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SYNTHESIS OF *p*-NITROPHENYL 6^5-*O*-BENZYL-α-MALTOPENTAOSIDE, A SUBSTRATE FOR ALPHA AMYLASES

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ABSTRACT

p-Nitrophenyl α-maltopentaoside, having a benzyl group on O-6 of the terminal (nonreducing) D-glucosyl group was prepared by use of a reductive ring-opening reaction. Highly regioselective reduction of *p*-nitrophenyl *O*-(2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-α-D-glucopyranosyl)-(1→4)-tris[*O*-(2,3,6-tri-*O*-benzoyl-α-D-glucopyranosyl)-(1→4)]-2,3,6-tri-*O*-benzoyl-α-D-glucopyranoside by dimethylamine–borane and *p*-toluenesulfonic acid, followed by debenzoylation, gave *p*-nitrophenyl *O*-(6-*O*-benzyl-α-D-glucopyranosyl)-(1→4)-tris[*O*-α-D-glucopyranosyl-(1→4)]-α-D-glucopyranoside. An experiment was done on the mode of action of human pancreatic and salivary alpha amylases on this derivative. The compound is suitable as a substrate for the assay of alpha amylase when used with glucoamylase and α-D-glucosidase as coupling enzymes.

INTRODUCTION

The quantitative measurement of alpha amylase activity in human serum and urine is useful as part of the diagnosis of pancreatic disease. During the past 80 years, several methods for the assay of alpha amylase activity have been developed. First, native and modified long-chain substrates were used in assays based on various principles[1–4]. Malto-oligosaccharides or their *p*-nitrophenyl derivatives having defined structures have also been used as substrates in coupled enzymic assays[5–7]. However, use of these substrates presents some problems, particularly the partial hydrolysis of the substrates by the α-D-glucosidase used as a coupling enzyme.

Chemically modified malto-oligosaccharides that are resistant to α-D-glucosidase and glucoamylase are useful for assay of alpha amylase activity[8–16]. Among these modified malto-oligosaccharides, pyridylamino derivatives can be

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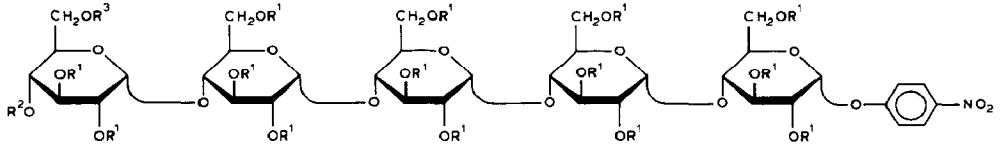
used for the differential assay of two human alpha amylases by taking advantage of the difference in the modes of action on them[9,11,15], but the yield in the preparation of these substrates was low. Benzylidene and ethylidene derivatives of *p*-nitrophenyl α-maltoheptaoside were used as the substrates for the assay, but they were hydrolyzed by alpha amylases to give many products[17,18].

We wanted, therefore, to develop a simple, alternative route in order to prepare a substrate that was modified at the nonreducing-end D-glucosyl group and hydrolyzed by two human alpha amylases at a single D-glucosidic linkage in a similar manner.

There have been many reports of the reductive ring-opening of the acetals of monosaccharides[19–22]. We found that, when the usual acetal-ring-opening reaction was used on derivatives of malto-oligosaccharides, the yields of the products were very low. For this reason, we sought better reaction conditions. We now report the synthesis of *p*-nitrophenyl *O*-(6-*O*-benzyl-α-D-glucopyranosyl)-(1→4)-tris[*O*-α-D-glucopyranosyl-(1→4)]-α-D-glucopyranoside (*p*-nitrophenyl 6^5-*O*-benzyl-α-maltopentaoside) by reduction of the $4^5,6^5$-*O*-benzylidene derivative of *p*-nitrophenyl α-maltopentaoside, and also describe the use of the product in the assay of alpha amylase activity in human serum.

