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Isolation and characterization of di- and tri-mannosyl-cyclomaltoheptaoses (β-cyclodextrins) produced by reverse action of α-mannosidase from jack bean

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Abstract

Di- and tri-mannosyl-cyclomaltoheptaoses (β-cyclodextrins, βCDs), which were synthesized together with mono-mannosyl-βCD in a large-scale production by reverse action of α-mannosidase from jack bean, were isolated and purified by HPLC. The structures of seven isomers of di-mannosyl-βCD and six isomers of tri-mannosyl-βCD were elucidated by FABMS and NMR spectroscopy, and by enzymatic methods. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: Di-mannosyl-cyclomaltoheptaose; Tri-mannosyl-cyclomaltoheptaose; Jack bean α-mannosidase; HPLC; FABMS; NMR; Enzymatic method

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1. Introduction

To develop new applications different from those associated with conventional cyclomalto-oligosaccharides (cyclodextrins, CDs) and homogeneous branched CDs such as glucosyl-CD and maltosyl-CD, we have synthesized heterogeneous branched CDs. In our previous papers, we reported that various α- and β-galactosidases and α-mannosidases produced galactosyl- and mannosyl-transfer products of CDs, i.e., glucosyl-CDs or maltosyl-CDs, respectively [1–5]. These heterogeneous branched CDs are expected to be useful as the possible drug carriers in drug delivery systems because D-galactose and D-mannose on the non-reducing terminal of sugar chains are recognized by animal lectins [6,7], and thus the galactosyl and mannosyl side chains of those heterogeneous branched CDs can be utilized as ligands for targeting of drugs. Recently we found that an α-mannosidase from jack bean linked the mannosyl residue directly to the CD ring by reverse action, and we synthesized novel branched CDs [8]. As multibranched CDs seem to be more interesting from the standpoint of cluster ligands for animal lectin studies, this paper deals with isolation and

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characterization of di- and tri-mannosyl-βCDs which were synthesized together with mono-mannosyl-βCD in large-scale production by reverse action of α-mannosidase from jack bean.

2. Experimental

Materials.—βCD was used as an acceptor substrate and was supplied by Bio Research Corporation of Yokohama. Mannose was used as a donor substrate and was purchased from Wako Pure Chemical. A crude α-mannosidase was prepared from jack bean meal (Sigma Chemical) as follows: 20 kg of jack bean meal was suspended in 40 L of distilled water, stirred mechanically at 5 °C overnight, and then centrifuged. The precipitates were suspended with 40 L of distilled water once more, stirred at 5 °C for 2 h, and centrifuged. To the supernatant (75 L), ethanol (18 L) was added in an ice-bath. After 1 h, precipitates were collected by centrifugation and dissolved in 16 L of 50 mM citrate buffer (pH 4.5). α-Mannosidase activity of this solution was 80.3 U/mL. Cyclomaltodextrin glucanotransferase (CGTase, EC 2.4.1.19)(2000 U/mL) from *Bacillus circulans* was prepared and purified by the previously reported method [9]. An α-glucosidase preparation from *Aspergillus niger*, 'Transglucosidase Amano' used for analyses by enzymatic methods, was purchased from Amano Pharmaceutical. Reagent-grade organic solvents used for chromatography were freshly distilled before use. Water used in solvent preparations was distilled, deionized, and redistilled.

Preparation and isolation of di- and tri-mannosyl-βCDs.—Mannose (15 kg) and βCD (5 kg) in 30 L of 50 mM citrate buffer (pH 4.5) were incubated with 401,250 U of jack bean α-mannosidase at 55 °C for 11 days. Then, the reaction mixture was heated at 100 °C for 10 min to stop the enzyme action. Using an ODS column (360 × 26 mm i.d., Organo) with water, mannose and manno-oligosaccharides were removed from the reaction mixture, and then βCD and branched βCDs were eluted from the column with 1:9 ethanol–water. After removing most of βCD by crystallization, a mannosyl-βCD mixture (462 g) containing mono-, di-, and tri-mannosyl-βCDs in ratios of 65.7%:24.0%:5.9%, was obtained by preparative HPLC with a Hitachi L-6000 pump, using a YMC-pack ODS-AQ column (500 × 100 mm i.d., YMC) with methanol–water. The mixture was fractionated by HPLC on a TSKgel Amide-80 column (300 × 21.5 mm i.d., Tosoh) to mono-, di-, and tri-mannosyl-βCDs.

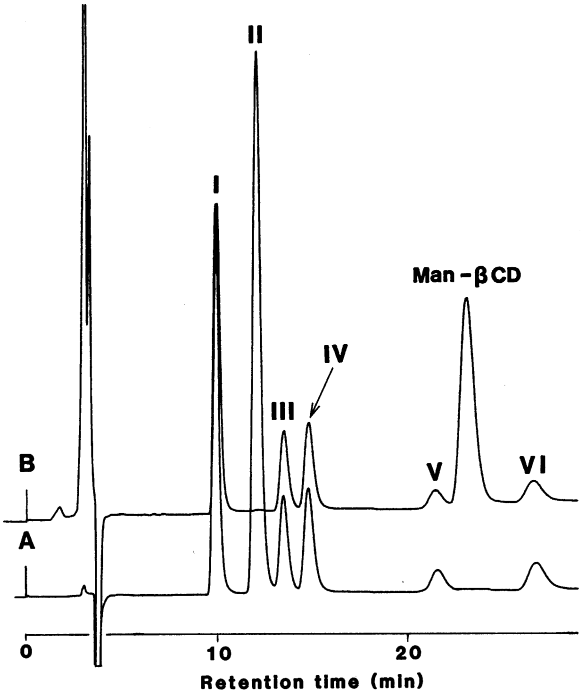


Fig. 1. Chromatograms of a mixture of dimannosyl-substituted βCDs, (A) original, and (B) after treatment with an α-glucosidase preparation from *A. niger*. Chromatographic conditions: column, Daisopak ODS-BP (150 × 6 mm i.d.); eluent, 5:95 CH_3OH–H_2O; flow rate, 1 mL/min; temperature, 30 °C.

