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SYNTHESIS OF BRANCHED CYCLOMALTO-OLIGOSACCHARIDES USING *Pseudomonas* ISOAMYLASE

JUN-ICHI ABE, NAOKI MIZOWAKI, SUSUMU HIZUKURI*,
Faculty of Agriculture, Kagoshima University, 21-24, Korimoto-1, Kagoshima 890 (Japan)

KYOKO KOIZUMI, AND TOSHIKO UTAMURA
Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 4-16, Edagawa-cho, Nishinomiya 663 (Japan)

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ABSTRACT

Branched cyclomalto-oligosaccharides (cyclodextrins) were synthesised from cyclomalto-oligosaccharides and maltose or maltotriose through the reverse action of *Pseudomonas* isoamylase. The reaction rate was greater with maltotriose than with maltose, and with increasing size of the cyclomalto-oligosaccharide ($cG_6 < cG_7 < cG_8$). Maltotriose is effective as both a side-chain donor and acceptor, and three isomers of 6-*O*-α-maltotriosylmaltotriose (branched G_6) were formed through mutual condensation, but maltose was effective only as a side-chain donor. Each branched cyclomalto-oligosaccharide and G_6 was purified by liquid chromatography, and their structures were determined by chemical, enzymic, and ^{13}C-n.m.r. spectroscopic analyses.

INTRODUCTION

Branched cyclomalto-oligosaccharides (cyclomaltaoses, cyclodextrins) have been isolated[1,2] from starch syrups prepared with cyclodextrin glucosyltransferase. However, the procedures used for the isolation were tedious and gave low yields. Also, only limited varieties of branched cyclomalto-oligosaccharides (GcG_n) were obtained, because the cyclomalto-oligosaccharides were separated from large amounts of linear and branched malto-oligosaccharides after their conversion into D-glucose with glucoamylase which also shortened the long side-chains of the branched cyclomalto-oligosaccharides. We now report a convenient method for the synthesis of branched cyclomalto-oligosaccharides through the reverse action of *Pseudomonas* isoamylase[3], which is better than pullulanase[4] for the preparation of maltotriosyl cyclomalto-oligosaccharides. The properties and the debranching action of *Pseudomonas* isoamylase on polysaccharides and oligosaccharides have been investigated in detail[5–12], but the reverse action (condensation) of the enzyme

*To whom correspondence should be addressed.

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was unreported hitherto. Isoamylase shows strong debranching activity towards chains longer than maltotriose and very low activity towards a maltose branch.

EXPERIMENTAL

Materials. — Crude *Pseudomonas* isoamylase, maltose (G_2, grade HHH), and maltotriose (G_3) were kindly donated by Hayashibara Co. and the saccharides were used without further purification. Cyclomaltohexaose (cG_6), cyclomaltoheptaose (cG_7), and cyclomalto-octaose (cG_8) were gifts from Nihon Shokuhinkako Co., and cG_7 was recrystallised three times from hot water. 6-*O*-α-D-Glucosylcyclomaltoheptaose (GcG_7) was prepared as reported previously[1]. CM-Toyopearl 650M was obtained from Toyo Soda. cG_6-Coupled Sepharose 6B was prepared by the method of Sundberg and Porath[13]. Other chemicals were of reagent grade.

Purification of isoamylase. — The enzyme was purified by the published[7,14] procedure with slight modifications. The crude enzyme was dissolved in 10mM sodium acetate buffer (pH 3.5) and, after removal of insoluble materials by centrifugation, the solution was applied to a column of cross-linked amylose gel. The column was washed with the above buffer containing 0.5M sodium chloride, and the enzyme was then desorbed with the buffer containing 5% of a mixture of maltooligosaccharides. The enzyme solution was concentrated with an Amicon filter PM-10 and then applied to a column of CM-Toyopearl 650M. The column was washed with the above buffer until no carbohydrate was detected, and the enzyme was then eluted with a linear gradient of sodium chloride (0→0.3M) in the buffer. The appropriate fractions were combined and applied to a column of cG_6-coupled Sepharose 6B. The column was washed with the above buffer containing 0.5% of cG_6, and the enzyme was then eluted with the buffer containing 2% of cG_6. The active fractions were applied to the column of CM-Toyopearl 650M and the enzyme was eluted with a linear gradient of sodium chloride, as described above, after the column had been washed with the buffer. The purified enzyme was concentrated with an Amicon PM-10 filter, precipitated by the addition of solid ammonium sulfate, and stored in 0.1M sodium acetate buffer (pH 3.5) half-saturated with ammonium sulfate.

