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Separation and characterization of five positional isomers of trimaltosyl-cyclomaltoheptaose (trimaltosyl-β-cyclodextrin) *

Yasuyo Okada [a], Kyoko Koizumi [a,†] and Sumio Kitahata [b]

[a] *Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 11-68, Koshien Kyuban-cho, Nishinomiya, 663 (Japan)*
[b] *Osaka Municipal Technical Research Institute, 1-6-50, Morinomiya, Jyoto-ku, Osaka, 536 (Japan)*

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ABSTRACT

Trace amounts of trimaltosyl-cyclomaltoheptaoses (trimaltosyl-β-cyclodextrins, trimaltosyl-βCDs) were found in a mixture of maltosyl-cyclomaltoheptaoses (maltosyl-β-cyclodextrins, maltosyl-βCDs) prepared from maltose and cyclomaltoheptaose (β-cyclodextrin, βCD) through the reverse action of *Klebsiella pneumoniae* pullulanase. Five positional isomers of trimaltosyl-βCD were isolated by high-performance liquid chromatography (HPLC) on a reversed-phase column and a graphitized carbon column. For the structural analysis of 6^1, 6^2, 6^3-, 6^1, 6^2, 6^5-, and 6^1, 6^3, 6^5-tri-*O*-maltosyl-βCDs, an enzymic method using glucoamylolysis, followed by hydrolysis with *Bacillus subtilis* saccharifying α-amylase, was applied. Although 6^1, 6^2, 6^4- and 6^1, 6^2, 6^6-substituted isomers were indistinguishable by this method, these isomers were distinguished clearly by digestion of branched oligosaccharides produced from each isomer by the aforesaid method, with *B. stearothermophilus* neopullulanase or with glucoamylase. The resulting hydrolysates were analyzed by HPLC on an amino derivatized column and by fast-atom bombardment spectrometry (FABMS). The chromatographic behavior and spectral data (^{13}C NMR and FABMS) of five positional isomers of trimaltosyl-βCD are described.

INTRODUCTION

A number of papers relating to the preparation, properties, and analysis of a wide variety of branched cyclomaltooligosaccharides (cyclodextrins, CDs), including monoglucosyl-, diglucosyl-, monomaltosyl-, and dimaltosyl-CDs have been published[2]. Application of branched CDs will be expanded to the fields of foods, cosmetics, and pharmaceuticals, etc., because these compounds have advantages in solubility and hemolytic activity, etc., over nonbranched CDs[3–5]. Today, we are able to commercially obtain a mixture of maltosyl cyclomaltoheptaoses (maltosyl-β-cyclodextrins, maltosyl-βCDs) that are produced from maltose and βCD through

* For a preliminary report, see ref 1.
† Corresponding author.

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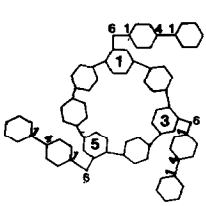
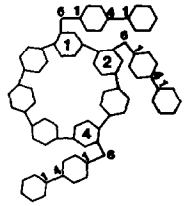
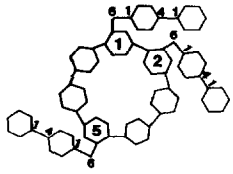


$6^1,6^3,6^5$-Tri-O-maltosyl-βCD (1)



$6^1,6^2,6^4$-Tri-O-maltosyl-βCD (2)



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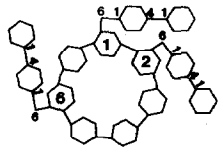
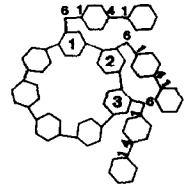


$6^1,6^2,6^6$-Tri-O-maltosyl-βCD (4)



$6^1,6^2,6^3$-Tri-O-maltosyl-βCD (5)

: Glucose

the condensation action of *Klebsiella pneumoniae* pullulanase (EC 3.2.1.41). We have already isolated and characterized three positional isomers of dimaltosyl-βCD from this mixture, in addition to monomaltosyl-βCD[6]. Through further detailed examination of this maltosyl-βCDs mixture, the presence of trace amounts of additional multibranched βCDs such as trimaltosyl-cyclomaltoheptaoses (trimaltosyl-β-cyclodextrins, trimaltosyl-βCDs) became apparent.

This paper deals with the separation of five positional isomers of trimaltosyl-βCD, their structural analysis by enzymic methods, ^{13}C NMR and FABMS spectra, and chromatographic behavior.

