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Preparation, isolation, and characterization of novel heterogeneous branched cyclomalto-oligosaccharides having β-D-galactosyl residue(s) on the side chain

Kyoko Koizumi [a], Toshiko Tanimoto [a], Koki Fujita [b], Koji Hara [b], Nobuhiro Kuwahara [b] and Sumio Kitahata [c]

[a] *Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Koshien Kyuban-cho, Nishinomiya, 663 (Japan)*
[b] *Carbohydrate Research Laboratory, Ensuiko Sugar Refining Co., Ltd., 13–46 Daikoku-cho, Tsurumi-ku, Yokohama, 230 (Japan)*
[c] *Osaka Municipal Technical Research Institute, 1-6-50 Morinomiya, Jyoto-ku, Osaka, 536 (Japan)*

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ABSTRACT

Transgalactosylated products of branched cyclodextrins (glucosyl-αCD, -βCD, -γCD, and maltosyl-αCD, -βCD, -γCD) were synthesized by β-D-galactosidases from *Bacillus circulans* and *Penicillium multicolor* using lactose as a donor substrate and branched CDs as acceptors. Eighteen β-D-galactosylated branched CDs were isolated and purified by HPLC. Their structures were elucidated by FABMS and ^{13}C NMR spectroscopies, and methylation analysis. The chromatographic behavior of these novel heterogeneous branched CDs on three HPLC columns of different separation modes was compared.

INTRODUCTION

Recently transgalactosylated products of branched cyclomalto-oligosaccharides (cyclodextrins, CDs) were synthesized by various β-D-galactosidases (β-D-galactoside galactohydrolase, EC 3.2.1.23) using lactose as a donor substrate and branched CDs as acceptors[1]. The branched CD having a galactosyl residue which can specifically bind to a receptor on a hepatocyte may be used as a drug carrier to the liver parenchymal cell.

In this paper we report methods of preparing and isolating eighteen β-D-galactosylated branched CDs, their structural analyses, and their chromatographic behavior on three high-performance liquid chromatography (HPLC) columns of different separation modes.

Correspondence to: Professor K. Koizumi, Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Koshien Kyuban-cho, Nishinomiya, 663, Japan.

EXPERIMENTAL

Materials.—Glucosyl (G1)-αCD, -βCD, -γCD and maltosyl (G2)-αCD, -βCD, -γCD were supplied by Ensuiko Sugar Refining Co., Ltd. β-Galactosidase preparations from *Bacillus circulans* and *Penicillium multicolor* were supplied by Daiwa Kasei (Osaka, Japan) and K·I Chemical Industry Co., Ltd. (Shizuoka, Japan), respectively. All reagents were of analytical grade. Reagent-grade organic solvents used for chromatography were dried and freshly distilled before use. Water used in solvent preparations was distilled, deionized, and redistilled.

Assay of β-D-galactosidase activity.—An enzyme solution (50 μL) was incubated with 400 μL of 5 mM *p*-nitrophenyl β-D-galactoside in 25 mM buffer (optimum pH of each enzyme) at 40°C for 10 min. The reaction was stopped by adding 0.5 mL of 0.2 M Na_2CO_3, and the release of *p*-nitrophenol from the substrate was measured spectrophotometrically. One unit of the enzyme activity is defined as the amount of enzyme that liberates 1 μmol of *p*-nitrophenol per min.

Preparation and isolation of D-galactosylated branched CDs.—*B. circulans* and *P. multicolor* β-D-galactosidases (50 units/g of lactose) were separately incubated with a mixture of lactose and branched CD (1:1 w/w) in 50 mM acetate buffer (pH 6.0 for *B. circulans* and pH 4.5 for *P. multicolor*) at 40°C for 1–2 h. The reaction mixtures were heated at 100°C for 15 min to inactivate the enzyme and centrifuged to remove the insoluble materials. After removing mono- and acyclic oligo-saccharides from the reaction mixture by preparative HPLC on an ODS column (100 × 26.4 cm i.d.) with H_2O, CDs were eluted from the column with 10% EtOH. Composition of the CD fractions obtained was determined by HPLC on a TSK-Gel Amide-80 (250 × 4.6 mm i.d.) with 63:37 MeCN–H_2O at 35°C. The desired galactosylated branched CDs were separated from the fraction of CDs by HPLC on a YMC-Pack SH-345-5 ODS column (500 × 20 mm i.d.) with 5–8% MeOH and purified on a YMC-Pack AQ-312-3 ODS column (150 × 6 mm i.d.) with 7–8% MeOH or on an Asahipak NH2P-50 column (250 × 10 mm i.d.) with 60% MeCN.

Analyses.—Optical rotations were determined with a JASCO digital polarimeter, model DIP 360.

HPLC analyses were performed with a JASCO 880-PU pump and a Showa Denko SE-61 RI monitor. The columns used were a YMC-Pack A-312 (3 μm, 150 × 6 mm i.d.) (YMC), an Asahipak NH2P-50 (250 × 4.6 mm i.d.) (Asahi Kasei) and a Hypercarb (100 × 4.7 mm i.d.) (Shandon). HPLC analyses at constant temperature were conducted using a column oven CO-1093C (Uniflows).

FABMS was performed with a JEOL JMS-DX 303 mass spectrometer using Xe atoms having a kinetic energy equivalent to 6 kV at an accelerating voltage of 3 kV. The mass marker was calibrated with perfluoroalkylphosphazine (Ultra Mark), and glycerol was used as the matrix.

NMR spectra data were recorded for solutions in D_2O with a JEOL GSX-500 spectrometer. Chemical shifts were expressed in ppm downfield from the signal of

TABLE I

Conditions for NMR measurement

	^{13}C NMR	^{1}H–^{1}H COSY	^{1}H–^{13}C COSY ^{1}H–^{13}C HOHAHA [a]
Concentration (%)	6–10	6–10	6–10
Temperature (°C)	50	50	50
Frequency range (Hz)	7002.8	1000	7002.8
Acquisition time (s)	4.679	1.024	0.292
Data point	65536	2048	4096
Column point		1024	512
Column frequency		1000	1000

[a] The mixing period consisted of 32.5 ms MLEV-17 cycles.

Me_4Si referred to external 1,4-dioxane (67.40 ppm). The conditions for NMR measurement are summarized in Table I.