The enzyme was free from alpha-amylase and alpha-glucosidase, since an excess did not hydrolyse G_2, G_3, or cyclomalto-oligosaccharides, and did not decrease the Blue Value of potato amylose. Its specific activity was 274 U/mg at 45° (202 U/mg at 40°). Isoamylase activity was assayed by the method of Kato *et al.*[14] at 45° with waxy corn starch as the substrate. Protein was determined by the method of Lowry *et al.*[15].

Preparation and purification of the reversion products. — The products of the enzymic reaction, branched cyclomalto-oligosaccharides and maltohexaose (G_6), were separated from the reaction mixture and purified by procedures similar to those used for the purification of GcG_7. For example, the reaction mixture (80 mL), which had been incubated at 45° with G_3 (40.3 g), cG_7 (7.3 g), and isoamylase (694

U) in 50mM sodium acetate buffer (pH 3.5) for 48 h, was heated for 15 min at ~100° to inactivate the enzyme. The solution was then concentrated to 75% of its original volume and cooled to 0°, and the precipitated cG_7 was removed by centrifugation (10,000*g*, 1 h). The supernatant solution was applied to a column (6.0 × 115 cm) of Sephadex G-15 and eluted with water. The branched G_6 was found in the first small peak, which was followed by a large peak of G_3. The G_3cG_7 eluted together with the latter half of the G_3, but was well separated from cG_7, which was eluted after G_3cG_7. The fractions containing branched G_6 and G_3cG_7 were collected, concentrated, and further purified on a column (2.6 × 95 cm) of Bio-Gel P-2 by elution with water to give branched G_6 (462 mg) and G_3cG_7 (644 mg), each of which was almost homogeneous (99%) in h.p.l.c. on TSK gel NH_2-60. The other branched cyclomalto-oligosaccharides were prepared by similar procedures. These preparations were further purified by h.p.l.c. on LiChroprep RP-18 or Asahipak GS-320[2].

Analyses. — Total carbohydrate and reducing sugars were determined by the anthrone–sulfuric acid method[16] and by the modified Park–Johnson method[17], respectively. The non-reducing, terminal residue was measured by means of rapid Smith-degradation[18], but the incubation time was extended to 3 h for complete oxidation, due to the slow oxidation of cyclomalto-oligosaccharides.

H.p.l.c. was conducted at room temperature with an HLC-803D pump (Toyo Soda) and an RI-8 differential refractometer (Toyo Soda) connected to a C-R3A computer (Shimadzu Chromatopac), and a column (4.6 × 250 mm) of TSK gel NH_2-60 (Toyo Soda) with acetonitrile–water (65:35) as the eluant at 0.8 mL/min. The range of the refractometer was set at 32×10^{-6} RIU/FS. A JASCO TRI-ROTAR SR-1, with a Shodex SE-31 differential refractometer (Showa Denko), and a column of YMC AL-312 ODS eluted with water at 1 mL/min were used for the isolation and analysis of branched hexaose isomers.

Methylation was carried out by the method of Prehm[19], and the products were hydrolysed, converted into the corresponding alditol acetates, and then analysed[20] with a Hitachi 663–30 gas chromatograph fitted with a flame-ionisation detector and a column (3 mm × 2 m) of 0.3% OV-275–0.4% GE XF-1150 on Uniport HP (80–100 mesh).

^{13}C-N.m.r. spectra (50.10 MHz) were recorded at 50° on 2–3% solutions in D_2O, using a JEOL JNM-FX 200 spectrometer. The F.t.-n.m.r. conditions were as follows: spectral width, 3000 Hz; pulse flipping angle, 45°; number of data points, 16,384. The delay time Δ for the INEPT method[21] was 5.1 ms (3/4J). A micro cell was used and chemical shifts are expressed in p.p.m. downfield from the signal of Me_4Si referenced to internal 1,4-dioxane (67.40 p.p.m.).

