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Characterization of five isomers of branched cyclomaltoheptaose (β CD) having degree of polymerization (d.p.) = 9: Reinvestigation of three positional isomers of diglucosyl-β CD

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ABSTRACT

It has been confirmed by methylation analyses and chemical syntheses that three isomers of branched cyclomaltoheptaose (β CD) isolated from the mother liquors of a large-scale preparation of β CD with *Bacillus ohbensis* cyclomaltodextrin glucanotransferase are $6^1,6^4$-di-*O*-(α-D-glucopyranosyl)-cyclomaltoheptaose (**1**), $6^1,6^3$-di-*O*-(α-D-glucopyranosyl)-cyclomaltoheptaose (**2**), and 6-*O*-(α-isomaltosyl)-cyclomaltoheptaose (**4**) instead of $6^1,6^2$-di-*O*-(α-D-glucopyranosyl)-cyclomaltoheptaose (**3**), which was erroneously characterized in an earlier paper[1]. Compound **3** has been newly isolated from a glucosyl-β CD mixture prepared by hydrolysis with glucoamylase of a maltosyl-β CD mixture, synthesized from maltose and β CD through the reverse action of pullulanase. Chromatographic behavior and spectral data (^{13}C-n.m.r. and f.a.b.-m.s.) of these isomers of branched β CD (**1–4**), as well as those of another isomer prepared by the reverse action of hydrolytic enzymes, 6-*O*-(α-maltosyl)-cyclomaltoheptaose (**5**), were compared.

INTRODUCTION

In the previous paper[1] we reported that the three positional isomers of cyclomaltoheptaose (β-cyclodextrin, β CD) derivatives having two D-glucose branches in the molecule were isolated from the mother liquors of a large-scale preparation of β CD by action of *Bacillus ohbensis* cyclomaltodextrin glucanotransferase [(1→4)-α-D-glucan 4-D-glucosyltransferase (cyclising), EC 2.4.1.19 (CGTase)] on potato starch, followed by hydrolysis with glucoamylase [glucan (1→4)-α-D-glucosidase, EC 3.2.1.3] to shorten the side chains of the branched CDs. It was confirmed by both f.a.b.-m.s. and ^{13}C-n.m.r. spectroscopy that these three compounds have the same molecular weight, 1458 daltons, and that they have two α-(1→6) linkages in the molecule, respectively. Although the structures of two of the three isomers were established to be $6^1,6^4$-di-*O*-(α-D-glucopyranosyl)-cyclomaltoheptaose (**1**), and $6^1,6^3$-di-*O*-(α-D-glucopyranosyl)-cyclo-

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maltoheptaose (**2**) by h.p.l.c. analysis of their partial hydrolyzates, the structure of the third isomer was not firmly established, as the amount of sample originally obtained was too small to subject it to fragmentation analysis (yield only 1.8%). The third isomer was presumed to be $6^1,6^2$-di-*O*-(α-D-glucopyranosyl)-cyclomaltoheptaose (**3**) and not to be isomaltosyl-β-CD, which also has a molecular weight of 1458 and two α-(1→6) linkages. The latter compound cannot be formed by this preparation from potato starch with pure CGTase, according to the general knowledge of the structure of starch. However, one additional isomer having the same d.p. of 9 and two α-(1→6) linkages in the molecule has been newly isolated from a glucosyl-β-CD mixture prepared by glucoamylolysis of a maltosyl-β-CD mixture, which was commercially produced from maltose and β-CD through the reverse action of pullulanase [α-dextrin endo-(1→6)-α-D-glucosidase, EC 3.2.1.41]. This isomer has been identified with the true **3** prepared by an unambiguous chemical synthesis[2]. Consequently, the third branched β-CD isolated from the mother liquors of a large-scale preparation of β-CD should be 6-*O*-(α-isomaltosyl)-cyclomaltoheptaose (**4**), which was confirmed by a chemical synthesis. Moreover, the structures of all isomers of branched β-CDs having d.p. 9, prepared by the action of CGTase on starch and of the hydrolytic enzyme on maltose and β-CD, *i.e.*, compounds **1**–**4** and 6-*O*-(α-maltosyl)-cyclomaltoheptaose (**5**), were firmly established by methylation analysis. Furthermore, their ^{13}C-n.m.r. spectra, chromatographic behavior, and f.a.b.-m.s. were compared.

