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Isolation and Characterization of Hexakis(2,6-di-*O*-methyl)cyclomaltohexaose and Octakis(2,6-di-*O*-methyl)cyclomalto-octaose, and Their Over-methylated Homologues

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The methylation of cyclomaltohexaose (α-CD) and cyclomalto-octaose (γ-CD) gave two major components in each case. These methylated compounds were isolated by high-performance liquid chromatography and characterized by carbon-13 nuclear magnetic resonance spectroscopy, fast-atom bombardment mass spectrometry, and fragmentation analysis. The two major products from α-CD were hexakis(2,6-di-*O*-methyl)cyclomaltohexaose and pentakis(2,6-di-*O*-methyl)-mono(2,3,6-tri-*O*-methyl)cyclomaltohexaose and those from γ-CD were octakis(2,6-di-*O*-methyl)cyclomalto-octaose and heptakis(2,6-di-*O*-methyl)-mono(2,3,6-tri-*O*-methyl)cyclomalto-octaose.

Keywords hexakis(2,6-di-*O*-methyl)cyclomaltohexaose; pentakis(2,6-di-*O*-methyl)-mono(2,3,6-tri-*O*-methyl)cyclomaltohexaose; octakis(2,6-di-*O*-methyl)cyclomalto-octaose; heptakis(2,6-di-*O*-methyl)-mono(2,3,6-tri-*O*-methyl)cyclomalto-octaose; ^{13}C-NMR; HPLC; FAB-MS; solubility

Methylated cyclomalto-oligosaccharides (CDs) have unique physicochemical properties, exhibiting very high solubilities in both water and organic solvents compared with the parent CDs.[1] Therefore, they have become of interest in many fields.[2-11] Some research groups have reported the preparation of 2,6-di-*O*-methyl CDs.[2,12-15] Casu *et al.*[12] have prepared hexakis(2,6-di-*O*-methyl)cyclomaltohexaose (DM-α-CD) and heptakis(2,6-di-*O*-methyl)cyclomaltoheptaose (DM-β-CD) by partial methylation of α-CD and β-CD with dimethyl sulfate, but later they corrected their previous report, finding that 10% of hydroxyl groups at C-3 in both methylated compounds was also methylated.[14] Boger *et al.*[13] and Szejtli *et al.*[15] reported that DM-α-CD[13] and DM-β-CD[13,15] were obtained in a pure state by methylation of α-CD and β-CD with dimethyl sulfate. However, we proved that DM-β-CD prepared by Szejtli *et al.*'s method[15] was a mixture of DM-β-CD, hexakis(2,6-di-*O*-methyl)-mono(2,3,6-tri-*O*-methyl)-cyclomaltoheptaose, and several minor over-methylated homologues.[16] DM-α-CD prepared by both methods[13,15] was also ascertained to be a mixture. On the other hand, little is known concerning octakis(2,6-di-*O*-methyl)cyclomalto-octaose (DM-γ-CD). Pitha[2] investigated the enhancement of water solubility of vitamins by complexation with DM-γ-CD, but the details of the isolation and the physical constants of DM-γ-CD were not given.

We now report an isolation of pure DM-α-CD and DM-γ-CD, and moreover, of their over-methylated homologues by semi-preparative high-performance liquid chromatography (HPLC), and we present their physicochemical properties.