F.a.b.-m.s. of branched cG_7s was performed in the positive-ion mode, using a JEOL JMS-DX 303 mass spectrometer with xenon atoms having a kinetic energy equivalent to 6 kV. The mass marker was calibrated with perfluoroalkyl phosphazine (ULTRA MARK), and glycerol was used as the matrix solution.

RESULTS AND DISCUSSION

H.p.l.c. on NH_2-bonded silica was useful for analyses of the products and the substrates[22] (Fig. 1). Plots of log T_{Glc} (retention time relative to that of glucose) against d.p. for G_1-, G_2-, and G_3-cG_7 were linear and parallel to that for malto-oligosaccharides G_1–G_7, but not with that for cG_6, cG_7, and cG_8 (Fig. 2). The same relationship was observed for the derivatives of cG_6 and cG_8.

When a mixture of G_3 and cG_7 was incubated with isoamylase, two products,

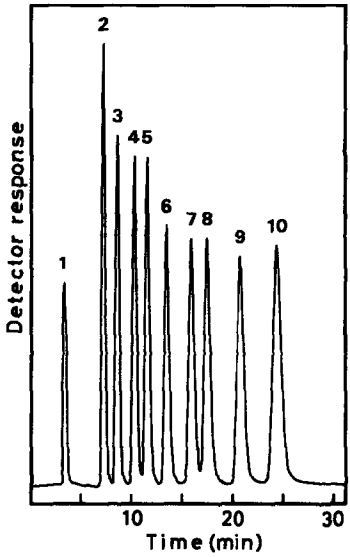


Fig. 1. H.p.l.c. on NH_2-bonded silica (see Experimental) of a mixture of D-glucose, G_2, G_3, cG_n, and branched cG_7s: 5 μL of a mixture containing 30 mg/mL of each compound was injected: 1, H_2O; 2, D-glucose; 3, G_2; 4, G_3; 5, cG_6; 6, cG_7; 7, cG_8; 8, GcG_7; 9, G_2cG_7; 10, G_3cG_7.

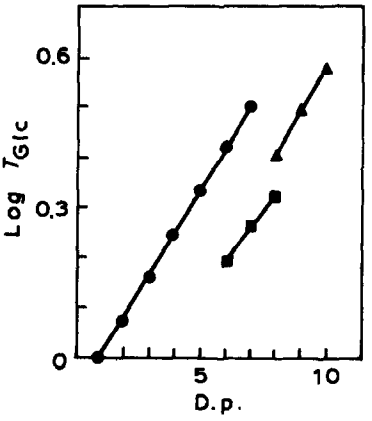


Fig. 2. Relationship between log T_{Glc} and d.p. for malto-oligosaccharides and cyclomalto-oligosaccharides in h.p.l.c.: ——●——, malto-oligosaccharides; ——■——, cG_n: ——▲——, branched cG_7s.

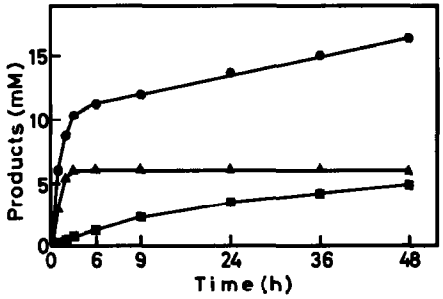


Fig. 3. Time courses of the formation of branched G_6, G_3cG_7, and G_2cG_7. A solution of 40mM cG_7, 570 mM G_2 or G_3, and 8.7 U of isoamylase in a final volume of 1 mL of 50mM sodium acetate buffer (pH 3.5) was incubated at 45°: —●—, branched G_6; —▲—, G_3cG_7; —■—, G_2cG_7.