Methylation was carried out by the method of Prehm[2]. The products were hydrolysed, converted into the corresponding alditol acetates, and then analyzed with a Shimadzu GCMS-QP2000 gas chromatograph–mass spectrometer on a ULBON HR-SS-10 column (50 m × 0.25 mm i.d.) (Shinwa Kako).

RESULTS AND DISCUSSION

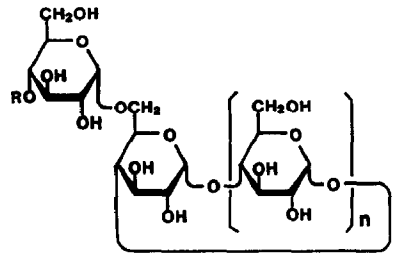
Preparation.—Two kinds of transgalactosylated branched CDs (**1** and **2** from each G1-CD, and **4** and **5** from each G2-CD) by *B. circulans* β-D-galactosidase and one (**3** from each G1-CD, and **6** from each G2-CD) by *P. multicolor* β-D-galactosidase were obtained from each starting branched CD. Elongation of the reaction time by *B. circulans* β-D-galactosidase led to the production of **3** or **6** in small amounts. In Table II are listed the yields (%) of transgalactosylated branched CDs calculated from the amount of each starting branched CD (mol). The yields of **3** or **6** by *P. multicolor* β-5D-galactosidase decreased in the reaction if left running for 2 h because of the hydrolysis of **3** or **6** that was produced.

Isolation.—Most of galactosylated branched CDs could be isolated by HPLC on a semipreparative ODS column. In the cases of **α2**, **α4**, **α5**, **β2**, **β4**, and **β5**, purification by HPLC on a more efficient ODS column packed with 3-μm C_{18}-bonded silica gel or on an amino column (a polyamine-bonded vinyl alcohol copolymer) was indispensable.

Structural analyses.—The FABMS spectra of the galactosylated G1- and G2-CDs suggested that **1**, **3**, **4**, and **6** each had one galactosyl residue bonded on the side chain of the G1- or G2-CD, and **2** and **5** each had two galactosyl residues. The molecular-ion peak in the negative mode $[M - H]^-$ and primary fragment ions derived on one cleavage of the side chain of the molecule are listed in Table III.

^{13}C NMR spectroscopy of galactosylated G1- and G2-CDs offered further corroborating evidence relating to their structures. The ^{13}C resonances of all

carbons in the spectrum of galactosyl G1-βCD (**β1**) were assigned using COSY, $^{1}H-^{13}C$ COSY, and $^{1}H-^{13}C$ HOHAHA (heteronuclear relayed Hartmann–Hahn spectroscopy[3] methods. Fig. 1 shows the $^{1}H-^{13}C$ COSY spectrum of **β1** in D_2O at 50°C. The spectra of the others were similar to that of **β1**, and hence assignments of signals could be made by analogy. It is known that a substituent on the oxygen atom attached to any carbon of the sugar affects the chemical shift of that carbon



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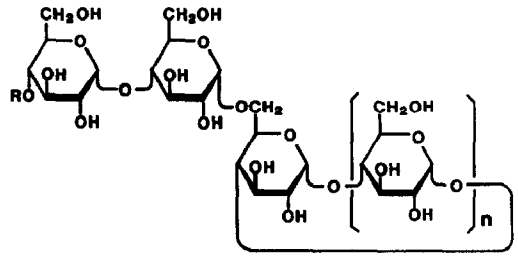

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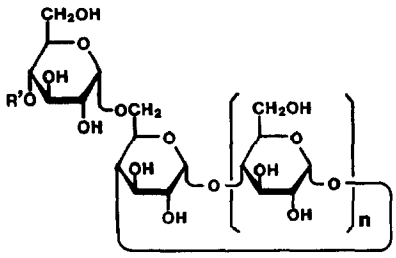

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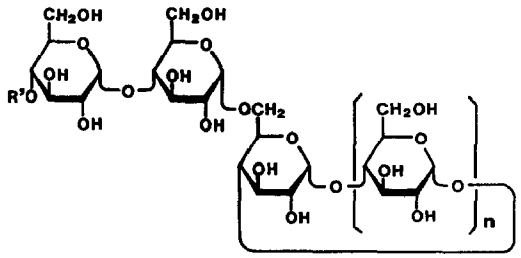

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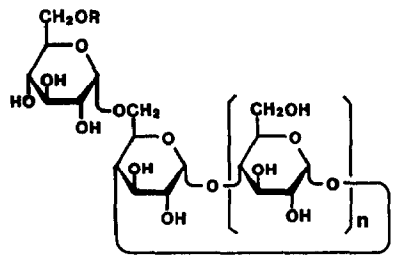

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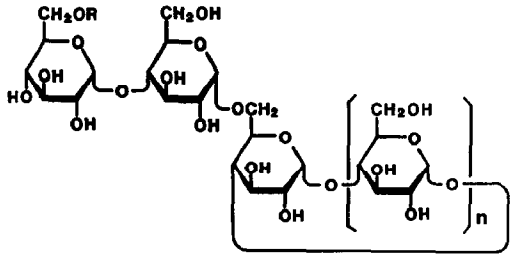

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R = β-D-galactopyranosyl
R' = 4-*O*-(β-D-galactopyranosyl)-β-D-galactopyranosyl

αCD series (n = 5): α1, α2, α3, α4, α5, α6
βCD series (n = 6): β1, β2, β3, β4, β5, β6
γCD series (n = 7): γ1, γ2, γ3, γ4, γ5, γ6

Scheme 1.

