Syntheses of 2-Chloro-4-nitrophenyl β-D-Maltopentaosides with Bulky Modification and Their Application to the Differential Assay of Human α-Amylases

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Four novel maltopentaosides, 2-chloro-4-nitrophenyl *O*-(6-*O*-*p*-toluenesulfonyl-α-D-glucopyranosyl)-(1→4)-tris[*O*-α-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside (4), 2-chloro-4-nitrophenyl *O*-[6-*O*-(*tert*-butyldimethyl)silyl-α-D-glucopyranosyl]-(1→4)-tris[*O*-α-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside (5), 2-chloro-4-nitrophenyl *O*-[6-deoxy-6-(phenyl)sulfonyl-α-D-glucopyranosyl]-(1→4)-tris[*O*-α-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside (10), and 2-chloro-4-nitrophenyl *O*-(6-deoxy-6-phthalimido-α-D-glucopyranosyl)-(1→4)-tris[*O*-α-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside (11) were synthesized. Substrates 4, 5, 10, and 11 were hydrolyzed by human pancreatic α-amylase (HPA) from 1.1 to 2.9-fold faster than by human salivary α-amylase (HSA). Taking advantage of the difference in the hydrolytic rate of 5 (2.9-fold faster), we developed a new method for the differential assay of these two human α-amylases.

Keywords 2-chloro-4-nitrophenyl β-D-maltopentaosides; bulky modification; α-amylase activity; pancreatic; salivary; differential assay

Two kinds of human α-amylases (EC 3.2.1.1) which are salivary α-amylase (HSA) and pancreatic α-amylase (HPA) are known to exist in serum and urine as normal components. Since changes in the activities of each amylase are also known to be in correlation with certain diseases (ex. acute pancreatitis, parotitis), the differential assay of the two amylases is very important for making an accurate diagnosis.[1] Several differential assay methods using electrophoresis,[2] isoelectric focusing,[3] chromatography,[4] amylase inhibitor,[5,6] and modified α-D-maltopentaosides as the substrate to measure the ratio of the products in the hydrolysis[7–9] have been reported at present; but these methods still remain problematic.

We have been studying convenient and useful substrates for enzymes assay,[10] and our recent studies on the influence of various modifications at a terminal (non-reducing-end) D-glucosyl group of maltopentaosides on α-amylase hydrolysis have utilized many systematically synthesized substrates.[11,12] Our finding that most substrates having modification at the non-reducing-end were hydrolyzed by HPA and HSA at an approximately equal rate, except for derivatives with a small modification (6^5-deoxy and 6^5-deoxy-6^5-fluoro-maltopentaosides), which were hydrolyzed by HPA 2 to 4-fold slower than by HSA, led us to expect that maltopentaosides with bulky modification at the terminal glucosyl moiety were hydrolyzed at a higher rate by HPA than HSA. Such compounds would then be potential substrates for the differential assay of the two amylases.

The object of the present work was to synthesize maltopentaosides with bulky modification and to identify a potential substrate for the differential assay. In this report we describe the synthesis of four novel 2-chloro-4-nitrophenyl β-D-maltopentaosides, the interesting mode of actions of the two amylases on the compounds, and a new method of the differential assay using one of them.

