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Note

Isolation and Identification of Novel Tri- and Tetra-saccharides Synthesized by *Thermoanaerobacter brockii* Kojibiose Phosphorylase

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Abstract: Novel tri- and tetra-saccharides were synthesized by glucosyltransfer from β-D-glucose 1-phosphate (β-D-G1P) to palatinose using *Thermoanaerobacter brockii* kojibiose phosphorylase. There saccharides were isolated using carbon-Celite column chromatography and preparative high performance liquid chromatography. Gas liquid chromatography analysis of methyl derivatives, MALDI-TOF MS and NMR measurements were used for structural confirmation of the saccharides. The ^{1}H and ^{13}C NMR signals of the saccharides were assigned using 2D-NMR including COSY, HSQC, HSQC-TOCSY and HMBC. These oligosaccharides were identified as 2^{G}-α-D-glucopyranosyl-palatinose; *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-D-fructofuranose and 2^{G}(2-α-D-glucopyranosyl)$_2$-palatinose; *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-D-fructofuranose.

Key words: Kojibiose phosphorylase, oligosaccharide, Palatinose, NMR

Oligosaccharides are widely known to have benefical health effects. They are classified into homooligosaccharides such as xylooligosaccharide, maltooligosaccharide and inulooligosaccharide, and heterooligosaccharides such as fructooligosaccharide, galactooligosaccharide, soybeenoligosaccharide, raffinose and palatinose. Thus, they are thought to selectively increase bifidobacteria,[1)] enhance immune responses, [2)] promote the absorption of minerals, [3)] and more recently, prevent diseases such as a heart diseases, hypertension, diabetes and osteoporosis.

Palatinose is produced by treatment of sucrose with *Protaminobacter rubrum,*[4)] and is used in various foods as sweeteners not causing dental caries.[5)] Although palatinose is hydrolyzed more slowly than sucrose, this saccharide is finally absorbed in the small intestine. Changing this saccharide into a non-digestible oligosaccharide is useful to improve the function.

In recent years, the synthesis of oligosaccharides having various functions has been actively performed. These oligosaccharides are mostly synthesized using hydrolases and glycosyltransferases. The phosphorylases belonging to glycosyltransferases are also expected to give good results due to their reversible reaction, although there have been few studies.

We have previously studied the production of indigestible oligosaccharides such as inulooligosaccharide,[6,7)] fructooligosaccharide,[8)] fructosylxyloside[9)] and fructosyllactosucrose[10)] using *Penicillium purpurogenum* inulinase,[6,7)] *Scopulariopsis brevicaulis* fructosyltransferase[8,9)] and asparagus 1^{F}-fructosyltransferase.[10−12)] These oligosaccharides were shown to suppress the rise of serum glucose and insulin responses in rats,[13)] and serum cholesterol[14)] and triacylglycerol[15)] in humans, and to have prebiotic effects.[15,16)]

Recently, our studies have involved the synthesis of novel oligosaccharides elongated with one, two or three additional glucose units by glucosyltransfer from β-D-glucose 1-phosphate to isokestose, nystose,[17)] raffinose and stachyose[18)] using *Thermoanaerobacter brockii* kojibiose phosphorylase.

Now, we report the structural analysis and functional analysis of two novel oligosaccharides synthesized by glucosyltransfer from β-D-glucose 1-phosphate to palatinose using kojibiose phosphorylase.

MATERIALS AND METHODS

Saccharides. Crystalline palatinose (*O*-α-D-glucopyranosyl-(1→6)-D-fructofuranose) were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Kojibiose and β-D-glucose 1-phosphate (β-D-G1P) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Enzyme. Kojibiose phosphorylase was purified from a cell-free extract of *T. brockii ATCC 35047*.[19)]

High performance anion-exchange chromatography (HPAEC). The oligosaccharides was analyzed using a Dionex Bio LC Series apparatus equipped with a carbohydrate column (Carbo Pack PA-1, inert styrene divinylbenzene polymer) and a pulsed amperometric detector (PAD).[20,21)] The mobile phase consisted of eluent A (150 mM NaOH) with a sodium acetate gradient as follows: 0−

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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 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 −0.6 V[22] respectively, and the output range was 1μC.