TABLE II

Transgalactosylated products of glucosyl (G1)-CDs and maltosyl (G2)-CDs by two β-galactosidases, and their yields

β-galactosidase from	Acceptor	Product	Yield (%)
B. circulans [a]	G1-αCD	**α1**	20.2
		α2	5.7
		α3	1.1
	G2-αCD	**α4**	21.8
		α5	5.3
		α6	2.6
	G1-βCD	**β1**	20.0
		β2	4.2
		β3	1.2
	G2-βCD	**β4**	22.0
		β5	5.3
		β6	2.3
	G1-γCD	**γ1**	17.3
		γ2	3.2
		γ3	0.0
	G2-γCD	**γ4**	20.8
		γ5	8.2
		γ6	1.6
P. multicolor [b]	G1-αCD	**α3**	6.8
	G2-αCD	**α6**	10.3
	G1-βCD	**β3**	13.7
	G2-βCD	**β6**	12.6
	G1-γCD	**γ3**	9.5
	G2-γCD	**γ6**	8.0

[a] Reaction time, 2 h. [b] Reaction time, 1 h.

atom, moving it downfield by 8–11 ppm[4]. The large downfield shift of one and two C-4 signal(s) was observed in each spectrum of transgalactosylation products by *B. circulans* β-D-galactosidase, **α1**, **β1**, **γ1**, **α4**, **β4**, **γ4**, and **α2**, **β2**, **γ2**, **α5**, **β5**, **γ5**, respectively. On the other hand, each C-6 signal of the transgalactosylation products from *P. multicolor* β-D-galactosidase, **α3**, **β3**, **γ3**, **α6**, **β6**, **γ6**, shifted downfield. The assignments of the C-6 signals were confirmed by the distortionless enhancement by polarization transfer (DEPT) method[5]. The C-1 resonances of the glucose residues of the CD ring and side chain, which were α(1 → 4)-linked, and side-chain glucose, which were α(1 → 6)-linked to the CD ring appeared at δ ~ 102.2, ~ 100.8, and ~ 99.4 ppm, respectively. Whereas β(1 → 4)- or β(1 → 6)-linked C-1 signals of the galactose residues were observed at lower fields than the C-1 signals of the CD ring (Tables IV–IX). In the cases of branched γ-CDs having a side-chain of (1 → 4)-linked tri- or tetra-saccharide, viz., **γ2**, **γ4**, and **γ5**, the signals of the C-6 atom of the ring glucose unit (G-6′) and C-1 atom of the side-chain glucose unit (G′-1), both involved in the linkage of branch point, were broadened considerably at 50°C, and the broadening was improved somewhat at

TABLE III

The molecular ion (●) and primary fragments (○) in the FABMS spectra of galactosylated glucosyl- and maltosyl-cyclomalto-oligosaccharides in the negative-ion mode

Compound	*m/z*						
	971	1133	1295	1457	1619	1781	1943
α1	○	○	●				
α2	○	○	○	●			
α3	○	○	●				
α4	○	○	○	●			
α5	○	○	○	○	●		
α6	○	○	○	●			
β1		○	○	●			
β2		○	○	○	●		
β3		○	○	●			
β4		○	○	○	●		
β5		○	○	○	○	●	
β6		○	○	○	●		
γ1		○	○	●			
γ2		○	○	○	●		
γ3		○	○	●			
γ4		○	○	○	●		
γ5		○	○	○	○	●	
γ6		○	○	○	●		

80°C (Fig. 2). These facts suggest that free rotation of the linkage is hindered. On the other hand, those signals of **γ6** which has a side chain of trisaccharide made up of one (1 → 4)- and one (1 → 6)-linkage, were normal. These phenomena were also observed in the spectra of maltotriosyl-γCD (broadening) and panosyl-γCD (normal).

To make sure that in the cases of 6-*O*-galactosylated maltosyl-CDs (**α6**, **β6**, and **γ6**), the galactosyl residue substituted on the oxygen at C-6 of the nonreducing end of the side-chain maltose, methylation analysis was carried out. The result of GLC–MS of the partially methylated alditol acetates obtained by the sequence of methylation, hydrolysis, reduction, and acetylation of the sample showed clearly the production of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol.

The structures of eighteen β-D-galactosylated branched CDs elucidated by FABMS and ^{13}C NMR spectroscopies, and methylation analysis are presented in Scheme 1, and their specific rotations are listed in Table X.

Chromatographic behavior.—The eighteen β-galactosylated G1- and G2-CDs showed characteristic chromatographic behavior on three HPLC columns of different separation modes.

Fig. 3 shows their elution profiles on a reversed-phase column, YMC-Pack A-312 (3 μm). The separation mechanism of the reversed-phase column is probably an example of hydrophobic chromatography, namely, increased retention time with decreasing solubility in water[6]. γ-CD Derivatives were shown to move most

