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Note

Enzymatic α-glucosaminylation of maltooligosaccharides catalyzed by phosphorylase

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ABSTRACT

This paper describes phosphorylase-catalyzed enzymatic α-glucosaminylation for the direct incorporation of a 2-amino-2-deoxy-α-D-glucopyranose unit into maltooligosaccharides. When the reaction of 2-amino-2-deoxy-α-D-glucopyranosyl 1-phosphate as the glycosyl donor with maltotetraose as a glycosyl acceptor was performed in the presence of phosphorylase, glucosaminylated oligosaccharides were produced, which were characterized by MALDI-TOF MS measurement after N-acetylation of the crude products. The N-acetylated derivative of the main product in this system was isolated by using HPLC, and its structure was confirmed by MS and ^{1}H NMR spectra. Furthermore, glucoamylase-catalyzed reaction of the isolated compound provided support that the α-glucosamine unit is positioned at the non-reducing end of the oligosaccharide.

Oligosaccharides in glycoproteins, glycolipids, and other glycoconjugates have important roles and functions in biological processes. For example, differences in oligosaccharide structures are responsible for blood group activities, and are involved in ontogenesis and oncogenesis as differentiation antigens.[1] Among them, oligosaccharides containing D-glucosamine (2-amino-2-deoxy-D-glucopyranose, GlcN) and *N*-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucopyranose, GlcNAc) units serve key functions in living organisms such as cell–cell recognition and immune responses. The preparation of saccharide chains containing 2-amino-2-deoxy-sugar residues, therefore, has been frequently required for glycoscience applications. Because highly selective glycosylation reactions are a promising approach to supply such substrates with well-defined structures,[2] much effort has been focused on glycosylations using glycosyl donors derived from GlcNAc and N-derivatized GlcN residues, such as the oxazoline glycosylation.[3] However, the glycosylation of a GlcN donor with a free amino group has hardly been achieved.

On the basis of the above points, we focused on enzymatic glycosylation[4] to incorporate an α-GlcN unit directly into oligosaccharide chains. In particular, we have focused on reactions catalyzed by phosphorylase (EC 2.4.1.1). The GlcN unit has been enzymatically incorporated into chitooligosaccharides linked via the β-(1→4)-configuration by bovine β-(1→4)-galactosyltransferase (EC 2.4.1.38).[5] To the best of our knowledge, enzymatic α-glycosylation using a GlcN donor with the free amino group, however, has not been reported. Phosphorylase is the enzyme that catalyzes the reversible phosphorolysis of α-(1→4)-glucans, such as glycogen and amylose, at the non-reducing end giving an α-D-glucopyranosyl 1-phosphate (Glc-1-P).[6] Due to the reversibility of the reaction, α-(1→4)-glucosidic linkages can be prepared by the phosphorylase-catalyzed chain-elongation using Glc-1-P as a glycosyl donor.[7] To initiate the reaction, a maltooligosaccharide, which is used as the glycosyl acceptor, is required. The smallest substrates typically accepted for phosphorolysis and for chain-elongation are maltopentaose and maltotetraose, respectively. Phosphorylase often expresses loose specificity for the recognition of substrates. For example, α-D-mannopyranosyl 1-phosphate, 2-deoxy-α-D-glucopyranosyl 1-phosphate, and α-D-xylopyranosyl 1-phosphate were used as the glycosyl donor for the phosphorylase-catalyzed reaction, giving α-mannosylated,[8a] 2-deoxy-α-glucosylated,[8b] and α-xylosylated[8c] oligosaccharides, respectively. These reports inspired us to examine the possibility of the recognition of 2-amino-2-deoxy-α-D-glucopyranosyl 1-phosphate (GlcN-1-P) as the glycosyl donor by this enzyme, leading to a facile and highly selective glycosylation for the formation of α-(1→4)-glucosaminyl linkage. In this note, we report the enzymatic α-glucosaminylation of maltooligosaccharides using GlcN-1-P catalyzed by phosphorylase (Scheme 1).

