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Phosphorylase-catalyzed *N*-formyl-α-glucosaminylation of maltooligosaccharides

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ABSTRACT

This paper describes the phosphorylase-catalyzed enzymatic *N*-formyl-α-glucosaminylation of maltooligosaccharides for direct incorporation of 2-deoxy-2-formamido-α-D-glucopyranose units into maltooligosaccharides. When the reaction of 2-deoxy-2-formamido-α-D-glucopyranose-1-phosphate (GlcNF-1-P) as the glycosyl donor and maltotetraose as a glycosyl acceptor was performed in the presence of phosphorylase, the *N*-formyl-α-D-glucosaminylated pentasaccharide was produced, as confirmed by MALDI-TOF MS. Furthermore, the glucoamylase-catalyzed reaction of the crude products supported that the 2-deoxy-2-formamido-α-D-glucopyranoside unit was positioned at the non-reducing end of the pentasaccharide. The pentasaccharide was isolated from the crude products and its structure was further determined by the ^{1}H NMR analysis. On the other hand, when the phosphorylase-catalyzed reactions of maltotriose and maltopentaose using GlcNF-1-P were conducted, no *N*-formyl-α-glucosaminylation took place in the former system, whereas the latter system gave *N*-formyl-α-D-glucosaminylated oligosaccharides with various degrees of polymerization. These results could be explained by the recognition behavior of phosphorylase toward maltooligosaccharides.

1. Introduction

In addition to naturally occurring oligo- and polysaccharide structures displayed on cell surfaces that are involved in molecular recognition processes mediated by binding events,[1] unnatural saccharide structures can be expected to show new functions and applications in glycoscience such as potential as drug candidates.[2] Therefore, approaches for the efficient syntheses of unnatural building blocks for the assembly of complex oligosaccharides composed of multiple different monosaccharide units still receive significant attention. Oligosaccharides containing 2-amino-2-deoxy-D-glucopyranose (D-glucosamine, GlcN) units and its derivatives, such as 2-acetamido-2-deoxy-D-glucopyranose (*N*-acetyl-D-glucosamine, GlcNAc), serve key functions in living organisms such as in cell–cell recognition and immune responses. The preparation of saccharide chains containing GlcN derivatives, therefore, has been frequently required for the various studies in glycoscience. Because highly selective glycosylations are promising approaches to supply such substrates with well-defined structures, much effort has been focused on glycosylation using glycosyl donors derived from GlcNAc and other N-substituted GlcN residues, such as the oxazoline glycosylation.[3]

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.[4] Among the enzymes involved in the formation of glycosidic bonds, phosphorylases have the potential to be employed in the practical synthesis of saccharide chains.[5] α-Glucan phosphorylase (EC 2.4.1.1, simply phosphorylase) is the most extensively studied phosphorylase. This enzyme catalyzes the reversible phosphorolysis of α-(1→4)-glucans such as glycogen and amylose, giving α-glucose 1-phosphate (Glc-1-P). Due to the reversibility of the reaction, α-(1→4)-glucosidic linkages can be prepared by the phosphorylase-catalyzed chain-elongation (glycosylation) using Glc-1-P as a glycosyl donor.[6] To initiate the reaction, a maltooligosaccharide as a glycosyl acceptor is required.

The smallest substrates typically accepted for phosphorolysis and glycosylation by phosphorylase are maltopentaose (Glc_5) and maltotetraose (Glc_4), respectively. Because enzymes often express loose specificity for substrate structures, extension of the enzymatic chain-elongation or glycosylation using different substrates is useful to obtain unnatural oligosaccharides. Previously, α-D-mannopyranose-1-phosphate, 2-deoxy-α-D-glucopyranose-1-phosphate, and α-D-xylopyranose-1-phosphate were used as the glycosyl donors for phosphorylase-catalyzed glycosylation, giving α-mannosylated,[7a] 2-deoxy-α-glucosylated,[7b] and α-xylosylated[7c]

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oligosaccharides, respectively. These results indicate that phosphorylase does not strictly recognize the hydroxy groups at positions 2 and 6 in the donor.