Isolation of oligosaccharides synthesized from palatinose and β-D-G1P by kojibiose phosphorylase. The reaction mixture (50.0 mL) which contained kojibiose phosphorylase (10.0 U), palatinose (2.0 g), β-D-G1P (1.34 g) and acetate buffer (0.05 M, pH 5.5), was incubated at 50 °C for 48 h. After terminating the reaction by heating in a boiling water bath for 5 min, the reaction mixture was loaded onto a carbon-Celite [1:1; charcoal (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and Celite-535 (Nakarai Chemical Industries, Ltd., Osaka, Japan)] column (3.5×29 cm) and successively eluted with water, 5% ethanol and 10% ethanol. These fractions, which contained saccharides **1** and **2**, were concentrated and purified using preparative HPLC. A portion of the saccharide **1** and **2** mixture was purified using an HPLC system (JASCO GULLIVER, Tokyo, Japan) equipped with an ODS column (TSKgel ODS-80Ts, 20 mm×25 cm, Tosoh, Tokyo, Japan) at 35°C, and eluted with water at 3.5 mL/min, using refractive index detection. Saccharides **1** (841 mg) and **2** (74.9 mg) were obtained by repeated HPLC purification.

Methylation and methanolysis. Methylation of the oligosaccharides was carried out by the method of Hakomori.[23] The permethylated saccharides were methanolysed 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 *in vacuo* to dryness. The resulting methanolysate was dissolved in a small volume of methanol and analysed using gas chromatography.

Gas liquid chromatography (GC). For the analysis of the methanolysate, GC was carried out using a Shimadzu GC8A 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.

Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS). MALDI-TOF-MS spectra were measured using a Shimadzu-Kratos mass spectrometer (KOMPACT Probe).

NMR measurements. Saccharides **1** (5 mg) and **2** (5 mg) were dissolved in 0.5 mL D_2O. NMR spectra were recorded at room temperature 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,[24,25] and HSQC[26] were obtained using gradient selected pulse sequences. The phase sensitive HSQC-TOCSY spectra were determined with the sequence including inversion of direct resonance (IDR).[27] HMBC spectra[28] were obtained using the pulse sequences of CT-HMBC proposed by Furihata and Seto.[29]

Digestivity by intestinal α-glucosidases. α-Glucosidases were prepared from rat intestinal acetone powder (Sigma Chemical Co.). 500 mg of the acetone powder was homogenized in 10 mL of potassium phosphate buffer (pH 6.1), and centrifuged (25,000×*g*, 15 min). The supernatant was used as enzyme preparation. The reaction mixture of the enzyme assay, consisting of 0.1 mL of 20 mM saccharide **1** and 0.1 mL of enzyme solution in 60 mM potassium phosphate buffer (pH 6.1) was incubated at 37°C. The glucose liberated was determined by the glucose oxidase-peroxidase method.[30,31]

RESULTS AND DISCUSSION

Saccharides **1** and **2** were produced from palatinose and β-D-glucose 1-phosphate using kojibiose phosphorylase. A HPAEC chart of saccharides **1** and **2** produced after 48 h reaction is shown in Fig. 1. As shown in Fig. 2, the production of saccharide **1** increased rapidly and reached a maximum at 10 h after reaction. However the production of saccharide **2** increased more slowly. The yield of saccharides **1** and **2** were 34.2% (mol/mol) and 7.1% (mol/mol) against palatinose, respectively. From the reaction mixture, saccharides **1** and **2** were isolated by successive chromatographic procedures using carbon-Celite and ODS columns, and finally obtained as white powder.

