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Isolation, Purification, and Characterization of Cyclomaltodecaose (ε-Cyclodextrin), Cyclomaltoundecaose (ζ-Cyclodextrin) and Cyclomaltotridecaose (θ-Cyclodextrin)

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Cyclomaltodecaose (ε-cyclodextrin, ε-CD), cyclomaltoundecaose (ζ-CD) and cyclomaltotridecaose (θ-CD) are a series of cyclomalto-oligosaccharides composed of ten, eleven and thirteen α-(1→4)-linked D-glucopyranose units, respectively. ε-CD, ζ-CD and θ-CD were purified from the commercially available CD powder mainly using the combined treatment of HPLC and column chromatographies. The molecular weights of ε-CD, ζ-CD and θ-CD were determined by FAB-MS, and their cyclic structures were identified by ^{1}H-NMR and ^{13}C-NMR. Furthermore, the ^{13}C-NMR chemical shift of large-ring cyclodextrins composed of several α-(1→4)-linked D-glucopyranose units, in which numbers 9, 10, 11, 12, and 13 are named δ-, ε-, ζ-, η-, θ-CD, were elucidated and compared with those of conventional α-, β-, and γ-CD.

Key words large-ring cyclodextrin; ε-cyclodextrin; ζ-cyclodextrin; θ-cyclodextrin; isolation; purification

The best known cyclomalto-oligosaccharides are conventional α-, β-, and γ-cyclodextrin (CD). Along with their derivatives such as methylated, hydroxyalkylated, and branched CD, they have been well studied and used in various industries.[1-4] For example, they are useful to improve drug stability, solubility, dissolution rate and bioavailability, and to reduce the side effects of drugs in pharmaceutics.[5,6] Earlier papers by French *et al.* provided the first definitive evidence for the existence of large-ring CDs (LR-CDs) composed of several α-(1→4)-linked D-glucopyranose units, in which numbers 9, 10, 11, 12 and 13 are named δ-, ε-, ζ-, η-, θ-CD, respectively.[7] Because of difficulties in the isolation and purification of large-ring cyclomaltoses, there are no reports except for studies in which LR-CDs are enzymatically synthesized with bacterial α-amylase by Nishimura *et al.*[8] and potato D-enzyme by Takaha *et al.* in 1996.[9] In addition, studies of LR-CDs in the pharmaceutical field yielded poor results because of their expected high solubility in water and predicted weak complex-forming ability.[3,6] However, previously,[10,11] we reported the isolation and purification of cyclomaltononaose (δ-CD) and cyclomaltododecaose (η-CD), and in particular, the lower aqueous solubility and much weaker hemolytic activity of δ-CD than α-CD or γ-CD, giving it a complex-forming ability for some drugs. The isolation, purification, and characterization of cyclomaltodecaose (ε-CD), cyclomaltoundecaose (ζ-CD), and cyclomaltotridecaose (θ-CD) are reported herein. Furthermore, the ^{13}C-NMR chemical shifts of five kinds of LR-CDs (δ-CD—θ-CD) were elucidated and compared with those of conventional α-, β-, and γ-CD.

Experimental

Materials CD powder (Dexy Pearl K-50), β-amylase [α-(1→4)-glucan maltohydrolase], and glucoamylase [α-(1→4)-glucan glucohydrolase] were purchased from Ensuiko Sugar Refining Co. (Yokohama, Japan), Tokyo Kasei Kogyo Co. (Tokyo, Japan), and Seikagaku Kogyo Co. (Tokyo, Japan), respectively. Novo Nordisk Bioindustry Co. (Chiba, Japan) donated pullulanase [α-(1→6)-glucosidase, Promozym 200L™]. All other chemicals were from reliable commercial sources and were used without further purification. Milli-Q water (Millipore Co., Milford, MA, U.S.A.) was used as the purified water in all preparations and purifications.