Table 1
The molecular ion (●) and primary fragments (○) in the FABMS spectra of dimannosyl-βCDs in the negative-ion mode

Compound	*m/z*			Side chain
	113	1295	1457	
I		○	●	dimannosyl-
II	○	○	●	mannobiosyl-
III		○	●	dimannosyl-
IV	○	○	●	mannobiosyl-
V	○	○	●	mannobiosyl-
VI	○	○	●	mannobiosyl-

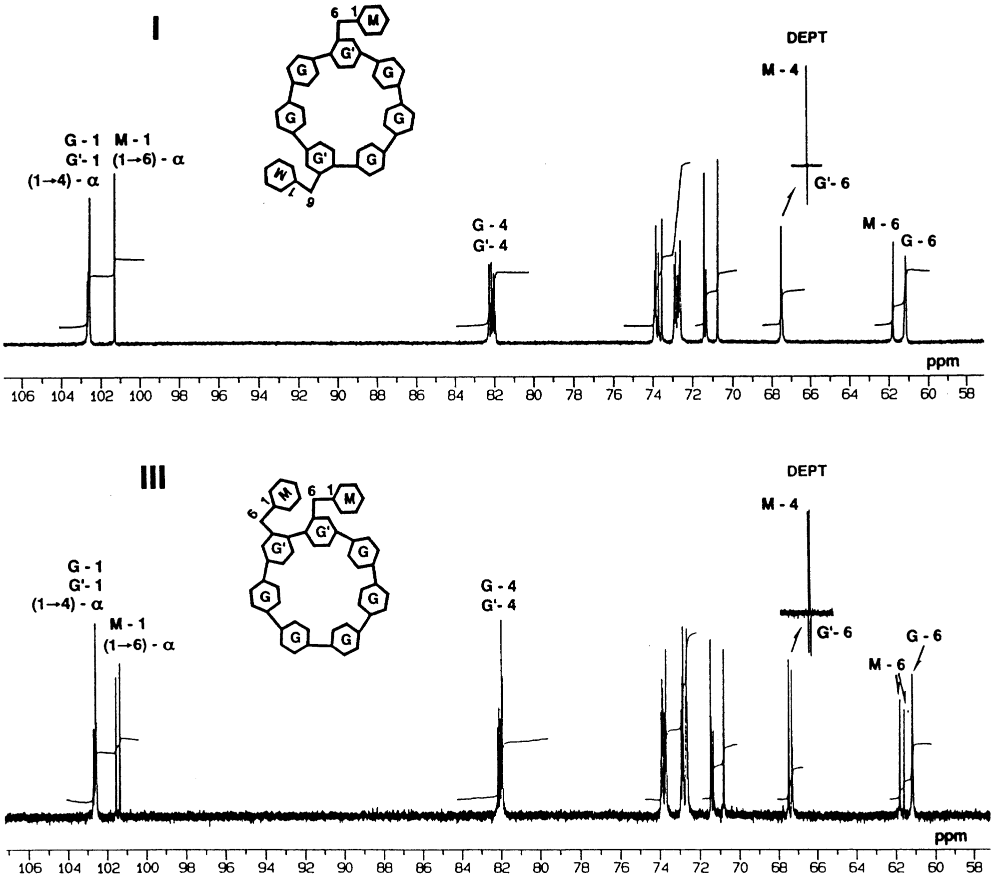


Fig. 2. ^{13}C NMR spectra of **I** and **III** in D_2O at 50 °C. G-1, -4, and -6 are signals of C-1, -4, and -6 atoms of the ring D-glucopyranose units. G′ is the ring D-glucopyranose unit on which the mannopyranosyl residue is linked. M-1, -4, and -6 are signals of C-1, -4, and -6 atoms of the side chain D-mannopyranose unit.

Analysis.—HPLC analyses of the reaction products were performed with a Jasco 980-PU pump and a Shodex RI-71 monitor. The columns used were a TSKgel amide-80 (250 × 4.6 mm i.d., Tosoh), a YMC-pack polyamine-II (150 × 4.6 mm i.d., YMC) and a Daisopak ODS-BP (150 × 6 mm i.d., Daiso). For semipreparative HPLC, a Daisopak ODS-BP (5 μm, 250 × 20 mm i.d., Daiso) and a YMC-Pack A-323-3 (3 μm, 250 × 10 mm i.d., YMC) were used. HPLC analyses at constant temperature were conducted using a CO-1093C column oven (Uniflows). A Shimadzu C-R6A chromatopac was used to calculate peak areas.

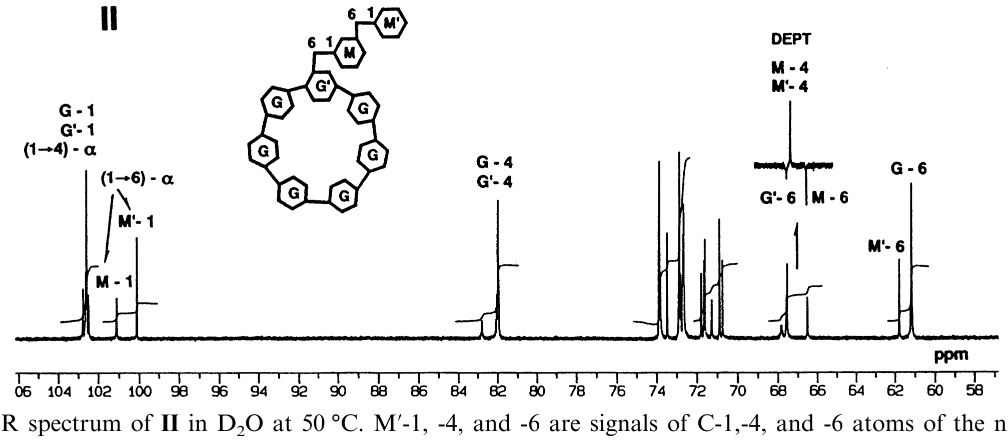


Fig. 3. ^{13}C NMR spectrum of **II** in D_2O at 50 °C. M′-1, -4, and -6 are signals of C-1,-4, and -6 atoms of the non reducing end mannopyranose unit. Other symbols are the same as in Fig. 2.