A and *B*, which were later identified as branched G_6 and G_3cG_7, were detected on the chromatogram, whereas only one product (*C*), later identified as G_2cG_7, was found when G_2 and cG_7 were incubated together. Thus, the reaction catalysed by the isoamylase was reversible. The equilibrium constant for the condensation between G_3 and cG_7 was ~0.286 at 45°, and ΔG^0 was calculated to be 790 cal/mol. The time courses of the above reaction indicated the reaction with G_3 and cG_7 to be faster than that with G_2 and cG_7 (Fig. 3).

Structure of O-*α-maltotriosyl-(1→6)-maltotriose* (A). — Compound *A* was also produced from G_3 alone with the enzyme, the peak for *A* in h.p.l.c. on NH_2-bonded silica was behind but close to that of G_6, and its d.p. was shown to be 6.0 by determination of total carbohydrate and reducing power. Glucoamylase completely degraded *A* into 6.0 mol of D-glucose, and isoamylase hydrolysed it into 2 mol of G_3. Thus, *A* was α-(1→6)-linked maltotriosylmaltotriose and appeared to be a mixture of isomers. H.p.l.c. on YMC AL-312 ODS of borohydride-reduced *A* gave OS1–OS3 in the ratios 1:1:4.5 (Fig. 4). Each compound was purified by rechromatography and then analysed by ^{13}C-n.m.r. spectroscopy. Peak assignments were made on the basis of comparison with the spectra of maltotriose[23] and D-glucitol[24] (Fig. 5, see Scheme 1). The CH_2 signals were easily recognised as negative peaks by the INEPT (Δ = 3/4J) method. The C-6 signals of the glucopyranose residues (M′, M″, S, S′, S″; see Scheme 1) normally appeared at the highest field (δ ~61.5). The peak at δ 63.76 that is common to OS1–OS3 could be attributed to C-1 of the M residue (M-1); consequently, the peaks at δ 63.28 of OS2 and OS3 must have been due to M-6. The M-6 resonance of OS1 was shifted to lower field (δ 69.65). Usui *et al.*[23] pointed out that glycosylation of a hydroxyl group effects a downfield shift, similar to the methylation shift (8–11 p.p.m.), of the resonance of the carbon directly attached (*i.e.*, α). The M″-6 resonance of OS2 and the M′-6 resonance of OS3 were also shifted downfield by ~7 p.p.m. from the usual range for a pyranose C-6. The large downfield shifts of the peaks of M-6 (OS1), M″-6 (OS2), and M′-6 (OS3) indicated that branched maltotriose residues were linked to the respective residues. The structure of OS2 was confirmed by comparison of the C-4 resonances of its two non-reducing end residues (M″ and S″) with those of OS1

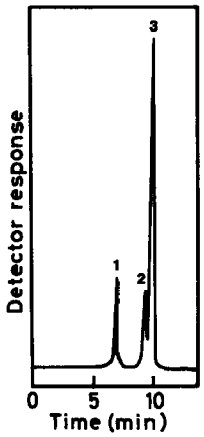


Fig. 4. Fractionation of borohydride-reduced, branched G_6 on YMC AL-312 ODS: 1, OS1; 2, OS2; 3, OS3.

