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ISOLATION AND CHARACTERIZATION OF BRANCHED CYCLODEXTRINS

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ABSTRACT

Three branched cyclodextrins (CDs) were isolated by high-performance liquid chromatography (l.c.) from the mother liquors of a large-scale preparation of the unbranched CDs with *Bacillus ohbensis* cyclomaltodextrin glucanotransferase. Evidence from chromatographic behavior on three l.c. columns of different separation modes, fragmentation analysis, ^{13}C-n.m.r. spectroscopy, methylation analysis, and fast-atom bombardment–mass spectrometry (f.a.b.–m.s.) indicated that these compounds were 6-*O*-α-D-glucopyranosylcyclomaltohexaose (**1**), 6-*O*-α-D-glucopyranosylcyclomaltoheptaose (**2**), and 6,6‴-di-*O*-α-D-glucopyranosylcyclomaltoheptaose (**3**).

INTRODUCTION

Branched cyclodextrins (cyclomalto-oligosaccharides, cycloamyloses) were discovered by French *et al.*[1] in cyclodextrin (CD) mixtures produced by action of *Bacillus macerans* amylase on starch. Since then, various branched CDs having purely α-(1→4)-linked rings with an α-(1→6) linked D-glucose or (1→4)-α-D-glucooligosaccharide side chain have been reported[2–7]. However, the separation techniques used in these studies were mainly gel-permeation chromatography and paper chromatography, and exact separation of mixtures of CDs into their components was therefore very difficult.

Recently, we found that a C_{18}-bonded silica column eluted by methanol–water was very useful for fine separations of cyclic oligo- and poly-saccharides[8,9]. Application of this technique for resolution of a CD mixture produced by action of cyclomaltodextrin glucanotransferase (CGTase), prepared from a culture filtrate of *Bacillus ohbensis*[10] acting on potato starch led to isolation of three branched CDs, 6-*O*-α-D-glucopyranosylcyclomaltohexaose (**1**), 6-*O*-α-D-glucopyranosylcyclomaltoheptaose (**2**), and 6,6‴-di-*O*-α-D-glucopyranosylcyclomaltoheptaose (**3**).

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The third compound, **3**, has two D-glucose branches in the molecule, and was not entirely unexpected.

Compounds **1–3** crystallized from water. Their structures were established by chromatographic behavior on three l.c. columns of different separation modes, by l.c. of partial hydrolyzates, by ^{13}C-n.m.r. spectroscopy, and by methylation analysis. Their molecular weights were confirmed by f.a.b.–m.s.

EXPERIMENTAL

Materials. — Potato starch was purchased from Tokai Starch Co., Ltd. Glucoamylase (GNL-2, *Aspergillus niger*) used for digestion of a mixture of CDs and malto-oligosaccharides was kindly donated by Amano Pharmaceutical Co., Ltd. CGTase was prepared from the culture filtrate of *Bacillus ohbensis* as reported previously[10]. All reagents were of analytical-reagent grade. Reagent-grade organic solvents used for chromatography were dried and freshly distilled before use. Water used in solvent preparations was deionized and distilled.

General methods. — Melting points were measured with a micro m.p. apparatus (Yanagimoto, Kyoto, Japan) and are uncorrected. Optical rotations were determined with a digital polarimeter DIP-4 (Jasco, Tokyo, Japan).

L.c. analysis of CDs and their partial hydrolyzates were performed with a Tri Rotar SR-1 pump, a VL-614 variable-loop injector (both from Jasco), and an SE-31 refractive index (r.i.) monitor (Showa Denko, Tokyo, Japan). Preparative l.c. was performed with a Twincle pump and a VL-611 variable-loop injector (Jasco) with an SE-11 r.i. monitor (Showa Denko) or a KHD-W-104 mini-micro pump (Kyowa Seimitsu, Tokyo, Japan) with a Waters RI-403 detector (Waters Assoc., Milford, MA, U.S.A.). The columns used for l.c. were a Hibar LiChrosorb NH_2 (250 × 4 mm i.d.), a Hibar LiChrosorb RP-18 (250 × 4 mm i.d.) (both from Merck, Darmstadt, F.R.G.), an Asahipak GS-320 (500 × 7.6 mm i.d.) (Asahi Kasei, Tokyo, Japan), and an ERC-NH-1171 (200 × 6 mm i.d.) (Erma Optical Works, Tokyo, Japan). For preparative chromatography, a column packed with LiChroprep RP-18 (5–20 μm, 30 × 2 cm i.d.) (Merck) was used. A column (120 × 1.8 cm i.d.) packed with Toyopearl HW-40S (Toyo Soda, Tokyo, Japan) was also used for pre-fractionation of CDs with a peristaltic pump AC-2120 (Atto, Tokyo, Japan). L.c. analyses at constant temperature were conducted by the use of a column oven TU-300 (Jasco).