Apparatus and Columns for Preparative and Analytical Methods The HPLC on a preparative octadecyl bonded silica (ODS) column was performed with a SSC Flow System 3100J pump (Senshu Kagaku, Tokyo, Japan), with a Degasys DA-1200 degasser (Uniflows, Tokyo, Japan), and an ERC-7530 refractive index (RI) monitor (Erma Optical Works, Tokyo, Japan). The HPLC on a preparative amino bonded (NH$_2$) column and HPLC analyses of CDs were performed using a PU-986 intelligent pump (Jasco, Tokyo, Japan), with a DG-980-50 3-line degasser (Jasco),

Table 1. ^{13}C-NMR Chemical Shifts of CDs

Carbon	δ value (ppm)							
	α-CD	β-CD	γ-CD	δ-CD	ε-CD	ζ-CD	η-CD	θ-CD
1	102.19	102.58	102.42	100.96	99.74	99.83	100.17	100.34
2	72.61	72.67	73.19	73.08	72.65	72.56	72.53	72.51
3	74.21	73.89	73.82	73.74	73.68	73.66	73.67	73.64
4	82.07	81.94	81.33	79.26	78.03	78.31	78.87	78.97
5	72.91	72.89	72.69	72.34	71.78	71.74	71.82	71.90
6	61.37	61.17	61.21	61.29	61.49	61.53	61.54	61.43

ppm downfield from external tetramethylsilane at 50 °C in D$_2$O soln.

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and RI-930 intelligent RI detector (Jasco) with 807-IT integrator (Jasco). In HPLC experiments, the column temperature was held constant with a water bath and a column oven. For preparative chromatography, the columns used for HPLC were a Senshu Pak ODS-5251-SS (250 mm × 20 i.d., Senshu Kagaku) and an Asahipak NH2P-50 (250 mm × 10 i.d., Showa Denko, Tokyo, Japan). The columns used for analytical HPLC were Hibar LiChroCart LiChrospher 100 RP-18 (e) (250 mm × 4 i.d., Cica-Merck, Darmstadt, Germany) and Asahipak NH2P-50 (250 mm × 4.5 i.d., Showa Denko).

Preparation of LR-CD Mixture LR-CD mixture was prepared by a method developed by Kobayashi *et al.*[12] The process was carried out as follows. Commercially available CD powder was dissolved in an acetate buffer (pH 5.2) and incubated at 30 °C for 3 d with a mixture of β-amylase, pullulanase, and yeast. Through this process, dextrins and branched CDs in the CD powder underwent a breakdown to D-glucose and CDs by an enzyme-catalyzed reaction with β-amylase and pullulanase, and the free D-glucoses were digested by the yeast. Following centrifugation, the α-, β-, and γ-CD were removed from the supernatant as insoluble complexes of α-CD with tetrachloroethane and β-CD and γ-CD with bromobenzene. Uncomplexed dextrins were precipitated by ethanol and removed by centrifugation, giving a supernatant that appeared to contain many kinds of LR-CDs. The supernatant was subjected to deionization, decolorization, and hydrolysis of residual dextrins using glucoamylase. Finally, the LR-CD mixture was precipitated by acetone. The details of the procedure, including all experimental conditions, have been reported previously.[10,11]

Isolation and Purification of ε-CD, ζ-CD and θ-CD LR-CD mixture was redissolved in water and was roughly separated into 4 fractions by HPLC on a preparative ODS column with methanol–water (6:100) at a flow rate of 6.0 ml/min (Fig. 1). Fraction HO-2, which LR-CDs suggested to be rich, was further fractionated into 4 fractions by HPLC on a semi-preparative Asahipak NH2P-50 with acetonitrile–water (55:45) at a flow rate of 2.0 ml/min. Finally, ε-CD was purified by recrystallization after the fractionation of fr. NH-1 by HPLC on the semi-preparative Asahipak NH2P-50, and ζ-CD and θ-CD were purified by HPLC on the semi-preparative Asahipak NH2P-50 from fr. NH-2.

Purity of ε-CD, ζ-CD and θ-CD by HPLC Their purities were checked by HPLC on a reverse phase column (LiChrospher 100 RP-18 (e)) and a normal phase column (Asahipak NH2P-50). HPLC was conducted under the following conditions: (1) column, LiChrospher 100 RP-18 (e); eluent, methanol–water (5:95); flow rate, 0.8 ml/min; column temperature, 25 °C, and, (2) column, Asahipak NH2P-50; eluent, acetonitrile–water (55:45); flow rate, 0.7 ml/min; column temperature, 20 °C. The HPLC apparatus was identical to that described in the section of Apparatus and Columns for Preparative and Analytical Methods.

Characterization of ε-CD, ζ-CD and θ-CD by Mass Spectrum FAB-MS spectra were measured in a positive ion mode by an SX-102A mass spectrometer (JEOL, Tokyo, Japan) using glycerin as a matrix. The acceleration voltage was 10 kV.