Experimental

General Methods Melting points were measured with a Yanagimoto micro melting-point apparatus and are uncorrected. Optical rotations were determined with a JASCO digital polarimeter, model DIP 360. Thin-layer chromatography (TLC) was performed on Silica gel 60 TLC plates (Merck) with benzene–acetone–methanol (7:4:1) by spraying with sulfuric acid. HPLC was conducted with a TRI ROTAR SR-1 (JASCO), as SE-61 refractive index monitor (Showa Denko), and a column oven SSC-3510C (Senshu). The columns used were (A) YMC-Pack A-312 ODS (150 × 6 mm i.d.), (B) YMC-Pack AQ-323 ODS (250 × 10 mm i.d.), and (C) YMC-Pack SH-343-5 AQ ODS (250 × 20 mm i.d.) (Yamamura Chemical). Preparative liquid chromatography (LC) was performed on a Lobar prepacked column packed with LiChroprep Si 60 (40—63 μm), size C (Merck). The structures of the partially methylated derivatives were confirmed by fragmentation analyses,[16] involving successive hydrolysis, reduction, acetylation and characterization of the resulting partially methylated D-glucitol acetates by gas-liquid chromatography-mass spectrometry (GLC-MS). The determination of the molecular weights of the partially methylated CDs by fast-atom bombardment (FAB)-MS was also carried out under the same conditions as described previously.[16] Carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra were recorded for solutions in D_2O and $CDCl_3$, using a JEOL JNM-FX 200 (50.10 MHz) spectrometer. A micro cell was used and chemical shifts are expressed in ppm downfield from the signal of Me_4Si referred to external 1,4-dioxane (67.40 ppm). The Fourier transform (FT)-NMR conditions were as follows: spectral width, 3000 Hz; pulse flipping angle, 45°; number of data points, 16384. The delay time for the insensitive nuclei enhanced by polarization transfer (INEPT) method[17] was 5.1 ms (3/4 J). ^{1}H [^{1}H]-shift-correlated two dimensional (2D) NMR spectroscopy (COSY) and ^{13}C[^{1}H]-shift-correlation (C–H COSY) spectra were recorded with a JEOL GSX-500 spectrometer. The conditions for COSY (and C–H COSY) measurement were as follows: spectral width, 1100 Hz (6002 Hz), pulse flipping angle, 90° (90°); matrix size, 2K × 1K (512 × 4K).

Materials α-CD and γ-CD were kindly supplied by Sanraku Ltd. and Nihon Shokuhin Kako Ltd., respectively. The purity of CDs was confirmed by HPLC examinations and if necessary, they were recrystallized from water. Reagent-grade organic solvents used for chromatography were distilled and water used in the solvent preparations was distilled, deionized and redistilled. Solvents used for synthesis were dried and freshly distilled before use.

Methylation Method A[13]: A mixture of α-CD (0.97 g, 1.0 mmol) or γ-CD (1.30 g, 1.0 mmol), barium oxide (2 g), and barium hydroxide (2 g) in dimethyl sulfoxide (6 ml) and *N*,*N*-dimethylformamide (6 ml) was treated with dimethyl sulfate (4.0 ml, 42 mmol for α-CD; 5.3 ml, 56 mmol for γ-CD) for 48 h at 0—2 °C. To the resultant suspension, 2 ml of concentrated ammonia solution was added. The mixture was stirred for 3 h and diluted with chloroform, then the inorganic materials were filtered off on a pad of Celite. The chloroform layer was washed with water, dried, and concentrated. The yields of methylated α-CDs and γ-CDs were 1.09 g and 0.52 g, respectively.

Method B[18]: Barium oxide (4.5 g) and barium hydroxide (2.3 g) were added to a stirred solution of CD (3.0 g, 3.1 mmol for α-CD, 2.3 mmol for γ-CD) in *N*,*N*-dimethylformamide (50 ml), and the mixture was cooled to 0 °C, then 3.4 ml (36.6 mmol) of methyl iodide was added in portions with stirring while the temperature was maintained below 5 °C. The mixture was stirred for 3 h at 19—20 °C and then diluted with chloroform. The inorganic materials were filtered off on a pad of Celite. The filtrate was neutralized with diluted sulfuric acid and concentrated. The chloroform layer was washed successively with water, aqueous sodium thiosulfate, and water, dried, and concentrated (methylated α-CDs, 3.49 g; methylated γ-CDs, 2.95 g).

Isolation of Partially Methylated CD Derivatives The isolation of each component of partially methylated α-CDs and γ-CDs was performed by LC. A mixture of methylation products was first pre-fractionated on a Lobar LiChroprep Si 60 column with benzene–acetone (1 : 1) as the eluent. The fractions were checked by TLC and then HPLC on a column (A) eluted with 1-propanol–water (15 : 85). Each component was isolated from

appropriate fractions by semi-preparative HPLC on a column (B or C) with 1-propanol–water (15:85—18:82).