On the basis of the above-mentioned considerations, we have focused on the use of enzymatic glycosylation to incorporate α-GlcN derivatives directly into oligosaccharide chains, catalyzed by phosphorylase. The aforementioned previous studies on the extension of the phosphorylase-catalyzed glycosylation using different substrates inspired us to examine the possibility of the recognition of 1-phosphates of GlcN derivatives as the glycosyl donor by this enzyme, because GlcN is a sugar having an amino group at position 2 in the place of a hydroxy group of a glucose. This would allow the facile and highly selective glycosylation for the formation of α-(1→4)-glucosaminyl linkages. In our previous study in this area, we found that 2-amino-2-deoxy-α-D-glucopyranose-1-phosphate (GlcN-1-P) was recognized as the glycosyl donor by phosphorylase, leading to direct incorporation of a GlcN unit at the non-reducing end of maltooligosaccharides.[8] To the best of our knowledge, this report was the first example of the enzymatic α-glycosaminylation using the GlcN donor with a free amino group. However, we also confirmed that phosphorylase did not recognize 2-acetamido-2-deoxy-α-D-glucopyranose- 1-phosphate (GlcNAc-1-P) as the glycosyl donor. This is probably because the bulky acetamido group prevented approach to the phosphorylase active site. We have continued to study the extension of the phosphorylase-catalyzed α-glucosaminylation by investigating the use of 1-phosphates of other GlcN derivatives as glycosyl donors. Construction of the new saccharide chains containing different GlcN units has the potential to lead to the development of new applications of unnatural substrates, such as drug candidates. In this paper, we report that 2-deoxy-2-formamido-α-D-glucopyranose-1-phosphate (GlcNF-1-P), which has a smaller substituent, that is, a formamide group rather than the acetamide of GlcNAc-1-P, is recognized as a glycosyl donor by phosphorylase. This allows the glycosylation of maltooligosaccharides to give *N*-formyl-α-D-glucosaminylated maltooligosaccharides.

2. Results and discussion

We examined the enzymatic *N*-formyl-α-glucosaminylation using GlcNF-1-P as the glycosyl donor and Glc_4 as the glycosyl acceptor catalyzed by phosphorylase (from potato) in sodium acetate buffer solution (pH 6.2) at 40 °C for 4 days. Maltotetraose (Glc_4) is the smallest glycosyl acceptor for the phosphorylase-catalyzed glycosylation and five equivalents of GlcNF-1-P was used relative to Glc_4. After the reaction mixture was lyophilized, the

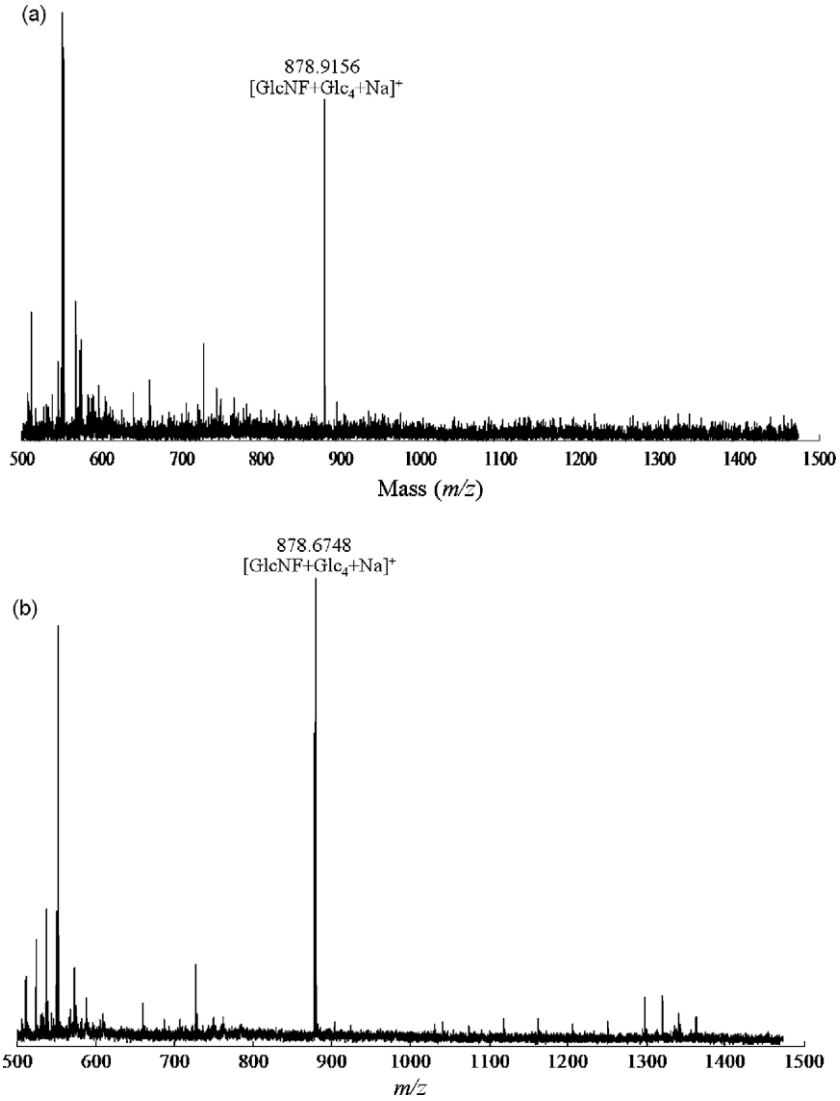


Figure 1. MALDI-TOF MS spectra of the crude products by enzymatic reaction of maltotetraose (a) and the hydrolyzed products by glucoamylase (b).

