Heptakis(2,6-di-*O*-methyl-3-*O*-acetyl)-β-cyclodextrin: A Water-Soluble Cyclodextrin Derivative with Low Hemolytic Activity

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Abstract □ Acetyl groups were introduced to the hydroxyl groups of heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CyD), and the resulting heptakis(2,6-di-*O*-methyl-3-*O*-acetyl)-β-CyD (DMA-β-CyD) was evaluated for the inclusion property and hemolytic activity. It was confirmed by means of NMR and mass spectroscopies that in the DMA-β-CyD molecule, all seven hydroxyl groups at the 3-position were substituted by acetyl groups. Thus, it has the degree of substitution (DS) of 7, whereas DMA4-β-CyD with the lower substitution (DS 3.8) was a mixture of components with different DS. The aqueous solubility of DMA-β-CyD was higher than those of β-CyD, DM-β-CyD, and heptakis(2,3,6-tri-*O*-methyl)-β-CyD (TM-β-CyD). The hydrophobicity of the whole molecule, assessed from measurements of surface tension, increased in the order of DM-β-CyD < DMA-β-CyD < TM-β-CyD. The half-life of DMA-β-CyD for hydrolysis in pH 9.5 and 60 °C was about 19 h, and there was only slight liberation of acetic acid in rabbit plasma and carboxylesterase (EC 3.1.1.1) at 37 °C. DMA-β-CyD had an inclusion ability similar to that of TM-β-CyD for *p*-hydroxybenzoic acid esters with different alkyl chain lengths and an antiinflammatory drug, flurbiprofen, although it was inferior to that of DM-β-CyD. The hemolytic activity and rabbit muscular irritation of DMA-β-CyDs were much weaker than those of DM-β-CyD: no hemolysis was observed even in the presence of 0.1 M DMA-β-CyD with DS 7. The results suggest that the water-soluble CyD derivative with superior bioadaptability and inclusion ability can be prepared by properly designing substituents at the 3-position and by optimally controlling their degree of substitution.

Introduction

Cyclodextrins (CyDs) are cyclic oligosaccharides usually consisting of six to eight glucose units and are used successfully as drug carriers with improved levels of solubility, stability, and bioavailability, etc.[1−3] Recently, various kinds of CyD have been prepared in order to improve physicochemical properties and inclusion capacities of parent CyDs.[4−6] For example, hydrophilic CyD derivatives such as methylated, hydroxyalkylated, and branched CyDs are useful for the improvement of low solubility, dissolution rate, and bioavailability of poorly water-soluble drugs.[7,8] On the other hand, hydrophobic CyDs such as ethylated and acylated derivatives have potential as sustained release carriers for water-soluble drugs.[9,10] Among these derivatives, heptakis(2,6-di-*O*-methyl)-β-CyD (DM-β-CyD) is of interest because of its powerful solubilizing ability for the majority of lipophilic drugs such as steroid hormones,[11] vitamins A, E, and K,[12,13] cyclosporin A,[14] and long chain fatty acids.[15] However, one of the drawbacks of DM-β-CyD is its membrane toxicity, causing tissue irritation and hemolysis in a concentration-dependent manner.[16] For example, the concentration of DM-β-CyD to induce 50% hemolysis of human erythrocytes is lower than that of the so-called bioadaptable CyD derivatives such as 2-hydroxypropyl-β-CyD, sulfobutyl ether of β-CyD, and maltosyl-β-CyD.[17−23] The hemolytic activity of CyDs is associated with the extraction of membrane components, mainly through inclusion complexation with cholesterol. In this study, we have attempted to reduce the membrane toxicity of DM-β-CyD, through a chemical modification of hydroxyl groups at the 3-position of glucose units of DM-β-CyD. As a first step of the modification, acetyl groups were introduced to the hydroxyl groups, and the resulting heptakis(2,6-di-*O*-methyl-3-*O*-acetyl)-β-CyD (DMA-β-CyD) was evaluated for the inclusion property, the hemolytic activity, and muscular tissue irritation.

Experimental Section

Materials—β-CyD, DM-β-CyD, and heptakis(2,3,6-tri-*O*-methyl)-β-CyD (TM-β-CyD) were supplied by Japan Maize Co. (Tokyo, Japan). Flurbiprofen, 2-(2-fluoro-4-biphenylyl)propionic acid, was donated by Mitsubishi Kasei Co. (Tokyo, Japan), and *p*-hydroxybenzoic acid esters with different alkyl groups were purchased from Tokyo Kasei Co. (Tokyo, Japan). All other chemicals and solvents were of analytical reagent grade, and deionized double-distilled water was used throughout the study.