RESULTS AND DISCUSSION

Chemical synthesis. — The reaction of *p*-nitrophenyl α-maltopentaoside (**1**) with benzaldehyde dimethyl acetal and *p*-toluenesulfonic acid (*p*-TsOH) in *N,N*-dimethylformamide (DMF) afforded the $4^5,6^5$-*O*-benzylidene derivative (**2**) of *p*-nitrophenyl α-maltopentaoside. This intermediate was treated with benzoyl chloride in pyridine, to give the perbenzoylated $4^5,6^5$-*O*-benzylidene derivative (**3**). The perbenzoate (**4**) of the 6^5-*O*-benzyl derivative was obtained from **3** by reduction with dimethylamine–borane (Me_2NH–BH_3) and *p*-TsOH in tetrahydrofuran (THF). Removal of the benzoyl groups from **4** gave compound **5**, which was



	R^1	R^2	R^3
1	H	H	H
2	H	Bzd	
3	Bz	Bzd	
4	Bz	H	Bn
5	H	H	Bn

Bn = $PhCH_2$

Bzd = Ph—CH<

Bz = PhCO

purified by hydrophobic chromatography (yield, 16%). Reduction of **3** with sodium cyanoborohydride and hydrogen chloride gave **5** in ~2% yield.

Monosaccharide derivatives, namely, *p*-nitrophenyl 4,6-*O*-benzylidene-α-D-glucopyranoside and *p*-nitrophenyl 2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-α-D-glucopyranoside, were reduced with Me_2NH–BH_3 and *p*-TsOH in order to investigate the effect of substituents on O-2 and O-3. The products in these reactions were all 6-*O*-benzyl derivatives; no 4-*O*-benzyl derivatives were formed. These results showed that the direction of the reductive opening was not affected by the benzoylation of O-2 and O-3. Benzoylation was employed in order to obtain a derivative of compound **2** that is soluble in THF.

Confirmation of the structure of **5**. — The elution positions of compounds **1**, **2**, and **5** on a h.p.l.c. column were all different (data not shown). Compound **5** was not hydrolyzed by glucoamylase or α-D-glucosidase, because the nonreducing-end D-glucosyl group of **5** was substituted. On methanolysis followed by g.l.c. analysis, the molar ratios of glucose: 6-*O*-benzylglucose: *p*-nitrophenol for **5** were found to be 3.8:0.93:1.0, and no 4-*O*-benzylglucose was detected (see Fig. 1). The presence of a 6-*O*-benzyl group in **5** was also confirmed by comparison of the ^{13}C-n.m.r. spectrum of **5** with those of methyl 4-*O*-benzyl-α-D-glucopyranoside and methyl 6-*O*-benzyl-α-D-glucopyranoside. The 69.7-p.p.m. signal arising from the benzyloxy-substituted 6-carbon atom[22] was detected in the spectrum of **5**, but the 78-p.p.m. signal arising from the substitution of a benzyloxy group on carbon 4 was not detected. Thus, the nonreducing-end residue of **5** was found to be 6-*O*-benzylglucose. The molecular weight of **5** as determined by f.a.b.-mass spectrometry had the expected value. These results indicated clearly that **5** is *p*-nitrophenyl 6^5-*O*-benzyl-α-maltopentaoside.

Patterns of action of two human alpha amylases on **1**, **2**, *and* **5**. — The action

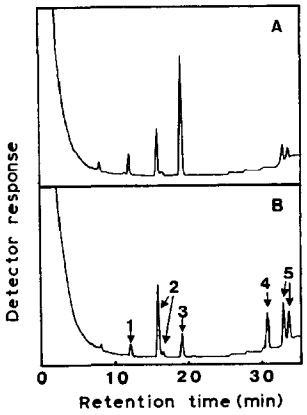


Fig. 1. Component analysis of compound **5** by g.l.c. The compound was methanolyzed and trimethylsilylated prior to g.l.c. as described in the Experimental section. A, **5**; B, standard mixture of 1, *p*-nitrophenol; 2, D-mannose (standard); 3, D-glucose; 4, methyl 4-*O*-benzyl-α-D-glucopyranoside; and 5, methyl 6-*O*-benzyl-α-D-glucopyranoside.