MALDI-TOF MS measurement was conducted to confirm the transfer of the GlcNF unit to the maltooligosaccharides.

In the MALDI-TOF MS spectrum of the crude products (Fig. 1a), only a significant peak corresponding to the mass of a pentasaccharide containing one GlcNF unit (GlcNF-Glc_4) is observed (*m/z* 878.9). This data indicates that the transfer of one GlcNF residue to Glc_4 from GlcNF-1-P occurred under these conditions. However, it was not evident from this data that the GlcNF unit was positioned at the non-reducing end of the produced pentasaccharide as shown in Scheme 1. Consequently, the hydrolysis of the crude products was performed using glucoamylase (GA, EC 3.2.1.3, Wako Pure Industries) to reveal whether the GlcNF unit was positioned at the non-reducing end. Glucoamylase catalyzes the *exo*-type hydrolysis from the non-reducing end of α-(1→4)-glucans. In the MALDI-TOF MS spectrum of the hydrolyzed products (Fig. 1b), the peak assigned to the molecular mass of the produced pentasaccharide remained intact, supporting that the GlcNF unit is positioned at the non-reducing end. If the transfer of one GlcNF residue to Glc_4 from GlcNF-1-P proceeds once, further *N*-formyl-α-glucosaminylation is probably suppressed because GlcNF-Glc_4 is not recognized as a glycosyl acceptor by phosphorylase.

For further analysis, the formation of *N*-formyl-α-D-glucosaminylated oligosaccharides versus reaction time in the phosphorylase-catalyzed glycosylation using GlcNF-1-P and Glc_4 (5:1) was evaluated and compared with that using GlcN-1-P as well as Glc-1-P under the same conditions. The total yields of the glycosylated products were calculated on the basis of the amounts of inorganic phosphate produced from the glycosyl donor by the phosphorylase-catalyzed glycosylation, which were measured by a modified Fiske–Subbarow assay.[9] A reaction time of 4 days in the glycosylation using GlcNF-1-P gave 33% yield of the products based on the amount of Glc_4, whereas the yield was 72% in the glycosylation using GlcN-1-P under the same conditions (Fig. 2). These data indicate that phosphorylase recognizes GlcN-1-P more efficiently than GlcNF-1-P. However, the reaction using Glc-1-P was much faster than such two reactions and the yield reached almost 100% in 45 min, because Glc-1-P is the native substrate for phosphorylase.

Moreover, GlcNF-Glc_4 was the nearly sole product, as judged by the MALDI-TOF MS spectra (Fig. 1). This compound was isolated from the hydrolyzed crude products by the following subsequent procedures; acetylation, column chromatography, and deacetylation. Thus, the isolated product was analyzed by the ^{1}H NMR measurement. Besides the signals due to the anomeric proton of the reducing ends at δ 5.22 ppm (H-1α) and δ 4.64 ppm (H-1β) in the ^{1}H NMR spectrum (Fig. 3), two kinds of the anomeric signals are observed at around δ 5.34–5.37 (H-1′) and δ 5.39 ppm (H-1″, J = 3.7 Hz), which are assignable to the α-D-glucopyranoside and

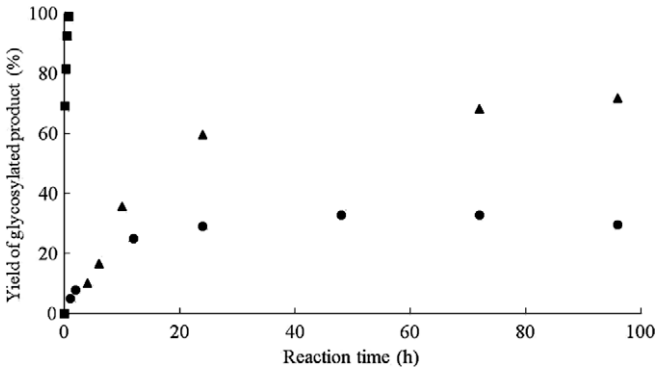
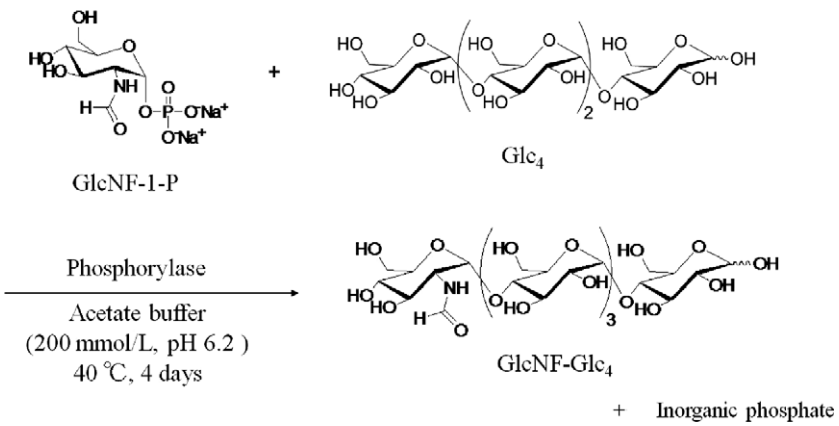


