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Physicochemical and biological properties of $6^1,6^3,6^5$-tri-*O*-α-maltosyl-cyclomaltoheptaose ($6^1,6^3,6^5$-tri-*O*-α-maltosyl-β-cyclodextrin)

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Abstract—A unique multibranched cyclomaltooligosaccharide (cyclodextrin, CD) of $6^1,6^3,6^5$-tri-*O*-α-maltosyl-cyclomaltoheptaose [$6^1,6^3,6^5$-tri-*O*-α-maltosyl-β-cyclodextrin, $(G_2)_3$-βCD] was prepared. The physicochemical and biological properties of $(G_2)_3$-βCD were determined together with those of monobranched CDs (6-*O*-α-D-glucopyranosyl-α-cyclodextrin (G_1-αCD), 6-*O*-α-D-glucopyranosyl-β-cyclodextrin (G_1-βCD), and 6-*O*-α-maltosyl-β-cyclodextrin (G_2-βCD)). NMR spectra of $(G_2)_3$-βCD were measured using various 2D NMR techniques. The solubility of $(G_2)_3$-βCD in water and MeOH–water solutions was extremely high in comparison with nonbranched βCD and was about the same as that of the other monobranched βCDs. The formation of an inclusion complex of $(G_2)_3$-βCD with stereoisomers (estradiol, retinoic acid, quinine, citral, and glycyrrhetinic acid) depends on the cis–trans isomers of guest compounds. The cis isomers of estradiol, retinoic acid, and glycyrrhetinic acid were included more than their trans isomers, while the trans isomers of citral and quinine fit more tightly than their cis isomers. $(G_2)_3$-βCD was the most effective host compound in the cis–trans resolution of glycyrrhetinic acid. Among the branched βCDs, $(G_2)_3$-βCD exhibited the weakest hemolytic activity in human erythrocytes and showed negligible cytotoxicity in Caco-2 cells up to 200 μM. These results indicate unique characteristics of $(G_2)_3$-βCD in some biological responses of cultured cells.

Keywords: Branched cyclodextrin; $6^1,6^3,6^5$-Tri-*O*-α-maltosyl-cyclomaltoheptaose; $6^1,6^3,6^5$-Tri-*O*-α-maltosyl-β-cyclodextrin; Stereoisomer; Inclusion complex; NMR; Cytotoxicity

1. Introduction

Cyclomaltooligosaccharides (cyclodextrins, CDs) basically consist of three well-known family members such as cyclomalto-hexaose, -heptaose, and -octaose (α, β, and γCD, respectively), which comprise six, seven, and eight glucopyranose units, respectively. Various derivatives of CDs have been synthesized to improve their solubility and stability in aqueous solutions, as well as their availability and cytotoxicity in biological systems. It is generally accepted that branched CDs have many advantages over nonbranched CDs in terms of greater solubility in water and less hemolytic activity.[1–6]

We have prepared a novel βCD derivative, $6^1,6^3,6^5$-tri-*O*-α-maltosyl-cyclomaltoheptaose ($6^1,6^3,6^5$-tri-*O*-α-maltosyl-β-cyclodextrin ($G_2)_3$-βCD), from the reaction products of maltose and β-cyclodextrin (βCD) produced by the action of *Klebsiella pneumoniae* pullulanase [EC 3.2.1.41].[7,8] This multibranched CD comprises three maltose side chains attached to the βCD core ring by α-(1→6)-linkages. The physicochemical and biological properties of this unique branched βCD are now determined.

Ideally, CD derivatives for biological experiments have good solubility and little cytotoxicity as well as hemolytic activity. In the current study, the solubility

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and cytotoxicity of branched βCDs were examined in cultured Caco-2 cells. Further, the cis–trans resolution of several stereoisomeric compounds determined through the formation of inclusion complexes with branched βCDs was investigated.

