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Chemo-Enzymatic Synthesis of Galactosylmaltooligosaccharidonolactone as a Substrate Analogue Inhibitor for Mammalian α-Amylase[1]

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We performed chemo-enzymatic transformation of maltooligosaccharides into both end-modified oligosaccharidonolactones of potential use as substrate analogue inhibitors for mammalian α-amylases. Enzymatic modification of the non-reducing end glucosyl residue of the maltooligosaccharide was first performed by transglycosylation with β-D-galactosidase from *Bacillus circulans*. When maltotriose and maltotetraose were the acceptors, the enzyme regioselectively synthesized 4^3-*O*-β-D-galactosyl maltotriose (LG3) and 4^4-*O*-β-D-galactosyl maltotetraose (LG4) from lactose as a donor. LG4 was further selectively hydrolyzed with a specific α-amylase to afford 4^2-*O*-β-D-galactosyl maltose (LG2). The anomer hydroxyl groups of LG2 and LG3 were chemically oxidized to give the corresponding lactones, 4^2-*O*-β-D-galactosyl maltobionolactone (LG2O) and 4^3-*O*-β-D-galactosyl maltotrionolactone (LG3O), respectively. LG2O and LG3O, which are competitive inhibitors for mammalian α-amylases, exhibited K_i values of the order of 2.8-18.0 μM, with *p*-nitrophenyl α-maltopentaoside (G5P) as the substrate. On ^{1}H-NMR analysis, these oligosaccharidonolactones were shown to be transformed into the corresponding aldonic acid forms with time in an aqueous solution. In this case, the lactone form was essential for the occurrence of the α-amylase inhibitor.

Key words: α-amylase inhibitor, β-D-galactosidase, galactosyl maltooligosaccharide, oligosaccharidonolactone, transglycosylation.

Various models (*1-4*) have been proposed for the reaction mechanisms of glycosidases in the transition-state, but a reasonable model remains to be established. The oxocarbonium ion model has been applied to interpret the reaction mechanisms of many glycosidases, including lysozyme (*5, 6*), glucoamylase (*7*), and glucosidase (*8*). In these mechanisms, when a glycosidic bond is cleaved, a glycosyl oxocarbonium ion intermediate is formed for catalysis. Evidence of the role of the transition-state intermediates came from inhibition studies involving lactones as transition-state analogues, because of their structural similarity (*9*). Based on this hypothesis, many researchers have performed inhibition studies on glycosidases with aldonolactones (*10-16*), which were derived from the corresponding sugars. Maltooligosaccharidonolactone and its analogue are also expected to exhibit α-amylase inhibition activity (*17, 18*). However, little information is available about the effect of modification at the nonreducing end of maltooligosaccharidonolactone on amylase activity.

On the other hand, we have already reported that the both end-blocked maltooligosaccharides, *p*-nitrophenyl 4^5-*O*-β-D-galactosyl-α-maltopentaoside (LG5P) and *p*-nitrophenyl 3^5-*O*-β-*N*-acetylglucosaminyl-α-maltopentaoside, are very suitable as novel substrates for the analytical use of human α-amylase in serum through a conjugated reaction involving glucoamylase and α-D-glucosidase (*19-21*). From such an aspect, both end-modified maltooligosaccharide derivatives, which would be expected to exhibit tolerance as to digestion with the digestive enzymes, α-glucosidase and glucoamylase, were designed as substrate analogue inhibitors for mammalian α-amylases. Systematic trends in inhibition studies on the structural modification of substrates would be helpful in revealing with the requirements for binding and catalytic specificity.

The present paper describes a preparative synthetic method involving the chemo-enzymatic transformation of maltooligosaccharides into β-D-galactosyl maltooligonolactones, and the latters' effectiveness as substrate analogue inhibitors for mammalian α-amylases.

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Abbreviations: LG2O, 4^2-*O*-β-D-galactosyl maltobionolactone; LG2, 4^2-*O*-β-D-galactosyl maltose; LG3O, 4^3-*O*-β-D-galactosyl maltotrionolactone; LG3, 4^3-*O*-β-D-galactosyl maltotriose; G2O, maltobionolactone; G3O, maltotrionolactone; G4O, maltotetraonolactone; G5P, *p*-nitrophenyl α-maltopentaoside; LG5P, *p*-nitrophenyl α-4^5-*O*-β-D-galactosyl maltopentaoside; HAS, human salivary α-amylase; PPA, porcine pancreatic α-amylase; HPA, human pancreatic α-amylase.