Figure 2. Total yield of glycosylated products versus reaction time in the phosphorylase-catalyzed glycosylation using GlcNF-1-P, GlcN-1-P, and Glc-1-P as the glycosyl donors and Glc_4 as a glycosyl acceptor (glycosyl donor–glycosyl acceptor 5:1): ●; GlcNF-1-P, ▲; GlcN-1-P, ■; Glc-1-P.

2-formamido-2-deoxy-α-D-glucopyranoside residues, respectively. The integrated ratio of the signals due to H-1α + β:H-1′:H-1″ is 1:3:1, supporting the pentasaccharide structure. Furthermore, there is no signal (δ 3.40 ppm) assigned to the H-4 position of a glucose residue at the non-reducing end of Glc_4, whereas there is a signal (H-4″, δ 3.51 ppm) ascribed to the free H-4 position of GlcNF. This observation indicates that the GlcNF unit is positioned at the non-reducing end, probably bound with the α-(1→4)-linkage. Moreover, the two signals at δ 8.04 and 8.17 ppm are observed, which are assigned to *E*- and *Z*-*N*-formyl groups due to hindered rotation of the formyl C–N bond (two rotamers).[10] The above-mentioned NMR analysis fully supports the GlcNF-Glc_4 structure of the isolated oligosaccharide.

We also examined the phosphorylase-catalyzed reaction using GlcNF-1-P as a glycosyl donor and maltotriose (Glc_3) or maltopentaose (Glc_5) as a glycosyl acceptor under the same conditions. In the MALDI-TOF MS spectrum of the crude products using Glc_3, no peaks assignable to the molecular masses of oligosaccharides having a GlcNF unit are observed (Fig. 4a), indicating no occurrence of *N*-formyl-α-glucosaminylation of Glc_3 by GlcNF-1-P. Because the smallest glycosyl acceptor for the phosphorylase-catalyzed glycosylation is Glc_4, Glc_3 was not recognized by phosphorylase. In the MALDI-TOF MS spectrum of the crude products using Glc_5, on the other hand, several peaks separated by *m/z* = 162 are observed (Fig. 4b), which correspond to the molecular masses of pentasaccharides–octasaccharides containing one GlcNF unit. This finding indicates occurrence of both the *N*-formyl-α-glucosaminylation by GlcNF-1-P and glucosylation by Glc-1-P when Glc_5 was used



Scheme 1. Enzymatic *N*-formyl-α-glucosaminylation of maltotetraose using GlcNF-1-P.

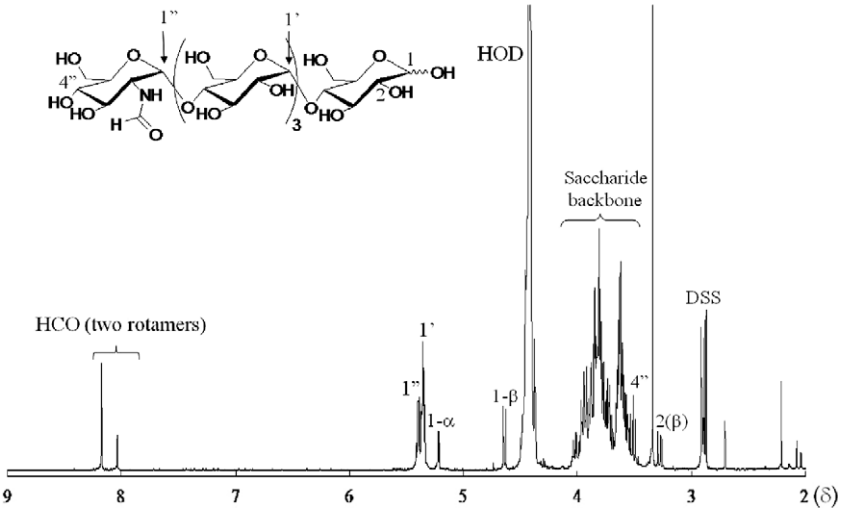


Figure 3. ^{1}H NMR spectrum of the isolated material (D_2O).

