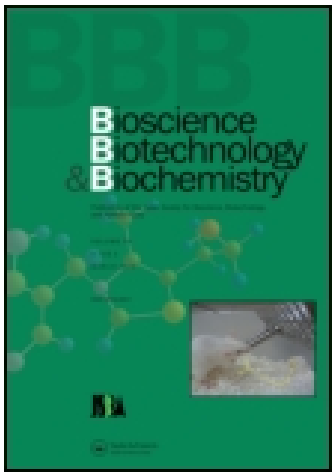

Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

Enzymatic Synthesis of Oligosaccharides Containing Galβ→4Gal Disaccharide at the Non-Reducing End Using β-Galactanase from Penicillium citrinum

Hiroshi Fujimoto[a], Hirofumi Nakano[ab], Megumi Isomura[a], Sumio Kitahata[ab] & Katsumi Ajisaka[a]

[a] Meiji Institute of Health Science, Naruda Odawara 250, Japan,

[b] Osaka Municipal Technical Research Institute, Osaka 536, Japan

Published online: 12 Jun 2014.

To cite this article: Hiroshi Fujimoto, Hirofumi Nakano, Megumi Isomura, Sumio Kitahata & Katsumi Ajisaka (1997) Enzymatic Synthesis of Oligosaccharides Containing Galβ→4Gal Disaccharide at the Non-Reducing End Using β-Galactanase from Penicillium citrinum, Bioscience, Biotechnology, and Biochemistry, 61:8, 1258-1261, DOI: 10.1271/bbb.61.1258

To link to this article: http://dx.doi.org/10.1271/bbb.61.1258

Biosci. Biotech. Biochem., **61** (8), 1258–1261, 1997

Enzymatic Synthesis of Oligosaccharides Containing Galβ→4Gal Disaccharide at the Non-Reducing End Using β-Galactanase from *Penicillium citrinum*

Hiroshi FUJIMOTO, Hirofumi NAKANO,* Megumi ISOMURA, Sumio KITAHATA,* and Katsumi AJISAKA

Meiji Institute of Health Science, Naruda Odawara 250, Japan,
**Osaka Municipal Technical Research Institute, Osaka 536, Japan*
Received November 27, 1996

The transglycosylation reaction was done with a β-galactanase from *Penicillium citrinum*. The regioselectivity in the transglycosylation reaction was studied using soy bean arabinogalactan as a donor and mono- or disaccharide derivatives containing β-galactosyl residue as acceptors. We also synthesized oligosaccharides containing Galβ1→4Gal sequence such as Galβ1→4Galβ1→4Glc, Galβ1→4Galβ1→3GlcNAc, Galβ1→4Galβ1→4GlcNAc, Galβ1→4Galβ1→6GlcNAc, and Galβ1→4Galβ1→3GalNAc for use in the total synthesis of complex sugar chains.

Key words: β-galactanase; *Penicillium citrinum*; transglycosylation

A great number of β-galactosidases (EC 3.2.1.23) of different origins have been characterized and their hydrolysis specificities have been identified.[1] β-Galactosidase can also catalyze the synthesis of galactosyl oligosaccharides by two mechanisms; reverse hydrolysis reaction and transglycosylation reaction. We have so far synthesized a wide variety of biologically important galactosyl oligosaccharides by using both reaction modes.[2-7] The transglycosylation reaction is generally known to have the following advantages in comparison with reverse hydrolysis; high specificity and high yield in a short period.

Although the transglycosylation reaction using β-galactosidase has been reported successfully by many researchers, there are still a few difficulties.[8-11] First, the transglycosylation products are formed *via* transfer of a galactose residue to an acceptor, however the hydrolysis reaction begins at the moment of oligosaccharide formation. Therefore the time to stop the reaction is critical to get a high yield of a target product. Secondly, if the acceptor contains a β-galactosyl linkage at the non-reducing end, the galactosyl linkage will also be hydrolyzed since it can be another substrate.

To overcome these drawbacks, we used endo-1,4-β-galactanase (EC 3.2.1.89) instead of β-galactosidase. The β-galactanases have been purified from various microorganisms.[12-15]; *Penicillium citrinum*, *Bacillus subtilis*, *Aspergillus niger*, and *Rhizopus niveus*. These β-galactanases have been reported to hydrolyze β1→3 or β1→4 linked galactan to release galactosyl oligosaccharides. β-Galactanases from *P. citrinum* and *B. subtilis* have been reported to transfer galactosyl oligosaccharide to alcohols and sugars.[16,17] However, little is known about the transglycosylation reaction using the β-galactanase.

