Preparation of Heptakis(2,6-di-*O*-ethyl)-β-cyclodextrin and Its Nuclear Magnetic Resonance Spectroscopic Characterization

Fumitoshi Hirayama,[1] Masahiko Kurihara,[1] Yasuhide Horiuchi,[1] Tadanobu Utsuki,[1] Kaneto Uekama,[1,3] and Masaki Yamasaki[2]

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Heptakis(2,6-di-O-ethyl)-β-cyclodextrin (DE-β-CyD) was prepared and its ^1H and ^{13}C nuclear magnetic resonance (NMR) signals in DMSO-d_6 were unequivocally assigned by two-dimensional COSY and ROESY. The results on ^1H coupling constants indicated that all ethylated glucose units are in a $^4C_1$ chair conformation. The average spin-lattice relaxation times (T_1) of ring carbons of DE-β-CyD were only slightly shorter, and their standard deviations from the mean T_1 value were larger, than those of β-cyclodextrin (β-CyD) and heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CyD), suggesting the presence of slightly irregular internal motion in the ethylated glucose units. The temperature dependence of chemical shift of DE-β-CyD in DMSO-d_6 suggested that the C3 hydroxyl protons may participate as proton donor in the intramolecular hydrogen bond to the C2 ethoxyl groups of neighboring glucose, and the intramolecular hydrogen bond of DE- and DM-β-CyDs is much stronger than that of β-CyD, suggesting the stable macrocyclic ring structure of DE-β-CyD.

KEY WORDS: heptakis(2,6-di-*O*-ethyl)-β-cyclodextrin; nuclear magnetic resonance spectroscopy; ^{13}C spin-lattice relaxation; intramolecular hydrogen bonding.

INTRODUCTION

Chemically modified cyclodextrins (CyDs) have received considerable attention regarding enzyme and receptor models, reaction-control agents, chromatographic separating agents, and drug delivery carriers (1–3). The physicochemical properties of CyDs are markedly altered when ethyl groups are introduced onto the hydroxyls of CyDs, and ethylated β-CyDs are promising candidates for release-control carriers for water-soluble drugs because of the formation of a less-soluble complex (4). For example, ethylated β-CyDs were surface active, slightly soluble in water, and less hygroscopic than parent β-CyD. The release of water-soluble drugs, such as diltiazem hydrochloride (5,6), isosorbide dinitrate (7), and buserelin acetate (8), from the complexes with ethylated β-CyDs was significantly decelerated, thereby maintaining constant drug levels in blood after *in vivo* administrations. In this paper, we report on the preparation and purification of heptakis(2,6-di-*O*-ethyl)-β-cyclodextrin (DE-β-CyD), a β-CyD derivative in which C2 and C6 hydroxyls of glucose units are ethylated. Further, we compare its NMR spectra with those of parent β-CyD and heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CyD).

MATERIALS AND METHODS

Materials

β-CyD was supplied by Nihon Shokuhin Kako Co. (Tokyo) and recrystallized from water. DM-β-CyD was supplied by Toshin Chemical Co. (Tokyo) and purified by repeated recrystallizations from methanol, which was effective to purify DM-β-CyD from the chemically related mixtures. DM-β-CyD was then recrystallized from water and checked to be a single component by high-performance liquid chromatography (HPLC) (9). Dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) were dried over molecular sieves (4 Å; Merck). Other materials and solvents were of analytical reagent grade.