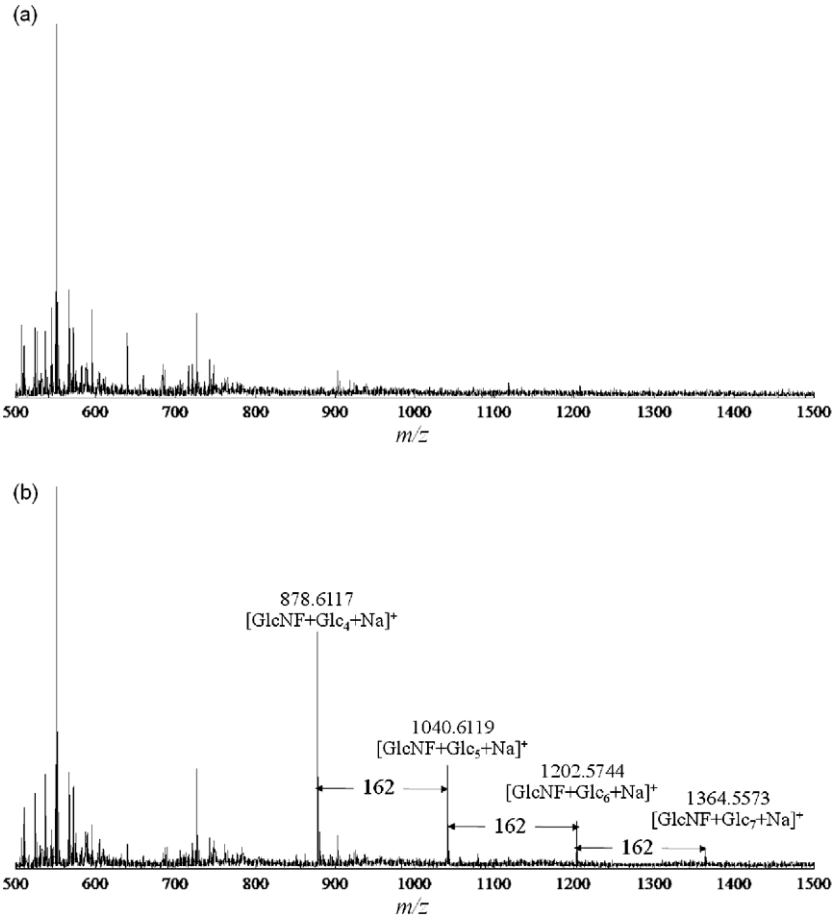


Figure 4. MALDI-TOF MS spectra of the crude products by enzymatic reaction of maltotriose (a) and maltopentaose (b).

as the glycosyl acceptor. Because Glc_5 is the smallest substrate for the phosphorolysis in the presence of inorganic phosphate, Glc-1-P is possibly produced by phosphorolysis of Glc_5 with simultaneously production of Glc_4 at an early stage of the reaction, where inorganic phosphate is formed by the transfer of a GlcNF unit to Glc_5. Then, Glc-1-P is recognized more efficiently by the enzyme than GlcNF-1-P. Thus, the maltooligosaccharides with larger degrees of polymerization (DPs), such as Glc_6 and Glc_7, are produced by the transfer of the glucose residues to Glc_4 or Glc_5 from Glc-1-P. Furthermore, if *N*-formyl-α-glucosaminylation of the formed maltooligosaccharides by GlcNF-1-P proceeds, subsequent glycosylation is probably suppressed because the *N*-formyl-α-D-glucosaminylated oligosaccharides are less efficiently recognized by phosphorylase.

In the reaction using Glc_5, therefore, multiple oligosaccharides of the GlcNF-Glc_{4-7} are produced in contrast to the reaction with Glc_4. The total yield of the multiple *N*-formyl-α-glucosaminylated oligosaccharides in the reaction using Glc_5 after ~50 h was estimated as 16% based on the determination of produced inorganic phosphate during the reaction. Then, the obtained crude products were subjected to high performance anion exchange chromatography (HPAEC) analysis after glucoamylase-catalyzed hydrolysis (40 °C for 18 h) to determine the ratio of each oligosaccharide. The ratio of peak areas corresponding to GlcNF-G_4, GlcNF-G_6, and GlcNF-G_7 in the HPAEC chart was 1:0.30:0.15. However, a peak area of GlcNF-G_5 could not be calculated under this HPAEC conditions because the corresponding peak was completely overlapped with a peak due to residual GlcNF-1-P.