EXPERIMENTAL

General methods. — H.p.l.c. was performed with a JASCO 880-PU pump, a Waters U6K universal injector, and a Showa Denko SE-61 r.i. monitor. The columns used were a YMC-Pack A-312 (150 × 6 mm i.d.) (YMC) and Asahipak NH2P-50 (250 × 4 mm i.d.) (Asahi Kasei). For preparative chromatography, YMC-Pack A-323 (250 × 10 mm i.d.) and SH-343-5 AQ (250 × 20 mm i.d.) columns were used. H.p.l.c. analyses at constant temperature were conducted using a column oven SSC 3510C (Senshu Scientific Co.). A Shimazu Chromatopac C-R3A digital integrator was used for integration of peak areas.

General procedures for chemical syntheses: melting points were measured with Yanagimoto micro melting-point apparatus and are uncorrected; optical rotations were determined with a JASCO digital polarimeter, model DIP 360; T.l.c. was performed on Silica Gel 60 t.l.c. plates (E. Merck) with appropriate developing solvents by spraying with sulfuric acid; a Harrison Centrifugal Thin-Layer Chromatotron, model 7924, was used for centrifugal chromatography; column chromatography was performed on a Lobar prepacked column packed with LiChroprep Si 60 (40–63 μm), size C (E. Merck).

F.a.b.-m.s. was performed with 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. Branched β-CDs (d.p. 9) were analyzed in the negative-ion mode.

^{13}C-N.m.r. spectra (125.65 MHz) were recorded at ambient temperature on 2–3% solutions in D_2O with JEOL GSX-500 spectrometer. Chemical shifts were expressed in p.p.m. downfield from the signal of Me_4Si using 1,4-dioxane (67.40 p.p.m.) as the external standard. The delay time for the insensitive nuclei enhanced by the polarization-transfer (INEPT) method[3] was 5.1 ms (3/4*J*). The conditions for ^{1}H[^{1}H]-shift-correlated 2D n.m.r. spectroscopy (COSY) and ^{13}C[^{1}H]-shift-correlation (C-H COSY) measurements were as follows: spectral width 1100 Hz (6002 Hz), pulse flipping angle 90° (90°), matrix size 2k × 1k (512 × 4k).

Materials. — The glucosyl-β CD mixture was that commercially produced by Ensuiko Sugar Refining Co., Ltd. and contained mainly a monoglucosyl-β CD and diglucosyl-β CDs, together with triglucosyl-β CDs as minor components (Fig. 1). Maltosyl-β CD was also commercially produced by Ensuiko Sugar Refining Co., Ltd. from maltose and β CD through the reverse action of pullulanase. A mixture of branched β CDs, prepared by action of CGTase on potato starch as previously reported[4], was kindly donated by Sanraku 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.

Methylation analysis. — Methylation of branched β CDs was performed by the method of Prehm[5]. The products were purified by h.p.l.c. on a YMC-Pack A-323 column with 25:75 1-propanol–water, hydrolyzed, converted to their alditol acetates, and then analyzed with a Hitachi gas chromatograph Model 063 fitted with a flame-