Thin-layer chromatography (t.l.c.) was performed on Silica gel 60 TLC plates (Merck) with 6:2:5:3 1-propanol–ethyl acetate–water–25% ammonia. After two ascents, chromatograms were sprayed with 1% methanolic I_2 solution. When treated in this way, cyclomaltohexaose ("α-CD") gave a characteristic purple color, and cyclomaltoheptaose ("β-CD"), and cyclomaltooctaose ("γ-CD") gave yellow spots.

Fragmentation analysis. — A CD sample (3 mg) was partially hydrolyzed in 1 mL of 0.5M trifluoroacetic acid for 45–90 min at 100°. The solution containing the hydrolyzate was evaporated to dryness under diminished pressure. The residue was dissolved in 100 μL of water and aliquots (2 μL) were analyzed by l.c.

N.m.r. spectra. — ^{13}C-N.m.r. spectra were recorded with a JNM-FX 200 Fourier-transform n.m.r. spectrometer (Jeol, Tokyo, Japan). The samples were dissolved in $^{2}H_2O$ at a concentration of 2–3% in a micro cell. Chemical shifts are expressed in p.p.m. downfield from that of tetramethylsilane, using 1,4-dioxane (67.40 p.p.m.) as the internal standard. The machine parameters induced a spectrum frequency of 50.10 MHz, 16 K memory, 45° pulse, 0.682 s between pulses, a sweep width of 12,004 Hz, 16,384 data points, and a recycle of 5,000. The spectra were recorded at ambient temperature. The delay time Δ in the INEPT (Insensitive Nuclei Enhanced by Polarization Transfer) method[11] was 5.1 ms (3/4J).

Methylation analysis. — A branched CD sample (10 mg) was methylated by the Hakomori procedure[12] and then hydrolyzed. The hydrolyzates were converted into the corresponding alditol acetates[13] and analyzed by gas chromatography–mass spectrometry (g.l.c.–m.s.). G.l.c.–m.s. was conducted with an MS-SL05 gas chromatograph, coupled directly to the source of a JMS DX-300 mass spectrometer (Jeol). The gas chromatograph was equipped with a 2 m × 2.6 mm (i.d.) glass column filled with 3% SE-30 on Chromosorb W (AW-DMCS, 80–100 mesh) and the column temperature was maintained at 180°. Low-resolution e.i. mass spectra were obtained at an ionizing energy of 20 eV, an ionization current of 300 μA, an accelerating voltage of 3 kV, and an ion-source temperature of 270°.

F.a.b.–m.s. was performed with a JMS-HX100 mass spectrometer (Jeol) using xenon atoms having a kinetic energy equivalent to 6 keV. The mass marker was calibrated with CsI and glycerol was used as the matrix solution. The branched CDs were analyzed in the positive-ion and negative-ion modes.

Preparation of cyclodextrins. — A 10% (w/v) solution of potato starch (100 kg) and CGTase (10 units/g of starch) were incubated at 55° and pH 7.6 for 40 h in a 2-kL tank. The enzyme reaction was stopped by boiling for 10 min, and the broth was concentrated to 50 L by evaporation and cooled to ~10°. Cyclomaltoheptaose crystallized (21 kg) and was removed by filtration. The filtrate was treated with 0.01% (w/v) of glucoamylase GNL-2 for 5 h at 40° and then concentrated to 5 L by a reverse-osmosis apparatus RO-B (Ulvac Service, Tokyo, Japan) with an AS-215 module (Koch Membrane Systems Inc. Wilmington, MA, U.S.A.) into which substances of molecular weight <500 can permeate. By this treatment, cyclomaltooctaose and the higher homologs were degraded, as the glucoamylase used was impure. Acetone (10 L) was added to the concentrated broth and the cycloheptaamylose precipitate was discarded by filtration. An additional 15 L of acetone was added and the resulting precipitate was collected by filtration and dried: the yield of crude CD mixture was 500 g.