In conclusion, we examined the phosphorylase-catalyzed enzymatic *N*-formyl-α-glucosaminylation of maltooligosaccharides using GlcNF-1-P. The results of the reactions depended on the DPs of maltooligosaccharides used as the glycosyl acceptor. When Glc_4 and Glc_5 were used as the glycosyl acceptor, *N*-formyl-α-glucosaminylation occurred to produce the sole product of GlcNF-Glc_4 and the multiple products of GlcNF-Glc_{4-7}, respectively. The former product was isolated and the structure was further determined by the ^{1}H NMR analysis. On the other hand, the use of Glc_3 as the glycosyl acceptor did not afford any *N*-formyl-α-glucosaminylated products. As reported in our previous paper,[8] it should be noted that GlcNAc-1-P was not recognized by phosphorylase. The smaller substituent of the formamido group in GlcNF-1-P, compared with the more bulky acetamido group in GlcNAc-1-P, probably allows the approach of the substrate to the active site of the enzyme.

3. Experimental

3.1. Materials

Phosphorylase (from potato) was supplied by Ezaki Glico Co. Ltd.[11] Glucoamylase was purchased from Wako Pure Chemical Industries (Osaka, Japan). Other reagents and solvents were used as received.

3.2. Synthesis of 2-deoxy-2-formamido-α-D-glucopyranose 1-phosphate disodium salt (GlcNF-1-P)[12]

Under argon, a solution of sodium methoxide (3.60 g, 66.6 mmol) in CH_3OH (100 mL) was added to a suspension of 2-amino-2-deoxy-D-glucopyranose hydrochloride (10.0 g, 46.3 mmol) in CH_3OH (100 mL) at room temperature and the mixture was stirred for 10 min. After triethylamine (6.00 mL, 46.3 mmol) was added to the mixture, methyl formate (40.0 mL, 324 mmol) was added with vigorous stirring at 0 °C. After the mixture was stirred at room temperature overnight, the resulting precipitate was isolated by filtration, washed with CH_3OH, and dried under reduced pressure to give 2-deoxy-2-formamido-D-glucopyranose (7.46 g, 36.9 mmol) in 80% yield. ^{1}H NMR (D_2O, α:β = 0.6:0.4) δ 3.17–3.98 (m, 6H, H-2,3,4,5,6), 4.72, 4.75 (2d, J = 8.3, 8.2 Hz, respectively, 0.4H, H-1(β)), 5.21, 5.25 (2d, J = 3.6, 3.7 Hz, respectively, 0.6H, H-1(α)), 8.02, 8.04, 8.17, 8.21 (4s, 1H, HCO (two pairs of rotamers of α and β anomers)).

Under argon, acetyl chloride (26.0 mL, 366 mmol) was added to 2-deoxy-2-formamido-D-glucopyranose (7.30 g, 36.1 mmol) at 0 °C and the mixture was stirred at room temperature overnight. After the reaction mixture was diluted with $CHCl_3$, the solution was washed with saturated aq $NaHCO_3$, dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was subjected to column chromatography on silica gel (hexane–EtOAc 3:2 (v/v)) to give 3,4,6-tri-*O*-acetyl-2-deoxy-2-formamido-α-D-glucopyranosyl chloride (5.00 g, 14.2 mmol) in 40% yield. ^{1}H NMR ($CDCl_3$) δ 2.04, 2.06, 2.11 (3s, 9H, CH_3), 4.14 (d, J = 10.5 Hz, 1H, H-6a), 4.28–4.32 (m, 2H, H-5,6b), 4.63 (ddd, J = 4.1, 8.7, 10.6 Hz, 1H, H-2), 5.23 (t, J = 10.1 Hz, 1H, H-4), 5.37 (t, J = 10.6 Hz, 1H, H-3), 5.95 (d, J = 8.7 Hz, 1H, NH), 6.20 (d, J = 4.1 Hz, 1H, H-1α), 8.18 (s, 1H, HCO).

To a solution of 3,4,6-tri-*O*-acetyl-2-deoxy-2-formamido-α-D-glucopyranosyl chloride (2.18 g, 6.20 mmol) in $CHCl_3$ (10.0 mL) were added pyridine (0.500 mL, 6.20 mmol) and water (0.110 mL, 620 mmol) at room temperature and the mixture was stirred overnight. The reaction mixture was concentrated and the residue was subjected to column chromatography on silica gel ($CHCl_3$–CH_3OH 80:1 (v/v)) to give 3,4,6-tri-*O*-acetyl-2-deoxy-2-formamido-α-D-glucopyranose (1.03 g, 3.12 mmol) in 50.3% yield. ^{1}H NMR ($CDCl_3$) δ 2.05, 2.10, 2.18 (3s, 9H, CH_3), 3.67 (br s, 1H, OH), 4.13–4.27 (m, 3H, H-5,6), 4.40 (dt, J = 3.6, 10.1 Hz, 1H, H-2), 5.15 (t, J = 9.6 Hz, 1H, H-4), 5.30–5.36 (m, 2H, H-1α,3), 6.01 (d, J = 9.6 Hz, 1H, NH), 8.17 (s, 1H, HCO).

