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Carbohydrate Research 339 (2004) 2875–2881
Carbohydrate RESEARCH

Isolation and characterization of two positional isomers of novel heterogeneous branched cyclomaltohexaoses (α-cyclodextrins) having a D-galactobiosyl residue on the side chain

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Received 13 August 2004; accepted 13 October 2004
Available online 11 November 2004

Abstract—Transgalactosylated products, 2-*O*-α-D-galactobiosyl-cyclomaltohexaoses (α-cyclodextrins, αCDs), were synthesized by α-galactosidase from coffee bean using melibiose and αCD as a donor substrate and an acceptor, respectively. Two positional isomers of 2-*O*-α-galactobiosyl-αCDs were isolated and purified by HPLC, and their structures were elucidated by FABMS and NMR spectroscopies, as well as by an enzymatic degradation method. The chromatographic behavior of these novel galactosylated αCDs was compared on three HPLC columns with different separation modes.
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Keywords: 2-*O*-α-Galactobiosyl-α-cyclodextrin; Positional isomer; Separation; Structural analysis; NMR spectroscopy

1. Introduction

Cyclomalto-oligosaccharides (cyclodextrins, CDs) have been extensively used to improve the solubility, stability, and bioavailability of various water-insoluble compounds by employing their complexating properties. To develop broader applications different from the conventional CDs and homogeneous branched CDs such as glucosyl-CDs and maltosyl-CDs, heterogeneous branched CDs of galactosyl-CDs were produced by transgalactosylation with α-galactosidase (EC 3.2.1.22).[1] The preparation was confirmed to contain 6-*O*-α-D-galactosyl-CDs as the main products and 2-*O*-α-D-galactosyl-CDs as minor components.

The isolation and characterization of two new positional isomers of 2-*O*-α-D-galactobiosyl-cyclomaltohexaoses (α-cyclodextrins, αCDs) are described in this study. Until now, information about branched CDs has related to 6-*O*-glycosyl-CDs, and no data on 2-*O*-glycosyl-CDs has been reported. 2-*O*-α-Galactobiosyl-αCDs are potential drug carriers in delivery systems because their terminal galactosyl residues are recognized by animal lectins,[2,3] and thus the galactobiosyl side chains of 2-*O*-α-galactobiosyl-αCDs can be utilized as ligands for the targeting of drugs. Studies on 2-*O*-glycosyl-CDs could open the way to the application of branched CDs.

2. Experimental

2.1. Materials and methods

A mixture of galactosyl-CDs was prepared as described previously.[1] Briefly, melibiose (0.2 M) and αCD (0.1 M) were dissolved in 50 mM acetate buffer (pH 6.5) and incubated with coffee bean α-galactosidase (36 units/melibiose 1 g) at 40 °C for 48 h. Subsequently, the mixture was heated at 100 °C for 10 min to stop the reaction, and the products were separated with HPLC.

Cyclomaltodextrin glucanotransferase (CGTase, EC 2.4.1.19) (2,000 units/mL) from *Bacillus circulans* was prepared and purified by a method previously reported.[4]

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doi:10.1016/j.carres.2004.10.007

All reagents were of analytical grade. HPLC-grade acetonitrile and methanol were used for chromatography. The water employed in HPLC was purified using an ultra-pure water system, CPW-100 (Advantec).

2.2. Isolation of galactobiosyl-αCDs

The fraction of galactobiosyl-αCDs was separated from a mixture of galactosyl-αCDs by HPLC on a ChemcoBond 5NH_2 column (250 × 20 mm id) (Chemco) with 57:43 acetonitrile–water, at a flow rate of 3.0 mL/min.

