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Note

Isolation and structural confirmation of the oligosaccharides containing α-D-fructofuranoside linkages isolated from fermented beverage of plant extracts

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ABSTRACT

Fermented beverage of plant extracts was prepared from the extracts of approximately 50 types of vegetables and fruits. Natural fermentation was carried out mainly by lactic acid bacteria (*Leuconostoc* spp.) and yeast (*Zygosaccharomyces* spp. and *Pichia* spp.). Two oligosaccharides containing an α-fructofuranoside linkage were detected in this beverage and isolated using carbon–Celite column chromatography and preparative HPLC. The structural confirmation of the saccharides was determined by methylation analysis, MALDI-TOF-MS, and NMR measurements. These saccharides were identified as α-D-fructofuranosyl-(2→6)-D-glucopyranose, which was isolated from a natural source for the first time, and a novel saccharide β-D-fructopyranosyl-(2→6)-α-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside.

We had previously investigated the preparation of a fructopyranoside series of saccharides from the fermented beverage of plant extracts including β-D-fructopyranosyl-(2→6)-D-glucopyranose,[1] β-D-fructopyranosyl-(2→6)-β-D-glucopyranosyl-(1→3)-D-glucopyranose,[2] β-D-fructopyranosyl-(2→6)-[β-D-glucopyranosyl-(1→3)]-D-glucopyranose,[2] β-D-fructopyranosyl-(2→6)-D-fructofuranose,[3] β-D-fructopyranosyl-(2→1)-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside,[3] and β-D-fructopyranosyl-(2→6)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside.[3] The characteristics of β-D-fructopyranosyl-(2→6)-D-glucopyranose included non-cariogenicity and low digestibility. This saccharide was selectively utilized by the beneficial bacteria, *Bifidobacterium adolescentis* and *Bifidobacterium longum*, but it was not utilized by the non-beneficial bacteria, *Clostridium perfringens*, *Escherichia coli*, and *Enterococcus faecalis*, which can produce mutagenic substances.[4,5] Furthermore, these novel saccharides were confirmed to be produced by fermentation of the plant extract. In addition, we previously reported the synthesis of β-D-fructopyranosyl-(2→6)-D-glucopyranose from D-glucose and D-fructose using a thermal treatment process.[6]

Recently, we isolated and identified novel saccharides from the beverage that possessed linkage features, including β-D-glucopyranosyl-(1→1)-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside, β-D-galactopyranosyl-(1→1)-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside,[7] and β-D-fructofuranosyl-(2↔1)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranoside.[8]

In this paper, we report the isolation and structural confirmation of two oligosaccharides containing α-fructofuranoside linkage and fructopyranoside residue, which are unusual in the fermented beverage of plant extracts (Fig. 1).

Saccharides **1** and **2** were isolated from fermented beverage of plant extracts using carbon–Celite column chromatography, and they were shown to be homogeneous by anion exchange HPLC [t_R, sucrose (relative retention time; retention time of sucrose = 1.0, 5.24 min): 1.82 and 1.21, respectively]. The retention times of saccharides **1** and **2** did not correspond with the following known saccharides: glucose, fructose, sucrose, maltose, trehalose,

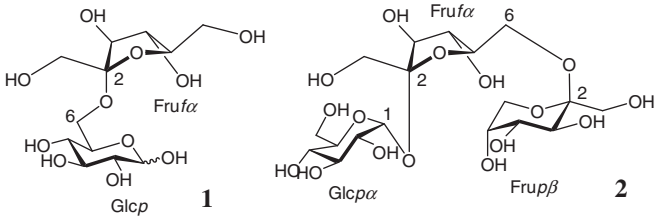

Figure 1. Structures of α-D-fructofuranosyl-(2→6)-D-glucopyranose (**1**) and β-D-fructopyranosyl-(2→6)-α-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside (**2**).