Results and Discussion

Preparation and Isolation Methylation of anhydrous α-CD in the same manner as described for β-CD by the method of Szejtli *et al.*[15] with dimethyl sulfate in *N*,*N*-dimethylformamide–dimethyl sulfoxide (1:1) in the presence of barium oxide and barium hydroxide gave a similar result to methylation of β-CD.[16] However, methylation of γ-CD gave mainly octakis(2,3,6-tri-*O*-methyl)cyclomalto-octaose (TM-γ-CD), and DM-γ-CD could not be detected on TLC.

Boger *et al.*[13] reported that DM-α-CD was obtained by methylation of α-CD with the same reagents as mentioned above when the reaction temperature was controlled at 0 °C (method A). This method made it possible to produce DM-γ-CD, as well as DM-α-CD.

Recently, Takeo[18] found that a mild methylation of α-CD, β-CD, and γ-CD with methyl iodide, barium oxide, and barium hydroxide in *N*,*N*-dimethylformamide for 3 h at room temperature gave DM-α-CD, DM-β-CD, and DM-γ-CD (method B). The methylation of α-CD and γ-CD with both methods (A and B) gave almost the same results.

Fig. 1. Chromatograms of Methylation Products of α-CD and γ-CD Using Method B on a Silica Gel 60 TLC Plate (15 cm) Developed with Benzene–Acetone–Methanol (7:4:1)

(1), methylated α-CDs; (2), TM-α-CD; (3), methylated γ-CDs; (4), TM-γ-CD.

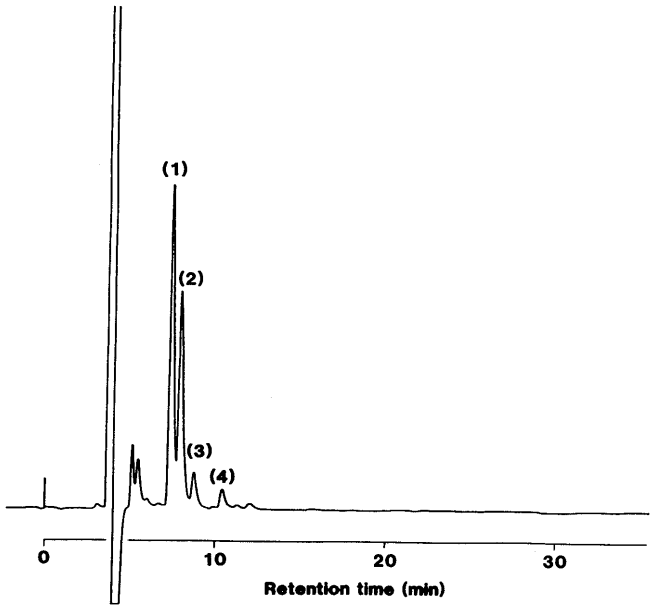

Fig. 2. Separation of Partially Methylated α-CDs Prepared by Method B by HPLC

(1), hexakis(2,6-di-*O*-methyl)cyclomaltohexaose (α-I); (2), pentakis(2,6-di-*O*-methyl)-mono(2,3,6-tri-*O*-methyl)cyclomaltohexaose (α-II); (3) and (4), tetrakis(2,6-di-*O*-methyl)-bis(2,3,6-tri-*O*-methyl)cyclomaltohexaoses (α-III and α-IV). Chromatographic conditions: Column, YMC-Pack A-312 ODS (150 × 6 mm i.d.); eluent, 1-propanol–water (15:85); flow rate, 0.8 ml/min; temperature, 35°C.