2. Experimental

2.1. Materials

The mono- and multibranched CDs used as host compounds were G_1-αCD, G_1-βCD, G_2-βCD, and $(G_2)_3$-βCD. G_1-αCD, G_1-βCD, and G_2-βCD, which were supplied by Bio Research Corporation of Yokohama (Yokohama, Japan), were further purified by semipreparative HPLC on a YMC-Pack SH-343-5 ODS column (250 × 20 mm i.d.) (YMC, Kyoto, Japan) at a flow rate of 3 mL/min and a column temperature of 30 °C, eluting with an MeOH–H_2O system containing an optimal concentration of MeOH, that is, 6:94 MeOH–H_2O for G_1-αCD and 8:92 MeOH–H_2O for G_1-βCD and G_2-βCD. Each fraction was analyzed by HPLC on a YMC-Pack A-312 ODS column (150 × 6 mm i.d.) (YMC) by eluting with 5:95 MeOH–H_2O for G_1-αCD and 6:94 MeOH–H_2O for G_1-βCD and G_2-βCD, respectively, at a flow rate of 0.7 mL/min and a column temperature of 30 °C. $(G_2)_3$-βCD was isolated and purified by HPLC as described previously.[8] Nonbranched αCD and βCD and all the guest compounds were obtained from commercial sources. Nonbranched αCD and βCD were used after their recrystallization from water. The stereoisomeric compounds used as the guest compounds were as follows: 17α-estradiol (trans conformation of 13-CH_3/17-OH) and 17β-estradiol (cis conformation of 13-CH_3/17-OH); 18α-glycyrretinic acid (trans conformation of the D/E rings) and 18β-glycyrrhetinic acid (cis conformation of the D/E rings); 13-*cis*-retinoic acid and all *trans*-retinoic acid; citral A (cis) and citral B (trans); and quinine (trans conformation of the 8–9 bond/vinyl group) and quinidine (cis conformation of the 8–9 bond/vinyl group). All HPLC solvents were of HPLC-grade, filtered through a 0.45-μm filter, and degassed before use. All other chemicals were of reagent grade.

2.2. Separation of stereoisomers by HPLC

HPLC analyses of the guest compounds were performed with a Jasco PU-980 pump and a Jasco UV-970 variable-wavelength UV spectrometer (Jasco, Tokyo, Japan). Chromatographic conditions are listed in Table 1. Figure 1 shows the separation of each cis and trans isomers of stereoisomers. HPLC analyses at constant temperature were conducted using an SSC 3510C column oven (Senshu Scientific Co., Tokyo, Japan).

2.3. Solubility of $(G_2)_3$-βCD

The solvent (water or various concentrations of MeOH–H_2O solution) was carefully added in portions of 0.01–0.1 mL to a glass vessel containing 500 mg of the dried $(G_2)_3$-βCD. The volume of solvent required for the complete dissolution of $(G_2)_3$-βCD within 30 min, with vigorous shaking for 30-s periods at 5-min intervals, was individually measured at 25 ± 1, 40 ± 1, and 55 ± 1 °C.

2.4. NMR spectroscopy of $(G_2)_3$-βCD

The NMR spectra were obtained in a solution of $(G_2)_3$-βCD (39.5 mg) in D_2O (0.75 mL) at 5 °C and 20 °C with a Varian Unity-600 NMR spectrometer. Spectra in the TOCSY, DQF-COSY, ROESY, HMQC, and HMBC modes were measured at 599.59 and 150.77 MHz for ^1H and ^{13}C nuclei, respectively. Chemical shifts are given in parts per million downfield from external Me_4Si.

2.5. Phase-solubility analysis

Solubility measurements were carried out according to the method of Higuchi and Connors.[9] Excess amounts of the guest compounds (ratio of cis and trans isomers, 1:1) were added to solutions containing various concentrations of CDs and were shaken at 30 °C. After equilib-

Table 1. Chromatographic conditions for the HPLC of stereoisomers[a]

Guest compounds	Column	Eluent	Detection at UV (nm)	Retention time (min)	
				Cis	Trans
Estradiol	A	CH_3OH–H_2O (60:40)	280	16.5	18.1
Retinoic acid	B	CH_3CN–0.05% H_3PO_4 (75:25)	345	15.5	17.1
Quinidine and quinine	B	CH_3CN–0.01% H_3PO_4 (88:12)	232	8.4	9.7
Citral	B	CH_3OH–H_2O (60:40)	245	15.5	17.7
Glycyrretinic acid	C	CH_3CN–0.01% H_3PO_4	248	27.4	25.4

[a] Column: (A) YMC-Pack J'sphere ODS-H80 (150 × 4.6 mm i.d.), (B) YMC-Pack J'sphere ODS-M80 (150 × 4.6 mm i.d.), (C) YMC-Pack J'sphere ODS-L80 (150 × 4.6 mm i.d.); flow rate, 0.7 mL/min; column temperature, 30 °C.