2.3. General instrumental methods

HPLC was performed with a Jasco PU 980 pump, a Rheodyne 7125 injector, a RID-10A refractive index detector, and a C-R6A chromatopac (Shimadzu). HPLC analyses at constant temperature were conducted with an SSC 3510C column oven (Senshu Scientific Co.). The columns employed were a Daisopak SP-120-5-ODS-BP (250 × 20 mm id) (Daiso), a Wakosil-II5C22 (250 × 4.6 mm id) (Wako), a ChemcoBond 3.5NH_2 column (150 × 4.6 mm id) (Chemco), and a Hypercarb (100 × 4.6 mm id and 100 × 10 mm id) (ThermoQuest).

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, Inc., San Jose, CA, USA) fitted with the Finnigan electrospray-ionization (ESI) interface. The mass spectrometer was operated in the positive-ion mode; the ESI voltage was set to 4.5 kV, and the capillary temperature was 250 °C. The pressure of the sheath gas was 70 psi, and the auxiliary gas was 15 units. Total ion monitoring was performed by scanning the range *m/z* 500–1500 with a scan rate of 3 s/scan.

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 perfluoroalkylphosphazine (UltraMark), and glycerol was used as the matrix solution.

NMR spectral data were recorded for 5–10% solutions in D_2O at 50 °C with a JNM-ECP 400 spectrometer (JEOL). Chemical shifts were expressed in ppm downfield from the signal of Me_4Si referenced to external 1,4-dioxane (67.4 ppm). The other conditions for ^{13}C NMR, ^1H–^1H COSY, and ^1H–^{13}C COSY measurements were the same as in a previous paper.[5]

2.4. Enzymatic degradation

Each sample (0.2 mg) of the galactobiosyl-αCDs (**A** and **B**) in 60 μL of 10 mM acetate buffer (pH 6.0) was individually incubated with CGTase (20 μL, 40 units) at 40 °C for 24 h in order to digest them completely. After inactivation of the enzyme, each hydrolysate was analyzed by HPLC and LC–MS on a ChemcoBond 3.5NH_2 column (150 × 4.6 mm id) (Chemco).

3. Results and discussion

3.1. Isolation

The fraction of galactobiosyl-αCDs transgalactosylated by coffee bean α-galactosidase using melibiose as a donor substrate and αCD as an acceptor, was further separated by semipreparative HPLC on a Daisopak SP-120-5-ODS-BP column with 6:94 methanol–water, at a flow rate of 2.5 mL/min, followed by a graphitized carbon column (Hypercarb) with 12.5:87.5 acetonitrile–water, at 2.5 mL/min. Two components (**A** and **B**) were purified, giving 30 and 75 mg samples, respectively. The molar ratio of **A**:**B** of galactobiosyl-αCDs was 1:2.5.

3.2. FABMS

FABMS studies of the branched CDs can be used not only to estimate the molecular weights, but also to determine the number of branched points on the CD ring. A molecular ion at *m/z* 1295 was observed in each negative-ion FABMS spectrum of **A** and **B**. Accordingly, it was confirmed that they were comprised of αCD and two galactosyl residues. A fragment ion at *m/z* 971 $[M - 2Gal - H]^-$ formed through cleavage of the galactobiosyl side chain, in addition to a fragment ion at *m/z* 1133 $[M - Gal - H]^-$ that was also detected. These data suggest that **A** and **B** are the positional isomers of galactobiosyl-αCDs having only one branched point on the αCD ring.

3.3. ^{13}C NMR spectroscopy

Figure 1 shows the ^1H–^{13}C COSY spectra of galactobiosyl-αCD**s** (**A** and **B**). Their NMR spectra were assigned using ^1H–^1H COSY and ^1H–^{13}C COSY methods and by referring to the spectrum of 2-*O*-α-D-galactosyl-αCD.[1] 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.[6] The assignments of the C-6 signals were confirmed by the distortion less enhancement with the polarization transfer (DEPT) method.[7]

By FABMS analysis, it has been estimated that **A** and **B** are galactobiosyl-αCDs having another galactosyl residue attached to the branched galactose of galactosyl-αCD. The NMR spectra provide information on the mode of linkage between galactobiosyl and the αCD ring, and between two galactosyl residues of galactobiose. In the spectrum of **A**, four different signals for