Apparatus—Nuclear magnetic resonance (NMR) spectra were taken on a JEOL JNM-α 500 instrument (Tokyo, Japan) operating at 500.16 MHz for protons at 25 °C. The concentration of DMA-β-CyDs was 1.0×10^{-2} M in deuterated chloroform ($CDCl_3$), and the chemical shifts were given as parts par million (ppm) downfield from that of tetramethylsilane. Fast atom bombardment mass (FAB-MS) spectra were measured in a negative mode at 25 °C by a JEOL JMS-DX 303 mass spectrometer (Tokyo, Japan) using the matrix (methanol/glycerol/*m*-nitrobenzyl alcohol). Ultraviolet (UV), fluorescence, and circular dichroism (CD) spectra were measured at 25 °C using Hitachi U-2000 UV and F-4010 fluorescence spectrometers (Tokyo, Japan) and a Jasco J-720 polarimeter (Tokyo, Japan), respectively. Surface tension was measured at 25 °C by a Shimadzu duNouy surface tensionmeter (Kyoto, Japan).

Preparation of DMA-β-CyD—Dried DM-β-CyD (10 g, 7.5 mmol) was dissolved in dried pyridine (50 mL), acetic anhydride (10.7 g, 105 mmol) was added dropwise for 2−3 h, and the mixture was stirred at 80 °C for about 24 h. The reaction was terminated by addition of ice−water, and the resulting oil was extracted with chloroform. The organic phase was washed with 2 mM sodium carbonate and in turn with water, dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was subjected to silica gel chromatography (Kieselgel 60, 0.063−0.2 mm, 70−200 mesh) with an eluent of methanol/chloroform increasing the methanol concentration from 0 to 12% v/v. DMA-β-CyD (DS 7) was recrystallized from water and obtained as white crystals (yield 60%). Mp 126 °C; TLC $R_f = 0.3$ (silica gel 60 F_{254}, methanol/chloroform 2:15 v/v); FAB MS (negative mode) *m*/*z* 1777 [M + *m*-nitrobenzyl alcohol (matrix) − H)$^-$; ^{1}H NMR ($CDCl_3$) δ 5.16 (t, 1H, CyD H-3), 5.00 (d, 1H, CyD H-1), 3.91−3.87 (m, 2H, CyD H-5 and H-6b), 3.79 (t, 1H, CyD H-4), 3.54 (d, 1H, CyD H-6a), 3.37 (s, 3H, 6-CH_3), 3.33 (s, 3H, 2-CH_3), 3.21 (dd, 1H, CyD H-2), 2.04 (s, 3H, 3-CH_3). DMA4-β-CyD with a low degree of substitution

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(DS) was prepared by using small amounts of the acid anhydride (4.6 g, 45 mmol) versus DM-β-CyD (10 g, 7.5 mmol). The other condition of preparation was identical to that described above, except for the recrystallization due to the fact that it was a mixture of components with different DS. The residue after the extraction and evaporation was subjected to the silica gel chromatography described above, and the factions containing acetylated DM-β-CyDs (TLC $R_f = 0.4-0.5$, silica gel 60 F_{254}, methanol/chloroform 2:15 v/v) were collected and evaporated under reduced pressure. DMA4-β-CyD was obtained as white powder (yield 80%). It was confirmed that DMA4-β-CyD contains neither DM-β-CyD (R_f = 0.7) nor DMA-β-CyD (DS 7, $R_f = 0.3$) by TLC analysis and no solvents such as methanol and chloroform by ^{1}H-MMR spectroscopy. The DS value was determined by a peak ratio of the CyD anomeric or skeleton protons and the methyl proton of acetyl groups in ^{1}H NMR spectra (see Supporting Information), and was 3.8. This value coincided with that (DS = 3.9 ± 0.1) determined by the amount of acetic acid released after alkaline hydrolysis in 2.0 N NaOH solution. Unfortunately, the mass spectroscopic determination of DS was difficult because of the partial degradation of DMA4-β-CyD to DM-β-CyD during the ionization.

Solubility Measurements—The solubility method was carried out according to the method of Higuchi and Connors.[24] The screw-capped vials containing drugs in excess amounts in aqueous CyD solutions at various concentrations ($1.0-5.0 \times 10^{-2}$ M) were shaken at 25 °C. After equilibrium was attained (about 5 days), the solution was centrifuged at 800*g* force for about 5 min, and the supernatant was filtered through a membrane filter (Advantec DISMIC-3CP (TOYO−Roshi), Tokyo, Japan) and analyzed for drugs by high-performance liquid chromatography (HPLC) under the following condition: a Hitachi L-6000 pump and an L-4000 UV detector (Tokyo, Japan) at 256 nm, a YMC AM-312 column (6.0 × 150 mm), flow rate of 1.0 mL/min. The mobile phases for the analysis of flurbiprofen and *p*-hydroxybenzoic acid esters were methanol/0.1 M acetic acid (7:3 v/v) and methanol/water (7:3 v/v), respectively. The stability constants ($K_{1:1}$) of 1:1 complexes were calculated from the slope and intercept of straight line of the phase solubility diagram according to the equation of Higuchi and Connors.[24] In the case of phase solubility diagrams with positive curvature, the diagrams were analyzed according to the method of Kristiansen[25] to obtain the 1:1 and 1:2 ($K_{1:2}$, guest:host) stability constants.