We first examined the enzymatic reaction using GlcN-1-P as the glycosyl donor and maltotetraose (Glc_4) as the glycosyl acceptor catalyzed by phosphorylase (from potato, 300 U/mL) in sodium acetate buffer solution (pH 5.9) at 40 °C. In the reaction, 5 equiv

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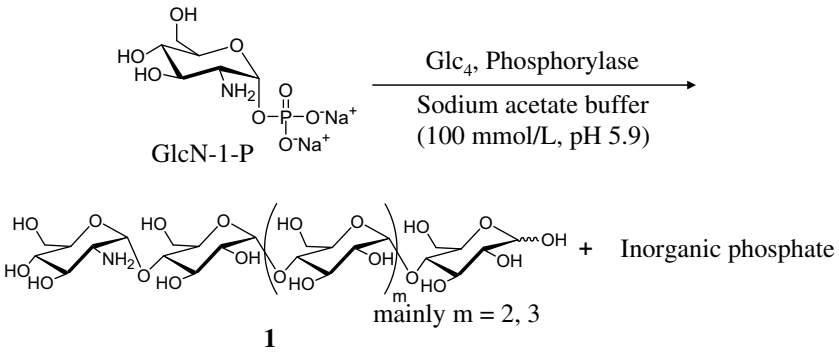


Scheme 1. Enzymatic α-glucosaminylation of maltooligosaccharide using GlcN-1-P.

of GlcN-1-P relative to Glc_4 was used. After the reaction mixture was lyophilized, N-acetylation was carried out using acetic anhydride, and the transfer of a GlcN unit to the maltooligosaccharides was evaluated by MALDI-TOF MS measurement. Because the difference in the molecular masses of the anhydroglucose and anhydroglucosamine units is only 1 (162 and 161, respectively), which can be made larger by the N-acetylation of the latter unit, these investigations were done on the N-acetylated material. In the MALDI-TOF MS spectrum of the N-acetylated crude products (Fig. 1), significant peaks corresponding to the masses of a pentasaccharide and a hexasaccharide containing one GlcNAc unit are observed (*m/z* 892.7 and 1054.7, respectively). These data indicate the glucosaminylation of Glc_4 or maltopentaose (Glc_5); the latter was possibly produced by the glucosylation of Glc-1-P to Glc_4. Indeed, the other peaks assignable to the molecular masses of maltotriose Glc_3 (*m/z* 527.5) and Glc_4 (*m/z* 689.6) were also present. The former is produced by the formation of Glc-1-P by phosphorolysis of Glc_4 at early stage of the reaction; the product Glc-1-P then acts as the glycosyl donor for the glucosylation of Glc_4, giving rise to Glc_5. The above analytical results of the N-acetylated crude products show that the main reaction in this system is the transfer of one GlcN residue to maltooligosaccharides from GlcN-1-P. However, it was not evident that the GlcN unit was positioned at the non-reducing end of the glucosaminylated products as the structure of **1** in Scheme 1. Consequently, the hydrolysis of the N-acetylated crude products was performed by glucoamylase (GA, EC 3.2.1.3, Wako Pure Industries), which catalyzes an exo-type hydrolysis at the non-reducing end of α-(1→4)-glucans, to reveal whether the GlcNAc unit was positioned at the non-reducing end. In the MALDI-TOF MS spectrum of the hydrolyzed products (Fig. 2), the peaks due to the molecular masses of the Glc_3 and Glc_4 disappear, whereas the peaks assigned to the molecular masses of the glucosaminylated oligosaccharides remain intact, thus supporting that the GlcNAc unit is positioned at the non-reducing end. If the transfer of one GlcN residue to maltooligosaccharides from GlcN-1-P proceeds once, further glycosylation is probably suppressed because **1** is not recognized as the acceptor by phosphorylase. For further analysis, we evaluated the formation of **1** versus reaction time as the ratios of Glc_4 to GlcN-1-P were varied from1:5 to 1:3 to 1:1 (Fig. 3). The total concentration of **1** was calculated based on the amount of inorganic phosphate produced from GlcN-1-P by the enzyme-catalyzed glucosaminylation, which was measured by the method described by Saheki et al.[9a] (modified from the Fiske–Subbarow method[9b]). A reaction time of 24 h gave highest yields in each case, which were approximately 80% (1:5), 60% (1:3), and 40% (1:1) based on the amounts of Glc_4 used (5 mM).