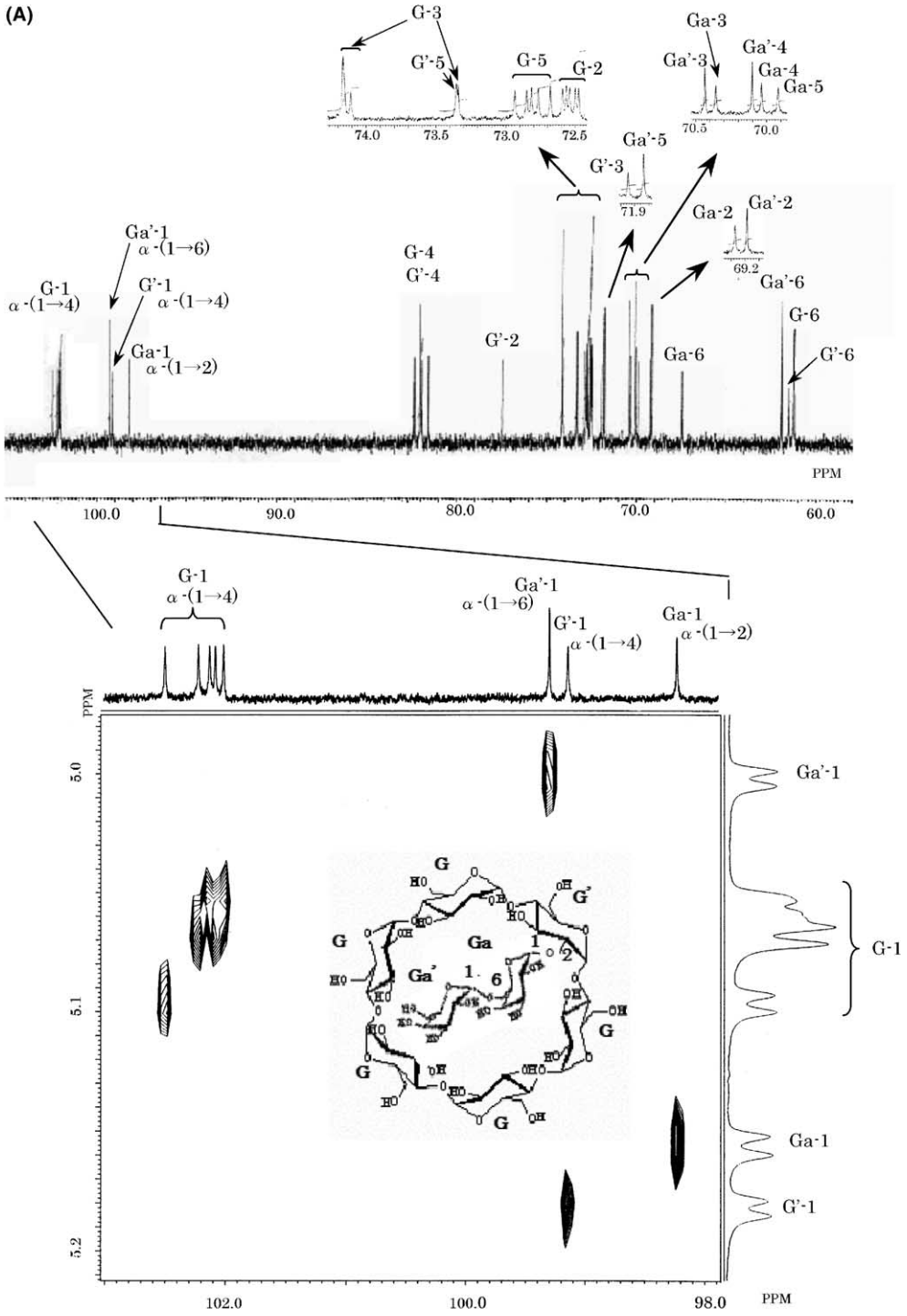

Figure 1. ^{1}H–^{13}C COSY spectra of galactobiosyl-αCD (**A** and **B**) in D_2O at 50 °C. G-1, -2, -3, -4, -5, and -6 are signals of C-1, -2, -3, -4, -5, and -6 atoms of the αCD ring D-glucopyranose units. G′ is the αCD ring D-glucopyranose unit on which the D-galactopyranose residue is α-(1→2)-linked. Ga-1, -2, -3, -4, -5, and -6 are signals of C-1, 2, -3, -4, -5, and -6 atoms of the D-galactopyranose unit on which α-(1→2) is linked directly to CD. Ga′ is the terminal D-galactopyranose unit of the galactobiosyl side chain on αCD.

C-6 were observed; C-6 signals of αCD ring glucoses (G-6, δ 61.29– 61.33), one C-6 signal (G′-6, δ 61.6) of the branched point of αCD ring glucose (G′), one C-6 signal (Ga′-6, δ 61.9) of the terminal galactose residue (Ga′), and the large downfield-shifted C-6 signal (Ga-6, δ 67.5) of another galactose residue (Ga). The relative intensities of G-6, G′-6, Ga′-6, and Ga-6 were 5:1:1:1. Further, the Ga-substituted C-2 signal (G′-2) of G′ shifted considerably downfield to δ 77.5, the adjacent α-(1→4)-linked C-1 signal (G′-1) shifted upfield to δ