EXPERIMENTAL

Materials.—A mixture of maltosyl-βCDs was donated by Ensuiko Sugar Refining Co., Ltd. (Yokohama, Japan). A crystalline glucoamylase (EC 3.2.1.3) (37.8 U/mg) was obtained from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Purified bacterial saccharifying α-amylase (BSA, EC 3.2.1.1) from *Bacillus subtilis* was prepared as previously described[7]. Neopullulanase[8] found in *B. stearothermophilus* was kindly donated by Dr. T. Kuriki (Ezaki Glico Co., Ltd.). 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.

General methods.—Optical rotations were measured with a Jasco digital polarimeter, model DIP 360. HPLC was performed with a Jasco 880 PU pump, a Waters U6K universal injector, and a Showa Denko SE-61 RI monitor. HPLC

analyses at constant temperature were conducted with an SSC 3510C column oven (Senshu Scientific Co.). A Shimadzu Chromatopac C-R3A digital integrator was used for integration of peak areas. The columns used were a YMC-Pack AQ-312-3 ODS (octadecylsilyl) (150 × 6 mm i.d.) (YMC), a Carbonex (100 × 4.6 mm i.d.) (TONEN) and two YMC-Pack Polyamine-II (amine) (250 × 6 mm i.d., Lot nos. 062504036 and 062504240). The larger size columns, a YMC-Pack AQ-343-5 ODS (250 × 20 mm i.d.) and a Carbonex (100 × 10 mm i.d.) were used for preparative separation of trimaltosyl-βCDs from a mixture of maltosyl-βCDs.

FABMS was performed in the negative-ion mode using a Jeol JMS-DX 303 mass spectrometer with Xe having a kinetic energy equivalent to 6 kV at an accelerating voltage of 3 kV. The mass marker was calibrated with perfluoroalkyl phosphazine (Ultra Mark), and glycerol was used as the matrix solution.

^{13}C NMR spectra (125.65 MHz) were recorded for 2–3% solutions in D_2O at 50°C, using a Jeol GSX-500 spectrometer. Chemical shifts are expressed in ppm downfield from the signal of Me_4Si referenced to external 1,4-dioxane (67.40 ppm).

Structural analysis by enzymic hydrolysis.—Trimaltosyl-βCDs (**1–5**) were first converted into triglucosyl-βCDs (**1′–5′**, corresponding to compounds **1–5**). The conversion was done with crystalline glucoamylase as follows. The trimaltosyl-βCD sample (10 mg) and glucoamylase (2 mg) in distilled water (1 mL) were incubated at 40°C for 20 h, and the enzyme was then inactivated by heating at 100°C for 10 min. The product was subjected to HPLC to confirm that the conversion of trimaltosyl-βCD into triglucosyl-βCD was complete. To obtain the fragments digested by BSA, compounds **1′–5′** (1 mg each) in 0.2 mL of 10 mM acetate buffer (pH 6.0) were individually incubated with BSA (5 mg) at 40°C for 48 h. After inactivation of the enzyme, the products of BSA digestion were analyzed by HPLC on an amino-bonded silica gel column that allowed the prediction of the degree of polymerization (dp). Two kinds of dp 9 branched oligosaccharides (**9** and **10**) were obtained from compounds **2′** and **4′**, respectively. In order to elucidate the structures of these two types of branched oligosaccharides isolated by HPLC, each sample (0.2 mg) and glucoamylase (0.1 mg) in distilled water (50 μL) were incubated at 40°C for 24 h. Furthermore, each sample (0.2 mg) in 20 mM acetate buffer (pH 6.0, 20 μL) and neopullulanase solution (20 μL) was incubated at 50°C for 6 h. After inactivation of the enzymes, each hydrolysate was analyzed by HPLC.

RESULTS AND DISCUSSION

Separation.—Fig. 1 shows a chromatogram of a mixture of maltosyl-βCDs produced from maltose and βCD through the reverse action of pullulanase. We confirmed previously that peaks I and II were dimaltosyl-βCDs having three positional isomers; peak I contained 6^1, 6^3- and 6^1, 6^4-di-*O*-maltosyl-βCDs, peak II was 6^1, 6^2-di-*O*-maltosyl-βCD, and peak III was monomaltosyl-βCD[6]. It was