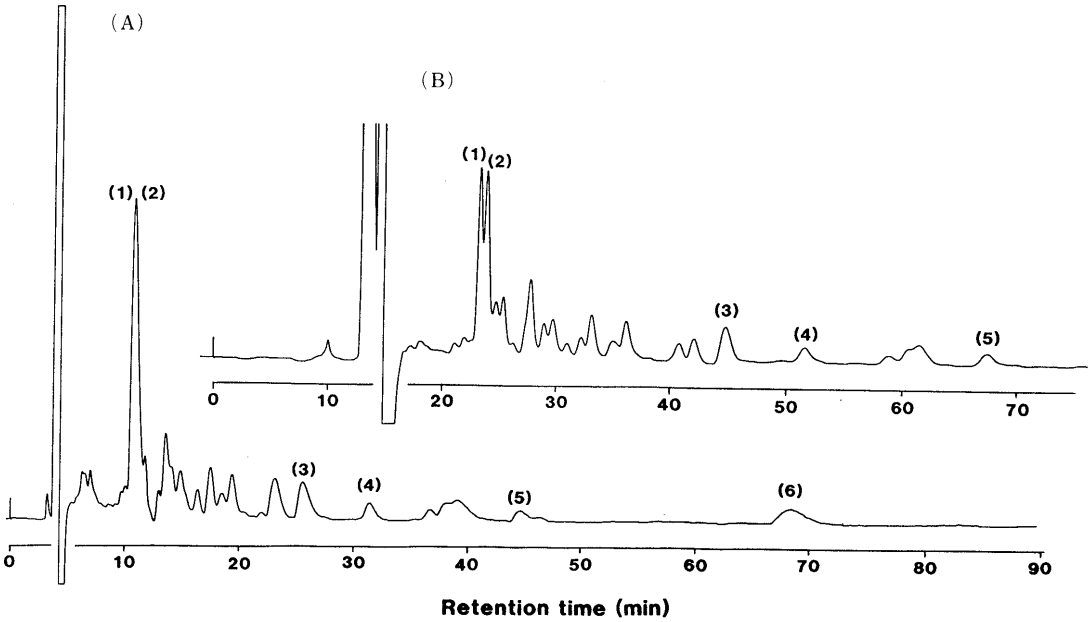

Fig. 3. Separation of Partially Methylated γ-CDs Prepared by Method B by HPLC

(1), octakis(2,6-di-*O*-methyl)cyclomalto-octaose (γ-I); (2), heptakis(2,6-di-*O*-methyl)-mono(2,3,6-tri-*O*-methyl)cyclomalto-octaose (γ-II); (3) and (4), bis(2,6-di-*O*-methyl)-hexakis(2,3,6-tri-*O*-methyl)cyclomalto-octaoses (γ-III and γ-IV); (5), tris(2,6-di-*O*-methyl)-pentakis(2,3,6-tri-*O*-methyl)cyclomalto-octaose (γ-V); (6), mono(2,6-di-*O*-methyl)-heptakis(2,3,6-tri-*O*-methyl)cyclomalto-octaose (γ-VI). Chromatographic conditions: (A) as in Fig. 2; (B) column, YMC-Pack AQ-323 ODS (250 × 10 mm i.d.); eluent, 1-propanol–water (17:83); flow rate, 1 ml/min; temperature, 35 °C.

However, method B using methyl iodide had many advantages over method A with dimethyl sulfate; a much shorter reaction time, smaller amounts of barium oxide and barium hydroxide, and easier control of the reaction temperature.

The methylation products of α-CD gave one major and a few minor spots on TLC (Fig. 1). The HPLC elution profile shows that the product contained two major and at least two minor components (Fig. 2). The relative ratio of major components 1 to 2 was 1.3 to 1.0.

On the other hand, methylation of γ-CD gave one major product together with a number of by-products on TLC (Fig. 1). Figure 3 shows elution profiles of the methylation products of γ-CDs on two ODS columns of different sizes. The components 1 and 2 could not be separated on column (A) eluted with 1-propanol–water (15 : 85) at 0.8 ml/min (Fig. 3 A). The use of a bigger column (B) made it possible to separate them by elution with 1-propanol–water (17 : 83) at 1.0 ml/min (Fig. 3B).

The results revealed much higher reactivity of the hydroxyl groups at C-3 in γ-CD than in α-CD and β-CD, and therefore in the preparation of DM-γ-CD, by-production of significant quantities of over-methylated homologues could not be avoided.

Each component was isolated by semi-preparative HPLC on a 10 or 20 mm i.d. column of YMC-Pack AQ ODS and the partially methylated α-CD derivatives (α-I—α-IV) corresponding to the peaks from 1 to 4 in Fig. 2, and six partially methylated γ-CD derivatives (γ-I—γ-VI) corresponding to the peaks from 1 to 6 in Fig. 3 were obtained in a chromatographically pure state.