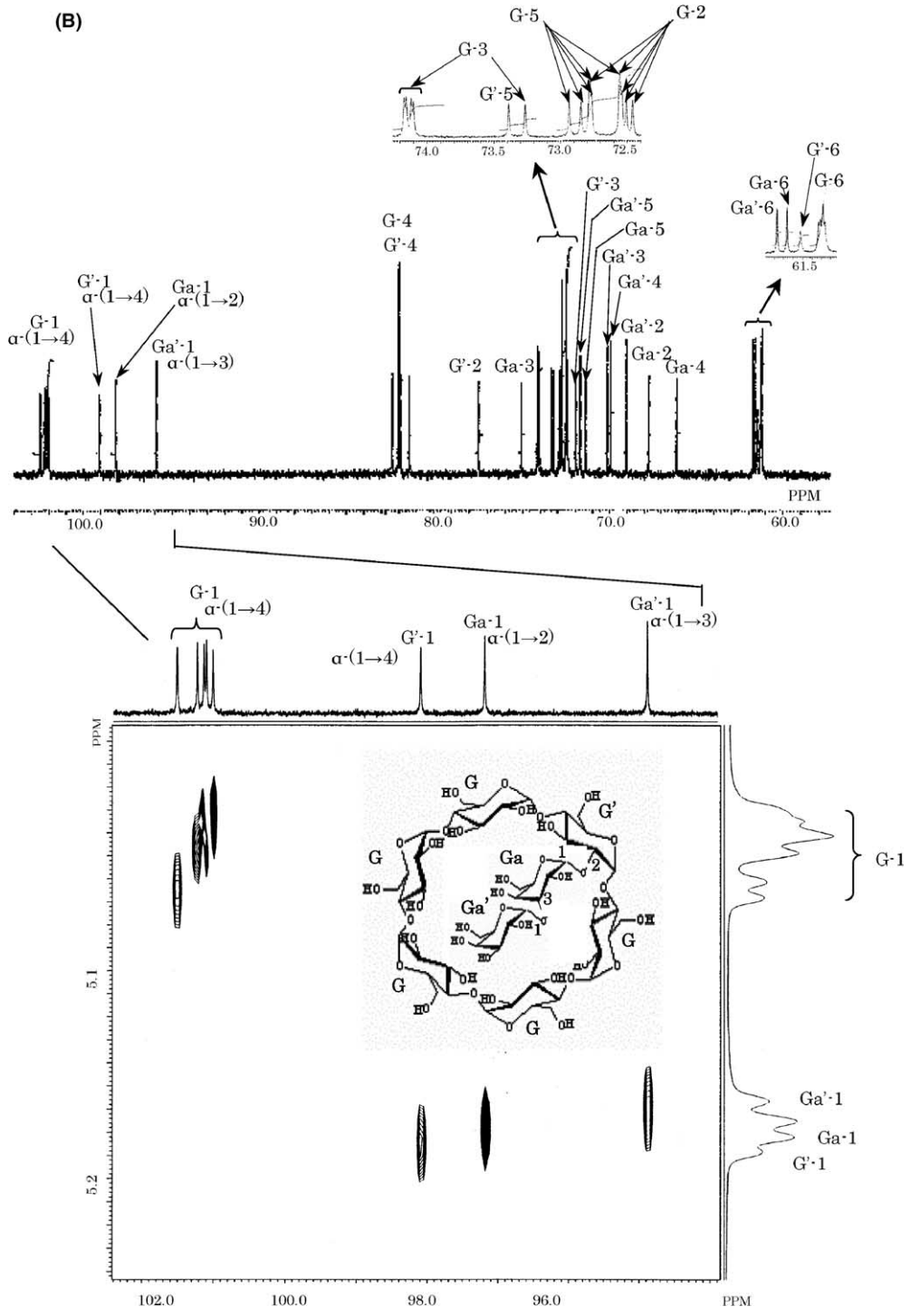

Figure 1 (*continued*)

99.1 under the influence of Ga substitution on C-2 (effect), and the α-(1→2)-linked C-1 signal (Ga-1, δ 98.2) was observed more upfield than G′-1. Moreover, the α-(1→6)-linked C-1 signal (Ga′-1, δ 99.3) of Ga′, appeared more upfield than G-1 (δ 102.0–102.2), and the relative intensities of G-1, G′-1, Ga-1, and Ga′-1 were 5:1:1:1. Therefore, it was determined that **A** was 2-*O*-(6-*O*-α-D-galactopyranosyl)-α-D-galactopyranosyl-αCD [α-D-Gal-(1→6)-α-D-Gal-(1→2)-αCD].

Similarly, in the spectrum of **B**, four kinds of signals for C-6 were observed in the chemical shifts of δ 61.26 to δ 61.8, that is, C-6 signals of αCD ring glucoses (G-6, δ 61.26–61.32), one C-6 signal (G′-6, δ 61.52) of G′, one C-6 signal (Ga′-6, δ 61.8) of Ga′, and the C-6 signal

(Ga-6, δ 61.7) of Ga. The relative intensities of G-6, G′-6, Ga′-6, and Ga-6 were 5:1:1:1. The most characteristic Ga' substituted C-3 signal (Ga-3) of Ga was observed well downfield at δ 75.1, and the corresponding α-(1→3)-linked C-1 signal (Ga′-1, δ 95.9) of Ga′, appeared more upfield than the other C-1 signals. Further, the Ga-substituted C-2 signal (G′-2) of G′ shifted considerably downfield to δ 77.5, the adjacent α-(1→4)-linked C-1 signal (G′-1) shifted upfield to δ 99.1 under the influence of Ga substitution on C-2 (β effect), and the α-(1→2)-linked C-1 signal (Ga-1, δ 98.2) was observed more upfield than G′-1. Therefore, it was determined that **B** was 2-*O*-(3-*O*-α-D-galactopyranosyl)-α-D-galactopyranosyl-αCD [α-D-Gal-(1→3)-α-D-Gal-(1→2)-αCD].