MATERIALS AND METHODS

Materials—Crude β-D-galactosidase (5,420 IU/g, *Bacillus circulans*) was obtained from Daiwa Kasei (Tokyo) and used without further purification. A saccharogenic α-amylase of *Thermononospora viridis* (2,000 U/ml, TVA) was purified according to the method of Muramatsu *et al.* (*22*). Glucoamylase of *Rhizopus* sp. (44 U/mg, GA-AMANO) was obtained from Amano Seiyaku (Tokyo). A series of *p*-nitrophenyl α-maltooligosaccharides was purchased from Calbiochem-Boehring (USA). Maltose, maltotriose and maltotetraose were products of Nihon Shokuhin Kako (Tokyo). Human salivary α-amylase (HSA) and porcine pancreatic α-amylase (PPA) were obtained from Sigma Chemical (USA), and purified further by the methods of Mayo and Carlson (*23*) and Schramn and Loyter (*24*), respectively. Isozyme II (PPA-II) was isolated by the method of Sakano *et al.* (*25*). Human pancreatic α-amylase (HPA), which was purified by the method of Matsuura *et al.* (*26*), was a generous gift from Dr. Watazu of Kokusai Siyaku (Tokyo). Other chemicals were of the highest quality available commercially.

Analytical Methods—HPLC analysis for sugars was performed on a Shodex column DC-613 (6.0×150 mm) or Asahipak column GS-220HQ (7.5×300 mm) in a Hitachi 6000-series liquid chromatograph equipped with a Hitachi L-4000 ultraviolet detector and an L3350 RI monitor. Elution of the former column was performed with 30:70 (v/v) H_2O-acetonitrile at room temperature, and that of the latter with distilled water at 65°C. The flow rate in both cases was 0.9 ml/min. Analysis of oxidized sugars was also performed under the following conditions: Shodex DC-613 column (6.0×150 mm): mobile phase, 30:70 (v/v) H_2O-acetonitrile containing 0.1 M $NaNO_3$; flow rate, 0.8 ml/min; temperature, 30°C; and detection, RI. Before injection, 0.2 ml of the reaction digest containing about 2 mg of a lactone was mixed thoroughly with 0.2 ml of a 0.2 M $NaNO_3$ solution, and then the mixture was injected into the apparatus immediately.

^{13}C and ^{1}H NMR spectra were obtained with a JEOL JNM-EX 270 spectrometer operating at 68 MHz in the pulsed Fourier-transform mode with complete proton decoupling, and at 270 MHz, respectively. Chemical shifts are expressed in ppm relative to sodium 3-(trimethylsilyl) propanesulfonate sodium salt (TPS) as an internal standard. NMR signal assignments were made by carbon-proton shift correlation spectroscopy (CH-COSY). FABMS analysis was carried out in the positive- and negative-ion modes using a JEOL DX-303 HF mass spectrometer, coupled to a JEOL DA-500 data system operating at the full accelerating potential (3 kV). The molecular weight of a sample was estimated from the *m/z* value of the quasi-molecular-ion $[M+1]^+$ peak. Specific rotation was determined with a Digital Automatic Polarimeter PM-101 apparatus (Union Giken).

Enzyme Activity Assays—Beta-D-galactosidase activity was assayed as follows. A mixture comprising 2 mM *o*-nitrophenyl β-D-galactopyranoside in 0.9 ml of 50 mM Na_3PO_4 buffer (pH 6.0) and an appropriate amount of the enzyme, in a total volume of 0.1 ml, was incubated for 10 min at 30°C. The reaction was stopped by adding 0.1 M Na_2CO_3 (2 ml), and then the liberated *o*-nitrophenol was determined spectrophotometrically at 420 nm. One unit of activity was defined as the amount of the enzyme releasing 1 μmol of *o*-nitrophenol per min.

Glucoamylase activity was determined as follows (*27*). A substrate solution (1 ml) comprising 0.5% soluble starch in 50 mM phosphate buffer (pH 6.0) and an appropriate amount of the enzyme solution (0.1 ml) was incubated at 37°C for 15 min. The reaction was stopped by heating at 95°C for 3 min and then cooling down to room temperature. An aminoantipyrine-peroxidase solution (3 ml) and 0.1 ml of the reaction solution were mixed well and kept at 37°C for 15 min, and then the absorbance at 505 nm of the solution was measured. The aminoantipyrine-peroxidase solution was prepared as follows. Three thousand two handred units of peroxidase (Amano Pharmaceutial) and 3,200 units of glucose oxidase (Amano Pharmaceutial) were diluted with 50 ml of 0.1 M phosphate buffer (pH 7.0), and then 3.2 ml of the 4-aminoantipyrine solution (5 mg/ml), 2 ml of a 5% phenol solution and 0.4 ml of a 5% Triton X-100 solution were added. Then the solution was made up to 200 ml with 0.1 M phosphate buffer. One unit is defined as the amount of enzyme that produced 1 μmol of glucose per min. TVA activity was determined according to the method of Muramatsu *et al.* (*22*).