Separation of CDs. — The crude CDs (500 mg) were fractionated by gel-permeation chromatography on a column of Toyopearl HW-40S with water at a flow-rate of 0.4 mL/min. The CDs in 6-mL fractions were monitored by t.l.c., and fractions 22–23, which contained CDs giving yellow spots, and fractions 25–29 which contained CDs giving purple spots were combined, respectively, and evaporated: the yield of dry powder from fractions 22–23 (product B) was 145.3 mg and

from fractions 25–29 (product A) was 204.8 mg. Product A was mainly composed of a branched CD (compound **1**), with small amounts of cyclomaltohexaose and noncyclic oligosaccharides as contaminants. Product B was a mixture of two branched CDs (compounds **2** and **3**) in the respective ratios of ~1:2. Each product was subjected to preparative l.c. on a column of Hibar LiChroprep RP-18 with 2–3% methanol at a flow rate of 5 mL/min, and the branched CDs isolated were further purified by semipreparative l.c. on a column of Asahipak with water for **1** and with 5% methanol for **2** and **3**.

RESULTS AND DISCUSSION

The mother liquors from a large-scale preparation of cyclomaltoheptaose using CGTase from *Bacillus ohbensis* were treated with glucoamylase to hydrolyze the noncyclic dextrins and oligosaccharides, and shorten the side chains of the branched CDs. After removing the D-glucose by reverse osmosis, the remaining substances were divided by gel-permeation chromatography into two fractions, one of which contained cyclomaltohexaose derivatives and the other cyclomaltoheptaose derivatives. Each fraction was subjected to preparative l.c. on a C_{18}-bonded silica column and three branched CDs (compounds **1, 2,** and **3**) were isolated. In order to remove minor, unknown contaminants and non-cyclic dextrins that had escaped the digestion, a column of unique separation mode, Asahipak GS-320, was used. The Asahipak column is a gel-permeation column for l.c., packed with a synthetic polymer type of hydrophilic hard gel having many hydroxyl groups. The exclusion limit (pullulan) of GS-320 is 40,000. It interacts weakly with solutes and permits the separation of solutes having similar molecular weights from each other.

The chromatographic behavior of the branched CDs was compared with that of the well-recognized cyclomalto-hexa-, hepta-, and octa-oses. Figs. 1, 2, and 3 show the l.c. elution profiles of a standard mixture of CDs on Hibar Lichrosorb NH_2 (Fig. 1), on Hibar LiChrosorb RP-18 (Fig. 2), and on Asahipak GS-320 (Fig. 3). The elution sequence with the NH_2-bonded silica and acetonitrile–water system follows the order of molecular size. The elution time gives qualitative information about the molecular size of sugar[14]. Compound **1** probably has the same number of glucose units as cyclomaltoheptaose, seven, **2** has eight glucose units like cyclomaltooctaose, and **3** has one more glucose units than **2**, nine. In contrast, C_{18}-bonded silica is a reversed phase and so the separation mechanism is probably an example of hydrophobic chromatography, namely, increased retention with decreasing solubility in water[15]. It may be predicted from the retention times in Fig. 2 that **1** would be more soluble in water than cyclomaltooctaose, known as the most soluble CD, and the solubilities of **2** and **3** are intermediate between those of cyclomalto-hexa- and hepta-ose and **3** having a larger molecular size is more soluble than the analogue, **2**. The elution profile of CDs on Asahipak GS-320 resembles that on C_{18}-bonded silica, except the elution order of **1** and cyclomaltooctaose is reversed, but **1** and non-cyclic maltohexaose, which are eluted together from the

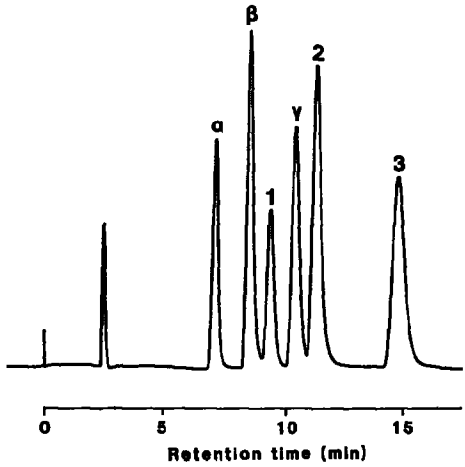


Fig. 1. Separation of a standard mixture of cyclodextrins on NH_2-bonded silica. Compounds were **1, 2, 3,** and cyclomalto-hexaose ("α"), -heptaose ("β"), and -octaose ("γ"). Chromatographic conditions: column; Hibar LiChrosorb NH_2 (250 × 4 mm i.d.), eluent; 3:2 acetonitrile–water, flow-rate; 1 mL/min, detector; Shodex RI SE-31 at 2×10^{-5} RI units full scale, temperature; ambient.

