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Structural Analysis and Characteristics of Oligosaccharides Isolated from Fermented Beverage of Plant Extract

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ABSTRACT

A fermented beverage of plant extract was prepared from about 50 kinds of fruits and vegetables. Natural fermentation was conducted by lactic acid bacteria (*Leuconostoc* spp.) and yeast (*Zygosaccharomyces* spp. and *Pichia* spp.). Eighteen kinds of oligosaccharides were isolated from this beverage, and their structures were confirmed by methylation analysis, MALDI-TOF-MS and NMR measurements. In these saccharides, eight novel oligosaccharides were found to be constructed by di- and trisaccharides with the fructosyl residue of pyranose form, and other trisaccharides with fructosyl residues of sucrose bond with the β-D-galactose and β-D-glucose. The characteristics of one of novel saccharide, *O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose (F*p*2-6G) were investigated, and it was shown to be non-cariogenicity and have low digestibility. Furthermore, the saccharide was selectively used by beneficial bacteria, *Bifidobacterium adolescentis* and *B. longum*, but was not used by unfavorable bacteria, *Clostridium perfringens*, *Escherichia coli* and *Enterococcus faecalis* that produce mutagenic substances.

Keywords: β-D-fructopyranoside, natural fructopyranoside, novel oligosaccharide
Abbreviations: F*p*2-6G, *O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose

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INTRODUCTION

The extract from 50 kinds of fruits and vegetables was fermented to produce a new beverage (Okada *et al.* 2005, 2006). The juices were extracted using sucrose-osmotic pressure in a cedar barrel for one week and were fermented by lactic acid bacteria (*Leuconostoc* spp.) and yeast (*Zygosaccharomyces* spp. and *Pichia* spp.). The fermented beverage showed scavenging activity against 1,1'-diphenyl-2-picrylhydrazyl (DPPH) radical, and reduced significantly the ethanol-induced damage of gastric mucosa in rat (Okada *et al.* 2005). Analysis by high performance anion-exchange chromatography (HPAEC) showed that this beverage contained high levels of saccharides, estimated between 550 and 590 gL^{-1}, mainly glucose and fructose, and a small amount of undetermined oligosaccharides. Recently, it was reported that different positions of the glycosidic linkage of oligosaccharide isomers affect the isomer's physiological and physical properties (Kohmoto *et al.* 1988; Murosaki *et al.* 1999, 2002). The development of HPLC analyses with high sensitivity and separation ability enable the detection and isolation of oligosaccharides in fermented beverages. We already reported that 18 kinds of oligosaccharides were isolated from this beverage, in these saccharides, eight novel oligosaccharides [saccharide **2** (Okada *et al.* 2006), **10**, **14** (Kawazoe *et al.* 2008a), **12**, **13** (Kawazoe *et al.* 2008b), **8**, **9**, **16** (Okada *et al*. 2009)] were found.

In this paper, we report on the isolation and structural confirmation of the saccharides from the fermented beverage of plant extracts.

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Invited Review

METHODOLOGY

Preparation of fermented beverage of plant extract

For preparation of initial juice, 50 kinds of fruits and vegetables were used to produce the final extract as detailed in Okada *et al*. (2005). Fruits and vegetables, obtained from local markets of Hokkaido region, were sorted for the absence of defects, washed and immediately processed for juice extraction. The fruits and vegetables were cut, sliced or diced into small pieces, mixed and placed in cedar barrels. Afterwards, the equivalent weight of sucrose was added to samples, mixed well to allow high samples-sucrose contact, and then barrels were left at room temperature for one week. The juice exudates were then separated – without compression – from solids and used for fermentation. Almost all sucrose was hydrolyzed to glucose and fructose during fermentation (glucose 30%, fructose 25%). The fermented beverage was obtained by incubation of the juice at 37°C in the dark by natural fermentation using yeast (*Zygosaccharomyces* spp. and *Pichia* spp.) and lactic acid bacteria (*Leuconostoc* spp.). After 7 days, the fermented beverage was kept in a closed enameled tank (5,000 L) at 37°C for 6-10 months for additional maturation and aging to finally obtain a brown and slightly sticky liquid (Super Ohtaka, Ohtakakohso, Co., Ltd. Hokkaido).

The pH of plant extract reached about 3.2 after 7 days fermentation (**Fig. 1**).

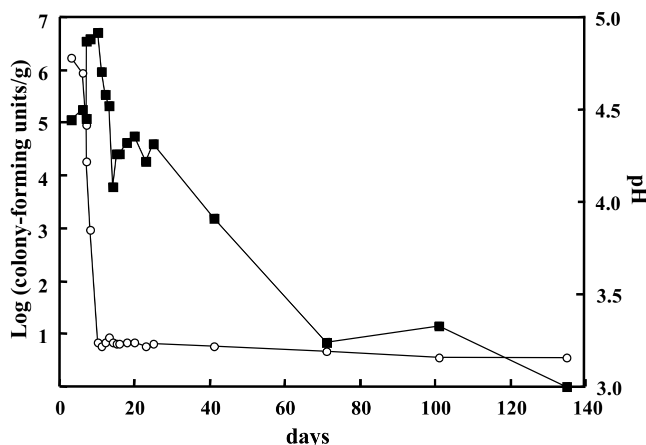

Fig. 1 Fermentation process of plant extracts. Symbols: ○, pH; ■, colony-forming units/g.

High performance anion-exchange chromatography (HPAEC)

The oligosaccharides were analyzed using a Dionex Bio LC Series apparatus (Sunnyvale, CA) equipped with an HPLC carbohydrate column (Carbo Pac PA1, inert styrene divinyl benzene polymer, Sunnyvale, CA) and a pulsed amperometric detector (PAD: Rocklin and Pohl 1983; Johnson 1986). The gradient was established by mixing eluant A (150 mM NaOH) with elunat B (500 mM sodium acetate in 150 mM NaOH) as follows: 0-1 min, 25 mM; 1-2 min, 25-50 mM; 2-20 min, 50-200 mM; 20-22 min, 500 mM; 22-30 min, 25 mM; using a flow rate through the column of 1.0 mL/min. The applied PAD potentials for E1 (500 ms), E2 (100 ms) and E3 (50 ms) were 0.1, 0.6 and -0.6V, respectively, and the output range was 1 µC.

p-Aminobenzoic acid ethyl ester conversion method

Conversion of the saccharides at the reducing end with *p*-Aminobenzoic acid ethyl ester (ABEE: Seikagaku Biobusiness Co., Tokyo) was carried out according to a prior method (Yasuno *et al.* 1997; Kobayashi *et al.* 2002). Ten µL of standard saccharide solution was added to an ABEE reagent solution (40 µL). The mixture was incubated at 80°C for 1 h. Distilled water (0.2 mL) and chloroform (0.2 mL) were added and mixture was centrifuged at 2,000 × *g* for 1 min, aqueous layer was diluted (100-fold) with water and subjected to HPLC analysis. ABEE-converted saccharide was detected by UV at 305 nm.