Preparation of DE-β-CyD

β-CyD (50 g) was dissolved in 1000 ml of DMSO/DMF (1:1, v/v), BaO (100 g), and $Ba(OH)_2 \cdot 8H_2O$ (100 g) were added at 0°C, and the mixture was stirred for 30 min under argon. Diethyl sulfate (200 ml) was added dropwise over 2 hr at 0°C, and the reaction mixture was further stirred for 48 hr at 7°C. The reaction was terminated by the addition of aqueous ammonium hydroxide (100 ml) and stirring at room temperature (about 27°C) for 3 hr. After evaporation of the solvent under reduced pressure, ethylated β-CyDs were extracted with ethyl acetate. The organic phase was washed with water containing sodium hydrosulfite, then with water several times, and dried over sodium sulfate. After evaporation of the solvent under reduced pressure, the residue was applied to silica gel column chromatography: crude ethylated β-CyDs (21 g), Wakogel C-300 (500 g, 200–300 mesh; Wako Pure Chemical Co., Osaka, Japan), and ethyl acetate/toluene (13:7, by volume, 1000 ml; then 5:2, 1000 ml) as eluants. Overethylated products and DE-β-CyD were eluted by 13:7 and 5:2 ethyl acetate/toluene, respectively. Recrystallization from methanol afforded DE-β-CyD as white needle crystals (yield about 20% based on β-CyD, after recrystallization); m.p. 255°C; R_f 0.385, Kieselgel 60F254 plate (Merck; ethyl acetate as eluant). Retention time on HPLC was 7.89 min under the following conditions: an Hitachi 655A pump (Tokyo) with a Shodex SE-51 differential refractometer (Tokyo); a YMC packed column AQ312 (5 μm, 150 × 6 mm; Kyoto); a mobile phase of acetonitrile/methanol, 1:4; a flow rate of 1.5 ml/min; MS (positive ion FAB), 1527 for $[M + H^+]^+$; *Anal.* Calc. for $C_{70}H_{126}O_{35}$: C, 55.0; H, 8.3. Found: C, 54.9; H, 8.2.

NMR Spectroscopy

NMR spectra of β-CyDs were measured in solutions of DMSO-d_6 or $CDCl_3$ using Jeol JNM EX-400 or GX-400 spectrometers (Tokyo), operating at 399.65 and 100.40 MHz for

[1] Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862, Japan.

[2] Department of Bioengineering, Yatsushiro National College of Technology, 2627 Hirayama-shinmachi, Yatsushiro, Kumamoto 866, Japan.

[3] To whom correspondence should be addressed.

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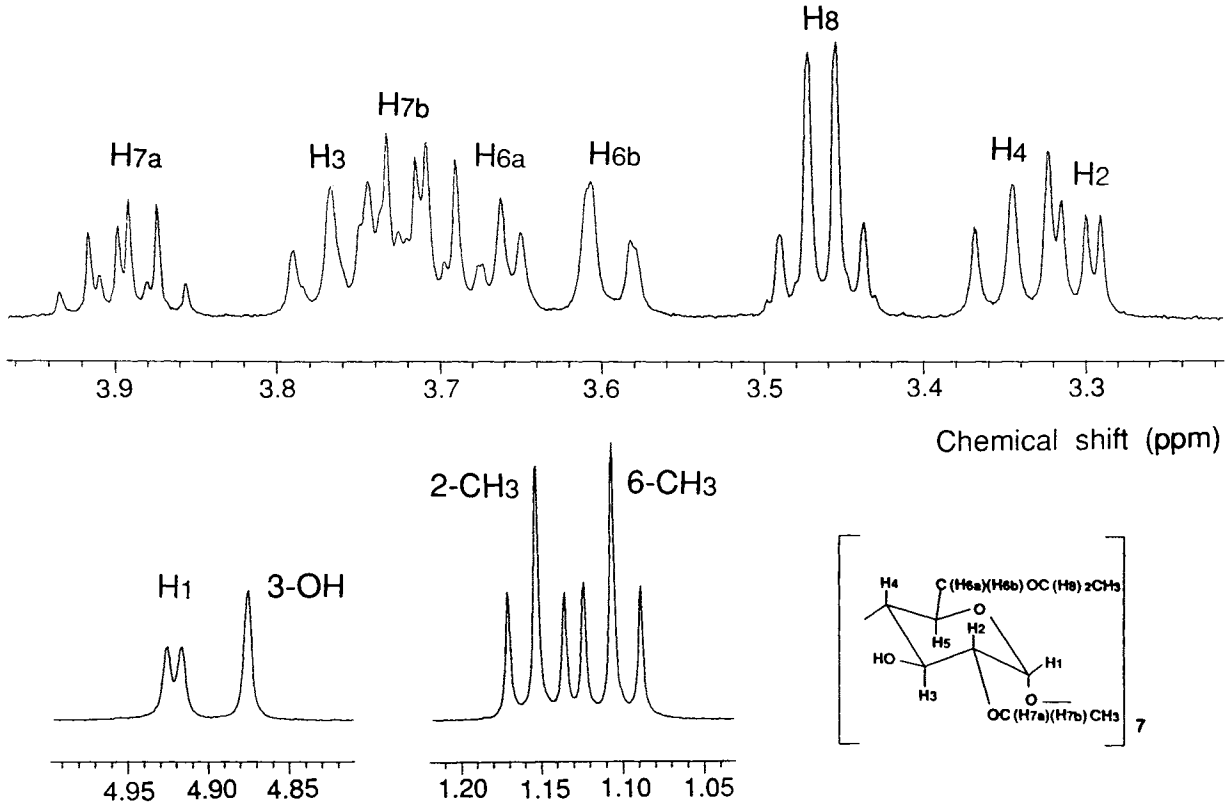