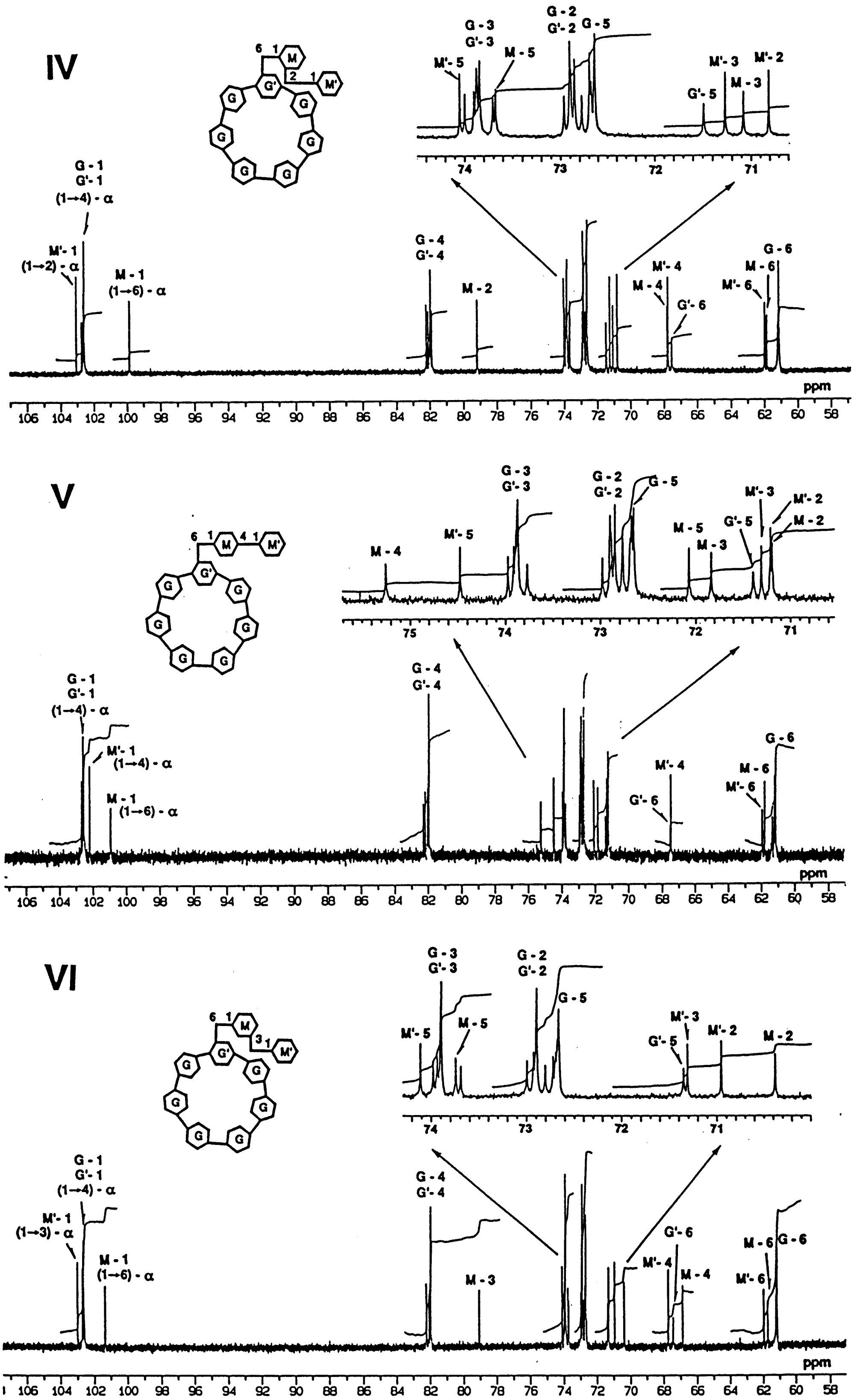


Fig. 4. ^{13}C NMR spectra of **IV**, **V** and **VI** in D_2O at 50 °C. G-1, -2, -3, -4, -5 and -6 are signals of C-1, -2, -3, -5, -4 and -6 atoms of the ring D-glucopyranose units. G′ is the ring D-glucopyranose unit on which the mannopyranosyl residue is linked. M- and M′-1, 2, 3, 4, 5 and 6 are signals of C-1, -2, -3, -4, -5 and -6 atoms of the side chain D-mannopyranose units.

LC–MS was carried out using a Hewlett–Packard HP1050 series model HP79852A pump interfaced to a Finnigan TSQ-7000 triple-stage quadrupole mass spectrometer (Finnigan MAT Instruments, San Jose, CA) fitted with the Finnigan electrospray ionization (ESI) interface. The mass spectrometer was operated in the positive-ion mode: the ESI capillary voltage was set to 4.5 kV, and the ESI current was 0.34 μA. The capillary temperature was 230 °C. The pressure of the sheath gas was 70 psi, and the auxiliary gas was 15 u. Total-ion monitoring was done by scanning which covered the range *m/z* 800–1500 with a scan rate of 2 s/scan.

FABMS was performed in negative-ion mode on a Jeol JMS-DX 303 mass spectrometer using xenon 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 6–10% solutions in D_2O at 50 °C with a Jeol GSX-500 spectrometer. Chemical shifts were expressed in ppm downfield from the signal of Me_4Si referenced to external 1,4-dioxane (67.40 ppm). The other conditions for ^{13}C NMR, ^1H–^1H COSY and ^1H–^{13}C COSY measurements were the same as reported in the previous paper [5].

Discrimination of positional isomers of 6^I,6^n-disubstituted derivatives by enzymatic degradation.—Each sample (0.2 mg) of 6^I,6^n-disubstituted derivatives in 70 μL of 10 mM acetate buffer (pH 6.0) was individually incubated with CGTase (20 μL, 24 U) at 40 °C for 24 h. Then the enzyme was inactivated by placing the sample tube in a boiling water bath for 10 min. Fragments produced by digestion of each sample with CGTase were analyzed by HPLC and LC–MS on a YMC-pack polyamine-II (150 × 4.6 mm i.d.).