Saccharides **1**($[\alpha]_D^{20}$+143.0) and **2**($[\alpha]_D^{20}$+148.9) were shown to be homogenous using HPAEC [t_R, retention time of sucrose=1.00; 1.56 and 1.81]. The degrees of polymerization were established as 3 (saccharide **1**), and 4 (saccharide **2**), as shown by measurements of $[M+Na]^+$

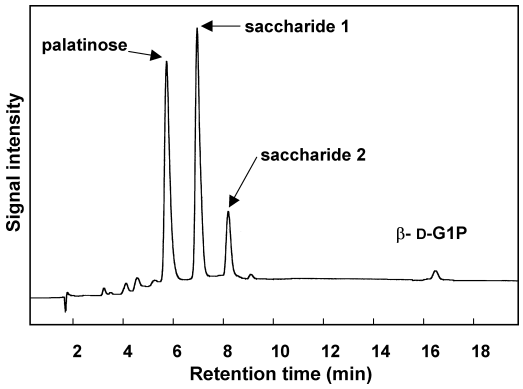

Fig. 1. HPAEC of saccharides produced from palatinose and β-D-G1P by kojibiose phosphorylase.

The enzyme reaction was carried out with 0.12 M palatinose and 0.10 M β-D-G1P in the mixture at 50°C for 48 h.

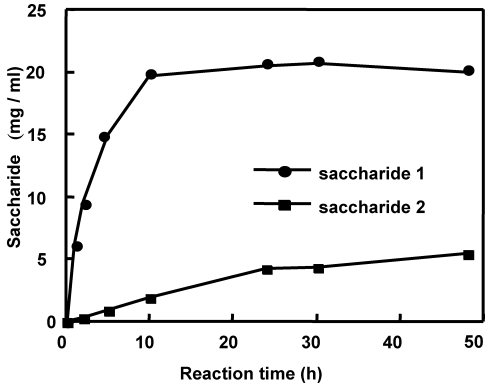

Fig. 2. Time course of formation of saccharides **1** and **2**.

ions (m/z: 527, **1**: 689, **2**) using TOF-MS, and analysis of the molar ratios of D-glucose and D-fructose in the acid hydrolysates of the oligosaccharides.

From the GC analysis, relative retention times of the methanolysates of the permethylated saccharides were investigated [t_R, retention time of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside=1.0 (retention time, 9.05 min)]. The methanolysate of permethylated saccharide **1** exhibited seven peaks corresponding to methyl 2,3,4,6-tetra-*O*-methyl-D-glucoside (t_R, 1.00 and 1.39), methyl 3,4,6-tri-*O*-methyl-D-glucoside (t_R, 2.88, 3.44), and methyl 1,3,4-tri-*O*-methyl-D-fructoside (t_R, 1.80, 2.06, 2.39). The methanolysate of permethylated saccharide **2** also exhibited seven peaks, which corresponded to the same methyl glycosides as those from saccharide **1**. Two peaks corresponding to methyl-3,4,6-tri-*O*-methyl-D-glucoside (t_R, 2.96 and 3.53) from the methanolysate of permethylated saccharide **2** were larger than those of permethylated saccharide **1**. The peak of methyl-3,4,6-tri-*O*-methyl-D-glucoside indicating 1→2 glucosyl linkage of each saccharide was increased by additional units of glucose.

From these findings, saccharides **1** and **2** were proved to be 2^G-α-D-glucopyranosyl-palatinose and 2^G(2-α-D-glucopyranosyl)$_2$-palatinose, respectively (Fig. 3).

The structural confirmation of saccharides **1** and **2** according to ^{1}H, ^{13}C NMR analyses and the subsequent complete assignment of ^{1}H, ^{13}C NMR signals were carried out using 2D-NMR techniques, including COSY,[24,25] HSQC,[26] HSQC-TOCSY[27] and HMBC.[28,29]

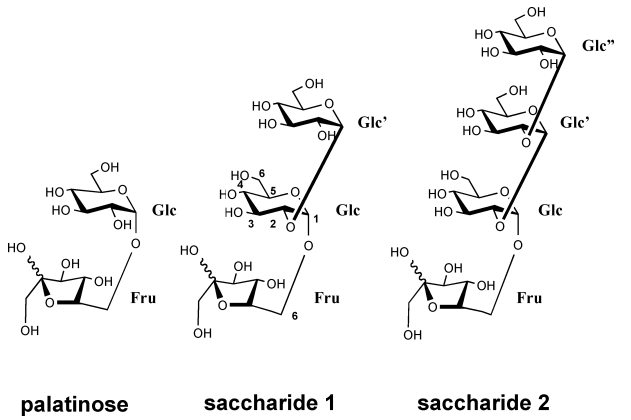

Fig. 3. Structures of saccharides **1** and **2** formed by kojibiose phosphorylase.