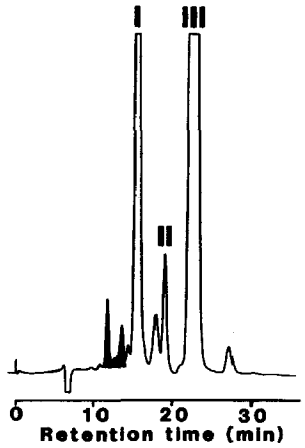


Fig. 1. Chromatogram of a mixture of maltosyl-βCDs commercially produced by Ensuiko Sugar Refining Co., Ltd. Peak I contains 6^1, 6^3- and 6^1, 6^4-di-*O*-maltosyl-βCDs. Peaks II and III are 6^1, 6^2-di-*O*-maltosyl-βCD and monomaltosyl-βCD, respectively. The shaded peaks contain trimaltosyl-βCDs. Chromatographic conditions: column, YMC-Pack AQ-312-3 ODS (150×6 mm i.d.); eluent, 1:9 methanol–water; flow rate, 0.5 mL/min; temperatures, 25°C.

expected that trimaltosyl-βCDs would be present in the fraction eluted earlier than the dimaltosyl-βCDs. This order was confirmed by HPLC on an amino-bonded column. The shaded peaks in Fig. 1 correspond to trimaltosyl-βCDs. The ratio of mono-, di-, and tri-maltosyl-βCDs was 16:8:1 as measured from this chromatogram. Analysis using a larger column, a YMC-Pack AQ-343 ODS (250 × 20 mm i.d.) indicated that many components existed in the peaks containing trimaltosyl-βCDs as shown in Fig. 1 (see Fig. 2). Each peak was fractionated, and each fraction was analyzed on an amino-bonded column that showed that trimaltosyl-βCDs were present in peaks A–D. Furthermore, it was shown by HPLC analysis on a graphitized carbon column (as Carbonex), that peak B contained two components (**2** and **3**) (Fig. 3). Compounds **1–5** were purified by HPLC with both an ODS and a graphitized carbon columns. The ratio of compounds **1**, **2**, **3**, **4** and **5** was 17:5:5:5:1, as determined by integrating the peak areas in the chromatograms shown in Figs. 2 and 3.

Determination of molecular weight.—It was confirmed by negative-ion FABMS that these five compounds (**1–5**) had identical molecular weights (2106), indicating a compound made up of 13 glucose residues. The FABMS spectra of these five positional isomers were essentially identical.

Structural analysis.—Structural analyses of the five positional isomers of trimaltosyl-βCD (**1–5**) were carried out essentially by the method by which Yoshimura et al.[9] elucidated the structures of three positional isomers of dimaltosyl-βCD. Thus compounds **1–5** were first converted into the corresponding triglucosyl-βCDs (**1′–5′**) with glucoamylase to facilitate the analysis of the product by BSA digestion. Possible fragments produced by digestion of the five positional isomers of trigluco-

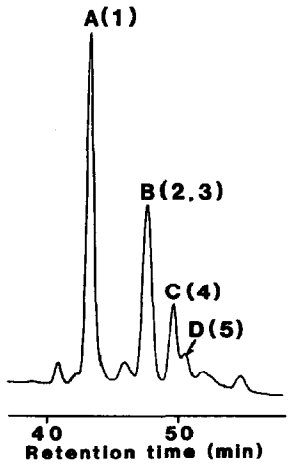


Fig. 2. Chromatogram of trimaltosyl-βCDs. Chromatographic conditions: column, YMC-Pack AQ-343-5 ODS (250×20 mm i.d.); eluent, 11:89 methanol–water; flow rate, 2 mL/min; temperature, 30°C.

syl-βCD with BSA are summarized in Fig. 4. Fragments from positional isomers other than 6^1, 6^2, 6^4- and 6^1, 6^2, 6^6-substituted ones have different dp's. Consequently, structural analysis of trimaltosyl-βCDs could be solved by determination of the dp's of fragment oligosaccharides using HPLC and FABMS. Although 6^1, 6^2, 6^4- and 6^1, 6^2, 6^6-tri-*O*-glucosyl-βCDs produce different fragments, the fragments have the same dp (dp 9); therefore, these two isomers are indistinguishable by HPLC analysis of the BSA degradation products. Fig. 5 shows the chromatograms of the digestion products from compounds **1′–5′** with BSA. As the retention times of saccharides on the amino-bonded column increase with the increase of their molecular weights, it seems that the molecular weights of compounds **6–10** and **12** are **6** < **7** < **8** < **9** = **10** < **12**. Compound **11** coincides with

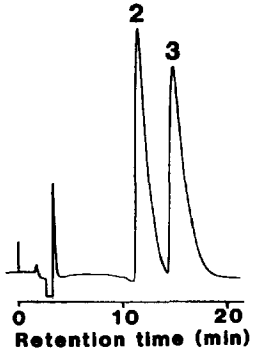


Fig. 3. Rechromatography of peak B in Fig. 2 on a graphitized carbon column. Chromatographic conditions: column, Carbonex (100×4.6 mm i.d.); eluent, 16:84 acetonitrile–water; flow rate, 0.4 mL/min; temperature, 20°C.