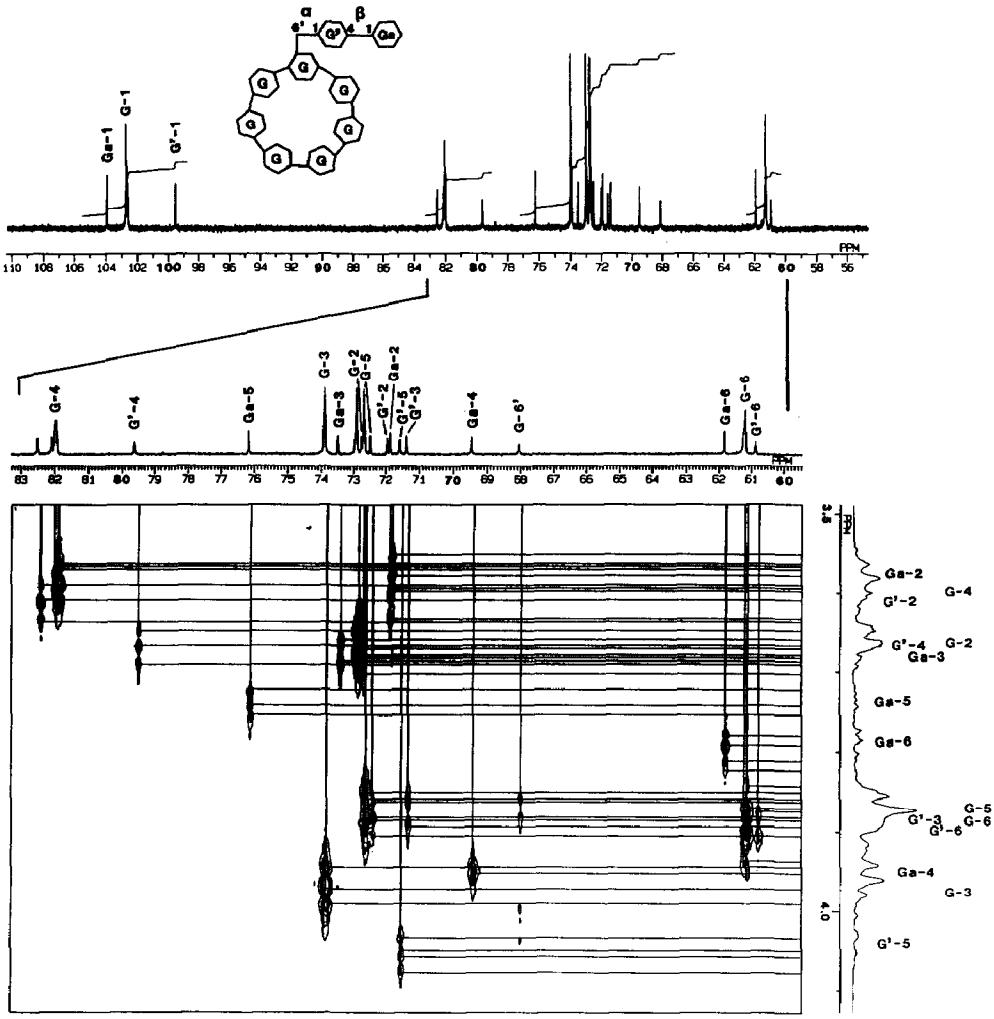

Fig. 1. $^{1}H-^{13}C$ COSY spectrum of *O*-β-D-galactopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 6)-cyclomaltoheptaose (**β1**) in D_2O at 50°C.

rapidly, while the β-CD derivatives eluted too slowly with the same eluent (6:94 methanol–water), for example, the retention time (t_R) of **β1** was 47.6 min, and hence, a stronger eluent, 7:93 methanol–water, was required for βCD derivatives. The elution order of each member in the three series was the same, namely the 6-*O*-galactosylated G1- and G2-CDs (**3** and **6** in each series) eluted faster than the 4-*O*-galactosylated isomers (**1** and **4**). Compound **3** was shown to elute most quickly. Among the 4-*O*-galactosylated derivatives, the G2-CD derivative eluted faster than the G1-CD derivative, and the monogalactosylated derivative eluted faster than the digalactosylated compound. In a search for chromatographic conditions, it was found that the efficiency of the ODS column was considerably affected by column temperature. The theoretical number (N) of plates for a

TABLE IV

Chemical shifts of C-1, C-4, and C-6 in the ^{13}C NMR spectra (125.65 MHz, in D_2O at 50°C) of galactosylated glucosylcyclomaltohexaoses (**α1**, **α2**, and **α3**)

Atom [a]	**α1**		**α2**		**α3**	
	δ(ppm)	Integral	δ(ppm)	Integral	δ(ppm)	Integral
Ga′-1			105.04	1		
Ga-1	103.83	1	103.77	1	104.08	1
G-1	102.22	6	102.21	6	102.23	6
	102.19		102.16		102.19	
	102.17				102.15	
	102.16				102.13	
G′-1	99.39	1	99.36	1	99.80	1
G-4 [b]	82.46	6	82.47	6	82.37	6
	82.14		82.14		82.28	
	82.06		82.06		82.05	
	82.04		82.04		81.93	
	82.02		82.02			
	81.98		81.97			
G′-4	79.53 [b]	1	79.53 [b]	1	70.15	1
Ga-4	69.43	1	77.96 [b]	1	69.54	1
Ga′-4			69.49	1		
G-6′ [b]	68.08	1	68.08	1	68.01	1
Ga′-6			61.82			
Ga-6	61.82	1	61.57	1	61.87	1
G-6	61.34	5	61.34	5	61.32	5
	61.33		61.27			
	61.30					
	61.27					
G′-6	60.82	1	60.78	1	68.90 [b]	1

[a] G-1, -4, and -6 are C-1, -4, and -6 atoms of the ring D-glucopyranose units. G-6′ is C-6 atom of the ring D-glucopyranose unit involved in branching. G′ and G″-1, -4, and -6 are the carbon atoms of the side chain D-glucopyranose units. Ga and Ga′-1, -4, and -6 are the carbon atoms of D-galactopyranose units. [b] C-4 and C-6 atoms involved in linkages.

YMC-Pack A-312-3 ODS column, measured using βCD as the sample, was 9520 and 25°C, 9120 at 30°C, and 8270 at 35°C. Furthermore, the difference in the number of plates on another ODS column having a lower carbon loading (%C), a YMC-Pack AQ-312-3, was even more significant, i.e., 9500 at 25°C, 8160 at 30°C, and 6180 at 35°C. This pronounced dependence on temperature was recognized more or less on ODS columns from other manufacturers, and, on larger sized columns, the difference in N with temperature was more significant.

Chromatograms of β-galactosylated G1- and G2-CDs on an Asahipak NH2P-50 column are shown in Fig. 4. the Asahipak NH2P-50 is a so-called "amino column" packed with chemically polyamine-bonded vinyl alcohol copolymer gel (5 μm), and the packing stability is much better than aminopropyl-bonded silica[7]. It is known that the elution sequence with the aminopropyl-bonded silica column, the typical amino column, and an acetonitrile-water system follows the order of molecular

TABLE V

Chemical shifts of C-1, C-4, and C-6 in the ^{13}C NMR spectra (125.65 MHz, in D_2O at 50°C) of galactosylated maltosylcyclomaltohexaoses (**α4**, **α5**, and **α6**)

Atom [a]	**α4**		**α4**		**α6**	
	δ(ppm)	Integral	δ(ppm)	Integral	δ(ppm)	Integral
Ga′-1			105.11	1		
Ga-1	103.79	1	103.73	1	104.16	1
G-1	102.25	6	102.23	6	102.25	6
	102.19		102.20		102.19	
			102.17		102.16	
			102.14			
G″-1	100.82	1	100.78	1	100.95	1
G′-1	99.44	1	99.39	1	99.37	1
G-4 [b]	82.45	6	82.41	6	82.47	6
	82.23		82.16		82.16	
	82.22		82.09		82.06	
	82.16		82.08		82.04	
	82.11		82.06			
			82.05			
G′-4 [b]	79.31	1	79.29 [c]	1	79.13	1
G″-4	79.31 [b]	1	79.26 [b,c]	1	70.19	1
Ga-4	69.51	1	78.10 [b]	1	69.55	1
Ga′-4			69.51	1		
G-6′ [b]	68.08	1	68.05	1	68.12	1
Ga′-6			61.84	1		
Ga-6	61.89	1	61.60	1	61.86	1
G-6 and	61.50	6	61.42	6	61.54	6
G′-6	61.49		61.39		61.36	
	61.41		61.35		61.30	
	61.37		61.29			
	61.36					
G″-6	60.94	1	60.82	1	69.39 [b]	1