Assaying of Inhibition Activity—The inhibition of α-amylase activity by some oligonolactones was determined under the following conditions. The reaction mixture (1.0 ml) comprising 0.25 mM or 0.10 mM G5P, 20 mM PIPES buffer (pH 6.9), 40 mM NaCl, and 5 mM $CaCl_2 \cdot 2H_2O$ was incubated at 37°C for 7 min preliminarily. The reaction was performed with about 0.08 U of α-amylase in the presence of 0-40 μM various lactones. Samples (200 μl) were taken at intervals (0, 1, 2, 3, and 5 min) and inactivated by adding 400 μl of 1 N acetic acid for analysis by HPLC. The amounts of *p*-nitrophenyl α-maltooligosaccharides (G*n*P: *n*=1-4) formed from the initial substrate during the reaction were determined. One unit of α-amylase activity was defined as the amount of enzyme that hydrolyzed 1 μmol of G5P per min. The K_m values (mM) with G5P were 0.25 for HSA, 0.29 for HPA, and 0.14 for PPA, respectively (*28*). The inhibition constants (K_i) for various oxidized sugars were calculated from Dixon and Webb plots (*29*), and the mode of inhibition was reconfirmed by the method of Lineweaver-Burk (*30*).

Preparation of LG2O—(a) Crude 4⁴-O-β-D-galactosyl maltotetraose (LG4): Lactose (3.5 g) and maltotetraose (8.5 g) were dissolved in 20 ml of 50 mM phosphate buffer (pH 7.0), and then β-D-galactosidase (35 U) from *B. circulans* was added. The mixture was incubated for 5 h at 40°C. The pH of the reaction mixture was adjusted to 3.0 with 4% HCl and then the reaction was terminated by heating for 10 min. Then 300 ml of H_2O was added to the reaction mixture and the diluted solution was adjusted to pH 5.0 with 1 N NaOH. The solution was incubated with glucoamylase (210 U) from *Rhizopus* sp. at 50°C for 20 h in order to remove unreacted maltotetraose. After termination of the reaction by heating at 95°C for 10 min, a 1/4 volume of the reaction mixture was loaded onto a column (φ2.6×90 cm) of Toyopearl HW40S. Elution was monitored by measurement at 485 nm (carbohydrate contents, determined by the phenol-sulfuric acid method). The eluate (10 ml fractions) gave one main peak (F-I: tubes 21-24) as a transglycosylation product. The remaining aliquots

were similarly worked up as above. The fractions corresponding to F-I were combined, concentrated and lyophilized to afford crude LG4 (1.2 g). The crude LG4 was contaminated by about 10% of its isomer, 6^4-*O*-β-D-galactosyl maltotetraose (LG4-i), but was used for the next step without further purification.

(b) 4^2-O-β-D-Galactosyl maltose (LG2): Crude LG4 (900 mg) dissolved in 18 ml of 80 mM 3,3-dimethylglutaric acid (pH 6.9) containing 40 mM NaCl and 5 mM $CaCl_2 \cdot 2H_2O$ was incubated with TVA (6.8 U) for 9 h at 40°C. After termination of the reaction by heating for 10 min, the insoluble material was removed by centrifugation and the supernatant solution was loaded onto a column (ϕ4.0×98 cm) of charcoal-Celite (1:1 by weight). The column was first washed with water (1.5 liters) and then eluted with a linear gradient of 0 (3 liters)-40% (3 liters) ethanol. Elution was monitored by measurement at 485 nm (carbohydrate contents, determined by the phenol-sulfuric acid method). The eluate (20 ml fractions) gave one main peak (F-I: tubes 21-40) was collected and lyophilized to afford LG2 (390 mg).

F-I: $[\alpha]_D^{25}+103.1° \rightarrow +100.7°$ (c1, H_2O) and *m/z* 505 $[M+H]^+$. NMR data (D_2O): ^1H, δ5.24 (1H, d, J=3.62 Hz, H-1α), 4.66 (1H, d, J=7.92 Hz, H-1β), 5.43 (1H, d, J=3.96 Hz, H-1′), 4.47 (1H, d, J=7.58 Hz, H-1″). ^{13}C-NMR data (D_2O): 105.78 (C-1″), 102.27 (C-1′α), 102.18 (C-1′β), 98.70 (C-1β), 94.82 (C-1α), 81.02 (C-4′), 79.87 (C-4α), 79.67 (C-4β), 79.11 (C-3β), 78.28 (C-5″), 77.44 (C-5β), 76.94 (C-2β), 76.11 (C-3′), 75.48 (C-3α, C-3″), 74.30 (C-5′), 74.22 (C-2′), 74.12 (C-2α), 73.90 (C-2″), 72.82 (C-5α), 71.49 (C-4″), 63.96 (C-6″), 63.59 (C-6β), 63.46 (C-6α), 62.76 (C-6′).