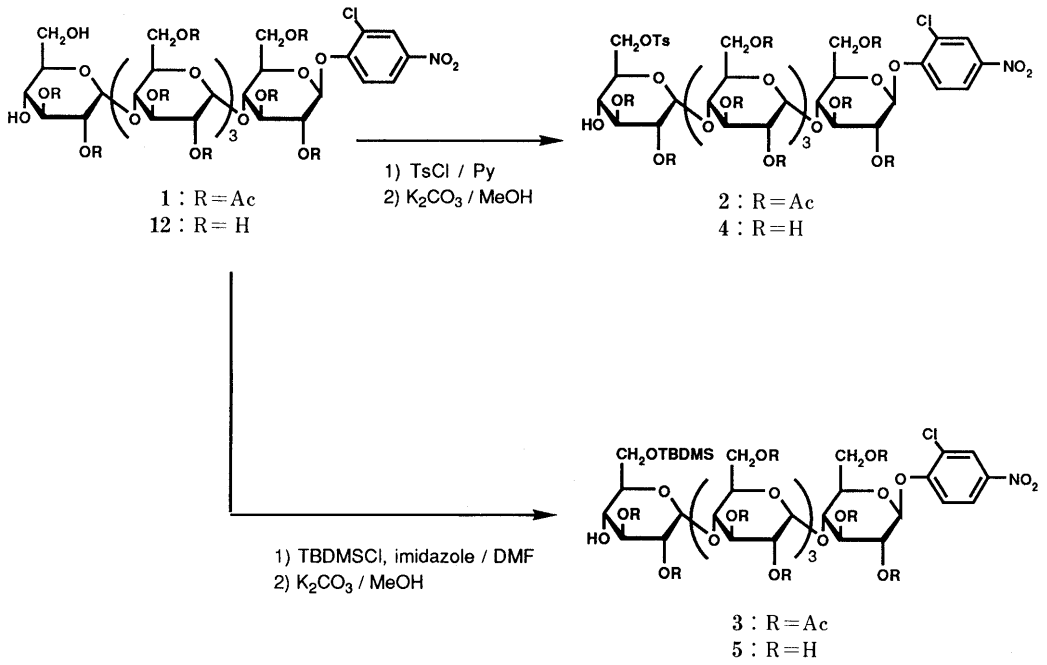

Chart 1

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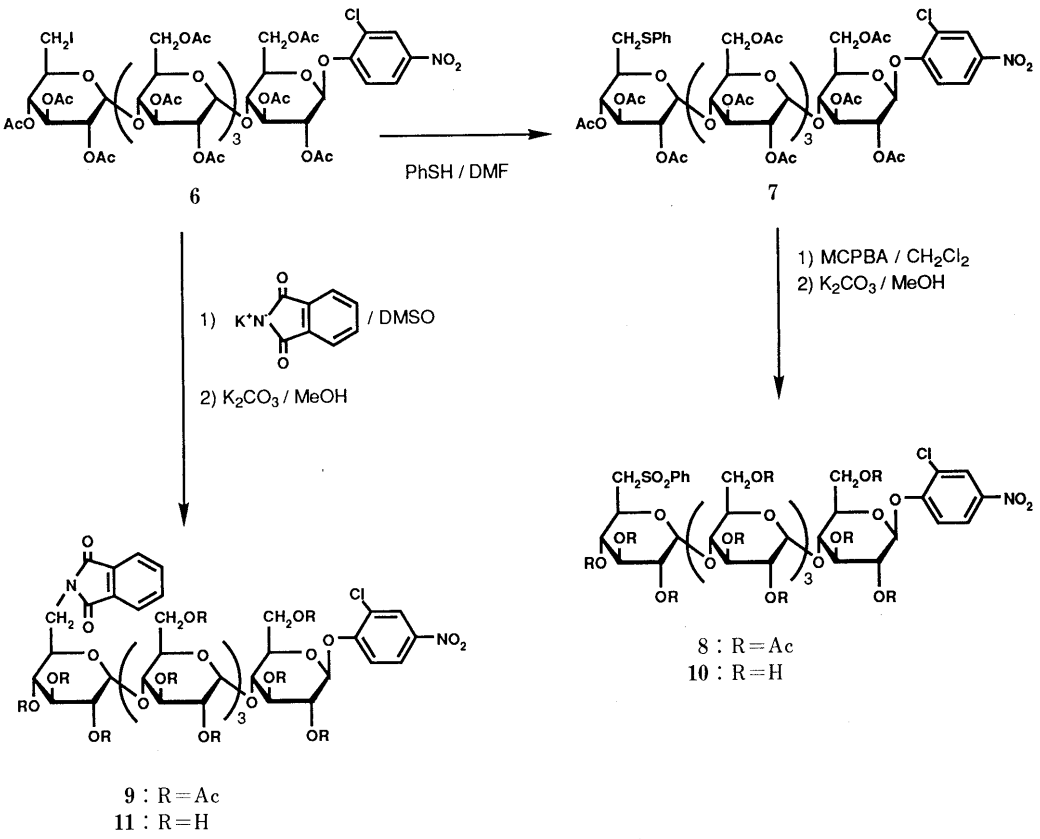

Chart 2

Synthesis 6^5-*O*-Modified derivatives having bulky groups were first synthesized as follows. Selective *p*-toluenesulfonylation of diol (**1**)[11] with TsCl (15 eq) in pyridine at room temperature gave 6^5-*O*-Ts derivative (**2**) in a 51% yield. The reaction of **1** and *tert*-butyldimethylsilyl chloride (TBDMS-Cl) using imidazole as a catalyst in dimethylformamide (DMF) carried out selective silylation to afford 6^5-*O*-TBDMS derivative (**3**) in an 86% yield. *O*-De-acetylation of **2** and **3** with K_2CO_3 in MeOH gave 2-chloro-4-nitrophenyl *O*-(6-*O*-toluenesulfonyl-α-D-glucopyranosyl)-(1→4)-tris[*O*-α-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside (**4**, yield 81%) and 2-chloro-4-nitrophenyl *O*-[6-*O*-(*tert*-butyldimethyl)silyl-α-D-glucopyranosyl]-(1→4)-tris[*O*-α-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside (**5**, yield 86%), respectively.

The second target compounds were 6^5-deoxy-6^5-substituted derivatives, which were prepared to investigate the effects of the absence of the oxygen atom at the 6^5-position. The reaction of 6^5-iodide (**6**)[11] with thiophenol and triethylamine (Et_3N) in DMF gave 6^5-(phenyl)thio derivative (**7**) in a 66% yield. Then, **7** was oxidized by *m*-chloroperbenzoic acid (MCPBA) to afford the desired 6^5-(phenyl)sulfonyl derivative (**8**) in an 80% yield. The reaction of **6** and potassium phthalimide in dimethyl sulfoxide (DMSO) gave 6^5-phthalimido derivative (**9**) in a 75% yield. Compounds **8** and **9** were *O*-de-acetylated by the same method as **2** to give 2-chloro-4-nitrophenyl *O*-[6-deoxy-6-(phenyl)sulfonyl-α-D-glucopyranosyl]-(1→4)-tris[*O*-α-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside (**10**, yield 74%) and 2-chloro-4-nitrophenyl *O*-(6-deoxy-6-phthalimido-α-D-glucopyranosyl)-(1→4)-tris[*O*-α-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside (**11**, yield 65%), respectively.

TABLE I. Kinetic Parameters of the Action of Two Human α-Amylases on Maltopentaosides

Compd. No.	K_m (mM)		Relative rate of hydrolysis		
	HPA	HSA	HPA	HSA	V_{HPA}/V_{HSA}
4	0.07	0.09	1.08	0.940	1.14
5	0.10	0.03	0.360	0.125	2.88
10	0.21	0.14	0.484	0.288	1.67
11	0.37	0.32	0.463	0.209	2.22
12	0.29	0.37	1.00[a]	1.00[a]	1.00

a) The relative rates of hydrolysis assume the value of unity for **12**.

Confirmation of Structures of Maltopentaosides The structures of **4**, **5**, **10**, and **11** were established by spectral data and elemental analyses as described in the experimental section. Except for the signals due to each substituent at the terminal-end, the ^{1}H-NMR spectrum of the four compounds showed similar signal patterns due to the hydrogens of β-2-chloro-4-nitrophenyl maltopentaoside to those of a series of maltopentaosides previously reported by us,[11,12] designating that they have the same basic structures.

Kinetic Parameters and Patterns of Action of Two Human α-Amylases on Modified Maltopentaosides Maltopentaosides (**4**, **5**, **10**, and **11**) were tested to determine the kinetic parameters and patterns of action of HPA and HSA by the same methods as described before.[12] These results are shown in Tables I and II. As a matter of convenience, it is assumed that the active site of the enzymes includes nine subsites per glucosyl moiety (G) numbered S_1—S_9 from the

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TABLE II. Patterns of the Action of Two Human α-Amylases on Modified Maltopentaosides

Compd. No.	Ratio of products with HPA				Ratio of products with HSA			
	G_4-CNP :	G_3-CNP :	G_2-CNP :	G_1-CNP	G_4-CNP :	G_3-CNP :	G_2-CNP :	G_1-CNP
4	0 :	0.01 :	0.96 :	0.03	0 :	0 :	0.99 :	0.01
5	0 :	0 :	0.57 :	0.43	0 :	0 :	0.71 :	0.29
10	0 :	0 :	0.87 :	0.13	0 :	0 :	0.89 :	0.11
11	0 :	0 :	0.78 :	0.22	0 :	0 :	0.86 :	0.14

Abbreviations: G_4-CNP, 2-chloro-4-nitrophenyl β-D-maltotetraoside; G_3-CNP, 2-chloro-4-nitrophenyl β-D-maltotrioside; G_2-CNP, 2-chloro-4-nitrophenyl β-D-maltoside; and G_1-CNP, 2-chloro-4-nitrophenyl β-D-glucopyranoside.