Characterization of ε-CD, ζ-CD and θ-CD by NMR Spectrum ^{1}H-NMR, ^{13}C-NMR and two-dimensional ^{1}H–^{13}C correlation (H–C COSY) NMR spectra were taken on a JMN-LA500 spectrometer (500 MHz ^{1}H, JEOL) at 50 °C. The samples were dissolved in 99.8% deuterium oxide. Chemical shifts were reported in δ-units (ppm) downfield from the signal of external tetramethylsilane [$(CH_3)_4Si$].

Results and Discussion

Isolation and Purification of ε-CD, ζ-CD and θ-CD Figure 1 shows the chromatogram of the LR-CD mixture by HPLC on a preparative ODS column. In the first process to obtain a LR-CD-rich fraction, the LR-CD mixture was separated into 4 fractions by the preparative ODS column, and fr. HO-2 was collected, since fr. HO-3 is a δ-CD rich fraction[10] and other LR-CDs were speculated to have higher aqueous solubility than that of δ-CD. Figure 2 shows the chromatogram of fr. HO-2 by HPLC on an Asahipak NH2P-50. In the next purification step, fr. HO-2 was further separated into 4 fractions by HPLC on the semi-preparative Asahipak NH2P-50, as shown in Fig. 2, because the LR-CDs were separated more clearly on the NH_2 column than on the ODS column and because the Asahipak NH2P-50 (chemically polyamine bonded vinyl copolymer gel column) gave a better separation than LiChrospher 100 NH_2 (aminopropyl-bonded silica column). Finally, ε-CD was purified by recrystallization after the separation of fr. NH-1 by HPLC on the semi-preparative Asahipak NH2P-50, and ζ-CD

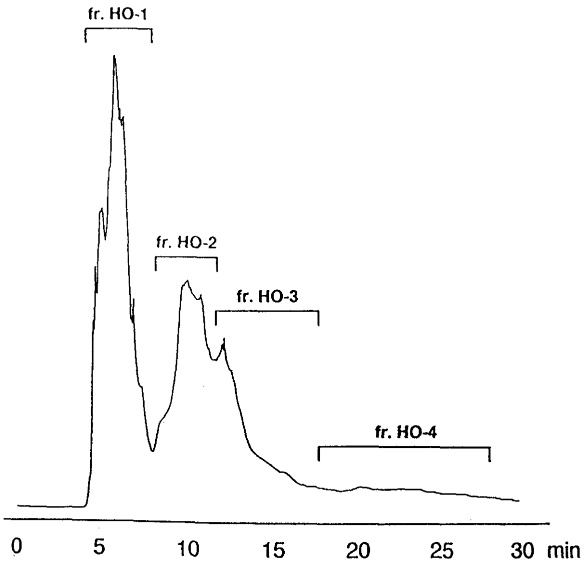

Fig. 1. Chromatogram of LR-CD Mixture on Preparative ODS Column

Conditions: column, Senshu Pak ODS-5251-SS; eluent, CH_3OH–H_2O (6:100); flow rate, 6.0 ml/min; column temperature, room temperature.

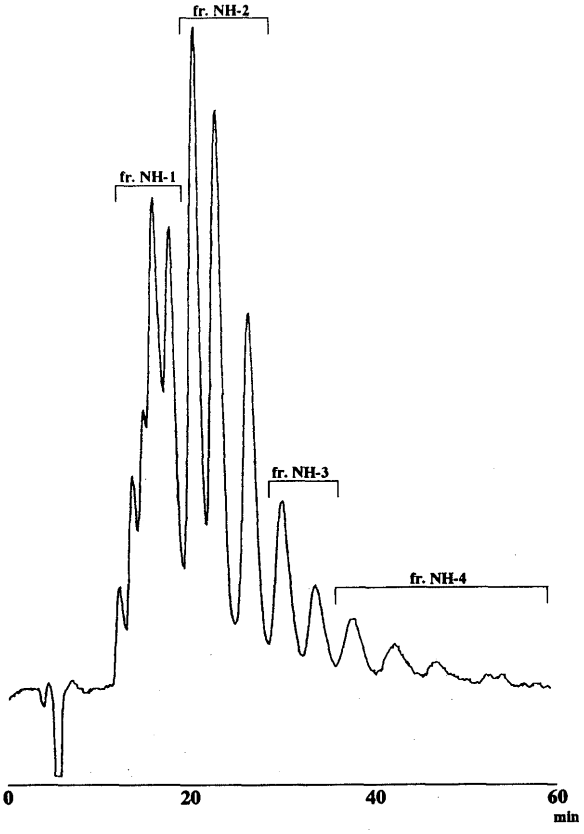

Fig. 2. Chromatogram of Fr. HO-2 on an Asahipak NH2P-50

Conditions: column, Asahipak NH2P-50; eluent, CH_3CN–H_2O (55:45); flow rate, 0.7 ml/min; column temperature, 20 °C.