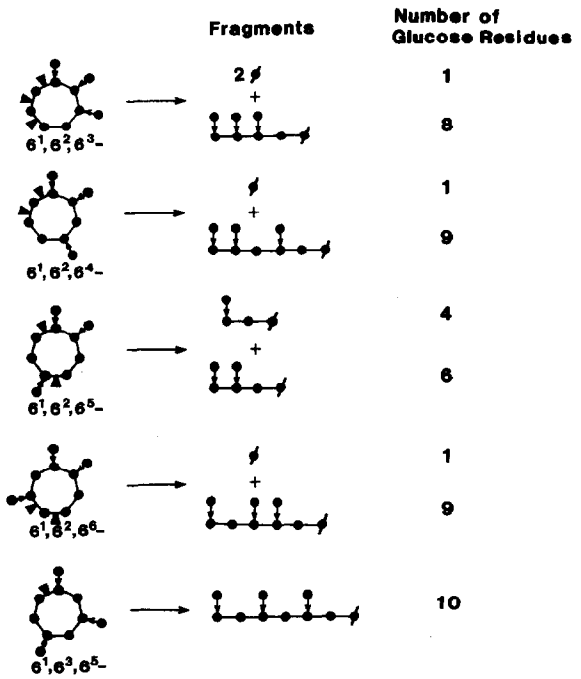


Fig. 4. Possible structures of fragments produced by digestion of triglucosyl-βCDs with BSA. 6^1, 6^2, 6^3-: 6^1, 6^2, 6^3-tri-*O*-glucosyl-βCD; 6^1, 6^2, 6^4-: 6^1, 6^2, 6^4-tri-*O*-glucosyl-βCD; 6^1, 6^2, 6^5-: 6^1, 6^2, 6^5-tri-*O*-glucosyl-βCD; 6^1, 6^2, 6^6-: 6^1, 6^2, 6^6-tri-*O*-glucosyl-βCD; 6^1, 6^3, 6^5-: 6^1, 6^3, 6^5-tri-*O*-glucosyl-βCD; Symbols: ●, glucose; ◆, glucose with reducing end; –, α-(1 → 4)-glucosidic linkage; ↓, α-(1 → 6)-glucosidic linkage; ▲, point of attack.

the starting material **1′**, 6^1, 6^3, 6^5-tri-*O*-glucosyl-βCD. In order to confirm the exact molecular weights of compounds **6–10** and **12**, they were isolated under the conditions described in Fig. 5, and their molecular weights were measured by FABMS. The molecular weight of compounds **6**, **7**, **8**, and **12** was 666 (four glucose residues), 990 (six glucose residues), 1314 (eight glucose residues), and 1638 (ten glucose residues), respectively, and that of compounds **9** and **10** was 1476 (nine glucose residues). From the above results, it is apparent that compounds **1**, **3**, and **5** are 6^1, 6^3, 6^5-, 6^1, 6^2, 6^5-, and 6^1, 6^2, 6^3-tri-*O*-maltosyl-βCDs, respectively, and that compounds **2** and **4** are either 6^1, 6^2, 6^4- or 6^1, 6^2, 6^6-tri-*O*-maltosyl-βCDs.

Next, in order to distinguish between the 6^1, 6^2, 6^4- and 6^1, 6^2, 6^6-isomers, enzymic hydrolysis of the branched oligosaccharides (**9** and **10**) with glucoamylase or neopullulanase were attempted, and the fragments so obtained were analyzed by HPLC on an amino-bonded column.