Fig. 1. ^{1}H-NMR spectrum of DE-β-CyD (1.0 w/v%) in DMSO-d_6 at 80°C and its proton numbering.

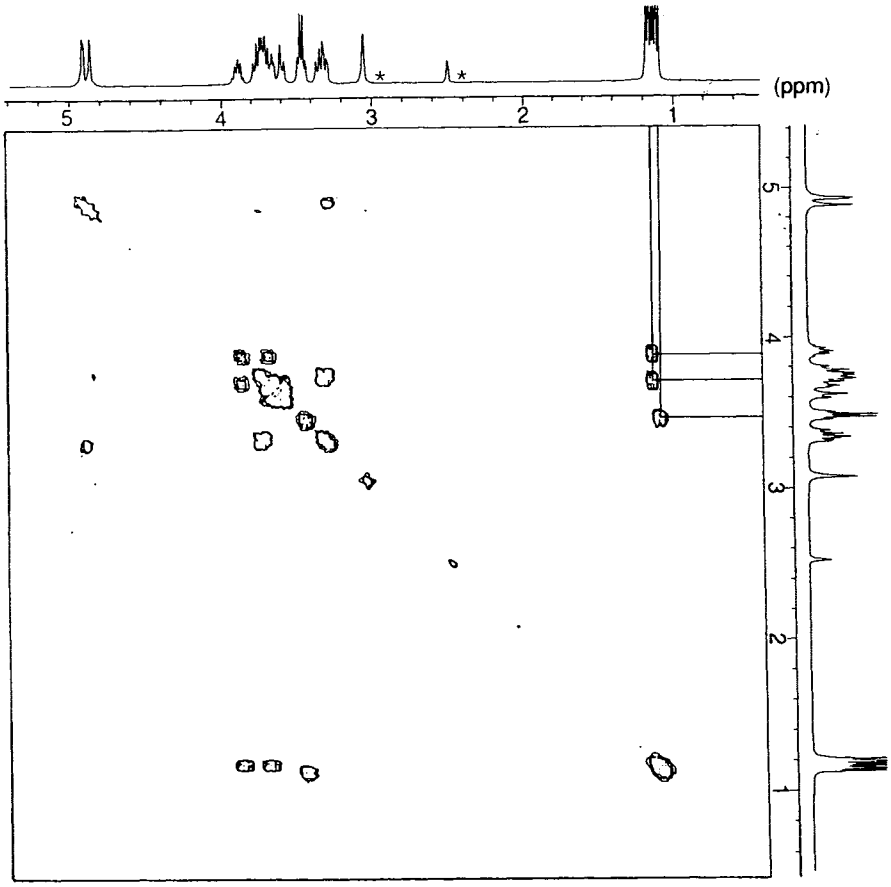

Fig. 2. ^{1}H–^{1}H COSY spectrum of DE-β-CyD (1.0 w/v%) in DMSO-d_6 at 80°C. Connectivities between the methyl and the methylene protons in ethyl groups are indicated by solid lines. (*) Peaks due to solvent (HDO and DMSO).