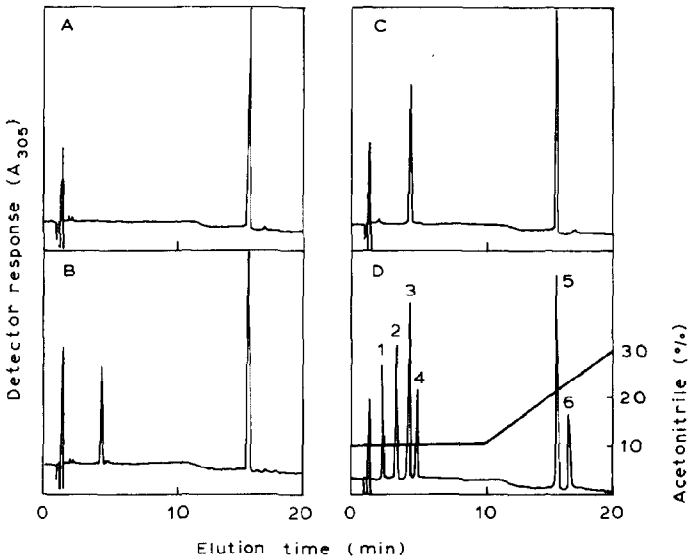


Fig. 2. Analysis by h.p.l.c. of the alpha amylase digests of **5**. The column (4.6 × 150 mm) was eluted with a linear gradient of acetonitrile from 10 to 30% (see D). The compounds were detected by measurement of the absorbance at 305 nm. A, **5**; B, pancreatic alpha amylase digest of **5**; C, salivary alpha amylase digest of **5**; D, standard mixture of 1, *p*-nitrophenyl α-maltotetraoside; 2, *p*-nitrophenyl α-maltotrioside; 3, *p*-nitrophenyl α-maltoside; 4, *p*-nitrophenyl α-D-glucopyranoside; 5, **5**; and 6, *p*-nitrophenol.

of human pancreatic and salivary alpha amylases on compounds **1, 2,** and **5** was studied, to ascertain the effect of the modification. Samples were incubated with the two alpha amylases as described in the Experimental section, and aliquots were analyzed by h.p.l.c., to identify the products. Compound **1** was hydrolyzed to *p*-nitrophenyl (R) α-maltotrioside (G-G-G-R), *p*-nitrophenyl α-maltoside (G-G-R), and *p*-nitrophenyl α-D-glucopyranoside (G-R) as shown in Table I, but compound **2** was not hydrolyzed at all, because of the presence of the benzylidene group. Compound **5** was hydrolyzed by both alpha amylases at a single glucosidic linkage,

TABLE I

PRODUCTS OF THE INITIAL STAGE OF THE ACTION OF TWO HUMAN ALPHA AMYLASES ON SUBSTRATE OLIGOSACCHARIDES

Substrate	*Products, as fraction of the total product*					
	Salivary alpha amylase			*Pancreatic alpha amylase*		
	G-G-G-R[a]	*G-G-R*	*G-R*	*G-G-G-R*	*G-G-R*	*G-R*
1	0.11	0.83	0.06	0.12	0.73	0.15
5	0	0.98	0.02	0	0.95	0.05

[a]G = α-D-glucosyl group or residue; R = $C_6H_4NO_2$-*p*.