C_{18}-bonded silica column, are separable on Asahipak GS-320; maltohexaose elutes faster than cyclomaltooctaose.

L.c. analyses of partially hydrolyzed CDs offered further corroborating evidence relating to the structure of branched CDs. Figs. 4[1], 4[2], 5[1], and 5[2] illustrate separations of the partial hydrolyzates of cyclomaltohexaose **1**, cyclomaltoheptaose, and **2**. As may be seen from Figs. 4[1] and 5[1], there is a relation-

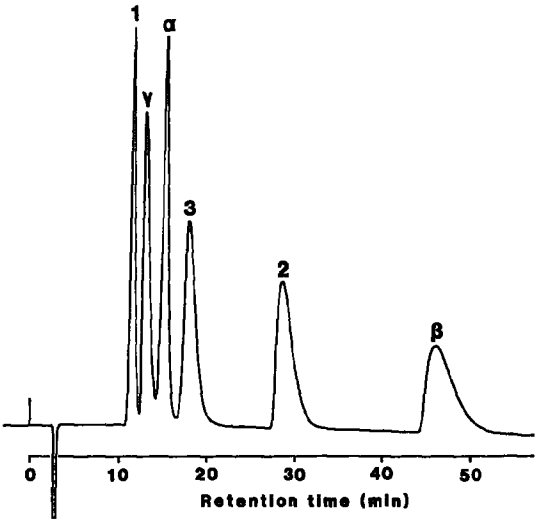


Fig. 2. Separation of a standard mixture of cyclodextrins on C_{18}-bonded silica. Chromatographic conditions: column; Hibar LiChrosorb RP-18 (250 × 4 mm i.d.), eluent; 2:23 methanol–water, temperature; 28°, other conditions as in Fig. 1.

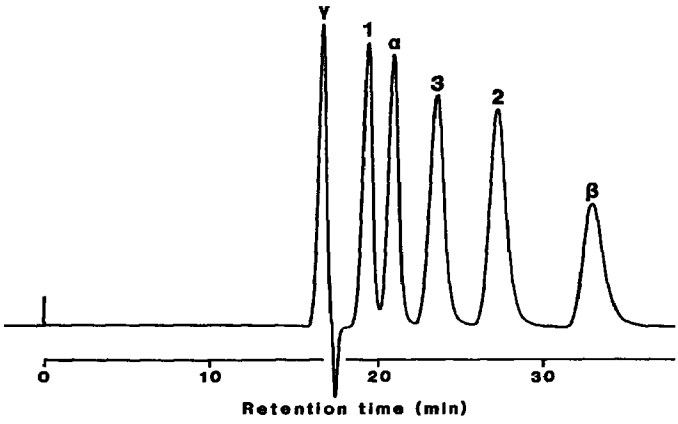
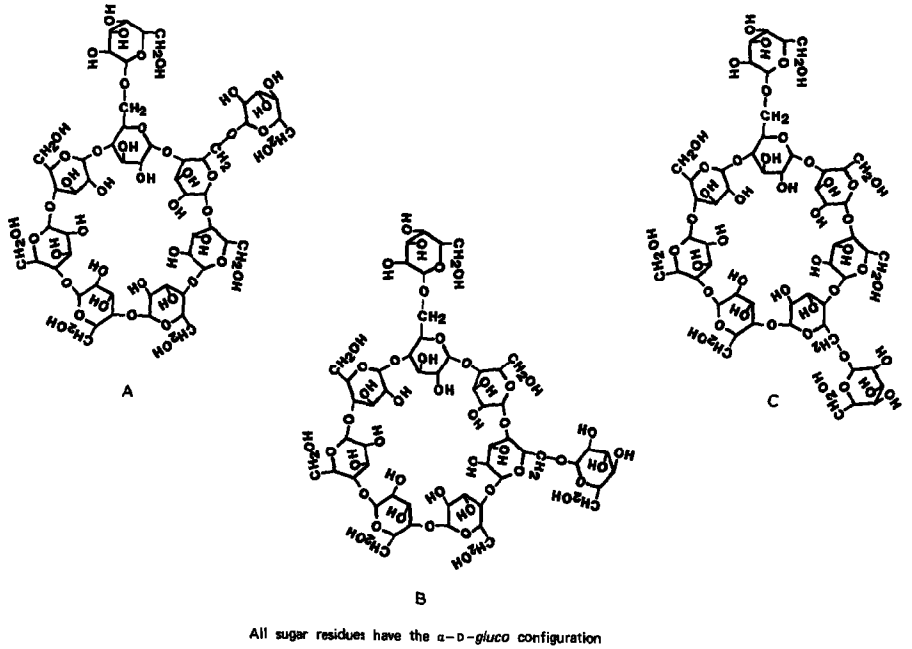