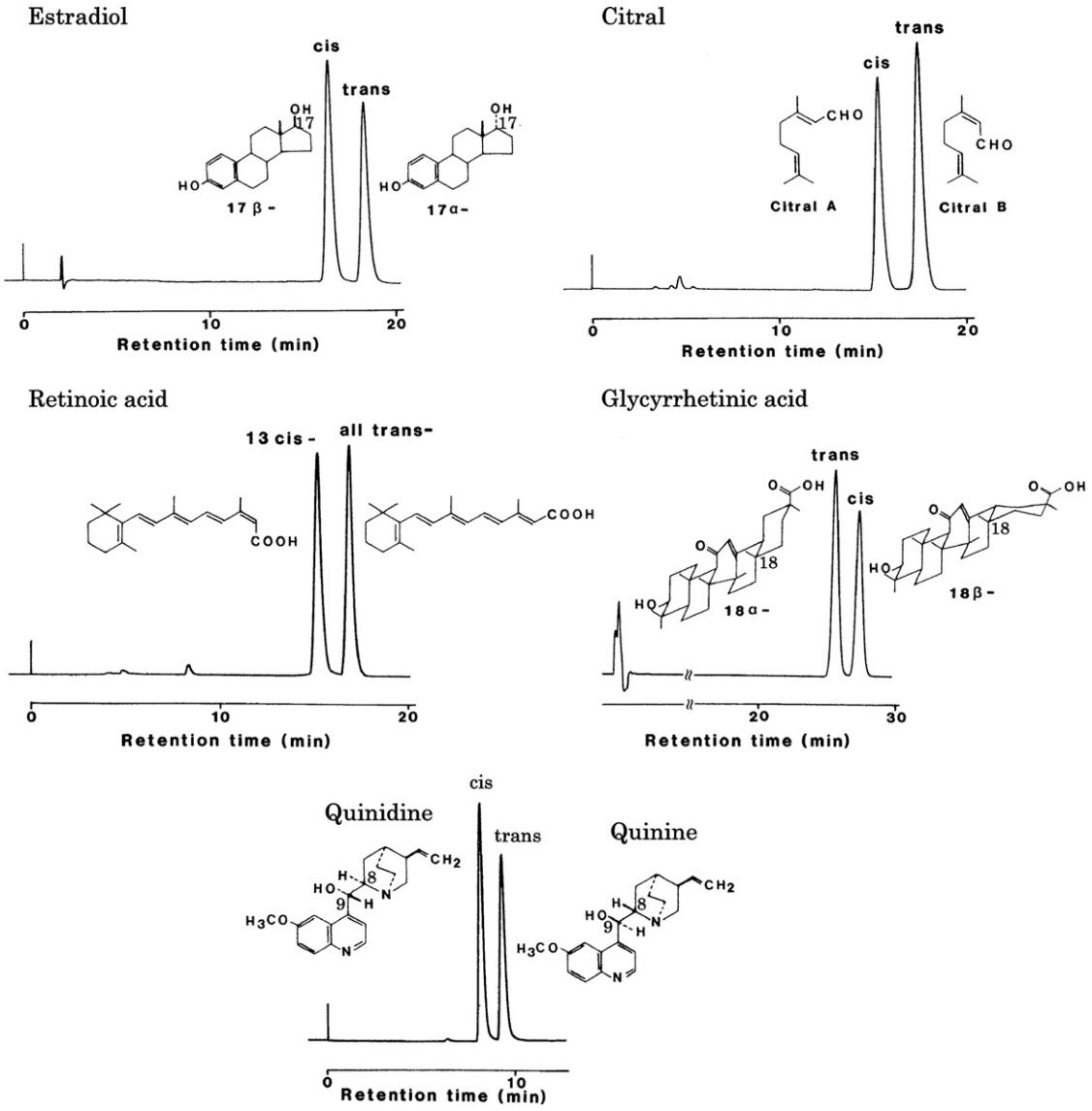

Figure 1. Separation of the stereoisomers by HPLC.

rium was attained, an aliquot was pipetted through a 0.2-μm membrane filter, and the amounts of cis and trans isomers dissolved in CD solution were analyzed by HPLC. The ratio of these isomers was also determined.

2.6. Hemolysis assays

A 0.2% (v/v) human erythrocyte suspension (1.0 mL) in 100 mM of isotonic phosphate buffer (pH 7.4) was added to 1.0 mL of phosphate buffer containing various concentrations of CDs. The mixture was incubated at 37 °C for 30 min and then centrifuged at 1300*g* for 10 min. The release of hemoglobin from the erythrocytes was measured at 541 nm using a V-550 spectrophotometer (Jasco). Hemolytic activity was expressed as a percentage of the total efflux value obtained using water instead of phosphate buffer.

2.7. Caco-2 cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 50 units/mL penicillin G, and 50 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. The monolayer cultures were subcultured at 80% confluency by treatment with 0.05% trypsin and 0.2% EDTA in phosphate-buffered saline (PBS) and less than 15 passages were used in the experiments.