(c) 4^2-O-β-D-Galactosyl maltobionolactone (LG2O): LG2 was oxidized according to the method of Kobayashi *et al.* (*31, 32*). LG2 (200 mg, 0.4 mmol) was dissolved in water (4.2 ml) and then added to an iodine (274 mg) solution in methanol (5.26 ml) at 40°C. At this temperature, a 4% w/v potassium hydroxide solution in methanol was added dropwise with magnetic stirring for 50 min until the color of the iodine disappeared. The solution was cooled externally in an ice bath. The precipitated crystalline product was collected by centrifugation, and the product was washed with cold methanol and recrystallized from a mixture of methanol and water (5:1 v/v). The resulting potassium lactonate was then converted into the free acid by passing the aqueous solution through a column of Amberlite IR-120B(H^+) (ϕ3.5×10 cm). The acidic eluate was collected, concentrated and lyophilized to afford compound LG2O (89 mg) as a white powder.

LG2O: $[\alpha]_D^{25}+85.4° \rightarrow +82.1°$(c1, H_2O), *m/z* 503 and *m/z* 521 $[M+H]^+$ attributed to the lactone and its aldonic acid form, respectively. NMR data (D_2O): ^1H, δ5.36 (1H, d, J=3.63 Hz, H-1′), 4.43 (1H, d, J=7.92 Hz, H-1″): anomeric protons attributed to the lactone form. δ5.15 (1H, d, J=3.30 Hz, H-1′), 4.45 (1H, d, J=7.92 Hz, H-1″): anomeric protons attributed to the aldonic acid form. The ^{13}C-NMR data are shown in Table I.

Preparation of LG3O—(a) 4^3-O-β-D-Galactosyl maltotriose (LG3): The preparation of LG3 was performed by the following procedure. The reaction mixture (30.5 ml)

TABLE I. **Chemical shifts of LG2O and LG3O in D_2O.**

Gal β1→4 Glc α1→4 Lactone (III, II, I) ⇄ Gal β1→4 Glc α1→4 GluA (III, II, I)

		C-1	C-2	C-3	C-4	C-5	C-6
I	Lactone	176.26	83.58	73.94	78.29	72.87	63.22
	GluA	178.45	74.91	74.72	84.47	74.32	64.78
II	Lactone	102.44	73.87	75.35	80.83	74.14	62.50
	GluA	103.06	73.87	75.45	80.83	74.14	62.50
III		105.81	73.76	75.35	71.36	78.15	63.84

Gal β1→4 Glc α1→4 Glc α1→4 Lactone (IV, III, II, I) ⇄ Gal β1→4 Glc α1→4 Glc α1→4 GluA (IV, III, II, I)

		C-1	C-2	C-3	C-4	C-5	C-6
I	Lactone	176.26	83.61	73.94	78.35	72.83	63.23
	GluA	178.41	74.91	74.72	84.38	74.25	64.78
II	Lactone	102.57	73.76	75.42	79.41	74.25	63.05
	GluA	102.30	73.76	75.42	79.41	74.25	63.05
III		103.00	73.76	76.12	80.81	74.11	62.61
IV		105.62	73.76	75.33	71.36	78.15	63.84

comprising 3.6 g of lactose and 10.0 g of maltotriose in 50 mM K phosphate buffer (pH 7.0) was incubated with 0.86 U of β-D-galactosidase for 5 h at 40°C. After termination of the reaction, the resulting insoluble material was centrifuged off. A 1/2 volume of the supernatant was loaded onto the same column of charcoal-Celite. The column was eluted with a linear gradient of 0 (5 liters)-50% (5 liters) ethanol. The eluate (60 ml fractions) gave a sharp peak (tubes 68-82) at about 24% of ethanol as a transglycosylation product. The remaining aliquots were similarly worked up to as above. The fractions corresponding to the main peak were combined, concentrated and lyophilized to afford LG3 (1.32 g).

LG3: $[\alpha]_D^{25}+126.0° \rightarrow +124.1°$(c1, H_2O) and *m/z* 667 $[M+H]^+$. NMR data (D_2O): ^1H, δ5.21 (1H, d, $J=3.63$ Hz, H-1α), 4.64 (1H, d, $J=8.24$ Hz, H-1β), 5.36 (1H, d, $J=3.63$ Hz, H-1′, H-1″), 4.46 (1H, d, $J=7.59$ Hz, H-1‴). ^{13}C-NMR data (D_2O): 105.64 (C-1‴), 102.25 (C-1″, C-1), 98.56 (C-1β), 94.68 (C-1α), 80.83 (C-4″), 79.78 (C-4′), 79.59 (C-4β), 79.51 (C-4α), 78.98 (C-3β), 78.17 (C-5‴), 77.32 (C-5β), 76.99 (C-3″β), 76.78 (C-2β), 76.10 (C-3′α), 75.99 (C-3″α), 75.35 (C-3‴, C-3α), 74.27 (C-2α, C-5′, C-5″), 74.12 (C-2′), 73.91 (C-2″), 73.76 (C-2‴), 72.70 (C-5α), 71.36 (C-4‴), 63.84 (C-6‴), 63.45 (C-6β), 63.32 (C-6α), 63.18 (C-6′), 62.61 (C-6″).

