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Enzymatic synthesis of a 2-*O*-α-D-glucopyranosyl cyclic tetrasaccharide by kojibiose phosphorylase

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Abstract—The glucosyl transfer reaction of kojibiose phosphorylase (KPase) from *Thermoanaerobacter brockii* ATCC35047 was examined using *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→} (CTS) as an acceptor. KPase produced four transfer products, saccharides **1**–**4**. The structure of a major product, saccharide **4**, was 2-*O*-α-D-glucopyranosyl-CTS, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-[α-D-Glc*p*-(1→2)]-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→}. The other transfer products, saccharides **1**–**3**, were 2-*O*-α-kojibiosyl-, 2-*O*-α-kojitriosyl-, and 2-*O*-α-kojitetraosyl-CTS, respectively. These results showed that KPase transferred a glucose residue to the C-2 position at the ring glucose residue of CTS. This enzyme also catalyzed the chain-extending reaction of the side chain of 2-*O*-α-D-glycopyranosyl-CTS.

Keywords: Cyclic tetrasaccharide; *Thermoanaerobacter brockii*; Kojibiose phosphorylase; Transglucosylation

1. Introduction

A cyclic tetrasaccharide, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→} (abbreviated as CTS), has a unique structure consisting of four glucose residues joined by alternate α-(1→3)- and α-(1→6)-linkages. Côté and co-workers first reported that CTS was produced from a dextran-like polysaccharide, alternan, by its degradation enzyme.[1,2] Recently, we found a new enzymatic system to synthesize this saccharide from maltodextrins by a joint reaction of two glycosyltransferases, 6-α-D-glucosyltransferase and 3-α-isomaltosyltransferase.[3] We also succeeded in the mass production of CTS from starch in a high yield using both enzymes.[4,5] CTS has tolerance to the hydrolytic activity of glycosidases such as amylase or α-glucosidase; therefore, this saccharide is expected to be used as a low-calorie sweetener. Single-crystal X-ray structure analysis has shown that CTS has a shallow cavity in the center of its cyclic structure.[6] The cavity can bind with small inorganic ions.[7] CTS binds ethanol on both the concave and convex sides.[8] These properties of CTS open the potential for further applications, for example, as a carrier in drug delivery systems or a base that removes toxic metal cations.

Kojibiose phosphorylase (EC 2.4.1.230: KPase) catalyzes the reversible phosphorolysis of kojibiose (α-D-glucopyranosyl-(1→2)-D-glucopyranose) as follows: β-D-glucose-1-phosphate (β-G1P) + D-glucose ⇌ kojibiose + inorganic phosphate (Pi). We have reported on the purification and properties of KPase from *Thermoanaerobacter brockii* ATCC35047.[9] This enzyme also catalyzes transglucosylation using β-G1P as glucosyl donor to the appropriate acceptor. Therefore, KPase is expected to catalyze transglucosylation to CTS.

2. Results

2.1. Transglucosylation to CTS by KPase

A reaction mixture (1 mL) containing KPase (34.2 U/mmol for β-G1P), β-G1P (73 mM), CTS (154 mM)

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in 50 mM sodium acetate buffer (pH 5.5) was incubated at 50 °C for 64 h. The reaction was stopped by heating in a boiling water bath for 10 min. A sample (50 μL) of the reaction mixture was removed for analysis by HPLC. As shown in Figure 1, KPase gave four transfer products, saccharide **1** (T_R 14.2 min: the HPLC retention time = T_R), **2** (T_R 15.6 min), **3** (T_R 17.4 min), and **4** (T_R 32.5 min). Figure 2 shows the time course of the reaction for the syntheses of saccharides **1**–**4**. Saccharide **4** seemed to be generated earlier than the other saccharides. The yield of saccharides **1**–**4** at a reaction time of 64 h reached 5.1%, 3.5%, 1.3%, and 22.5%, respectively.