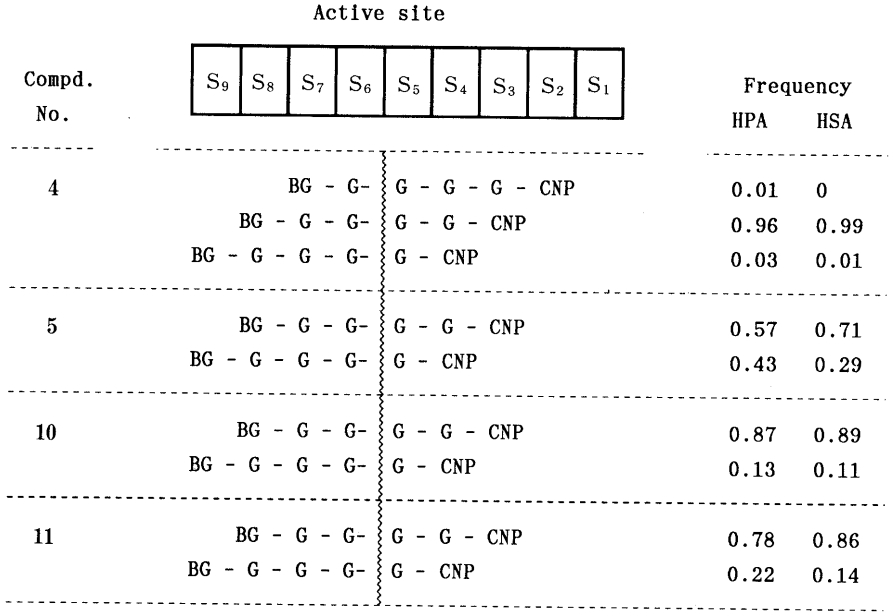

Fig. 1. Schematic Representation of Substrate Binding to Subsites of Two Human α-Amylases

Abbreviations: BG, modified glucosyl moiety; G, glucosyl moiety; CNP, β-2-chloro-4-nitrophenyl moiety.

reducing end,[13] and that glucosidic bonds of the substrates are split between S_5 and S_6 (Fig. 1). The binding modes of the substrates to the active sites on the course of hydrolysis can therefore be estimated from the action patterns of the enzymes on the substrates.

In agreement with our presumption described in the introduction, Table I indicates that there was a tendency; bulky modification made at the 6^5-position of maltopentaosides afforded a high ratio of the relative rate of hydrolysis by the two amylases (V_{HPA}/V_{HSA}). In the case of 6^5-*O*-TBDMS derivative (**5**) the tendency was the most remarkable, where the substrate was hydrolyzed by HPA 2.9-fold faster than by HSA. Taking advantage of this difference in the hydrolytic rate, a new method for the differential assay was developed, as described below. It was also found that a substrate which had the higher V_{HPA}/V_{HSA} value tended to give more 2-chloro-4-nitrophenyl β-D-glucopyranoside (G_1-CNP) as a hydrolyzed product and to have a lower rate of the hydrolytic reaction. This finding suggested that the subsite of S_8, which seemed to have hydrophobic amino acid residues,[11,14] tended to attract not only the non-reducing-ended D-glucosyl group but also the second D-glucosyl group from the terminal of a substrate, because the hydrophobic area of the modification was so large. In the case of the most characteristic substrate **5** it was considered that; since K_m values for it were small, meaning that α-amylase had a high affinity for the substrate, the low reaction rate did not result from the difficulty of forming an ES-complex but from weak repulsion between the subsites and cleft products, especially with respect to HSA. Additionally, it was designated that the absence of the oxygen atom at the 6^5-position effected an enlargement of the V_{HPA}/V_{HSA} value, since the value of **10**, which did not have the oxygen atom, was remarkably higher than that of **4** even though both of them had a similar arylsulfonyl group.

Application of 5 to Differential Assay of HPA and HSA When a sample containing HPA and/or HSA hydrolyzes each substrate **5** and non-modified derivative (**12**, Chart 1),[15] equations of the straight line of increments of absorbance are:

$$A_1 = k_1 \cdot a_p + k_2 \cdot a_s \quad (1)$$

$$A_2 = k \cdot (a_p + a_s) \quad (2)$$

where A_1 and A_2 are respectively the increase in absorbance at 400 nm per minute (ΔA) of enzymatic reaction with **5** and **12** as a substrate, a_p and a_s are HPA and HSA activities (U/l) in a sample, k_1 and k_2 are the rate constants of hydrolysis of **5** by HPA and HSA, and k is the rate constant of hydrolysis of **12** by each amylase. Since $k_1 = 0.360 \cdot k$ and $k_2 = 0.125 \cdot k$, as shown in Table I, Eqs. 1 and 2 can be

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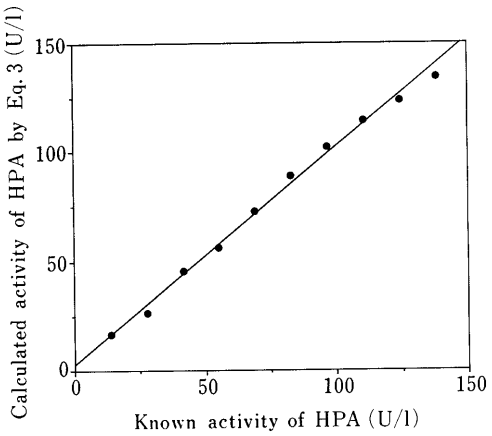

Fig. 2. Relation between Known Activity and Activity Calculated by Eq. 3 of HPA in Prepared Samples

$y=2.2586+0.99588x$. $r=0.997$. $n=11$.