protons and carbons, respectively. Chemical shifts are given as parts per million (ppm) downfiled from that of external tetramethylsilane, with an accuracy of about 0.005 and 0.01 ppm for ^1H and ^{13}C signals, respectively. The ^1H–^1H and ^1H–^{13}C COSY spectra were obtained using the standard pulse sequences and procedures (10). For ^1H–^1H COSY spectra, 24 free-induction decays were acquired, with a 2000-Hz sweep width and 1K data points, and 256 increments of 0.5 msec each were used for the incremented delay (t_1). The data were processed with zero-filling (final matrix, 512 × 1K) and with a sine-bell window in both dimensions. For ^1H–^{13}H COSY spectra, 160 free-induction decays were acquired, with 128 data points for ^1H (sweep-width 2000 Hz) and 2K data points for ^{13}C (sweep width, 11,000 Hz), and the data were expanded to the matrix 512 × 2K dimensions by zero-filling. Phase-sensitive ROESY spectra were measured under the following conditions: sweep width, 2500 Hz; carrier frequency, 2.95 ppm; spin-lock field, 4 kHz; mixing time, 200 msec; 32 scans for each t_1 point with a pulse delay of 1.5 sec; and data matrix, 2 × 256 × 1K. ^{13}C Spin-lattice relaxation times (T_1) were measured by the inversion-recovery method (11), (-180°-*t*-90°-*T*-) pulse sequence with $T > 5T_1$, on a Jeol JNM FX-270 spectrometer (Tokyo) operating at 67.94 MHz for ^{13}C carbons. The estimated error in T_1 values was within 5%. The least-squares adjustments of ^1H chemical shifts and coupling constants spectra were carried out using a LAOCOON III program (12) on a Fujitsu FM-16β personal computer (Tokyo).

Viscosity Measurements

Macroscopic viscosity of β-CyDs in DMSO was measured with an Epprecht Rheomat-15 viscometer (Contraves AG, Switzerland) at 25°C. The viscosities of β-CyD, DM-β-CyD, and DE-β-CyD solutions were 3.18 ± 0.03, 2.95 ± 0.02, and 3.03 ± 0.02 cp, respectively, at a concentration of 5.0 (w/v%).

RESULTS AND DISCUSSION

Figure 1 shows the ^1H NMR spectrum of DE-β-CyD in DMSO-d_6. The assignment of the peaks was carried out by two-dimensional ^1H COSY (Fig. 2) and ROESY (Fig. 3), and all peaks were unequivocally assigned except for the peak of H_5, which overlapped with that of H_{7b}. The geminal methylene protons (δ = 3.89 and 3.69 ppm) of one ethyl group were magnetically nonequivalent and were coupled with the methyl protons (δ = 1.14 ppm). On the other hand, the methylene protons (δ = 3.45 ppm) of another ethyl group were magnetically equivalent and were coupled with the methyl protons (δ = 1.10 ppm), as is apparent from the COSY spectrum. However, it was difficult to determine whether the ethyl group binds to the C2 or C6 hydroxyl group, on the