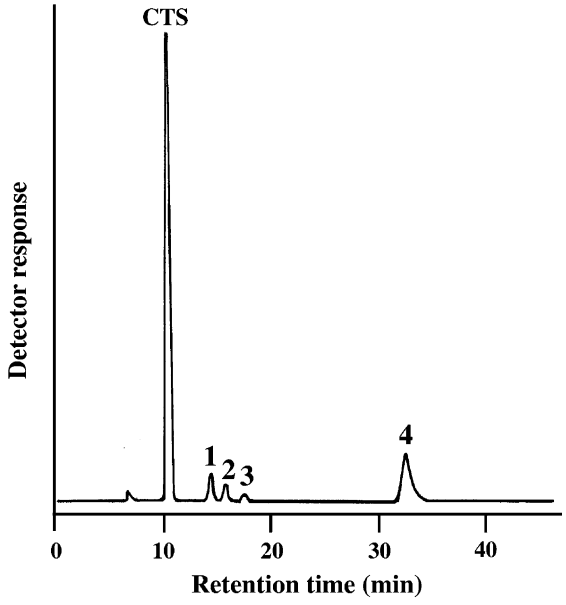

Figure 1. HPLC profile of reaction products on a mixture of β-G1P as the donor and CTS as the acceptor by KPase.

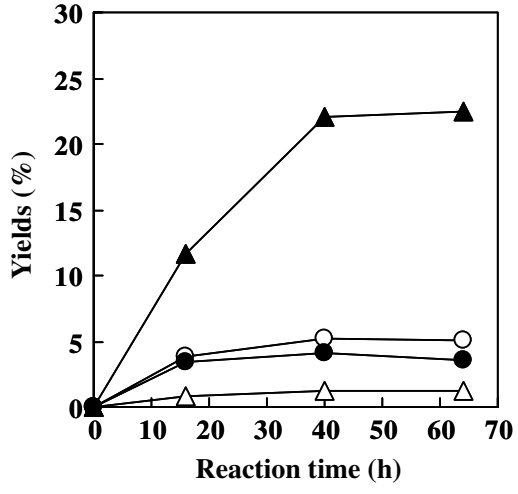

Figure 2. Time-course of formation of saccharides **1**–**4**. The yields of saccharides **1**–**4** are shown except β-G1P. ○, Saccharide **1**. ●, Saccharide **2**. △, Saccharide **3**. ▲, Saccharide **4**.

2.2. Preparation and isolation of saccharides 1–4

To prepare saccharides **1**–**4** on the gram scale, a reaction mixture containing 154 mM of CTS, 73 mM of β-G1P, and KPase (34.2 U/mmol for β-G1P) in 800 mL of 50 mM sodium acetate buffer (pH 5.5) was incubated at 50 °C for 64 h. The yields of saccharides **1**–**4** reached 5.1%, 3.5%, 1.3%, and 22.2%, respectively. After the enzyme reaction was stopped by heating, the mixture was centrifuged, and then the resultant supernatant was desalted by passing it through ion-exchange resins, 100 mL of Diaion SK1B (Mitsubishi Chem. Co., Tokyo, Japan), and 200 mL of Amberlite IRA411S (Japan Organo, Tokyo, Japan). The eluent was concentrated to 400 mL by evaporation at 40 °C. An 18-mL portion of the saccharide solution was put through a repeated preparative HPLC on an ODS-AQ R-355-15-AQ column (50 × 500 mm, YMC). Saccharides **1**–**4** were separated from CTS by elution with water as a solvent at a flow rate of 30 mL/min at 35 °C. The fractions containing saccharides **1**–**4** were separately collected and then evaporated at 40 °C. Saccharides **1**, **2**, and **4** were easily crystallized from their aqueous solutions. Saccharide **3** was obtained as a white powder by lyophilization. The amounts of purified saccharides **1**–**4** were 3.1 g (purity: 98.3%), 1.7 g (95.2%), 0.42 g (96.3%), and 18.6 g (98.3%), respectively.

2.3. Arsenolysis of saccharides 1–4 by KPase

KPase catalyzes the phosphorolysis of kojibiose. When arsenate is used in place of phosphate, kojibiose is converted into 2 mol of glucose.[10] As shown in Table 1, saccharide **4** was arsenolyzed to the equimolar of glucose and CTS. This result showed that saccharide **4** had a structure containing one glucose residue attached to CTS. When saccharides **1**–**3** were arsenolyzed, saccharide **4** was generated in addition to glucose and CTS. Therefore, saccharides **1**–**3** should have saccharide **4** in their structures.