Methylation and methanolysis

Methylation of the oligosaccharides was carried out by the method of Hakomori (Hakomori 1964).

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^- : Organo Co., Tokyo) to remove hydrochloric acid, and evaporated *in vacuo* to dryness. The resulting methanolysate was dissolved in a small volume of methanol and analyzed using gas liquid chromatography (GLC).

Gas liquid chromatography

For the analysis of the methanolysate, GLC was carried out 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.

GC-MS analysis

GC-MS analysis (Ciucanu and Costello 2003) was performed using JMS-AX500 mass spectrometer (JEOL, Japan) using a DB-17HT capillary column (30 m × 0.25 mm I.D., J&W Scientific, USA). Injection temperature was 200°C. The column temperature was kept at 50°C for 2 min after sample injection, increased to 150°C at 50°C/min, kept at 150°C for 1 min, then increased to 250°C at 4°C/min. The mass spectra were recorded in the electron ionization (EI) mode.

MALDI-TOF-MS

MALDI-TOF-MS spectra were measured using a Shimadzu-Kratos mass spectrometer (KOMPACT Probe) in positive ion mode with 2, 5-dihydroxybenzoic acid as a matrix. Ions were formed by a pulsed UV laser beam (nitrogen laser, 337 nm). Calibration was done using 1-kestose as an external standard. 1-Kestose was prepared from sucrose using a *Scopulariopsis brevicaulis* enzyme (Takeda *et al.* 1994).

NMR measurement

The saccharides were dissolved in 0.4 mL D_2O. NMR spectra were recorded at 27°C with a Bruker AMX 500 spectrometer (^1H 500 MHz, ^{13}C 125 MHz) equipped with a 5 mm diameter C/H dual (1D spectra) and TXI probe (2D spectra). Chemical shifts of ^1H (δ_H) and ^{13}C (δ_C) in ppm were determined relative to the 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 (Aue *et al.* 1975; von Kienlin *et al.* 1991), HSQC (Willker *et al.* 1993), HMBC (Bax and Summers 1986; Hurd and John 1991), and CT-HMBC (Bax and Summers 1986; Hurd and John 1991) spectra were obtained using gradient selected pulse sequences. The phase-sensitive HSQC-TOCSY (Domke 1991; Willker *et al.* 1993) and CH_2-selected E-HSQC-TOCSY (Yamamori *et al.* 2002) spectra were determined with the sequence including inversion of direct resonance (IDR). The TOCSY mixing time (108 ms) was composed of MLEV-17 composite pulses guarded by a trim pulse (2.5 ms). The coupling patterns of overlapped ^1H were analyzed by SPT method (Pachler and Wessels 1973; Uzawa and Yoshida 2004).

Cariogenicuty of *O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose (F*p*2-6G)

The cariogenicity was performed according to the method of Miyamura (1973). The oral cavity of a subject was rinsed with tap water and then the subject gargled with distilled water. The saliva naturally secreted was collected and well shaken at room temperature. A 0.5 mL aliquot of 1% F*p*2-6G, palatinose, glucose or distilled water was then added to a mixture of 1.5 mL of this fresh saliva and 0.5 mL of a brain heart infusion broth (Eiken Chemical Co., Ltd. Tokyo). These mixtures were incubated at 37°C to show the time-dependent pH variation of the cariogenicity. The same experiment was conducted with *Streptococcus mutans* JCM 5705 instead of saliva (the same experiment was repeated three times).

RESEARCH FINDINGS

Isolation of saccharides from fermented beverage of plant extract

The fermented beverage of plant extract (5 kg) was loaded onto to a carbon-Celite [1: 1; Charcoal (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and Celite-535 (Nakalai Chemical Industries, Ltd., Osaka, Japan)] column (4.5 × 35 cm) and successively eluted with water (14 L), 5% ethanol (30 L) and 30% ethanol. Almost all of glucose and fructose were eluted with water (4L) and then saccharides **1** and **2** were eluted with water (5-6L, **Fig. 2**a). Fractions including saccharides **1** and **2** were concentrated *in vacuo* and freeze-dried to give 1.25 g. Finally, the yield and purity of saccharide **2** were 49 and 98%, respectively. Saccharides **3**, **4** and **5** were eluted with 1 L of 5% ethanol (**Fig. 2**b). Saccharide **6** was obtained by additional elution (1L) of 5% ethanol (**Fig. 2**c). Both fractions containing saccharides **5** and **6** were also concentrated *in vacuo* and freeze-dried to give 0.97 and 1.70 g, respectively. Saccharides **7-18** were eluted with 30% ethanol (1-2L, **Fig. 2**d, 0.84 g). Subsequently, those fractions were successfully purified repeatedly using an HPLC system (Tosoh, Tokyo, Japan) equipped with an Amide-80 column (7.8 mm × 30 cm, Tosoh, Tokyo, Japan) at 80°C, and eluted with 80% acetonitrile at 2.0 mL/min, and using refractive index detection. Furthermore, those saccharides were purified using a HPLC with an ODS-80Ts column (4.6 mm × 25 cm, Tosoh, Tokyo, Japan) at room temperature.

Structure analysis of saccharides

The saccharides isolated from the fermented beverage were identified using high performance anion-exchange chromatography (HPAEC: Shiomi *et al.* 1991; Okada *et al.* 2003), MALDI-TOF-MS and NMR.