(b) 4³-O-β-D-Galactosyl maltotrionolactone (LG3O): LG3 (200 mg) was similarly oxidized to LG3O, in a yield of 123 mg, as a lyophilized white powder.

LG3O: $[\alpha]_D^{25}+118.5° \rightarrow +154.5°$ (c1, H_2O), *m/z* 665 and *m/z* 683$[M+H]^+$ attributed to the lactone and its aldonic acid form, respectively. NMR data (D_2O): ^1H, δ5.39 (1H, d, $J=3.63$ Hz, H-1′, H-1″), 4.43 (1H, d, $J=7.59$ Hz, H-1‴): anomeric protons attributed to the lactone form. δ5.17 (1H, d, $J=3.96$ Hz, H-1′): anomeric protons attributed to the aldonic acid form. The ^{13}C-NMR data are shown in Table I.

Maltose, maltotriose and maltotetraose were similarly oxidized to maltobionolactone (G2O), maltotrionolactone (G3O), and maltotetraonolactone (G4O), in yields of 42.1, 90.3, and 87.9% based on those of the corresponding maltooligosaccharides, respectively.

RESULTS AND DISCUSSION

Chemo-Enzymatic Synthesis of LG2O and LG3O—The present paper describes the chemo-enzymatic synthesis of both endo-modified derivatives LG2O and LG3O as substrate analogue inhibitors of mammalian α-amylases. We have already reported the regioselective synthesis of LG5P, through *B. circulans* β-D-galactosidase-mediated transglycosylation from lactose to G5P (*19-21*). Following the developed methodology, galactosyl maltooligosaccharides LG4 and LG3 were firstly prepared by enzymatic modification of the nonreducing end glucosyl residues of maltotetraose and maltotriose, respectively, using the β-D-galactosidase. In the process of the chromatographic separation of LG4, the fraction containing the desired compound was contaminated by about 10% of its β-(1,6) linked isomer, LG4-i, but was used for the preparation of LG2 without further purification. Because specific amylase TVA selectively hydrolyzed LG4 to LG2 but did not hydrolyze LG4-i, smooth chromatographic separation of LG2 from the unreacted LG4-i resulted. LG3 was obtained in a yield of 13.2% based on the acceptor added. LG2 and LG3 were then oxidized with potassium hypoiodate in water at 40°C, and the resulting oligosaccharidosyl aldonate potassium salts were neutralized by passage through an Amberlite IR-120 (H^+) column to obtain LG2O and LG3O in yields of 44.5 and 61.5%, respectively.

Characterization of LG2O and LG3O—The structures of LG2O and LG3O were elucidated from their ^1H and ^{13}C-NMR spectra. NMR signal assignments were made by carbon proton shift correlation spectroscopy (CH-COSY). In an aqueous solution a lactone is known to be readily hydrolyzed to its aldonic acid form. In the present study, the oxidized product, LG2O, existed in both forms, *i.e.* the lactone and aldonic acid (4²-O-β-D-galactosyl maltobionic acid) forms, even immediately after dissolution. The ^1H-NMR spectra of LG2O with time were correlated for the glycosidic protons, as shown in Fig. 1, because the glycosidic-proton signals at lower field could be more clearly differentiated. On comparison of the chemical shifts of LG2O with those of the corresponding LG2, the peaks at δ5.36 (A), 5.15 (B), and 4.47 (C) ppm were attributed to glycosidic protons involved in H-1′ due to the lactone form, H-1′ due to the aldonic acid one and H-1″ due to a galactosyl residue, respectively. There were significant variations in their intensities with time. Immediately after dissolution in D_2O, the ratio of the relative intensities of peaks A and B was about 1.0 : 1.0, respectively. After incubation for 24 h in D_2O at 25°C, peak A was remarkably decreased and peak B increased in relative intensity. As a result, the ratio

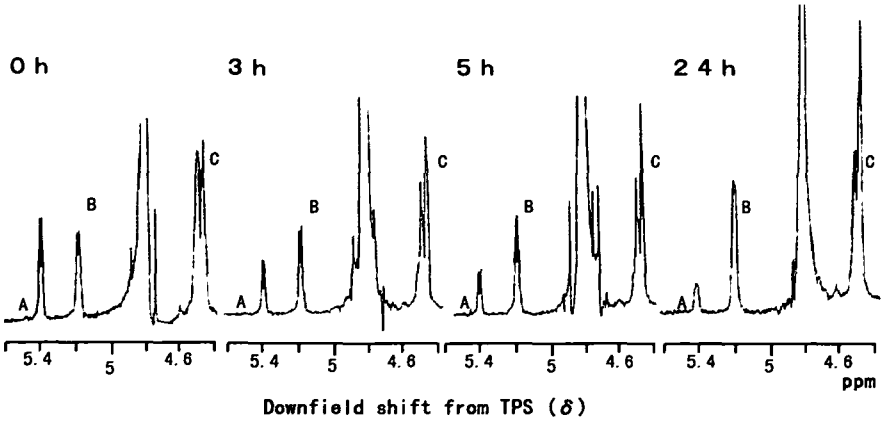

Fig. 1. **Time course of transformation of the lactone into the aldonic acid form of LG2O by ^1H-NMR.** LG2O was analyzed in D_2O (0.85% w/v) at 25°C. The proportions of the lactone and aldonic acid forms of LG2O were determined from the relative intensities of peaks A and B, respectively.