2.4. Characterization of saccharide 4

The molecular mass of **4** was found to be 810 Da by measuring the $[M + Na]^+$ ion (*m*/*z* 833) by ESIMS. This value was identical to that of CTS linked to one glucose

Table 1. Arsenolysis of saccharides **1**, **2**, **3**, and **4**

Saccharide	Sugar composition (%, mol)[a]					
	Glucose	CTS	1	2	3	4
1	52.9	9.1	0.0	Nd	Nd	38.0
2	65.8	4.2	0.8	0.0	Nd	29.2
3	68.9	3.0	2.8	5.4	0.1	19.8
4	30.6	28.2	Nd	Nd	Nd	41.2

[a] Nd = not detected.

residue. Methylation of saccharide **4** gave one molecular equivalent of 2,3,4,6-tetra-*O*-methyl product derived from a nonreducing end glucose, as shown in Table 2. Compared with CTS, one molecular equivalent of the 2,3,4-tri-*O*-methyl product was lost, and a corresponding amount of the 3,4-di-*O*-methyl product was generated. Therefore, saccharide **4** would be expected to have a structure containing one glucose residue attached to CTS by a (1→2) linkage. To confirm this structure, NMR measurements were carried out. The ^{13}C NMR spectrum of saccharide **4** contained 30 signals (Table 3), indicating that it should be a pentamer of glucose. ^{1}H–^{1}H and ^{1}H–^{13}C COSY showed the α-configuration of the nonreducing end glucose residue in saccharide **4** (Glc-V in Fig. 3a) that was confirmed by the *C*-1 signal of this residue at 98.5 ppm {δ_1H, 5.2 ppm (d, $J_{1,2}$ 3.5 Hz)}. Furthermore, the interresidual HMBC correlation between the anomeric proton of Glc-V and the *C*-2 signal (δ_{13}C, 77.6 ppm) of one of the 6-*O*-glycosylated ring residues (Glc-III in Fig. 3a) was observed in the spectra. From these results, saccharide **4** was deduced to be 2-*O*-α-D-glucopyranosyl-CTS, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-[α-D-Glc*p*-(1→2)]-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→} (Fig. 3a).

2.5. Characterization of saccharide 1

Mass spectrum of saccharide **1** showed its molecular weight to be 972 Da by measuring the $[M + Na]^+$ ion (*m*/*z* 995). This value was identical to that of CTS linked to two glucose residues. Methylation analysis showed that saccharide **1** contains one kojibiose residue linked by a (1→2) linkage to one of the two α-(1→6)-glycosylated residues of CTS (Table 2). Therefore, saccharide **1** was deduced to be 2-*O*-α-kojibiosyl-CTS, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-[α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→2)]-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→} (Fig. 3b).

2.6. Characterization of saccharide 2

Mass spectrum of saccharide **2** showed an $[M + Na]^+$ ion peak with a *m*/*z* ratio of 1157, for an apparent mass of 1134 Da. This value was identical to that of CTS linked to three glucose residues. From the result of the methylation analysis of saccharide **2**, there was an increase of two molecular equivalents of the 3,4,6-tri-*O*-methyl product compared with that of saccharide **4** (Table 2). Therefore, saccharide **2** was deduced to be 2-*O*-α-kojitriosyl-CTS, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-[α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→2)]-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→} (Fig. 3b).

Table 3. ^{13}C NMR chemical shift data for saccharide **4**[a]

Residue[b]	Carbon atom	Saccharide **4**	CTS[c]
I	C-1	99.7	99.0
	C-2	74.4	74.0
	C-3	75.5	75.1
	C-4	73.3	73.0
	C-5	72.6	72.5
	C-6	70.3	69.9
II	C-l	101.7	100.8
	C-2	72.7	72.3
	C-3	77.9	76.9
	C-4	73.6	73.3
	C-5	74.2	73.9
	C-6	62.9	62.5
III	C-l	97.2	99.0
	C-2	77.6	74.0
	C-3	74.6	75.1
	C-4	73.4	73.0
	C-5	72.7	72.5
	C-6	70.6	69.9
IV	C-l	101.5	100.8
	C-2	72.7	72.3
	C-3	78.4	76.9
	C-4	73.6	73.3
	C-5	74.3	73.9
	C-6	63.0	62.5
V	C-1	98.5	
	C-2	74.1	
	C-3	75.5	
	C-4	72.1	
	C-5	73.8	
	C-6	63.1	

[a] NMR spectra data were recorded for solutions in D_2O at 27 °C. The chemical shifts are expressed in ppm downfield from the signal of 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (TPS), which was used as an internal standard.
[b] Roman numerals indicate the positions of the hexose residues in the saccharide **4** shown in Figure 3.
[c] Data taken from Côté and Biely (Ref. 1).

