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Effects of various saccharides on cycloinulo-oligosaccharide fructanotransferase reaction: production of β-inulotriosyl-α-D-mannopyranoside and 1-*O*-β-inulotriosyl-α-L-sorbopyranose

Mishio Kawamura *, Hiroshi Nakai, Takao Uchiyama

Department of Biology, Osaka Kyoiku University, 4-698-1 Asahigaoka, Kashiwara, Osaka 582-8582, Japan

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Abstract

The effects of various saccharides on the reaction of cycloinulo-oligosaccharide fructanotransferase with cycloinulohexaose were examined. In addition to β-D-fructofuranosides and methyl α-D-glucopyranoside, D-mannose and L-sorbose were found to be effective acceptors in the reactions, and they enhanced the hydrolytic activity as effectively as methyl α-D-glucopyranoside. Hetero-tetrasaccharides were isolated as the major transfer products from both reaction mixtures. The isolates were identified by NMR spectroscopy as β-inulotriosyl-α-D-mannopyranoside and 1-*O*-β-inulotriosyl-α-L-sorbopyranose. Methyl β-D-glucopyranoside was slightly effective and methyl α-D-mannopyranoside was not effective at all as the acceptor, but these saccharides strongly enhanced the hydrolytic activity. D-Glucosamine inhibited the enzyme activity. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Cycloinulo-oligosaccharide fructanotransferase; Intermolecular transfructosylation; β-Inulotriosyl-α-D-mannopyranoside; 1-*O*-β-Inulotriosyl-α-L-sorbopyranose; NMR spectroscopy

1. Introduction

Cycloinulo-oligosaccharide fructanotransferase (CFTase) from *Bacillus circulans* OKUMZ 31B catalyzes the decomposition of inulin into cycloinulo-oligosaccharides by intramolecular transfructosylation [1]. The enzyme also catalyzes intermolecular transfructosylation with β-D-fructofuranosyl compounds as acceptors, involving disproportionation and a coupling reaction, and the hydrolysis of β-(2→1) fructo-oligosaccharides [2]. Such multifunctional activity has been found in cyclomalto-oligosaccharide glucanotransferase (EC 2.4.1.19, CGTase) [3]. CGTase has been utilized for the synthesis of various glucosides since it has wide specificity for acceptors [4]. Recently, we have reported that CFTase employed methyl α-D-glucopyranoside as an acceptor in its intermolecular transfructosylation [5]. In the presence of the high-concentration acceptor and cycloinulohexaose (CF_6), methyl 6-*O*-β-inulohexaosyl-α-D-glucopyranoside was initially produced by the coupling reaction. Methyl 6-*O*-β-inulotriosyl-α-D-glucopyranoside (**1**) finally accumulated as a major product through the multiple actions of the enzyme. From this result, CFTase is expected to have a similar wide specificity for acceptors to CGTase.

* Corresponding author. Tel./fax: +81-6-729-78-3377.
E-mail address: kawamuria@cc.osaka-kyoiku.ac.jp (M. Kawamura)

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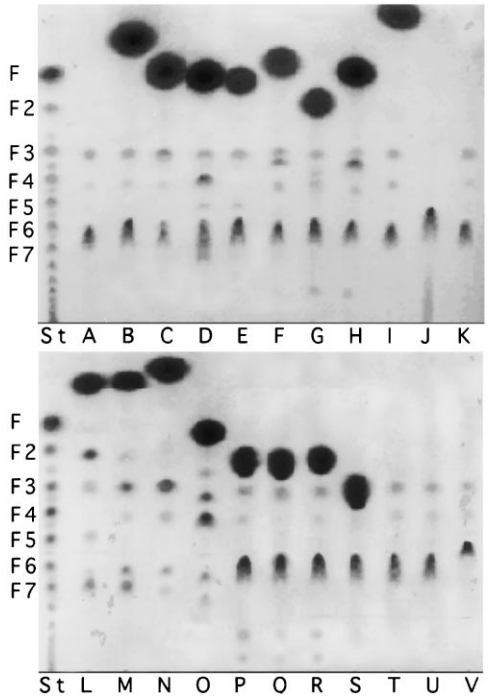