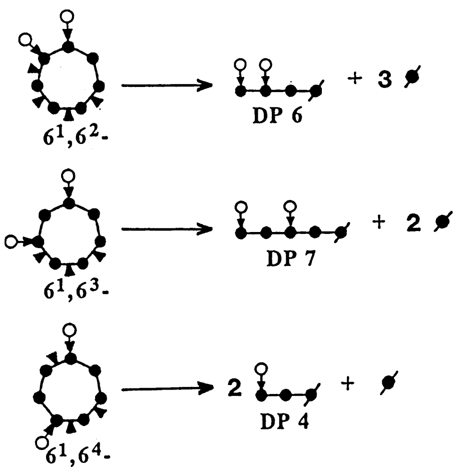
Shortening of mannobiosyl and mannotriosyl side chains with an α-glucosidase preparation, 'Transglucosidase Amano'.—A mixture of dimannosyl-βCDs or of trimannosyl-βCDs (4 mg) in 320 μL of 5 mM citrate buffer (pH 4.5) was incubated with 80 μL of 10 × diluted solution of 'Transglucosidase Amano', which has α-mannosidase activity at 40 °C for 4 h, then the reaction mixture was heated at 100 °C for 10 min to stop the enzyme action.

3. Results and discussion

Isolation and characterization of dimannosyl-βCDs.—*Isolation.* Fig. 1(A) shows the chromatogram of the fraction containing dimannosyl-βCDs on a Daisopak ODS-BP column (5 μm, 150 × 6 mm i.d.) with 5:95 MeOH–water. The ratios of components (**I**–**VI**) calculated from each peak area were 25.9, 45.0, 9.1, 11.5, 3.4 and 5.1%, respectively. These six components were isolated by semipreparative HPLC, using Daisopak ODS-BP (5 μm, 250 × 20 mm i.d.) with 8:92 MeOH–water at flow rate of 3 mL/min.

Characterization. Each component was analyzed at first by FABMS. In each FABMS spectrum of **I**–**VI** the molecular-ion peak in the negative-ion mode $[M-H]^-$ was observed at *m/z* 1457. Consequently, their degrees of polymerization (DP) were all 9; that is, they

6I,6n-di-mannosyl-βCDs



6-mannobiosyl-βCD

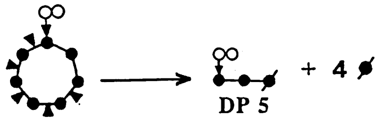


Fig. 5. Models of reaction on three positional isomers of 6^I, 6^n,-dimannosyl-βCD and 6-mannobiosyl-βCD with CGTase. Symbols: ●, glucose; ⊘, glucose with reducing end; ○, mannose; ●–●, α-(1 → 4)-glucosidic linkage;○→● , α-(1 → 6)-mannosidic linkage; ○○, mannobiose; ▲, point of attack.

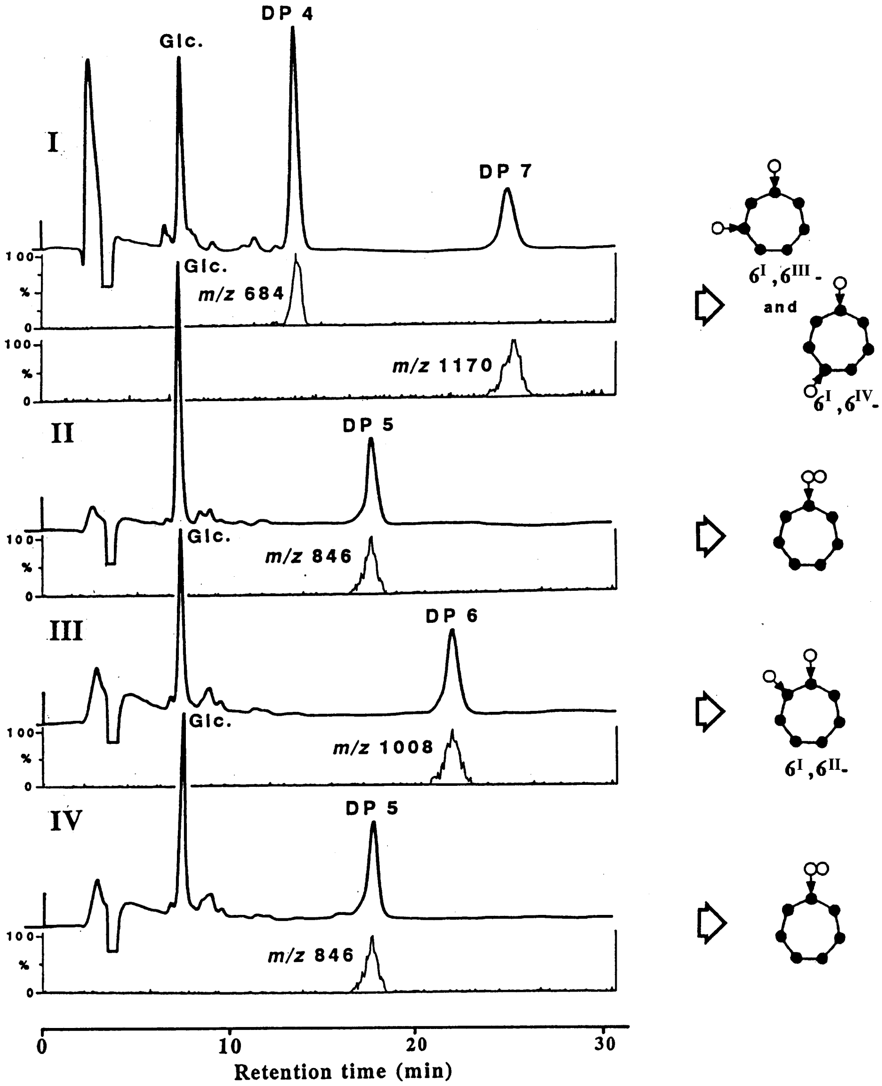


Fig. 6. Chromatograms of the degradation products from **I**, **II**, **III** and **IV** with CGTase and mass chromatograms of their ammonium adducts. Chromatographic conditions: column, YMC-pack polyamine-II (150 × 4.6 mm i.d.); eluent, 57:43 CH_3CN–10 mM CH_3COONH_4; flow rate, 0.5 mL/min; temperature, 30 °C. Each upper chromatogram was detected by RI.

were dimannosyl-substituted βCDs. Moreover, primary fragments suggested that **I** and **III** were $6^{I},6^{n}$-dimannosyl-βCDs, and **II**, **IV**, **V**, and **VI** were 6-mannobiosyl-βCDs (Table 1).