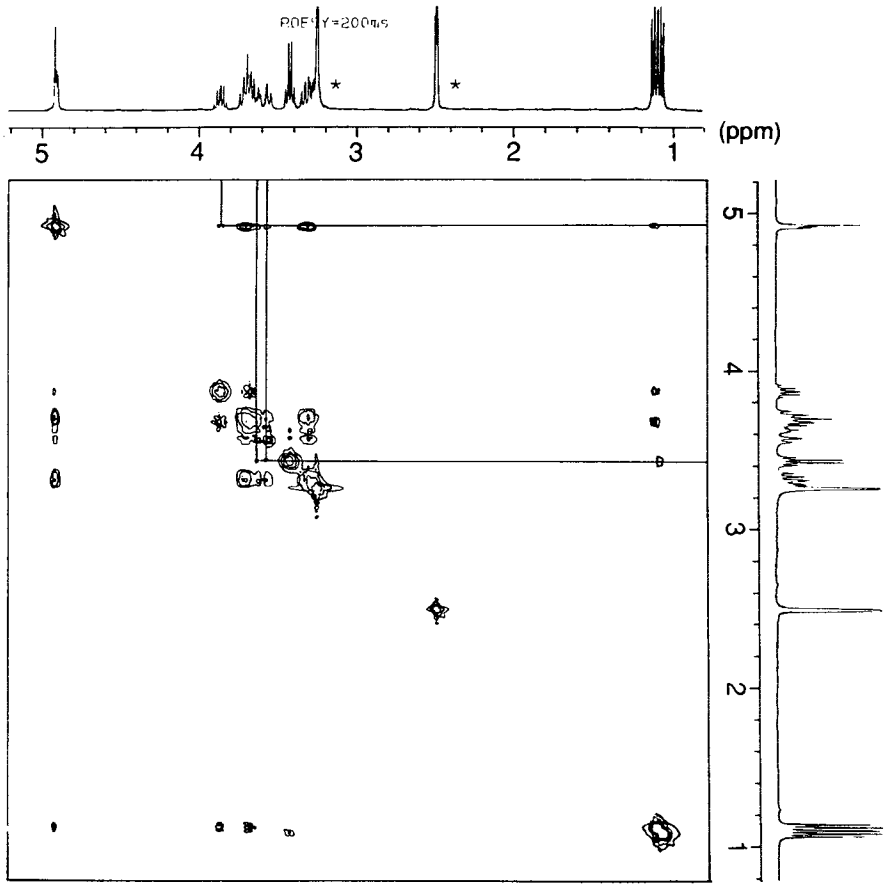

Fig. 3. ROESY spectrum of DE-β-CyD (3.0 w/v%) in DMSO-d_6 at 25°C. Connectivities between some protons are indicated by solid lines. (*) Peaks due to solvent (HDO and DMSO).

basis of only the COSY spectrum. Further, there have been conflicting reports concerning the assignment of alkyl groups of C2 and C6 positions (13,14). Therefore, the unequivocal assignments of the ethyl groups were conducted on the basis of the ROESY spectrum, which serves to detect the nuclei that are in proximity (10). As shown in Fig. 3, the methylene signal at 3.45 ppm gave two cross peaks to the H_{6a} and H_{6b} proton signals at 3.65 and 3.58 ppm, whereas the methylene signal at 3.89 ppm gave a cross peak to the C3 hydroxyl proton at 4.90 ppm. Similar cross peaks were also observed in the NOESY spectrum. These results indicate that the ethyl group with magnetically unequivalent methylene protons is introduced onto the C2 hydroxyl group and the other ethyl group is attached to the primary C6 hydroxyl group. The splitting pattern of the geminal protons was not changed with temperature change (25–80°C), suggesting that it does not arise from the restriction of free rotation of the ethyl group but, rather, from the diastereotropic environment, because the distance between the methylene protons and the chiral C2 center is shorter than that to the chiral C5 center. Chemical shifts (δ) and coupling constants (*J*) of DE-β-CyD refined using the LAOCOON spin simulation program (12) are listed in Table I. The J_{12}, J_{23}, and J_{34} values for the glucose ring were in good agreement with the corresponding values for β-CyD and DM-β-CyD (15), indicating the $^4C_1$ chair conformation of the glucose ring in DE-β-CyD. In $CDCl_3$ solution, all peaks of DE-β-CyD shifted slightly to downfield. The H_3 proton experienced a large shielding in $CDCl_3$ because it locates on the inner surface of the cavity (16). The H_{6a} and H_{6b} protons of DE-β-CyD were also magnetically nonequivalent. By analyzing the magnitude of J_{56a} and J_{56b} values according to the method of Streefkerk *et al.* (17), the population of conformers (Fig. 4) around the C5–C6 bond of DE-β-CyD was estimated to be about 65 and 35% for the gg-conformer and the gt- or tg-conformer, respectively. In the case of DM-β-CyD, populations of the gg-conformer and gt- or tg-conformer were 70–80 and 17–18%, respectively. These results suggest that oxygens of the alkyl groups at the C6 position are oriented predominantly away from CyD cavities.