Glucoamylase hydrolyzes both α-(1 → 4)- and α-(1 → 6)-glucocidic linkages in starch, glycogen, and their hydrolysates. But the hydrolysis rates of the α-(1 → 6)-linkage, especially when adjacent to another α-(1 → 6)-linkage, is slower than that of the α-(1 → 4)-linkage so that intermediate products are observed during the

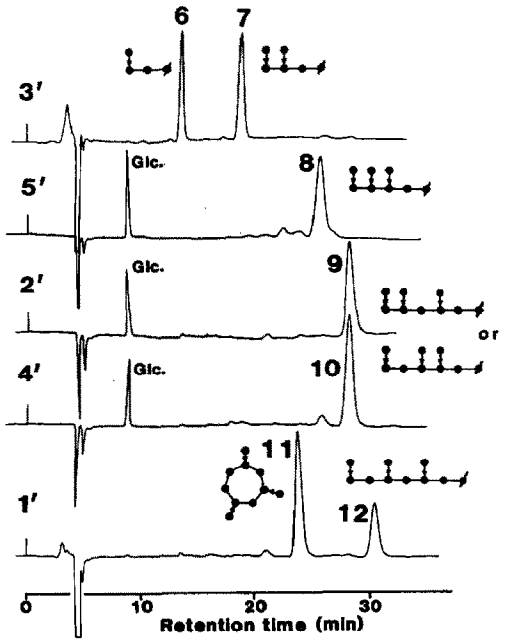


Fig. 5. Chromatograms of digestion products from compounds 1′–5′ with BSA. Chromatographic conditions: column, YMC-Pack Polyamine-II (250×6 mm i.d., Lot no. 062504036); eluent, 55:45 acetonitrile–water; flow rate, 1 mL/min; temperature, 25°C.

enzymic reaction[10]. Kobayashi et al.[11] have already reported that 6^4, 6^5-*O*-diglucosyl maltopentaose and 6^5, 6^6-*O*-diglucosyl maltohexaose were considerably resistant to the action of glucoamylase. On the other hand, Imanaka and Kuriki[12] reported that *B. stearothermophilus* neopullulanase hydrolyzes pullulan and 6^3-*O*-glucosyl maltotriose to produce panose. Thus the enzyme hydrolyzes the second glucosidic linkage toward the reducing end from the branch-point in the branched saccharides.

On the basis of these reaction specificities, we propose different models of action of glucoamylase[10,11] and neopullulanase[8,12] on two kinds of branched oligosaccharides that have the same number of glucose residues but different branch-points (**9** and **10**, Figs. 6 and 7). Advantageously, the reaction mechanisms of glucoamylase on these two kinds of branched oligosaccharides are not identical, and the mode of action of neopullulanase is also different. Fig. 8 shows chromatograms of the hydrolysates of compounds **9** and **10** with glucoamylase. Only glucose was detected as a hydrolysate of compound **9** by glucoamylase, and a considerable amount of unreacted compound **9** still remained. On the other hand, glucose and characteristic B6 were observed as the hydrolysates of compound **10**, and the starting material was not detected under the same conditions of digestion for compound **9**. On the basis of the decomposition pathways shown in Fig. 6 and

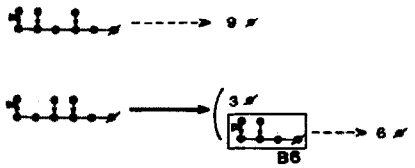


Fig. 6. Models of reaction on branched oligosaccharides with glucoamylase. Symbols: B6, 6^3, 6^4-di-*O*-glucosyl maltotetraose; ▲, easily attacked point; △, point attacked with difficulty; other symbols as in Fig. 4. A full line and dashed lines indicate relatively fast and slow enzyme reactions in the system, respectively. The oligosaccharides enclosed by rectangles are the accumulated products in each system.

the chromatograms in Fig. 8, it is apparent that compound **2** is 6^1, 6^2, 6^4-tri-*O*-maltosyl-β-CD and compound **4** is 6^1, 6^2, 6^6-tri-*O*-maltosyl-β-CD, respectively.

In addition, the results of the enzymic hydrolysis of compounds **9** and **10** with neopullulanase supported the above conclusions. As shown in the chromatograms given in Fig. 9, a large amount of B3 and B5 existed in hydrolysates of compound **9** with neopullulanase, but digestion products from compound **10** were mainly B8 and glucose, and only a very small amount of B3 and B5 were detected.

In conclusion, it was determined that compounds **1**, **2**, **3**, **4**, and **5** were 6^1, 6^3, 6^5-, 6^1, 6^2, 6^4- 6^1, 6^2, 6^5-, 6^1, 6^2, 6^6-, and 6^1, 6^2, 6^3-tri-*O*-maltosyl-β-CDs, respectively.