In this study, we studied the regioselectivity in the transglycosylation reaction using β-galactanases from *P. citrinum* towards acceptors of different structure. Furthermore, we studied the efficient synthesis of the oligosaccharide blocks containing galactosyl β1→4 linkage as components for the total synthesis of sugar chains.

Materials and Methods

Materials. Galβ1→3GlcNAc, Galβ1→4GlcNAc, Galβ1→6GlcNAc, and Galβ1→3GalNAc were products of Sigma. Soy bean arabinogalactan was prepared by the procedure of Morita.[18]

Enzymes. β-Galactanase from *P. citrinum* was prepared and purified as described before.[12]

HPLC. A HPLC system with an Asahipak NH2P50 column (4.6×250 mm, Showa Denko Co., Ltd., Tokyo, Japan) was used with 70% acetonitrile as a solvent. An ELSD-MK III (Alltech Associates, Inc., U.S.A.) was used as an evaporated light scattering detector. A L-4200 (Hitachi, Japan) and a 875-UV (JASCO, Japan) were used as an UV detector.

^{13}C-NMR measurement. ^{13}C-NMR spectra were measured at 125 MHz on a Varian Unity-500 spectrometer using D_2O as a solvent and a small amount of acetonitrile (δ 1.27 ppm) as an internal standard.

Typical transglycosylation reaction and isolation process. A reaction mixture containing arabinogalactan (100 mg), β-galactoside or disaccharide (100 mg), 1 M sodium acetate buffer (pH 5.0, 200 μl), and purified β-galactanase (1 unit) in 2 ml of water was incubated at 37°C. After 48 h, the enzyme was inactivated by heating the reaction mixture in a boiling water bath for 5 min. The remaining arabinogalactan was removed by the addition of 4 ml of ethanol to the mixture. After the removal of ethanol by evaporation, 2 ml of water was added. The solution was put on an activated carbon column (1.6×40 cm). The products were eluted using a gradient from zero to 30% aqueous ethanol solution (1 liter each), at a flow rate of 2 ml/min. The eluent was collected in 20 ml fractions. In all the experiments listed in Table I and II, products were isolated by the column chromatography using activated carbon and the structure was confirmed by ^{13}C-NMR spectroscopy. The yields were calculated from the moles of product relative to that of acceptor.

Results and Discussion

Regioselectivity of the transglycosylation reaction to β-galactoside

β-Galactanase from *P. citrinum* has been reported to catalyze the hydrolysis of arabinogalactan, Galβ1-*O*-*o*NP, and Galβ1-*O*-*p*NP. This enzyme is also known to have a transglycosylation activity when arabinogalactan[16] or Galβ1-*O*-*o*NP[19] are used as donors, but it has broad specificity for an acceptor.[16] The structure of the transfer

Abbreviations: Galβ1-*O*-*o*NP, *o*-nitrophenyl β-D-galactopyranoside; Galβ1-*O*-*p*Np, *p*-nitrophenyl β-D-galactopyranoside; Galα1-OMe, methyl α-D-galactopyranoside; Galβ1-OMe, methyl β-D-galactopyranoside.

products towards β-galactosides has not been investigated in detail.

Therefore, we studied at first the transfer reaction to β-galactosides of various structure such as Galα1-OMe, Galβ1-OMe, and Galβ1-*O*-*p*NP using arabinogalactan as a donor. Results are summarized in Table I. When Galα1-OMe was used as an acceptor, Galβ1→4Galα1-OMe and Galβ1→4Galβ1→4Galα1-OMe were obtained regioselectively. In this case, the regioselectivity to form β1→4 linkages was strictly high. In contrast, when Galβ1-OMe was used as an acceptor, disaccharide derivatives of β1→3 and β1→4 linkage were obtained, indicating low regioselectivity. Moreover, it is confusing that Galβ1→4Galβ1-*O*-*p*NP and Galβ1→4Galβ1→4Galβ1-*O*-*p*NP were obtained regioselectively, when Galβ1-*O*-*p*NP was used as an acceptor. Thus the regioselectivity of transglycosylation reaction depended on the aglycon structure when β-galactosides was used as an acceptor.