The structures of **I**–**VI** were elucidated by NMR spectroscopy. Fig. 2 shows the ^{13}C NMR spectra of positional isomers of $6^{I},6^{n}$-di-*O*-α-D-mannopyranosyl-βCD (**I** and **III**). Based on splitting of signals it was presumed that **I** was $6^{I},6^{IV}$-di-*O*-α-D-mannopyranosyl-βCD and **III** was $6^{I},6^{II}$-di-*O*-α-D-mannopyranosyl-βCD. However, it was elucidated by an enzymatic method, which will be mentioned later, that **I** was a mixture of $6^{I},6^{III}$- and $6^{I},6^{IV}$-di-*O*-α-D-mannopyranosyl-βCDs. In the ^{13}C NMR spectrum of one of the 6-mannobiosyl-βCDs, **II** (Fig. 3), the downfield shift of C-6 signal (M-6) of one mannosyl residue was confirmed by the distortionless increase by polarization transfer (DEPT) method [10]. Consequently, **II** was 6-*O*-α-(6-*O*-α-D-mannopyranosyl)-D-mannopyranosyl-βCD.

The ^{13}C resonances of all carbons in the spectra of **IV**, **V** and **VI** were assigned using COSY and ^{1}H–^{13}C COSY methods. Fig. 4 shows the ^{13}C NMR spectra of **IV**, **V** and **VI**. In all of these three spectra, each C-6 signal of mannosyl-substituted glucose in the CD ring (G′-6, $\delta \sim 67.6$) shifted downward from the other C-6 signals (G-6, $\delta \sim 61.2$). In addition, the ^{13}C NMR spectrum of **IV** shows a large

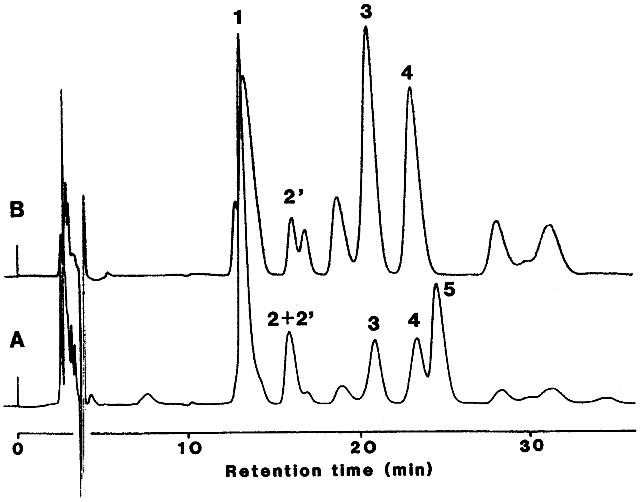


Fig. 7. Chromatograms of a mixture of trimannosyl-substituted βCDs, (A) original, and (B) after treatment with an α-glucosidase preparation from *A. niger*. Chromatographic conditions: column, Daisopak ODS-BP (150 × 6 mm i.d.); eluent, 3:97 MeOH–H_2O; flow rate, 1 mL/min; temperature, 30 °C.

downfield shift of one signal for the C-2 of mannosyl residue (M-2, δ 79.2) from another C-2 (M′-2, δ 70.8). Therefore, **IV** was 6-*O*-α-(2-*O*-α-D-mannopyranosyl)-D-mannopyranosyl-βCD. Similarly in the ^{13}C NMR spectra of **V** and **VI**, each C-4 signal (M-4) and C-3 signal (M-3) of the mannosyl residue shifted downfield by ~ 8 ppm, indicating that **V** was 6-*O*-α-(4-*O*-α-D-mannopyranosyl)-D-mannopyranosyl-βCD and **VI** was 6-*O*-α-(3-*O*-α-D-mannopyranosyl)-D-mannopyranosyl-βCD.

The structures of the positional isomers of the $6^{I},6^{n}$-di-substituted βCDs were elucidated by the analysis of fragments produced by digestion with CGTase [11]. Possible fragments produced by digestion of the three positional isomers of dimannosyl-βCD and mannobiosyl-βCD for reference with CGTase from *B. circulans* are summarized in Fig. 5. It should be noted that CGTase from *B. stearothermophilus*, which showed the same mode of action as in Fig. 5 for glucosyl- and galactosyl-CD derivatives [5,11,12], acted more strongly on the dimannosyl-βCDs and degraded them to smaller fragments. Fig. 6 shows the chromatograms by RI detection and the mass chromatograms of the digestion products of **I**–**IV** with CGTase. In the LC–MS analysis, the mass chromatograms were monitored as adduct ions with the ammonium ion $[M + NH_4]^+$ in the positive-ion ESI mode. The adduct ions at *m/z* 684, 846, 1008 and 1170 correspond to fragments of DP 4, 5, 6 and 7, respectively. Consequently, **I** contained two components that gave an enzymatic digest of DP 4 or 7 and, therefore, **I** was a mixture of $6^{I},6^{III}$- and $6^{I},6^{IV}$-di-*O*-α-D-mannopyranosyl-βCDs and their ratio calculated from the peak area of DP 7:0.5 peak area of DP 4 was 4:6. As the degradation product of DP 6 from **III** was obtained, **III** was proved to be the expected $6^{I},6^{II}$ -di-*O*-α-D-mannopyranosyl-βCD. Both mannobiosyl-βCDs, **II** and **IV**, gave the same chromatogram comprised of glucose and a fragment of DP 5, and mannobiosyl-βCDs were distinguishable from dimannosyl-βCDs by the enzymatic degradation with CGTase.

Table 2
The molecular ion (●) and primary fragments (○) in the FABMS spectra of trimannosyl-βCDs in the negative-ion mode and the action of α-mannosidase in an α-glucosidase preparation 'Transglucosidase Amano'

Compound	*m/z*				Action of α-mannosidase	Side chain[a]
	1133	1295	1457	1619		
1		○	○	●	+	Man and Man(1 → 6)Man
2		○	○	●	+	Man and Man(1 → 6)Man
2′		○	○	●	−	Man and Man(1 → *m*)Man
3	○	○	○	●	−	Man(1 → *m*)Man(1 → *n*)Man
4		○	○	●	−	Man and Man(1 → *m*)Man
5	○	○	○	●	+	Man(1 → 6)Man(1 → 6)Man

[a] Man, mannosyl; Man(1 → 6)Man and Man(1 → *m*)Man, mannobiosyl, *m* = 1, 2, 3 or 4; Man(1 → *m*)Man(1 → *n*)Man and Man(1 → 6)Man(1 → 6)Man, mannotriosyl, *n* = 2, 3, 4 or 6.