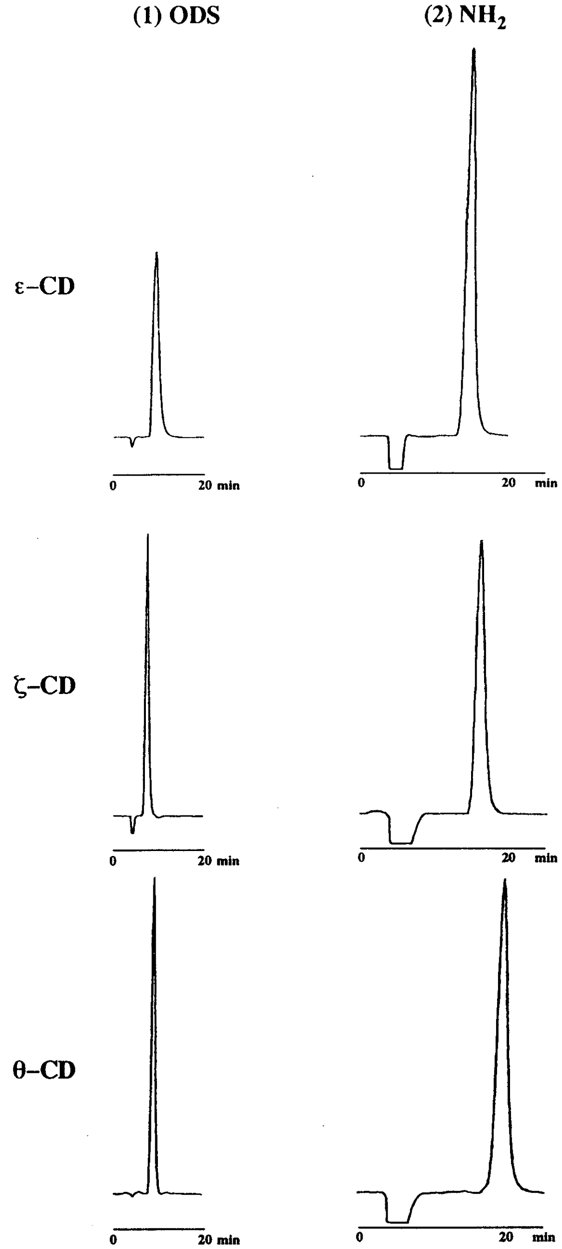

Fig. 3. Chromatograms of ε-CD, ζ-CD and θ-CD on an ODS and an NH_2 Column

Conditions: (1) column, LiChrospher 100 RP-18 (e); eluent, CH_3OH-H_2O (5:95); flow rate, 0.8 ml/min; column temperature, 25 °C. (2) column, Asahipak NH2P-50; eluent, CH_3CN-H_2O (55:45); flow rate, 0.7 ml/min; column temperature, 20 °C.

and θ-CD were purified by HPLC on the semi-preparative Asahipak NH2P-50 from fr. NH-2.

Purity of ε-CD, ζ-CD and θ-CD To check contaminants, for example other CDs, and external and internal branched CDs, the obtained fractions were analyzed by HPLC on a normal phase column (Asahipak NH2P-50) and reverse phase column (LiChrospher 100 RP-18 (e)). Figure 3 showed that they had >98% purity. We obtained about 160 mg of ε-CD and ζ-CD and about 370 mg of θ-CD.

Characterization of ε-CD, ζ-CD and θ-CD by HPLC, FAB-MS and NMR To obtain the elution sequence of LR-CDs, the freshly prepared three LR-CDs, δ-CD and η-CD were subjected to HPLC under the same condition.