2.8. Cytotoxicity

The release of intracellular lactate dehydrogenase (LDH) enzyme activity was measured using a

colorimetric cytotoxicity assay kit (LK 100) from Oxford Biomedical Research (USA). Caco-2 cells seeded on 24-well plates (Iwaki, Tokyo, Japan) at a density of 3×10^4 cells/cm^2 were cultured, washed with Hanks' balanced salt solution (HBSS), and then incubated with 500 μL of DMEM with or without CDs or Triton X-100 at 37 °C for 24 h. Caco-2 cells were observed under a phase-contrast microscope. Thereafter, cells were lysed, and cellular LDH activity in the supernatant was assayed according to the procedure outlined in the manual provided. Cytotoxicity of CDs was expressed as a percentage of the result of a negative control (without CDs and Triton X-100).

3. Results and discussion

3.1. Solubility

Table 2 shows the solubility of $(G_2)_3$-βCD in water (A) at 25, 40, and 55 °C, and in MeOH–water solutions (B), together with data on G_1-βCD, G_2-βCD, and the parent nonbranched βCD. The solubility values of $(G_2)_3$-βCD are much higher than those of nonbranched βCD, but lower than those of G_1-βCD and G_2-βCD. For this reason, it is thought that $(G_2)_3$-βCD will be applicable to a variety of fields along with the other branched CDs.

3.2. NMR spectroscopy

$(G_2)_3$-βCD is the $6^1,6^3,6^5$-position isomer of trimaltosyl-βCDs for which there are five positional isomers. We have already determined the structures of the four related isomers ($6^1,6^2,6^3$-, $6^1,6^2,6^4$-, $6^1,6^2,6^5$-, and $6^1,6^2,6^6$-isomers) with $(G_2)_3$-βCD ($6^1,6^3,6^5$-isomer) by ^{13}C NMR spectroscopy, FABMS, and an enzymic method. Herein are recorded the 1D ^{1}H NMR spectra of individual D-glucopyranosyl residues constituting the $(G_2)_3$-βCD ($6^1,6^3,6^5$-isomer) in the TOCSY mode. In the cases of nonbranched αCD and βCD, only a single set of NMR signals corresponding to a glucopyranose constituent is observed, because the six or seven D-glucopyranosyl residues are equivalent due to the presence of an apparently symmetric axis; furthermore, there is rapid intramolecular motion in solution.[10,11] On the other hand, $(G_2)_3$-βCD gave an extensively overlapping ^{1}H NMR spectrum (see Fig. 2I). This complexity is due to the D-glucopyranosyl residues of the three maltosyl side chains that perturb the symmetric nature of the D-glucopyranosyl residues, which form the macrocyclic βCD ring. The combination of various 2D NMR techniques (TOSY, DQF-COSY, ROESY, HMQC, and HMBC) makes it possible to assign individual D-glucopyranosyl residues that constitute $(G_2)_3$-βCD (assignments are given with the spectra in Fig. 2II–IX). In Figure 2 there are eight sets of independent spin network systems arising from eight different types of glucopyranose residues, which can be assigned, respectively, to the A–H units illustrated in Figure 2.

Table 2. Solubility of CDs in water (panel A) and MeOH–water solutions at 25 °C (panel B)

CDs	Solubility (mmol/100 mL)		
	25 °C	40 °C	55 °C
Panel A			
$(G_2)_3$-βCD	65	75	95
βCD	1.6	3	4.4
G_1-βCD	77	77	133
G_2-βCD	104	104	122
	10% (v/v)	30% (v/v)	50% (v/v)
Panel B			
$(G_2)_3$-βCD	70	60	50
βCD	1	0.5	0.3
G_1-βCD	92	86	77
G_2-βCD	82	76	69

3.3. Phase-solubility analysis

Figure 3 shows the phase-solubility diagrams obtained for the stereoisomers of some branched CDs in water at 30 °C. In most cases, the solubility of the guest compounds increases with a rise in concentration of the branched CDs. These solubility curves can be classified as type A, except for the citral–G_1-βCD system. The solubilizing effect of nonbranched βCD on the guest compounds was minor, because it formed insoluble complexes at lower βCD concentrations. $(G_2)_3$-βCD included much more 17β-estradiol (cis), 13-*cis* retinoic acid, quinine (trans), and citral B (trans) than 17α-estradiol (trans), all-trans retinoic acid, quinidine (cis), and citral A (cis), respectively (Fig. 3). These results indicate that the former isomers compared to the latter compound are fitted tightly into the cavity of branched βCDs. However, the ability of $(G_2)_3$-βCD was the same as that of G_1-βCD and G_2-βCD in every guest compound, although the ratio of cis and trans isomers included with each of the branched βCDs was different. This indicates that the resolution of cis–trans isomers of these guest compounds did not depend on the kind or number of saccharide side chains of branched βCDs.