The major oligosaccharide in the N-acetylated crude mixture was estimated to be 2-acetamido-2-deoxy-α-D-glucopyranosyl-Glc_4 (**2**) by HPLC chromatography of the hydrolyzed crude products by GA, which was isolated by HPLC using an ODS column. In the MALDI-TOF MS spectrum of the isolated material (Fig. 4), only the peak assignable to the molecular mass of **2** was observed. Besides the signals due to the anomeric proton of the reducing ends

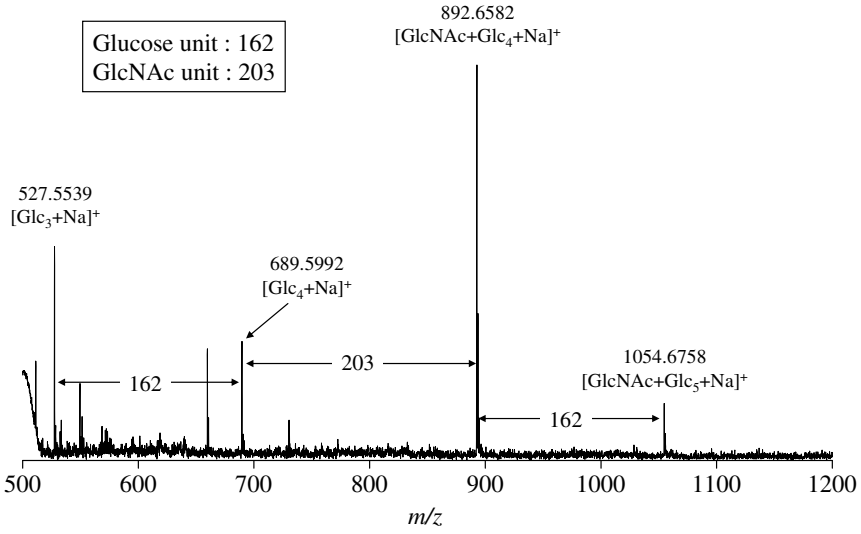


Figure 1. MALDI-TOF MS spectrum of the N-acetylated crude products.

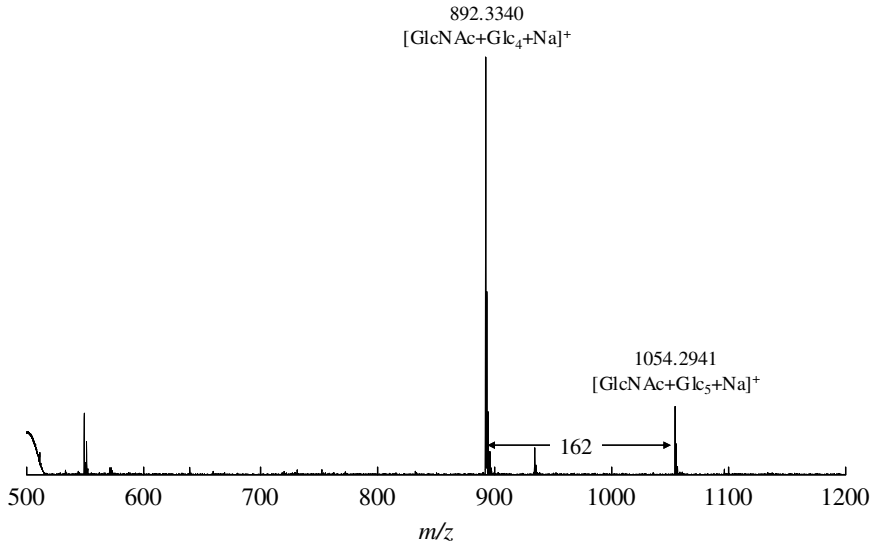


Figure 2. MALDI-TOF MS spectrum of the hydrolyzed products.