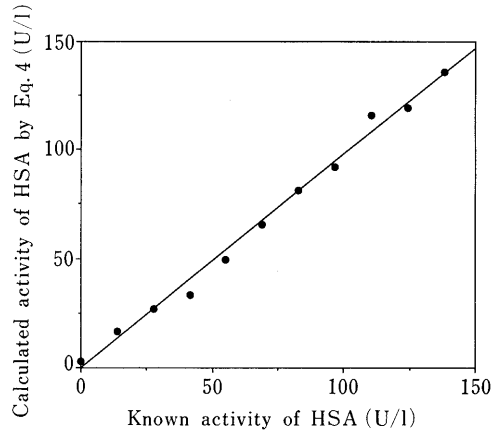

Fig. 3. Relation between Known Activity and Activity Calculated by Eq. 4 of HSA in Prepared Samples

$y=-0.70455+0.98250x$. $r=0.995$. $n=11$.

transformed to:

$$a_p=(10\cdot A_1-1.25\cdot A_2)/2.35\cdot k \quad (3)$$

$$a_s=(3.60\cdot A_2-10\cdot A_1)/2.35\cdot k \quad (4)$$

Since k is the constant determined by this assay procedure used, Eqs. 3 and 4 indicate that only two measurable amounts (A_1 and A_2) can give the value of a_p and a_s, that is the differential assay itself. When 1 unit (U) is the activity of the enzyme which hydrolyzes 1 μmol of **12** per minute at 37 °C and ε of 2-chloro-4-nitrophenol is 16100,[16] k is given to be 1.24×10^{-3} $(1\cdot\Delta A/U)$ under the conditions described in the experimental section.

We first tested the accuracy of the method for the differential assay. Activities of the commercially available standard HPA and HSA were determined by the usual method using **12**[17] as the substrate, and samples containing various known activities of each amylase were prepared. Each sample was incubated with **5** or **12** as the substrate in the presence of coupled enzymes in phosphate buffer (pH 7.0) at 37 °C as described in the experimental section. From obtained amounts of A_1 and A_2, values of a_p and a_s were calculated using Eqs. 3 and 4. These results are summarized in Figs. 2 and 3, both of which indicated highly linear

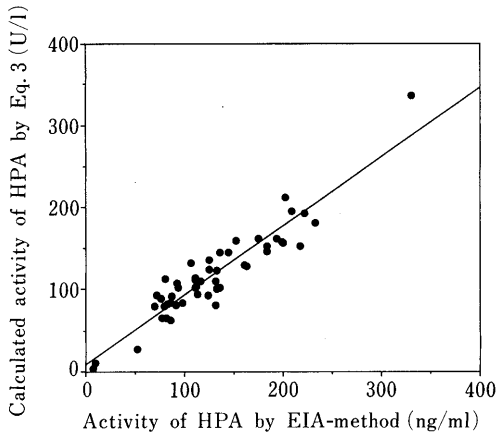

Fig. 4. Correlation of Our Method with EIA Method for the Determination of HPA in Human Sera

$y=8.3123+0.84389x$. $r=0.934$. $n=50$.

relationships, thus demonstrating the accuracy of the method. Secondly, we examined the usefulness of the method for the differential assay. Values of a_p in sera from 50 healthy persons were calculated in the same way as described; data were compared by the differential assay method (enzyme immunoassay method) using monoclonal antibodies of HSA (Fig. 4). There is a clear interrelation between our method and the enzyme immunoassay method.

We therefore concluded that this new differential assay method, which is the first one developed based on the difference in the hydrolytic rate by the two amylases using **5** and **12** as substrates, is applicable to the measurement of HPA and HSA in human serum. The method has been proved to be convenient, accurate, and is very suitable for routine clinical use.

Experimental

Reagents and Materials All chemicals were of reagent grade unless otherwise noted. α-Amylases (from human pancreatic juice and saliva) were obtained from International Reagents Corp., Japan. α-Glucosidase (from yeast) and β-glucosidase (from sweet almond) were obtained from Toyobo Co., Ltd., Japan. The enzyme immunoassay-kit of HPA (right assay P-Amylase) was obtained from Sanko Junyaku Co., Ltd., Japan.

Apparatus All melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were determined with a JASCO DIP-360 digital polarimeter at 25°. Infrared (IR) spectra were taken with a JASCO A-202 spectrometer. ^{1}H-NMR spectra were taken at 199.5 MHz and ^{13}C-NMR spectra were taken at 50.10 MHz with a JEOL JNM-FX200 spectrometer and Me_4Si as an internal standard. The following abbreviations are used for the signal patterns; s = singlet, d = doublet, t = triplet, m = multiplet, and br = broad. High performance liquid chromatography (HPLC) was performed on a [A] Cosmosil C_{18} column (4.6 mm i.d. × 150 mm) or a [B] TSK gel Amide-80 column (4.6 mm i.d. × 250 mm) with a flow rate of 1.0 ml/min using a JASCO pump (880-PU) and an ultraviolet (UV, 280 nm) detector (JASCO UVIDEC-100-V) at room temperature. Visible spectra (400 nm) were recorded with a Hitachi M-80 spectrometer. Column chromatography was performed on Merck Kiesel gel 60 (SiO_2, 230—400 mesh) and YMC-gel ODS-AQ (120-S50, from Yamamura Chemical Laboratories Co., Ltd., Japan).

2-Chloro-4-nitrophenyl *O*-(2,3-Di-*O*-acetyl-6-*O*-*p*-toluenesulfonyl-α-D-glucopyranosyl)-(1→4)-tris[*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1→4)]-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (2) TsCl (21.1 g, 110 mmol) was added to a solution of 2-chloro-4-nitrophenyl *O*-(2,3-di-*O*-acetyl-α-D-glucopyranosyl)-(1→4)-tris[*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1→4)]-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside[11] (**1**, 11.6 g, 7.38 mmol) in pyridine (300 ml), and the mixture was stirred for 5 h at room temperature. MeOH (5.0 ml) was added to the mixture and the