^{13}C NMR spectroscopy.—Fig. 10 shows the ^{13}C NMR spectra of trimaltosyl-β-CDs (**1**–**5**). Assignment of signals in the spectra were made by comparison to those in the spectra of dimaltosyl-β-CDs[6]. It is known that a substituent on the oxygen atom attached to any carbon atom of the sugar moiety affects the chemical shift of the carbon atom, moving it downfield by 8–11 ppm[13]. The large downfield shift of

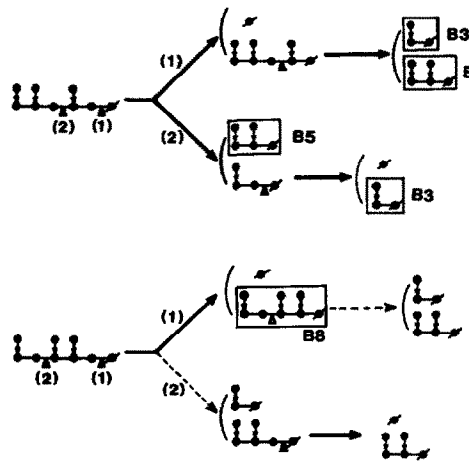


Fig. 7. Models of reaction on branched oligosaccharides with neopullulanase. Symbols: B3, panose; B5, 6^2, 6^3-di-*O*-glucosyl maltotriose; B8, 6^2, 6^3, 6^5-tri-*O*-glucosyl maltopentaose; other symbols as in Figs. 4 and 6.

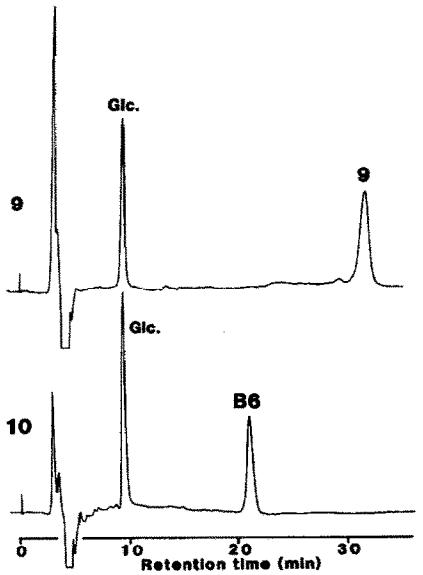


Fig. 8. Chromatograms of hydrolysates of compounds **9** and **10** with glucoamylase. Chromatographic conditions: column, YMC-Pack Polyamine-II (250×6 mm i.d., Lot no. 062504240), other conditions as in Fig. 5.

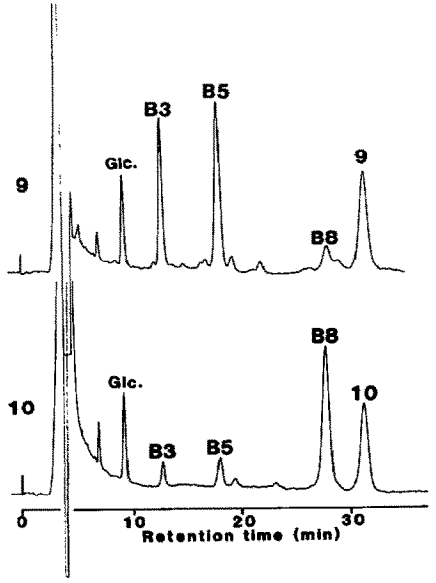


Fig. 9. Chromatograms of hydrolysates of compounds **9** and **10** with neopullulanase. Chromatographic conditions as in Fig. 8.