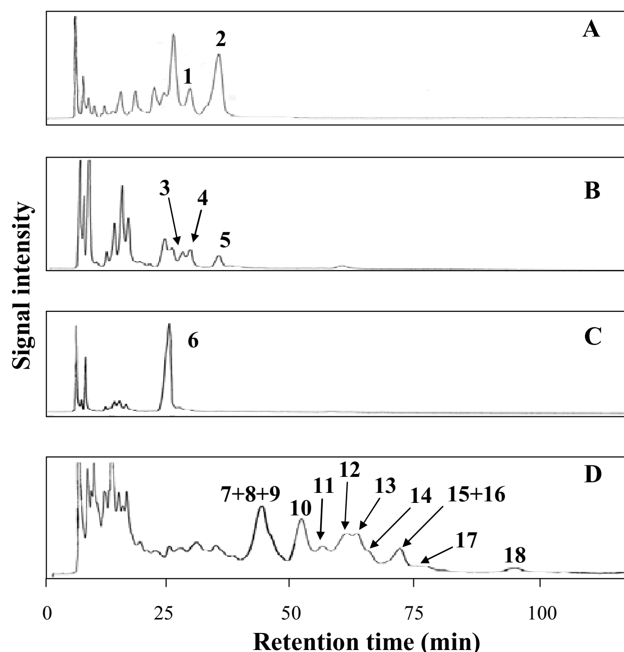

Fig. 2 Isolation of saccharides from plant extract by Amide-80 column chromatography. Fermented beverage of plant extract was loaded onto to a carbon-Celite column and successively eluted with water (**A**), 5% ethanol (**B**, **C**) and 30% ethanol (**D**).

Saccharides **1**, **3**, **4**, **5**, **6**, **7**, **11**, **15**, **17** and **18** corresponded to authentic saccharides, *O*-β-D-fructofuranosyl-(2→6)-glucose, maltose, sophorose, gentiobiose, laminaribiose, maltotriose, raffinose, panose, *O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→3)-D-glucopyranose and gentiotriose, respectively. Sophorose, gentiobiose and laminaribiose were already known to be synthesized enzymatically under highly concentrated solution of glucose and the presence of yeast and *Penicillium* β-glucosidase (Villa and Phaff 1979; Fujimoto *et al.* 1988; Rastall *et al.* 1992). *O*-β-D-Glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→3)-D-glucopyranose was also reported to be synthesized by β-glucosidase (Kawamura *et al.* 1971). The saccharides isolated from the fermented beverage of plant extracts were not detected before fermentation. Therefore, these saccharides were estimated to be synthesized by action of yeast β-glucosidase during fermentation. Although fructofuranosyl-(2→6)-glucose was obtained from partial hydrolysate of neokestose, it was presumed to be produced by reverse-reaction of invertase of yeast.

The $t_{R,sucrose}$ values of HPAEC of saccharides **2**, **8**, **9**, **10**, **12**, **13**, **14** and **16** did not correspond to those of any authentic saccharides. The degree of polymerization (DP) of

Table 1 Gas-liquid chromatographic analysis of methanolysates of permethylated saccharides.

Methanolysate origin	Relative retention time a)												
Saccharide 2	0.99				1.46			2.44			3.50		
Saccharide 8		1.00	1.07	1.30		1.48		2.59			3.50		
Saccharide 9			1.07	1.29				2.58		3.22	3.59		4.73
Saccharide 10	0.97				1.43			2.51		3.26	3.63		4.77
Saccharide 12		1.01				1.41			2.64			3.94	
Saccharide 13		1.01				1.42	1.74		2.64			3.97	
Saccharide 14	0.92	1.04			1.46	1.46							
Saccharide 16		0.97				1.47							
Fructose	0.93		1.07	1.30	1.42								
1-Kestose			1.08	1.28		1.49			2.65			3.96	
Raffinose			1.04	1.34			1.69	2.63			3.74		
Laminaribiose		0.98				1.49				3.31			4.88
Methyl-2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside		1.00											
Methyl-2,3,4,6-tetra-*O*-methyl-β-D-galactopyranoside							1.76						

a) Retention time of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside=1.

saccharide **2** was established as 2 by measurements of [M+Na] ions (*m/z*: 365) using TOF-MS and similar analyses of saccharides **8**, **9**, **10**, **12**, **13**, **14** and **16** established all their DP as 3 (*m/z*:527). From the GC analysis, relative retention times of the methanolysate of the permethylated saccharide were investigated [t_R (relative retention time; retention time of methyl 2, 3, 4, 6-tetra-*O*-methyl-β-D-glucopyranoside = 1.0; retention time, 9.60 min)]. The methanolysate of permethylated saccharide **2** exhibited four peaks (**Table 1**), two of which corresponded to methyl 2,3,4-tri-*O*-methyl-D-glucopyranoside (t_R, 2.44 and 3.50). From this finding, saccharide **2** was presumed to be fructosyl (2→6) glucose. However, other peaks (t_R, 0.99 and 1.46) did not correspond to methyl 1,3,4,6-tetra-*O*-methyl-D-fructofuranoside (t_R, 1.04 and 1.34) from permethylated raffinose. Because the peaks were estimated to correspond to methyl 1,3,4,5-tetra-*O*-methyl-D-fructopyranoside, we tried to prepare methyl fructopyranoside from-D-fructose. The permethylated fructose and methanolysate of permethylated saccharide **2** were analyzed by GC-MS. The permethylated fructose gave four peaks, that were confirmed to be methyl 1,3,4,6-tetra-*O*-methyl-β- and α-D-fructofuranoside (retention time, 8.34 and 8.56 min) and methyl 1,3,4,5-tetra-*O*-methyl-β- and α-D-fructopyranoside (9.14 and 10.57 min), respectively from the retention time and pattern of fragmentation (Ciucanu and Costello 2003). The methanolysate from permethylated saccharide **2** exhibited two peaks (9.14 and 10.63 min) corresponding to the α/β isomers methyl 1,3,4,5-tetra-*O*-methyl-D-fructopyranoside. No peaks corresponding to methyl 1,3,4,5-tetra-*O*-methyl-D-fructopyranoside were detected from the methanolysate of permethylated raffinose. From these findings as above, saccharide **2** was proved to be D-fructopyranosyl-(2→6)-D-glucose.

Subsequently, NMR analysis was done. The HSQC-TOCSY spectrum revealed the ^{1}H and ^{13}C signals of each β-Glc, α-Glc, and Fru residues. The COSY spectrum assigned the spin systems of these residues; from H-1 to H-6 in β-Glc, from H-1 to H-5 of α-Glc, and from H-4 to H-6 in Fru. The corresponding ^{13}C signals were assigned by HSQC spectrum. The assignment of the remaining signals, the position of the glycosidic linkage, and the pyranoside form of fructose were analyzed as follows. There was one methylene carbon not assigned yet, which was estimated as the C-1 of Fru (**Fig. 3** (a)). Its protons showed HMBC correlations to methine carbon at δ_C 69.29 and the only one quaternary carbon (δ_C 101.52) (**Fig. 3** (b)), which was assigned as C-3 and C-2 of Fru, respectively. The C-2 of Fru was also correlated to H-6 of Glc and H-6 of Fru (**Fig. 3** (b)). These results revealed that saccharide **2** had a fructopyranoside residue and a Fru*p* 2→6 Glc linkage, and all ^{1}H and ^{13}C NMR signals were assigned. Due to strong coupling between H-3 and H-4 in β-Glc, H-3 and H-4 in Fru, H-5 and H-6 in Fru, and H-5 and H-6 in α-Glc, these couplings could not be analyzed in the first order. The δ_C values of Fru indicated its β anomer form, by comparing those of α and β