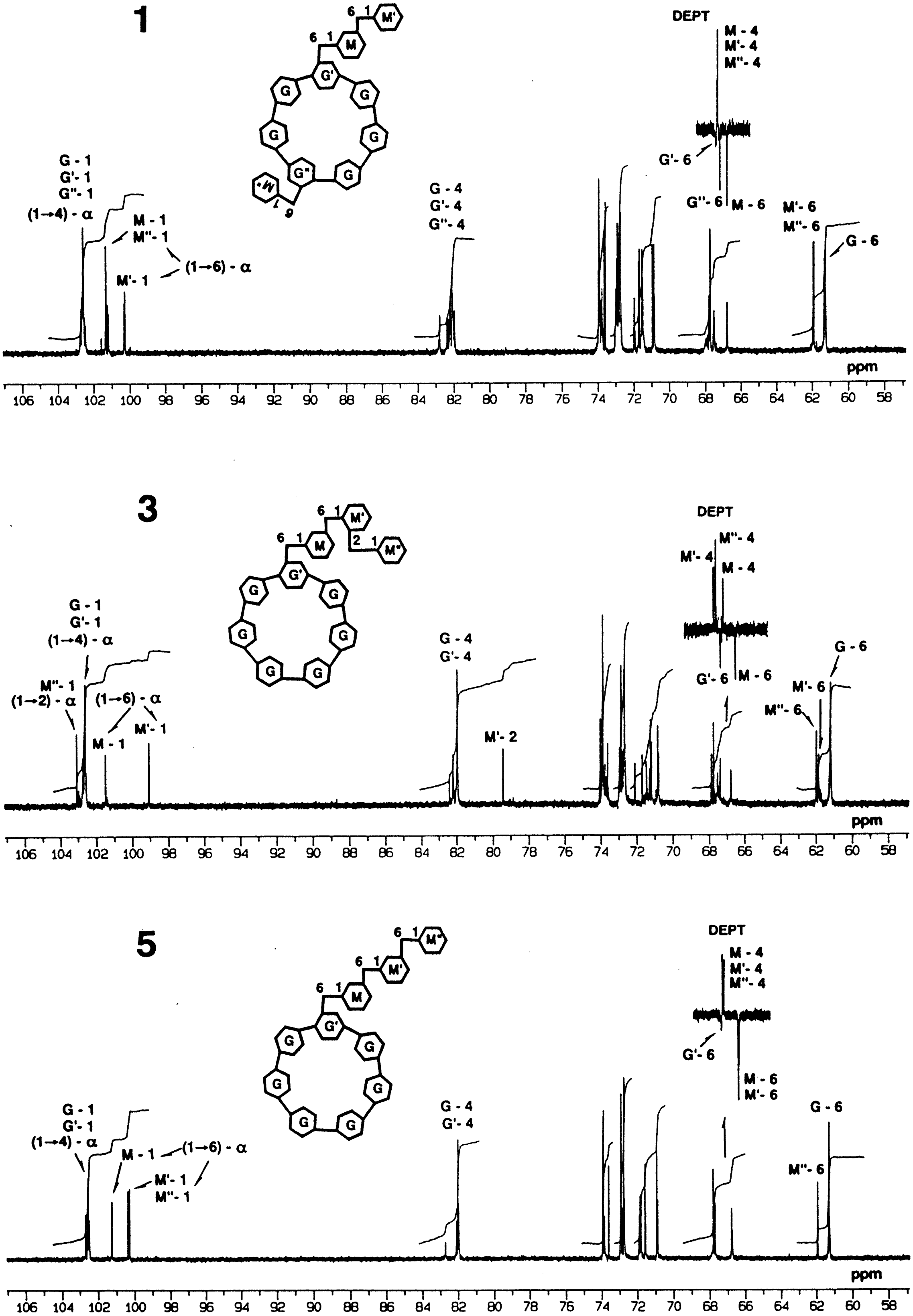


Fig. 8. ^{13}C NMR spectra of trimannosyl-βCDs (**1**, **3** and **5**) in D_2O at 50 °C. M″-1, 4, and 6 are signals of the C-1, -4, and -6 atoms of the side-chain D-mannopyranose unit. Other symbols are the same as in Fig. 4.

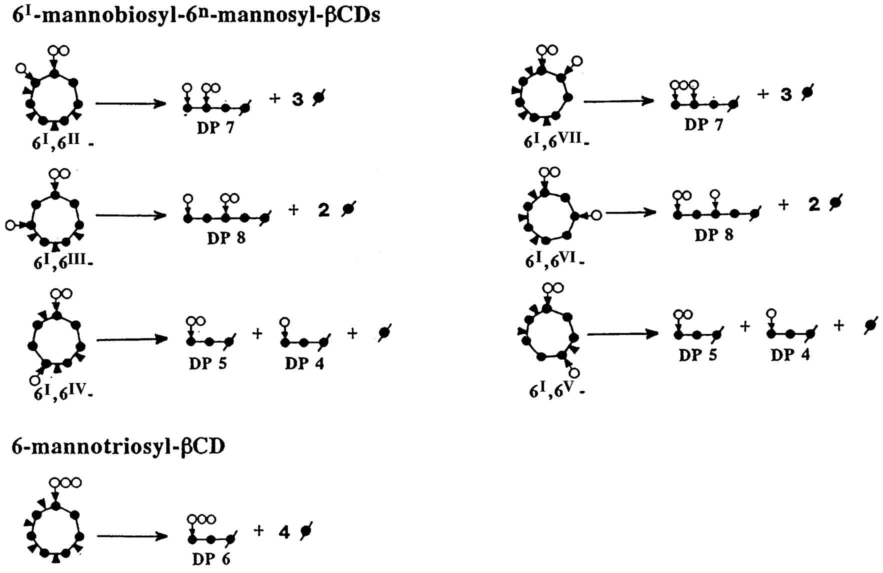


Fig. 9. Models of reaction on six positional isomers of 6^{I}-mannobiosyl, 6^{n}-mannosyl-βCD and 6-mannobiosyl-βCD with CGTase. Symbols: ○○○, mannotriose; others symbols are the same as in Fig. 5.