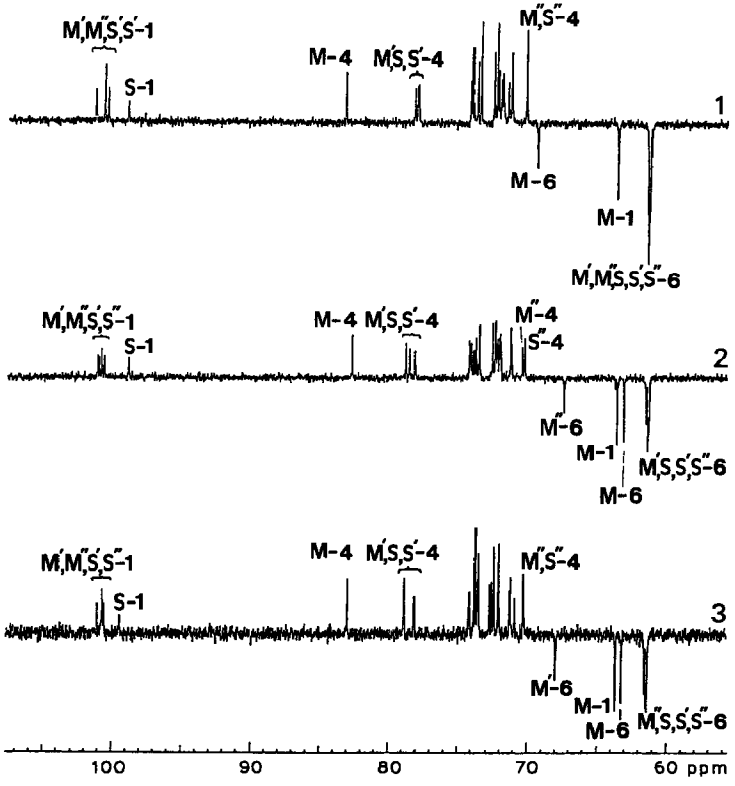
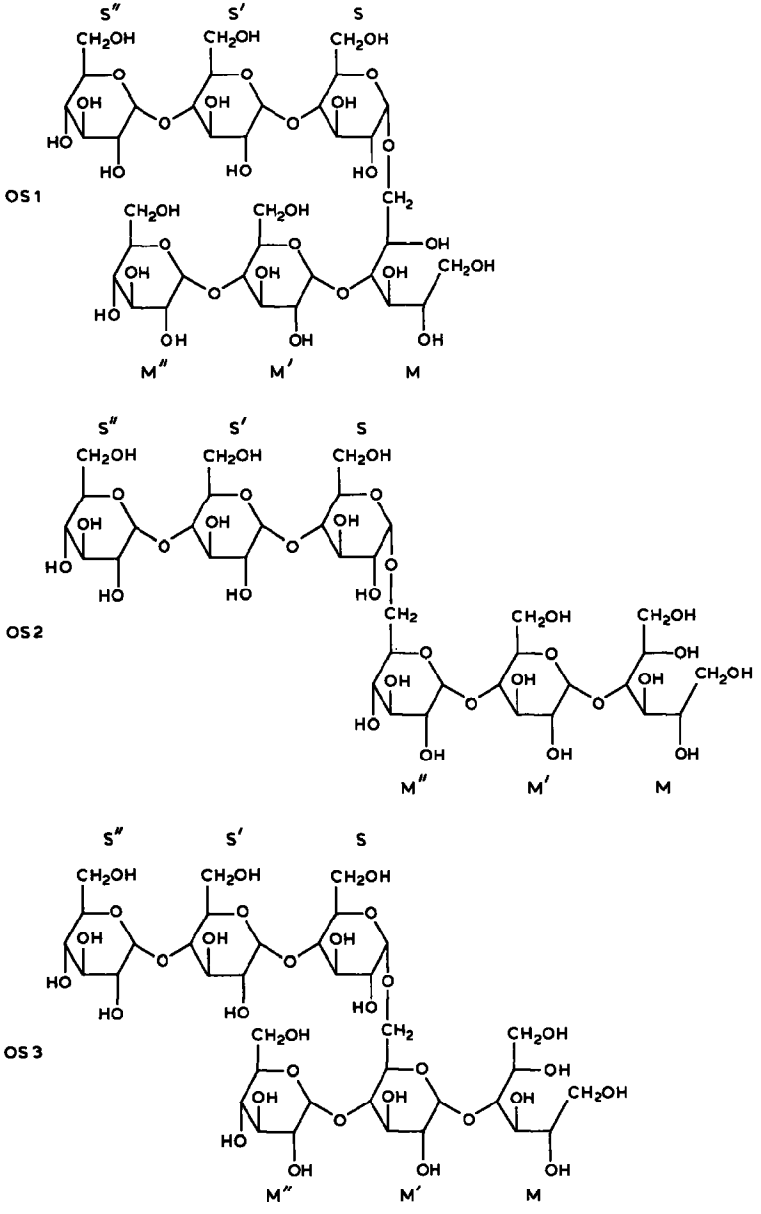


Fig. 5. ^{13}C-N.m.r. spectra of branched G_6 with the INEPT/complete decoupling method: 1, OS1; 2, OS2; 3, OS3.