2.7. Characterization of saccharide 3

In the mass analysis of saccharide **3**, a molecular ion $[M + Na]^+$ peak at *m*/*z* 1319 was observed. Therefore, the molecular weight of saccharide **3** was indicated to

Table 2. Methylation analysis of saccharides **1**, **2**, **3**, and **4**

Saccharide	2,3,4,6-Tetra-*O*-methyl-Glc	3,4,6-Tri-*O*-methyl-Glc	2,4,6-Tri-*O*-methyl-Glc	2,3,4-Tri-*O*-methyl-Glc	3,4-Di-*O*-methyl-Glc
1	0.7	0.8	2.0	0.7	0.8
2	0.8	1.9	2.0	0.8	0.9
3	1.0	2.9	2.0	0.8	1.1
4	0.8	—	2.0	0.7	0.9
CTS	—	—	2.0	2.0	—

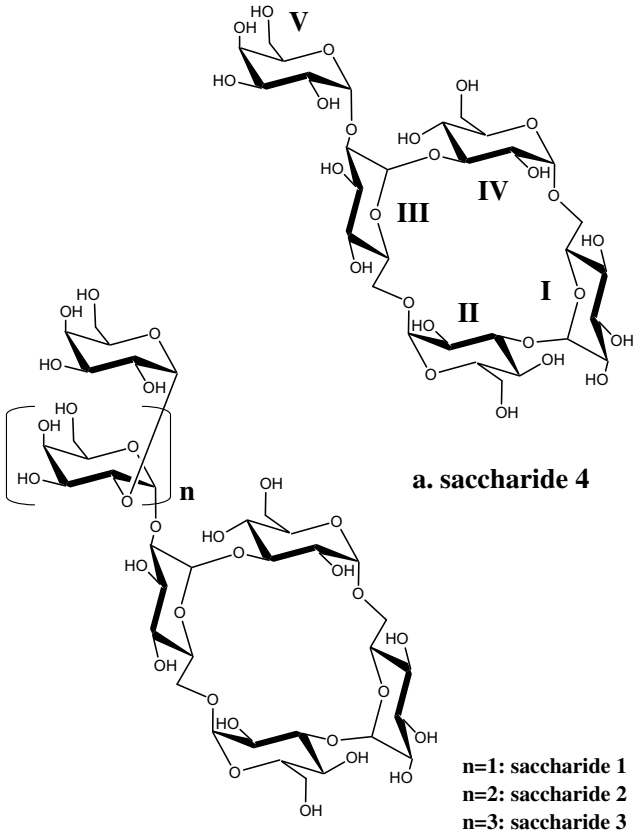

Figure 3. Structure of saccharides **4**(a) and **1**–**3**(b) formed by KPase.

be 1296 Da. This value is identical to that of CTS linked to four glucose residues. When methylation analysis of saccharide **3** was performed, there was an increase of three molecular equivalents of the 3,4,6-tri-*O*-methyl product compared with that of saccharide **4** (Table 2). Therefore, saccharide **3** was deduced to be 2-*O*-α-kojitetraosyl-CTS, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*- (1→6)-[α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→2)]-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→} (Fig. 3b).

2.8. Thermal analysis and solubility of 2-*O*-α-D-glucopyranosyl-CTS

A broad endothermic DTA signal was detected from 50 to 80 °C. Four molecular equivalents of H_2O were released in this temperature range, meaning that the crystals of 2-*O*-α-D-glucopyranosyl-CTS were a tetrahydrate. After dehydration, the anhydrous compound was shown to be heat-degraded around 340 °C.

The solubility of 2-*O*-α-D-glucopyranosyl-CTS was examined in water at 25 °C together with CTS. The solubilities of 2-*O*-α-D-glucopyranosyl-CTS and CTS were 10 and 75 mmol in 100 g of water. By a glucose attached to the 2-OH of one of the 6-glycosylated ring residue of CTS, the solubility was much lower than that for CTS itself.