Glucose and fructose residues of these saccharides are represented as Glc, Glc′, Glc″ and Fru, as shown in Fig. 3. The proton and carbon positions in a particular residue are represented by H-1-Glc and C-1-Fru, respectively.

First, the NMR spectra of palatinose as a common basic unit were analysed. Palatinose, saccharide **1** and saccharide **2** were anomer mixtures of fructose. NMR analysis of these oligosaccharides was performed about β anomer, the predominant form. The signal of α anomer and β anomer could be determined from the chemical shift of carbon.[32] From one anomeric proton (δ_H 4.95 ppm, d, 3.7 Hz) and one carbon (δ_C 99.06 ppm) in palatinose, one glucosyl residue was assigned by ^{1}H-^{1}H COSY and J_{HH}. The intra residual HMBC correlation between one of the anomeric protons (δ_H 4.95 ppm) and one of the carbons (δ_C 68.59 ppm) assigned these protons and carbons to H-1-Glc and C-6-Fru, respectively. The HMBC correlations between C-2-Fru and H-1-Fru (δ_H 3.52 ppm) and between C-1-Fru (δ_C 63.44 ppm) and H-3-Fru (δ_H 4.10 ppm, d, 8.1 Hz) as well as ^{1}H-^{1}H-COSY correlations enabled the assignments of fructose residue. The characteristic J (H-1, H-2) values of the Glc (J=3.7 Hz) determined both glucosyl bonds were α forms.

The palatinose unit in saccharide **1** was determined in the same manner as in palatinose. As shown in Fig. 4, the conectivity of Glc′(1→2)Glc was reduced from HMBC correlations between C-2-Glc (δ_C 76.18 ppm) and H-1-Glc′ (δ_H 5.09 ppm). The characteristic J (H-1, H-2) values of the Glc′ (J=3.9 Hz) determined both glucosyl bonds were α forms. These δ_C and δ_H values agreed well with those of the kojibiose (Glc′α1→2Glc)[33] and palatinose (Glcα1→6Fru).

The trisaccharide unit of **1** in saccharide **2** was determined in the same manner as in saccharide **1**. As shown in Fig. 4, further glucosyl linkages Glc″(1→2)Glc′ in saccharide **2** were determined by additional HMBC correlation between H-1-Glc″ (δ_H 5.10 ppm, d, 3.6 Hz) and C-2-Glc′ (δ_C 75.84 ppm) in saccharide **2**. Moreover, HMBC correlation peaks nearer part of the chemical shifts of carbon were separated by CT-HMBC, which has sharp line