Regioselectivity of the transglycosylation reaction to disaccharides.

Next, we examined the transfer reaction to disaccharides, because one of the merits of using β-galactanase is for the transglycosylation to oligosaccharides that have β-galactosyl residues in the non-reducing end. We tried transglycosylation using five kinds of disaccharides as acceptors, each containing β-galactosyl residue at non-reducing end. Figure 1 shows an HPLC chart of the reaction products using lactose as an acceptor. In the trisaccharide region, only the peak of Galβ1→4Galβ1→4Glc was observed together with a small peak corresponding to tetrasaccharide.

Similarly, in the reactions that β1→3, β1→4, and β1→6 linked Gal-GlcNAc were used as acceptors, galactose residue was transferred selectively to the 4-position of the galactose residue of acceptor disaccharides. Moreover, when Galβ1→3GalNAc was used as an acceptor, Galβ1→4Galβ1→3GalNAc was produced predominantly. These results indicated that the linkage and the structure of the reducing end sugar are not very important for the regioselectivity of glycosylation.

As summarized in Table II, in the reaction that disaccharides containing β-galactosyl linkage at non-reducing end were used as acceptors, only β1→4 linkage was formed regardless of the residue at the reducing end.

Table I. Summary of the Synthesis of Galactooligosaccharides (1)

Acceptor	Yield (%)[a]			
	Disaccharide		Trisaccharide	
	β1→3	β1→4	β1→3[b]	β1→4[b]
Galα1-OMe	n.d.[c]	18.8	n.d.[c]	3.5
Galβ1-OMe	7.0	15.5	n.d.[c]	2.5
Galβ1-*O*-*p*NP	n.d.[c]	14.0	n.d.[c]	7.5

[a] Yield was calculated as [(moles of the isoilated products)/(moles of acceptor)] × 100.
[b] β1→3 and β1→4 denote Galβ1→4Galβ1→3Galβ1-*O*-R and Galβ1→4Galβ1→4Galβ1-*O*-R, respectively.
[c] Not detected.

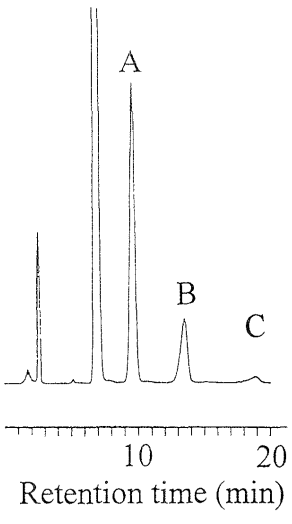

Fig. 1. HPLC Analysis of the Transglycosylation Reaction Using β-Galactanase from *P. citrinum*.
A, Galβ1→4Glc; B, Galβ1→4Galβ1→4Glc; C, Galβ1→4Galβ1→4Galβ1→4Glc.

Course of the reaction

In these transfer reactions, the enzyme used was endo-1,4-β-galactanase, which was assumed to transfer larger oligosaccharides. However the transfer products isolated in the above reactions were trisaccharide and a small amount of tetrasaccharide having β-galactoside and disaccharide containing β-galactosyl residue. To investigate the mechanism for the formation of tri- and tetrasaccharides, the course of the reaction was monitored for the reaction of 50 mg of arabinogalactan and 50 mg of Galβ1→4GlcNAc in the presence of 0.5 unit of β-galactanase. HPLC was measured at the appropriate times. Some of the HPLC chart is given in Fig. 2. At the initial stage of the reaction, tri- (A), tetra- (B), penta- (C), and hexasaccharide (D) peaks were observed in HPLC chart, then the peaks of penta- (C) and hexasaccharide (D) decreased with time and only the tri- (A) and tetrasaccharide (B) peaks remained after 48 h.

Similar results of the course of reaction were reported for the transglycosylation reaction using glycerol as an acceptor.[19] In that report, the reaction was monitored by its TLC pattern. Similarly to these results, the larger oligosaccharides observed at the initial stages of the reaction gradually decreased in the latter stage of the reaction.

It could be that galactosyl oligomers of various sizes might have been transferred to acceptor at the initial stage of the reaction, but the longer galactosyl oligosaccharides might be hydrolyzed with time to give, finally, tri- or tetrasaccharide.