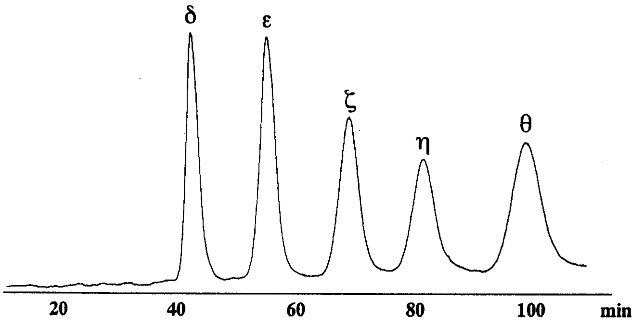

Fig. 4. Elution Profile of 5 LR-CDs on an Asahipak NH2P-50

Conditions: column, Asahipak NH2P-50; eluent, CH_3CN-H_2O (65:35); flow rate, 0.7 ml/min; column temperature, 20 °C.

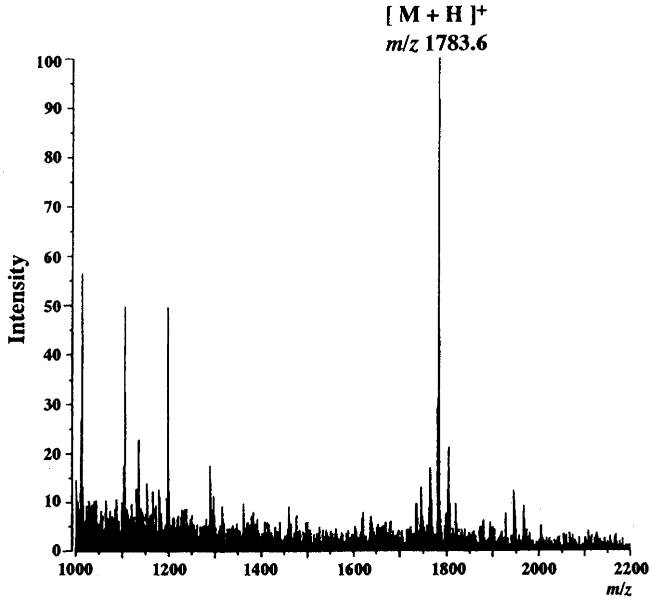

Fig. 5. FAB-MS Spectrum of ζ-CD

Matrix, glycerin; acceleration voltage, 10 kV.

Figure 4 shows the chromatogram of five kinds of LR-CDs on the Asahipak NH2P-50, and the same elution sequence was also observed by HPLC on the aminopropyl-bonded silica column. The elution sequence with the aminopropyl-bonded silica, the typical NH_2 column, and acetonitrile–water system follows the order of number of the glucopyranose unit.[13] This indicated that the numbers of glucopyranose units increased in the following order: δ-CD < ε-CD < ζ-CD < η-CD < θ-CD.

The molecular weight of each obtained fractions was calculated from the number of glucopyranose units. Figure 5 shows the FAB-MS spectrum of ζ-CD, as a representative example. Each FAB-MS spectrum gave the following: *m/z* peak 1621.7 $[M+H]^+$ from ε-CD, *m/z* peak 1783.6 $[M+H]^+$ from ζ-CD, and *m/z* peak 2107.8 $[M+H]^+$ from θ-CD. These findings were in agreement with the calculated molecular weight of ε-CD (Calcd for $(C_6H_{10}O_5)_{10}$: 1620), ζ-CD (Calcd for $(C_6H_{10}O_5)_{11}$: 1782) and θ-CD (Calcd for $(C_6H_{10}O_5)_{13}$: 2106), and indicated that each of the 3 obtained CDs were composed of 10, 11 and 13 glucopyranose units, respectively.

The ^1H-NMR, ^{13}C-NMR spectrum and two-dimensional ^1H–^{13}C correlation (H–C COSY) NMR spectrum