Dibenzyl *N,N*-diethylphosphoramidite (3.23 g, 10.2 mmol) was added to a solution of 3,4,6-tri-*O*-acetyl-2-deoxy-2-formamido-α-D-glucopyranose (0.680 g, 2.04 mmol) and 1,2,4-triazole (1.12 g, 16.3 mmol) in dry CH_2Cl_2 (20.0 mL) under argon at room temperature. The mixture was allowed to stir at room temperature for 5 h. The resulting solution was diluted with CH_2Cl_2 and then washed with saturated aq $NaHCO_3$, saturated aq NaCl, and water. The organic layer was dried over anhydrous Na_2SO_4 and concentrated. The residue was dissolved in dry CH_2Cl_2 (50.0 mL) and aq H_2O_2 (30%, 13.0 mL, 450 mmol) was added dropwise to the solution at −10 °C. After the mixture was stirred at room temperature for 1 h, the reaction mixture was diluted with CH_2Cl_2 and washed with saturated aq $NaHCO_3$, saturated aq NaCl, and water. The organic layer was dried over anhydrous Na_2SO_4 and concentrated. The residue was chromatographed by silica gel with hexane–EtOAc–triethylamine (500:400:1 (v/v/v)) to give dibenzyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-formamido-α-D-glucopyranose 1-phosphate (0.140 g, 0.230 mmol) in 11% yield. ^{1}H NMR ($CDCl_3$) δ 2.00, 2.01, 2.18 (3s, 9H, CH_3), 3.87 (dd, J = 2.3, 12.6 Hz, 1H, H-6a), 3.98–4.01 (m, 1H, H-5), 4.12 (dd, J = 4.1, 12.6 Hz, 1H, H-6b), 4.39–4.41 (m, 1H, H-2), 5.06–5.14 (m, 6H, H-3,4, CH_2–C_6H_5), 5.34 (d, J = 9.2 Hz, 1H, NH), 5.63 (dd, J = 3.2, 5.5 Hz, 1H, H-1α), 7.36–7.41 (m, 10H, C_6H_5), 7.81 (s, 1H, HCO); ^{31}P NMR ($CDCl_3$) δ −1.98.

Dibenzyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-formamido-α-d-glucopyranose 1-phosphate (0.137 g, 0.230 mmol) was hydrogenated over 10% Pd–C (27.4 mg) in dry CH_3OH (5.0 mL) for 1 h at room temperature. After the mixture was filtered, 1 M aq NaOH was added dropwise to the filtrate until it was turbid. The resulting mixture was poured into ethanol to precipitate the product, which was isolated by filtration and dried under reduced pressure to give 2-deoxy-2-formamido-α-D-glucopyranose-1-phosphate disodium salt (GlcNF-1-P, 0.0505 g, 0.150 mmol) in 65% yield. ^{1}H NMR (D_2O) δ 3.48 (t, J = 9.6 Hz, 1H, H-4), 3.74–3.82 (m, 2H, H-3,6a), 3.88 (dd, J = 1.4, 8.2 Hz, 1H, H-6a), 3.93–4.01 (m, 2H, H-2,5), 5.36, 5.45 (dd, J = 3.2, 7.8 and 3.5, 7.5 Hz, respectively, 1H, H-1α (two rotamers), 8.02, 8.17 (2s, 1H, HCO (two rotamers); ^{31}P NMR (D_2O) δ 2.20.

3.3. Enzymatic *N*-formyl-α-glucosaminylation of maltooligosaccharides

A typical experimental procedure for *N*-formyl-α-glucosaminylation was as follows. A mixture of GlcNF-1-P (0.070 g, 0.210 mmol) and maltotetraose (0.028 g, 0.042 mmol) in 200 mM sodium acetate buffer solution (7.0 mL, pH 6.2) was incubated in the presence of phosphorylase (42 U) at 40 °C for 4 days. After the reaction mixture was heated at 100 °C for 15 min and filtered, the filtrate was lyophilized. The residue was applied for MALDI-TOF MS analysis.