3.4. Enzymatic degradation

It is well known that bacterial saccharifying α-amylase (BSA, EC 3.2.1.1) and CGTase decompose α-(1→4)-glucosidic linkages except for the first and second glucosidic linkages toward the reducing end from the branching point (α-(1→6)-glucosidic linkage) in starch and branched dextrins, and produce 6^3-*O*-α-D-glucosylmaltotriose (degree of polymerization (DP) 4) as the smallest branched oligosaccharides. Using this degradation system, we have already elucidated the structures of many positional isomers of homogeneous and heterogeneous multibranched α, β, and γ CDs.[8–11] The highly rigid αCD ring, which has the smallest cavity, was decomposed with CGTase, but not with BSA. On the other hand, βCD and γCD rings were decomposed with BSA as well as with CGTase due to its more flexible structure and larger cavity than αCD. Moreover, it was confirmed that 2-*O*-α-D-galactosyl-αCD corresponding to the fundamental compound of the two isomers **A** and **B** of galactobiosyl-αCDs was finally decomposed to a DP 5 branched oligosaccharide and glucose with CGTase.

From these results, models of the digestion of galactobiosyl-αCDs (**A** and **B**) with CGTase are suggested together with that of 2-*O*-α-D-galactosyl-αCD in Figure 2. With CGTase, α-D-Gal-(1→3)-α-D-Gal-(1→2)-αCD and α-D-Gal-(1→6)α-D-Gal-(1→2)-αCD are finally decomposed to the same DP 6 of branched oligosaccharides and glucose. Figure 3 shows the chromatograms for RI detection and the mass chromatograms for the enzymatic degradation products of **A** and **B** with CGTase. In the LC–MS analysis, products **1** and **2** corresponding to the enzymatic digests of **A** and **B**, respectively, have different retention times, although the adduct ions with the ammonium ion $[M + NH_4]^+$ at *m/z* 1008 for both **1** and **2** were monitored in the positive-ion ESI mode. These results show that the products **1** and **2** are different

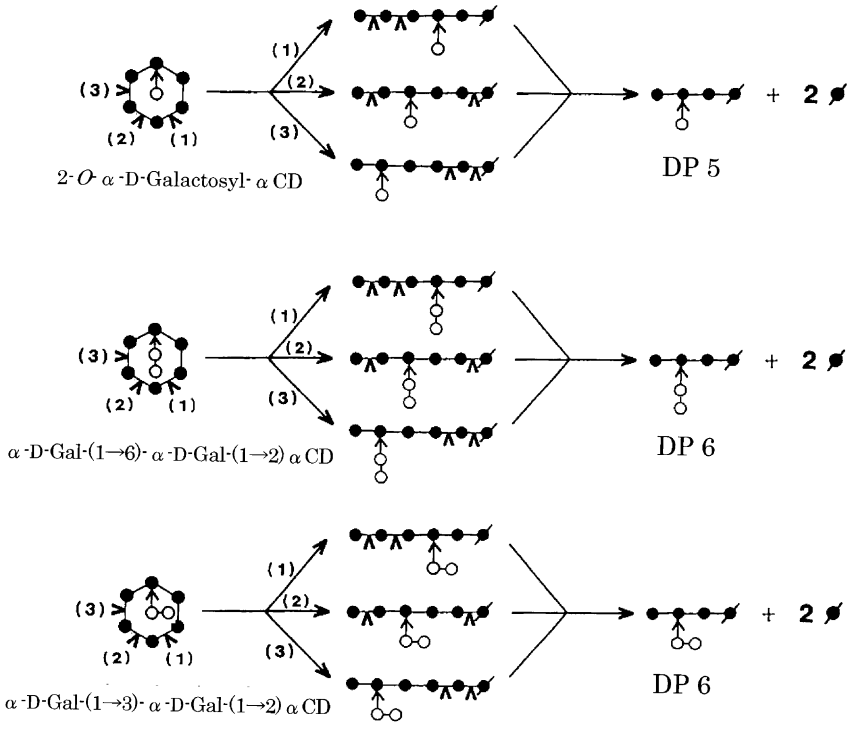

Figure 2. Models of the reaction of two positional isomers of galactobiosyl-αCDs and 2-*O*-α-D-galactosyl-αCD with CGTase. Symbols: ●, glucose; ◢, glucose with reducing end; ○, galactose; ●—●, α-(1→4)-glucosidic linkage; ⚲, α-(1→6)-galactosidic linkage; ○—○, α-(1→3)-galactosidic linkage; ↑, α-(1→2)-linkage between galactose and glucose; ∧, attack point of CGTase.