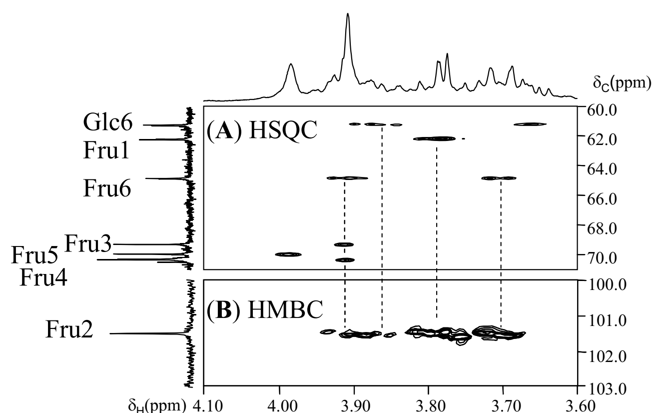

Fig. 3 Part of HSQC (A) and HMBC (B) spectra of saccharide 2.

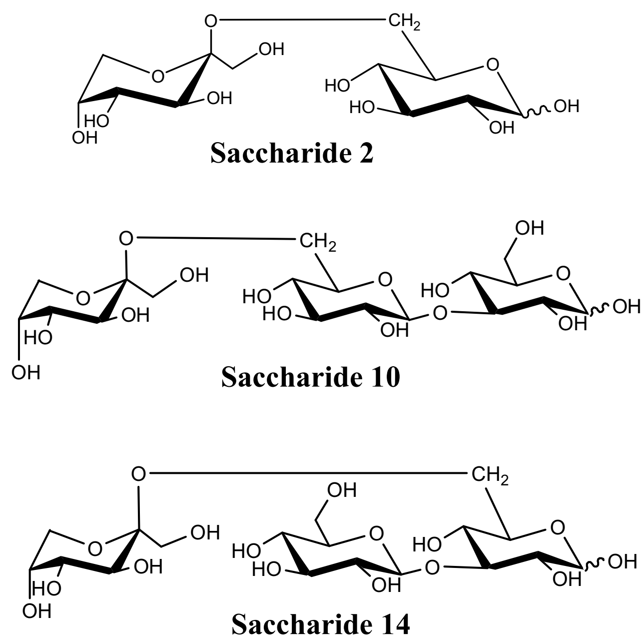

Fig. 4 Structures of novel saccharides containing fructopyranoside isolated from fermented beverage of plant extract.

form of methyl-D-fructopyranoside (Sinclaer 1988).

From these findings, the fructose residue of the non-reducing terminal of this saccharide was of a pyranose form, and saccharide **2** discovered in the fermented beverage of plant extracts was confirmed to be a novel saccharide, *O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose (**Fig. 4**, **Appendix 1**).

Saccharides **10** and **14** were similarly identified as follows by examination of partial acid hydrolysates, GLC analysis of methanolyzates and NMR measurement. Partial acid hydrolysates of saccharides **10** and **14** were liberated to glucose, fructose and laminaribiose. Saccharides **10** and **14** also had a fructose residue of the pyranose form. The methanolysate of permethylated saccharide **10** exhibited six peaks (**Table 1**), four of which corresponded to the α/β isomers of methyl 2,3,4-tri-*O*-methyl-D-glucopyranoside (t_R, 2.51 and 3.63) and methyl 2,4,6-tri-*O*-methyl-D-glucopyranoside (t_R, 3.26 and 4.77). The other two peaks corresponded to the α/β isomers methyl 1,3,4,5-tetra-*O*-methyl-D-fructopyranoside (t_R, 0.97 and 1.43).

The methanolysate of permethylated saccharide **14** exhibited three peaks, which corresponded to methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside (t_R, 1.04 and 1.46) and methyl 1,3,4,5-tetra-*O*-methyl-D-fructopyranoside (t_R, 0.92 and 1.46). No peaks corresponding to methyl 1,3,4,6-tetra-*O*-methyl-D-fructofuranoside were detected. The methanolysate of permethylated saccharide **14** gave two other peaks that were confirmed to be methyl 2,4-di-*O*-methyl-D-glucopyranoside from the retention time and pattern of fragmentation with GC-MS.

The ^{1}H and ^{13}C NMR signals of each saccharide were assigned using 2D-NMR including COSY, HSQC, HSQC-TOCSY, CH_2-HSQC-TOCSY, and CT-HMBC experiments (**Appendix 4** and **7**). Saccharides **10** and **14** were determined as new saccharides, *O*-β-D-fructopyranosyl-(2→6)-*O*-β-D-glucopyranosyl-(1→3)-D-glucopyranose and *O*-β-D-fructopyranosyl-(2→6)-[*O*-β-D-glucopyranosyl-(1→3)]-D-glucopyranose, respectively (**Fig. 4**). No saccharides containing fructopyranoside residues were found in natural resources excepting the saccharide in the fermented beverage of plant extract. Furthermore, synthesis of saccharides **2**, **10**, and **14** by fermentation of plant extracts was investigated by using the ABEE-method and these saccharides were produced during fermentation (**Fig. 5**).

Partial acid hydrolysate of saccharide **12** was liberated to glucose, fructose and sucrose, and that of saccharide **13** was liberated to galactose, glucose, fructose and sucrose.

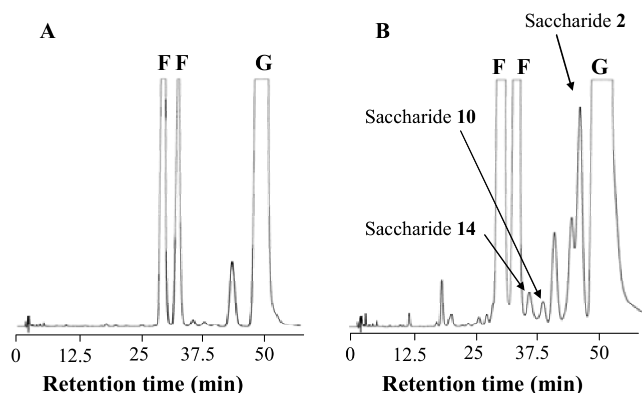

Fig. 5 High performance liquid chromatogram of fermentation products. HPLC analysis of saccharides produced during fermentation was done by ABEE conversion method. (**A**) Plant extract was fermented for 0 days. (**B**) Plant extract was fermented for 180 days. G: glucose; F: fructose.