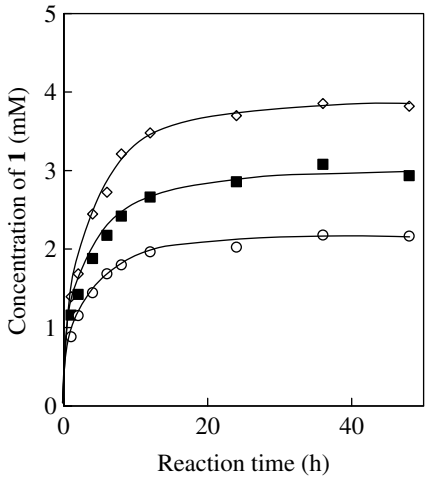


Figure 3. Concentration of **1** versus reaction time in the phosphorylase-catalyzed glucosaminylation. Reaction conditions: in acetate buffer 2 mL (100 mM, pH 5.9); amount of enzyme, 12 U; reaction temperature, 40 °C; initial concentration of Glc_4, 5 mM; Glc_4: GlcN-1-P = (◇) 1:5, (■) 1:3, (○) 1:1.

at δ 5.22 (α) and δ 4.63 (β) in the ^{1}H NMR spectrum (Fig. 5), two kinds of the anomeric signals are observed at around δ 5.35 (1′) and δ 5.32 (1″, J = 3.7 Hz), which are assignable to the α-glucopyranoside and 2-acetamido-2-deoxy-α-glucopyranoside, respectively. Furthermore, there is no signal (δ 3.40) assigned to the H-4 position of the glucose residue at the non-reducing end of Glc_4, whereas the signal (4″, δ 3.50) ascribed to the free H-4 position of GlcNAc is observed. This observation indicates that the GlcNAc unit is positioned at the non-reducing end, probably bound with the α-(1→4)-linkage. It was confirmed that isolated **2** was not hydrolyzed by GA, supporting further that the GlcNAc unit was positioned at the non-reducing end.

We also examined the phosphorylase-catalyzed reaction using 2-acetamido-2-deoxy-α-D-glucopyranosyl 1-phosphate (GlcNAc-1-P) as a glycosyl donor and Glc_4 as a glycosyl acceptor under the same conditions.[†] In the MALDI-TOF MS spectrum of the crude products, no peaks assignable to the molecular masses of oligosaccharides having a GlcNAc unit were observed. This result indicates that the GlcNAc-1-P is not recognized by phosphorylase, probably because the bulky acetamido group in GlcNAc-1-P blocks approach to the active site.

In summary, we examined the phosphorylase-catalyzed enzymatic α-glucosaminylation of maltooligosaccharides using GlcN-1-P. Glucosaminylation was confirmed by MALDI-TOF MS spectrometry of the N-acetylated crude products as well as the quantitative analysis of the produced inorganic phosphates by the modified Fiske–Subbarow method. The main product was isolated from N-acetylated crude mixture and characterized by the ^{1}H NMR and MALDI-TOF MS measurements, as well as GA-catalyzed hydrolysis, to be **2**. Additionally, it was confirmed that the phosphorylase-catalyzed transference of GlcNAc unit from GlcNAc-1-P did not proceed.

1. Experimental

1.1. Materials

Phosphorylase (from potato, 300 U/mL) 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.

1.2. Synthesis of 2-amino-2-deoxy-α-D-glucopyranosyl 1-phosphate disodium salt (GlcN-1-P)

To a solution of 2-azido-2-deoxy-α-D-glucopyranosyl 1-dibenzyl phosphate[12] (162 mg, 0.349 mmol) in dry MeOH (10 mL) was added $Pd(OH)_2$ (100 mg, 20 wt % on charcoal). After the mixture was stirred at room temperature under hydrogen atmosphere for 2 h, 1 M NaOH (1 mL) was added, the solution was filtered, and the filtered material was washed with water. The filtrate was concentrated and reprecipitated by EtOH. The resulting precipitate was isolated by filtration and dried in vacuo to give GlcN-1-P as a white powder (101 mg, 0.332 mmol, 95%). ^{1}H NMR (Fig. S1, D_2O) δ 5.50 (dd, $J_{1,P}$ = 7.5 Hz, $J_{1,2}$ = 3.3 Hz, 1H, H-1), 3.93–3.73 (m, 5H, H-3, 5, 6), 3.43 (t, 1H, H-4), 2.97 (d, $J_{2,3}$ = 9.2 Hz, 1H, H-2); ^{31}P NMR (D_2O) δ 0.911.