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laminaribiose, raffinose, 1-kestose, maltotriose, panose, nystose, β-D-fructopyranosyl-(2→6)-D-glucopyranose,[1] β-D-fructopyranosyl-(2→6)-β-D-glucopyranosyl-(1→3)-D-glucopyranose,[2] and β-D-fructopyranosyl-(2→6)-[β-D-glucopyranosyl-(1→3)]-D-glucopyranose.[2] The degree of polymerization of saccharide **1** was established as 2 based on measurements of $[M+Na]^+$ (*m/z*: 365) by MALDI-TOF-MS (Fig. 2) and the same analysis of saccharide **2** established a degree of polymerization of 3 (*m/z*: 527). This was supported by analyses of the molar ratios of D-glucose to D-fructose in the acid hydrolysates. The relative retention times of the methanolysates of the permethylated saccharides were investigated by gas liquid chromatography (GLC) analysis (t_R of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside = 1.0; actual retention time = 8.70 min). The methanolysate of permethylated saccharide **1** produced four peaks corresponding to methyl 1,3,4,6-tetra-*O*-methyl-D-fructoside (t_R, 1.09 and 1.31) and methyl 2,3,4-tri-*O*-methyl-D-glucoside (t_R, 2.49 and 3.52, Table 1). The methanolysate of permethylated saccharide **2** exhibited eight peaks corresponding to methyl 1,3,4-tri-*O*-methyl-D-fructoside (t_R, 1.87, 2.50, 3.92, and 4.55), methyl 1,3,4,5-tetra-*O*-methyl-D-fructopyranoside (t_R, 0.94 and 1.43), and methyl 2,3,4,6-tetra-*O*-methyl-D-glucoside (t_R, 1.06 and 1.53, Table 1). Based on these findings saccharides **1** and **2** were shown to be D-fructofuranosyl-(2→6)-D-glucose and D-fructopyranosyl-(2→6)-D-fructosyl-(2↔1)-D-glucoside, respectively.

The structural conformations of saccharides **1** and **2** were determined by ^1H and ^{13}C NMR analyses, followed by complete assignment of ^1H and ^{13}C NMR signals for the two saccharides using 2D NMR techniques. Each glucopyranose, fructofuranose, and fructopyranose residue of saccharides **1** and **2** was represented as Glc*p*, Fru*f*, and Fru*p* in Figure 1.

The NMR spectra of saccharide **1** indicated that it was a disaccharide containing Fru*f* and Glc*p*. It was an anomeric mixture of Glc*p* and the anomer configurations of α- (δ_H 4.64 ppm, 3.6 Hz) and β-Glc (δ_H 5.25 ppm, 8.1 Hz) were determined based on their *J* values. The HSQC-TOCSY spectrum revealed the ^1H and ^{13}C signals for each of the Fru*f*, Glc*pα*, and Glc*pβ* residues. The isolated methylene was assigned as H-1 and C-1 for Fru*f*. The COSY spectrum assigned the spin system of anomer units from H-4 to H-6 of Fru*f*, from H-1 to H-3 and H-5 to H-6 of Glc*pβ*, and from H-1 to H-4 of Glc*pα*. However, H-3 (δ_H 3.50 ppm) and H-4 (δ_H 3.50 ppm) of the Glc*pβ* signals suggested overlapping with a proton. The corresponding ^{13}C signals were assigned using the HSQC spectrum. The quaternary carbon was assigned as C-2 of Fru. Intra-residue HMBC correlation was found between the H-6 (δ_H 3.91 ppm and

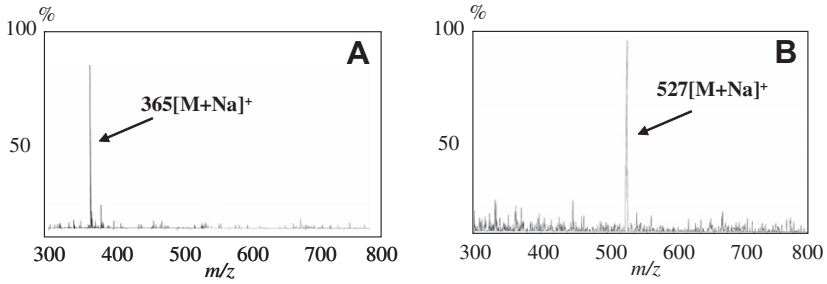

Figure 2. MALDI-TOF-MS spectra of saccharides **1** (A) and **2** (B).