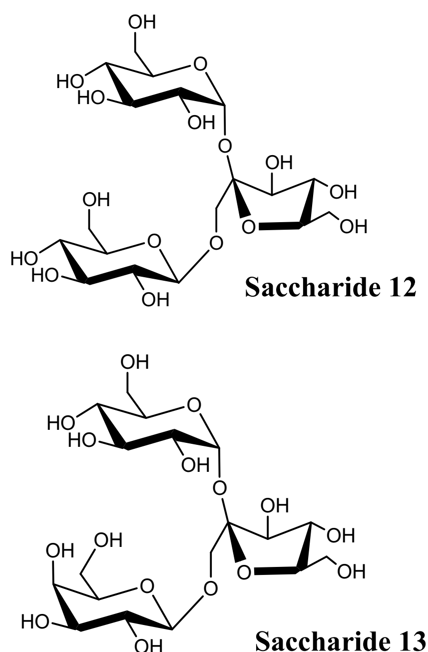

Fig. 6 Structures of novel saccharides isolated from fermented beverage of plant extract.

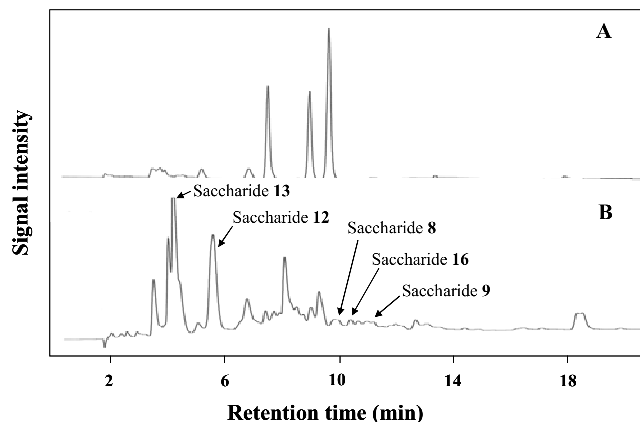

Fig. 7 High performance liquid chromatogram of fermentation products. Analysis of saccharides produced during fermentation was done by HPAEC. (**A**) Plant extract was fermented for 0 days. (**B**) Plant extract was fermented for 180 days. The beverage (100 mL) fermented for 0 or 180 days was added with 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.

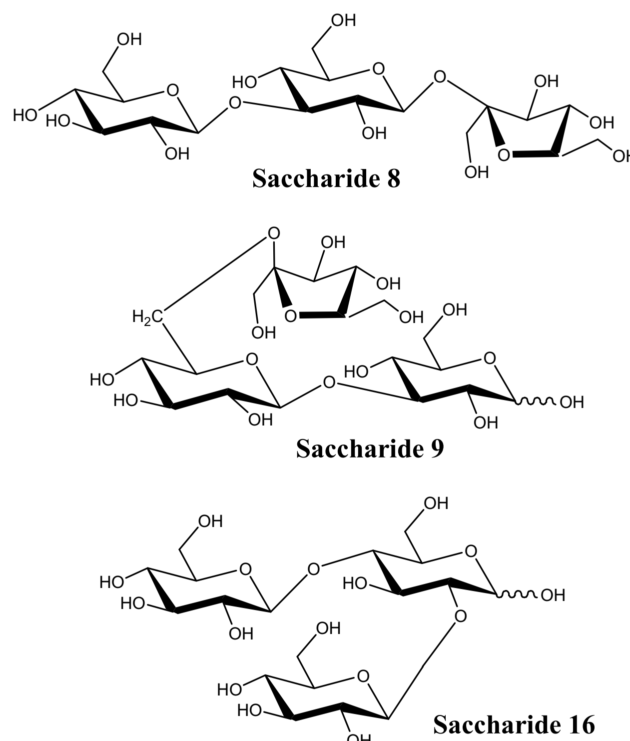

Fig. 8 Structures of novel trisaccharides isolated from fermented beverage of plant extract.

The methanolysate of permethylated saccharide **12** exhibited four peaks (**Table 1**) corresponding to methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside (t_R, 1.01 and 1.41) and methyl 3,4,6-tri-*O*-methyl-D-fructofuranoside (t_R, 2.64 and 3.94). The methanolysate of permethylated saccharide **13** also exhibited six peaks (**Table 1**) corresponding to methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside (t_R, 1.01 and 1.42), methyl 2,3,4,6-tetra-*O*-methyl-D-galactopyranoside (t_R, 1.74), and methyl 3,4,6-tri-*O*-methyl-D-fructofuranoside (t_R, 2.64 and 3.97).

From these findings as above, saccharides **12** and **13** were proved to be D-glucosyl-(1→1)-D-fructosyl-(2↔1)-D-glucoside and D-galactosyl-(1→1)-D-fructosyl-(2↔1)-D-glucoside, respectively. The ^{1}H and ^{13}C NMR signals of each saccharide were assigned using 2D-NMR including COSY, HSQC, HSQC-TOCSY, and HMBC experiments (**Appendix 5** and **6**).

The saccharide **12** and **13** were identified as new saccharides, *O*-β-D-glucopyranosyl-(1→1)-*O*-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside; 1^F-β-D-glucopyranosylsucrose and *O*-β-D-galactopyranosyl-(1→1)-*O*-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside; 1^F-β-D-galactopyranosylsucrose, respectively (**Fig. 6**). Although, melezitose, 1^F-α-galactosylsucrose and 6^F-β-galactosylsucrose, had already been reported (Pazur *et al.* 1958; Courtois and Ariyoshi 1960), 1^F-β-D-glucopyranosylsucrose and 1^F-β-D-galactopyranosylsucrose were found in natural resources for the first time. Synthesis of the saccharides by fermentation of plant extract was investigated using HPAEC.