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mixture was stirred for an additional 2 h. Then the mixture was evaporated *in vacuo* to leave a syrupy residue which was chromatographed on SiO_2 gel with AcOEt–MeOH–CH_2Cl_2 (50 : 1 : 100, v/v) to give **2** (6.43 g, 3.72 mmol, yield 50.5%), mp 109—113.5 °C (from Et_2O). $[\alpha]_D$ +88.0° (c = 0.650, 1,4-dioxane). *Anal.* Calcd for $C_{71}H_{88}ClNO_{44}S$: C, 49.38: H, 5.14; N, 0.81. Found: C, 49.14; H, 5.10; N, 0.79. IR ν_{max} cm^{-1}: 3490 (OH), 2970 (CH, aliph.), 1752 (C=O), 1586, 1486 (arom.), 1528, 1372 (NO_2), 1430, 1350, 1240, 1178, 1034 (SO_2, C–O). ^{1}H-NMR ($CDCl_3$) δ: 1.99—2.17 (42H, cluster of s, 14 OAc), 2.45 (3H, s, Ph–C$\underline{H}_3$), 3.50—4.80 (25H, m, H-2a—e, 4a—e, 5a—e, 6a—e), 5.10—5.50 (10H, m, H-1a—e, 3a—e), 7.27 (1H, d, J = 9.0 Hz, H-6 of CNP), 7.33 and 7.79 (each 2H, d, J = 8.5 Hz, Ph of Ts), 8.15 (1H, dd, J = 9.0, 2.7 Hz, H-5 of CNP), 8.29 (1H, d, J = 2.7 Hz, H-3 of CNP). t_R (column: [A], eluent: CH_3CN–H_2O, 7 : 3, v/v): 8.3 min.

2-Chloro-4-nitrophenyl *O*-[2,3-Di-*O*-acetyl-6-*O*-(*tert*-butyldimethyl)-silyl-α-D-glucopyranosyl]-(1→4)-tris[*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1→4)]-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (3) TBDMSCl (728 mg, 4.82 mmol) was added to a solution of **1** (1.50 g, 0.954 mmol) in DMF (30 ml) containing imidazole (654 mg, 9.60 mmol) and the mixture was stirred for 8 h at room temperature. Then toluene (1.0 l) was added to the reaction solution and the mixed solution was washed with water, dried (Na_2SO_4), and evaporated *in vacuo* to leave a syrupy residue which was chromatographed on SiO_2 gel with AcOEt–MeOH–CH_2Cl_2 (100 : 1 : 200, v/v) to give **3** (1.39 g, 0.824 mmol, yield 86.4%). mp 117—119 °C (from Et_2O). $[\alpha]_D$ +88.6° (c = 0.502, 1,4-dioxane). *Anal.* Calcd for $C_{70}H_{96}ClNO_{42}Si$: C, 49.84; H, 5.74; N, 0.83. Found: C, 49.57; H, 5.70; N, 0.75. IR ν_{max} cm^{-1}: 3430 (OH), 2950 (CH, aliph.), 1750 (C=O), 1584, 1484 (arom.), 1528, 1370 (NO_2), 1430, 1370, 1350, 1238, 1142, 1038 (C–O). ^{1}H-NMR ($CDCl_3$) δ: 0.09 (6H, s, 2 Si–C$\underline{H}_3$), 0.90 (9H, s, 3 Me of ^{t}Bu), 2.00—2.19 (39H, cluster of s, 13 OAc), 3.65—4.80 (25H, m, H-2a—e, 4a—e, 5a—e, 6a—e), 5.20—5.45 (10H, m, H-1a—e, 3a—e), 7.28 (1H, d, J = 9.0 Hz, H-6 of CNP), 8.16 (1H, dd, J = 9.0, 2.7 Hz, H-5 of CNP), 8.30 (1H, d, J = 2.7 Hz, H-3 of CNP). t_R (column: [A], eluent: CH_3CN–H_2O, 3 : 2, v/v): 16.4 min.

2-Chloro-4-nitrophenyl *O*-(6-*O*-*p*-Toluenesulfonyl-α-D-glucopyranosyl)-(1→4)-tris[*O*-(α-D-glucopyranosyl)-(1→4)]-β-D-glucopyranoside (4) K_2CO_3 (195 mg, 1.41 mmol) was added to a suspension of **2** (2.43 g, 1.41 mmol) in MeOH (100 ml) with stirring, and the reaction mixture was kept at room temperature for 5 h. Then 100 mM phosphate buffer (pH 6.5, 200 ml) was added to the solution, half of the solvent was evaporated *in vacuo*, and a solution of the residue in H_2O was obtained. ODS gel column chromatography (H_2O→5%→10%→20% CH_3CN, stepwise) of the solution gave pale yellow amorphous **4** (1.25 g, 1.14 mmol, yield 80.9%). $[\alpha]_D$ +78.9° (c = 0.502, 1,4-dioxane–H_2O, 1 : 1, v/v). *Anal.* Calcd for $C_{43}H_{60}ClNO_{30}S \cdot H_2O$: C, 44.66; H, 5.40; H, 1.21. Found: C, 44.56; H, 5.10; N, 1.11. IR ν_{max} cm^{-1}: 3410 (OH), 2920 (CH, aliph.), 1584, 1484 (arom.), 1520, 1350 (NO_2), 1276, 1152, 1078, 1024 (C–O). ^{1}H-NMR (DMSO-d_6–D_2O, 10 : 1, v/v) δ: 2.42 (3H, s, Ph–C$\underline{H}_3$), 3.00—3.80 (28H, m, H-2a—e, 4a—e, 5a—e, 6a—d), 4.13 (1H, dd, J = 10.1, 6.2 Hz, H-6e$_a$), 4.23 (1H, d, J = 10.1 Hz, H-6e$_b$), 5.04 (3H, br s, H-1), 5.12 (1H, d, J = 3.4 Hz, H-1), 5.26 (1H, d, J = 7.3 Hz, H-1a), 7.46 (1H, d, J = 9.0 Hz, H-6 of CNP), 7.47 and 7.78 (each 2H, d, J = 8.4 Hz, Ph of Ts), 8.19 (1H, dd, J = 9.0, 2.7 Hz, H-5 of CNP), 8.31 (1H, d, J = 2.7 Hz, H-3 of CNP). ^{13}C-NMR (DMSO-d_6–D_2O, 10 : 1, v/v) δ: 21.5 (Ph-$\underline{C}H_3$), 127.9, 130.4, 132.7, and 142.0 (–SO_2–Ph). t_R (column: [B], eluent: CH_3CN–H_2O, 3 : 1, v/v): 4.6 min.

