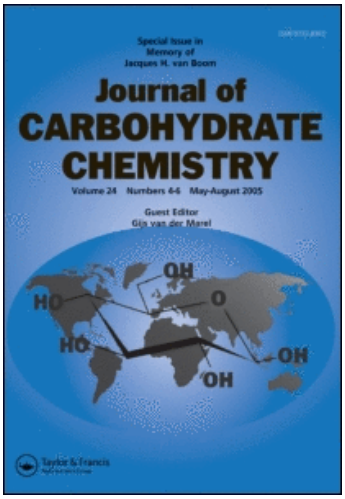

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Enzymatic Synthesis of α-D-Xylosylated Malto-oligosaccharides by Phosphorylase-catalyzed Xylosylation

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This paper describes the enzymatic synthesis of α-D-xylosylated malto-oligosaccharides by phosphorylase-catalyzed xylosylation of maltotetraose. When the xylosylation was carried out using α-D-xylose-1-phosphate as a glycosyl donor in the presence of phosphorylase, xylosylated oligosaccharides were produced with high conversion. α-D-Xylosyl-(1 → 4)-maltotetraose was isolated as the main product. Glucoamylase-catalyzed reaction of the isolated material revealed that one α-xyloside unit is positioned at the nonreducing end.

Keywords Enzymatic synthesis, Phosphorylase, Xylosylation, α-D-Xylosyl-(1 → 4)-malto-oligosaccharides, MALDI-TOF MS spectrum

INTRODUCTION

Hetero-oligosaccharides and heteropolysaccharides composed of multiple kinds of monosaccharide units play an important role in living systems, showing their multifunctional characteristics such as cell recognition and immune response.[1] Synthesis of novel heterosaccharide chains, therefore, is one of the most important research topics to develop new functions and applications in glycoscience. In order to supply such substrates with well-defined

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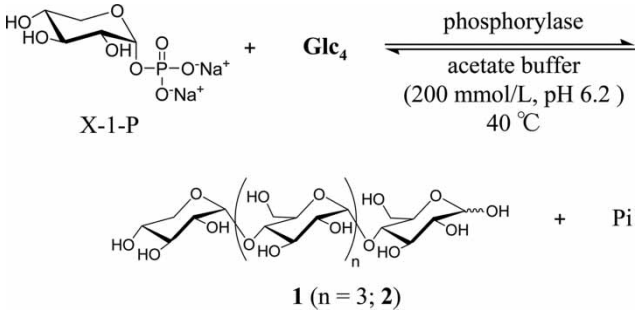
structures, highly selective glycosylations are in great demand.[2] Enzymatic glycosylation is a useful tool for the regio- and stereoselective construction of glycosidic bonds under mild conditions, where glycosyl donors and glycosyl acceptors can be employed in their unprotected forms, leading to the direct formation of unprotected saccharide chains in aqueous media.[3–7] In the enzymes involved in formation of glycosidic bonds, phosphorylases have potential to be employed in the practical synthesis of saccharide chains.[8]

Glycogen phosphorylase (starch phosphorylase or simply phosphorylase, EC 2.4.1.1) is the most extensively studied phosphorylase. This enzyme catalyzes the reversible catalytic phosphorolysis of α-1,4-glucans at the nonreducing end, such as glycogen and amylose, giving an α-D-glucose-1-phosphate (G-1-P).[8] By means of the reversibility of the reaction, α-1,4-glucans can be prepared by the phosphorylase-catalyzed chain elongation using G-1-P as a glycosyl donor.[9] At the beginning of the reaction, a malto-oligosaccharide primer is required. Since enzymes often have loose specificity for recognition of the substrate structure, extension of the enzymatic chain elongation or glycosylation using different substrates is useful to obtain new saccharide chains. Previously, α-D-mannose-1-phosphate and D-glucal were used as glycosyl donors for phosphorylase-catalyzed chain elongation, giving α-mannosylated and 2-deoxy-α-glucosylated oligosaccharides, respectively.[10,11] Schwarz et al. reported the kinetic analysis using G-1-P and α-D-xylose-1-phosphate (X-1-P) in the phosphorylase-catalyzed reaction to reveal the catalytic mechanism.[12] Although the phosphorylase displayed a very large kinetic selectivity ratio (i.e., $k_{catG1P}/k_{catX1P} = 20{,}000$ in the study), a possibility even for a little progress of the reaction using X-1-P may be denied. The detailed reaction and its product using X-1-P by phosphorylase have not been reported to date.