of the intensity of peak A to that of peak B changed to 1.0 : 4.4. This indicates that the lactone form was gradually hydrolyzed to its open-chain form. Such a conversion of the lactone form into the aldonic acid one was directly observed by this spectroscopic method. The ^{13}C NMR spectrum of LG2O immediately after dissolution (A) and that after 24 h (B) also provide useful information on their compositions, as shown in Fig. 2. All of the different carbon lines were resolved by comparison with the earlier data (*19-21*) and their chemical shifts are recorded in Table I. Twelve of the major signals of the α-D-lactosyl unit correspond well with the reported spectra of α-D-lactosyl oligosaccharides (*19-21*). On comparison of the relative intensities of spectra A and B, the peaks at δ176.26, 102.44, 83.58, 72.87, and 63.22 were observed to decrease with time. Such signals should be attributed to the resonances involved in the lactone residue. Confidence in the assignments of these signals is provided by the fact that they exhibit close similarity with those reported for glucono-δ-lactone (*33*). HPLC was also useful for determining the ratios of the ring and open-chain forms of oxidized compounds in an aqueous solution. The separation patterns of the lactone (L) and aldonic acid (A) forms of LG2O in D_2O with time at 25°C were investigated on a Shodex DC-613 column (data not shown). Thus, peak L was greater than peak A in the initial stage, but this relation was reversed after 24 h. A sequence of structural changes similar to these were also observed on the NMR analysis mentioned above, although the analytical

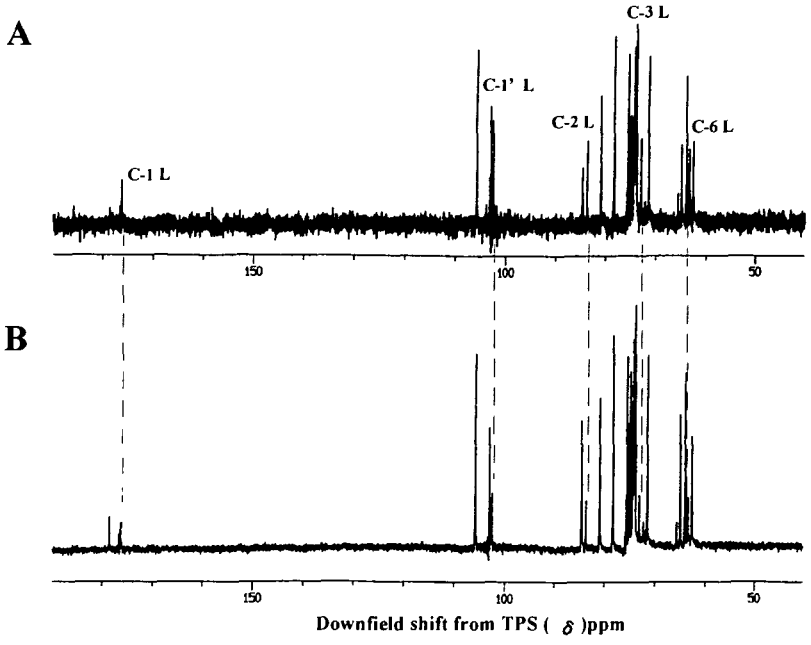

Fig. 2. **^{13}C-NMR spectra of LG2O in D_2O.** LG2O was analyzed in D_2O (2.54% w/v) at 25°C. (A) Immediately after dissolution, (B) after 24 h.