and OS3. For OS2, the C-4 resonances of the M″ and S″ residues appeared separately at δ 70.53 and 70.36, whereas those resonances of OS1 and OS3 were at δ 70.36 and an overlapped peak at δ 70.38, respectively. This fact indicates that the M″-4 and S″-4 in OS2 had a quite different magnetic environment, whereas those in OS1 and OS3 were the same or slightly different. The M′-4, S-4, and S′-4



Scheme 1. Designation of the glucose residues in the branched G_6 OS1–OS3.

resonances occurred at δ ~78, having undergone a glycosylation shift of ~8 p.p.m. downfield from the M″-4 and S″-4 resonances. The M-4 resonance appeared at δ ~83. The resonances of the glucosidic carbons linked to the C-4 nuclei appeared at δ ~101 and that of the carbon linked to the C-6 nucleus appeared at δ ~99.

The $J_{^{13}C,^{1}H}$ values of S-1 of OS1–OS3, determined by the INEPT/non-decoupling method, were 170.7, 170.5, and 170.7 Hz, respectively, indicating the (1→6) linkages to be α. Thus, the parents of OS1–OS3 were deduced to be 6^1-maltotriosylmaltotriose, 6^3-maltotriosylmaltotriose, and 6^2-maltotriosylmaltotriose, respectively. The preponderance of OS3 suggests that substrates having (1→6) linkages at inner residues are preferable to those having the linkages at terminal residues.