As shown in Figure 3, $(G_2)_3$-βCD mostly included the cis isomer of 18β-glycyrrhetinic acid among the three kinds of branched βCDs. However, the inclusion ability of the trans isomer of 18α-glycyrrhetinic acid was roughly the same in the three kinds of branched βCDs. The cis:trans ratios of isomers for glycyrrhetinic acid that include the 75 mM $(G_2)_3$-βCD, G_2-βCD, and G_1-βCD solutions were 75:25, 60:40, and 55:45, respectively. These results indicate that $(G_2)_3$-βCD appears to be a proper host compound in the inclusion of the cis

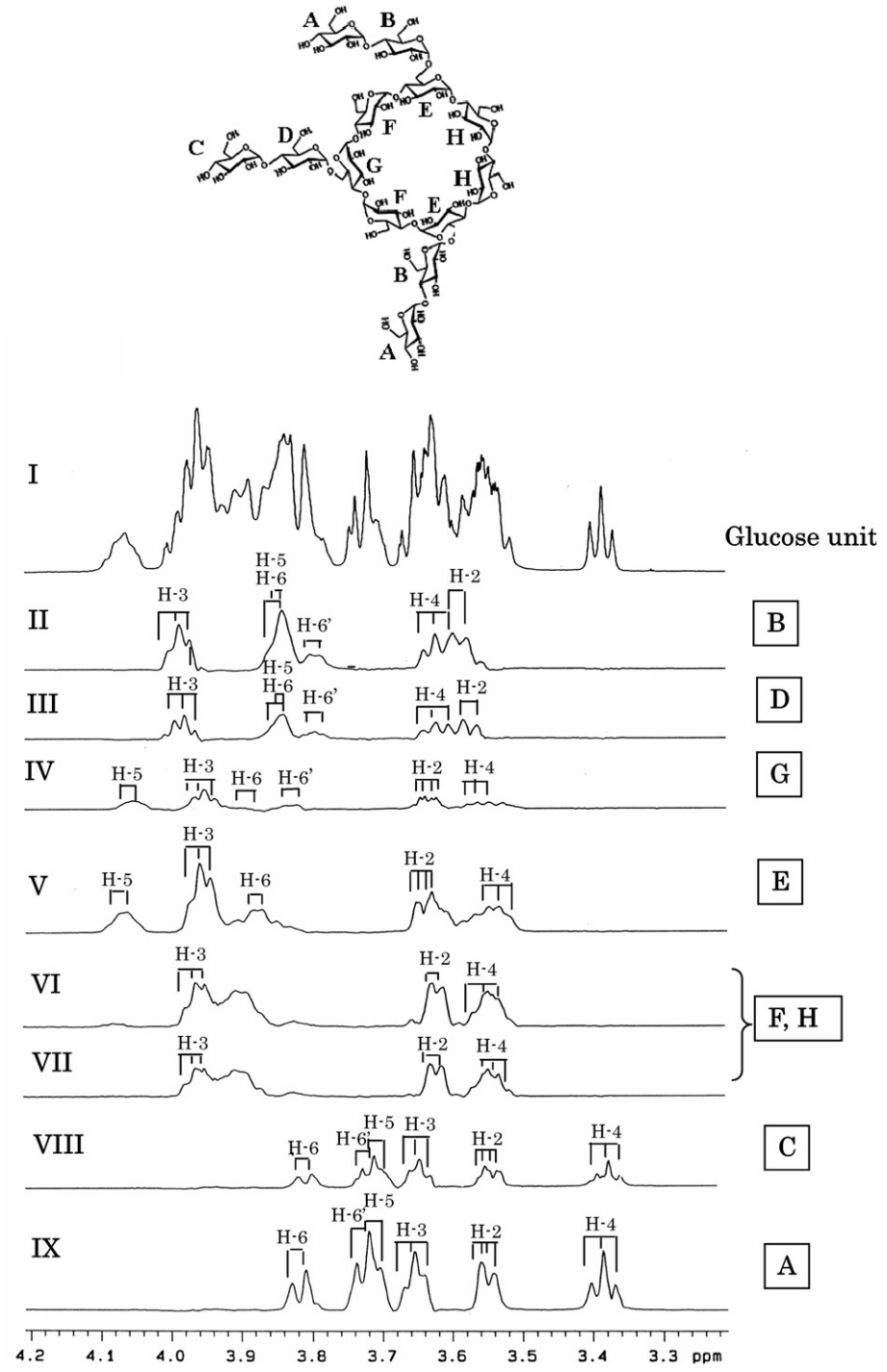

Figure 2. ^{1}H NMR spectrum (I) and corresponding TOCSY spectra (II–IX) of $(G_2)_3$-βCD in D_2O.

isomer of glycyrrhetinic acid. This ability of $(G_2)_3$-βCD increased with a rise in its concentration, and the solubility curves of glycyrrhetinic acid with $(G_2)_3$-βCD were characteristic of type Ap, particularly for the cis isomer. This suggests that the cis isomer of glycyrrhetinic acid is included, not only in the cavity of βCD, but also between the molecules of the branched βCDs. Thus $(G_2)_3$-βCD with a bulky side chain may provide a space suitable for glycyrrhetinic acid, especially for its cis isomer.