Hydrolysis Studies—Alkaline hydrolysis of DMA-β-CyD (1.0×10^{-2} M) was conducted in 0.1 M phosphate buffers (pH 8.5−11.5) at 60 °C. At timed intervals, the reaction solution (20.0 μL) was sampled and analyzed for the resulting acetic acid by HPLC under the following condition: a YMC AP-303 column (4.6 × 250 mm), a mobile phase of 0.1% phosphoric acid, a flow rate of 1.0 mL/min, a detection of 210 nm. The hydrolysis was monitored by measuring acetic acid at an early stage of the reaction (240 min, 150, 120, and 30 min at pH 8.5, 9.5, 10.5, and 11.5, respectively), because the pH of the solution changed due to the liberation of acetic acid. The rate constant (k) was calculated according to the following equation: $\ln(7C_0 - C) = -kt + \ln 7C_0$, where C_0 and C are the initial concentration of DMA-β-CyD and the concentration of the liberated acetic acid at time t, respectively. The total concentration of acetic acid at infinite time is supposed to be $7C_0$.

Carboxylesterase-catalyzed hydrolysis of DMA-β-CyD was conducted in 0.1 M *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES)/NaOH buffer solution (pH 7.4) at 37 °C. The concentrations of DMA-β-CyD and the enzyme (EC 3.1.1.1 obtained from porcine liver, purchased from Sigma Aldrich Japan, Tokyo) were 5.0×10^{-3} M and 0.25 or 25 units/mL, respectively. At timed intervals, an aliquot (20.0 μL) was sampled and analyzed for acetic acid by HPLC under the aforementioned condition.

Hydrolysis of DMA-β-CyD was conducted in 50% and 80% rabbit plasma at 37 °C. Rabbit blood was taken by heparinized injection syringe and centrifuged at 12000*g* for 5 min at 4 °C. DMA-β-CyD solution (1.0 mL, 2.0×10^{-2} M in pH7.4 isotonic phosphate buffer) was added to the plasma (1.0 mL). At appropriate times, an aliquot (0.3 mL) of the reaction solution was ultrafiltered using a membrane filter (Amicon Kit (Tokyo, Japan), a centrifugation of 2000*g* for 15 min at 4 °C), and the filtrate was analyzed for acetic acid by HPLC under the aforementioned condition.

Hemolysis Studies—Erythrocytes were separated by centrifugation of freshly drawn citrated rabbit blood at 1000*g* for 5 min, washed three times with phosphate buffer (0.154 M sodium chloride and 0.01 M phosphate, pH 7.4), and resuspended in the buffer solution to give a hematocrit of 5%. The cell suspension (0.1 mL) was added to the buffer solution (2.0 mL) containing β-CyDs at various concentrations. Each mixture was incubated for 30 min at 37 °C and centrifuged at 1000*g* for 5 min. The release of hemoglobin from the cells was measured spectrophotometrically at 543 nm. Results were expressed as percentages of the total efflux of hemoglobin which was obtained when water was used instead of the buffer solution. The morphological observation of erythrocytes was carried out as follows: the cell suspension (5%, 0.1 mL) was incubated with the buffer solution (2.0 mL) containing β-CyDs at 37 °C for 60 min, and fixed with 2% glutaraldehyde solution (5.0 mL). After standing for 1 h at room temperature, the fixed cells were washed three times with water, dried under reduced pressure for 16 h, coated with gold, and observed by scanning electron microscope (Akashi, MSM4C, Tokyo, Japan).

Determination of cholesterol: the cell suspension (5%, 0.2 mL) was incubated with the buffer solution (4.0 mL, pH 7.4) containing β-CyDs at 37 °C for 30 min. After centrifugation (1000*g*, 5 min), cholesterol in the supernatant (3.0 mL) was extracted with chloroform (5.0 mL), the organic phase was evaporated under reduced pressure, and cholesterol in the residue was determined by the cholesterol oxidase method with using a Cholesterol E-test kit (Wako, Osaka, Japan). Results were expressed as percentages of the total amount of cholesterol which was released when water was used instead of the buffer solution.

Intramuscular Irritation Studies—The intramuscular irritation study was carried out by the method of Shintani et al.[26] β-CyD solutions (100 mg/1.0 mL of normal sterile saline) were injected into *M. vastus lateralis* of three rabbits (2.5−3.0 kg) using a 23-gauge 0.5 in. needle. The rabbits were killed 2 days after the injection, the muscle was exposed and cut longitudinally, and the lesion were scored according to the method of Shintani et al.,[26] that is, score 0, no discernible gross reaction; score 1, slight hyperemia and discoloration; score 2, moderate hyperemia and discoloration; score 3, distinct discoloration in comparison with the color of surrounding area; score 4, brown degeneration with small necrosis; score 5, widespread necrosis.

Results and Discussion

Some Physicochemical Properties of DMA-β-CyD—It was confirmed by means of NMR and mass spectroscopies that in DMA-β-CyD molecule, all seven hydroxyl groups at the 3-position were substituted by acetyl groups, thus having DS 7. DMA4-β-CyD with the lower substitution (DS 3.8) was a mixture of components with different DS. In the following evaluation of physicochemical and inclusion properties, DMA-β-CyD with DS 7 was employed, and the lower DS form was used only for comparative studies of hemolysis and muscular irritation.