Fig. 3. Separation of a standard mixture of cyclodextrins on an Asahipak GS-320 column (500 × 7.6 mm i.d.). Chromatographic conditions: eluent; 1:19 methanol–water, other conditions as in Fig. 1.

ship between the degree of polymerization (d.p.) of a series of malto-oligosaccharides and their retention times (t_R). A plot of $\log t_R$ against d.p. gave a straight line (see Fig. 7). Previously[16], we found that other series of homogeneous D-gluco-oligosaccharides also showed individual linear relationships between $\log t_R$ and d.p., and saccharides having 1→6 linkages were retained longer than the corresponding (1→4)-linked saccharides. In the chromatogram of the partial hydrolyzates of **1** (Fig. 4[2]), the peak of cyclomaltohexaose can be observed. Consequently, **1** is a derivative of cyclomaltohexaose. This concept is also reinforced by the purple color of the iodine complex on t.l.c. In addition, another series of saccharides (d.p. 2–7) having a linear relationship between $\log t_R$ and d.p. can be seen together with the series of malto-oligosaccharides (d.p. 2–6) in Fig. 4[2]. Each saccharide in the former series may be a malto-oligosaccharide containing one 1→6 linkage. The information obtained from Fig. 4[2] suggests that **1** is 6-*O*-D-glucosylcyclomaltohexaose. The peaks of cyclomaltoheptaose, a series of malto-oligosaccharides (d.p. 2–7), and another series of malto-saccharides each containing one 1→6 linkage (d.p. 2–8) may be seen in Fig. 5[2]. Compound **2** therefore, seems to be 6-*O*-D-glucosylcyclomaltoheptaose. Fig. 6 shows the chromatogram of partial hydrolyzates of **3**, in which the peaks of cyclomaltoheptaose, **2**, and three series of linear saccharides may be found. Compound **3** should have the structure of **2**, with one more glucose unit attached. As treatment of **3** with glucoamylase does not give **2**, the side chain of **3** is not maltose. Compound **3** should be a diglucosylcyclomaltoheptaose whose structure is one of the three (A, B, and C) schematically illustrated. Among three series of linear saccharides in the partial hydrolyzates of **3**, the third series, each of which seems to contain two 1→6 linkages, consists of four members and the smallest member has d.p. 6; it was confirmed by changing the conditions of hydrolysis and chromatography that the member having d.p. 5 or 4 was apparently ab-



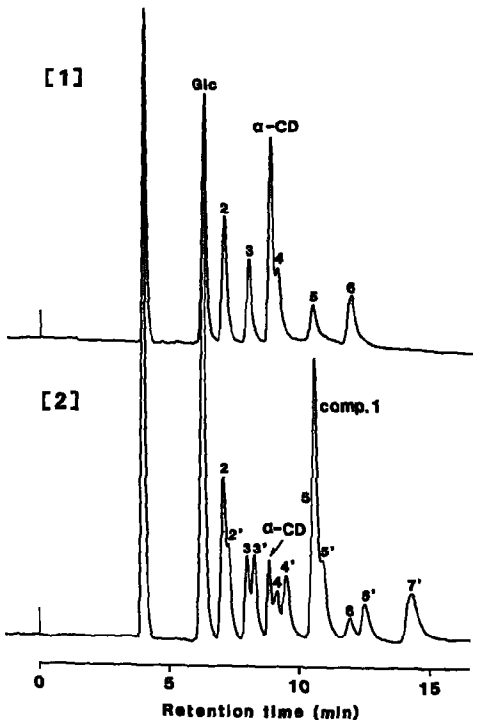


Fig. 4. Chromatograms of partial hydrolyzates of cyclomaltohexaose [1] and compound **1** [2]. Glc = glucose, 2–6 = malto-oligosaccharides (d.p. 2–6), 2′–7′ = malto-oligosaccharides each containing one 1→6 linkage (d.p. 2–7). Chromatographic conditions: column; ERC-NH-1171 (200 × 6 mm i.d.), eluent; 63:37 acetonitrile–water, other conditions as in Fig. 1.