Fig. 1. TLC patterns of reaction mixtures: no addition (A), D-xylose (B), L-arabinose (C), D-fructose (D), D-glucose (E), D-mannose (F), D-galactose (G), L-sorbose (H), *N*-acetyl glucosamine (I), D-glucosamine (J), L-rhamnose (K), methyl α-D-glucopyranoside (L), methyl β-D-glucopyranoside (M), methyl α-D-mannopyranoside (N), sucrose (O), maltose (P), cellobiose (Q), α,α-trehalose (R), lactose (S), D-mannitol (T), D-sorbitol (U), and inositol (V).

In this paper, we examine the effects of various saccharides on the CFTase reaction with CF_6 and look at the resulting structures of the hetero-oligosaccharides.

2. Materials and methods

Materials.—CFTase was purified from the cultured broth of *B. circulans* OKUMZ 31B, and the enzyme unit was determined based on the cyclizing activity as described elsewhere [6]. CF_6 was purified from the digestion of inulin with CFTase as described before [1]. Invertase from *Candida utilis* was purchased from Sigma Chemical Co. (St. Louis, MO). The other saccharides and chemicals were obtained from commercial sources.

General methods.—The reducing power was measured using the Somogyi–Nelson method [7]. The analysis of the sugar composition after invertase treatment, thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC) was carried out as described in a previous report [5]. NMR spectra were measured at 27 °C with a Bruker DPX 400S spectrometer. Tetramethylsilane and 3-(trimethylsilyl)-1-propanesulfonic acid (δ 0.007 for ^1H and δ −0.184 ppm for ^{13}C) were used as internal standards in Me_2SO-d_6 and in D_2O, respectively. Solutions containing 50-mg samples were measured. ^1H and ^{13}C NMR spectra were measured at 400 and 100 MHz, respectively. We recorded the two-dimensional spectra of ^1H–^1H correlation spectroscopy (COSY), homonuclear Hartmann–Hahn (HOHAHA) spectroscopy, ^1H–^{13}C correlation spectroscopy (H–C COSY), and ^1H–^{13}C correlation spectroscopy via long-range

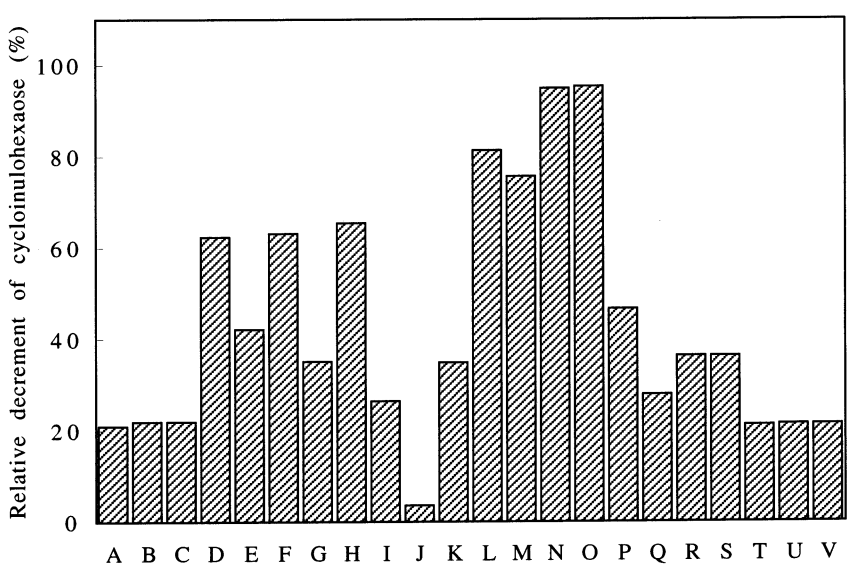

Fig. 2. Decrements of cycloinulohexaose in reactions (sugars the same as Fig. 1).

