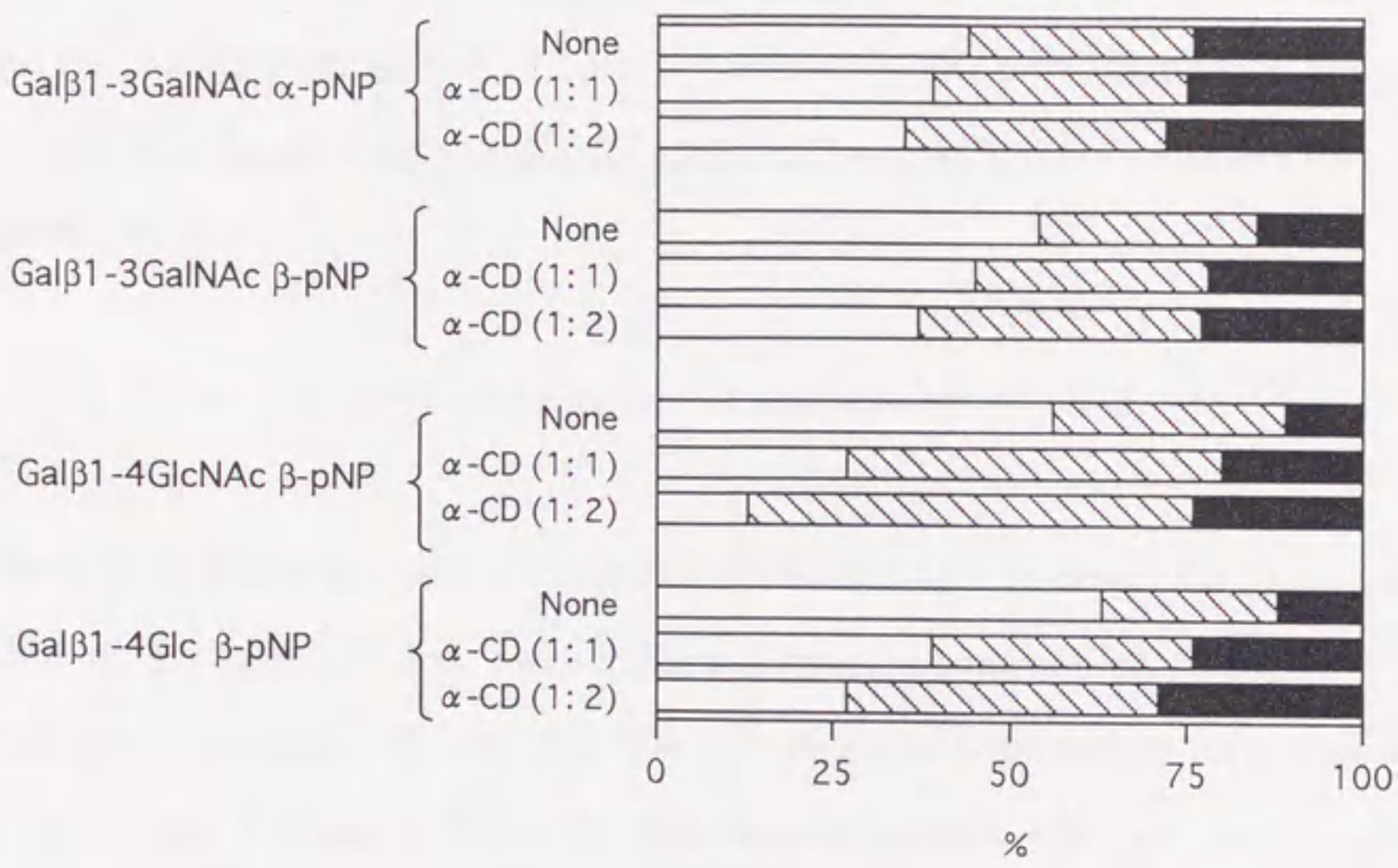


Fig. 32. Effect of α-CD on regioselectivity of GlcNAc-transfer reaction. Abscissa presents percentage of transfer products formed by transglycosylation at different molar ratios of α-CD to acceptor.

□, GlcNAc-transfer to *O*-6. ▨, GlcNAc-transfer to *O*-6'. ■, GlcNAc-transfer to *O*-3'.

with respect to O-6 and O-6' vs. 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 is not very influence on the regioselectivity at O-6 of 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.

3.9. Substrate specificity and kinetics of endo-α-GalNAc-ase

Endo-α-GalNAc-ase which hydrolyzes the linkage between GalNAc and Ser/Thr in glycoprotein has been isolated from a number of sources[68,73-77], hydrolyzes compound **15** into β-D-Gal-(1→3)-D-GalNAc and *p*-nitrophenol, that is, it can bypass a block of the disaccharide[68]. In this study, the substrate specificity of commercially available endo-α-GalNAc-ase-A from *Alcaligenes* sp.[74] was investigated using a series of synthetic *p*-nitrophenyl galactosyl-disaccharide glycosides (**4**, **5**, **6**, **7**, **8**, **15** and **16**). The enzyme products were detected by HPLC using a TSK-GEL G-Oligo-PW column. The enzyme was capable of liberating a reducing disaccharide only from **15**. This indicates that its specificity for the substrate is very high.