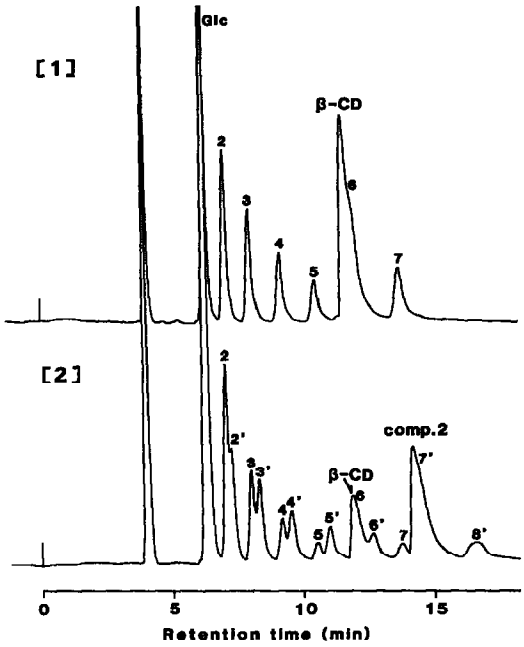


Fig. 5. Chromatograms of partial hydrolyzates of cyclomaltoheptaose [1] and compound **2** [2]. Glc = glucose, 2–7 = malto-oligosaccharides (d.p. 2–7), 2′–8′ = malto-oligosaccharides each containing one 1→6 linkage (d.p. 2–8). Chromatographic conditions as in Fig. 4.

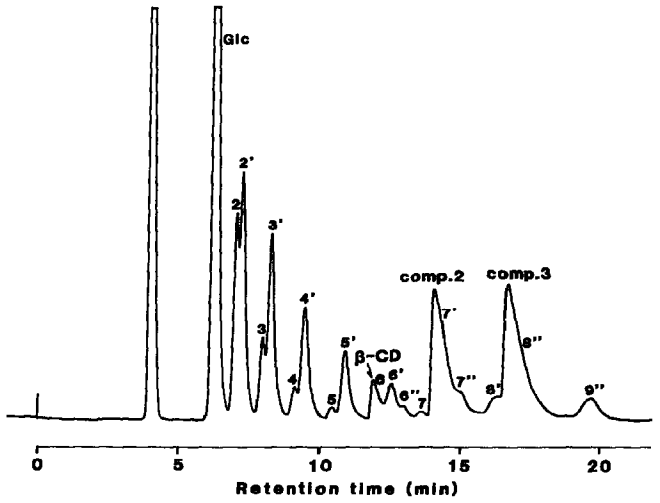


Fig. 6. Chromatogram of partial hydrolyzates of compound **3**. Glc = glucose, 2–7 = malto-oligosaccharides (d.p. 2–7), 2′–8′ = malto-oligosaccharides containing each one 1→6 linkage (d.p. 2–8), 6″–9″ = malto-oligosaccharides containing each two 1→6 linkages (d.p. 6–9). Chromatographic conditions as in Fig. 4.

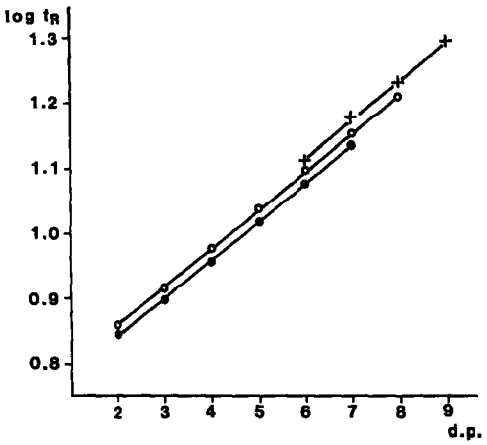


Fig. 7. Relationship between $\log t_R$ and d.p. ●——●; purely α-(1→4)-linked gluco-oligomers, ○——○; α(1→4)-linked gluco-oligomers each containing one 1→6 linkage, ×——×; α-(1→4)-linked gluco-oligomers each containing two 1→6 linkages. Chromatographic conditions as in Fig. 4.

TABLE I

CHEMICAL SHIFTS OF C-1 AND C-6 IN THE ^{13}C-N.M.R. SPECTRA OF BRANCHED CYCLODEXTRINS IN ^2H_2O

Compound	*C-1*[a]	*C-1'*[b]	*C-6*[a]	*C-6'*[b]	*C-6"*[c]
1	102.21	99.72	61.21	61.59	67.84
2	102.67	99.81	61.00	61.27	67.81
3	102.65	99.89	61.01	61.18	67.75

[a]C-1 and C-6: the carbon atoms of the ring D-glucose residue. [b]C-1' and C-6': the carbon atoms of the branched residue. [c]C-6": the carbon atom of the branch point.