[a] G-1, -4, and -6 are C-1, -4, and -6 atoms of the ring D-glucopyranose units. G-6′ is C-6 atom of the ring D-glucopyranose unit involved in branching. G′ and G″-1, -4, and -6 are the carbon atoms of the side chain D-glucopyranose units. Ga and Ga′-1, -4, and -6 are the carbon atoms of D-galactopyranose units. [b] C-4 and C-6 atoms involved in linkages. [c] Assignments may be interchanged.

size[8]. Therefore, it was presumed that isomers having the same molecular size should be difficult to separate from each other on the "amino column". In practice, however, the 4-*O*-galactosylated derivatives **1** and **4** moved faster than corresponding 6-*O*-galactosylated isomers **3** and **6**. Digalactosylated G1-CD **2** and monogalactosylated G2-CD **4**, which have the same molecular size, eluted almost at the same time. The digalactosylated derivatives **2** or **5** naturally eluted slower than the monogalactosylated derivatives **1** and **3** or **4** and **6** of the same parent branched-CD. The fact that the βCD derivatives moved much slower than the α- and γ-CD derivatives and consequently required an increase in the volume of water in the eluent, together with the fact that the t_Rs of CDs were somewhat

TABLE VI

Chemical shifts of C-1, C-4, and C-6 in the ^{13}C NMR spectra (125.65 MHz, in D_2O at 50°C) of galactosylated glucosylcyclomaltoheptaoses (**β1**, **β2**, and **β3**)

Atom [a]	**β1**		**β2**		**β3**	
	δ(ppm)	Integral	δ(ppm)	Integral	δ(ppm)	Integral
Ga'-1			105.07	1		
Ga-1	103.90	1	103.82	1	104.14	1
G-1	102.68	7	102.66	7	102.68	7
	102.66		102.63		102.66	
	102.64		102.58		102.64	
	102.61		102.55		102.62	
	102.60				102.60	
	102.58					
	102.57					
G'-1	99.47	1	99.42	1	99.95	1
G-4 [b]	82.53	7	82.52	7	82.45	7
	82.12		82.09		82.28	
	82.02		81.99		82.06	
	82.01		81.96		82.02	
	82.00		81.95		82.00	
	81.98				81.99	
	81.96				81.97	
G'-4	79.64 [b]	1	79.60 [b]	1	70.27	1
Ga-4	69.48	1	77.97 [b]	1	69.58	1
Ga'-4			69.51	1		
G-6' [b]	68.07	1	68.06	1	68.12	1
Ga'-6			61.84	1		
Ga-6	61.87	1	61.59	1	61.91	1
G-6	61.30	6	61.20	6	61.32	1
	61.27				61.25	
	61.23					
G'-6	60.89	1	60.81	1	69.00 [b]	1

[a] G-1, -4, and -6 are C-1, -4, and -6 atoms of the ring D-glucopyranose units. G-6' is C-6 atom of the ring D-glucopyranose unit involved in branching. G'-1, -4, and -6 are the carbon atoms of the side chain D-glucopyranose unit. Ga and Ga'-1, -4, and -6 are the carbon atoms of D-galactopyranose units. [b] C-4 and C-6 atoms involved in linkages.

decreased with increases in temperature, indicate that the elution mechanism on this column may involve some hydrophobic interactions.

Hypercarb is a graphitized carbon column, and the retention characteristics of carbohydrates on the column are essentially the result of an adsorption mechanism[9]. The unique resolving power of this column led to excellent separations of each of the positional isomers of 6^1, 6^n-di-*O*-(α-D-glucopyranosyl)-αCD[10], -βCD[11], and 6^1, 6^n-di-*O*-(α-maltosyl)-βCD[9] ($n = 3$ and 4). Interestingly, the t_Rs of αCD, βCD, γCD, G1-αCD, G1-βCD, and G1-γCD increased with an increase in temperature[9]. This reason may be due to the higher adsorptive activity of carbon at higher temperature. Fig. 5 shows the elution profiles of the eighteen β-D-galactosylated G1- and G2-CDs on this column. The difficulty in analysis on this column

TABLE VII

Chemical shifts of C-1, C-4, and C-6 in the ^{13}C NMR spectra (125.65 MHz, in D_2O at 50°C) of galactosylated maltosylcyclomaltoheptaoses (**β4**, **β5**, and **β6**)

Atom [a]	**β4**		**β5**		**β6**	
	δ(ppm)	Internal	δ(ppm)	Integral	δ(ppm)	Integral
Ga′-1			105.12	1		
Ga-1	103.77	1	103.76	1	104.15	1
G-1	102.66	7	102.66	7	102.66	7
	102.64		102.61		102.63	
	102.62		102.57		102.60	
	102.61		102.56		102.57	
	102.59		102.49		102.55	
	102.55					
	102.46					
G″-1	100.80	1	100.80	1	100.94	1
G′-1	99.43	1	99.45	1	99.44	1
G-4 [b]	82.31	7	82.35	7	82.42	7
	82.11		82.11		82.13	
	82.10		82.09		82.06	
	82.09		82.04		82.00	
	81.96		81.96		81.96	
	81.93		81.94		81.93	
			81.92		81.92	
G′-4 [b]	79.38 [c]	1	79.39 [c]	1	79.19	1
G″-4	79.33 [b,c]	1	79.37 [b,c]	1	70.17	1
Ga-4	69.44	1	78.11 [b]	1	69.55	1
Ga′-4			69.50	1		
G-6′ [b]	67.95	1	67.97	1	68.01	1
Ga′-6			61.83	1		
Ga-6	61.83	1	61.58	1	61.85	1
G-6 and	61.45	7	61.45	7	61.56	7
G′-6	61.31		61.31		61.29	
	61.27		61.30		61.26	
	61.26		61.29		61.18	
	61.23		61.25			
	61.18		61.22			
	61.15		61.18			
G″-6	60.87	1	60.83	1	69.38 [b]	1