Table II. Summary of the Synthesis of Galactooligosaccharides (2)

Acceptor	Product	Yield (%)[a]
Galβ1→4Glc	Galβ1→4Galβ1→4Glc	11.4
Galβ1→3GlcNAc	Galβ1→4Galβ1→3GlcNAc	11.2
Galβ1→4GlcNAc	Galβ1→4Galβ1→4GlcNAc	15.5
Galβ1→6GlcNAc	Galβ1→4Galβ1→6GlcNAc	17.6
Galβ1→3GalNAc	Galβ1→4Galβ1→3GalNAc	16.7

[a] See the footnote of Table I.

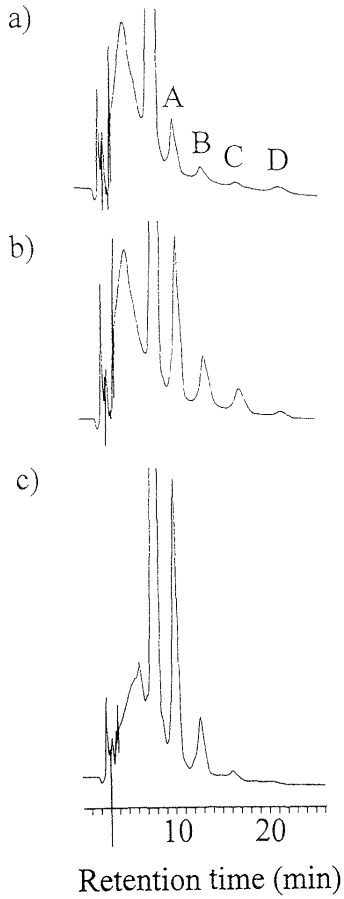

Fig. 2. Courses of the Transglycosylation Reaction after a) 0.5 h; b) 3 h; c) 48 h.

A, trisaccharide; B, tetrasaccharide; C, pentasaccharide; D, hexasaccharide.

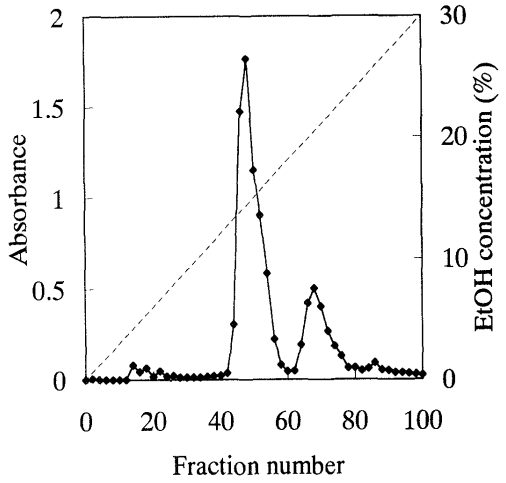

Fig. 3. Activated Carbon Column Chromatography of the Transglycosylation Reaction Products.

◆—◆, absorbance at 490 nm by phenol–sulfuric acid method; – – –, ethanol concentration; A, Galβ1→4Glc; B, Galβ1→4Galβ1→4Glc.

Factors controlling regioselectivity of the transglycosylation reaction by the β-galactanase

β-Galactanase acts on galactan in endo mode in the hydrolysis reaction, and can catalyze transglycosylation reaction. The regioselectivity was not high in the transfer reaction when monosaccharide derivatives were used as acceptors (Table I), but only β1→4 linkages were formed when disaccharide was used as an acceptor (Table II). The difference in the regioselectivity would be due to the difference between β-galactoside acceptors and disaccharide acceptors in the potentials to be recognized as a substrate by the β-galactanase. The result that disaccharide acceptors afforded β1→4 linkages with high regioselectivity may suggest that disaccharides containing β-galactosyl residues might be recognized as better substrates for the β-galactanase than β-galactosides.

In contrast, the transfer reaction towards various β-galactosides did not afford high regioselectivity. In the case of the reaction in which Galβ1-*O*-*p*NP was used as an acceptor, Galβ1-*O*-*p*NP might be recognized as a disaccharide, because phenyl group was large enough to be mistaken for a monosaccharide. On the other hand, methyl galactosides of both α- and β-structures might not be recognized as disaccharides, because the methyl group was too small to be mistaken for pyranose rings. Consequently, methyl galactosides would bind loosely with the enzyme and galactosyl donor attacked from two directions to form β1→3 linked and β1→4 linked disaccharides. The reaction in which Galα1-OMe was used as an acceptor might be an exceptional case, that the acceptor happened to bind with the enzyme so that the donor attacks the 4-position of the acceptor molecule.