3.4. Biochemical properties of $(G_2)_3$-βCD

The effect of $(G_2)_3$-βCD on the cellular activity of cultured Caco-2 cells was examined using a phase-contrast microscope (Fig. 4) and by measuring intracellular LDH activity. $(G_2)_3$-βCD showed negligible cytotoxicity in Caco-2 cells up to at least 200 μM for 24 h. Similar results were obtained in G_1-βCD- or G_2-βCD-treated Caco-2 cells. Next we compared the hemolytic activity

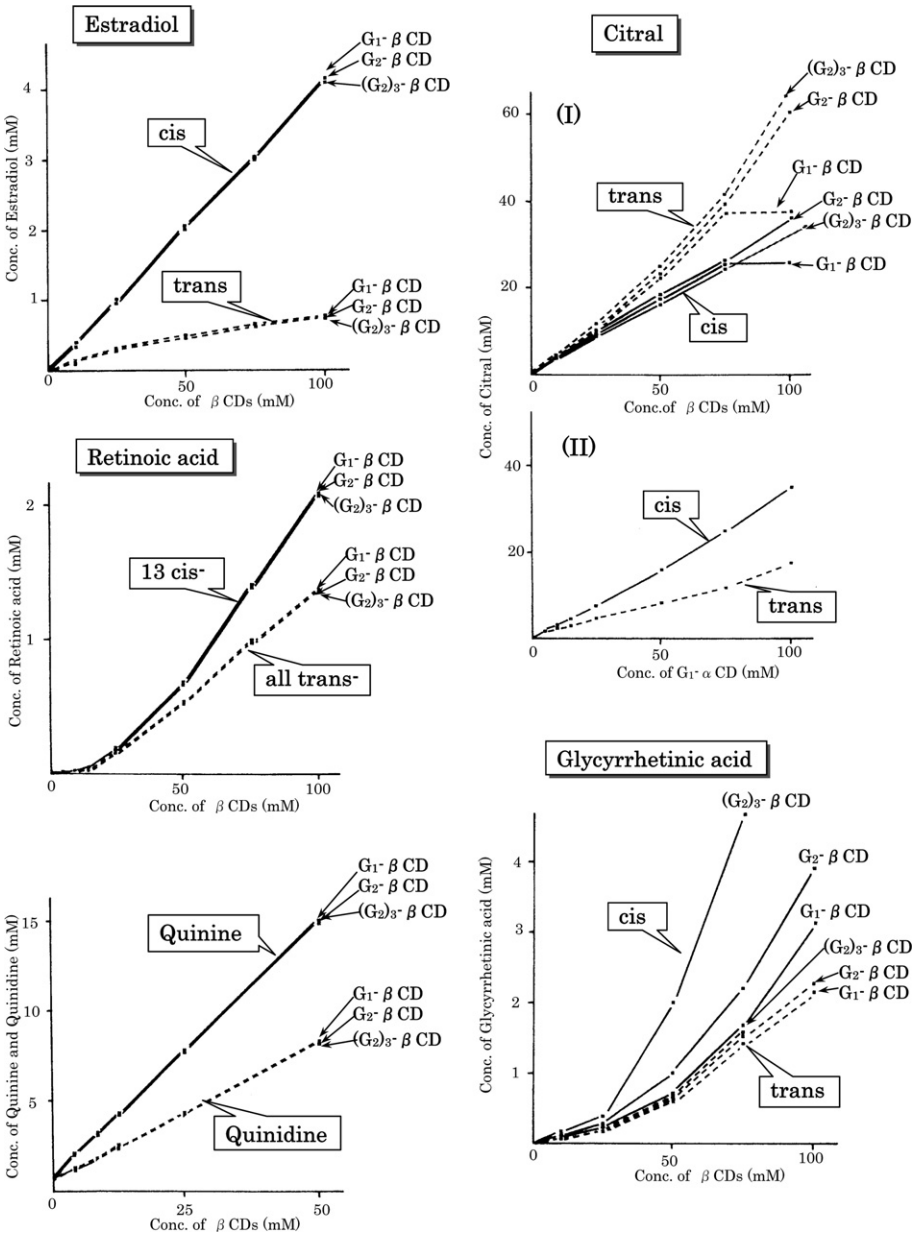

Figure 3. Phase-solubility diagrams of stereoisomers of branched CD systems in water at 30 °C.