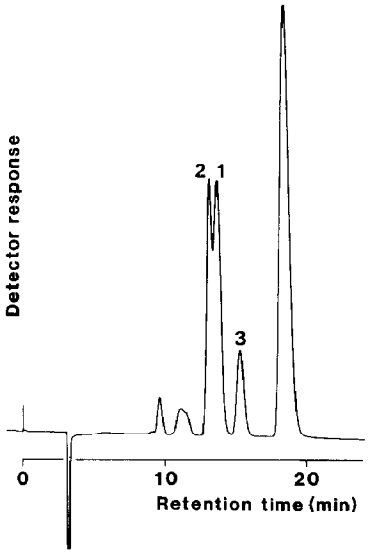

Fig. 1. Chromatogram of a glucosyl-β CD mixture (Lot. No. 89202) produced commercially by Ensuiko Sugar Refining Co., Ltd. Peaks 1, 2, and 3 are 6^1,6^4-, 6^1,6^3-, and 6^1,6^2-di-*O*-(α-D-glucopyranosyl)-β CDs, respectively. Chromatographic conditions: column, YMC-Pack A-312 (150 × 6 mm i.d.); eluent, 6:94 methanol–water; flow rate, 1 mL/min; detector, Shodex RI SE-61 at 2 × 10^{-5} r.i. units full scale; temperature, 30°.

ionisation detector on a column (2 m × 3 mm i.d.) of 0.3% OV-275–0.4% GE XF-1150 on Shimalite W (AW-DMCS, 80–100 mesh).

Chemical synthesis of 6-O-(α-isomaltosyl)-cyclomaltoheptaose (**4**). — *(2,3-Di-O-acetyl)hexakis(2,3,6-tri-O-acetyl)-cyclomaltoheptaose* (**6**). — According to the procedure of Fügedi *et al.*[6], the glycosyl acceptor **6** was prepared by reaction of β CD with *tert*-butyldimethylsilyl chloride in pyridine, followed by acetylation and desilylation.

Allyl 2,3,4-tri-O-acetyl-6-O-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)-β-D-glucopyranoside (**7**). — Phosphorus tribromide (0.91 mL) and water (0.35 mL) were added dropwise to a solution of octa-*O*-acetyl-β-isomaltose (6.5 g) in dichloromethane (10 mL). The mixture was stirred overnight at room temperature, then cooled, neutralized with potassium carbonate, and filtered. The filtrate was washed with cold water, dried, and evaporated. Hepta-*O*-acetyl-α-isomaltosyl bromide (6.7 g) thus obtained as a syrup was stirred together with mercuric cyanide (2.4 g) in dry allyl alcohol (40 mL) at 50° for 1 h[7], and the reaction mixture was then concentrated. The residual syrup was extracted with chloroform, and the extract was washed successively with water, aqueous potassium bromide, and water, dried, and concentrated. Column chromatography (4:1 hexane–acetone) of the product gave **7** (3.8 g, 58.6%), m.p. 130–131° (from ethanol), $[\alpha]_D^{28}$ +78.3° (*c* 1.2, chloroform); ^{13}C-n.m.r. data ($CDCl_3$): δ 133.5 and 117.6 ($CH=CH_2$), 99.5 (C-1), and 95.8 (C-1′).

Anal. Calc. for $C_{59}H_{80}O_{38}$: C, 51.48; H, 5.96. Found: C, 51.29; H, 6.09.

Allyl 2,3,4-tri-O-benzyl-6-O-(2,3,4,6-tetra-O-benzyl-α-D-glucopyranosyl)-β-D-glucopyranoside (**8**). — A solution of **7** (3.4 g) in 0.05N sodium methoxide (35 mL) was kept for 1 h at room temperature, neutralized with acetic acid, treated with Amberlite IR-120 (H^+) resin, filtered, and evaporated. Allyl 6-*O*-α-D-glucopyranosyl-β-D-glucopyranoside (1.9 g) that was obtained as a syrup was dissolved in *N,N*-dimethylformamide (40 mL) and cooled to 0°. Sodium hydride (2.5 g) was added, and the mixture was stirred for 1 h at room temperature. The mixture was again cooled to 0°, benzyl bromide was added dropwise (8.25 mL), and the mixture stirred overnight at room temperature. Methanol (2 mL) was added to decompose the excess hydride, the solvent was evaporated, and a solution of the residue in chloroform was washed with water, dried, and concentrated. Column chromatography (7:1 hexane–acetone) of the product gave **8** (4.4 g, 86.4%), m.p. 97–98° (from ethanol), $[\alpha]_D^{28}$ +37.0° (*c* 1.0, chloroform); ^{13}C-N.m.r. data ($CDCl_3$): δ 134.3 and 117.0 ($CH=CH_2$), 102.8 (C-1), and 97.3 (C-1′).