Table I. Melting Points and Specific Rotations for Methylated CD Derivatives

Compound	mp (°C)	$[\alpha]_D^{25}$ (°, $c=1.0$, H_2O)
α-I	301—307 (dec.)	+154.2
α-II	255—258 (dec.)	+154.9
γ-I	260—264 (dec.)	+180.0
γ-II	248—254 (dec.)	+158.1

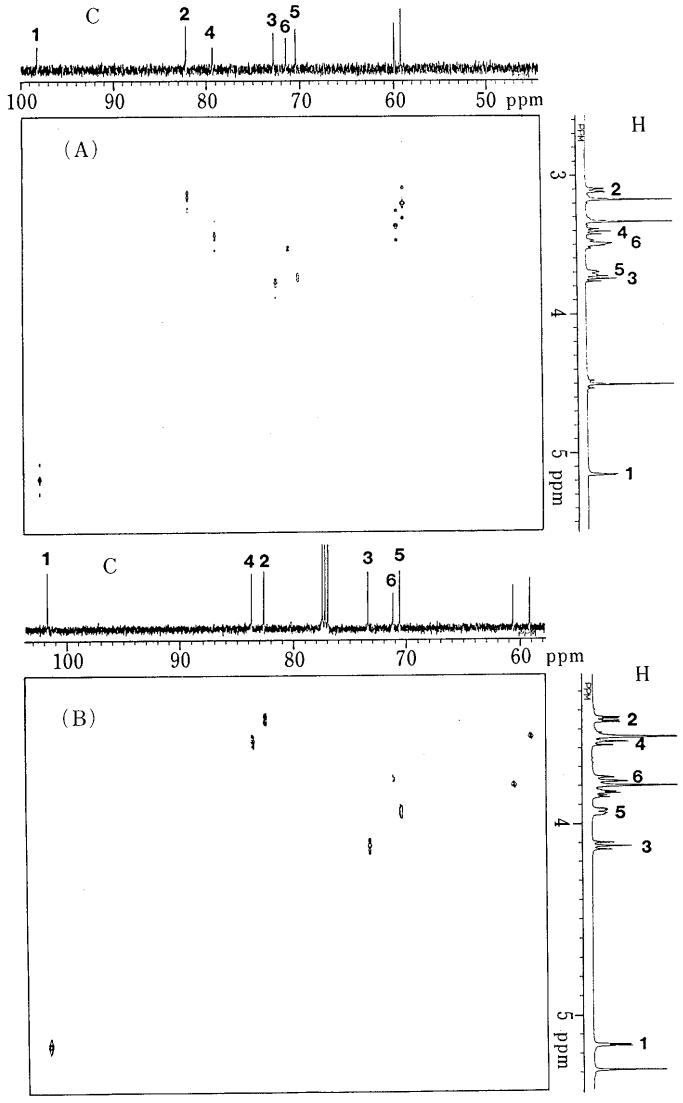

Fig. 4. C–H COSY Spectra of γ-I (DM-γ-CD) in D_2O (A) and in $CDCl_3$ (B) at 35°C

The ^1H- and ^{13}C-NMR spectra are shown along the F_1 and F_2 axes, respectively.