Table 1
Gas–liquid chromatographic analysis of methanolysates of permethylated saccharides **1** and **2**

Methanolysate origin	Relative retention time[a]					
	Methyl 1,3,4,5-tetra-*O*-methyl-D-fructopyranoside	Methyl 1,3,4,6-tetra-*O*-methyl-D-fructofuranoside	Methyl 2,3,4,6-tetra-*O*-methyl-D-glucoside	Methyl 2,3,4-tri-*O*-methyl-D-glucoside	Methyl 1,3,4-tri-*O*-methyl-D-fructoside	Methyl 3,4,6-tri-*O*-methyl-D-fructoside
Saccharide **1**		1.09 1.31		2.49 3.52		
Saccharide **2**	0.94 1.43		1.06 1.53		1.87 2.50 3.92 4.55	
β-F*p*2-6G[b]	0.99 1.46			2.44 3.50		
1-Kestose		1.08 1.28	1.08 1.49			2.65 3.96
Fructose	0.98 1.47	1.06 1.28				
Levan					1.90 2.50 3.92 4.49	
Methyl 2,3,4,6,-tetra-*O*-methyl-β-D-glucoside			1.00			

[a] Retention time of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside = 1.0; retention time, 8.70 min.
[b] β-F*p*2-6G: β-D-fructopyranosyl-(2→6)-D-glucopyranose.[5]

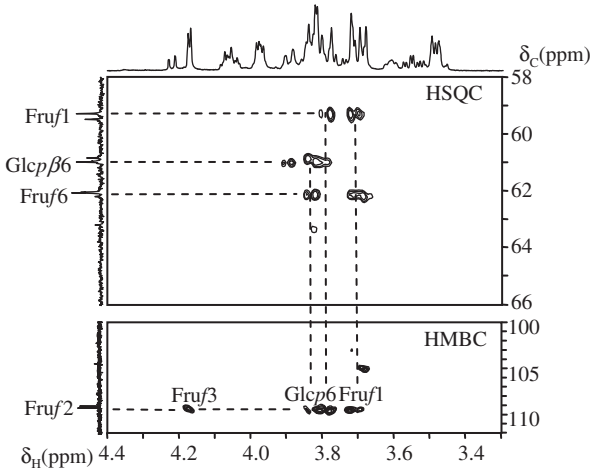

Figure 3. Part of HSQC and HMBC spectra of saccharide **1**.

3.81 ppm) of Glc*pβ* and C-4. Moreover, the selected $^{1}H–^{1}H$ coupling patterns of overlapping proton signals were extracted from the SPT difference spectrum. The Fru*f* residue was then assigned. Isolated methylene protons (δ_H 3.80 and 3.72 ppm) showed HMBC of C-3 of Fru*f*. This was estimated as the H-1 of Fru*f*. These protons also indicated HMBC to one quaternary carbon, which was assigned as C-2 of Fru*f*. H-3 to H-6 of Fru*f* were assigned in reverse using the COSY technique. It is known that α-fructoside produces larger chemical shifts compared with β-fructoside.[9] Fru*f* was determined as the α-furanoside type, because the C-2 (δ_C 109.20 ppm), C-3 (δ_C 81.15 ppm), C-4 (δ_C 78.19 ppm), and C-5 (δ_C 84.23 ppm) of Fru exhibited larger chemical shifts compared with the C-2 (δ_C 106.4 ppm), C-3 (δ_C 79.6 ppm), and C-4 (δ_C 77.1 ppm) of β-D-fructofuranosyl-(2→6)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside (neokestose).[10] The connectivity of saccharide residues was determined from the HMBC correlation between the C-2 of Fru and that of H-6 (δ_H 3.81 ppm, dd, 11.8 Hz and 5.7 Hz) of Glc*pβ* (Fig. 3). This chemical shift position overlapped with the methylene proton of Glc*pα*-6 (δ_H 3.86 ppm) and the H-6 of Fru. Fructose was determined as the α-furanoside type. Therefore, this correlation was not a correlation with a fructose intra-residue, so we were able to judge the correlation of the C-2 of Fru*f* (δ_C 109.20 ppm) and the H-6 of Glc*p* (δ_H 3.81 and 3.86 ppm).