Anal. Calc. for $C_{64}H_{68}O_{11}$: C, 75.87; H, 6.76. Found: C, 75.44; H, 6.86.

2,3,4-Tri-O-benzyl-6-O-(2,3,4,6-tetra-O-benzyl-α-D-glucopyranosyl)-α-D-glucopyranosyl trichloroacetimidate (**10**). — A solution of **8** (1.13 g), palladium chloride (0.30 g), and sodium acetate (0.70 g) in acetic acid–water (20:1, 21 mL) was stirred overnight at room temperature. The mixture was filtered through a Celite pad, and the combined filtrate and washings with methanol were concentrated. The residue was extracted with chloroform, and the extract was washed and concentrated. Centrifugal chromatography (5:1 hexane–acetone) of the residue gave amorphous *2,3,4-tri-O-benzyl-6-O-(2,3,4,6-tetra-O-benzyl-α-D-glucopyranosyl)-D-glucopyranose* (**9**, 0.62 g). A solution of **9** (0.62 g) and trichloroacetonitrile (2 mL) in absolute dichloromethane (10 mL) was stirred with sodium

hydride (0.20 g) for 2 h at room temperature[8]. Excess sodium hydride was filtered off on a Celite pad, and the filtrate was concentrated. The desired glycosyl donor **10** was purified by centrifugal chromatography (1:1 petroleum ether–ether) and obtained as a syrup (0.54 g, 74.4%); ^{13}C-n.m.r. data ($CDCl_3$): δ 161.3 (*C*(=NH)CCl_3), 97.5 (C-1'), 94.2 (C-1), 75.64, 75.58, 75.3, 74.9, 73.5, 72.9, 72.4, 68.7, and 65.9 (CH_2).