[a] G-1, -4, and -6 are C-1, -4, and -6 atoms of the ring D-glucopyranose units. G-6′ is C-6 atom of the ring D-glucopyranose unit involved in branching. G′ and G″-1, -4, and -6 are the carbon atoms of the side chain D-glucopyranose units. Ga and Ga′-1, -4, and -6 are the carbon atoms of D-galactopyranose units. [b] C-4 and C-6 atoms involved in linkages. [c] Assignments may be interchanged.

is that only under certain strictly controlled chromatographic conditions can good peak shapes be obtained. Consequently, in the simultaneous separation of each of the six members in each of the three series, it was impossible to get satisfactory results for all members in each series. In order to establish the best conditions for separation of the six members in each series, the concentration of organic modifier in the eluent, the flow rate, and the column temperature were all varied. The

TABLE VIII

Chemical shifts of C-1, C-4, and C-6 in the ^{13}C NMR spectra (125.65 MHz, in D_2O at 50°C) of galactosylated glucosylcyclomaltooctaoses (**γ1**, **γ2**, and **γ3**)

Atom [a]	**γ1**		**γ2** [b]		**γ3**	
	δ(ppm)	Integral	δ(ppm)	Integral	δ(ppm)	Integral
Ga′-1			105.12	1		
Ga-1	103.89	1	103.84	1	104.12	1
G-1	102.56	8	102.49	8	102.49	8
	102.53		102.46		102.46	
	102.51		102.44		102.43	
	102.46		102.42		102.42	
	102.42		102.37		102.39	
	102.38		102.33		102.36	
			102.28			
G′-1	99.42	1	99.47	1	99.91	1
G-4 [c]	82.06	8	81.00	8	81.80	8
	81.50		81.48		81.57	
	81.41		81.42		81.30	
	81.39		81.39			
	81.36		81.38			
	81.32		81.34			
			81.33			
G′-4	79.62 [c]	1	79.92 [c]	1	70.21	1
Ga-4	69.45	1	78.01 [c]	1	69.53	1
Ga′-4			69.60	1		
G-6′ [c]	68.05	1	68.10	1	68.00	1
Ga′-6			61.86	1		
Ga-6	61.85	1	61.60	1	61.86	1
G-6	61.23	7	61.35	7	61.22	7
	61.16		61.32		61.19	
			61.31		61.16	
			61.26		61.13	
G′-6	60.89	1	61.07	1	68.98 [c]	1

[a] G-1, -4, and -6 are C-1, -4, and -6 atoms of the ring D-glucopyranose units. G-6′ is C-6 atom of the ring D-glucopyranose unit involved in branching. G′-1, -4, and -6 are the carbon atoms of the side chain D-glucopyranose unit. Ga and Ga′-1, -4, and -6 are the carbon atoms of D-galactopyranose units. [b] Measured at 80 °C. [c] C-4 and C-6 atoms involved in linkages.

conditions for βCD series (eluent, 15 : 85 acetonitrile–water; flow rate, 0.6 mL/min; temperature, 30°C) gave t_Rs that were too short to separate the αCD series, and hence the temperature was raised to 50°C for the αCD series. The members of the γCD series which were retained longer than those of the βCD series, were chromatographed at 40°C and 0.7 mL/min flow rate with 16 : 84 acetonitrile–water. The elution order of each member in the three series was the same: the 4-*O*-galactosylated derivatives followed the order of molecular size, but the behavior of the 6-*O*-galactosylated derivatives was unique in that the G1-CD derivative **3** was the fastest moving, whereas G2-CD derivative **6** was the last to elute. This result

TABLE IX

Chemical shifts of C-1, C-4, and C-6 in the ^{13}C NMR spectra (125.65 MHz, in D_2O at 50°C) of galactosylated maltosylcyclomaltooctaoses (γ4, γ5, and γ6)

Atom [a]	γ4 [b]		γ5 [b]		γ6	
	δ(ppm)	Integral	δ(ppm)	Integral	δ(ppm)	Integral
Ga′-1			105.17	1		
Ga-1	103.85	1	103.81	1	104.16	1
G-1	102.42	8	102.43	8	102.53	8
	102.35		102.37		102.48	
	102.33		102.35		102.43	
	102.30		102.33		102.41	
	102.29				102.39	
	102.23					
G″-1	100.87	1	100.87	1	101.04	1
G′-1	99.56	1	99.59	1	99.50	1
G-4 [c]	81.91	8	81.93	8	81.95	8
	81.57		81.57		81.60	
	81.50		81.49		81.45	
	81.47		81.39		81.42	
	81.38		81.34		81.32	
	81.36		81.33		81.29	
	81.34				81.28	
G′-4 [c]	79.71	1	79.70 [d]	1	79.37	1
G″-4	79.71 [c]	1	79.69 [c,d]	1	70.16	1
Ga-4	69.50	1	78.16 [c]	1	69.55	1
Ga′-4			69.58	1		
G-6′ [c]	68.20	1	68.20	1	68.06	1
Ga′-6			61.85	1		
Ga-6	61.81	1	61.61	1	61.85	1
G-6 and	61.58	8	61.58	8	61.57	8
G′-6	61.42		61.41		61.29	
	61.41		61.40		61.24	
	61.32		61.33		61.16	
	61.27		61.27		61.13	
G″-6	61.12	1	61.07	1	69.38 [c]	1

[a] G-1, -4, and -6 are C-1, -4, and -6 atoms of the ring D-glucopyranose units. G-6′ is C-6 atom of the ring D-glucopyranose unit involved in branching. G′ and G″-1, -4, and -6 are the carbon atoms of the side chain D-glucopyranose units. Ga and Ga′-1, -4, and -6 are the carbon atoms of D-galactopyranose units. [b] Measured at 80 °C. [c] C-4 and C-6 atoms involved in linkages. [d] Assignments may be interchanged.

may reflect the stereochemical disposition of these compounds, that is, planar molecules are generally more strongly retained than are nonplanar molecules.

In conclusion, among the three HPLC columns having different separation modes, the ODS column gave the best separation. However, it was found that the other two columns were also very useful for the characterization of novel heterogeneous branched CDs, as these derivatives showed very unique behavior on each column.