The melting point decreased in the order, DM-β-CyD (295−300 °C), the parent β-CyD (280 °C), TM-β-CyD (157 °C), and DMA-β-CyD (126 °C). The aqueous solubility of DMA-β-CyD (>60 g/dL at 25 °C) was much higher than those of the parent β-CyD (1.85 g/dL), DM-β-CyD (57 g/dL), and TM-β-CyD (31 g/dL); however, DMA-β-CyD ≥ 70%w/v gave a viscous solution. DMA-β-CyD exhibited an exothermic dissolution in water in the similar manner as DM- and TM-β-CyDs[27] and thus precipitated at higher temperature. The hydrophobicity of DMA-β-CyD was assessed by measurements of the surface tension, because the oil/water partition method is affected by the inclusion of organic solvents in the CyD cavity. As shown in Figure 1, the surface tension of DMA-, DM-, and TM-β-CyDs decreased as the concentration increased, and the hydrophobicity of the whole molecule increases in the order of DM-β-CyD < DMA-β-CyD < TM-β-CyD. This order was in accordance with that of the π value of the substituents introduced at the hydroxyl groups, that is, −0.67 (OH of DM-β-CyD) < −0.64 (OCOCH$_3$ of DMA-β-CyD) < −0.02 (OCH$_3$ of TM-β-CyD).[28] The hydrolysis of DMA-β-CyD in alkaline solution was studied by monitoring the resulting acetic acid, because it was difficult to determine partially hydrolyzed

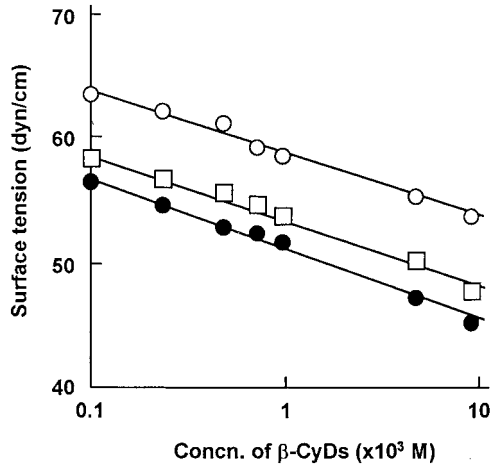
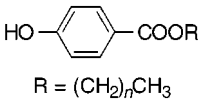


Figure 1—Surface tension of DMA-β-CyD (□), DM-β-CyD (○), and TM-β-CyD (●) in water at 25 °C.

DMA-β-CyDs having various regioisomers by means of HPLC. The hydrolysis was followed at an early stage of the reaction, because pH of the solution changed owing to the liberation of acetic acid. The appearance rates of acetic acid from DMA-β-CyD were 9.0 ($\pm$1.5) $\times$ 10^{-5} min^{-1} (mean ($\pm$SE), n = 3, pH 8.5), 6.2 ($\pm$0.2) $\times$ 10^{-4} min^{-1} (pH 9.5), 2.1 ($\pm$0.1) $\times$ 10^{-3} min^{-1} (pH 10.5), and 1.9 ($\pm$0.1) $\times$ 10^{-2} min^{-1} (pH 11.5) at 60 °C, giving the linear rate−pH profile (correlation coefficient = 0.990) with a slope of 0.75. The carboxylesterase-catalyzed hydrolysis of DMA-β-CyD was conducted at concentrations of 5.0 $\times$ 10^{-3} M of DMA-β-CyD and 0.25 or 25 units/mL of the esterase in HEPES buffer (pH 7.4) at 37 °C. No liberation of acetic acid (less than the detection limit of 3%) in the 0.25 units/mL enzyme solution was observed for 10 h, and the releases even in the 25 units/mL enzyme solution were less than 5% and 15% of the total amounts of acetic acid for 5 and 10 h, respectively. Furthermore, no liberation of acetic acid (less than 3%) was observed in 50% and 80% plasma of rabbits for 12 h, suggesting that DMA-β-CyD is chemically stable in biological fluids such as plasma.

Inclusion Property of DMA-β-CyD—The inclusion property of DMA-β-CyD was compared with those of the parent β-CyD and DM- and TM-β-CyDs. Table 1 shows the results of phase solubility diagrams of β-CyDs with *p*-hydroxybenzoic acid esters with different alkyl chains. The guest molecules with short alkyl chains showed A_L type phase solubility diagrams where the solubility of the guests increased linearly as a function of CyD concentrations (0−0.02 M), indicating the 1:1 complexation.[24] On the other hand, the ester with longer alkyl chains showed A_P type diagrams where the solubility curve deviated positively from a straight line, indicating the 1:2 (guest:host) complexation.[25] The stability constant of the 1:1 complexes increased as the alkyl chain lengthens up to the propyl ester, whereas the butyl ester formed the 1:2 complex with DMA-β-CyD and the hexyl eater formed the 1:2 complex with the three CyDs. The stability constants were generally in the order of DM-β-CyD ≫ TM-β-CyD ≈ DMA-β-CyD.