We now report the synthesis of unnatural α-D-xylosylated malto-oligosaccharides (**1**) by phosphorylase-catalyzed xylosylation of maltotetraose (Glc_4) using X-1-P. The xylosylated products were obtained in high conversions, and the main product was isolated by high-performance liquid chromatography (HPLC) after hydrolysis of general malto-oligosaccharides existing in the reaction mixture by glucoamylase (GA, EC 3.2.1.3). The structural characterization of the isolated material was conducted using the ^{1}H NMR and MALDI-TOF MS spectra. In relation to the present study, it was reported that cellodextrin phosphorylase (EC 2.4.1.49) was employed for enzymatic synthesis of β-(1 → 4)-hetero-D-glucose and D-xylose-based oligosaccharides using X-1-P as the substrate.[13]

RESULTS AND DISCUSSION

First, we examined the enzymatic reaction using X-1-P as a glycosyl donor and Glc_4 as a primer catalyzed by phosphorylase (Sch. 1). After the reaction was carried out using 10 equivalents of X-1-P for Glc_4 in acetate buffer (pH = 6.2)



Scheme 1: Enzymatic xylosylation of Glc_4 using X-1-P catalyzed by phosphorylase.

at 40°C for 4 d, the reaction mixture was lyophilized and analyzed. Figure 1 shows the ^{1}H NMR and MALDI-TOF MS spectra of the crude products. In the ^{1}H NMR spectrum (Fig. 1a), two significant signals due to the anomeric protons of the α-glucoside and α-xyloside units are observed at δ 5.35 (1b, 1b′) and δ 5.31 (1c, $J = 3.64$ Hz), respectively, in addition to the signals due to the anomeric protons of reducing ends (1a-α, 1a′-α, 1a-β, 1a′-β) and X-1-P.* Appearance of the α-xylosidic signal indicates progress of the xylosylation reaction, giving xylosylated malto-oligosaccharides. There are two possibilities for the assignment of the α-glucosidic signal, which is due to xylosylated and unxylosylated malto-oligosaccharides. In the MALDI-TOF MS spectrum (Fig. 1b), several peaks separated by $m/z = 162$ are observed, which correspond to the molecular masses of tetrasaccharide-octasaccharide containing one xylose unit. This finding indicates occurrence of both xylosylation by X-1-P and glucosylation by G-1-P, which is formed by phosphorolysis of Glc_4 at an early stage of the reaction. In fact, the other significant peak assignable to the molecular mass of maltotriose ($m/z = 527.6$) is also shown, which is obviously produced by phosphorolysis of Glc_4. Moreover, small peaks due to unspecified structures are observed at the region of high molecular masses (i.e., around $m/z = 1000-1800$). Although some of these peaks can be assigned to the molecular masses of the oligosaccharides consisting of two xylose units, the detailed structures have not yet been determined due to their very low yields. The analytical results for the crude products indicate that the main reaction in this system is transfer of one xylose residue to malto-oligosaccharides from X-1-P. However, the above data do not provide sufficient evidence to determine the structure **1** of the products, in which the xylose unit is positioned at the nonreducing end of the xylosylated

*The triplet signal due to an anomeric proton of β-D-xylose-1-phosphate is observed at δ 4.84, which was contained in the X-1-P as an impurity (α-X-1-P:β-X-1-P = 91:9). However, this impure material did not take part in the reaction.