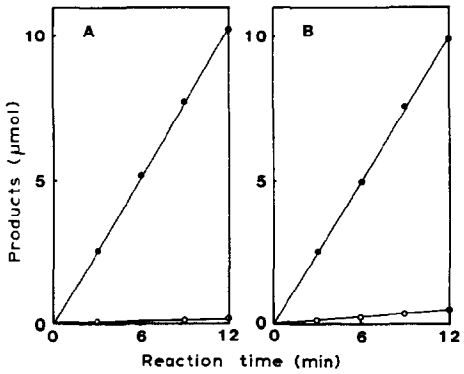


Fig. 3. Course of the digestion of compound **5** by two human alpha amylases. The reaction products were measured by h.p.l.c. The conditions for the digestion and analysis are described in the text. A, human salivary alpha amylase digestion; B, human pancreatic alpha amylase digestion. Symbols: ●, *p*-nitrophenyl α-maltoside; ○, *p*-nitrophenyl α-D-glucopyranoside.

to produce G-G-R (see Fig. 2 and Table I). These results indicated that the binding mode of **5** was influenced by the benzyl group on the nonreducing-end D-glucose residue of the substrate. This property of the substrate simplifies the assay of the total alpha amylases. The reaction courses of the two alpha amylases with **5** were linear during the period of measurement (see Fig. 3).

Kinetic parameters. — The rates of hydrolysis of **5** by the enzymes may also be influenced by the benzyl group. The Michaelis constant (K_m) and the rate of hydrolysis of **1** and **5** were examined by incubation with the alpha amylases (see Experimental section) and measurement, by h.p.l.c., of the amounts of products. The K_m values were calculated by the method of least squares with the use of a Lineweaver–Burk plot (see Table II). Modification of the nonreducing-end D-glucosyl residue caused a decrease in the K_m values and an increase in the relative rate of hydrolysis. The parameters of the action of the two human alpha amylases on **5** were quite similar. The substrate **5** is thus very suitable for measurements of the mixture of the two alpha amylases found in human fluids.

Assay of alpha amylase in human serum. — The activity of the alpha amylases

TABLE II

KINETICS OF HYDROLYSIS OF SUBSTRATES BY HUMAN ALPHA AMYLASES

Substrate	K_m *(mM)*		*Relative rates*	
	Salivary alpha amylase	*Pancreatic alpha amylase*	*Salivary alpha amylase*	*Pancreatic alpha amylase*
1	0.31	0.36	**1.0**[a]	**1.0**[a]
5	0.10	0.11	1.4	1.4

[a]Value taken as unity.

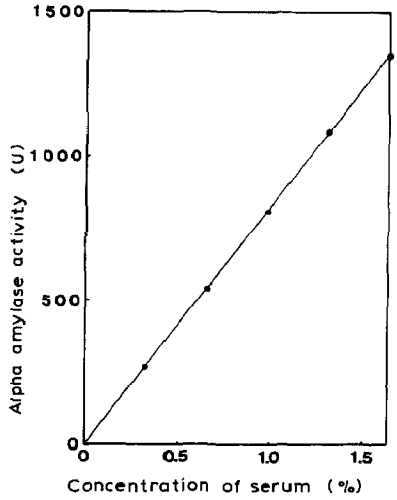


Fig. 4. Relationship between the concentration of serum (alpha amylase) and the initial rates of the enzymic reaction. Increases in absorbance at 405 nm with time were measured. Reaction conditions were as described in the text.

in human serum can be assayed by measurement of the amount of *p*-nitrophenol released from the amylolysis products by the action of glucoamylase and α-D-glucosidase, which are included in the assay mixture as coupling enzymes. The *p*-nitrophenol was determined by measuring the increase in absorbance at 405 nm; a recommended protocol is given in the Experimental section. It was found that the relationship between the concentration of alpha amylases and the release of *p*-nitrophenol was linear (see Fig. 4).