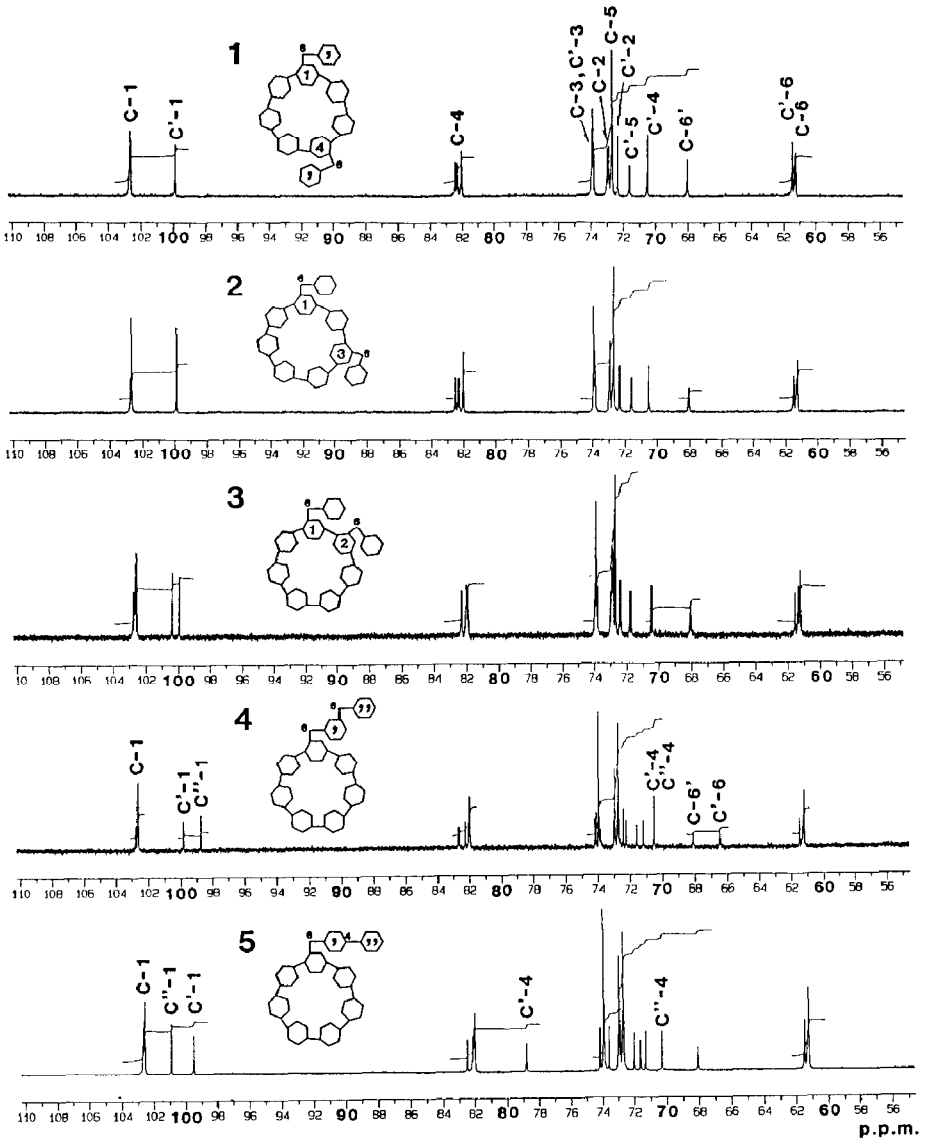

Fig. 2. ^{13}C-N.m.r. spectra of compounds **1–5** measured in D_2O at 125.65 MHz. C: the carbon atom of the ring D-glucose unit. C' and C'': the carbon atom of the branched unit. C-6': the carbon atom of the branch point.

6-O-(α-Isomaltosyl)-cyclomaltoheptaose (**4**). — A mixture of **6** (0.31 g) and powdered 4Å molecular sieves (0.25 g) in dry dichloromethane (5 mL) was stirred under nitrogen at −20°. A solution of **10** (0.54 g) in dichloromethane (5 mL) was added, followed after 30 min by a solution of trifluoromethanesulfonic acid (20 μL) in dichloromethane (3 mL). After 2 h, diethylamine (1 mL) was added, and the mixture was sequentially filtered through a Celite pad, washed with M sulfuric acid, saturated aqueous sodium hydrogencarbonate, and water, dried, and concentrated. Centrifugal chromatography (3:1) benzene–acetone) of the residue gave amorphous [2,3-di-*O*-acetyl-6-*O*-(2,3,4,2′,3′,4′,6′-hepta-*O*-benzyl-isomaltosyl)]hexakis(2,3,6-tri-*O*-acetyl-β-CD (0.12 g, 25.8%) which was subjected to catalytic debenzylation (Pd–C)[9] and deacetylation. The desired compound **4** was isolated from the resulting products by h.p.l.c. on a YMC-Pack A-323 column with 7:93 methanol–water, flow rate 2 mL/min at 35°; retention time 17.1 min. Compound **4** (0.035 g, 15%) failed to crystallize and was characterized by its chromatographic behavior on both C_{18}-bonded phase (Fig. 4) and

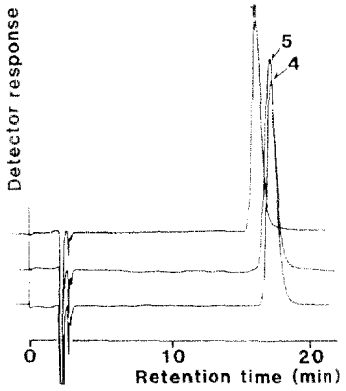

Fig. 3. Elution profiles of compounds **1**, **4**, and **5** on an Asahipak NH2P-50 (250 × 4 mm i.d.) column with 57:43 acetonitrile–water. Flow rate: 1 mL/min. Temp.: ambient. Detector: as in Fig. 1

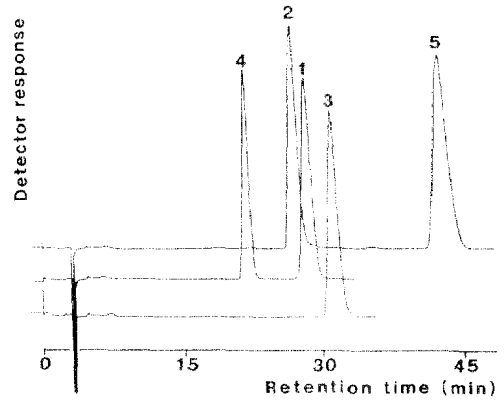

Fig. 4. Elution profiles of compounds **1**–**5** on a YMC-Pack A-312 (150 × 6 mm i.d.) column with 4:96 methanol–water. Flow rate 1 mL/min at 35°. Detector: as in Fig. 1.