On the other hand, acid hydrolysates of saccharides **8** and **9** were liberated to glucose and fructose, and that of saccharide **16** was liberated to glucose. The methanolysate of permethylated saccharide **8** exhibited six peaks (**Table 1**) corresponding to methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside (t_R, 0.94 and 1.48), methyl 2,4,6-tri-*O*-methyl-D-glucopyranoside (t_R, 3.27 and 4.81) and methyl 1,3,4,6-tetra-*O*-methyl-D-fructofuranoside (t_R, 1.06 and 1.32). The methanolysate of permethylated saccharide **9** also exhibited six peaks (**Table 1**) corresponding to methyl 2,3,4-tri-*O*-methyl-D-glucopyranoside (t_R, 2.58 and 3.59), methyl 2,4,6-tri-*O*-methyl-D-glucopyranoside (t_R, 3.22 and 4.73), and methyl 1,3,4,6-tetra-*O*-methyl-D-fructofuranoside (t_R, 1.07

and 1.29). On the other hand, the methanolysate of permethylated saccharide **16** exhibited two peaks (**Table 1**) corresponding to methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside (t_R, 0.97 and 1.47). GC-MS analysis of the retention times and fragmentation patterns of methyl glucosides showed two peaks (10.08 and 10.21 min) from the methanolysate of permethylated saccharide **16** to be methyl 3,6-di-*O*-methyl-D-glucopyranoside. The ^{1}H and ^{13}C NMR signals of each saccharide were assigned using 2D-NMR including COSY, HSQC, HSQC-TOCSY, CH_2-selected E-HSQC-TOCSY, HMBC and CT-HMBC experiments (**Appendix 2**, **3** and **8**).

The saccharides **8**, **9** and **16** were *O*-β-D-fructofuranosyl-(2→1)-[*O*-β-D-glucopyranosyl-(1→3)]-D-glucopyranoside, *O*-β-D-fructofuranosyl-(2→6)-*O*-β-D-glucopyranosyl-(1→3)-D-glucopyranose and *O*-β-D-glucopyranosyl-(1→2)-[*O*-β-D-glucopyranosyl-(1→4)]-D-glucopyranose, respectively (**Fig. 8**), and were also novel oligosaccharides.

Synthesis of saccharides by fermentation of plant extracts was investigated using HPAEC. Almost all monosaccharides were removed from the fermented and unfermented beverages of plant extracts by the batch method with charcoal. The saccharides **8**, **9**, **12**, **13** and **16** were observed in the fermented beverage, but not in the unfermented one. Therefore, these five saccharides were confirmed to be produced during fermentation of plant extracts (**Fig. 7**).

Characteristics of saccharide 2,*O*-β-D-fructopyranosyl-(2→6)-D-glucopyranose (F*p*2-6G)

The characteristics of F*p*2-6G were investigated. Sweetness was 0.2-fold compared with that of sucrose. Cariogenicity was performed according to the method of Miyamura (1973). As shown in **Fig. 9**, F*p*2-6G was a non-cariogenic sugar because *S. mutans* and oral bacteria produce no acid. Furthermore, F*p*2-6G was hydrolyzed by pig pancreatin and rat intestinal enzymes 3.5 and 12.2% compared with starch and sucrose, respectively, and the results showed that F*p*2-6G had low digestibility. LB broth was used as basal medium for testing utilization of the saccharides by intestinal bacteria. D-Glucose, sucrose, lactose, melibiose, palatinose, turanose, raffinose, 1-kestose, or F*p*2-6G was added to the LB medium at a final concentration of 0.5% (w/v). After incubation at 37°C for 72 h under anaerobic condition, bacterial growth was measured by pH analysis of the medium. The symbols "+++", "++", "+", "±" and "-" show the medium pH of 4.0-4.5, 4.5-5.0, 5.0-5.5, 5.5-6.0 and 6.0-7.0, respectively. *Bifidobacteria* and *Lactobacilli* are beneficial to both nutrition and health of human and animals, while some intestinal bacteria such as *Escherichia coli*, *Enterobactor cloaceae* and *Enterococcus faecalis* are detrimental intestinal bacteria.

Under the normal condition of *Bifidobacteria* growth, the pH of the medium added no saccharide (control), F*p*2-6G, 1-kestose, raffinose, turanose, palatinose, melibiose, lactose, sucrose or glucose was 6.51-6.68, 5.47-6.14, 4.08-4.40, 4.03-4.18, 4.13-6.05, 4.22-5.12, 4.00-4.16, 4.02-4.50, 4.12-4.94 or 4.00-4.40, respectively. On the other hand, F*p*2-6G as well as 1-kestose and turanose were not fermented by *Enterobacter cloacae*, *Escherichia coli* and *Enterococcus faecalis*. Moreover, F*p*2-6G was selectively used by four beneficial bacteria strains of *Bifidobacteria* (**Table 2**).

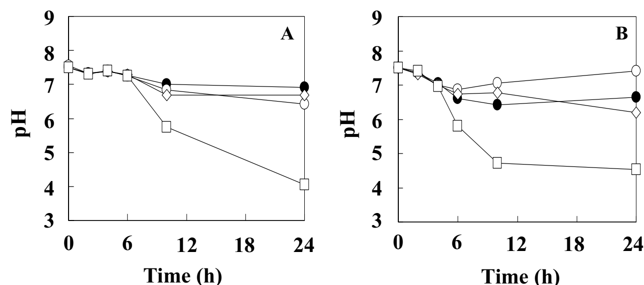

Fig. 9 Cariogenicity of F*p*2-6G. A 0.5 mL aliquot of 1% (w/v) F*p*2-6G, palatinose, glucose or distilled water was added to a mixture of 1.5 mL of fresh saliva or culture of *S. mutans*, and 0.5 mL of a brain heart infusion broth. (**A**) *Streptococcus mutans* JCM 5705. (**B**) Human saliva. Symbols: ○, Distilled water; □, Sucrose; ◇, Palatinose; ●, F*p*2-6G.

SUPPLEMENTATION

NMR-chemical shifts of saccharides (saccharides **2**, **8**, **9**, **10**, **12**, **13**, **14**, and **16**) are summarized as appendixes.

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Table 2 Utilization of F*p*2-6G and several other saccharides by some human intestinal bacteria.

Bacterial species	F*p*2-6G	1-Kestose	Raffinose	Turanose	Palatinose	Melibiose	Lactose	Sucrose	Glucose
Bifidobacterium adolescentis 2793	+	+++	+++	-	+++	+++	+++	+++	+++
B. bifidum 2777	±	+++	+++	++	++	+++	+++	+++	+++
B. breve 2776	-	+++	+++	++	+	+++	+++	+++	+++
B. infantis 2775	±	+++	+++	++	++	+++	+++	+++	+++
B. longum 2778	+	+++	+++	+++	+++	+++	+++	++	+++
Lactobacillus acidophilus 2243	-	+++	+	-	+++	+++	+++	+++	+++
L. casei 2036	-	+	++	-	±	+++	+++	++	+++
L. fermentum 2046	-	±	++	-	-	+++	+++	++	+++
Enterobacter cloacae 1180	-	-	++	-	±	+++	++	++	+++
Escherichia coli 1099	-	-	-	-	-	±	+	++	++
Enterococcus faecalis 2048	-	-	-	-	±	++	++	++	+++
Clostridium perfringens 1211	-	+	±	-	-	++	++	++	++

The symbols "+++", "++", "+", "±" and "-" show pH 4.0-4.5, 4.5-5.0, 5.0-5.5, 5.5-6.0 and 6.0-7.0, respectively.