Table 1—Stability Constants ($K_{1:1}$ and $K_{1:2}$, M^{-1})[a] and Types of Phase Solubility Diagrams for Inclusion Complexes of *p*-Hydroxybenzoic Acid Esters with β-CyDs in Water at 25 °C



	DM-β-CyD			TM-β-CyD			DMA-β-CyD		
ester	$K_{1:1}$	$K_{1:2}$	type	$K_{1:1}$	$K_{1:2}$	type	$K_{1:1}$	$K_{1:2}$	type
methyl	230	–	A_L	63	–	A_L	35	–	A_L
ethyl	960	–	A_L	120	–	A_L	140	–	A_L
propyl	5600	–	A_L	300	–	A_L	470	–	A_L
butyl	slope > 1[b]	–	A_L	290	–	A_L	46	110	A_P
hexyl	3400	90	A_P	1000	40	A_P	110	120	A_P

[a] Average of the values for duplicate measurements, which coincided with each other within ±5%. [b] Could not be determined due to the slope > 1.

Spectroscopic studies on the interaction of flurbiprofen with DMA-β-CyD was carried out, because the complexation of the drug with DM- and TM-β-CyDs had been investigated in detail.[29] Figure 2 shows the UV, CD, and fluorescence spectra of flurbiprofen in the absence and presence of DM-, TM-, and DMA-β-CyDs in pH 7.0 phosphate buffer. Flurbiprofen gave a UV absorption maximum (λ_{max}) at 246 nm, and by the addition of β-CyDs the intensity decreased with concomitant shifts to longer wavelength. This bathochromic shift was largest with DMA-β-CyD (λ_{max} 251 nm) compared with those of DM-β-CyD (249 nm), TM-β-CyD (249 nm), and β-CyD (247 nm),

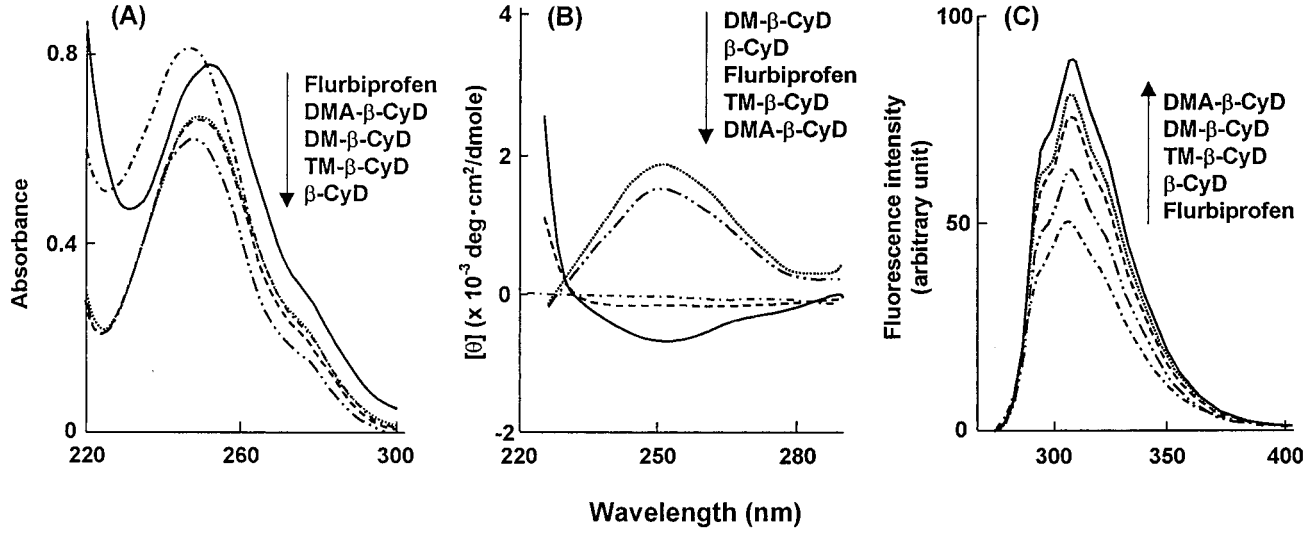


Figure 2—UV (A), CD (B), and fluorescence (C) spectra of flurbiprofen in the absence (–·–) and presence of DMA-β-CyD (—), β-CyD (–··–), DM-β-CyD (····) and TM-β-CyD (---) in 0.1 M phosphate buffer (pH 7.4, I = 0.2) at 25 °C. The concentrations of flurbiprofen and β-CyDs in UV and CD spectroscopic studies were 4.0 $\times$ 10^{-5} M and 4.0 $\times$ 10^{-3} M, respectively, and those in fluorescence spectroscopic studies were 1.0 $\times$ 10^{-6} M and 1.0 $\times$ 10^{-3} M, respectively. The excitation wavelength was 256 nm.