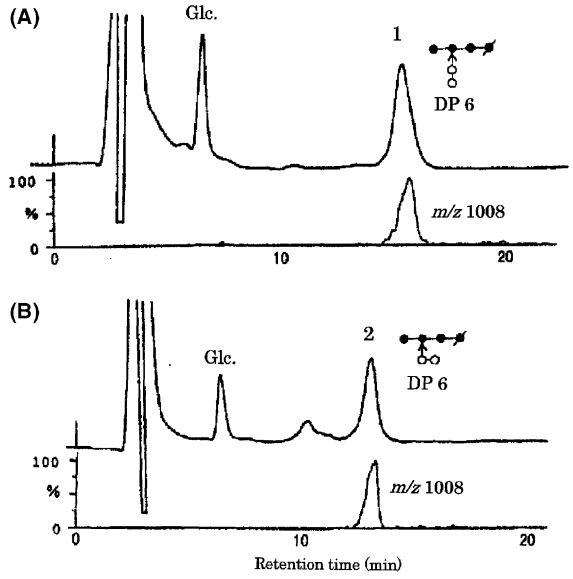

Figure 3. Chromatograms of degradation products from **A** and **B** with CGTase and mass chromatograms of their ammonium adducts. Chromatographic conditions: column, ChemcoBond 3.5NH_2 (150 × 4.6 mm id); eluent, 60:40 CH_3CN–H_2O; flow rate, 0.6 mL/min; temperature, 30 °C. Each upper chromatogram was detected by RI. Symbols as in Figure 2.

branched oligosaccharides having the same DP 6. Thus, it was readily determined that **A** and **B** were the positional isomers of 2-*O*-α-D-galactobiosyl-αCDs, and the results of enzymatic degradation were consistent with the structure determined previously with ^{13}C NMR spectroscopy and FABMS.

3.5. Chromatographic behavior

The two positional isomers (**A** and **B**) of 2-*O*-α-galactobiosyl-αCDs showed characteristic chromatographic behavior on three kinds of HPLC columns with different separation modes. Figure 4. [I] shows their elution profiles on a reversed-phase column: α-D-Gal-(1→6)α-D-Gal-(1→2)-αCD was eluted earlier than α-D-Gal-(1→3)-α-D-Gal-(1→2)-αCD on Wakosil II-5C22. On the other hand, on Hypercarb, which is a graphitized carbon column, the retention characteristics of carbohydrates are essentially the result of adsorption. The unique resolving power of this column led to the excellent separation of each of the positional isomers of several multi-branched CDs. The elution profiles of the two isomers of galactobiosyl-αCDs was the reverse of that on a reversed-phase column (see Fig. 4 [II]). The chromatograms of galactobiosyl-αCDs on ChemcoBond 3.5NH_2 are shown in Figure 4. [III] In general, an amino column with an acetonitrile–water system gives an elution sequence in the order of the molecular size of saccharides. Therefore, two isomers of galactobiosyl-αCDs having the same molecular size should be difficult to separate from each other on the amino column. However, the two isomers of galactobiosyl-αCDs were better separated on ChemcoBond 3.5NH_2. This would be likely due to the difference in the linkage mode of the terminal galactose residue of galactobiosyl-αCDs. The same phenomenon was observed in the chromatogram of products **1** and **2** having the same DP in the LC–MS analysis, that is, the isomer having the α-(1→6)-linkage was eluted later than the isomer having the α-(1→3)-linkage (see Fig. 3). The characteristic chromatographic behavior on three kinds of HPLC columns is useful to estimate the linkage mode of branched oligosaccharides.