By measuring the amount of *p*-nitrophenol as described in Materials and Methods, the initial velocities (v) were obtained directly from the initial slopes of the time-course plots (2, 4, 6 and 8 min) of the reaction. Six different substrate concentrations (0.05 ~ 0.8 mM) were used per experiment. The kinetic parameters for **15** of the enzyme assayed were calculated by the method of least squares with use of a [S]/v~[S] plot. The Michaelis constant (*K*m) and k_0/Km were 0.07 mM and 66.7 $mM^{-1} \cdot sec^{-1}$, respectively.

This indicates that **15** is a very sensitive substrate for the endo-α-GalNAc-ase-A. DiCioccio *et al.* have reported that the *K*m value of **15** for endo-α-GalNAc-ase-C from *C. perfringens* was 0.5 mM[68]. The *Km* value for this enzyme is much greater than that from *Alcaligenes* sp. Fan *et al.* have also reported that the activity of *Diplococcus pneumoniae* enzyme on DNS-Ser-GalNAc-Gal and DNS-Thr-GalNAc-Gal was lower than that of the *Alcaligenes* sp. enzyme[74]. This indicates that the reactivity of endo-α-GalNAc-ases using these substrates depends greatly on the enzyme source.

The substrate specificity of endo-α-GalNAc-ase-A and -D were further investigated using the compounds **17**, **18** and **19**. Because the specificity of these enzymes with regard to core 2 oligosaccharide has not been reported. The enzyme hydrolyzates were detected by TLC and HPLC. The enzymes did not show any activity on the compounds **17**, **18** and **19**. This suggests that the *O*-substituted GlcNAc groups show some resistance to attack by these enzymes, and its specificity for the substrate in very high. However, compound **17** would be useful as an exogenous substrate for endo-α-GalNAc-ase-S[69, 70], which released a tetrasaccharide β-D-Gal-(1→3)-[β-D-Gal-(1→4)-β-D-GlcNAc-(1→6)]-D-GalNAc from asialofetuin. The GlcNAc-containing trisaccharide glycosides synthesized in this study should be useful tool for examining of substrate specificity of endo-α-GalNAc-ases and for screening of the endo-α-GalNAc-ases which have a broader substrate specificity.

CONCLUSION

Author performed the preparation of biologically important di- and trisaccharide units through glycosidase-mediated transglycosylation. In this process, the regioselectivity on transglycosylation can be manipulated to some extent by devising the reaction system. The total yields (6-22%) were considerable to be sufficiently high for the practical method, because of the simplicity of enzymatic synthesis (Table 10). Such enzymes are readily available in large amounts. Excess of unreacted substrates are also recovered by straightforward column chromatography and reutilized for the synthesis. The approach described above should be applicable to a wide range of glycosidase-mediated oligosaccharide synthesis. Such well defined oligosaccharides would be useful as substrates for exo- and endogenous glycosidases and glycosyltransferases involved in glycoconjugates processing, as probes for lectin or selectin binding, and as common synthetic intermediates of antigens. Furthermore, the synthetic oligosaccharides are also useful as starting substances for glycopolymers, which are valuable tool for investigating biological recognition phenomena using lectins. Accordingly, the supply of enough amounts of oligosaccharides offers a promising prospect to construct novel glycomaterials. The present results were summarized in following three parts.

I . β-D-Gal-(1→3)-β-D-Glc-$OC_6H_4NO_2$-*p* (**1**) and its isomers (β-D-Gal-(1→4)-β-D-Glc-$OC_6H_4NO_2$-*p* (**2**) and β-D-Gal-(1→6)-β-D-Glc-$OC_6H_4NO_2$-*p*, **3**) were synthesized from lactose and β-D-Glc-$OC_6H_4NO_2$-*p*, using transglycosylation by the β-D-galactosidase from *B. circulans*. This reaction makes it possible to selectively synthesize **1** and **3** by controlling the reaction time. Porcine testes β-D-galactosidase catalyzed the synthesis of β-D-Gal-(1→3)-β-D-

Table 10. Glycosidase-mediated oligosacchareide synthyesis.