Identification of 6-O-α-maltotriosylcyclomaltoheptaose (B). — Compound *B*

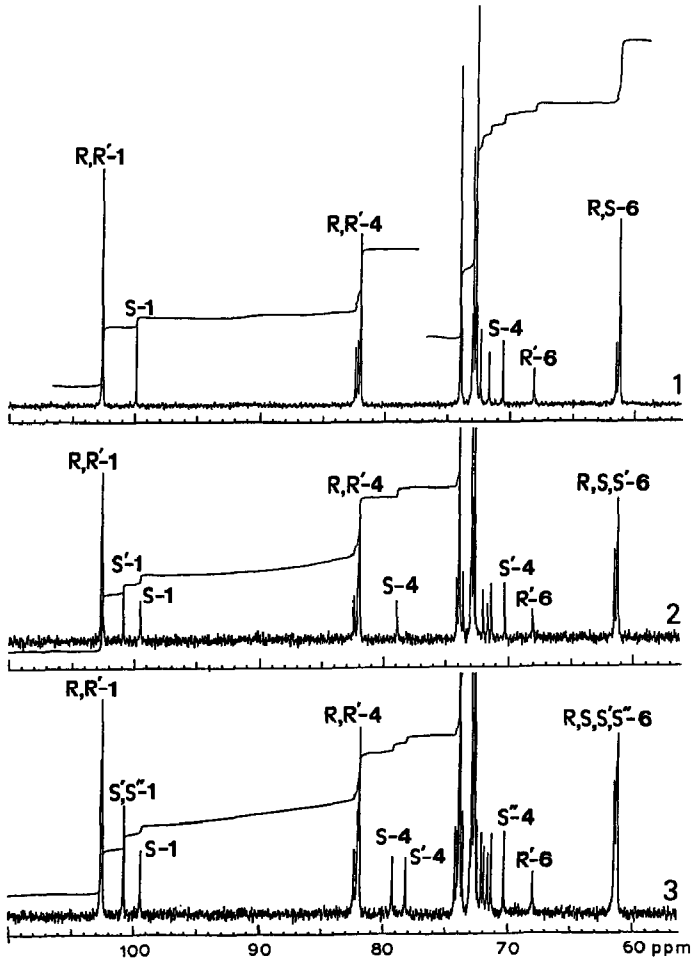


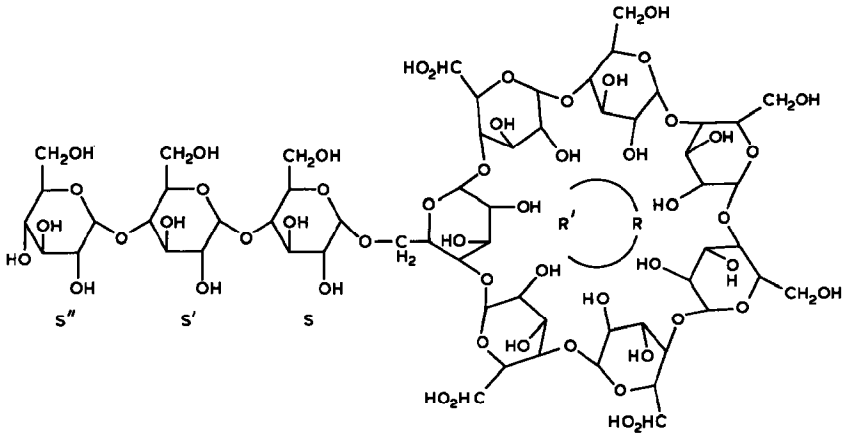
Fig. 6. ^{13}C-N.m.r. spectra of branched cG_7s with the complete decoupling method: 1, GcG_7; 2, G_2cG_7; 3, G_3cG_7.

was non-reducing, and was hydrolysed by isoamylase into G_3 and cG_7. With glucoamylase, it gave 2 mol of D-glucose and 1 mol of GcG_7, which is resistant to the action of glucoamylase[1]. Methylation analysis of *B* gave 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol, and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol in the molar ratios 1.0:8.0:1.0. Smith degradation of *B* yielded 0.97 mol of glycerol from 10 mol of D-glucosyl residues. F.a.b.-m.s. of *B* in the positive-ion mode gave a strong peak at 1621 for $[M + H]^+$. Thus, *B* was 6-*O*-α-maltotriosylcyclomaltoheptaose (G_3cG_7) (Scheme 2).

Identification of 6-O-α-*maltosylcyclomaltoheptaose* (C). — Compound *C*, obtained from G_2 and cG_7, was non-reducing and was hydrolysed into 1 mol each of D-glucose and GcG_7 by glucoamylase. F.a.b.-m.s. indicated the molecular weight of *C* to be 1458, and *C* was identified as 6-*O*-α-maltosylcyclomaltoheptaose (G_2cG_7).

^{13}C-N.m.r. spectra of branched cyclomalto-oligosaccharides. — Fig. 6 shows the ^{13}C-n.m.r. spectra of G_1-, G_2-, and G_3-cG_7. The signals for C-1,4,6 were assigned by comparing each spectrum with those of cG_7 and other branched cG_7's. Chemical shifts of the signals of C-1 and C-6 in the ^{13}C-n.m.r. spectrum of GcG_7 have already been reported[2]. The C-1 resonances of the glucose residues of the main ring (R-1 and R′-1), side-chain residues linked to the main ring (S-1), and the other side-chain residues (S′-1 and S″-1) of G_2cG_7 and G_3cG_7 (see Scheme 2) appeared separately at δ ~103, ~100, and ~101, respectively, and the relative intensities of these signals were 7:1:1 for G_2cG_7 and 7:1:2 for G_3cG_7. For each branched cyclomalto-oligosaccharide, the magnitude (170 Hz) of $J_{^{13}C,^1H}$ of the S residue was the same as that of the D-glucosyl residues of the main ring and the other side-chain residues, and hence the S residue was also α-linked to the main ring.

The resonance of the C-6 involved in glucosidic linkage occurred at δ ~68



Scheme 2. Designation of the glucose residues in G_3cG_7.

TABLE I

THE RATES[a] OF FORMATION OF BRANCHED CYCLOMALTO-OLIGOSACCHARIDES BY *Pseudomonas* ISOAMYLASE

Substrate	*Acceptor*[b]	cG_6		cG_7		cG_8	
	Donor[c]	G_2	G_3	G_2	G_3	G_2	G_3
$v^d \times 10^3$		5.74	209	56.4	891	137	1700
Ratio		1.0	36.3	9.8	155	23.7	296

[a]Each reaction mixture contained 40mM cG_n, 570mM G_2 or G_3, and 8.7 U of isoamylase in a final volume of 1 mL of 50mM sodium acetate buffer (pH 3.5) and was incubated at 45°. [b]For side chains. [c]For main chains. [d]μMol/min/mg of protein.

and those of the other C-6 atoms appeared at δ ~61, and the relative intensities of C-6 signals at δ ~61 and δ ~68 were 8:1 for G_2cG_7 and 9:1 for G_3cG_7. The assignments of these signals were confirmed by the INEPT method using $\Delta = 3/4J$. These data indicate that G_2 and G_3 bind to cG_7 through an α-(1→6) linkage.