[†] The GlcNAc units have enzymatically incorporated into the oligosaccharides by the β-glycosylation using their oxazoline derivertives.[10]

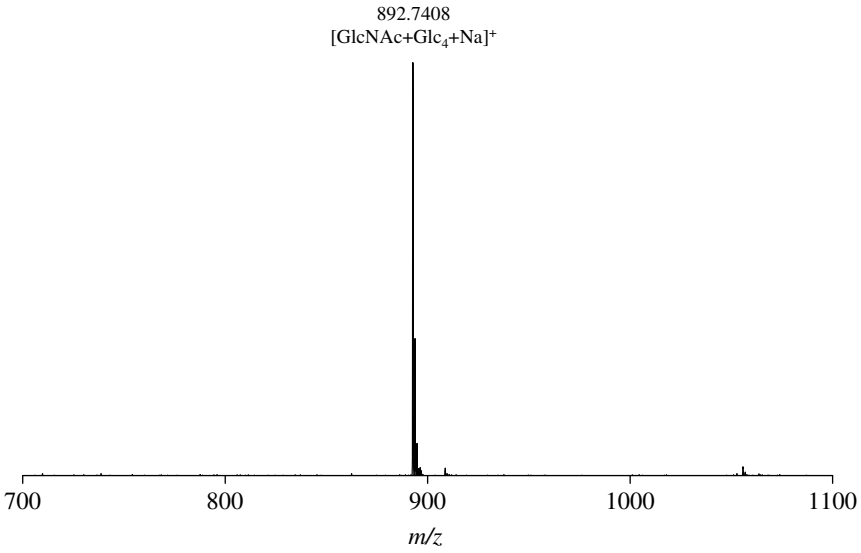
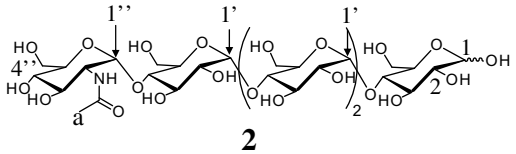


Figure 4. MALDITOF MS spectrum of the isolated material.



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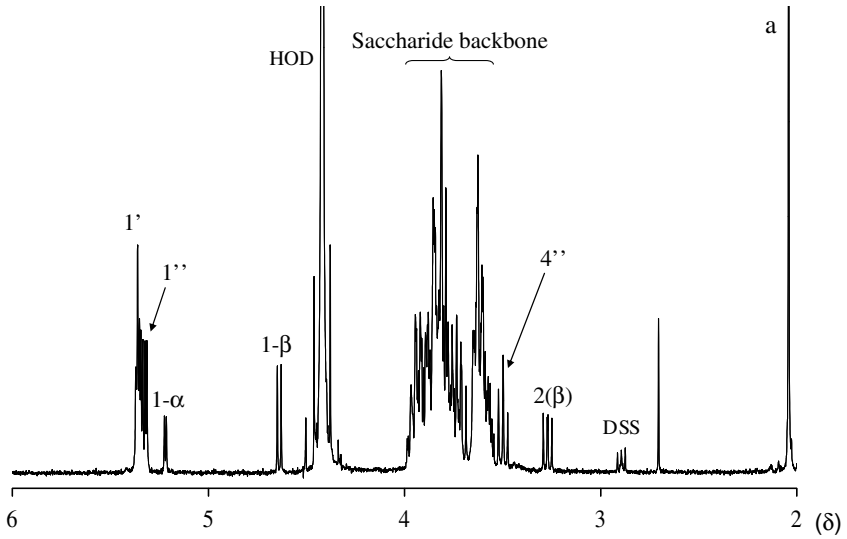

Figure 5. ^{1}H NMR spectrum of the isolated material (D_2O).

1.3. Synthesis of 2-acetamido-2-deoxy-α-D-glucopyranosyl 1-phosphate disodium salt (GlcNAc-1-P)

Acetic anhydride (6 μL, 0.0635 mmol) was added to a solution of GlcN-1-P (10 mg, 0.0329 mmol) in water (1 mL) in the presence of sodium carbonate (7 mg, 0.0660 mmol). The reaction mixture was stirred at room temperature for 30 min, and neutralized with Amberlite IRC76 (H^+ form). Lyophilization of the resulting solution afforded GlcNAc-1-P (9 mg), which contained sodium acetate in a low dose as an impurity. However, it was considered that the impurity did not affect the reaction. ^{1}H NMR (Fig. S2, D_2O) δ 5.35 (dd, $J_{1,P}$ = 7.6 Hz, $J_{1,2}$ = 3.2 Hz, 1H, H-1), 3.96–3.74 (m, 5H, H-2, 4, 5, 6), 3.47 (t, 1H, H-3), 2.09 (s, 3H, CH_3); ^{31}P NMR (D_2O) δ 0.804.