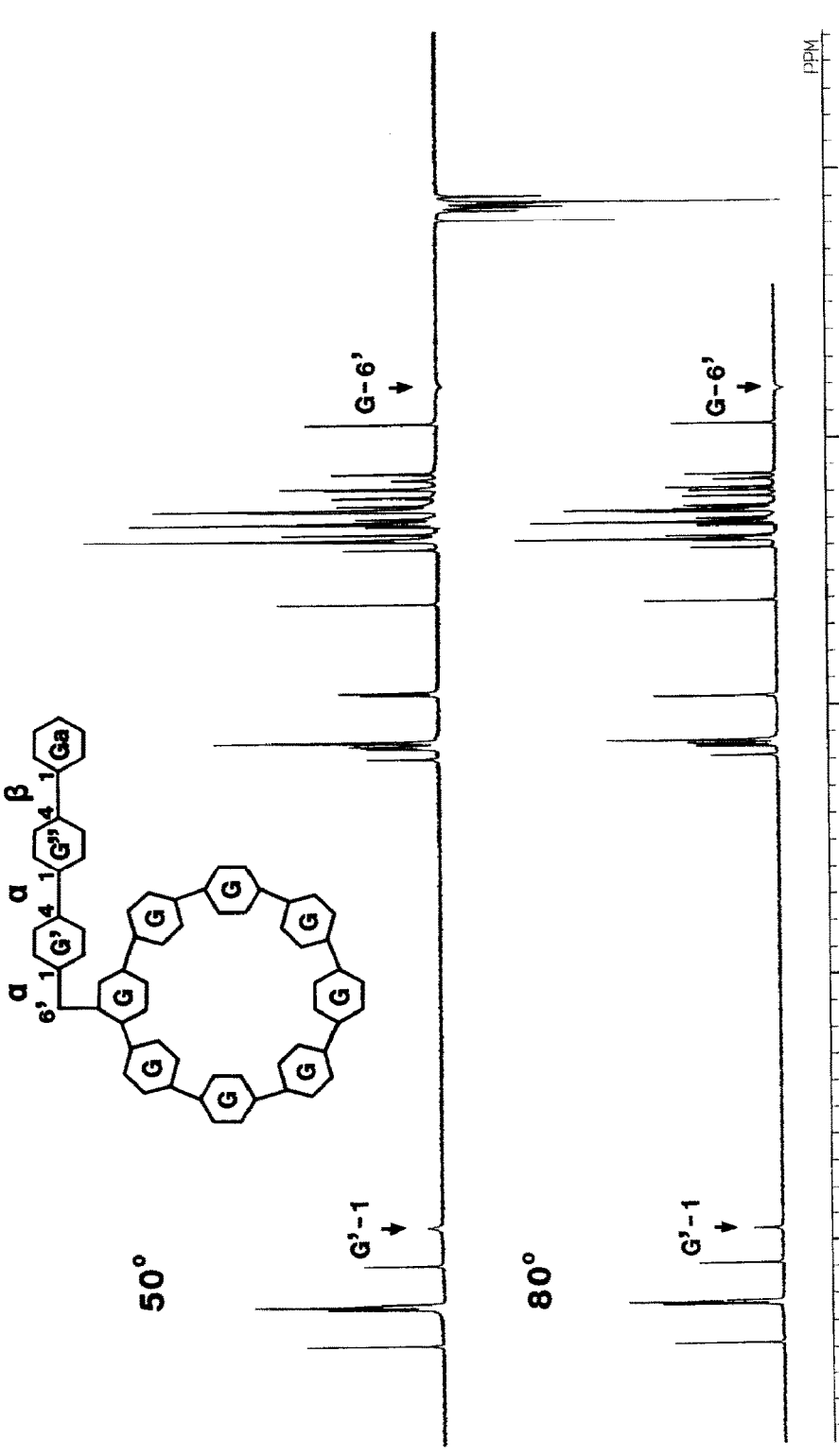

Fig. 2. ^{13}C NMR spectra of *O*-β-D-galactopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 6)-cyclomaltooctaose (γ4) with the DEPT method in D_2O at 50 and 80°C.

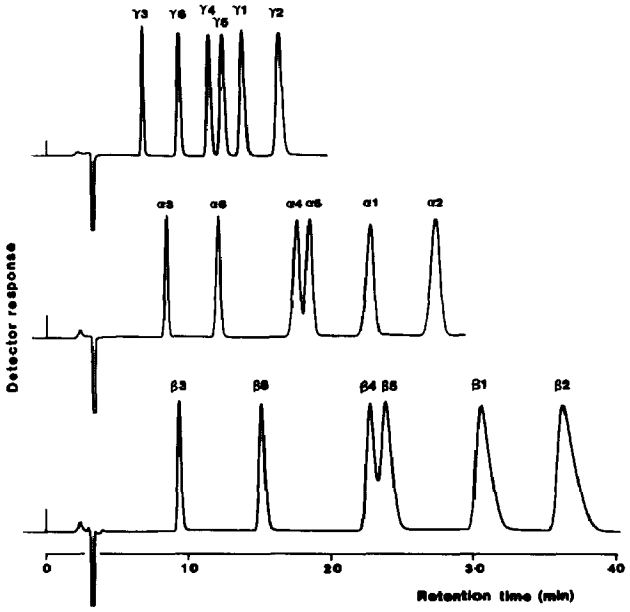

Fig. 3. Elution profiles of β-D-galactosylated glucosyl- and maltosyl-cyclomalto-oligosaccharides on a reversed-phase column, YMC-Pack A-312. Chromatographic conditions: eluent, MeOH–H_2O (6:94 for αCD- and γCD-series, and 7:93 for βCD-series); flow rate, 1 mL/min; temperature, 25°C.