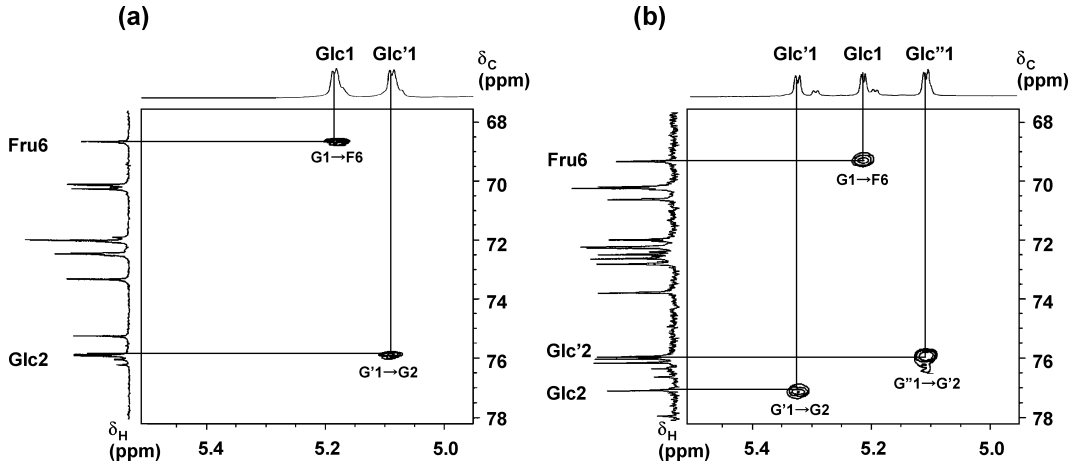

Fig. 4. Part of the HMBC spectra (a) and (b) of saccharides **1** and **2**.

Connectivities of Glc-＞Glc′ (a) or Glc-＞Glc′-＞Glc″ (b) and assignment of these free glucose residues were completed.

Table 1. ^{1}H and ^{13}C NMR chemical shifts of palatinose, saccharides **1** and **2**.

		Palatinose				Saccharide **1**				Saccharide **2**			
		δ_C	δ_H		J_{HH}	δ_C	δ_H		J_{HH}	δ_C	δ_H		J_{HH}
Glucose	1	99.06	4.95	d	3.7	96.25	5.19	d	3.4	96.03	5.21	d	3.2
	2	72.18	3.55	dd	10.6, 3.7	76.18	3.68	dd	10.0, 3.4	76.96	3.69	dd	9.3, 3.2
	3	73.82	3.74	dd	10.6, 9.7	72.11	3.85	dd	10.0, 8.5	72.16	3.87	dd	10.0, 9.3
	4	70.35	3.91	dd	9.7, 9.6	70.31	3.46	dd	9.4, 8.5	70.52	3.43	dd	10.0, 9.3
	5	72.67	3.74	m		72.55	3.74	m		72.38	3.78	m	
	6	61.37	3.86	m		61.35	3.88	m		61.41	3.87	m	
			3.74	m			3.76	m			3.76	m	
Glucose′	1					96.99	5.09	d	3.9	94.88	5.32	d	3.6
	2					72.11	3.54	dd	10.1, 3.9	75.84	3.66	dd	10.1, 3.6
	3					73.47	3.77	dd	10.1, 9.1	71.88	3.90	dd	10.1, 9.3
	4					70.16	3.44	dd	10.0, 9.1	70.08	3.49	dd	9.7, 9.3
	5					72.59	3.92	ddd	10.0, 4.4, 2.2	72.52	3.93	ddd	9.7, 4.4, 2.4
	6					61.10	3.83	dd	12.5, 2.2	61.03	3.83	dd	12.5, 2.4
							3.76	dd	12.5, 4.4		3.77	dd	12.5, 4.4
Glucose″	1									96.81	5.10	d	3.6
	2									72.13	3.56	dd	10.0, 3.6
	3									73.69	3.78	dd	10.0, 9.0
	4									70.13	3.43	dd	10.0, 9.0
	5									72.71	3.89	ddd	10.0, 5.3, 2.2
	6									61.14	3.83	dd	12.3, 2.2
											3.76	dd	12.3, 5.3
Fructose	1	63.44	3.52	d	12.2	63.40	3.59	d	12.2	63.40	3.58	d	12.3
			3.57	d	12.2		3.54	d	12.2		3.54	d	12.3
	2	102.50				102.58				102.73			
	3	76.06	4.10	d	8.1	76.14	4.11	d	8.0	76.04	4.10	m	
	4	75.29	4.18	dd	8.1, 7.7	75.47	4.20	dd	8.0, 7.6	75.91	4.04	m	
	5	79.71	3.95	ddd	7.7, 5.5, 2.5	79.77	3.98	ddd	7.6, 6.4, 2.8	76.96	4.04	ddd	10.1, 6.2, 3.0
	6	68.59	3.86	dd	11.6, 5.5	68.64	3.87	dd	11.1, 6.4	69.22	3.82	dd	11.1, 6.2
			3.68	dd	11.6, 2.5		3.72	dd	11.1, 2.8		3.77	dd	11.1, 3.0

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-(trimethysilyl)-propanoate in D_2O (δ_H 0.00 ppm) and 1,4-dioxane (δ 67.40, ^{13}C) in D_2O, respectively.

shape in F_1 (^{13}C) inherently.[27] The assignments of all ^{1}H and ^{13}C signals of these saccharides **1** and **2** are shown in Table 1.