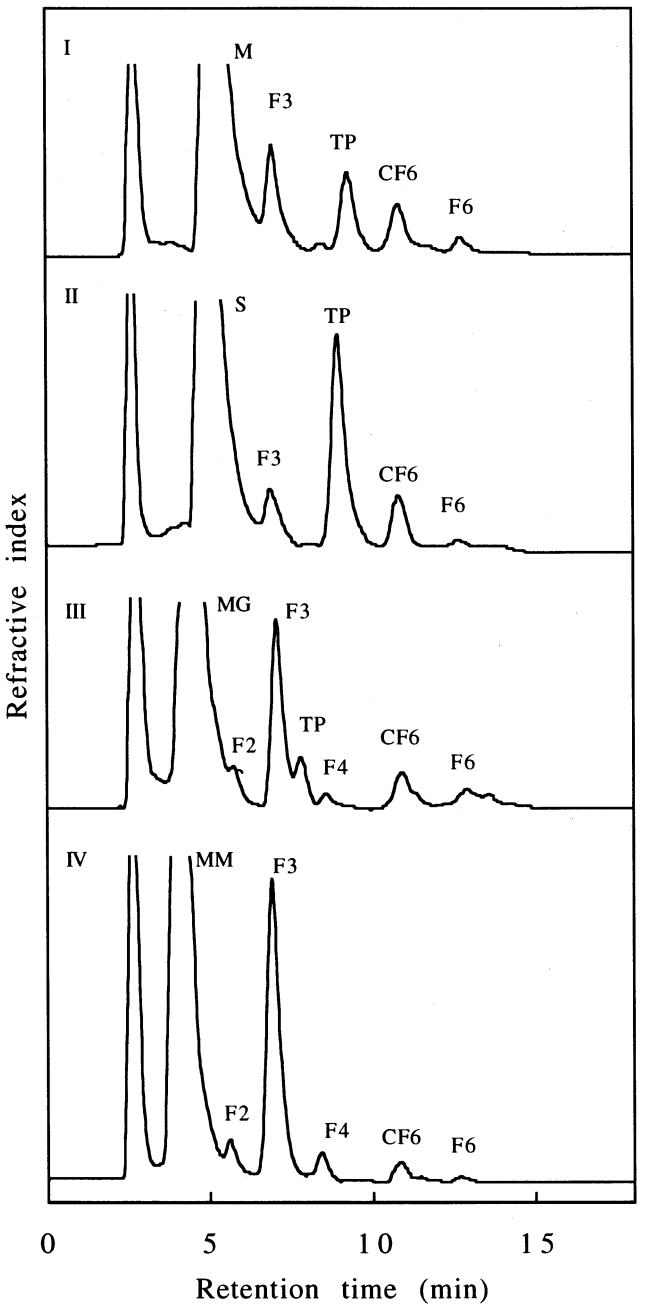

Fig. 3. HPLC patterns of oligosaccharides in reaction mixtures with D-mannose (I), L-sorbose (II), methyl β-D-glucopyranoside (III), and methyl α-D-mannopyranoside (IV). Abbreviations: M, D-mannose; S, L-sorbose; MG, methyl β-D-glucopyranoside; MM, methyl α-D-mannopyranoside; TP, transfer product; CF_6, cycloinulohexaose; F_2 to F_6, inulo-oligosaccharides from biose to hexaose.

coupling (COLOC) with the standard pulse programs of the XWIN-NMR™ software provided with the instrument.

Enzyme reaction and analysis of products.—Each reaction mixture containing 25 μL of CFTase solution (2.1 units/mL), 50 μL of 30% (w/v) saccharide solution, 10 μL of 10% CF_6 solution, and 15 μL of 20 mM Tris–citrate buffer, pH 7.5 was incubated at 30 °C for 24 h. The reaction was stopped by boiling for 5 min and then subjected to TLC analysis. An aliquot (20 μL) of each sample was then combined with 10 μL of invertase solution (10 units/mL in 50 mM acetate buffer, pH 5.0) and incubated at 40 °C for 60 min. A residual amount of CF_6 was measured by HPLC.