NH_2-bonded phase (Fig. 3), and by ^{13}C-n.m.r. spectroscopy in D_2O (Fig. 2 and see data of **3** in Table I of ref. 1): $[\alpha]_D^{30}$ + 166.1° (*c* 0.6, water). Production of the 6-*O*-β-isomaltosyl derivative could not be observed even by careful h.p.l.c. of the reaction product. In order to account for the low yield of **4**, it is presumed that a part of isomaltose derivative **10** was consumed as the glycosyl donor and/or the isomaltosyl β CD derivatives produced were hydrolyzed to glucose or glucosyl-β CD derivatives during glycosylation or debenzylation. In the chromatogram of the reaction product described above, 6-*O*-α- and β-D-glucosyl-β CDs were detected.

*Chemical synthesis of 6^1,6^2-di-*O*-(α-D-glucopyranosyl)-cyclomaltoheptaose* (**3**). — 6^1,6^n-Di-*O*-(*tert*-butyldimethylsilyl)-β CDs (n = 2, 3, and 4) were first prepared by reaction of dry β CD with 2.5 mol. equiv. of *tert*-butyldimethylsilyl chloride in pyridine. The three positional isomers were isolated by h.p.l.c. and characterized by ^{13}C-n.m.r. spectroscopy and by conversion to the known compounds, 6^1,6^n-di-*O*-(*p*-toluenesulfonyl)-β CDs[10].

Isolated 6^1,6^2-di-*O*-(*tert*-butyldimethylsilyl)-β CD (**11**) was acetylated and desilylated with 47% borontrifluoride etherate[11] to obtain the required glucosyl acceptor, bis(2,3-di-*O*-acetyl)pentakis(2,3,6-tri-*O*-acetyl)-β CD (**12**), $[\alpha]_D^{26}$ + 112.5° (*c* 0.9, chloroform). ^{13}C-N.m.r. data ($CDCl_3$): δ 97.1, 97.0 (7C, C-1), 63.3–61.7 (7C, C-6), and 20.7 (CH_3CO).

Compound **12** was then glucosylated by reaction with 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl trichloroacetimidate[8] and was post-treated in the same manner as described above. Details on these have been reported together with chemical syntheses of the other two positional isomers of diglucosyl-β CD[2]. The $[\alpha]_D^{30}$ value of **3** was + 165.8° (*c* 0.6, water).

RESULTS AND DISCUSSION

*Isolation and identification of 6^1,6^2-di-*O*-(α-D-glucopyranosyl)-β CD* (**3**) *from a glucosyl-β CD mixture.* — Fig. 1 shows a chromatogram of glucosyl-β CD mixture (Lot. No. 89 202). The component corresponding to peak 3 was relatively easily isolated from this mixture by semi-preparative h.p.l.c. on a YMC-Pack SH-343-5 AQ column with 13:87 methanol–water at a flow-rate of 5 mL/min. The product was identified with chemically synthesized[2] **3** by h.p.l.c. and by ^{13}C-n.m.r. spectroscopy. The molar ratio of **1:2:3** in this mixture was 2.8:2.3:1.0.

*Identification of 6-*O*-(α-isomaltosyl)-β CD* (**4**) *and mechanism of its production.* — The third branched β CD isolated from the mother liquors of a large-scale preparation of β CD by action of CGTase on potato starch was identified with chemically synthesized **4** by h.p.l.c. and by ^{13}C-n.m.r. spectroscopy.