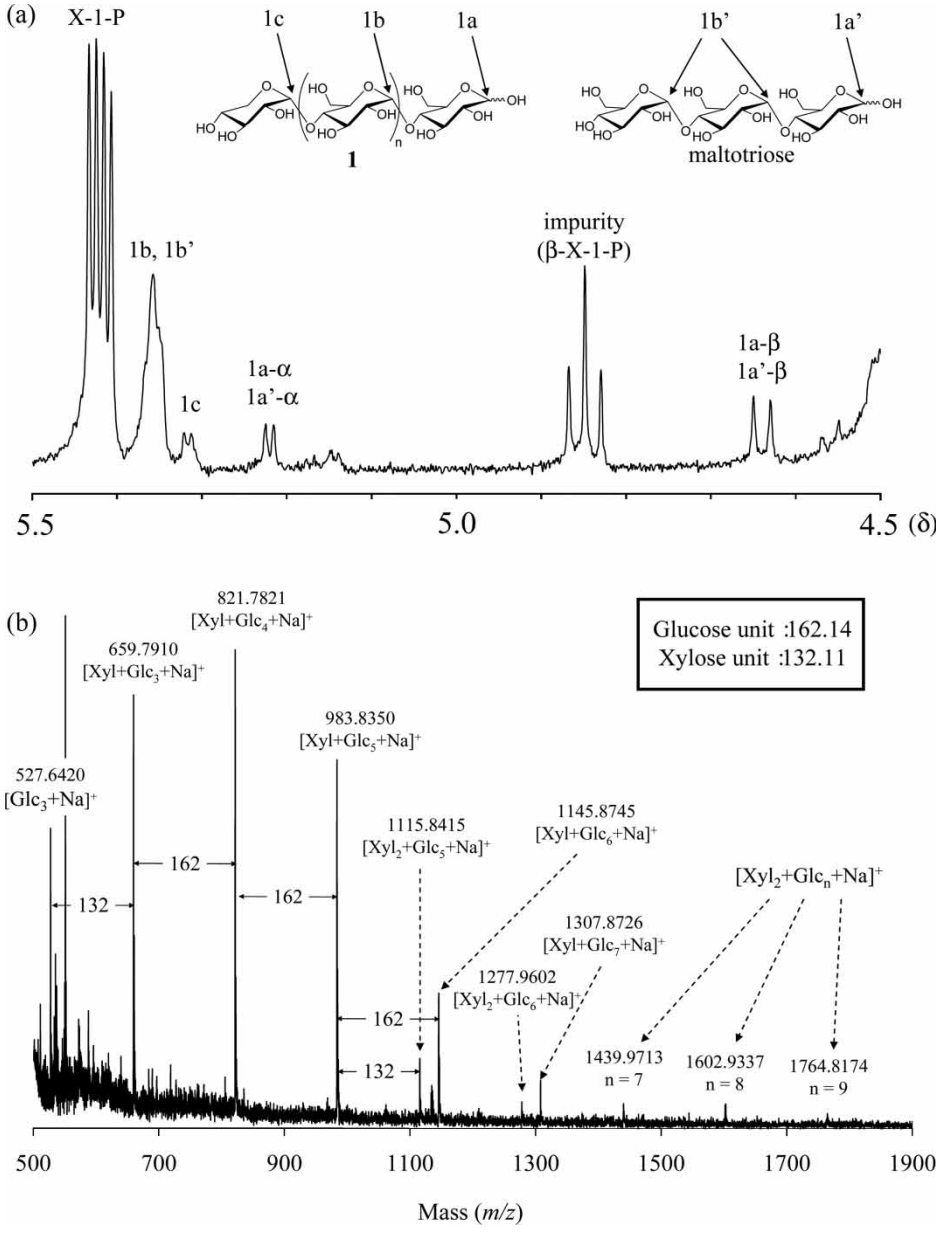


Figure 1: ^{1}H NMR (a) and MALDI-TOF MS (b) spectra of crude products.

oligosaccharides. Consequently, the hydrolysis of the reaction mixture was carried out with GA, which catalyzes an exowise hydrolysis at the nonreducing end of α-1,4-glucan. The hydrolysis of the crude products was carried out under the appropriate conditions in the presence of GA. In the ^{1}H NMR spectrum of