Preparation and isolation of transfer products.—Enzyme reactions with D-mannose or L-sorbose as acceptors were carried out in the same manner but scaled up 200 times. The transfer products were isolated by charcoal column chromatography according to a previous report [5]. The fractions containing only the transfer products with D-mannose and L-sorbose were combined and evaporated at 50 °C under reduced pressure, resulting in white powders; yield 120 and 180 mg, respectively. These samples were purified to homogeneity on TLC and HPLC.

3. Results and discussion

Effects of various saccharides on CFTase reaction.—Twenty-one types of saccharides were tested for their effects on the reaction of CFTase with CF_6. The products were analyzed by TLC (Fig. 1), and residual amounts of CF_6 were determined by HPLC after invertase treatment for quantification of the reactions (Fig. 2). In the control experiment, spots of inulotriose and inulotetraose were found on TLC as the result of the hydrolytic action of the enzyme, which resulted in a 20.9% decrease in CF_6 (column A). These spots were found in every test except that using D-glucosamine (column J), where the decrease in CF_6 was only 3.5%. This result suggests that D-glucosamine inhibits the enzyme activity. In the tests using D-mannose (column F) and L-sorbose (column H), additional spots were also found, and these zones seemed to be due to the transfer products and high consumption of CF_6, as is the case with methyl α-D-glucopyranoside (column L) and sucrose (column O). The high consumption was also observed in tests with methyl β-D-glucopyranoside

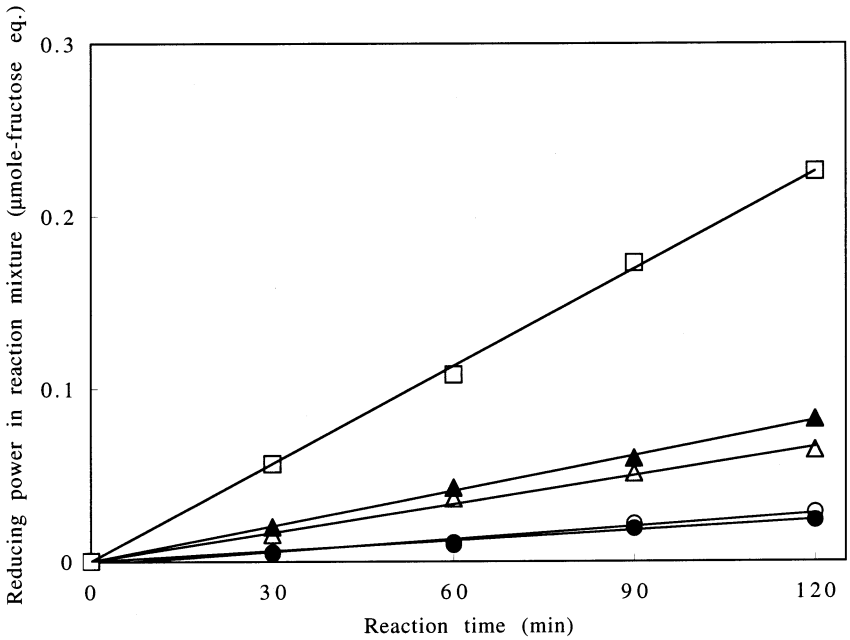

Fig. 4. Effect of methyl glycosides and sucrose on hydrolytic action of CFTase. Time course for no addition (○), methyl α-D-glucopyranoside (△), methyl β-D-glucopyranoside (▲), methyl α-D-mannopyranoside (□), or sucrose (●).