On the other hand, it was determined that α-mannosidase activity in the α-glucosidase preparation from *A. niger* hydrolyzed specifically the linkage of the nonreducing end of 6-*O*-α-D-mannosyl-D-mannose [Man(1→6)Man] and, therefore, HPLC of the hydrolyzates with this enzyme suggests the components that have side-chains which contain the linkage Man(1→6)Man (Fig. 1(B)). This method was found to be very useful for the analysis of trimannosyl-βCDs having much more complicated structures.

Isolation and characterization of trimannosyl-βCDs.—Isolation. Fig. 7(A) and (B) show, respectively, the chromatograms (Daisopak ODS-BP column, 5 μm, 150 × 6 mm i.d., 3:97 MeOH–water) for the fraction containing trimannosyl-βCDs of the original sample and the sample after treatment with the α-glucosidase preparation from *A. niger*. Sample (B) was prepared by removing monomannosyl- and dimannosyl-βCDs produced by hydrolysis of mannobiosyl and mannotriosyl side chains containing the linkage of Man(1→6)Man with the α-glucosidase preparation by HPLC on a TSKgel amide-80 column.

The major five components (**1**–**5**) were isolated by semipreparative HPLC, using a YMC-pack A-323-3 (3 μm, 250 × 10 mm i.d.) with 1:24 MeOH–water at a flow rate of 1 mL/min at 30 °C.

Characterization. Each component was analyzed by FABMS and their DPs were confirmed on the bases of the molecular-ion peak $[M-H]^-$ observed at *m/z* 1619 to be all 10; that is, they were trimannosyl-substituted βCDs. Moreover, primary fragments and the action of α-glucosidase preparation suggested the structures of their side-chains as in Table 2. There were no $6^{I},6^{x},6^{y}$-tri-*O*-α-D-mannopyranosyl-βCDs, which were all three mannosyl residues linked directly to βCD ring. This fact suggested that the third mannosyl residue attached preferentially to the side chain mannosyl residue. Moreover, although it was nothing strange that a mannobiosyl-βCD containing the linkage of Man(1→6)Man was not found in a mixture of dimannosyl-βCDs produced by hydrolysis of mannotriosyl-βCDs with the α-glucosidase preparation, mannobiosyl-βCDs containing the linkage of Man(1→2)Man or Man(1→3)Man also could not be detected. This fact suggested that the third mannosyl residue did not link to OH on C-6 of the side chain of these mannobiosyl-βCDs. Furthermore, it seemed that the third mannosyl residue could link to OH on C-6 of the side chain of Man(1→4) Man-βCD, but the chance might be very small.

Fig. 8 shows the ^{13}C NMR spectra of **1**, **3**, and **5**. These spectra and information obtained from studies of FABMS and hydrolysis with the α-glucosidase preparation indicated that **1** was 6^{I}-Man(1→6)Man,6^{n}-Man-βCD, **3** was 6-Man(1→2)Man(1→6)Man-βCD, and **5** was 6-Man(1→6)Man(1→6)Man-βCD.

Discrimination between positional isomers of 6^{I}-mannobiosyl,6^{n}-mannosyl-βCD was attempted by the analysis of fragments produced by digestion with CGTase. Possible fragments produced by digestion of the six positional isomers of 6^{I}-mannobiosyl,6^{n}-mannosyl-βCD and mannotriosyl-βCD for reference with CGTase from *B. circulans* are summarized in Fig. 9. Each of the three pairs of 6^{I}-mannobiosyl,6^{n}-mannosyl-βCDs ('6^{I},6^{II}- and 6^{I},6^{VII}-', '6^{I},6^{III}- and 6^{I},6^{VI}-', and '6^{I},6^{IV}- and 6^{I},6^{V}-') are indistinguishable. Fig. 10 shows the chromatograms by RI detection and the mass chromatograms of the digestion products of **1**–**4** with CGTase. The adduct ions at *m*/*z* 684, 846, 1008, 1170 and 1332 correspond to fragments of DP 4, 5, 6, 7 and 8, respectively. Consequently, **1** was 6^{I}-mannobiosyl,6^{IV}-mannosyl-βCD and/or 6^{I}-mannobiosyl,6^{V}-mannosyl-βCD, **3** was mannotriosyl-βCD, and **4** was 6^{I}-mannobiosyl,6^{II}-mannosyl-βCD and/or 6^{I}-mannobiosyl,6^{VII}-mannosyl-βCD. Compound **2** was hydrolyzed with the α-glucosidase preparation and gave 6^{I},6^{II}-di-*O*-α-D-mannopyranosyl-βCD. Therefore, **2** was 6^{I}-[Man(1→6)Man],6^{II}-Man-βCD and/or 6^{I} - [Man(1 → 6)Man],6^{VII} - Man - βCD, and hence the mannobiosyl residue of **4** was thought to be Man (1→2)Man. Compound **2′** was 6^{I}-mannobiosyl,6^{III}-mannosyl-βCD and/