TABLE II

RELATIVE RETENTION-TIMES[a] AT 180° AND PRIMARY FRAGMENTS IN THE MASS SPECTRA OF PARTIALLY METHYLATED GLUCITOL ACETATES, PRODUCTS OF METHYLATION ANALYSIS

Product	*Relative* t_R	m/z					
		45	*117*	*161*	*205*	*233*	*261*
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol	1.00	○	○	○	○		
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol	1.46	○	○	○		○	
1,4,5,6-Tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol	2.23		○				○

[a]Column: 3% SE-30 on Chromosorb W (AW-DMCS, 80–100 mesh).

TABLE III

PHYSICAL AND ANALYTICAL DATA[a] FOR THE BRANCHED CYCLODEXTRINS

Branched cyclodextrin	*M.p.* (°)	$[\alpha]_D^{22}$ *in* H_2O (°)	*Formula*	*Elemental analysis*				*Molecular weight*	
				Calc.		*Found*		*Calc.*	*Found by f.a.b.–m.s.*
				C	*H*	*C*	*H*		
6-*O*-α-D-Glucosyl-cyclomaltohexaose	>283[b] (dec.)	+151.1	$C_{42}H_{70}O_{35} \cdot 3\,H_2O$	42.42	6.44	42.13	6.35	162 × 7	1134
6-*O*-α-D-Glucosyl-cyclomaltoheptaose	>283[b] (dec.)	+164.3	$C_{48}H_{80}O_{40} \cdot 4\,H_2O$	42.11	6.48	41.92	6.40	162 × 8	1296
6,6‴-Di-*O*-α-D-Glucosyl-cyclomaltoheptaose	289–290 (dec.)	+169.7	$C_{54}H_{90}O_{45} \cdot 5\,H_2O$	41.86	6.51	41.78	6.28	162 × 9	1458

[a]For reference, the specific rotations of cyclomaltohexaose, cyclomaltoheptaose, and cyclomaltooctaose determined under the same conditions were +150.8°, +163.0°, and +174.7°, respectively. [b]Not clear.