of $(G_2)_3$-βCD toward human erythrocytes in isotonic phosphate buffer with that of G_1-βCD, G_2-βCD and the parent nonbranched βCD (Fig. 5). The hemolytic curve of $(G_2)_3$-βCD shifted markedly to higher concentrations, and the order of hemolytic activity was as follows: βCD > G_1-βCD > G_2-βCD ≫ $(G_2)_3$-βCD. The hemolytic effect of CD on human erythrocytes was profoundly correlated to the extraction of the various components of erythrocytes, cholesterol, phospholipids, and proteins from the membrane with CD, and the extraction of cholesterol with βCD may be of more importance than that of phospholipids or proteins.[12,13] Therefore, the inclusion complexation between cholesterol and the three kinds of branched βCDs was examined in order to elucidate the influence of the saccharide side chains on the hemolytic activity. The solubility of cholesterol in 30 mM G_1-βCD, G_2-βCD, and $(G_2)_3$-βCD was 2.2, 2.0, and 1.1 mM, respectively, and the ability to form a complex with cholesterol was enhanced in the order G_1-βCD > G_2-βCD ≫ $(G_2)_3$-βCD. This order is the same as that for the hemolytic activity in branched βCDs. The greatly depressed hemolytic activity of $(G_2)_3$-βCD may be due to three maltosyl side chains linked to the βCD ring that act to control the extraction of cholesterol from the erythrocyte membrane. The current results show that $(G_2)_3$-βCD as well as other branched βCDs could include cholesterol, indicating the possible application of βCD to modify