1.4. Enzymatic α-(1→4) glucosaminylation of maltooligosaccharides

The major steps of glucosaminylation were as follows. A mixture of GlcN-1-P (15 mg, 0.05 mmol) and maltotetraose (6.7 mg, 0.01 mmol) in 100 mM sodium acetate buffer solution (2 mL, pH 5.9) was incubated in the presence of phosphorylase (12 U) for the desired reaction time. After the reaction mixture was heated at 100 °C for 10 min and filtered, acetic anhydride (6 μL, 0.0635 mmol) was added to the solution. Then, the mixture was stirred in the presence of sodium carbonate for 1 h, and neutralized with Dowex 50 W ion exchange resin (H^+ form). The neutral solution was used in the MALDI-TOF MS analysis (Fig. 1).

1.5. Glucoamylase-catalyzed hydrolysis of the N-acetylated crude products

Glucoamylase (20 U) was added to the above neutral solution, 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 (Fig. 2).

1.6. The estimation of the total concentration of produced 1

The determination of produced inorganic phosphate in the glucosaminylation was as follows. The enzymatic glucosaminylation was carried out as described above, and the sample (8 μL) was taken out at each reaction time. The mixture was diluted with water (192 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. This mixture was incubated at 30 °C for 15 min, and the absorbance was measured at 850 nm. The sodium acetate buffer solution of GlcN-1-P and Glc_4 was used as a blank.

1.7. Isolation of 2-acetamido-2-deoxy-α-D-glucopyranosyl-(1→4)-maltotetraose (2)

For the isolation of the title product, a mixture of GlcN-1-P (30 mg, 0.100 mmol) and maltotetraose (13.2 mg, 0.02 mmol) in 100 mM sodium acetate buffer solution (4 mL, pH 5.9) was incubated in the presence of phosphorylase (24 U) at 40 °C for 2 days. Following, the resulting solution was heated at 100 °C for 10 min, and the precipitate was combed out. After acetic anhydride (10 μL, 0.106 mmol) was added to the resulting solution in the presence of sodium carbonate, the reaction mixture was stirred at room temperature for 1 h, and neutralized with Dowex 50 W ion exchange resin (H^+ form). The neutral solution was incubated at 40 °C for 1 h in the presence of glucoamylase (30 U), heated at 100 °C for 20 min, filtered, and lyophilized. The resulting powder was dissolved in water (2 mL), and 0.8 mL of the solution was subjected by HPLC with Waters μBondapack C18 column to isolate the title compound. The fraction of pentasaccharide was collected and lyophilized to give title compound as a white solid (2.1 mg, 2.4 μmol). ^{1}H NMR (Fig. 5, D_2O, α:β = 0.4:0.6) δ 5.37–5.33 (m, 3H, Glc-H-1), 5.32 (d, $J_{1,2}$ = 3.7 Hz, 1H, GlcNAc-H-1), 5.22 (d, $J_{\alpha 1,2}$ = 3.7 Hz, 0.4H, α-H-1), 4.63 (d, $J_{\beta 1,2}$ = 7.8 Hz, 0.6H, β-H-1), 3.97–3.56 (m, 28.4H, Glc-H-2 (expect a proton of a H-2(β) corresponding to the signal at δ 3.26),3,4,5,6, GlcNAc-H-2,3,5,6), 3.50 (t, 1H, GlcNAc-H-4), 3.26 (t, 0.6H, a Glc-H-2(β)).

1.8. Measurements

^{1}H 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 ^{1}H 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. HPLC analyses were performed on a JASCO PU-2080 apparatus using RI-2031 detector with Waters μBondapack C18 column (3.9 × 150 mm) with a water as the eluent (flow rate, 0.4 mL/min; room temperature). For the Fiske–Subbarow method, absorbance was measured at 850 nm using JASCO V-650Q1 spectrometer.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.08.013.

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