EXPERIMENTAL

Materials. — Human salivary alpha amylase [EC 3.2.1.1] was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Human pancreatic alpha amylase [EC 3.2.1.1] was purified from pancreatic juice by the method of Matsuura *et al.*[23]. The activity of human alpha amylase is defined as the amount of the enzyme that hydrolyzes 1 μmol of **5** per min in mM **5** at pH 7.6 and 37°. Glucoamylase [EC 3.2.1.3] (*Rhizopus delemar* 36.5 U/mg) and α-D-glucosidase [EC 3.2.1.20] (*Saccharomyces carlsbergensis* 121 U/mg) were purchased from Toyobo Co., Ltd., Osaka, Japan. The chromatographic adsorbent SP-800 (bead form) was from Mitsubishi Chemical Industries, Ltd., Tokyo, Japan. The other reagents used were of reagent grade, manufactured by Wako Pure Chemical Industries Ltd., Osaka, Japan.

G.l.c. analyses. — Glucose and benzyl derivatives of glucose in the substrate were analyzed by g.l.c. on a column (0.4 × 200 cm) of 2% of OV-17 on Chromosorb W after methanolysis with 1.4M HCl–methanol for 2 h at 90°, followed by trimethylsilylation with hexamethyldisilazane and chlorotrimethylsilane in

pyridine[24]. The temperature was programmed to rise from 110 to 250° at the rate of 4° per minute.

High-performance liquid chromatography (h.p.l.c.). — The chromatograph used for h.p.l.c. was a Shimadzu Model LC-6A (Kyoto, Japan). For the measurement of alpha amylase activity, a column (4.6 × 150 mm) packed with Wakosil $5C_{18}$ (reversed phase, Wako) was equilibrated with 10% acetonitrile in 0.1% acetic acid solution, and eluted with a linear gradient of acetonitrile from 10 to 30%. The flow rate was 1.5 mL/min. The compounds were monitored by measurement of the absorbance at 305 nm arising from the *p*-nitrophenyl group. The amounts of the products were calculated from their peak areas on the chromatogram.

H.p.l.c. analyses. — A mixture of 20 μL of alpha amylase solution (200 U/L) and 200 μL of 1.2mM substrate solution (**1**, **2**, or **5**) in 50mM BES [*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid]–NaOH buffer (pH 7.6), containing 20mM sodium chloride and 2mM calcium chloride, was incubated for 3, 6, 9, or 12 min at 37°. Then, 25 μL of the reaction was added to 500 μL of 5% acetic acid to stop the reaction. The sample (100 μL) was analyzed by h.p.l.c. to identify the products.

N.m.r. — ^{13}C–N.m.r. spectra of the compounds dissolved in dimethyl sulfoxide-d_6 were recorded with a Jeol GX-400 spectrometer (100 MHz, Nihon Denshi Co., Ltd., Tokyo, Japan).

F.a.b.-mass spectrometry. — F.a.b.-mass spectra were recorded with a Jeol HX-100 double-focusing mass spectrometer (Nihon Denshi Co., Ltd.,) fitted with a 2.33-T magnet, and equipped with a f.a.b. ion source and a post-accelerating system. A solution (1–2 μL) containing sample (10–20 μg), glycerol (0.5 μL), and M hydrochloric acid (0.5 μL) was bombarded with a xenon neutral-atom beam accelerated by a 7-keV potential. A Jeol DA-500 mass data analysis system was used as a processor, and the molecular weight of the sample was estimated from the *m/z* value of the quasimolecular-ion $(M + H)^+$ peak.

Assay of alpha amylase activity. — To a solution of 3 mL of **5** (mM) and the coupled enzymes (30 U/mL glucoamylase and 10 U/mL α-D-glucosidase) in 50mM BES/NaOH buffer (pH 7.6), containing 20mM sodium chloride and 2mM calcium chloride, was added 50 μL of serum from a patient with hyperamylasemia, and the mixture was incubated at 37°. The reaction was monitored by the increase in absorbance at 405 nm.