These results indicated a Fru*f*2α→6Glc*p* linkage and all the ^{1}H and ^{13}C NMR signals were assigned as shown in Table 2.

The NMR spectra of saccharide **2** were analyzed in the same manner as that of saccharide **1**. The 1D NMR spectra of saccharide **2** showed that it was a trisaccharide containing Fru, Fru′, and Glc*p*, and not an anomeric mixture. The Glc*p* was determined as an α-form based on the *J* value of H-1. The HSQC-TOCSY spectrum revealed the ^{1}H and ^{13}C signals for each of the Glc*pα*, Fru, and Fru′ residues. The isolated methylene was assigned as the H-1 and C-1 of Fru. The remaining three methylene carbons were assigned to C-6 in these residues. The COSY spectrum assigned the spin systems of these residues from H-1 to H-6 of Glc*p*. Two signals at 3.85 and 3.73 ppm were assigned to H-6 of Fru using HSQC-TOCSY, and the spin system of Fru was assigned to H-6 to H-3 by COSY. The corresponding ^{13}C signals were assigned based on the E-HSQC spectrum. The HMBC of C-3/H-1of Fru was used to assign this carbon. Fru was confirmed to be of the fructofuranose form based on the HMBC correlation between C-2 and H-5 of Fru, and the α-form based on its chemical shift.

The COSY spectrum was used to assign H-3 to H-6 of Fru′. The Fru′ protons indicated an HMBC with a methine carbon at δ_C 69.22 and only one quaternary carbon (δ_C 101.51), which was assigned as C-3 and C-2 of Fru′, respectively. The isolated methylene was assigned as the H-1 and C-1 of Fru′. Therefore, Fru′ was confirmed as the fructopyranose form based on the HMBC between the C-2 and H-6 of Fru′, and as the β-form based on its chemical shift. The positions of the glycosidic linkage and fructosidic linkage were analyzed as follows. One methylene carbon was unassigned,

Table 2
^{1}H and ^{13}C NMR spectral data (δ^{a} in ppm, *J* in Hz) of saccharides **1** and **2**

Saccharide **1**										Saccharide **2**				
		δ_C	δ_H	J_{HH}			δ_C	δ_H	J_{HH}			δ_C	δ_H	J_{HH}
Fru*f*α	1	59.29	3.80 d	12.6	Fru*f*α	1	59.48			Fru*f*α	1	62.22	3.79 s[b]	
			3.72 d	12.6										
	2	109.20				2	109.01				2	110.33		
	3	81.15	4.19 d	3.5		3	81.31				3	81.12	4.22 d	2.5
	4	78.19	3.99 dd	6.2, 3.5		4	78.05				4	78.55	4.06 dd	4.4, 2.5
	5	84.23	4.09 m[c]			5	83.94	4.07 m			5	84.62	4.26 ddd	7.4, 4.4, 3.5
	6	62.05	3.84 dd	12.2, 3.5		6					6	61.88	3.80 dd	11.7, 3.5
			3.72 dd	12.2, 5.4									3.68 dd	11.7, 7.4
Glc*p*β	1	96.81	4.67 d	8.1	Glc*p*α	1	92.99	5.25 d	3.6	Fru*p*	1	61.37	3.80 m	
	2	74.89	3.27 m			2	72.26	3.56 dd	10.1,3.6					
	3	76.57	3.50 m			3	73.65	3.73 dd	10.1,8.4		2	101.51		
	4	70.64	3.50 m			4	70.64	3.52 dd	10.8,8.4		3	69.22	3.91 m	
	5	75.30	3.62 m			5	71.01	3.99 m			4	70.38	3.91 m	
	6	60.98	3.91 m			6	60.83	3.86 m			5	69.89	4.00 m	
			3.81 dd	11.8, 5.7							6	64.94	3.85 d	13.5
													3.73 d	13.5
										Glc*p*α	1	91.89	5.36 d	4.0
											2	71.96	3.57 dd	9.5, 4.0
											3	74.16	3.69 dd	9.5, 9.4
											4	70.25	3.39 dd	9.9, 9.4
											5	73.43	3.91 m	
											6	61.29	3.85 m	
													3.73 m	