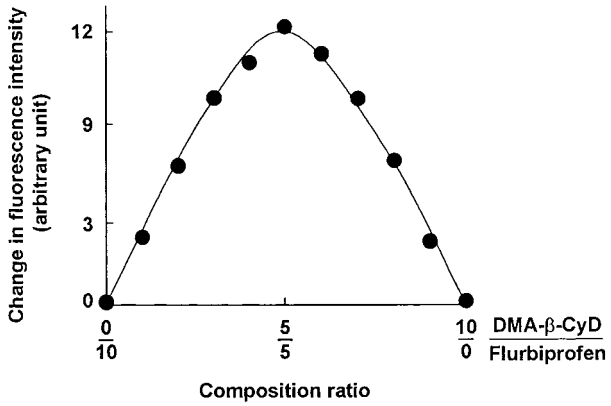


Figure 3—Continuous variation plot for flurbiprofen/DMA-β-CyD system (total concentration of the host and guest molecules = 5.0×10^{-6} M) in 0.1 M phosphate buffer (pH 7.4, $I = 0.2$) at 25 °C.

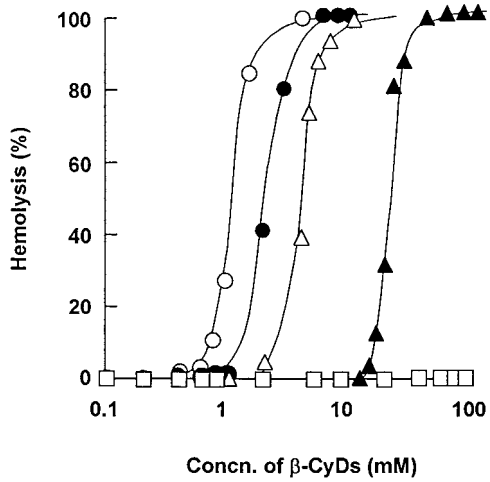


Figure 4—Hemolytic effects of β-CyDs on rabbit erythrocytes in isotonic phosphate buffer (pH 7.4) at 37 °C. Key: (□) DMA-β-CyD; (▲) DMA4-β-CyD; (△) β-CyD; (○) DM-β-CyD; (●) TM-β-CyD.

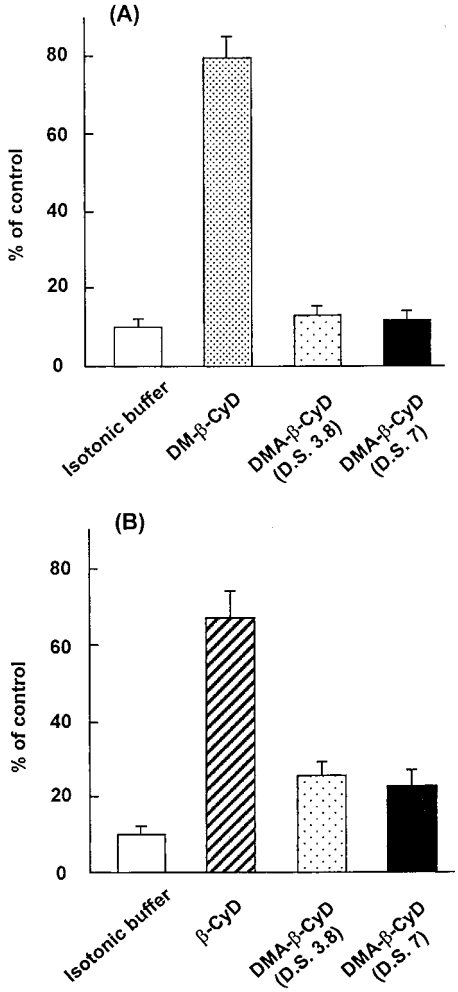


Figure 5—Cholesterol release from intact erythrocytes of rabbits treated with β-CyDs (A, 0.5 mM; B, 3.0 mM) in isotonic phosphate buffer (pH 7.4) at 37 °C. Each value represents the mean ± SE of three experiments.