Table I. ^{1}H NMR Chemical Shifts (δ; ppm)[a] and Coupling Constants (*J*; Hz) of DE-β-CyD, DM-β-CyD, and β-CyD in DMSO-D_6 at 60°C

δ or *J*	DE-β-CyD		DM-β-CyD	β-CyD
H_1	4.92	(4.92)[b]	4.97	4.84
H_2	3.31	(3.36)	3.20	3.32
H_3	3.75	(3.93)	3.74	—[c]
H_4	3.34	(3.42)	3.34	3.36
H_5	—[c]	(3.79)	3.70	3.60
H_{6a}	3.65	(—)[c]	[3.58[d]	[3.66[d]
H_{6b}	3.58	(3.64)		
H_{7a}	3.89	(4.05)	—	—
H_{7b}	3.69	(3.70)	—	—
H_8	3.45	(3.54)	—	—
2-CH_3	1.14	(1.24)	3.52	—
6-CH_3	1.10	(1.20)	3.27	—
2-OH	—		—	5.52
3-OH	4.90	(5.07)	4.90	5.52
6-OH	—		—	4.24
$J_{1,2}$	3.5	(3.7)	3.5	3.3
$J_{2,3}$	9.7	(9.6)	9.7	9.9
$J_{3,4}$	9.2	(9.2)	9.2	9.2
$J_{4,5}$	9.2	(9.2)	9.5	9.9
$J_{5,6a}$	4.9	(3.3)	3.3	—[c]
$J_{5,6b}$	1.6	(<1.5)	1.7	—[c]
$J_{6a,6b}$	−10.4	(−9.2)	—[c]	—[c]
$J_{7a,7b}$	−9.7	(−9.5)	—	—
$J_{2\text{-}CH3,7a}$	7.1	(7.0)	—	—
$J_{2\text{-}CH3,7b}$	7.1	(7.0)	—	—
$J_{6\text{-}CH3,8}$	7.0	(7.0)	—	—
$J_{2\text{-}OH,2}$	—		—	6.2
$J_{3\text{-}OH,3}$	0.0	(0.0)	0.0	2.9
$J_{6\text{-}OH,6}$	—		—	5.5

[a] Relative to external TMS; the concentration of β-CyDs was 1.0 (w/v%).

[b] Values in parentheses are ^{1}H chemical shifts of DE-β-CyD (1.0 w/v%) in $CDCL_3$ at 40°C.

[c] Could not be determined accurately because of the close proximity or overlap of other signals.

[d] One of H_{6a} and H_{6b} could not be determined.

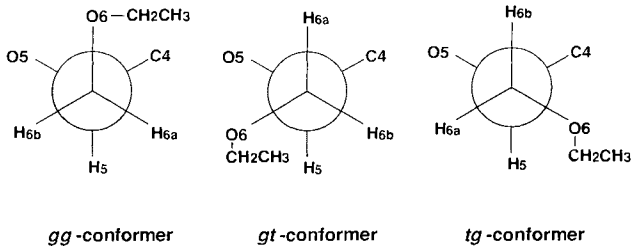

Fig. 4. The gg-, gt-, and tg-conformations around the C5–C6 bond.