the hydrolysis product (Fig. 2), the peak at δ 5.35 assigned to the anomeric protons of the α-glucoside units decreased, whereas the peak at δ 5.31 due to the anomeric protons of the α-xyloside units remains intact. These results indicate that only unxylosylated malto-oligosaccharides (i.e., mainly maltotriose) are hydrolyzed by GA to the exclusion of hydrolysis of xylose-containing products, supporting that the xylose unit is positioned at the nonreducing end of the xylosylated oligosaccharides. Furthermore, the structure of the main product is estimated by the peak ratio of the anomeric protons of the α-xyloside units (1c) to the peak due to that of the α-glucoside units (1b) to be α-D-xylosylated Glc_4. Figure 3 shows the total concentrations of **1** versus reaction times under the conditions as the ratios of Glc_4 to X-1-P = 1:10, 1:5, and 1:1. The concentrations of **1** were determined by the integrated ratios of the peak due to the anomeric protons of the α-xyloside units to the peaks due to those of the reducing end in the ^{1}H NMR spectra of the crude products. Highest yields of 44% (1:10), 25% (1:5), and 10% (1:1) based on the amounts of Glc_4 used were obtained after 48 h. According to the result of the MS spectrum of the crude products (Fig. 1b), the rest of the main products can be assumed to be maltotriose. For further analysis, the main product as a part of the peak with the highest intensity in the chromatographic profile was isolated by HPLC using an ODS column. In the ^{1}H NMR spectrum of the isolated material (Fig. 4a), no signal (δ 3.40) ascribed to the H-4 proton at the nonreducing end of Glc_4 is observed. Furthermore, if the xylose unit is linked to a glucose unit by a glycosidic bond besides the α-(1 $\rightarrow$ 4)-bond, such as the α-(1 $\rightarrow$ 6)-bond, the signal due to the H-4 proton of the glucose unit reasonably appears at around δ 3.40–3.50.[14] No appearance of the NMR

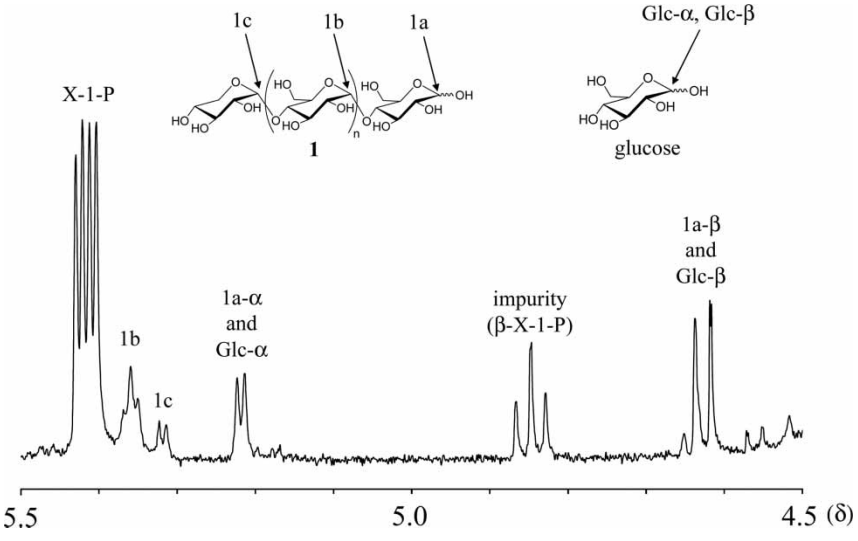


Figure 2: ^{1}H NMR spectrum of crude products after hydrolysis with glucoamylase.

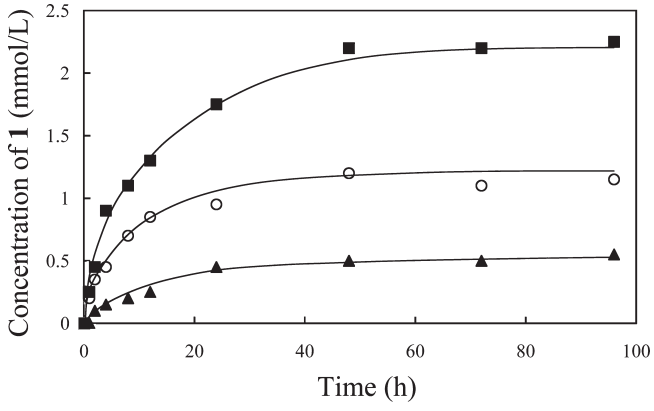


Figure 3: Concentration of **1** versus reaction time in phosphorylase-catalyzed xylosylation. Reaction conditions: in acetate buffer 2 mL (100 mmol/L, pH 6.2); amount of enzyme, 15 U/mL; reaction temperature, 40°C; initial concentration of a primer (Glc_4), 5 mmol/L; Glc4:X-1-P = (■) 1:10, (○) 1:5, (▲) 1:1.