although the intensity change was small with DMA-β-CyD. In the CD spectra, the optical activity of flurbiprofen was induced positively by the addition of DM-β-CyD and β-CyD, whereas it was induced negatively by DMA-β-CyD and TM-β-CyD where the effect of DMA-β-CyD was larger than that of TM-β-CyD. The sign of CyD-induced CD bands depends on a spatial relationship between the asymmetric center of CyD cavity and the perturbed chromophore of guest molecules. For example, the electronic transition of an included guest with a transition dipole moment, parallel to the z-axis of the CyD cavity, gives a positive CD, and that with a transition dipole moment perpendicular to the z-axis gives a negative CD.[30] Our crystallographic studies indicated that flurbiprofen is included in such a manner that the long molecular axis of the drug is parallel to the z-axis of the β-CyD cavity, that is, an axial inclusion, whereas the long axis of the drug is inclined in the TM-β-CyD cavity.[31,32] These dispositions of flurbiprofen in CyD cavities were reflected in the sign of induced CD bands, giving a positive CD with β-CyD and a small negative CD with TM-β-CyD. DMA-β-CyD gave a larger negative CD band than TM-β-CyD, suggesting that the drug is more inclined in the former cavity than in the latter cavity. The fluorescence intensity of flurbiprofen at 315 nm was markedly increased in the order of β-CyD < TM-β-CyD < DM-β-CyD < DMA-β-CyD. Since the fluorescence of the drug is known to be increased significantly in organic solvents such as ethanol, the CyD-induced fluorescence change suggested that the drug is included within the hydrophobic CyD cavity. The largest enhancement of the fluorescence by DMA-β-CyD may be attributable to the increase in hydrophobicity of the cavity, although the hydrophobicity of the whole molecule of DMA-β-CyD was lower than that of TM-β-CyD as is apparent from the surface tension. The methyl moieties of acetyl groups in the DMA-β-CyD molecule seem to be directed inside the cavity, while the carbonyl moieties are directed outside the cavity, providing a more hydrophobic environment of the cavity. On the other hand, TM-β-CyD is known to have the distorted macrocyclic ring in which the secondary rim is wider whereas the primary rim is narrower,[33] thus allowing water molecules easily to access the inside of the cavity. This may be a reason the fluorescence enhancement of TM-β-CyD was smaller than DMA-β-CyD, despite the higher hydrophobicity of TM-β-CyD as a whole molecule. Figure 3 shows a continuous variation plot of the fluorescence intensity at 310 nm of the flurbiprofen/DMA-β-CyD system. The plot gave a maximum at 0.5, indicating the 1:1 stoichiometry which was the same as that of β-CyD, DM-β-CyD, and TM-β-CyD complexes.[29] Therefore, the fluorescence change as a function of concentrations of the four β-CyDs was analyzed quantitatively by the Scott equation[34] to obtain the stability constant ($K_{1:1}$) of the 1:1 complexes. The $K_{1:1}$ values in pH 7.0 phosphate buffer at 25 °C were 3610 (±120) M^{-1} (mean (± SE), $n = 3$), 8060 (±600) M^{-1}, 1660 (±80) M^{-1}, and 1410 (±80) M^{-1} for the complexes with β-CyD, DM-β-CyD, TM-β-CyD, and DMA-β-CyD, respectively. Furthermore, the phase solubility diagram of flur-

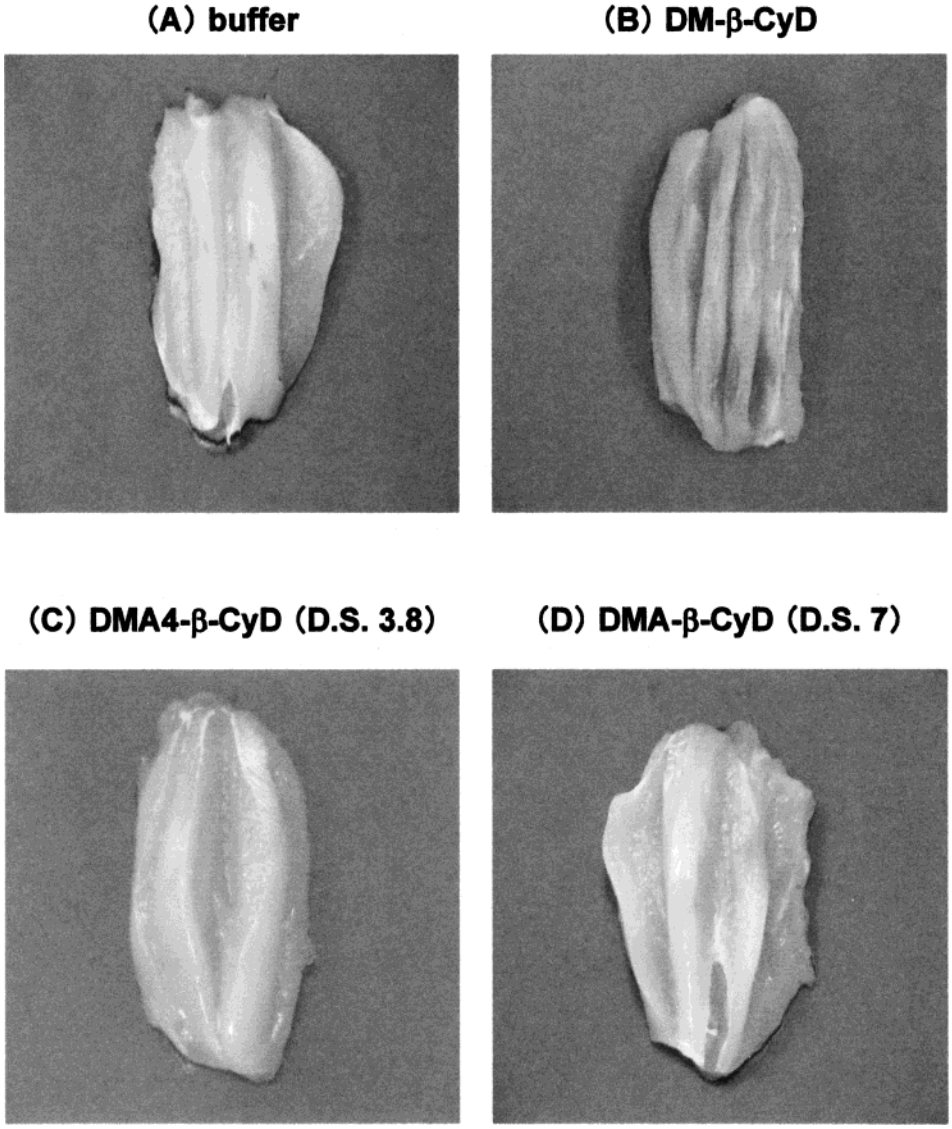


Figure 6—Macrographs of *M. vastus lateralis* after intramuscular injections of β-CyDs (100 mg/site, 1.0 mL) to rabbits. (A) Isotonic buffer; (B) DM-β-CyD; (C) DMA4-β-CyD; (D) DMA-β-CyD.