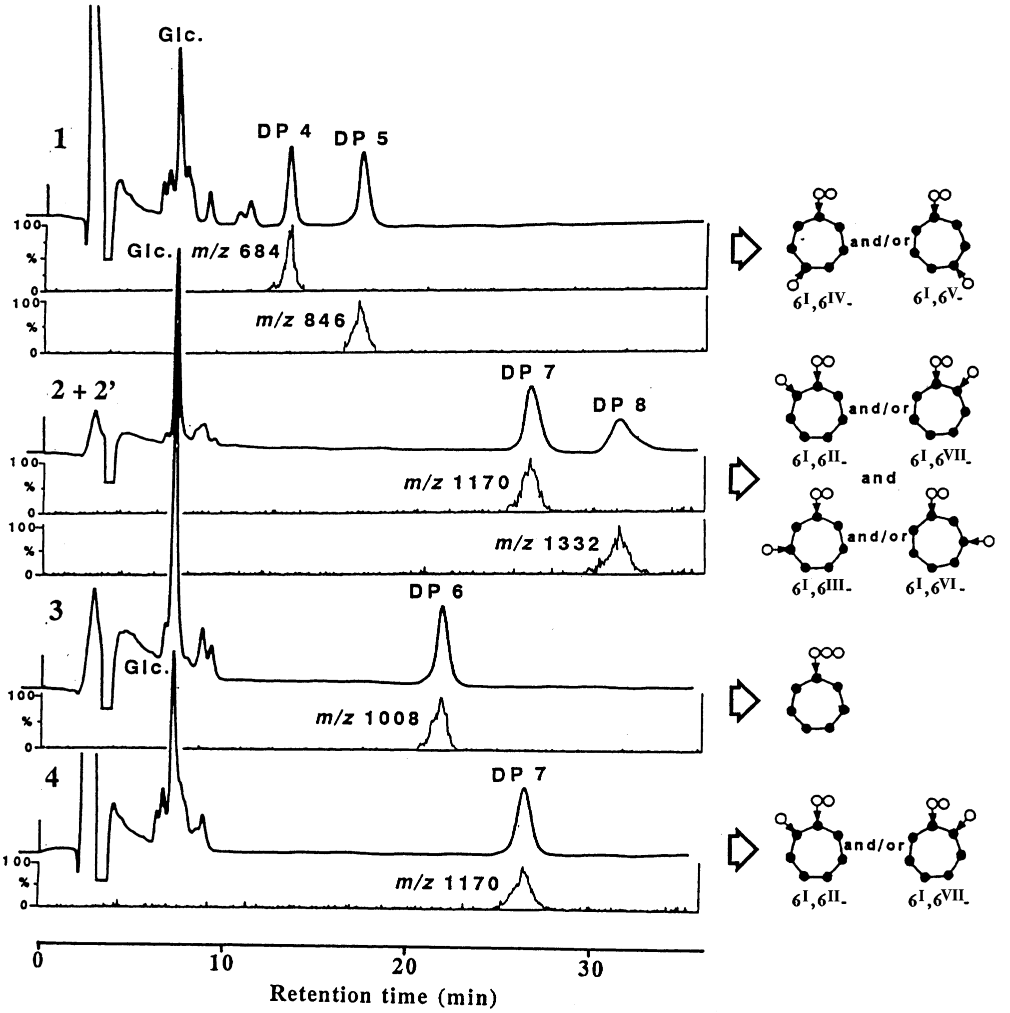


Fig. 10. Chromatograms of degradation products from **1**, **2**+**2**′, **3**, and **4** with CGTase and mass chromatograms of their ammonium adducts. Chromatographic conditions: column, YMC-pack polyamine-II (150 × 4.6 mm i.d.); eluent, 57:43 CH_3CN–10 mM CH_3COONH_4; flow rate, 0.5 mL/min; temperature, 30 °C. Each upper chromatogram was detected by RI.

or 6^{I}-mannobiosyl,6^{VI}-mannosyl-βCD and was not hydrolyzed with the α-glucosidase preparation. Thus, the mannobiosyl residue of **2′** may be Man(1 → 2)Man.

4. Conclusions

Di- and tri-mannosyl-βCDs in a mixture of mannosyl-βCDs, which were synthesized by reverse action of jack bean α-mannosidase on a mixture of a high concentration of mannose and βCD, were separated by HPLC on an amino-derivatized column.

Dimannosyl-βCDs comprised three isomers of $6^{I},6^{n}$-(Man)2-βCD ($n = 2$, 3, and 4) and four isomers of 6-Man(1 → m)Man-βCD ($m =$ 2, 3, 4 and 6), and their ratios suggested that the second mannosyl residue attached preferentially to OH on C-6 of the side chain mannose of 6-*O*-α-D-mannosyl-βCD.

The composition of trimannosyl-βCDs was very complicated, and thus only five major components were isolated. As it was elucidated that α-mannosidase activity in the α-glucosidase preparation from *A. niger*, 'Transglucosidase Amano' hydrolyzed specifically the linkage of Man(1 → 6)Man of the side chain mannobiose, this method was used for structural analysis of trimannosyl-βCDs together with FABMS and NMR analyses. The results suggested that there were no $6^{I},6^{x},6^{y}$-tri-*O*-α-D-mannopyranosyl-βCDs, and that the side chain(s) was (were) one mannosyl and one mannobiosyl, or mannotriosyl unit. This fact suggested that the third mannosyl residue attached preferentially to the side chain of the mannosyl or mannobiosyl residue.

A series of isomers of di- and tri-mannosyl-βCDs obtained will be used for studies on lectins as authentic samples. Although enzymatic synthesis has the limitation of complicated reaction products, fine separation techniques make it possible to isolate simultaneously a series of isomers.

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References

[1] S. Kitahata, K. Fujita, Y. Takagi, K. Hara, H. Hashimoto, T. Tanimoto, K. Koizumi, *Biosci. Biotech. Biochem.*, 56 (1992) 242–245.

[2] K. Koizumi, T. Tanimoto, K. Fujita, K. Hara, N. Kuwahara, S. Kitahata, *Carbohydr. Res.*, 238 (1993) 75–91.

[3] K. Hara, K. Fujita, N. Kuwahara, T. Tanimoto, H. Hashimoto, K. Koizumi, S. Kitahata, *Biosci. Biotech. Biochem.*, 58 (1994) 652–659.

[4] S. Kitahata, K. Hara, K. Fujita, N. Kuwahara, K. Koizumi, *Biosci. Biotech. Biochem.*, 56 (1992) 1518–1519.

[5] K. Koizumu, T. Tanimoto, Y. Okada, K. Hara, K. Fujita, H. Hashimoto, S. Kitahata, *Carbohydr. Res.*, 278 (1995) 129–142.

[6] G. Ashwell, A.G. Morell, *Adv. Enzymol.*, 41 (1974) 99–128.

[7] P. Chakraborty, A.N. Bhaduri, P.K. Das, *Biochem. Biophys. Res. Commun.*, 166 (1990) 404–410.

[8] K. Hamayasu, K. Hara, K. Fujita, Y. Kondo, H. Hashimoto, T. Tanimoto, K. Koizumi, H. Nakano, S. Kitahata, *Biosci. Biotech. Biochem.*, 61 (1997) 825–829.

[9] S. Okada, S. Kitahata, *Proc. Symp. Amylase*, 8 (1973) 21–27.

[10] D.M. Doddrell, D.T. Pegg, *J. Am. Chem. Soc.*, 102 (1980) 6388–6390.

[11] Y. Okada, Y. Okazaki, K. Koizumi, K. Hara, S. Kitahata, *Carbohydr. Res.*, 297 (1997) 301–307.

[12] Y. Okada, K. Koizumi, S. Kitahata, *Carbohydr. Res.*, 287 (1996) 213–223.