signals at the regions in Figure 4a, therefore, strongly indicates that the xylose unit is positioned at the nonreducing end, which is linked to the glucose unit by the α-(1 → 4)-bond in the isolated material. In the MALDI-TOF MS spectrum (Fig. 4b), the only peak that corresponds to the molecular mass of α-xylosylated Glc_4 is observed. These analytical results can be taken to support the structure of α-xylosyl-(1 → 4)-Glc_4 (**2**). Moreover, it was confirmed further that isolated **2** was not hydrolyzed with GA, strongly indicating that the xylose unit is positioned at the nonreducing end.

In conclusion, we examined the synthesis of α-D-xylosylated malto-oligosaccharides by phosphorylase-catalyzed xylosylation using X-1-P as a glycosyl donor and Glc_4 as a primer. The progress of the xylosylation was confirmed by the ^{1}H NMR and MALDI-TOF MS spectra of the crude products. Moreover, α-D-Xylosyl-(1 → 4)-maltotetraose (**2**) was isolated as a main product, which was characterized by the ^{1}H NMR and MALDI-TOF MS spectra as well as its GA-catalyzed reaction.

EXPERIMENTAL

General Method

The starting material for the synthesis of X-1-P was prepared following literature procedures.[15] Phosphorylase (300 U/mL) was supplied by Ezaki Glico Co. Ltd.[16] Other reagents and solvents were used as received. NMR spectra were recorded on a JEOL ECX400 spectrometer. MALDI-TOF MS measurements were carried out by using SHIMADZU Voyager Biospectrometry Workstation Ver. 5.1 with 2,5-dihydroxybenzoic acid as a matrix