[a] The chemical shifts of ^{1}H (δ_H) and ^{13}C (δ_C) in ppm were respectively determined relative to the external standard of sodium [2,2,3,3-$^{2}H_4$]-3-(trimethylsilyl)propanoate in D_2O (δ_H 0.00 ppm) and 1, 4-dioxane (δ_C 67.40 ppm) in D_2O.
[b] s, singlet.
[c] m, multiplet.

but it was estimated as the C-1 of Fru*p* (Fig. 4). Its protons indicated an HMBC with a methine carbon at δ_C 69.22 and only one quaternary carbon (δ_C 101.51, Fig. 4), which was assigned as the C-3 and C-2 of Fru*p*, respectively. The C-2 of Fru*f* had an HMBC with the H-1 of Glc*p*. The C-2 of Fru*p* correlated with H-6 of Fru*f*. These results indicated a Glc*p*1α↔α2Fru*f*6←β2Fru*p* linkage and all the ^{1}H and ^{13}C NMR signals were assigned as shown in Table 2.

Based on all of these findings, the saccharides **1** and **2** isolated from fermented beverage of plant extracts were confirmed as oligosaccharides containing an α-D-fructofuranoside linkage, α-D-fructofuranosyl-(2→6)-D-glucopyranose, and the novel saccharide, β-D-fructopyranosyl-(2→6)-α-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside (Fig. 1). No saccharide containing α-fructofuranoside linkage is known to have been isolated from natural sources, with the exception of these saccharides isolated from the fermented beverage of plant extracts. The chemical synthesis of saccharide **1** has already been reported,[11] but NMR analysis was not conducted. The present study performed complete assignment of the ^{1}H and ^{13}C signals.

The synthesis of the saccharides by the fermentation of plant extracts was investigated using high performance anion-exchange chromatography (HPAEC). Most of the monosaccharides were removed using charcoal from the fermented and unfermented plant extract beverage in a batch method. Saccharides **1** and **2** were identified in the fermented beverage, but they were not present in the unfermented one. Therefore, saccharides **1** and **2** were confirmed as fermentation products in the plant extract beverage (Fig. 5).

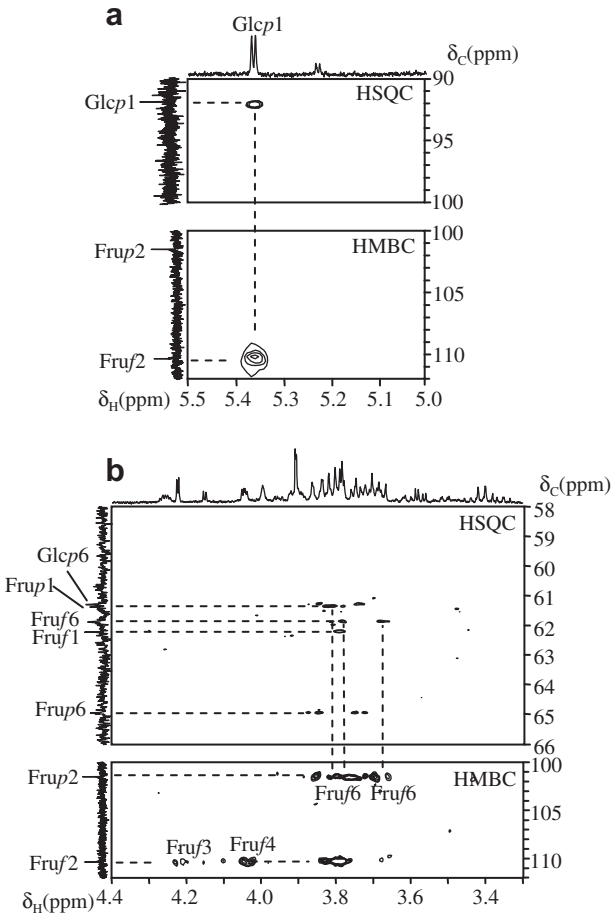

Figure 4. Part of HSQC and HMBC spectra of saccharide **2**.