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

		δ_C	δ_H		J_{HH}
Fru*p*	1	62.19	3.76	d	11.7
			3.79	d	11.7
	2	101.52			
	3	69.29	3.91	m[b]	
	4	70.34	3.91	m	
	5	70.00	3.98	br s[c]	
	6	64.83	3.91	m	
			3.70	m	
βGlc	1	96.72	4.61	d	7.7
	2	74.94	3.22	dd	9.4, 7.7
	3	76.31	3.45	m	
	4	70.34	3.45	m	
	5	75.88	3.56	dd	9.3, 5.7
	6	61.20	3.65	dd	10.8, 5.7
			3.88	d	10.8
αGlc	1	92.87	5.21	d	3.7
	2	72.24	3.52	dd	9.3, 3.7
	3	73.39	3.68	dd	9.8, 9.3
	4	70.51	3.39	dd	9.8, 9.3
	5	71.62	3.92	m	
	6	61.24	3.67	m	
			3.85	m	

[a] Chemical shifts of ^{1}H (δ_H) and ^{13}C (δ_C) in ppm were determined relatively 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, respectively.
[b] m: multiplet.
[c] br s: broad singlet

Appendix 2 ^{1}H and ^{13}C NMR spectral data (δ^a in ppm, *J* in Hz) of saccharide 8.

		δ_C	δ_H		J_{HH}
Fru*f*	1	62.02	3.79	d	12.6
			3.69	d	12.6
	2	105.47			
	3	76.61	4.29	d	8.6
	4	74.57	4.14	dd	8.6, 8.6
	5	82.47	3.94	ddd	8.6, 6.4, 3.2
	6	63.04	3.82	m[b]	
Glc	1	95.20	4.90	d	7.9
	2	73.44	3.55	dd	9.8, 7.9
	3	85.15	3.80	dd	9.8, 7.1
	4	68.87	3.52	m	
	5	76.11	3.51	m	
	6	61.33	3.89	m	
			3.74	dd	12.6, 4.4
Glc'	1	103.57	4.77	d	7.4
	2	74.29	3.36	dd	9.8, 7.4
	3	76.37	3.54	dd	9.8, 8.9
	4	70.42	3.42	dd	9.5, 8.9
	5	76.81	3.50	ddd	9.5, 5.7, 1.1
	6	61.53	3.93	m	
			3.74	dd	12.3, 5.7

[a] Chemical shifts of ^{1}H (δ_H) and ^{13}C (δ_C) in ppm were determined relatively 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, respectively.
[b] m: multiplet.

Appendix 3 ^{1}H and ^{13}C NMR spectral data (δ^{a} in ppm, *J* in Hz) of saccharide 9.

		δ_C	δ_H		J_{HH}			δ_C	δ_H		J_{HH}
Fru*f*	1	60.95	3.76	d	12.3	Fru*f*	1				
			3.68	d	12.3						
	2	104.53					2				
	3	[g]77.75	[c]4.17	d	8.1		3	[g]77.79	[c]4.17	d	8.2
	4	75.28	[d]4.11	dd	8.1, 7.8		4		[d]4.12	dd	8.2, 7.9
	5	81.93	3.87	m[b]			5				
	6	[h]63.11	3.83	m			6	[h]63.14			
			3.67	dd	12.3, 6.4						
Glc	1	104.04	4.68	d	7.9	Glc	1	104.12	4.67	d	7.9
	2	74.08	3.39	dd	9.5, 7.9		2	74.10	3.40	dd	9.6, 7.9
	3	76.24	[e]3.54	dd	9.5, 8.5		3		[e]3.54	dd	9.6, 8.9
	4	[i]70.59	3.46	dd	10.1, 8.5		4	[i]70.64			
	5	75.83	3.62	m			5				
	6	61.72	[f]4.08	dd	10.8, 4.4		6		[f]4.07	dd	10.8, 4.4
			3.70	dd	10.8, 6.4						
βGlc’	1	96.45	4.70	d	8.1	αGlc’	1	92.74	5.26	d	3.9
	2	74.15	3.44	dd	9.8, 8.1		2	71.33	3.73	dd	9.6, 3.9
	3	87.14	3.72	dd	9.8, 7.1		3	84.95	3.89	dd	9.6, 9.2
	4	[j]69.22	3.53	m			4	[j]69.19	3.53	m	
	5	76.27	3.53	m			5	71.91	3.89	m	
	6	61.54	3.90	m			6	61.36	3.84	dd	12.3, 2.2
			3.73	dd	12.1, 4.7				3.77	m	

[a] Chemical shifts of ^{1}H (δ_H) and ^{13}C (δ_C) in ppm were determined relatively 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, respectively.
[b] m: multiplet.
[c-j] interchangeable.

Appendix 4 ^{1}H and ^{13}C NMR spectral data (δ^{a} in ppm, *J* in Hz) of saccharide 10.

		δ_C	δ_H		J_{HH}
Fru*p*	1	62.18	3.79	m[b]	
			3.79	m	
	2	101.58			
	3	69.50	3.89	m	
	4	70.36	3.90	m	
	5	69.95	3.99	dd	3.2, 1.5
	6	64.86	3.87	m	
			3.72	m	
Glc’	1	103.95	4.67	d	7.7
	2	74.08	3.38	dd	9.8, 7.7
	3	76.19	3.53	m	
	4	70.45	3.47	m	
	5	75.80	3.63	m	
	6	61.20	3.93	m	
			3.67	m	
βGlc	1	96.44	4.68	d	8.0
	2	74.11	3.42	dd	9.4, 8.0
	3	87.02	3.69	m	
	4	69.16	3.50	m	
	5	76.26	3.50	m	
	6	61.51	3.88	m	
			3.72	m	
αGlc	1	92.71	5.25	d	3.7
	2	71.27	3.72	dd	9.6, 3.7
	3	84.83	3.85		
	4	69.13	3.50		
	5	71.92	3.87		
	6	61.34	3.82		
			3.76		

[a] Chemical shifts of ^{1}H (δ_H) and ^{13}C (δ_C) in ppm were determined relatively 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, respectively.
[b] m: multiplet.