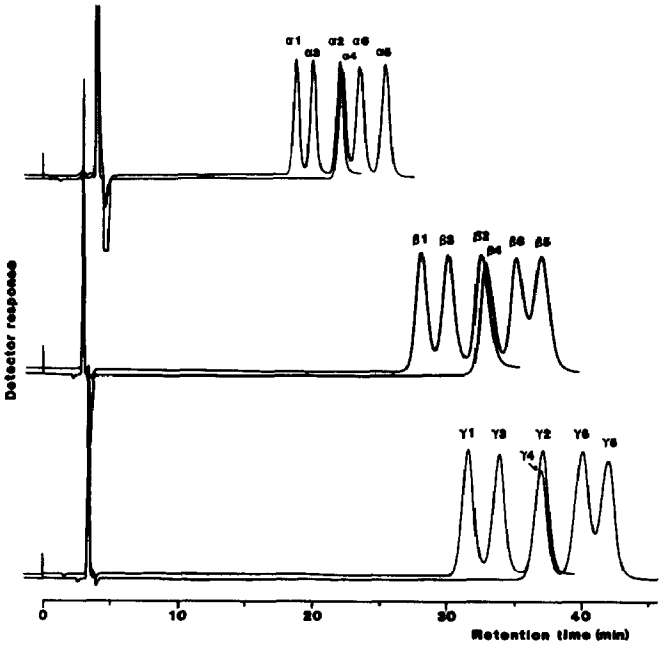

Fig. 4. Elution profiles of β-D-galactosylated glucosyl- and maltosyl-cyclomalto-oligosaccharides on an amino column, Asahipak NH2P-50. Chromatographic conditions for αCD-series: eluent, MeCN–H_2O (65:35); flow rate, 0.6 mL/min; for βCD-series: eluent, MeCN–H_2O (64:36); flow rate, 0.8 mL/min; for γCD-series: eluent, MeCN–H_2O (65:35); flow rate, 0.7 mL/min; temperature, 40°C for all series.

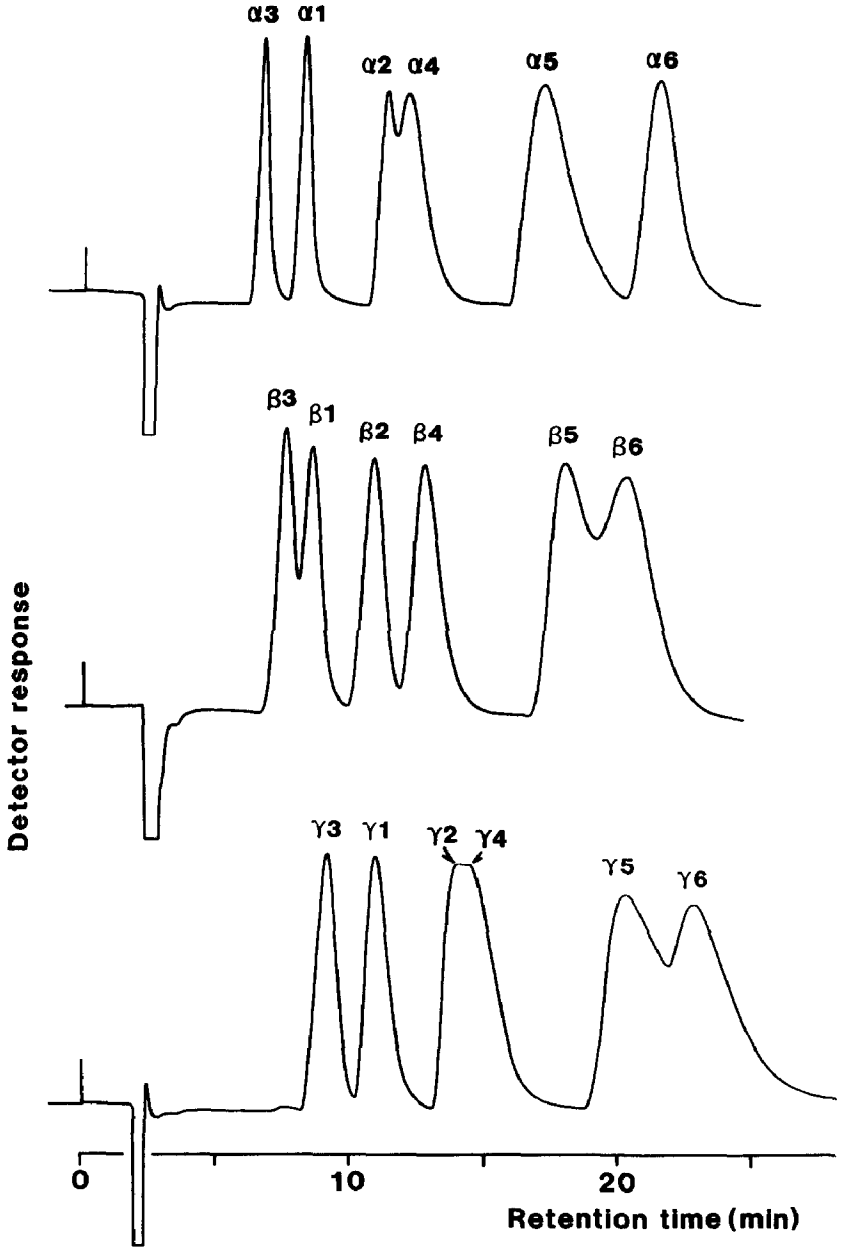

Fig. 5. Elution profiles of β-D-galactosylated glucosyl- and maltosyl-cyclomalto-oligosaccharides on a graphitized carbon column, Hypercarb. Chromatographic conditions for αCD-series: eluent, MeCN–H_2O (15:85); flow rate, 0.6 mL/min; temperature, 50°C; for βCD-series: eluent, MeCN–H_2O (15:85); flow rate, 0.6 mL/min; temperature, 30°C; for γCD-series: eluent, MeCN–H_2O (16:84); flow rate, 0.7 mL/min; temperature, 40°C.

TABLE X

Specific rotations of galactosylated glucosyl- and maltosyl-cyclomalto-oligosaccharides in water

Compound	$[\alpha]_D$		
	(°)	Temperature (°C)	Concentration (%)
α1	134.4	26	0.6
α2	127.3	26	0.9
α3	131.1	26	1.1
α4	142.1	28	1.0
α5	128.9	28	0.5
α6	137.0	24	1.2
β1	154.0	28	1.0
β2	135.0	28	1.0
β3	145.0	28	1.0
β4	157.8	23	1.2
β5	136.7	23	0.5
β6	150.0	26	1.1
γ1	160.8	21	1.3
γ2	143.2	23	0.8
γ3	156.6	26	1.0
γ4	167.1	21	1.3
γ5	150.4	24	0.7
γ6	157.3	21	0.8

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