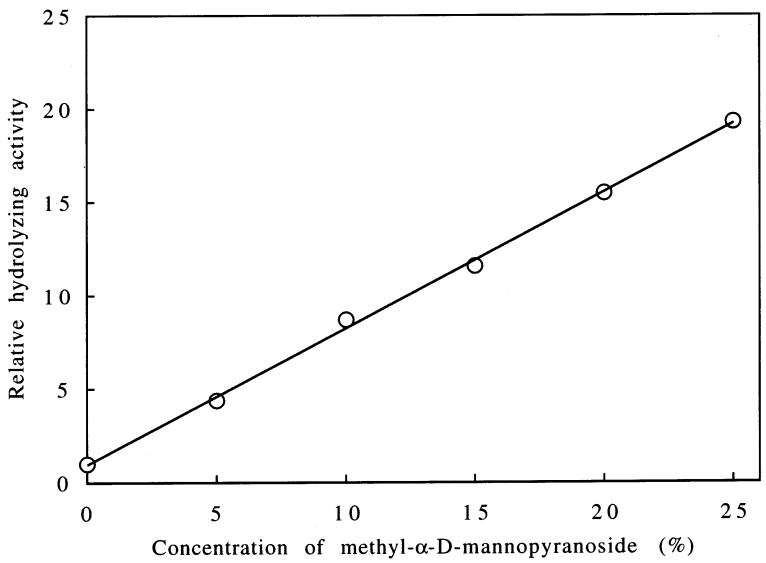

Fig. 5. Effect of concentration of methyl α-D-mannopyranoside on hydrolytic activity of CFTase.

Table 1
^{13}C NMR data for the transfer products of **2** and **3**

Products	Residues	C-1	C-2	C-3	C-4	C-5	C-6
2	D-Man	93.68	71.84	70.99	67.26	74.26	61.76
	D-Fru-1	61.97	104.09	77.68	75.09	82.10	63.01
	D-Fur-2	61.76	103.95	78.16	74.72	81.89	63.08
	D-Fur-3	61.35	104.52	77.59	75.16	81.87	62.90
3	L-Sor	64.49	98.20	71.69	74.36	70.11	62.54
	D-Fru-1	61.49	103.73	78.25	75.10	81.79	62.51
	D-Fur-2	61.54	103.73	78.51	75.10	81.87	62.51
	D-Fur-3	61.23	104.43	77.53	74.83	81.82	62.99

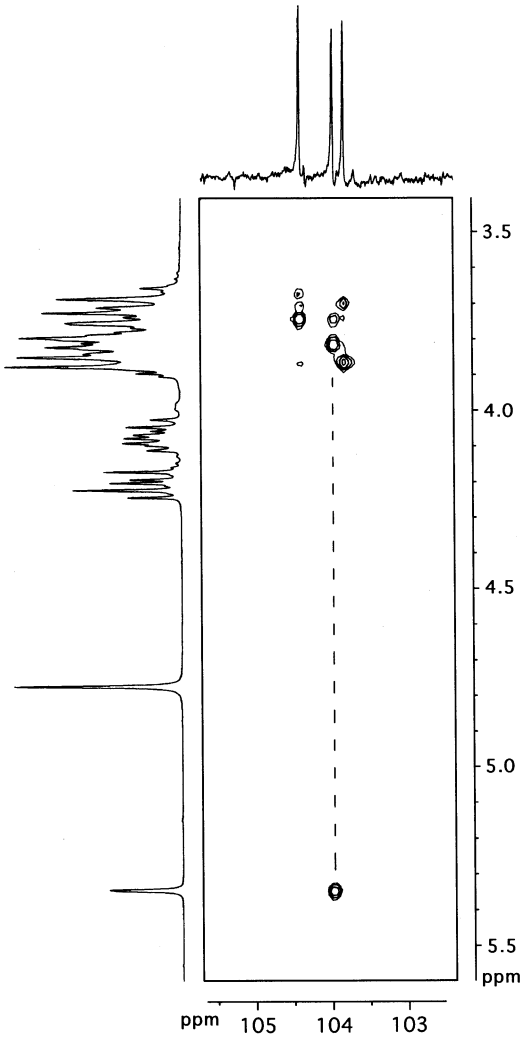

Fig. 6. COLOC spectrum of β-inulotriosyl-α-D-mannopyranoside solution in D_2O at 27 °C. Only the region with anomeric-carbon signals of D-fructosyl residues is shown.