Table II summarizes ^{13}C chemical shifts of DE-β-CyD in DMSO-d_6, in comparison with β- and DM-β-CyDs. The ^{13}C resonance peaks were unambiguously assigned to carbons of 2,6-diethyl glucose by means of ^{1}H–^{13}C COSY. DE-β-CyD gave only a set of resonance peaks of the ethylated glucose unit, where the terminal methyl signals were overlapped with each other, suggesting that all seven glucose units have the same conformation on NMR time scale. The C2 and C6 resonances of DM- and DE-β-CyDs shifted significantly to downfield (8–9 ppm), compared with β-CyD, while other ring carbons were little affected by the ethylation, supporting the introduction of ethyl group onto hydroxyl groups at the C2 and C6 positions of all glucose units.

To gain insight into the molecular motion of DE-β-CyD,

Table II. ^{13}C NMR Chemical Shifts (δ; ppm)[a] of DE-β-CyD, DM-β-CyD, and β-CyD in DMSO-D_6 at 27°C

δ	DE-β-CyD	DM-β-CyD	β-CyD
C1	100.73	100.10	101.92
C2	79.97	81.76	72.38
C3	72.86	72.74	73.02
C4	82.91	82.76	81.52
C5	69.86	69.83	72.01
C6	68.67	70.74	59.90
C7	67.22	—	—
C8	65.49	—	—
2-CH_3	15.09	59.60	—
6-CH_3	15.00	58.05	—

[a] Relative to external TMS; concentration of β-CyDs was 3.0 w/v%.

Table III. ^{13}C Relaxation Times (NT_1) for DE-β-CyD, DM-β-CyD, and β-CyD in DMSO-d_6 at 27°C[a]

	NT_1 (sec)									
β-CyDs	C1	C2	C3	C4	C5	C6	C7	C8	2-CH_3	6-CH_3
DE-β-CyD	0.126	0.136	0.142	0.135	0.133	0.173	0.742	0.730	2.80	3.06
DM-β-CyD	0.137	0.147	0.150	0.140	0.141	0.179	—	—	2.72	2.92
β-CyD	0.140	0.144	0.144	0.145	0.140	0.176	—	—	—	—

[a] *N*, number of hydrogen atoms attached to the carbon.

^{13}C spin-lattice relaxation times (T_1) were measured, and the results are summarized in Table III. The average NT_1 values ($\langle T_1 \rangle_{1-5}$) for the ring carbons (C1–C5) were in the order of β-CyD (0.143 sec) = DM-β-CyD (0.143 sec) > DE-β-CyD (0.134 sec), and the standard deviation from the average value increased in the order of β-CyD (σ = 0.0024) < DM-β-CyD (σ = 0.0053) = DE-β-CyD (σ = 0.0058). The larger standard deviation of DM- and DE-β-CyDs suggests the presence of irregular motions in the substituted glucose rings, probably because of the slightly faster internal motion at the C3 carbon. The effective correlation time (τ_{eff}) for isotropic molecular orientation was estimated to be 0.33, 0.34, and 0.37 nsec for DE-β-CyD, DM-β-CyD, and β-CyD, respectively, by analyzing the shortest NT_1 value in the ring carbons, i.e., the C1 carbon (11,18). The τ_{eff} can also be given by Eq. (1), on the basis of the Brownian diffusion model (19,20):

$$\tau_{eff} = \eta \cdot f \cdot V/(\kappa \cdot T) \quad (1)$$

where η and f are the viscosity of the solution and the microviscosity factor, respectively, and κ and T are Boltzmann's constant and the absolute temperature, respectively. The ratio of τ_{eff}, for a series of chemically related compounds, can be used as an estimate of molecular volume, if the environmental parameters (η, f, and T) remain unchanged. Since no difference in viscosity was observed among DMSO solutions of DE-β-CyD, DM-β-CyD, and β-CyD (5.0, w/v%) as described under Materials and Methods, τ_{eff} ratios were used for comparison of molecular volume of β-CyDs. The τ_{eff} ratios of DM- and DE-β-CyDs to parent β-CyD were 1.03 and 1.12, respectively, suggesting that the molecular dimension of β-CyD was not significantly changed by the alkylation, and the terminal methyl groups may be folded in compact forms. Internal motions of the terminal methyl group at the primary ethoxyl site were slightly faster than that at the secondary ethoxyl site.