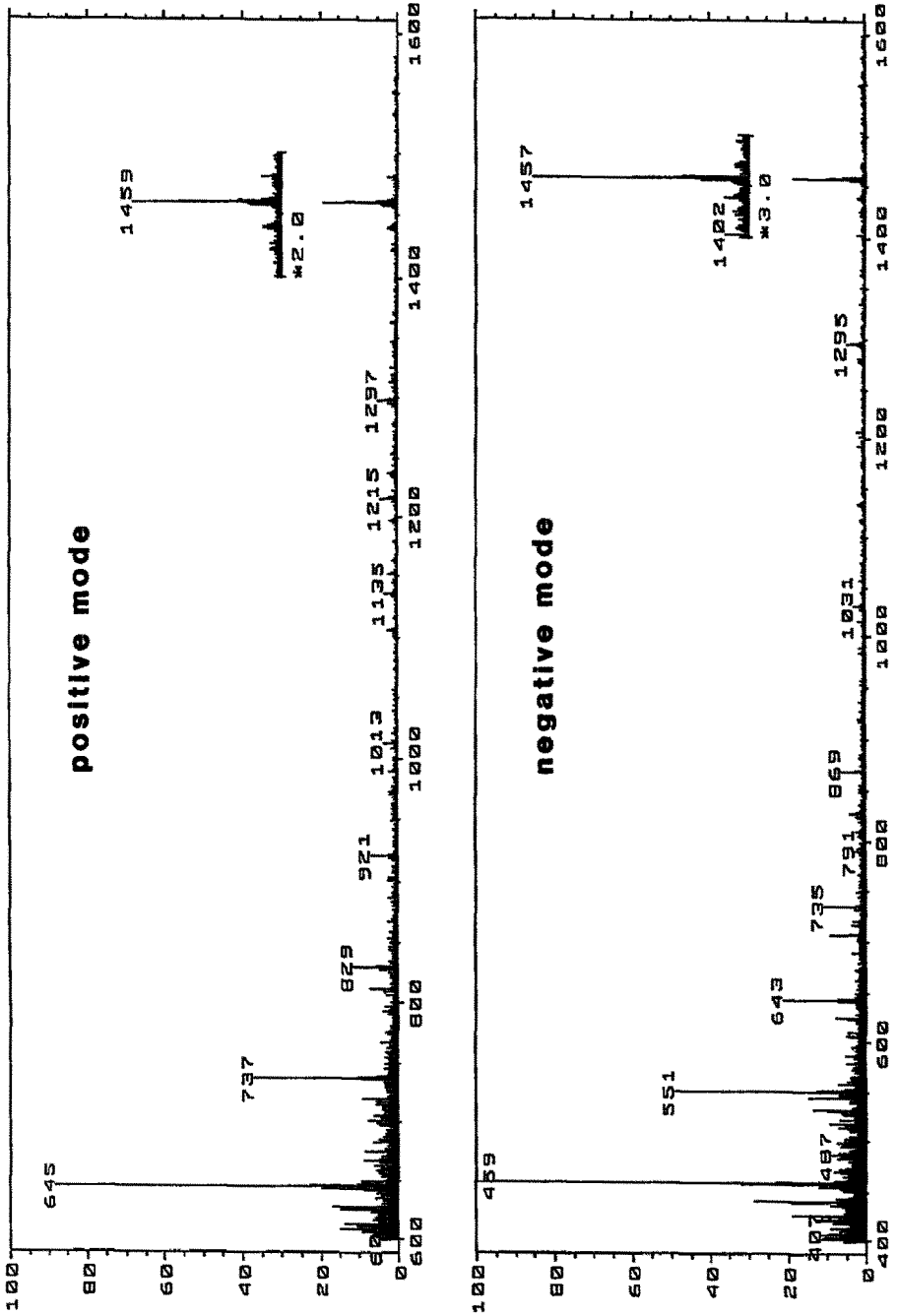


Fig. 8. F.a.b.–mass spectra of compound **3** in positive and negative modes.

sent. This fact indicates that the structure of **3** is **c**, namely 6,6‴-di-*O*-D-glucosylcyclomaltoheptaose.

In the ^{13}C-n.m.r. spectrum of each of the branched CDs, two kinds of C-1 signal and three kinds of C-6 signal were observed. Assignments of these signals are given in Table I. Coupling constants ($J_{^{13}C-^{1}H}$) of both C-1 atoms are 170 Hz and therefore, the configuration of C-1 in the branched residue is α, as well as that of C-1 in the CD ring. The integrated ratios in the intensities of ring C-1 signal and side-chain C-1 signals are 6:1 for **1**, 7:1 for **2**, and 7:2 for **3**. The assignments of three kinds of C-6 signals were confirmed by the INEPT method, using $\Delta = 3/4J$. A large downfield shift of one C-6 signal (~68 p.p.m.) indicates that the side-chain D-glucose residue is attached to oxygen on this carbon atom; it is known that a substituent on the oxygen atom attached to any carbon atom of sugar affects[17] the chemical shift of that carbon atom, moving it downfield by 8–11 p.p.m. The ratios in the signal intensities of ring C-6, side chain C-6, and branch-point C-6 were 5:1:1 for **1**, 6:1:1 for **2**, and 5:2:2 for **3**.

The partially methylated D-glucitol acetates obtained by the sequence of methylation, hydrolysis, reduction, and acetylation of the branched CDs were characterized by g.l.c.–m.s.[18] (Table II). The products from three branched CDs were the same: 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, and their molecular ratios were 1.00:5.23:1.04 for **1**, 1.00:6.14:0.95 for **2**, and 2.00:5.13:1.86 for **3**.

Fig. 8 shows typical f.a.b. mass spectra of **3** in the positive and negative modes. As may be seen in Fig. 8, the production of strong $(M + H)^+$ and $(M - H)^-$ made it easy to identify the molecular weight. In the same manner, the molecular weights of **1** and **2** were unambiguously determined (Table III).

CONCLUSION

Evidence from chromatographic behavior, fragmentation analysis, ^{13}C-n.m.r. spectroscopy, methylation analysis, and f.a.b.–m.s. were in harmony with the concept that the branched CDs (**1**, **2**, and **3**) isolated from the mother liquors of a large-scale preparation of β-CD are 6-*O*-α-D-glucosylcyclomaltohexaose, 6-*O*-α-D-glucosylcyclomaltoheptaose, and 6,6‴-di-*O*-α-D-glucosylcyclomaltoheptaose. Their physical and analytical data are summarized in Table III.

Thus far, 6-*O*-α-D-glucosylcyclomaltohexaose[6] and 6-*O*-α-D-glucosylcyclomaltoheptaose[7] have been isolated, but their purities are suspect. In this work we have obtained them, together with a novel branched CD, 6,6‴-di-*O*-α-D-glucosylcyclomaltoheptaose, in a high state of purity. The novel CD, having two branches in a molecule, crystallizes very readily from water.

ACKNOWLEDGMENTS

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