Substrate specificity of Pseudomonas *isoamylase.* — The above data show that isoamylase catalyses the condensation of G_2 or G_3 with cG_7, and G_3 with G_3, at practical rates. However, no branched G_4 was produced from G_2. In addition, no branched G_4 or GcG_n was formed from D-glucose and G_3, and D-glucose and cG_n, respectively. This implies that G_2 is the smallest side-chain donor, but that it is not an acceptor of side chains. Thus, *Pseudomonas* isoamylase requires at least three D-glucose residues for a main chain and two residues for a side chain.

The initial rates of the reaction with G_2 and G_3 as donors of side chains, and cG_6, cG_7, and cG_8 as acceptors are listed in Table I. The specific activities of the condensation reactions between G_3 and cG_n were several times higher than those for pullulanase[25]. This is advantageous for the synthesis of G_3cG_n. The reaction rates for G_3 were much higher than those for G_2 (36 times for cG_6, 16 times for cG_7, and 12.5 times for cG_8). The ratios of the reaction rates for cG_6, cG_7, and cG_8 were 1.0:9.8:24 and 1.0:4.3:8.2, using G_2 and G_3 as side-chain donors, respectively. Thus, larger-sized acceptors showed higher reaction rates, and greater differences were observed for G_2 as a donor than for G_3. It is concluded that the reaction rate with *Pseudomonas* isoamylase depends on the sizes of both the acceptor and the donor. These relative reaction rates are considered to be the same as those of the hydrolytic reactions because the reactions are reversible and the amount of branched G_6 is negligible initially. The relative reaction rates for G_2 and G_3, using cG_7 and cG_8 as acceptors, were 16- and 12.5-fold, respectively, and these values were similar to those (9.7- and 13.4-fold[5]) for the hydrolysis rates of maltose and maltotriose branches of beta-limit dextrin of glycogen and amylopectin, respectively. Thus, the maltose residues of substrates are difficult to hydrolyse. In fact, a 10-fold amount of the enzyme and a longer incubation period were needed for the complete hydrolysis of G_2cG_7 than for G_3cG_7 (Fig. 7).

The rate of formation of G_3cG_8 was ~1/150 of the rate for hydrolysis of waxy

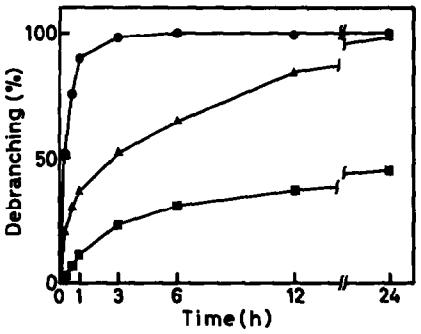


Fig. 7. Hydrolysis of G_3cG_7 and G_2cG_7 with *Pseudomonas* isoamylase. Reaction mixtures contained 11.3mM G_3cG_7 (●) and 0.62 U of isoamylase, or 12.6mM G_2cG_7 and 6.2 (▲) or 0.62 U (■) of the enzyme in 1 mL of 50mM sodium acetate buffer (pH 3.5) at 45°. Debranching (%) was calculated from the amount of cG_7 released.

corn starch, suggesting the low but significant occurrence of reversion in the hydrolysis of starch at a high concentration. Precautions should be taken against reversion during structural analysis of glycogen, amylopectin, and related oligosaccharides, and in the industrial production of maltose and other oligosaccharides. Attention must also be paid to the fact that the extent of hydrolysis of the maltose residues of the substrate depends on the amount of enzyme used. A similar warning about the amount of isoamylase in the determination of the A:B chain ratio has been given[26].

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