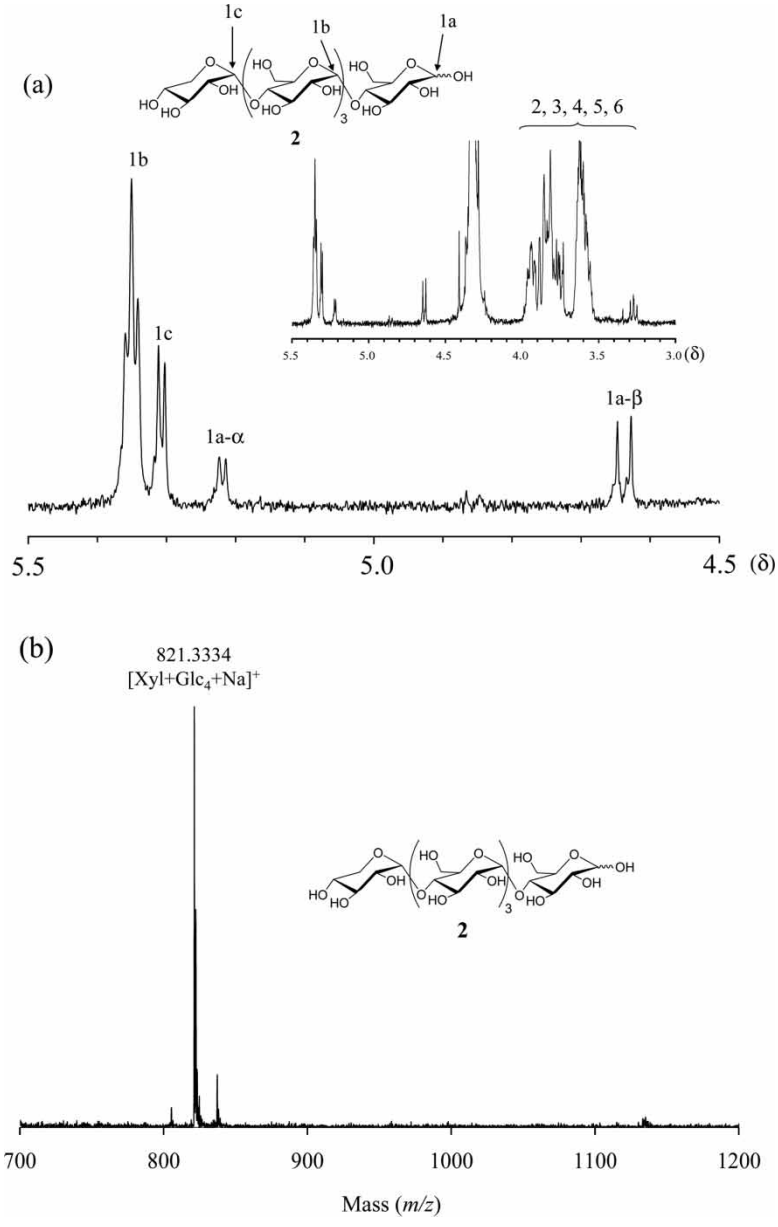


Figure 4: ^{1}H NMR (a) and MALDI-TOF MS (b) spectra of **2**.

containing 0.05% trifluoroacetic acid under positive ion mode. HPLC analyses were performed on a HITACHI L-2130 apparatus using L-2490 RI detector with Waters μBondapack C18 column (3.9 $\times$ 150 mm) with water as the eluent (flow rate, 0.3 mL/min; rt).

Synthesis of X-1-P [17,18]

Peracetylated α-D-xylose (0.6876 g, 2.16 mmol)[15] was dissolved in anhydrous phosphoric acid (2.1686 g, 21.6 mmol) in anhydrous THF (10 mL) and the solvent was evaporated to give the syrupy residue, which was stirred further under reduced pressure at 40°C for 2 h. Under argon, 1 mol/L aqueous solution of lithium hydroxide (180 mL) was added to the residue and the milky suspension was stirred overnight at rt. After it was filtered, the filtrate was neutralized with Amberlite IRC76 (H^+ form) ion exchange resin. The neutral solution was concentrated and poured into ethanol to precipitate the product, which was filtered and dried. After the resulting white powder was dissolved in water, the solution was treated with IRC76 (Na^+ form) ion exchange resin, concentrated, and reprecipitated into ethanol. The precipitate was isolated by filtration and dried in vacuo to give X-1-P as white powder (0.1781 g, 0.65 mmol) in 30% yield (α:β = 91:9). ^{1}H NMR (400 MHz, D_2O): δ 5.39 (dd, 1H, $J_{1,2}$ = 3.2 Hz, $J_{1,P}$ = 7.0 Hz, α-H-1), 3.78–3.43 (m, 5H, H-2, 3, 4, 5). ^{31}P NMR (162 MHz, D_2O): δ 1.61.

Enzymatic Xylosylation of Malto-oligosaccharides

A typical xylosylation procedure was as follows. A mixture of X-1-P (0.0275 g, 0.1 mmol) and maltotetraose (0.0067 g, 0.01 mmol) in 200 mmol/L acetate buffer solution (2 mL) was incubated with phosphorylase (30 U) for 4 d at 40°C. After the reaction, the reaction mixture was heated for 30 min at 100°C, filtered, and lyophilized to give the crude products.

*Isolation of α-D-Xylosyl-(1 → 4)-maltotetraose (**2**)*

For the isolation of the main product, a mixture of X-1-P (0.2748 g, 1 mmol) and maltotetraose (0.0667 g, 0.1 mmol) in 200 mmol/L acetate buffer solution (5 mL) was incubated with phosphorylase (60 U) for 4 d at 40°C, followed by addition of glucoamylase (92 U). After incubation further for 1 h at 40°C, the solution was heated for 30 min at 100°C, filtered, and lyophilized. The resulting powder was dissolved in water (3 mL) and 0.5 mL of the solution was subjected by HPLC with Waters μBondapack C18 column to isolate the main product. The fractions due to pentasaccharide were collected and dried in vacuo to give **2** as a white solid (0.5 mg, 3.7%). ^{1}H NMR (400 MHz, D_2O): δ 5.35 (m, 3H, Glc-H-1), 5.30 (d, 1H, $J_{1,2}$ = 3.64 Hz, Xyl-H-1), 5.22 (d, 0.4H, $J_{\alpha 1,2}$ = 3.64 Hz, α-H-1), 4.62 (d, 0.6H, $J_{\beta 1,2}$ = 7.8 Hz, β-H-1), 3.90–3.27 (m, 29H, Glc-H-2,3,4,5,6, Xyl-H-2,3,4,5).

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