(column M) and methyl α-D-mannopyranoside (column N). No additional products were indicated, but the spots of inulo-oligosaccharides were apparently enhanced. Additional faint spots and greater consumption of CF_6 than the control were found with D-glucose (column E), D-galactose (column G), maltose (column P), cellobiose (column Q), and α,α-trehalose (column R). This result suggests that CFTase can use these saccharides as acceptors. The other saccharides, pentoses and sugar alcohols, had no effect.

The four saccharides, D-mannose, L-sorbose, methyl β-D-glucopyranoside, and methyl α-D-mannopyranoside, were newly found to have pronounced effects on the CFTase reaction. Fig. 3 shows the HPLC patterns of their reaction mixtures. In the presence of D-mannose or L-sorbose, the transfer product was detected as the largest peak, and the inulotriose produced by hydrolysis was obvious. Such enhanced hydrolysis has been reported with methyl α-D-glucopyranoside [5]. On the other hand, methyl β-D-glucopyranoside only showed a small peak due to a transfer product, and methyl α-D-mannopyranoside showed no such peak. However, most of the CF_6 was hydrolyzed into inulotriose or into inulobiose and inulotetraose via inulohexaose. Therefore, we examined the effects of the methyl glycosides and sucrose on the hydrolyzing activity of CFTase with the same reaction mixtures as above (Fig. 4). The reducing power linearly increased during the 2-h incubation in all of the reaction mixtures, and the rate with all methyl glycosides increased compared with that without. Although two anomers of methyl D-glucopyranosides had distinct reactivities as acceptors, they showed similar enhancement in the hydrolysis. The highest enhancement occurred with methyl α-D-mannopyranoside, which was ten times greater than the control. In contrast, sucrose showed no effect or a slight tendency to suppress the hydrolysis. Subsequently, we examined the effect of the concentration of methyl α-D-mannopyranoside on the hydrolytic activity. The activity linearly accelerated depending on the concentration up to 25% (w/v) (in Fig. 5, the experiments above this concentration were limited by the solubility of the saccharide in water). At a 25% (w/v) concentration, the hydrolyzing activity was estimated at 0.27 μmol-fructose equivalents/min/mL of enzyme solution, which corresponded to 13% efficiency of the cyclizing activity. Such enhancement was considered to be due to an irregular conformational change of the enzyme caused by the interaction of the irregular substrates or analogs. In this situation, water may more readily approach the catalytic site.

Structure analysis of transfer products.— The transfer products produced by the reactions with D-mannose and with L-sorbose were isolated from the reaction mixtures, so we analyzed their sugar compositions. Both sugars were hetero-tetrasaccharides that consisted of one acceptor and three D-fructose residues. Thus, β-inulotriosyl D-mannose

(MF_3) and β-inulotriosyl L-sorbose (SF_3) were obtained. Tests of the reducing power showed the former was a nonreducing sugar and the latter was a reducing sugar.

The ^{13}C NMR signals of MF_3 and SF_3 in D_2O were assigned based on the 2D-NMR spectra of C–H COSY, COLOC, and HOHAHA (Table 1). MF_3 was obviously β-inulotriosyl D-mannoside judging from its nonreducing character. This structure was supported by the COLOC spectrum, since we observed a cross peak between one of three signals due to anomeric carbons of β-D-fructofuranosyl residues and an H-1 signal in the D-mannose residue ($M_{H\text{-}1}$) (Fig. 6). The ^{13}C NMR spectrum and the chemical shift of $M_{H\text{-}1}$ (δ 5.34 in D_2O) suggest that the D-mannose residue takes the configuration of α-D-mannopyranose [8–10]. MF_3 was thus identified as β-inulotriosyl α-D-mannopyranoside (**2**).