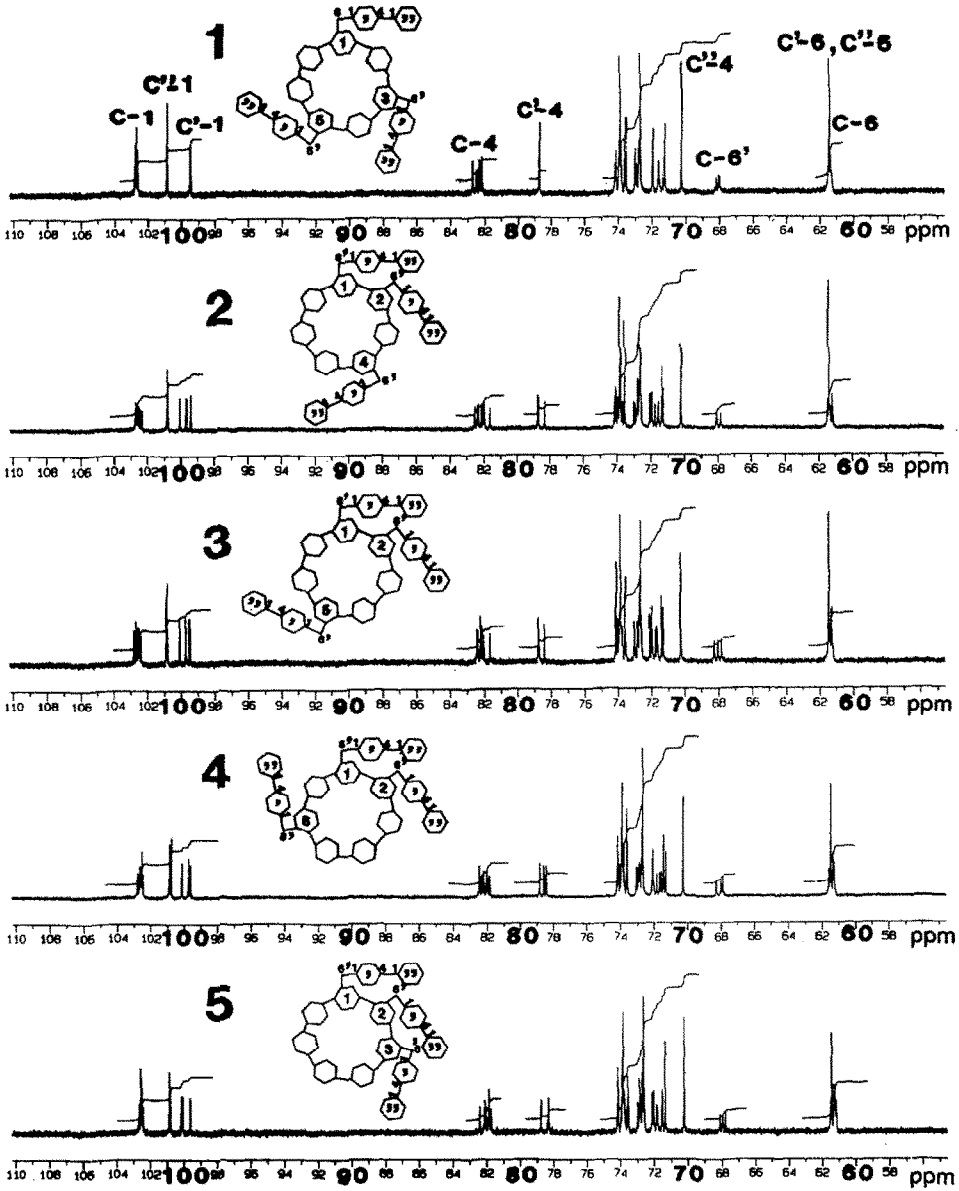


Fig. 10. ^{13}C NMR spectra of five positional isomers of trimaltosyl-βCD (**1–5**) measured in D_2O at 125.65 MHz: C, the carbon atom of the ring D-glucose residue; C′ and C″; the carbon atoms of the branched residue; C-6′, the carbon atom of the branch-point.

three C-6 signals indicates that the side-chain maltose residues are attached to oxygen atoms on these carbon atoms. The assignments of the C-6 signals were confirmed by the distortionless enhancement by polarization transfer (DEPT)

method[14]. The relative signal intensity ratio of the CD ring C-6 (at δ ~ 61.3 ppm), the side-chain C-6 (at δ ~ 61.5 ppm, C′- and C″-6), and the branch-point C-6 (at δ 67.7–68.3 ppm, C-6′) was 4:6:3. That of the CD ring C-4 (at δ 81.6–82.7 ppm), and the side-chain C-4s contained an α-(1 → 4)-linkage (at δ 78.3–78.9 ppm, C′-4), and at the nonreducing end (at δ ~ 70.3 ppm, C″-4) was 7:3:3, and that of the CD ring C-1 (at δ 102.3–102.8 ppm), and side-chain C-1s contained an α-(1 → 4)-linkage (at δ ~ 100.9 ppm, C″-1) and an α-(1 → 6)-linkage (at δ 99.4–100.2 ppm, C′-1) was also 7:3:3. In the all spectra, the C-6′ signal is split into three peaks. (The three peaks of the C-6′ signal of compound **2** were confirmed by an expanded spectrum.) These facts indicate that compounds **1–5** each have three maltose residues as the side chains on the βCD ring. In the spectrum of compound **1**, the C′-4 and C′-1 signals were observed as single peaks, although these signals of the other four isomers were split into two or three peaks. These phenomena are due to the differences in magnitude of the interactions among the three side-chain maltosyl residues in each molecule of compounds **1–5**.