2-Chloro-4-nitrophenyl *O*-[6-*O*-(*tert*-Butyldimethyl)silyl-α-D-glucopyranosyl]-(1→4)-tris[*O*-(α-D-glucopyranosyl)-(1→4)]-β-D-glucopyranoside (5) *O*-De-acetylation of **3** (1.33 g, 0.791 mmol) with K_2CO_3 as described for **4**, gave pale yellow amorphous **5** (742 mg, 0.676 mmol, yield 85.5%). $[\alpha]_D$ +78.8° (c = 0.500, MeOH). *Anal.* Calcd for $C_{42}H_{68}ClNO_{28}Si \cdot 3/2H_2O$: C, 44.82; H, 6.36; N, 1.24. Found: C, 44.66; H, 6.18; N, 1.11. IR ν_{max} cm^{-1}: 3420 (OH), 2940 (CH, aliph.), 1586 (arom.), 1524, 1350 (NO_2), 1274, 1256, 1154, 1082, 1032 (C–O). ^{1}H-NMR (DMSO-d_6–D_2O, 10 : 1, v/v) δ: 0.04 (6H, s, 2 Si–Me), 0.87 (9H, s, 3 Me of ^{t}Bu), 3.05—3.85 (30H, m, H-2a—e—6a—e), 5.05 (3H, br s, H-1), 5.10 (1H, d, J = 5.4 Hz, H-1), 5.25 (1H, d, J = 7.3 Hz, H-1a), 7.47 (1H, d, J = 9.0 Hz, H-6 of CNP), 8.18 (1H, dd, J = 9.0, 2.7 Hz, H-5 of CNP), 8.30 (1H, d, J = 2.7 Hz, H-3 of CNP). ^{13}C-NMR (DMSO-d_6–D_2O, 10 : 1, v/v) δ: 5.2 (Si-CH_3), 18.1 (C-$\underline{C}H_3$), 26.0 ($\underline{C}$-Me_3). t_R (column: [B], eluent: CH_3CN–H_2O, 3 : 1, v/v): 4.3 min.

2-Chloro-4-nitrophenyl *O*-[2,3,4-Tri-*O*-acetyl-6-deoxy-6-(phenyl)thio-α-D-glucopyranosyl]-(1→4)-tris[*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1→4)]-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (7) Thiophenol (715 μl, 6.96 mmol) and Et_3N (969 μl, 6.96 mmol) were added to a stirred solution of 2-chloro-4-nitrophenyl *O*-(2,3,4-tri-*O*-acetyl-6-deoxy-6-iodo-α-D-glucopyranosyl)-(1→4)-tris[*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1→4)]-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside[11] (**6**, 1.21 g, 0.701 mmol) in DMF (120 ml) and the resulting mixture was stirred for 3 h at room temperature. Toluene (700 ml) was added to the reaction solution, and the mixed solution was washed with water, dried (Na_2SO_4), and evaporated *in vacuo* to leave a syrupy residue which was chromatographed on SiO_2 gel with AcOEt–MeOH–CH_2Cl_2 (30 : 1 : 99, v/v) to give **7** (790 mg, 0.463 mmol, yield 66.4%). mp 112—115 °C (from Et_2O), $[\alpha]_D$ +90.4° (c = 0.810, 1,4-dioxane). *Anal.* Calcd for $C_{72}H_{88}ClNO_{42}S \cdot 3/4H_2O$: C, 50.26; H, 5.24; N, 0.81. Found: C, 50.21; H, 5.25; N, 0.71. IR ν_{max} cm^{-1}: 2960 (CH, aliph.), 1750 (C=O), 1584, 1484 (arom.), 1526, 1370 (NO_2), 1430, 1350, 1238, 1162, 1124, 1038 (C–O). ^{1}H-NMR ($CDCl_3$) δ: 1.98—2.19 (45H, cluster of s, 15 OAc), 3.06—3.11 (2H, ABX, H-6e), 3.80—4.35 (23H, m, H-2a—e, 4a—e, 5a—e, 6a—d), 5.03—5.50 (10H, m, H-1a—e, 3a—e), 7.15—7.40 (6H, m, H-6 of CNP and Ph), 8.16 (1H, dd, J = 9.0, 2.7 Hz, H-5 of CNP), 8.29 (1H, d, J = 2.7 Hz, H-3 of CNP). t_R (column: [A], eluent: CH_3CN–H_2O, 3 : 1, v/v): 7.9 min.

2-Chloro-4-nitrophenyl *O*-[2,3,4-Tri-*O*-acetyl-6-deoxy-6-(phenyl)sulfonyl-α-D-glucopyranosyl]-(1→4)-tris[*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1→4)]-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (8) MCPBA (224 mg, 1.30 mmol) was added to a stirred solution of **7** (790 mg, 0.463 mmol) in CH_2Cl_2 (70 ml) and the resulting mixture was stirred for 4 h at room temperature. The mixture was evaporated *in vacuo* to leave a syrupy residue which was chromatographed on SiO_2 gel with AcOEt–MeOH–CH_2Cl_2 (50 : 1 : 99, v/v) to give **8** (637 mg, 0.370 mmol, yield 79.9%). mp 124—128 °C (from Et_2O), $[\alpha]_D$ +88.9° (c = 0.406, 1,4-dioxane). *Anal.* Calcd for $C_{72}H_{88}ClNO_{44}S$: C, 49.73; H, 5.10; N, 0.81. Found: C, 49.52; H, 5.13; N, 0.59. IR ν_{max} cm^{-1}: 2960 (CH, aliph.), 1752 (C=O), 1586, 1488 (arom.), 1532, 1372 (NO_2), 1430, 1350, 1238, 1150, 1038 (C–O). ^{1}H-NMR ($CDCl_3$) δ: 1.97—2.18 (45H, cluster of s, 15 OAc), 3.15 (1H, dd, J = 12.5, 2.5 Hz, H-6e$_a$), 3.34 (1H, dd, J = 12.5, 7.5 Hz, H-6e$_b$), 3.85—4.90 (23H, m, H-2a—e, 4a—e, 5a—e, 6a—d), 5.15—5.50 (10H, m, H-1a—e, 3a—e), 7.28 (1H, d, J = 9.0 Hz, H-6 of CNP and Ph), 7.52—7.72 (3H, m, H-3—5 of SO_2Ph), 7.89 (2H, br d, J = 8.5 Hz, H-2 and H-6 of SO_2Ph), 8.16 (1H, dd, J = 9.0, 2.7 Hz, H-5 of CNP), 8.31 (1H, d, J = 2.7 Hz, H-3 of CNP). t_R (column: [A], eluent: CH_3CN–H_2O, 3 : 1, v/v): 5.5 min.