The two saccharides formed by glucosyltransfer from β-D-G1P to palatinose using *Thermoanaerobacter brockii* kojibiose phosphorylase were confirmed to be new oligosaccharides, 2^{G}(2-α-D-glucopyranosyl)$_m$-palatinose: m=1 (saccharide **1**) and 2 (saccharide **2**); *O*-α-D-glucopyranosyl-(1→[2-*O*-α-D-glucopyranosyl-1]$_n$→2-*O*-α-D-glucopyranosyl-(1→6)-D-fructofuranose: n=0 (saccharide **1**) and 1 (saccharide **2**).

The digestion of saccharide **1** was examined using intestinal α-glucosidase. As shown Fig. 5, hardly any saccharide **1** was hydrolyzed, although palatinose was hydrolyzed slowly until 60 min. From this result, saccharide **1** was identified to be a non-digestible oligosaccharide.

Palatinose is known to have various functions such as non-cariogenicity. However, it is inadvisable to use palatinose in patients having diseases such as diabetes, because palatinose is hydrolyzed slowly in the small intestine. Furthermore, oligosaccharides of low molecular weight such as palatinose may have the negative effect of high osmotic pressure. The two novel oligosaccharides synthesized in this study are expected to be more indigestible than palatinose and to have lower osmotic pressure than palatinose, making intestinal conditions better. Further studies are needed to clarify the nutritional functions of these oligosaccharides, including non-cariogenicity.

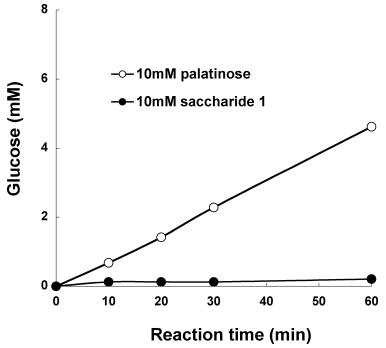

Fig. 5. Time course of hydrolysis of saccharide **1** by intestinal α-glucosidases.

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Thermoanaerobacter brockii Kojibiose Phosphorylase によって合成された新規三および四糖の分離および同定

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パラチノースは抗う蝕性のようなさまざまな機能をもつことが知られている．しかしながら，パラチノースはゆっくりではあるが小腸で加水分解をうけるため，糖尿病のような疾患をもつ患者への使用は薦められない．さらに，この糖のような低分子のオリゴ糖は比較的高浸透圧になりやすいため生体にとってよくない影響を及ぼすことがある．本研究では，二糖であるパラチノースを用い，*Thermoanaerobacter brockii* kojibiose phosphorylase のグルコシル転移作用を利用し，グルコース 1 リン酸とパラチノースから新規オリゴ糖を合成した．反応は糖 1 および糖 2 が効率よく生成した 48 時間で止めた (Fig. 1)．また，転移生成物である糖 1 は反応 10 時間で最大となった (Fig. 2)．活性炭-セライトカラムおよび調製用 HPLC を用いて糖 1 および糖 2 を単離し，MALDI-TOF-MS 分析およびメチル誘導体のガスクロマトグラフィー分析を行い構造の推定を行った (Fig. 3)．さらに COSY，HSQC，HSQC-TOCSY および HMBC (Fig. 4) の各手法を用いた 2 次元 NMR 解析により糖 1 を 2^G-α-D-glucopyranosyl-palatinose; *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-D-fructofuranose，糖 2 を 2^G(2-α-D-glucopyranosyl)$_2$-palatinose; *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-D-fructofuranose と同定した (Table 1)．今後，これらの糖の栄養機能について明らかにする必要がある．