p-*Nitrophenyl* 6^5-O-*benzyl-α-maltopentaoside* (**5**). — A mixture of *p*-nitrophenyl α-maltopentaoside (**1**, 3 g), benzaldehyde dimethyl acetal (4.3 mL), and *p*-TsOH (anhydrous) (0.45 g) in DMF (45 mL) was kept for 3 h at 50°. After evaporation of DMF *in vacuo*, compound **2** was purified by gel-permeation chromatography on a column (30 × 1500 mm) of Bio-gel P-2. For the preparation of compound **5**, the DMF solution was mixed with pyridine (70 mL) and benzoyl chloride (50 mL), and kept for 1 h at room temperature. The mixture was extracted with chloroform (100 mL), and the extract was washed with water, dried, and evaporated, to yield **3** (6 g, 75%). A solution of **3** (3 g) in THF (12 mL) containing

molecular sieves was cooled in an ice bath, and Me_2NH–BH_3 (12 g) in THF (20 mL) and *p*-TsOH (anhydrous) (27 g) in THF (80 mL) were simultaneously added. Stirring was continued for 1 h. The mixture was extracted with chloroform (50 mL), and the extract was washed with water, dried, and evaporated. To the resulting syrup was added 0.2% sodium methoxide in methyl alcohol (70 mL), and the mixture was stirred for 5 h. After neutralization of the base with 6M hydrochloric acid, the mixture was evaporated *in vacuo*. A solution of the syrup in water (50 mL) was put on a column (1 × 60 cm) of SP-800. The column was eluted with 9:11 ethanol–water to yield compound **5** (260 mg, 16%); $[\alpha]_D$ +194.6° (*c* 1, H_2O); Calc. for $C_{43}H_{61}NO_{28}$: 1039.3; Found by f.a.b.-m.s., $(M + H)^+$: 1040.3.

Anal. Calc.: C, 49.65; H, 5.87; N, 1.35. Found: C, 49.46; H, 5.58; N, 1.49.

Methyl 2,3-di-O-benzoyl-4,6-O-benzylidene-α-D-glucopyranoside. — Methyl α-D-glucopyranoside (20 g) was converted into the title compound (36 g, 70%), first by treatment with benzaldehyde dimethyl acetal and then with benzoyl chloride, as already described; m.p. 152–153°, $[\alpha]_D$ +92.9° (*c* 1, $CHCl_3$); lit.[25] m.p. 154°, $[\alpha]_D$ +94 ±2° (*c* 1.51, $CHCl_3$).

Anal. Calc. for $C_{28}H_{26}O_8$: C, 68.56; H, 5.34. Found: C, 68.67; H, 5.32.

Methyl 4-O-benzyl-α-D-glucopyranoside. — Methyl 2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-α-D-glucopyranoside (3 g) was treated with lithium aluminum hydride (1 g) and aluminum chloride (3 g) in 1:1 ether–dichloromethane by the procedure of Lipták *et al.*[19] (0.4 g, 23%); m.p. 126–127°, $[\alpha]_D$ 154.5° (*c* 1, $CHCl_3$); ^{13}C-n.m.r. data (100 MHz, Me_2SO-d_6): δ 54.3, 60.5, 71.3, 72.2, 73.5, 73.6, 78.2, 99.6, 127.2, 127.4, 128.0, and 139.1.

Anal. Calc. for $C_{14}H_{20}O_6$: C, 59.14; H, 7.09. Found: C, 58.96; H, 7.07.

Methyl 6-O-benzyl-α-D-glucopyranoside. — Methyl 2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-α-D-glucopyranoside (1 g) was reduced with sodium cyanoborohydride (1.7 g) and hydrogen chloride in THF[20]. Debenzoylation of the product gave the title compound (0.34 g, 84%); m.p. 57–58°, $[\alpha]_D$ +107.2° (*c* 1, $CHCl_3$); ^{13}C-n.m.r. data (100 MHz, Me_2SO-d_6): δ 54.3, 69.8, 70.4, 71.1, 71.9, 72.2, 73.4, 99.6, 127.2, 127.3, 128.1, and 138.6

Anal. Calc. for $C_{14}H_{20}O_6$: C, 59.14; H, 7.09. Found: C, 59.02; H, 7.08.

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