Chromatographic behavior of trimaltosyl-βCDs.—Five positional isomers of trimaltosyl-βCD showed characteristic chromatographic behavior on three HPLC columns of different separation modes. The elution of the 6^1, 6^3, 6^5-isomer was especially distinct from those of the other four positional isomers examined on the columns.

As shown in Fig. 2, the trimaltosyl-βCDs other than the 6^1, 6^2, 6^4- (**2**) and 6^1, 6^2, 6^5- (**3**) isomers were efficiently separated on a YMC-Pack AQ-343-5 ODS column. Their elution order was 6^1, 6^3, 6^5- (**1**), 6^1, 6^2, 6^4- (**2**) and 6^1, 6^2, 6^5- (**3**), 6^1, 6^2, 6^6- (**4**), and 6^1, 6^2, 6^3- (**5**).

YMC-Pack Polyamine-II is an amino-bonded silica column, and it has been reported that positional isomers having the same molecular size are difficult to separated from each other on such a column, because of the fact that the elution

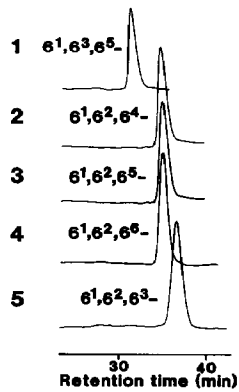


Fig. 11. Elution profiles of the isomeric trimaltosyl-βCDs (**1–5**) on an amino-bonded column. Chromatographic conditions as in Fig. 5.

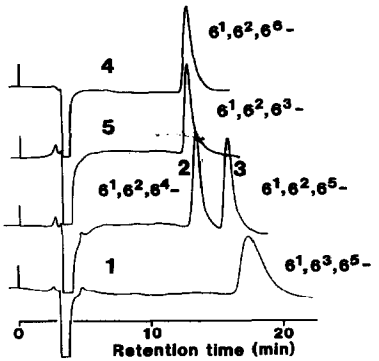


Fig. 12. Elution profiles of the isomeric trimaltosyl-βCDs (1–5) on a graphitized carbon column. Chromatographic conditions as in Fig. 3.

sequence of saccharides with an amino column and an acetonitrile–water system essentially follows the order of molecular size[15]. However, positional isomers of trimaltosyl-βCD were moderately well separated on a YMC-Pack Polyamine-II column with 55:45 acetonitrile–water as the eluent. This evidently depends upon the influence of the three side-chain maltosyl residues (Fig. 11).

The third column, Carbonex, is classified as a graphitized carbon column. The elution mechanism on this type of column involves mainly adsorption as well as some hydrophobic interactions[16,17]. Its unique resolving power allowed an excellent separation of 6^1, 6^2, 6^4- and 6^1, 6^2, 6^5-isomers that could not be separated on either the ODS or amino-bonded columns. Elution profiles of trimaltosyl-βCDs on the Carbonex column were significantly different from those on either the ODS or amino-bonded columns (Fig. 12). Trimaltosyl-βCDs on a graphitized carbon column are roughly divided into two groups. One group consists of the 6^1, 6^2, 6^3-, 6^1, 6^2, 6^4-, and 6^1, 6^2, 6^6-isomers, and the other group is made up of the 6^1, 6^2, 6^5- and 6^1, 6^3, 6^5-isomers. It should be pointed out that planar molecules are generally better retained than nonplanar molecules because the surface of the graphitized carbon column is flat[17]. It is supposed that 6^1, 6^3, 6^5- and 6^1, 6^2, 6^5-isomers, which are retained longer on this column, have essentially more planar structures than the other isomers.

Optical rotations.—The $[\alpha]_D^{25}$ values in water were +170.8° (*c* 0.86) for **1**, +176.8° (*c* 1.0) for **2**, +176.2° (*c* 0.94) for **3**, +163.8° (*c* 0.90) for **4**, and +155.2° (*c* 0.75) for **5**.

CONCLUSIONS

Five positional isomers of trimaltosyl-βCD from a mixture of maltosyl-βCDs were isolated by HPLC using both a conventional ODS column and a unique graphitized carbon column.

The structures of these compounds were elucidated by enzymic methods using the characteristic action specificity of BSA, glucoamylase, and neopullulanase on a variety of branched oligosaccharides.

These five compounds will serve as standards for the structural analysis of other multibranched βCDs which may prepared by chemical synthesis and enzymic reaction. In addition, we are greatly interested in the inclusion behavior and the biological function of trimaltosyl-βCDs.

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