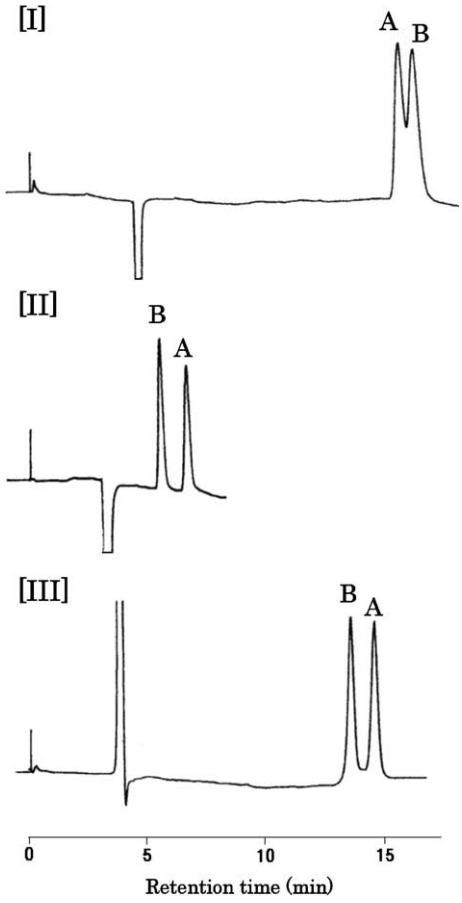

Figure 4. Elution profile of two isomers of galactobiosyl-αCD on HPLC. **A**, α-D-Gal-(1→6)-α-D-Gal-(1→2)-αCD; **B**, α-D-Gal-(1→3)-α-D-Gal-(1→2)-αCD. Chromatographic conditions: [I] column, Wakosil II-5C22 (250 × 4.6 mm id); eluent, 4:96 MeOH–H_2O; flow rate, 0.7 mL/min; temperature, 30 °C; [II] column, Hypercarb (150 × 4.6 mm id); eluent, 16:84 CH_3CN–H_2O; flow rate, 0.7 mL/min; temperature, 30 °C;. [III] column, ChemcoBond 3.5NH_2 (250 × 4.6 mm id); eluent, 63:37 CH_3CN–H_2O; flow rate, 0.8 mL/min; temperature, 30 °C.

4. Conclusions

2-*O*-(6-*O*-α-D-Galactopyranosyl)-α-D-galactopyranosyl-αCD and 2-*O*-(3-*O*-α-D-galactopyranosyl)-α-D-galactopyranosyl-αCD were synthesized at a molar ratio of 1:2.5 from αCD and melibiose by transgalactosylation

of coffee bean α-galactosidase, with no 2-*O*-(4-*O*-α-D-galactopyranosyl)-α-D-galactopyranosyl-αCD in the reaction products. The result was identical to that for a series of 6-*O*-α-galactobiosyl-αCDs reported already.[1] That is, 2-*O*-α-D-galactosyl-αCD as the intermediate is formed by the first transgalactosylation with coffee bean α-galactosidase and the second galactosyl residue in the digalactosylation is linked to OH on C-3 or C-6 of the side chain galactose of monogalactosyl-αCD, not to OH on C-4.

It is likely that two novel positional isomers of 2-*O*-α-galactobiosyl-αCDs will be used for studies on lectins as authentic samples together with a series of 6-*O*-α-galactobiosyl-αCDs.

Acknowledgements

The authors thank Dr. S. Horiyama and Ms. K. Suwa (Mukogawa Women's University) for measuring the NMR and FABMS spectra. We are grateful to Bio Research of Yokohama for generously providing the galactosyl-αCD mixture.

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