As **4** cannot be formed by this preparation from potato starch with pure CGTase, production of **4** was presumed to be due to a reverse action of glucoamylase, used for shortening of the side chains of the branched CDs produced. In order to prove this presumption, a substrate solution containing 2 mL of 50% 6-*O*-(α-maltosyl)-β CD in 0.1M acetate buffer (pH 4.5) and 1 mL of enzyme solution containing 300 U of

TABLE I

Methylation analyses of compounds **1–5**

Product	*Molar ratio*				
	1	**2**	**3**	**4**	**5**
1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol	2	2	2	1	1
1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol				1	
1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol	5	5	5	6	7
1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol	2	2	2	1	1

glucoamylase (One unit of glucoamylase is defined as the amount of enzyme that forms 10 mg of glucose from soluble starch in 30 min at 40° and pH 4.5) from *Rhizopus niveus* were incubated for 5 h at 55°. After the glucoamylase was inactivated in boiling water for 10 min, the resulting solution was examined by h.p.l.c., and the production of trace amounts of **4** was confirmed.

Methylation analysis. — The results of methylation analyses of **1–5** indicated that **1**, **2** and **3** are diglucosyl-β CDs, **4** is isomaltosyl-β CD, and **5** is maltosyl-β CD (Table I).

^{13}C-N.m.r. spectroscopy. — In Fig. 2 ^{13}C-n.m.r. spectra of **1–5** are compared. The ^{13}C-resonances of ring carbons in the spectrum of **1** were assigned using the 2D method. The spectra of isomers are similar to that of **1**, and hence assignments of signals could be definitively made by analogy. The assignments of C-6 signals were confirmed by the INEPT method, using $\Delta = 3/4J$. Although the spectra of **1**, **2**, and **3** are similar, the side-chain C-1, C-4, and C-5 signals of **3** are each split into two peaks, whereas those signals of **1** appear individually as a single peak. In the expanded spectra of the 66 to 72.5 p.p.m. region, those signals of **1** remained unaltered as single peaks, respectively, while the side chain C-4 and C-5 signals of **2** were slightly split into two peaks each[1]. These facts indicate the difference of magnitude of interaction between two side-chain D-glucose units in each molecule of **1**, **2**, and **3**.

The most remarkable features in the spectra of **4** and **5** are signals of $\alpha(1\rightarrow6)$-linked C-1 and C-6 in the side-chain of **4**, and $\alpha(1\rightarrow4)$-linked C-1 and C-4 in the side-chain of **5**, respectively.

Chromatographic behavior. — Fig. 3 shows the elution profiles of **1**, **4**, and **5** on an Asahipak NH2P-50 column with 57:43 acetonitrile–water as an eluent. Chromatographic behavior of three positional isomers of diglucosyl-β CD (**1**, **2**, and **3**) in this system was the same, while **4** and **5** eluted a little more slowly. The elution sequence on the NH_2-bonded stationary phase generally follows the order of molecular size[12]. While isomers having the same molecular size are difficult to separate from each other, it became apparent that branched β CDs with one side-chain are distinguishable from the isomers having two side-chains.

On the other hand, the retention time on the reversed-phase column increases with decreasing solubility in water[13]. Chromatographic behavior of the five isomers