1. Experimental

1.1. Materials

Glucose, fructose, sucrose, maltose, trehalose, laminaribiose, raffinose, maltotriose, panose, and levan were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Crystalline 1-kestose (β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside) and nystose (β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside) were prepared from sucrose using a *Scopulariopsis brevicaulis* enzyme.[12] β-D-fructopyranosyl-(2→6)-D-glucopyranose, β-D-fructopyranosyl-(2→6)-β-D-glucopyranosyl-(1→3)-D-glucopyranose, and β-D-fructopyranosyl-(2→6)-[β-D-glucopyranosyl-(1→3)]-D-glucopyranose were prepared according to methods described previously.[1,2] All other chemicals used in this study were of analytical grade.

The fermented beverage was prepared from extracts of fruits and vegetables and naturally fermented using yeast (*Zygosaccharomyces psedrouxii* and *Pichia anomala*) and lactic acid bacteria (*Leuconostoc* spp.), as described in previous reports.[1,8,13]

1.2. HPAEC

The oligosaccharides were analyzed using a Dionex Bio LC Series system (Sunnyvale, CA) equipped with an HPLC carbohydrate column (Carbo Pack PAI, inert styrene divinyl benzene polymer, Sunnyvale, CA) and pulsed amperometric detection (PAD).[14,15] The mobile phase consisted of eluent A (150 mM NaOH) and eluent B (500 mM sodium acetate in 150 mM NaOH) with a sodium acetate gradient as follows: 0–1 min, 0–5 mM; 1–20 min, 5–100 mM; 20–22 min, 500 mM; 22–30 min, 0 mM. The flow rate through the column was 0.5 mL/min. The applied PAD potentials for E1 (500 ms), E2 (100 ms), and E3 (50 ms) were 0.1, 0.6, and −0.6 V, respectively, and the output range was 1 μC.[16]

1.3. Isolation of saccharides

Fermented plant extract beverage (1000 g) was loaded onto a 4.5 × 35 cm carbon–Celite column, [1:1; charcoal (Wako Pure

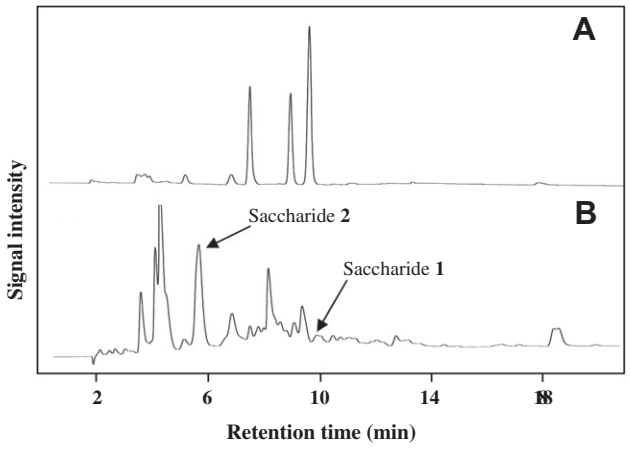

Figure 5. High performance liquid chromatogram of fermentation products. Analysis of saccharides produced during fermentation was done by HPAEC. (A) Plant extracts were fermented for 0 days. (B) Plant extracts were fermented for 180 days. The beverage (100 mL) fermented for 0–180 days was added to charcoal (10 g), stirred for 3 h. and filtered. The charcoal was extracted with 30% ethanol (500 mL) three times. The ethanol extracts were combined, concentrated to dryness, and solubilized with one ml of distilled water. The sugar solution was analyzed by HPAEC.