Appendix 5 ^{1}H and ^{13}C NMR spectral data (δ^{a} in ppm, *J* in Hz) of saccharide **12**.

		δ_C	δ_H		J_{HH}
αGlc	1	93.32	5.43	d	3.9
	2	71.96	3.54	dd	10.5, 3.9
	3	73.44	3.74	dd	10.5, 9.2
	4	70.09	3.46	dd	10.0, 9.2
	5	73.27	3.84	ddd	10.0, 3.7, 2.7
	6	60.98	3.81	m[b]	
Fru*f*	1	69.76	4.05	d	11.1
			3.80	d	11.1
	2	103.82			
	3	77.29	4.31	d	8.5
	4	74.53	4.06	dd	8.9, 8.5
	5	82.14	3.91	ddd	8.9, 6.4, 4.2
	6	63.02	3.81	m	
βGlc	1	103.60	4.51	d	8.0
	2	73.88	3.32	dd	9.2, 8.0
	3	76.44	3.50	dd	9.2, 9.2
	4	70.42	3.40	dd	9.8, 9.2
	5	76.82	3.45	ddd	9.8, 5.7, 2.0
	6	61.52	3.91	dd	12.4, 2.0
			3.73	dd	12.4, 5.7

[a] Chemical shifts of ^{1}H (δ_H) and ^{13}C (δ_C) in ppm were determined relatively 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, respectively.
[b] m: multiplet.

Appendix 6 ^{1}H and ^{13}C NMR spectral data (δ^{a} in ppm, J in Hz) of saccharide **13**.

		δ_C	δ_H		J_{HH}
αGlc	1	93.36	5.44	d	3.9
	2	71.99	3.55	dd	10.1, 3.9
	3	73.45	3.75	dd	10.1, 9.3
	4	70.11	3.47	dd	9.9, 9.3
	5	73.29	3.85	ddd	9.9, 3.5, 3.0
	6	60.99	3.81	m[b]	
Fru*f*	1	69.81	4.07	d	11.2
			3.80	d	11.2
	2	104.19			
	3	77.43	4.33	d	8.9
	4	74.56	4.06	dd	8.9, 8.4
	5	82.13	3.92	ddd	8.4, 6.4, 2.7
	6	63.03	3.81	m	
βGal	1	104.19	4.44	d	7.9
	2	71.57	3.56	dd	9.6, 7.9
	3	73.48	3.66	dd	9.6, 3.4
	4	69.50	3.93	d	3.4
	5	76.10	3.70	dd	8.4, 4.6
	6	61.85	3.80	dd	12.1, 8.4
			3.78	dd	12.1, 4.6

[a] Chemical shifts of ^{1}H (δ_H) and ^{13}C (δ_C) in ppm were determined relatively 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, respectively.
[b] m: multiplet.

Appendix 7 ^{1}H and ^{13}C NMR spectral data (δ^{a} in ppm, J in Hz) of saccharide **14**.

		δ_C	δ_H		J_{HH}
Fru*p*	1	62.16	3.79		
			3.79	m[b]	
	2	101.55			
	3	69.28	3.92	m	
	4	70.33	3.92	m	
	5	69.95	3.99	br s[c]	
	6	64.86	3.93	m	
			3.72	m	
Glc’	1	103.58	4.74	d	8.0
	2	74.26	3.35	dd	9.4, 8.0
	3	76.37	3.52	dd	9.4, 8.7
	4	70.39	3.41	dd	8.9, 9.7
	5	76.83	3.47	m	
	6	61.52	3.92	m	
			3.72	m	
βGlc	1	96.50	4.66	d	8.1
	2	74.63	3.43	dd	8.3, 8.1
	3	85.03	3.73	dd	8.3, 8.3
	4	68.91	3.56	m	
	5	75.57	3.59	m	
	6	61.23	3.90	m	
			3.69	m	
αGlc	1	92.79	5.23	d	3.6
	2	71.81	3.73	dd	9.6, 3.6
	3	82.83	3.90	dd	9.6, 9.6
	4	69.03	3.55		
	5	71.46	3.96		
	6	61.27	3.87		
			3.69		

[a] Chemical shifts of ^{1}H (δ_H) and ^{13}C (δ_C) in ppm were determined relatively 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, respectively.
[b] m: multiplet.
[c] br s: broad singlet.

Appendix 8 ^{1}H and ^{13}C NMR spectral data (δ^{a} in ppm, J in Hz) of saccharide 16.

		δ_C	δ_H		J_{HH}
αGlc	1	92.35	5.43	d	4.0
	2	81.12	3.69	dd	9.7, 4.0
	3	71.22	3.96	dd	9.7, 9.7
	4	79.09	3.69	dd	10.1, 9.7
	5	70.70	3.95	ddd	10.1, 6.9, 1.0
	6	60.67	3.86	m[b]	
Glc’	1	104.74	4.63	d	7.6
	2	74.11	3.35	dd	9.6, 7.6
	3	76.40	3.49	m	
	4	70.29	3.40	m	
	5	76.62	3.44	m	
	6	61.44	3.72	dd	12.3, 5.6
			3.89	dd	12.3, 1.2
Glc’’	1	103.26	4.51	d	7.6
	2	73.98	3.31	dd	9.6, 7.6
	3	76.33	3.50	m	
	4	70.29	3.41	m	
	5	76.86	3.48	m	
	6	61.44	3.73	dd	12.3, 6.2
			3.91	dd	12.3, 1.2
βGlc	1	95.32	4.73	d	8.0
	2	81.72	3.55	dd	9.1, 8.0
	3	75.39	3.83	dd	9.4, 9.1
	4	79.09	3.68	dd	9.6, 9.4
	5	75.59	3.59	m	
	6	60.87	3.81	dd	11.7, 5.2
			3.95	dd	11.7, 1.2
Glc’	1	103.51	4.80	d	7.6
	2		3.31	dd	9.6, 7.6
	3				
	4				
	5				
	6		3.70	dd	12.2, 6.5
			3.92	dd	12.2, 1.2
Glc’’	1	103.32	4.50	d	7.8
	2		3.30	dd	9.6, 7.8
	3				
	4				
	5				
	6		3.72	dd	12.3, 6.2

[a] Chemical shifts of ^{1}H (δ_H) and ^{13}C (δ_C) in ppm were determined relatively 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, respectively.
[b] m: multiplet.