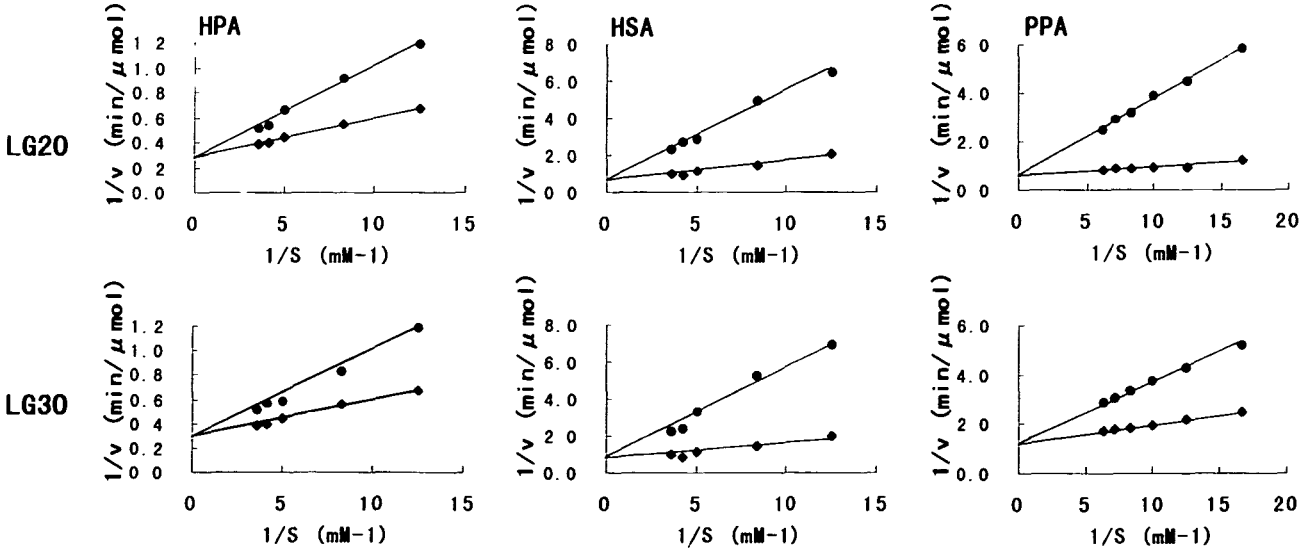

Fig. 3. **Lineweaver-Burk plots of inhibition of α-amylases by LG2O and LG3O.** The inhibition assay was described under "MATERIALS AND METHODS." ●, with an inhibitor ([I] = 0.025 mM); ◆, no inhibitor.

conditions were entirely different from each other. The same phenomenon was also observed with LG3O.

Inhibition Analysis—The novel synthetic LG2O and LG3O were designed as inhibitors of mammalian α-amylase catalyzed hydrolysis. Both the compounds take the corresponding lactone and aldonic acid forms in an aqueous solution, as mentioned above. Therefore, the conversion ratios of their two forms with time were determined by HPLC prior to the α-amylase inhibition study. When the inhibition reaction was followed for 5 min, the extent of the conversion corresponded to less than 5% of the initial lactone content. On the basis of the data, it was performed, assuming that the proportions of the lactone forms of LG2O and LG3O are constant in the initial stage of reaction (5 min). The lactone contents of both compounds were determined by HPLC because no inhibitory effect was detected with the corresponding aldonic acid forms (*vide infra*). Kinetic studies have been performed on the effect of inhibition on the hydrolysis of G5P, which was shown to be a suitable substrate for mammalian α-amylases (*34*). The mode of inhibition of LG2O and LG3O for HSA, HPA and PPA were shown to be competitive by the Lineweaver-Burk plots in Fig. 3. The modes of inhibition of maltobionolactone (G2O), maltotrionolactone (G3O), and maltotetraonolactone (G4O), as control samples, were also of the competitive types. Furthermore, the K_i values of LG2O and LG3O for these α-amylases were graphically determined by the method of Dixon. The two compounds were shown to be strong competitive inhibitors with K_i values of 2.8–18.0 μM, as shown in Table II. Despite the introduction of a galactosyl group at the terminal position, the K_i values of LG2O and LG3O for HSA and HPA were close to those of G3O and G4O, as control samples, respectively. In this process, LG2O and LG3O were not hydrolyzed by these amylases. Furthermore, maltotriose and maltotetraose did not show any inhibitory activity against these α-amylases up to 10^{-3} M addition. From these results, it is evident that a lactone form with a certain glucose-chain length is essential for enzyme inhibition. Accordingly, the actual K_i values of LG2O and LG3O should be as high as they appear, because they exist in the active lactone and inactive aldonic acid forms. This was further confirmed by the following experiments. The time course of the lactone content of LG2O was compared with that of the relative inhibition activity, as shown in Fig. 4. There was a distinct correlation between the lactone content and the inhibition activity with time. The LG2O inhibition activity showed a remarkable decrease (about half the initial value) within 5 h, but only a gentle decrease during the subsequent reaction. Its profile well corresponded to that of the lactone content with respect to time. This added strong support for the suggestion that the lactone form plays a essential role in the enzyme inhibition. No inhibitory activity of LG2O was observed on incubation in the same buffer at 50°C for 48 h, because it was completely converted to the aldonic acid form.