biprofen with DMA-β-CyD ($0.0-5.0 \times 10^{-2}$ M) in water at 25 °C showed the A_L type and gave the stability constant of 2020 ($\pm$90) M^{-1}. This value was the same as that (2200 ($\pm$110) M^{-1}) of the TM-β-CyD complex, but was smaller than that (14700 ($\pm$900) M^{-1}) of the DM-β-CyD complexes determined under the same conditions. The stability constants determined by the fluorescence method in pH 7.0 phosphate buffer were smaller than those determined by the solubility method in water, which may be due to the acid-ionization of flurbiprofen (pK_a 3.8). These results suggested that DMA-β-CyD has the same inclusion ability as TM-β-CyD, although it is inferior to that of DM-β-CyD, and the hydrophobicity of the DMA-β-CyD cavity is higher than that of TM-β-CyD.

Hemolysis and Muscular Irritation of DMA-β-CyD—Hemolytic activity of DMA-β-CyDs was compared with that of other CyD derivatives in order to estimate its local irritation. Figure 4 shows hemolysis profiles of β-CyD, DM-β-CyD, TM-β-CyD, and DMA-β-CyDs with different DS (7 and 3.8), measured under the same conditions. It is apparent that the hemolytic activity of DMA-β-CyDs was weaker than those of β-CyD, DM-β-CyD, and TM-β-CyD. For example, the hemolysis began at about 2 mM, 0.5 mM, and 1 mM, and the concentrations to induce 50% hemolysis were about 4 mM, 1 mM, and 2 mM for β-CyD, DM-β-CyD, and TM-β-CyD, respectively. On the other hand, the hemolysis of DMA4-β-CyD with DS 3.8 began at about 12 mM, and its 50% hemolysis concentration was about 22 mM. In the case of DMA-β-CyD with DS 7, no hemolysis was observed up to 100 mM. Scanning electron micrographic studies indicated no change in the shape of erythrocytes even at a concentration of 100 mM DMA-β-CyD, that is, they maintained the discocyte shape same as that in the buffer control (Supporting Information). One of the causes of CyD-induced hemolysis is known to be extractions of cholesterol and phospholipids from erythrocytes through the inclusion complex formation.[16] Therefore, the cholesterol release behavior from rabbit erythrocytes by the addition of DMA-β-CyDs was investigated and compared with those of β-CyD and DM-β-CyD. Figure 5 shows the released amounts of cholesterol from the intact erythrocytes of rabbits treated with β-CyDs in 0.1 M phosphate buffer (pH 7.4) at 37 °C. DM-β-CyD induced about 80% release of cholesterol at a concentration of 0.5 mM at which the hemolysis only slightly occurred (see Figure 4). On the other hand, DMA-β-CyDs induced only 10% release of cholesterol at the same concentration, and this release was the same as that of the control experiment conducted in isotonic buffer. Similarly, the 3 mM parent β-CyD solution induced 65% release of cholesterol, whereas

DMA-β-CyDs induced only about 20−25% release of cholesterol. These results indicated that the hemolytic activity of DMA-β-CyDs, particularly the DS 7 derivative, is much weaker than those of the parent β-CyD and the methylated β-CyDs.

Figure 6 shows macrographs of *M. vastus lateralis* of rabbit dissected 2 days after the single injection of DM-β-CyD and DMA-β-CyDs with DS 7 and 3.8 at a dose of 100 mg/site. In the case of DM-β-CyD, a discoloration of the muscle with hemorrhage was observed, and the irritation score according to Shintani et al.[26] was 4.0 ± 1.0. On the other hand, both DMA- and DMA4-β-CyD gave negligible damage to the muscle, giving the irritation score of 0.3 ± 0.7 which was the same as that of the saline injection.

In conclusion, the hemolytic activity and the muscular tissue irritation of DM-β-CyD were significantly reduced by acetylating hydroxyl groups at the 3-position of the glucose units, and the inclusion ability of DMA-β-CyD was comparable to that of TM-β-CyD. These results suggest that water-soluble CyD derivatives with superior bioadaptability and inclusion ability can be prepared by pertinently designing substituents at the 3-position and by optimally controlling their degree of substitution.

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Supporting Information Available—Three figures of ^{1}H-NMR and FAB mass spectra of DMA-β-CyD and SEM of erythrocytes. This material is available free of charge via the Internet at http://pubs.acs.org.

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