2-Chloro-4-nitrophenyl *O*-(2,3,4-Tri-*O*-acetyl-6-deoxy-6-phthalimido-α-D-glucopyranosyl)-(1→4)-tris[*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1→4)]-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (9) Potassium phthalimide (214 mg, 1.16 mmol) was added to a stirred solution of **6** (1.00 g, 0.580 mmol) in DMSO (50 ml) and the resulting mixture was stirred for 1 h at 95 °C. Toluene (500 ml) was added to the reaction solution, and the mixed solution was washed with water, dried (Na_2SO_4), and evaporated *in vacuo* to leave a syrupy residue which was chromatographed on SiO_2 gel with AcOEt–MeOH–CH_2Cl_2 (50 : 1 : 99, v/v) to give **9** (755 mg, 0.433 mmol, yield 74.7%), mp 111—113 °C (from Et_2O), $[\alpha]_D$ +90.9° (c = 0.514, 1,4-dioxane). *Anal.* Calcd for $C_{74}H_{87}ClN_2O_{44}$: C, 50.97; H, 5.03; N, 1.61. Found: C, 51.22; H, 5.19; N, 1.36. IR ν_{max} cm^{-1}: 2960 (CH, aliph.), 1750, 1720 (C=O), 1588 (arom.), 1530, 1370 (NO_2), 1428, 1370, 1236, 1038 (C–O). ^{1}H-NMR ($CDCl_3$) δ: 1.96—2.17 (45H, cluster of s, 15 OAc), 3.75—4.90 (25H, m, H-2a—e—6a—e), 5.15—5.50 (10H, m, H-1a—e, 3a—e), 7.28 (1H, d, J = 9.1 Hz, H-6 of CNP and Ph), 7.71—7.89 (4H, m, Ph of phthalimido), 8.16 (1H, dd, J = 9.1, 2.7 Hz, H-5 of CNP), 8.29 (1H, d, J = 2.7 Hz, H-3 of CNP). t_R (column: [A], eluent: CH_3CN–H_2O, 3 : 1, v/v): 5.5 min.

2-Chloro-4-nitrophenyl *O*-[6-Deoxy-6-(phenyl)sulfonyl-α-D-glucopyranosyl]-(1→4)-tris[*O*-(α-D-glucopyranosyl)-(1→4)]-β-D-glucopyranoside (10) *O*-De-acetylation of **8** (680 mg, 0.394 mmol) with K_2CO_3 as described for **4** gave pale yellow amorphous **10** (323 mg, 0.292 mmol, yield 74.1%). $[\alpha]_D$ +70.8° (c = 0.500, MeOH). *Anal.* Calcd for $C_{42}H_{58}ClNO_{29} \cdot H_2O$: C, 44.78; H, 5.37; N, 1.24. Found: C, 44.45; H, 5.40; N, 1.07. IR ν_{max} cm^{-1}: 3400 (OH), 2930 (CH, aliph.), 1586, 1488 (arom.), 1520, 1352 (NO_2), 1446, 1352, 1276, 1150, 1040, 1024 (C–O). ^{1}H-NMR (DMSO-d_6–D_2O, 10 : 1, v/v) δ: 2.91—4.00 (30H, m, H-2a—e—6a—e), 5.05 (3H, br s, H-1), 5.08 (1H, d, J = 3.7 Hz, H-1), 5.26 (1H, d, J = 7.8 Hz, H-1a), 7.46 (1H, d, J = 9.3 Hz, H-6 of CNP), 7.60—7.73 (3H, m, H-3—5 of SO_2Ph), 7.91 (2H, br d, J = 7.6 Hz, H-2 and H-6 of SO_2Ph), 8.19 (1H, dd, J = 9.3, 2.7 Hz, H-5 of CNP), 8.31 (1H, d, J = 2.7 Hz, H-3 of CNP). ^{13}C-NMR (DMSO-d_6–D_2O, 10 : 1, v/v) δ: 57.7 ($\underline{C}H_2SO_2Ph$), 127.5, 129.3, 133.7, 140.5 (SO_2Ph). t_R (column: [B], eluent: CH_3CN–H_2O, 3 : 1, v/v): 5.9 min.

2-Chloro-4-nitrophenyl *O*-(6-Deoxy-6-phthalimido-α-D-glucopyranosyl)-(1→4)-tris[*O*-(α-D-glucopyranosyl)-(1→4)]-β-D-glucopyranoside (11) *O*-De-acetylation of **9** (499 mg, 0.286 mmol) with K_2CO_3 as described for **4**, gave pale yellow amorphous **11** (206 mg, 0.185 mmol, yield 64.7%). $[\alpha]_D$ +79.9° (c = 0.502, MeOH). *Anal.* Calcd for $C_{44}H_{57}ClN_2O_{29} \cdot 5/2H_2O$: C,

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45.62; H, 5.39; N, 2.42. Found: C, 45.41; H, 5.19; N, 2.31. IR ν_{max} cm^{-1}: 3400 (OH), 2920 (CH, aliph.), 1772, 1706 (C=O), 1632, 1584 (arom.), 1520, 1348 (NO_2), 1484, 1428, 1276, 1250, 1150, 1030 (C–O). ^{1}H-NMR (DMSO-d_6–D_2O, 10:1, v/v) δ: 2.95—4.05 (30H, m, H-2a—e—6a—e), 4.87 (1H, d, J=4.8 Hz, H-1), 4.92 (1H, d, J=5.2 Hz, H-1), 5.02 (1H, d, J=3.8 Hz, H-1), 5.10 (1H, d, J=3.7 Hz, H-1), 5.28 (1H, d, J=7.6 Hz, H-1a), 7.47 (1H, d, J=9.3 Hz, H-6 of CNP), 7.70—8.00 (4H, m, Ph of phthalimido), 8.19 (1H, dd, J=9.3, 2.7 Hz, H-5 of CNP), 8.31 (1H, d, J=2.7 Hz, H-3 of CNP). ^{13}C-NMR (DMSO-d_6–D_2O, 10:1, v/v) δ: 59.9 ($\underline{C}H_2$-phthalimido), 123.3, 131.8, 134.7, 157.9 (Ph of phthalimido), 179.8 (C=O). t_R (column: [B], eluent: CH_3CN–H_2O, 3:1, v/v): 6.2 min.