3. Discussion

In this study, we succeeded in synthesizing four kinds of novel branched CTS compounds using a transglucosylation reaction mediated by KPase. The major transfer product was 2-*O*-α-D-glucopyranosyl-CTS. Its structure was that of a glucose unit linked to the 2-OH group of the 6-linked glucosyl residue of CTS. Other minor products were a series of mono-branched CTSs that extended the branched part of 2-*O*-α-D-glucopyranosyl-CTS. CTS has four 2-OH groups in its structure; two are of the 3-linked glucosyl residues and the other two are of the 6-linked residues. The X-ray crystal structure of CTS showed that the two 2-OH groups of the 3-linked glucosyl residues were buried in the ring structure of CTS and that the two 2-OH groups of the 6-linked residues were exposed to the solvent space.[6] Therefore, the 2-OH group of the 6-linked residue is considered to be the acceptor site for the glucosylation reaction of KPase. In this experiment, we detected a series of mono-branched CTSs but not di-branched CTSs linked to both 2-OH groups. This result means that KPase can transfer glucose to the 2-OH of one 6-linked residue but cannot transfer to the 2-OH group of another 6-linked residue. Previously, we reported on the transglycosylation to CTS by cyclomaltodextrin glucanotransferase (EC 2.4.1.19: CGTase).[11] This enzyme transferred maltodextrins to both of the 4-OH groups of the 6-linked residues of CTS to generate a di-branched CTS. The tertiary structure of CGTase was determined, and the active site of CGTase was revealed.[12] The acceptor-binding site of CGTase locates in a loose structure; therefore, this enzyme might allow the mono-branched CTS to access the active center of the enzyme. On the other hand, KPase, of which the tertiary structure has not yet been determined, may have a rigid acceptor-binding site that disallows the mono-branched CTS to bind the site. Probably, the branched glucose residue of 2-*O*-α-D-glucopyranosyl-CTS becomes a steric hindrance to the acceptor-binding to KPase.

Generally, the glucosylation to low-soluble compounds improves their solubility. For example, 6-*O*-α-D-glucopyranosyl-cyclomaltoheptaose (Glucosyl-β-CD) dissolved approximately 40-fold over the nonbranched β-CD in water at 25 °C.[13] However, the solubility of 2-*O*-α-D-glucopyranosyl-CTS was found to be much lower than the nonbranched CTS. It is interesting that 2-*O*-α-D-glucopyranosyl-CTS showed the longest retention time on the reversed-phase HPLC column (Fig. 1). This long retention time suggests that 2-*O*-α-D-glucopyranosyl-CTS may be more hydrophobic than CTS

and other branched CTSs. In the center of its structure, CTS has a small cavity that is shallower than that of cyclomaltodextrins. Therefore, CTS holds only one water molecule in the space of depression, but forms no inclusion complex with phenolic compounds and other high-molecular-weight compounds. It is possible that the glucosyl residue of 2-*O*-α-D-glucopyranosyl-CTS may cause a deeper and more hydrophobic depression than the nonbranched CTS. We are now preparing the single crystal of 2-*O*-α-D-glucopyranosyl-CTS for X-ray analysis to explore this possibility.

Recently, various heterogeneous, branched CTSs have been synthesized by glycosyltransferases. Biely et al. reported the transfer of a α-D-galactopyranose to CTS by α-galactosidases.[14] From the result of the structural analysis of the α-D-galactopyranose-linked CTS, the position of the α-D-galactopyranose transfer was identified to be the 6-OH group of the 3-linked residue of CTS. On the other hand, we studied the transfer of a β-D-galactopyranose and a β-*N*-acetylglucosamine to CTS. The β-galactosidase from *Bacillus circulans* produced 3-*O*-β-D-galactopyranosyl-CTS.[15] The lysozyme from egg white synthesized 3-*O*-β-*N*-acetylglucosaminyl-CTS.[16] In both cases, the transfer position was the 3-OH group of the 6-linked residue of CTS. In this work, we found that KPase transferred α-D-glucopyranose directly to the 2-OH group of the 6-linked residue of CTS. We also found that 2-*O*-α-D-glucopyranosyl-CTS has a new character different from CTS. These branched-CTSs are expected to have various functions depending on the difference of the branched position or the kind of added saccharide.

4. Experimental

4.1. Carbohydrate and enzymes

CTS (purity: 99.9%) was prepared from starch with 6GT and IMT as described previously.[4] KPase and trehalose phosphorylase (TPase) were prepared from the recombinant *Escherichia coli* cells in which the genes were cloned from *T. brockii* ATCC35047.[17,18]

4.2. Assay of KPase activity

The reaction mixture contained 0.1% (w/v) kojibiose, McIlvaine buffer (pH 5.5 Pi concentration: 102 mM), and the enzyme solution in a total volume of 2.2 mL. After incubation at 60 °C for 30 min, the reaction was stopped by boiling for 10 min. The released glucose was measured by the glucose oxidase–peroxidase method.[19] One unit of the enzyme activity was defined as the amount of enzyme liberating 1 μmol glucose per min.