Robyt and French have reported that the active site of PPA contains five subsites for the binding of glucose units, in which the catalytic site is located between subsites 3 and 4 (subsite corresponding to the nonreducing end) (*35*). HSA and HPA have also been shown to have five binding sites for glucose residues of substrates (*36*). Taking into account these earlier data and the present inhibition kinetic results, we reasonably concluded that LG2O is bound to subsites geometrically complementary to three glucose residues, 1, 2, and 3, and LG3O to four glucose residues, 0, 1, 2, and 3, as shown in Fig. 5. Such binding is expected to result in competitive inhibition as to substrates. Thus, the lactone forms of both LG2O and LG3O are accepted because they have a more favorable conformation at subsite 3 for the induction of strong inhibition. As a result, the 4-*O*-substituted galactosyl group of LG2O interacts with subsite 1 at the terminal of the five binding subsites and mimics a

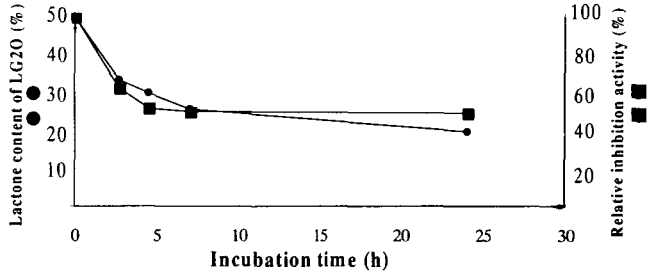

Fig. 4. **Time course relationship between the lactone content of LG2O and its inhibitory activity toward HSA.** The remaining lactone content of LG2O was analyzed by HPLC. The inhibition activity was conveniently determined as the amount of LG2O required to give 50% inhibition (IC_{50}) toward HSA by the Dixon plot method. The IC_{50} of LG2O immediately after dissolution was arbitrarily set at 100%. LG2O was dissolved in 20 mM PIPESS buffer containing 40 mM NaCl and 5 mM $CaCl_2$ (pH 6.9), and allowed to stand at 30°C. The inhibition analysis was performed with 0.1 mM G5P in the same buffer. ●, lactone content of LG2O (%); ■, relative inhibitory activity (%).

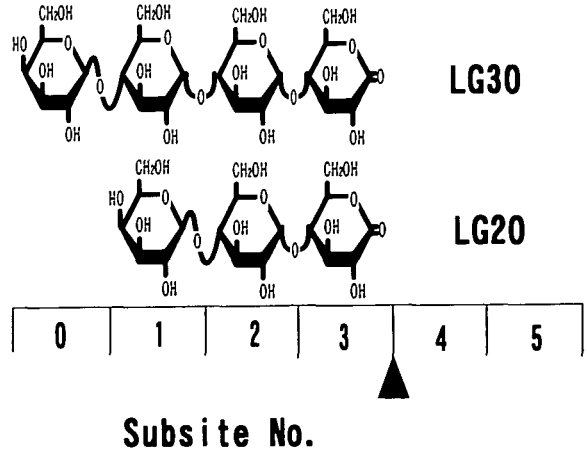

Fig. 5. **Binding models of synthetic oligonolactones on the subsites of α-amylases.** The arrow head indicates the catalytic site and each box numbered in order represents a subsite.

TABLE II. K_i (μM) values of mammalian α-amylases.

Lactone	HSA	HPA	PPA
G2O	45.4	NT[a]	135.9
G3O	5.0	16.7	0.9
G4O	2.6	9.5	0.9
LG2O	4.9	18.0	2.8
LG3O	3.4	11.4	2.9

[a]NT, not tested.

glucose residue, while that of LG3O shifts to subsite 0, because the 4-*O*-substituted galactosyl group at the non-reducing end glycosyl group does not act as a barrier for the binding with the enzymes. As a result, LG2O and LG3O act as strong competitive inhibitors, despite their unusual structures containing a lactosyl residue. As we expected, LG2O and LG3O were completely resistant to α-glucosidase and glucoamylase. The nonreducing end D-galactosyl group of both inhibitors is a blocking group against the action of such enzymes.

Two theories have been proposed as to the mechanism of glycoside hydrolysis by an enzyme like lysozyme. Phillips and his colleagues have proposed the lysozyme-subsite model, in which the glycosyl group at which cleavage occurs must first be distorted into the half chair conformation of an oxocarbonium ion intermediate in order to fit the enzyme (*5, 6*). On the other hand, Post and Karplus (*37*) indicated that the glycosyl group does not have to be distorted into the half chair conformation to fit the enzyme, because the higher-order structure of the enzyme protein is not fixed and the amino acid residues of the enzyme are able to move. The results of the present inhibition studies on LG2O and LG3O, with these α-amylases support the former theory strongly.

In conclusion, the new chemo-enzymatic process for obtaining the desired LG2O and LG3O is simple and the yields are sufficiently high to make the method practical. These both end-modified maltooligosaccharide derivatives, which exhibit tolerance to the digestive enzymes, α-glucosidase and glucoamylase, were shown to be useful as novel inhibitors of mammalian α-amylases.

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