Michaelis Constants (K_m) A solution of coupled enzymes (110 U/ml α-glucosidase and 13 U/ml β-glucosidase, 1.0 ml) in 50 mM phosphate buffer (pH 7.0, containing 40 mM NaCl and 2.0 mM $MgCl_2$) was added to a solution of HPA or HSA in H_2O (150 U/ml, 0.25 ml), and the enzymatic solution was incubated at 37 °C for 1 min. Then, a solution of **4**, **5**, **10**, or **11** (2.0 ml) in the same buffer was added to the enzymatic solution and the mixture was incubated. After 2 min, the reaction was monitored by the increase in absorbance at 400 nm for 2 min. For the blank, H_2O was added instead of the α-amylase solution. The K_m values of hydrolysis of the substrates were calculated by the method of least squares with the use of Lineweaver–Burk plot as shown in Table I.

Patterns of the Action A solution of **4**, **5**, **10**, or **11** (each 1.5 mM, 2.0 ml) in the same buffer was added to a solution of HPA or HSA in H_2O (150 U/ml, 0.25 ml), and the mixture was incubated at 37 °C for 15 min. Then 0.1 ml of the mixture was added to 0.9 ml of CH_3CN to stop the action. The sample (5 μl) was analyzed by HPLC. Patterns of the action of the substrates were summarized in Table II.

Relative Rate of Hydrolysis A solution of coupled enzymes (1.0 ml) described above was added to a solution of HPA or HSA in H_2O (0.25 ml), and the enzymatic solution was incubated at 37 °C for 1 min. Then a solution (2.0 ml) of **4** (0.45 mM), **5** (0.50 mM), **10** (1.05 mM), **11** (1.85 mM), or **12** (2.0 mM) in the buffer described above was added to the enzymatic solution and the mixture was incubated. After 2 min, the reaction was monitored by the increase in absorbance at 400 nm for 2 min. For the blank, H_2O was added instead of the α-amylase solution. Relative rate of hydrolysis were shown in Table I.

Differential Assay of HPA and HSA Using Eqs. 3 and 4 A solution of coupled enzymes described above was added to a sample solution (0.25 ml) containing known amounts of HPA and/or HSA in H_2O (total activity; 138 U/l, activity of HPA/HSA; 0/10—10/0, 11 samples) or a sample from human serum (50 persons), and the enzymatic solution was incubated at 37 °C for 1 min. Then, using a substrate solution (2.0 ml) of **5** (0.50 mM) or **12** (2.0 mM), the increase in absorbance at 400 nm was determined by the above method. From the obtained amounts of A_1 and A_2, values of a_p and a_s were calculated using Eqs. 3, 4, and k. These results are summarized in Figs. 2—4.

Differential Assay of HPA by the Method (Enzyme Immunoassay Method) Using Monoclonal Antibodies of HSA The assay was carried out followed by directions for use of the enzyme immunoassay-kit. These results are summarized in Fig. 4.

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References

1) T. Fujisawa, M. Otsuka, and S. Baba, *J. Jpn. Soc. Starch Sci.*, **35**, 49 (1988).
2) T. Takeuchi, T. Matsushima, T. Sugimura, T. Kozu, and T. Takemoto, *Clin. Chim. Acta*, **54**, 137 (1974); M. Otsuki, S. Saeki, H. Yuu, M. Maeda, and S. Baba, *Clin. Chem.*, **22**, 439 (1976).
3) T. Takeuchi, T. Matsushima, and T. Sugimura, *Clin. Chim. Acta*, **60**, 207 (1975).
4) L. Fridhandler, J. E. Berk, K. A. Montgomery, and D. Wong, *Clin. Chem.*, **20**, 547 (1974).
5) M. D. O'Donnell, O. FitzGerald, and K. F. McGeeny, *Clin. Chim. Acta*, **23**, 560 (1977); N. Matsuda, J. Makise, E. Saitoh, Y. Tanahashi, and M. Kanayama, *Rinsho Kensa*, **33**, 107 (1989).
6) T. E. Miffin, D. C. Benjamin, and D. E. Bruns, *Clin. Chem.*, **31**, 1283 (1985); M. Gerber, K. Najoks, H. Lenz, W. Gerhardt, and K. Wulff, *ibid.*, **31**, 1331 (1985); N. Minamiura and K. Ito, *J. Jpn. Soc. Starch Sci.*, **35**, 159 (1988).
7) K. Omichi and T. Ikenaka, *Clin. Chim. Acta*, **138**, 197 (1984).
8) K. Omichi and T. Ikenaka, *J. Biochem.*, **100**, 1353 (1986).
9) K. Ogawa, H. Nagai, H. Matsui, and T. Usui, Abstracts of Papers, 13th Japanese Carbohydrate Symposium, Higashi-Osaka, July 1991, pp. 67—68.
10) S. Tokutake, N. Yamaji, and M. Kato, *Chem. Pharm. Bull.*, **38**, 13 (1990); S. Tokutake, K. Kasai, T. Tomikura, N. Yamaji, and M. Kato, *ibid.*, **38**, 3466 (1990).
11) S. Tokutake, R. Uchida, K. Kotani, K. Saito, and N. Yamaji, *Carbohydr. Res.*, accepted.
12) S. Tokutake, T. Oguma, K. Tobe, K. Kotani, K. Saito, and N. Yamaji, *Carbohydr. Res.*, accepted; S.Tokutake, N. Yamaji, K. Saito, and K. Kotani, Abstracts of Papers, 111th Nippon Yakugakukai, Tokyo, March 1991, p. 109.
13) K. Ishikawa and H. Hirata, *Arch. Biochem. Biophys.*, **272**, 356 (1989).
14) K. Omichi, K. Shiosaki, K. Matsuba, and T. Ikenaka, *J. Biochem.*, **106**, 646 (1989).
15) K. Tobe and A. Maki, Japan Kokai Patent 78994 (1985) [*Chem. Abstr.*, **104**, 225181*a* (1986)].
16) J. Ohkawa and N. Handa, *Medical Technology*, **15**, 333 (1987).
17) H. Katsuta and K. Yoshii, *Rinsyo-kensa Kiki Shiyaku*, **8**, 161 (1985).

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