Doner	Acceptor	Product	Total yield (%)[a]
β-D-galactosidase			
Lactose	GlcβO*p*NP	Galβ1,3GlcβO*p*NP	21.6
		Galβ1,4GlcβO*p*NP	
		Galβ1,6GlcβO*p*NP	
lactose	GalNAcβO*p*NP	Galβ1,3GalNAcβO*p*NP	15.3
		Galβ1,6GalNAcβO*p*NP	
lactose	GlcNAcβO*p*NP	Galβ1,3GlcNAcβO*p*NP	20.9
		Galβ1,4GlcNAcβO*p*NP	
		Galβ1,6GlcNAcβO*p*NP	
Lactose	GalNAcαO*p*NP	Galβ1,3GalNAcαO*p*NP	22.1
		Galβ1,6GalNAcαO*p*NP	
β-NAHase			
$(GlcNAc)_2$	Galβ1,4GlcNAcβO*p*NP	GlcNAcβ1,3Galβ1,4GlcNAcβO*p*NP	6.2
		GlcNAcβ1,6Galβ1,4GlcNAcβO*p*NP	
		Galβ1,4(GlcNAcβ1,6)GlcNAcβO*p*NP	
$(GlcNAc)_2$	Galβ1,3GalNAcαO*p*NP	Galβ1,3(GlcNAcβ1,6)GalNAcαO*p*NP	14
		GlcNAcβ1,3Galβ1,3GalNAcαO*p*NP	
		GlcNAcβ1,6Galβ1,3GalNAcαO*p*NP	
$(GlcNAc)_2$	Galβ1,3GalNAcβO*p*NP	Galβ1,3(GlcNAcβ1,6)GalNAcβO*p*NP	8
		GlcNAcβ1,3Galβ1,3GalNAcβO*p*NP	
		GlcNAcβ1,6Galβ1,3GalNAcβO*p*NP	

[a]Yields were calculated based on acceptor substrates.

GalNAc-$OC_6H_4NO_2$-*p* (**4**) and its positional isomer β-D-Gal-(1→5)-β-D-GalNAc-$OC_6H_4NO_2$-*p* (**5**) from lactose and β-D-GalNAc-$OC_6H_4NO_2$-*p*. The enzyme formed the disaccharides **4** and **5** in 15.3% total yield (based on the acceptor) and in a ratio of 5 : 1, respectively. The enzyme also catalyzed the synthesis of β-D-Gal-(1→3)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**6**) and its isomers β-D-Gal-(1 → 4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**7**)and β-D-Gal-(1 → 6)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**8**). Compounds **6**, **7**, and **8** were observed in 20.9% total yield (based on the acceptor) and in a ratio of 48 : 20 : 32, respectively. These reactions were efficient enough to allow the one-pot preparation of galactosyl-glucoside from lactose.

II . β-*N*-acetyl-D-hexosaminidase from *N. orientalis* catalyzed the synthesis of β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**9**) and β-D-GlcNAc-(1→6)-β-D-Gal-(1→4)-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**10**) with its isomer β-D-Gal-(1→4)-[β-D-GlcNAc-(1→6)]-β-D-GlcNAc-$OC_6H_4NO_2$-*p* (**11**) through *N*-acetylglucosaminyl transfer from *N*-, *N'*-diacetylchitobiose to *p*-nitrophenyl β-*N*-acetyllactosaminide. The enzyme formed the mixture of trisaccharides **9**, **10**, and **11** in a ratio of 11 : 33 : 56. In this case, when an inclusion complex of *p*-nitrophenyl β-*N*-acetyllactosaminide with α-CD was used, compounds **9**, **10**, and **11** were formed in a ratio of 24 : 63 : 13. The regioselectivity of glycosidase-catalyzed formation of the trisaccharide glycosides was substantially changed. It resulted not only in a significant increase of the proportion of the desired compounds **9** and **10**, but also in the substantial increase of the overall yield of transfer products.

Ⅲ. A consecutive synthetic method for obtaining β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* (**15**) and β-D-Gal-(1 → 3)-[β-D-GlcNAc-(1 → 6)]-α-D-GalNAc-$OC_6H_4NO_2$-*p* (**17**), which are carbohydrate units of mucin-types 1 and 2 core, was established. When α-D-GalNAc-$OC_6H_4NO_2$-*p* was used as an acceptor, β-Galactosidase from porcine testes synthesized mainly **15** with its (1→6) linked isomer **16**. The use of an inclusion complex of the glycoside acceptor with β-CD increased the efficiency of the transglycosylation by increasing the solubility of the acceptor. An β-*N*-acetyl-D-hexosaminidase from *N. orientalis* catalyzed the synthesis of the desired compound **17** with its isomers β-D-GlcNAc-(1→6)-β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* (**18**) β-D-GlcNAc-(1→3)-β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p* (**19**) through *N*-acetylglucosaminyl transfer from *N,N'*-diacetylchitobiose to β-D-Gal-(1→3)-α-D-GalNAc-$OC_6H_4NO_2$-*p*. The enzyme formed the trisaccharides **17**, **18**, and **19** 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'. These reactions were efficient enough to allow the one-pot preparation of the desired carbohydrate units of mucin-types 1and 2 core.

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inches 1 2 3 4 5 6 7 8

cm 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Kodak Color Control Patches

Blue Cyan Green Yellow Red Magenta White 3/Color Black

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A 1 2 3 4 5 6 **M** 8 9 10 11 12 13 14 15 **B** 17 18 19