Production and isolation of biologically important trisaccharides

Galβ1→4Galβ1→4Glc is found in human blood cells.[20] We synthesized this trisaccharide by using lactose as an acceptor. The HPLC pattern and elution pattern of the activated carbon column chromatography are given in Fig. 1 and in Fig. 3, respectively. Galβ1→4Galβ1→4Glc (33.6 mg) was obtained from 200 mg of galactan and 200 mg of lactose. Figure 4a shows the ^{13}C-NMR spectrum of the purified trisaccharide. A peak at 77.2 ppm shows the Galβ1→4Gal linkage formed newly.

Galβ1→4Galβ1→4GlcNAc is also a partial structure of a glycolipid in eggs of Indian Medaka Fish, *Oryzias melastigma*.[21] This trisaccharide (132 mg) was also synthesized by a similar transglycosylation from 600 mg of galactan and 600 mg of Galβ1→4GlcNAc. The ^{13}C-NMR spectrum in Fig. 4b also shows the formation of Galβ1→4Gal linkages by the peak at 77.2 ppm. As we have reported previously, Galβ1→4GlcNAc can be synthesized regioselectively by the transglycosylation reaction using β-galactosidase from *Streptococcus pneumoniae* or *Bifidobacterium bifidum*. Therefore this trisaccharide can be synthesized by the successive use of β-galactosidase and β-galactanase.

Similarly, Galβ1→4Galβ1→3GalNAc is a partial structure of a mucin type glycoprotein in rainbow trout eggs.[22] This trisaccharide (15.8 mg) was also regioselectively obtained by the reaction from 66.6 mg of galactan and 66.6 mg of Galβ1→3GalNAc. The structure was confirmed also by the presence of a peak at 77.2 ppm in the ^{13}C-NMR spectrum as shown in Fig. 4c.

These oligosaccharides are difficult to synthesize by a simple transglycosylation reaction catalyzed by β-galactosidase, because simultaneous hydrolysis of β-galactosyl linkages in the acceptor is inevitable. As exemplified above, however, these trisaccharides were easily synthesized using β-galactanase without the hydrolysis of the acceptor. In all