Hydroxyl protons participating in the intramolecular hydrogen bond of CyDs are less affected by temperature change, compared with those of the intermolecular hydrogen bond, and the C3 hydroxyl protons of CyDs participate as proton donors in the intramolecular hydrogen bonding (21,22). Figure 5 shows ^1H chemical shift changes of hydroxyl protons of DE-β-CyD, DM-β-CyD and β-CyD in DMSO-d_6 over the temperature range of 25–80°C. The chemical shifts of hydroxyl protons shifted upfield with the increase in temperature, while those of protons attached to carbons changed little. In the case of DE-β-CyD and DM-β-CyD, the temperature dependence of the C_3 hydroxyl proton was much smaller than that of β-CyD, suggesting a strong hydrogen bond with the C_2 hydroxyl oxygen of neighboring glucose. Onda *et al.* (22) reported that the vicinal $J_{3\text{-OH.3}}$ value (C3 hydroxyl to H_3 protons) for DM-β-CyD is about 0 Hz and its dihedral angle is about −85°, which allows the C3 hydroxyl proton to contact closely the C2 ethoxyl group of the neighboring glucose. A similar orientation of the C3 hydroxyl proton may also be maintained in DE-β-CyD because of its $J_{3\text{-OH.3}}$ equal to approximately 0 Hz (singlet peak). This orientation of the primary ethoxyl oxygen was also supported by the ROESY spectrum (Fig. 3) of DE-β-CyD, since cross peaks between the C3 hydroxyl proton at about 4.9 ppm and the C2 ethyl group at about 1.1 and 3.85 ppm were observed.

CONCLUSION

As mentioned above, DE-β-CyD was prepared, where ethyl groups are introduced onto hydroxyl groups at the C2 and C6 positions of all glucose units, and the ^1H and ^{13}C NMR signals were unequivocally assigned. The results suggested no appreciable conformational change ($^4C_1$) of glucose units of DE-β-CyD by ethylation. The macrocylcic ring structure of β-CyD was maintained in the DE-β-CyD mole-

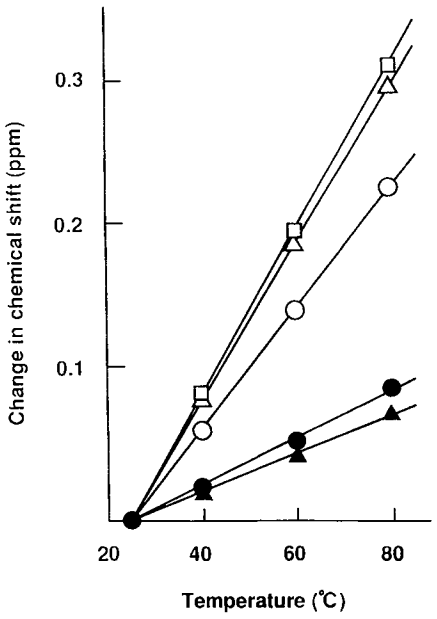

Fig. 5. Temperature dependences of chemical shifts for hydroxyl protons of β-CyD, DM-β-CyD, and DE-β-CyD (3.0 w/v%) in DMSO-d_6. DE-β-CyD: (▲) C3 hydroxyl proton. DM-β-CyD: (●) C3 hydroxyl proton. β-CyD: (△) C2 hydroxyl proton; (○) C3 hydroxyl proton; (□) C6 hydroxyl proton. The change in chemical shift refers to the upfield shift relative to the chemical shift observed at 25°C.

cule, which may be attributable at least partly to the strong intramolecular hydrogen bond between the C3 hydroxyl proton and the C2 ethoxyl group.

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