4.3. Assay of TPase activity

A reaction mixture consisting of 50 mM sodium phosphate buffer (pH 7.0), 1% (w/v) trehalose, and the enzyme solution in a total volume of 2.0 mL was incubated at 60 °C for 30 min. The reaction was stopped by boiling for 10 min. The released glucose was measured by the glucose oxidase–peroxidase method.[19] One unit of the enzyme activity was defined as the amount of enzyme liberating 1 μmol glucose per minute.

4.4. Preparation of β-G1P

A reaction mixture (150 L) containing 195 mM of trehalose and TPase (3.42 U/mmol for trehalose) in 500 mM sodium phosphate buffer (pH 7.0) was incubated at 55 °C for 72 h. The purification of β-G1P as done by the method of Ashby et al.[20] and we obtained 1580 g of β-G1P·2Na.

4.5. Arsenolysis by KPase

The reaction mixtures (1.0 mL) containing 10 mM of each of branched-CTSs, and KPase (10.0 U/mmol for branched-CTSs) in a 50 mM citrate–sodium arsenate buffer (pH 5.5) were incubated at 60 °C for 24 h. These reactions were stopped by boiling for 10 min. Samples (30 μL) of the reaction mixtures were removed for analysis by HPLC.

4.6. High-performance liquid chromatography (HPLC)

The amounts and purity of neutral saccharides in the samples were determined by HPLC. Samples were first treated by filtration using a filter kit, KC prep dura (0.45 μm, Katayama Chemical Co., Osaka, Japan) and by deionization using a micro acilyzer G0 (Asahi Chemical Co., Tokyo, Japan). HPLC analysis was performed with a LC-10AD pump, a RID-10A refractive index monitor, and a C-R7A data processor (Shimadzu Corporation, Kyoto, Japan) equipped with an ODS-AQ AQ-303 column (4.6 mm id × 250 mm; YMC Co., Ltd Kyoto, Japan) at a flow rate of 0.5 mL/min, using water as the solvent at 40 °C. Analysis of arsenolysis products by KPase was carried out with the same system, except that this time we used an MCI GEL CK04SS column (10 mm id × 200 mm × 2; Mitsubishi Chem. Co., Tokyo, Japan) at a flow rate of 0.4 mL/min at 80 °C.

4.7. Methylation analysis

Methylation analysis was performed according to the method of Hakomori.[21] The saccharide samples (100 μg) were methylated, and after extraction with $CHCl_3$, the methylated sample was hydrolyzed with 90% HOAc containing 0.5 N H_2SO_4 at 80 °C for 6 h.

The methylated monosaccharides were reduced with $NaBH_4$ and then acetylated with Ac_2O at 100 °C for 4 h. The resulting partially methylated alditol acetates were analyzed by GLC (GC-14B, Shimadzu Corporation) in a DB-5 capillary column (J&W, Folsom, CA) at 130–250 °C (5 °C/min).

4.8. MS analyses

The molecular masses were determined by an electrospray-ionization mass spectrometry (ESIMS). ESIMS was performed with an LCQ advantage ion trap mass analyzer (Thermo Electron Corporation, Kanagawa, Japan). The mass spectrometer was operated in the positive-ion mode; the ESI voltage was set to 5 kV, and the capillary temperature was 350 °C.

4.9. NMR measurements

NMR spectral data were recorded for 1–5% solutions in D_2O at 27 °C with a JNM-AL300 spectrometer (^1H 300.4 MHz, ^{13}C 75.45 MHz: JEOL, Tokyo, Japan). The chemical shifts were expressed in ppm downfield from the signal of 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (TPS) used as an internal standard.

4.10. Thermal analysis

Thermal analysis was examined with a SSC/5200H thermoanalytical system (TG/DTA220U: Seiko Instruments Inc., Chiba, Japan), with samples in aluminum pans under a constant nitrogen flow of 50 mL/min. The heating rate was 10 °C/min. Aluminum pans without samples were used as a DTA standard.

4.11. Measurement of solubility

CTS (10 g) and 2-*O*-α-D-glucopyranosyl-CTS (1.5 g) were separately added to a glass vessel containing the water (10 mL) and vigorously stirred at 25 °C. The mixtures were filtered at 24-h intervals. Solubility was calculated by measuring the solid content of the filtrate.

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