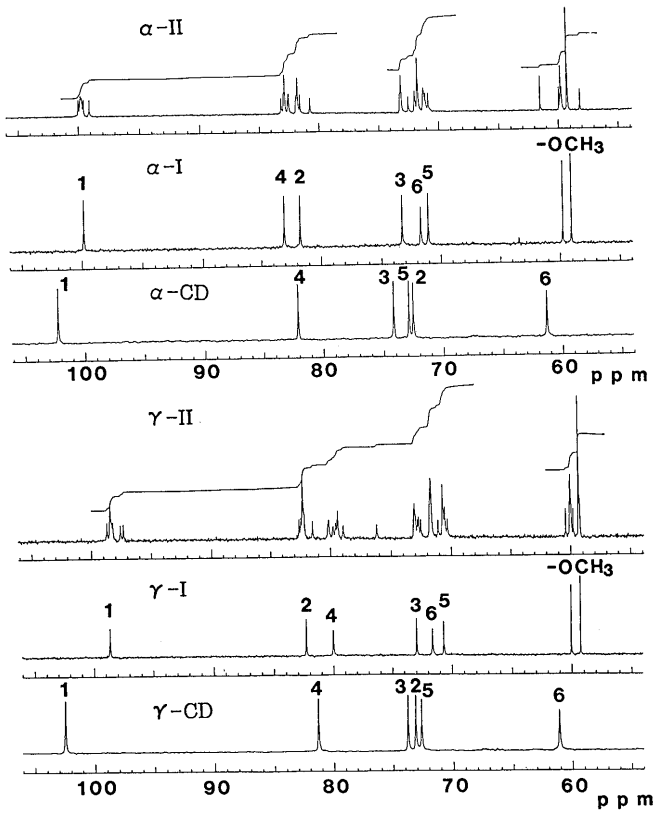

Fig. 5. ^{13}C-NMR Spectra of α-CD, α-I, α-II, γ-CD, γ-I, and γ-II in D_2O

Table II. ^{13}C-NMR Chemical Shifts[a] for CDs and Methylated CD Derivatives in D_2O and $CDCl_3$ at 35°C

Compound	C-1	C-2	C-3	C-4	C-5	C-6
In D_2O						
α-CD	102.23	72.57	74.17	82.15	72.92	61.33
γ-CD	102.47	73.12	73.77	81.30	72.66	61.09
α-I	99.82	81.75	73.27	83.09	71.08	71.72
γ-I	98.65	82.30	72.97	80.01	70.69	71.63
In $CDCl_3$						
α-I	100.91	81.87	73.59	83.62	70.66	71.34
γ-I	101.64	82.59	73.37	83.69	70.58	71.17

a) In ppm from the signal for Me_4Si.

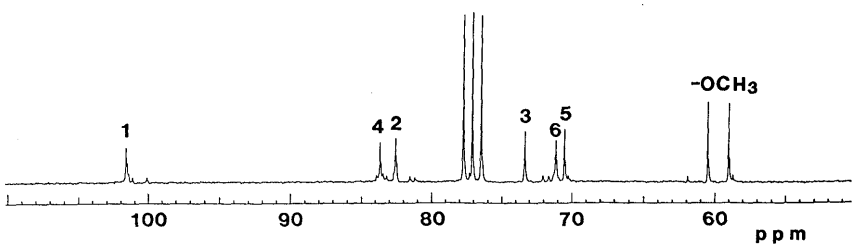

Fig. 6. ^{13}C-NMR Spectrum of a Mixture of Equal Proportion of γ-I and γ-II in $CDCl_3$

Characterization The structures of the partially methylated derivatives isolated were characterized by GLC-MS examination after hydrolysis, followed by conversion into the partially methylated D-glucitol acetates. There was only one product from α-I and γ-I, 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-D-glucitol (2,6-OCH_3). Among the products from other partially methylated derivatives 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol (2,3,6-OCH_3), other than 2,6-OCH_3, was found and the ratios of 2,6-OCH_3/2,3,6-OCH_3 were 5/1 for α-II, 4/2 for α-III and α-IV, 7/1 for γ-II, 2/6 for γ-III and γ-IV, 3/5 for γ-V, and 1/7 for γ-VI. Their molecular weights ($M+H^+$) determined by FAB-MS (m/z) were as follows; α-I (1141), α-II (1155), α-III and α-IV (1169), γ-I (1521), γ-II (1535), γ-III and γ-IV (1605), γ-V (1591), and γ-VI (1619). The results of GLC-MS and FAB-MS indicated that α-I is hexakis(2,6-di-*O*-methyl)cyclomaltohexaose, α-II is pentakis(2,6-di-*O*-methyl)-mono(2,3,6-tri-*O*-methyl)cyclomaltohexaose, α-III and α-IV are the positional isomers of tetrakis(2,6-di-*O*-methyl)-bis(2,3,6-tri-*O*-methyl)cyclomaltohexaose, γ-I is octakis(2,6-di-*O*-methyl)cyclomalto-octaose, γ-II is heptakis(2,6-di-*O*-methyl)-mono(2,3,6-tri-*O*-methyl)cyclomalto-octaose, γ-III and γ-IV are the isomers of bis(2,6-di-*O*-methyl)-hexakis(2,3,6-tri-*O*-methyl)cyclomalto-octaose, γ-V is tris(2,6-di-*O*-methyl)-pentakis(2,3,6-tri-*O*-methyl)cyclomalto-octaose, and γ-VI is mono(2,6-di-*O*-methyl)-heptakis(2,3,6-tri-*O*-methyl)cyclomalto-octaose.