The ^1H NMR spectrum of SF_3 in Me_2SO-d_6 showed one singlet located at the lowest magnetic field (δ 5.45). This signal was obviously due to the 2-OH of the L-sorbose residue ($S_{OH\text{-}2}$), which agrees with its reducing power. The ^{13}C chemical shifts imply that the L-sorbose residue takes the configuration of α-L-sorbopyranose [10]. Subsequently the H–C COSY spectrum in D_2O showed that the doublet signal of $S_{H\text{-}3}$ was located at the highest magnetic field in all of the proton signals. This signal was easily identified as a doublet–doublet signal in Me_2SO-d_6. Then the resonances

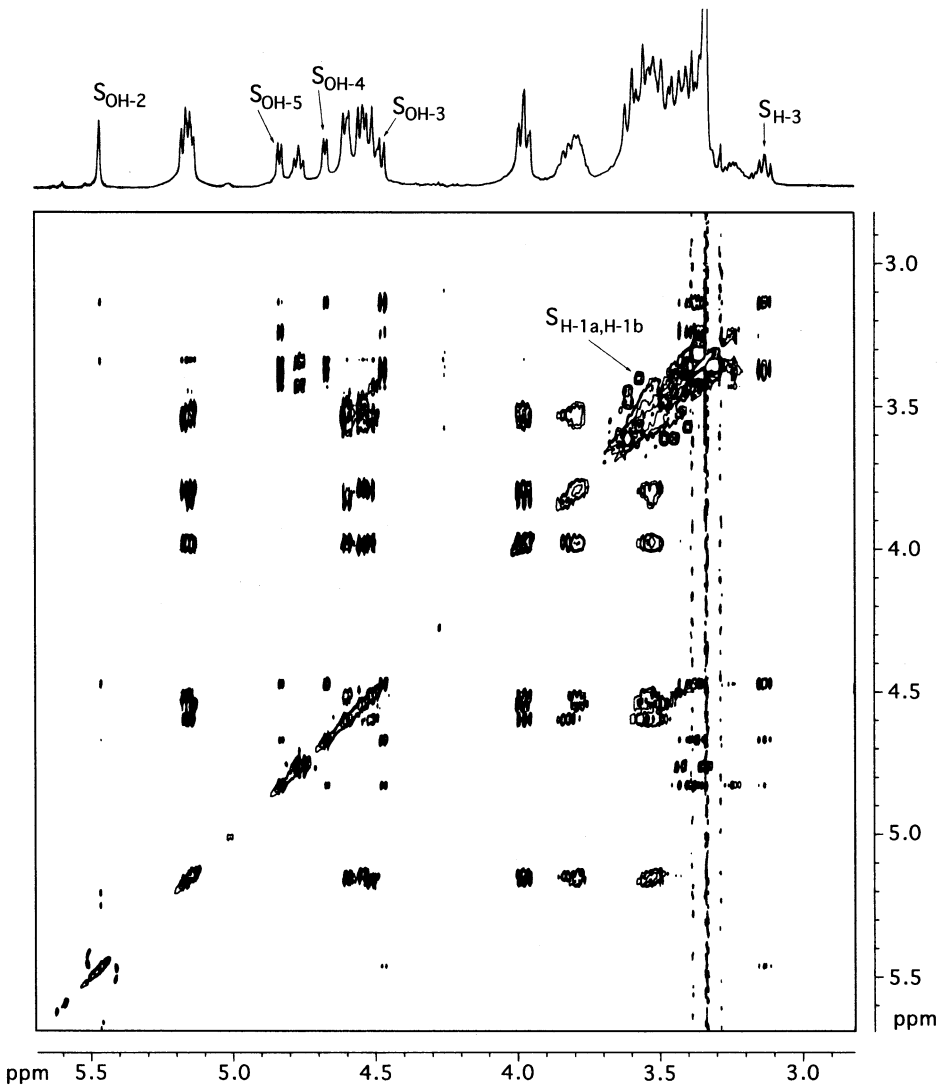

Fig. 7. HOHAHA spectrum of 1-*O*-β-inulotriosyl-α-L-sorbopyranose solution in Me_2SO-d_6 at 27 °C. Spin-lock for 120 ms done by MLEV-17 pulse sequences.