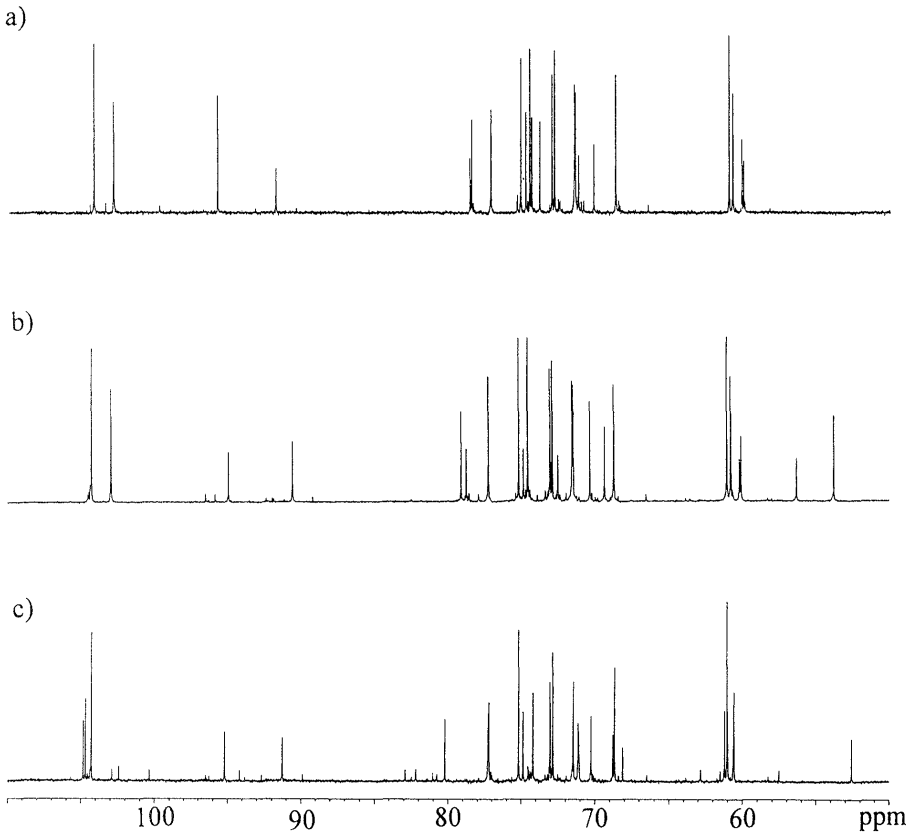

Fig. 4. ^{13}C-NMR Spectra of Trisaccharides.
a) Galβ1→4Galβ1→4Glc; b) Galβ1→4Galβ1→4GlcNAc; c) Galβ1→4Galβ1→3GalNAc.

of these reactions, yields are more than 10% and high enough for the production of aimed compounds. The trisaccharides synthesized here can be used as key intermediates for the total synthesis of glycoproteins or glycolipids.

Acknowledgments. This work was done as a part of the Research and Development Projects of Industrial Science and Technology Programs supported by NEDO (New Energy and Industrial Technology Development Organization).

References

1) K. Wallenfels and O. P. Malhotra, *Adv. Carbohydr. Chem.*, **16**, 239–298 (1961).
2) K. Ajisaka, H. Nishida, and H. Fujimoto, *Biotechnol. Lett.*, **9**, 387–392 (1987).
3) K. Ajisaka, H. Fujimoto, and H. Nishida, *Carbohydr. Res.*, **180**, 35–42 (1988).
4) K. Ajisaka and H. Fujimoto, *Carbohydr. Res.*, **185**, 139–146 (1989).
5) K. Ajisaka and H. Fujimoto, *Carbohydr. Res.*, **199**, 227–234 (1990).
6) K. Ajisaka and H. Fujimoto, and M. Isomura, *Carbohydr. Res.*, **259**, 103–115 (1994).
7) H. Fujimoto, M. Isomura, T. Miyazaki, I. Matsuo, R. Walton, T. Sakakibara, and K. Ajisaka, *Glycoconjugate J.*, **14**, 75–80 (1997).
8) T. Toba and S. Adachi, *J. Dairy Sci.*, **61**, 33–38 (1978).
9) T. Toba, A. Yokota, and S. Adachi, *Food Chem.*, **16**, 147–162 (1985).
10) R. E. Huber and K. Wallenfels, *Biochemistry*, **15**, 1994–2001 (1976).
11) V. Dumortier, J. Montreuil, and S. Bouquelet, *Carbohydr. Res.*, **201**, 115–123 (1990).
12) H. Nakano, S. Takenishi, and Y. Watanabe, *Agric. Biol. Chem.*, **49**, 3445–3454 (1985).
13) H. Nakano, S. Takenishi, S. Kitahata, and Y. Watanabe, *Eur. J. Biochem.*, **193**, 61–67 (1990).
14) P. Pellerin and J-M. Brillouet, *Carbohydr. Res.*, **264**, 281–291 (1994).
15) Y. Hashimoto, Y. Tsujisaka, and J. Fukumoto, *Nippon Nōgeikagaku Kaishi*, **43**, 831–836 (1969).
16) H. Nakano, S. Takenishi, and Y. Watanabe, *Agric. Biol. Chem.*, **52**, 1319–1322 (1988).
17) H. Nakano, S. Kitahata, H. Kinugasa, Y. Watanabe, H. Fujimoto, K. Ajisaka, and S. Takenishi, *Agric. Biol. Chem.*, **55**, 2075–2082 (1991).
18) M. Morita, *Agric. Biol. Chem.*, **29**, 564–573 (1965).
19) H. Nakano, S. Takenishi, and Y. Watanabe, *Agric. Biol. Chem.*, **52**, 1913–1921 (1988).
20) S. Hakomori, K. Stellner, and K. Watanabe, *Biochem. Biophys. Res. Commun.*, **49**, 1061–1068 (1972).
21) T. Taguchi, A. Seko, K. Kitajima, S. Inoue, T. Iwamatsu, K-H. Khoo, H. R. Morris, A. Dell, and Y. Inoue, *J. Biol. Chem.*, **268**, 2353–2362 (1993).
22) M. Iwasaki and S. Inoue, *Glycoconjugate J.*, **2**, 209–228 (1985).