Melting points and specific rotations of α-I, α-II, γ-I and γ-II are listed in Table I. The data of α-I agreed well with those of DM-α-CD synthesized by Takeo *et al.*[19]

Both α-I and α-II were soluble in methanol, the solubility of α-II being higher than that of α-I. α-I was slowly crystallized from methanol in plates. The solubility of γ-II in methanol was higher than that of γ-I, and γ-I could be partially purified by fractional extraction with methanol from solid mixtures and by fractional recrystallization from methanol. It has been reported that the aqueous solubility of the methylated CDs is, interestingly, related inversely to temperature. This property was more marked in the γ-series than in the α-series.

Assignments of the signals in the ^{1}H-NMR spectra of α-I (DM-α-CD) and γ-I (DM-γ-CD) were accomplished by means of a COSY experiment. The ring ^{13}C resonances could be unambiguously assigned by C–H COSY based on the assignment of the ^{1}H resonances. The C–H COSY spectra of γ-I in D_2O and $CDCl_3$ are illustrated in Fig. 4. Similar experiments were carried out on α-I in D_2O and $CDCl_3$, and on α-CD and γ-CD in D_2O and the ^{13}C-chemical shifts are summarized in Table II. Morris and Hall[20] and Yamamoto and Inoue[21] recently presented detailed 2D-NMR studies of α-CD in D_2O. Our assignments for α-CD are in agreement with their results. Comparing the ^{13}C-NMR spectra of α-I and γ-I measured in D_2O with those of α-CD and γ-CD, respectively, typical large downfield shifts, known as methylation shifts[22] were observed at C-2 (+9.18 ppm for α-I and γ-I) and at C-6 (+10.39 ppm for α-I and +10.54 ppm for γ-I), while the signals for C-3 (β-carbon) were shifted upfield by −0.8—−0.9 ppm, and larger upfield shifts were observed at other β-carbons, C-1 (−2.41 ppm for α-I and −3.82 ppm for γ-I) and C-5 (−1.84 ppm for α-I and −1.97 ppm for γ-I). The signal for the C-4 of γ-I was also shifted upfield (−1.29 ppm), whereas that of α-I moved downfield (+0.94 ppm) (Table II and Fig. 5).

It can be presumed that the above-mentioned large shifts of the C-1, C-4, and C-5 signals of α-I and γ-I are due to not only the substituent effect of methylation, but also changes of dihedral angles (ϕ and ψ) of the glycoside linkage and rotation around the C-5–C-6 bond.

The ^{13}C-NMR spectra of α-II and γ-II in D_2O compared with those of α-CD, α-I, γ-CD and γ-I in D_2O are also shown in Fig. 5. The spectra of α-II and γ-II in D_2O were very complicated owing to the methylation of one hydroxyl group at C-3. On the other hand, the spectra of α-II and γ-II measured in $CDCl_3$ were relatively simple and resembled those of α-I and γ-I, respectively. Therefore, the ^{13}C-NMR spectra recorded in $CDCl_3$ of α-I and γ-I contained α-II and γ-II, respectively, may imply that they are homogeneous compounds. For example, Fig. 6 shows the ^{13}C-NMR spectrum of a mixture of equal proportions of γ-I and γ-II in $CDCl_3$. Difficulty of distinction of the spectrum from that of pure γ-I (see Fig. 4B) increases with decreasing proportion of γ-II in the mixture.

Any method of methylation to obtain the 2,6-di-*O*-methyl CD derivative gave a mixture of DM-CDs and over-methylated homologues, so the purity of methylation products of CDs should always be checked by HPLC.

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