Chemical Industries, Ltd, Osaka, Japan) and Celite-535 (Nacalai Tesque Inc, Osaka, Japan)] and was successively eluted with water (14 L), 5% ethanol (30 L), and 30% ethanol (10 L). Most of the glucose and fructose were eluted with water (4 l) before saccharide **1** was eluted with water (5–6 L). The water fraction containing saccharide **1** was concentrated in vacuo and freeze-dried to yield 913 mg. The 30% ethanol fraction containing saccharide **2** was concentrated in vacuo and freeze-dried to yield 894 mg. The water fraction (10 mg) and 30% ethanol fraction (10 mg) were subsequently applied to an HPLC system (Tosoh, Tokyo, Japan) fitted with an Amide-80 column (7.8 mm × 30 cm, Tosoh, Tokyo, Japan), which was operated at 80 °C and eluted with 80% acetonitrile at 2.0 mL/min, and a refractive index detector. The chromatographic separation was repeated 60 times. Furthermore, saccharide fractions **1** (24.3 mg) and **2** (83.8 mg) were purified using an HPLC system with an ODS-100 V column (4.6 mm × 25 cm, Tosoh, Tokyo, Japan), which was operated at room temperature and eluted with water at 0.5 mL/min. The purified saccharides **1** (5.0 mg) and **2** (3.5 mg) were obtained as white powders.

1.4. Hydrolysis

The oligosaccharides (0.5 mg) were dissolved in 0.05 M oxalic acid (0.5 mL) and partially hydrolyzed by heating at 60 °C for 15 min.

1.5. Methylation and methanolysis

Methylation of the oligosaccharides was carried out according to the method of Hakomori.[17]

The permethylated saccharides were methanolyzed by heating with 1.5% methanolic hydrochloric acid at 96 °C for 10 or 180 min. The reaction mixture was treated with Amberlite IRA-410 (OH^-) to remove hydrochloric acid and evaporated to dryness in vacuo. The resulting methanolysate was dissolved in a small volume of methanol and analyzed using GLC.

1.6. GLC

The methanolysate was analyzed by GLC using a Shimadzu GC-8A gas chromatograph equipped with a glass column (2.6 mm × 2 m) packed with 15% butane 1,4-diol succinate polyester on acid-washed Celite at 175 °C. The flow rate of the nitrogen gas carrier was 40 mL/min.

1.7. MALDI-TOF-MS

MALDI-TOF-MS spectra were measured using a Shimadzu–Kratos mass spectrometer (KOMPACT Probe) in the positive ion mode using 2,5-dihydroxybenzoic acid as the matrix. Ions were formed using a pulsed UV laser beam (nitrogen laser, 337 nm). Calibration was conducted using 1-kestose as an external standard.

1.8. NMR measurement

The saccharides (ca. 0.5 mg of **1**, 0.8 mg of **2**) were dissolved in 0.06 mL (saccharide **1**) and 0.4 mL (saccharide **2**) of D_2O. NMR spectra were recorded at 27 °C using a Bruker AMX 500 spectrometer (^1H 500 MHz, ^{13}C 126 MHz) equipped with a 2.5 mm C/H dual probe (saccharide **1**), a 5 mm C/H dual probe (1D spectra of saccharide **2**), and a 5 mm TXI probe (2D spectra of saccharide **2**). Chemical shifts of ^1H (δ_H) and ^{13}C (δ_C) in ppm were determined relative to an external standard of sodium [2,2,3,3-$^2H_4$]-3-(trimethylsilyl) propanoate in D_2O (δ_H 0.00 ppm) and 1,4-dioxane (δ_C 67.40 ppm) in D_2O, respectively. ^1H–^1H COSY,[18,19] HSQC,[20] E-HSQC,[20] HSQC-TOCSY,[20,21] and HMBC[22,23] spectra were obtained using gradient selected pulse sequences. The TOCSY mixing period (0.1 s) was conducted using DIPSI-2. The coupling patterns of overlapped ^1H were analyzed by SPT method.[24,25]

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