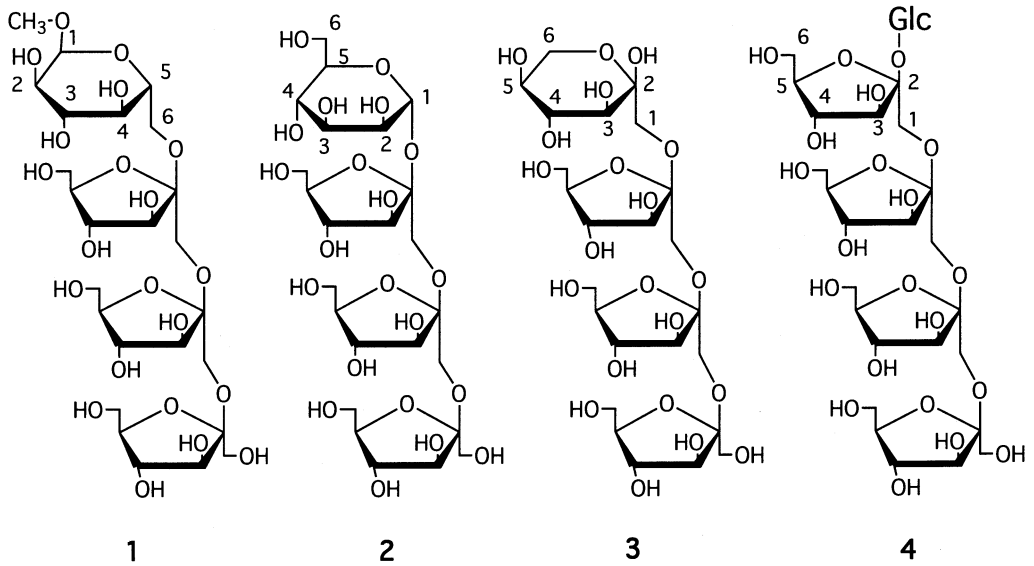

Fig. 8. Transfer product structures of **1**, **2**, **3**, and **4** depicted in Haworth projections. The methyl α-D-glucopyranoside moiety in **1** is represented as the reversed and rotated form. Carbon atoms numbered in acceptor moieties.

of S_{OH-3}, S_{OH-4}, and S_{OH-5} were assigned according to the correlation of $^{2}J_{H,H}$ and $^{3}J_{H,OH}$ in the COSY spectrum. Furthermore, the long-range couplings between these OH signals were confirmed in the HOHAHA spectrum (Fig. 7). There was no signal that correlated with the resonance due to S_{OH-1a} or S_{OH-1b}. The absence of S_{OH-1} was also demonstrated by the observation that the cross peak of $S_{H-1a,\ H-1b}$ was a coupled doublet in the shift-correlation spectra in Me_2SO-d_6. From these results, SF_3 was identified as 1-*O*-β-inulotriosyl-α-L-sorbopyranose (**3**).

A total of three hetero-oligosaccharides of **1**, **2**, and **3** were synthesized with CFTase. Fig. 8 shows a comparison of these structures with the structure of 1-*O*-β-D-fructofuranosyl-nystose (**4**), which was produced in the reaction with sucrose as an acceptor. In **1**, **3**, and **4**, a hydroxymethyl group was commonly the target for the transfer reaction, whereas in **2**, a ring OH of D-mannopyranose was the target. Thus, the intermolecular transfructosylation of the enzyme is not specific for a hydroxymethyl group on the acceptor, and a configuration among some OHs is considered important for an acceptor. As such, OH-4, OH-3, and OH-2 in α-D-glucopyranoside, OH-3, OH-4, and OH-6 in α-D-mannopyranose, and OH-3, OH-4, and OH-5 in α-L-sorbopyranose likely take a similar configuration to OH-3, OH-4, and OH-6 in β-D-fructofuranoside. However, this is not a sufficient condition for the acceptor, because methyl β-D-glucopyranoside did not behave in the same manner.

The structure of **2** suggests that methyl α-D-mannopyranoside is an acceptor analog in which the transfer position is methylated. Furthermore, it is considered to have the ability to convert an enzyme into a hydrolyzing enzyme. Compound **2** is an epimer of nystose, and we were unable to find any reports concerning it or related substances like D-fructosyl-D-mannoside, so its physiological function is of interest.

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