3.4. Estimation of the total yield of glycosylated products

The determination of produced inorganic phosphate in the phosphorylase-catalyzed glycosylation was as follows. The phosphorylase-catalyzed enzymatic reaction was carried out according to a similar experimental manner as that described in Section 3.3 using GlcNF-1-P, GlcN-1-P, or Glc-1-P, and the sample (6 μL) was taken out at each reaction time. The mixture was diluted with water (194 μL) and 800 μL of molybdate reagent (15 mM ammonium molybdate, 100 mM zinc acetate, pH 5.0) and 200 μL of ascorbic acid reagent (10% (w/v), pH 5.0) were added to the solution. The mixture was incubated at 30 °C for 20 min, and the absorbance was measured at 850 nm. The sodium acetate buffer solution of GlcNF-1-P, GlcN-1-P, or Glc-1-P and phosphorylase was used as a blank.

3.5. Glucoamylase-catalyzed hydrolysis of the crude products

A solution of glucoamylase (20 U) in 200 mM sodium acetate buffer solution (7.0 mL, pH 6.2) was added to the crude residue obtained by the above-mentioned enzymatic *N*-formyl-α-glucosaminylation (Section 3.3) and the mixture was incubated at 40 °C for 1 h. After the reaction solution was heated at 100 °C for 20 min, the precipitate was filtered. The filtrate was used for the MALDI-TOF MS analysis.

3.6. Isolation of 2-deoxy-2-formamido-α-D-glucopyranosyl-(1→4)-maltotetraose

The crude residue (0.280 g) obtained by the above-mentioned enzymatic hydrolysis by glucoamylase (Section 3.5) was dissolved in a mixed solvent of pyridine (4.0 mL) and DMF (4.0 mL). Acetic anhydride (4.70 mL, 49.5 mmol) was added to the solution and the mixture was allowed to stir at 60 °C overnight. The reaction mixture was diluted with $CHCl_3$, washed with 1 M aq H_2SO_4 saturated aq $NaHCO_3$ and water. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated. Thus the residue was subjected to column chromatography on silica gel (hexane–EtOAc 7:1 (v/v)) to give the pure acetylated product (0.0289 g). The resulting product was dissolved in CH_3OH (3.0 mL) and a solution of sodium methoxide (0.00102 g, 0.0189 mmol) in CH_3OH (2.0 mL) was added. After the mixture was stirred at room temperature for 1 h, the reaction mixture was neutralized with Dowex 50 W (H^+ form). The mixture was filtered and the filtrate was concentrated and dried under reduced pressure. This procedure was repeated twice for completion of deacetylation to give 2-formamido-2-deoxy-α-D-glucopyranosyl-(1→4)-maltotetraose (0.00710 g, 0.0083 mmol) in 44% yield (based on the amount of Glc_4 used for the enzymatic *N*-formyl-α-glucosaminylation). ^1H NMR (D_2O, α:β = 0.5:0.5) δ 3.27 (dd, *J* = 9.6, 7.8 Hz, 0.5H, Glc-H-2(β)), 3.51 (t, *J* = 9.2 Hz, 1H, GlcNF-H-4), 3.55–4.08 (m, 28.5H, Glc-H-2 (expect a proton of H-2(β) corresponding to the signal at δ 3.27),3,4,5,6, GlcNF-H-2,3,5,6), 4.64 (d, *J* = 7.8 Hz, 0.5H, H-1(β)), 5.22 (d, *J* = 3.7 Hz, 0.5H, H-1(α)), 5.34–5.37 (m, 3H, Glc-H-1), 5.39 (d, *J* = 3.7 Hz, 1H, GlcNF-H-1), 8.04, 8.17 (2s, 1H, HCO (two rotamers)).

3.7. Measurements

^1H, and ^{31}P NMR spectra were recorded at 400 and 162 MHz, respectively, on a JEOL ECX400 spectrometer, and chemical shifts were referenced to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, δ = 0.0 ppm) or tetramethylsilane (TMS, δ = 0.0 ppm) for ^1H NMR, and External 85% H_3PO_4 (δ = 0.0 ppm) for ^{31}P NMR. MALDI-TOF MS measurements were carried out by using Shimadzu Voyager Biospectrometry Workstation Ver.5.1 with 2.5-dihydroxybenzoic acid as matrix containing 0.05% trifluoroacetic acid under positive ion mode. HPAEC analysis was carried out with a DIONEX DX-300 system (Dionex, CA, USA) with a pulsed amperometric detector (model PAD-II, Dionex) and a Carbopac PA-100 column (4 mm × 250 mm). A sample (25 μL) was injected and eluted with a gradient of sodium acetate (0–1 min, 50 mM; 1–23 min, increasing from 50 mM to 350 mM with the installed gradient program 3; 23–25 min, 850 mM) in 150 mM NaOH with a flow rate of 1.0 mL/min. For Fiske-Subbarow method, absorbance was measured at 850 nm using a JASCO V-650Q1 spectrometer.

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