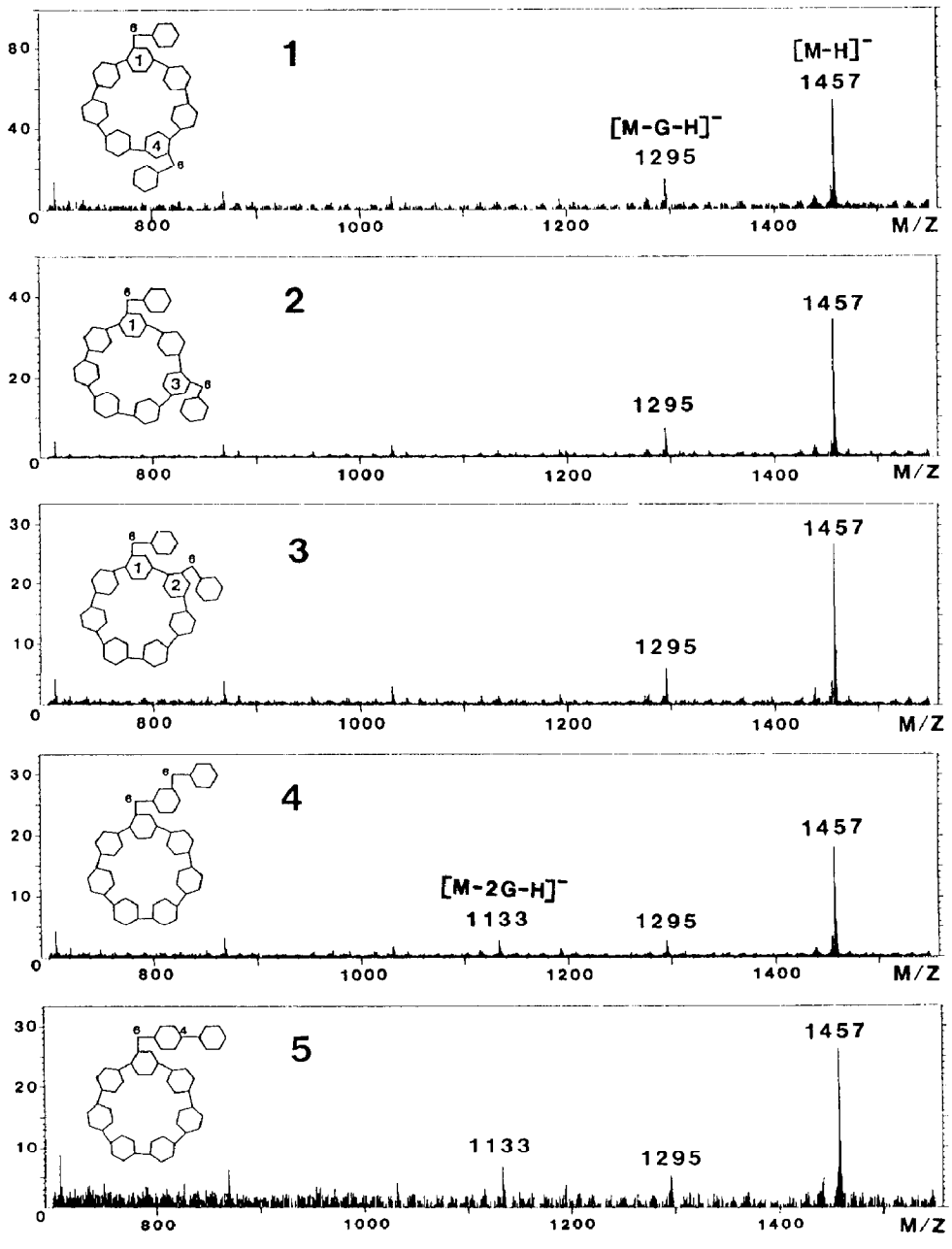

Fig. 5. F.a.b.-m.s. spectra of compounds **1**–**5** in the negative-ion mode.

(**1–5**) on a YMC-Pack A-312 ODS column differed from each other (Fig. 4), and hence they could be isolated by semi-preparative h.p.l.c. on a large-scale column of the same type. A few per cent of methanol in water was used as the eluent, and increasing methanol concentration in the eluent decreased retention times. The retention time of maltosyl-β CD having an $\alpha(1\rightarrow4)$ linkage in the side chain was more influenced by the concentration of methanol in the eluent than the isomers which have only $\alpha(1\rightarrow6)$ linkages in the side chain(s).

F.a.b.-m.s. — The f.a.b.-m.s. spectra of **1–5** in the negative mode were consistent with their structures (Fig. 5). The $[M - H]^-$ peak was clearly observed at *m/z* 1457. A peak for fragment ion $[M - G - H]^-$ (*m/z* 1295) was in all spectra, but $[M - 2G - H]^-$ (*m/z* 1133) peak was observed only in the spectra of **4** and **5**, the side chain of which is a disaccharide. All these fragment ions must be formed through one cleavage of the side chain (primary fragments).

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