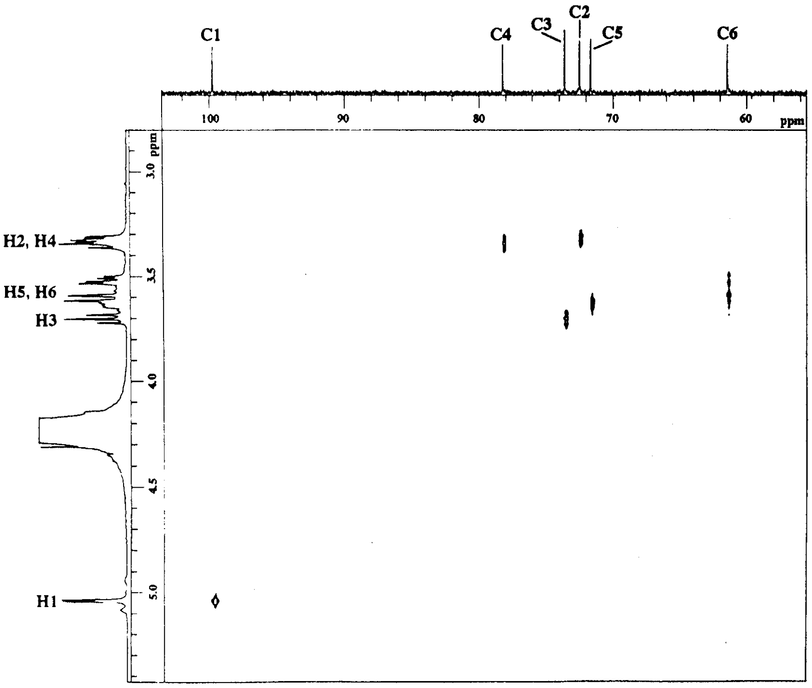

Fig. 6. ^1H–^{13}C COSY NMR Spectrum of ζ-CD

Solvent, deuterium oxide; temperature, 50 °C.

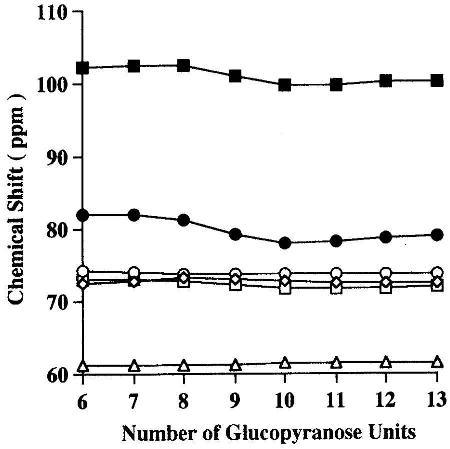

Fig. 7. Variation of ^{13}C Chemical Shifts of CDs with Number of Glucopyranose Units

■, C1; ◇, C2; ○, C3; ●, C4; □, C5; △, C6.

of the 3 obtained CDs were measured by the JMN-LA500 spectrometer at 50 °C in deuterium oxide. Figure 6 shows the ^1H–^{13}C correlation (H–C COSY) NMR spectrum of ζ-CD, as a representative example. Their assignments could be made from the two-dimensional ^1H–^{13}C correlation (H–C COSY) NMR spectrum, and a free –CH_2OH group could be discerned. Each spectrum of ε-CD, ζ-CD and θ-CD showed six clear, and distinct, single peaks attributed to equivalent glucopyranose units. Chemical shifts of each carbon obtained with ε-CD, ζ-CD and θ-CD were similar to those that had been attributed to the cyclic structures of conventional CDs and other LR-CDs (see Table 1). Figure 7 shows the upperfield shifts of the chemical shifts at C1 and C4 that were used for binding to two glucopyranose units, in comparison with the chemical shift of other carbons. These differences might be caused by a difference in the state of α-(1→4) glucosidic linkage between conventional CDs and LR-CDs. Conventional CDs and LR-CDs may have the same fundamental cyclic structure of α-(1→4) glucosidic linkage, but they had some differences in fine structure. NMR measurements suggested a distorted cyclic structure of LR-CDs and some of the linkages at the 1- and 4-positions were in a nonidentical state. This speculation was supported by the crystal and molecular structure of δ-CD $13.75H_2O$[14] and ε-CD $21H_2O$.[15] The detailed molecular structure of ε-CD will be published elsewhere in the near future.

In conclusion, we newly established a purification method for ε-CD, ζ-CD and θ-CD from commercially available CD powder and isolated them as a freeze-dried powder. We have already isolated and purified δ-CD[10] and η-CD.[11] Therefore, the existence of all LR-CDs (δ-, ε-, ζ-, η-, and θ-CD) reported by French *et al.*[7] was confirmed by up-to-date analytical techniques, including HPLC, FAB-MS, and one- and two-dimensional NMR. Furthermore, the ^{13}C-NMR data suggested that conventional CDs and LR-CDs had the same fundamental cyclic structure, but with slight differences in their fine structure.

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