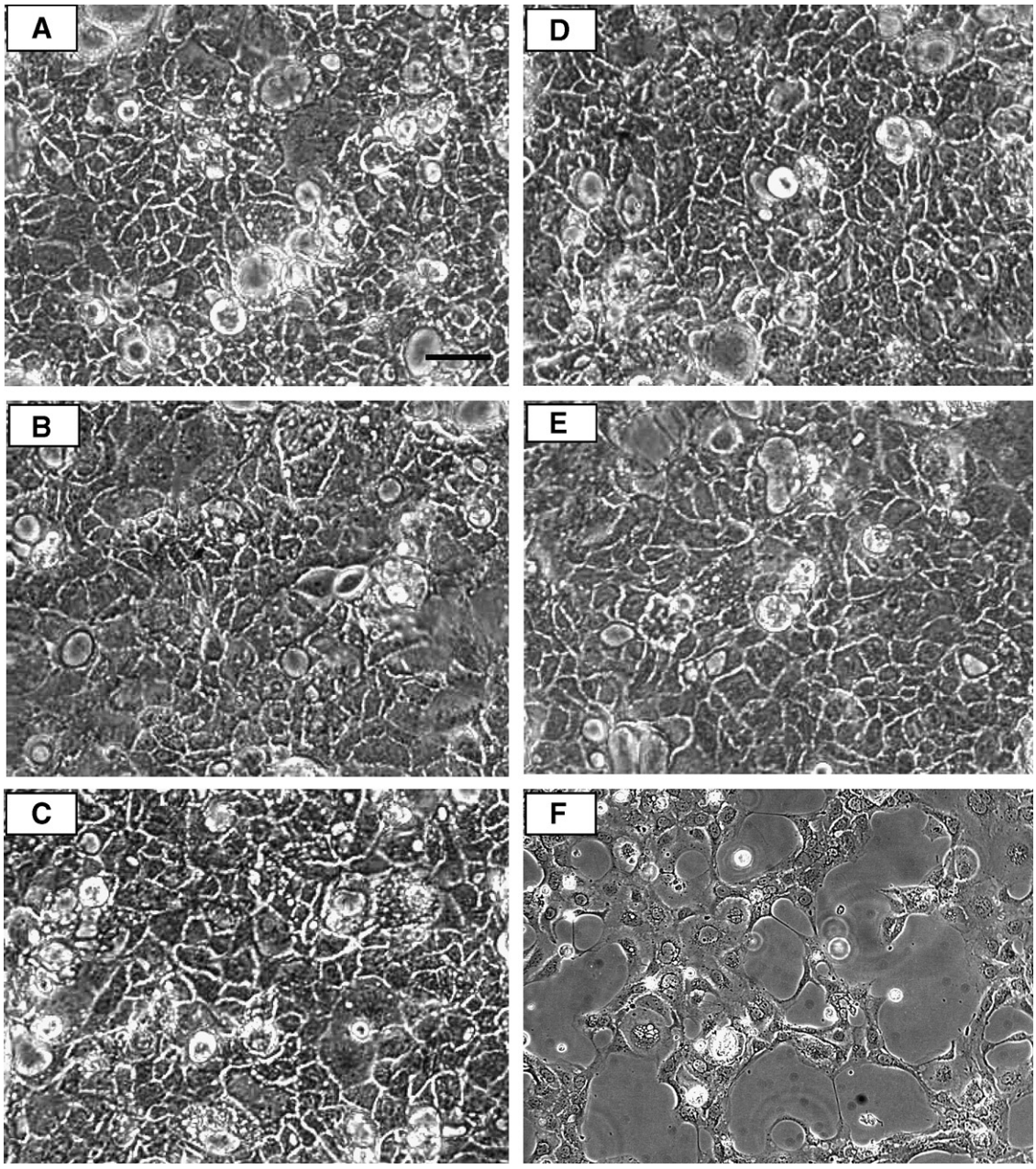

Figure 4. Morphology of Caco-2 cells incubated for 24 h with 200 μM each of the branched CDs. (A) Control; (B) $(G_2)_3$-βCD; (C) G_1-βCD; (D) G_2-βCD; (E) G_1-αCD; (F) Triton X-100 (25 μM).

membrane metabolism closely related to cholesterol. Cholesterol is known to be localized in microdomains of the membrane known as rafts.[14] Very recently, the extraction of cholesterol by methyl-βCD was reported to result in the stimulation of tissue factor procoagulant activity in HEK293 cells[15] in the disintegration of syntaxin 1 clusters that are involved in reducing the number of docked insulin granules necessary for insulin exocytosis[16] and play a role in the inhibition of the poliovirus entry process.[17] Cholesterol is abundant in lipid microdomains in membranes, which function in protein sorting, cell signaling, and synaptic transmission. On the other hand, methyl-βCD treatment altered the colocalization of Cav 2.1 with the proteins of the exocytic machinery and also impaired calcium influx in presynaptic terminals.[18] Therefore, $(G_2)_3$-βCD that has weak hemolytic activity and cytotoxic activity for Caco-2 cells may be effective in experiments on cellular events that involve cholesterol. This speculation is necessary for identification or removal of cholesterol fractions from erythrocytes or Caco-2 cell membranes. We have been conducting such experiments.

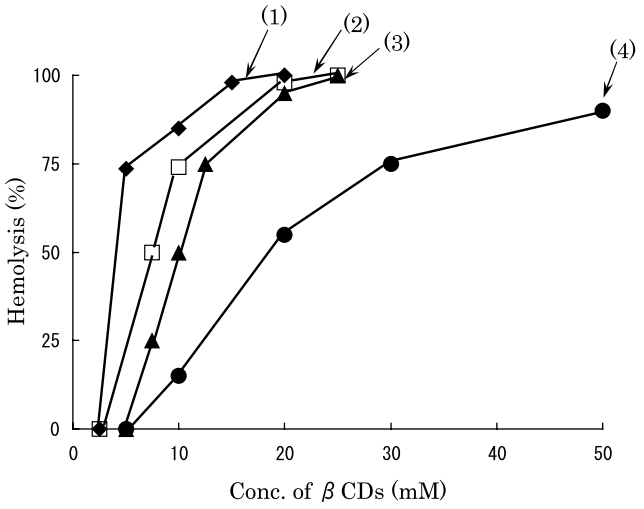

Figure 5. Hemolytic effects of βCDs on human erythrocytes in isotonic phosphate buffer (pH 7.4) at 37 °C. (1) βCD; (2) G_1-βCD; (3) G_2-βCD; (4) $(G_2)_3$-βCD.

4. Conclusions

The physicochemical and biological properties of a newly developed branched βCD, $(G_2)_3$-βCD, have been compared with those of the parent βCD, G_1-βCD, and G_2-βCD. The solubility of $(G_2)_3$-βCD is much higher than that of nonbranched βCD and about the same as that of the other monobranched βCDs. The formation of an inclusion complex of $(G_2)_3$-βCD with stereoisomers (estradiol, retinoic acid, quinine, citral, and glycyrrhetinic acid) depends on the cis–trans isomers of guest compounds. Notably, $(G_2)_3$-βCD is the most effective host compound in the cis–trans resolution of glycyrrhetinic acid. The hemolytic activity of $(G_2)_3$-βCD is weaker than that of βCD, G_1-βCD, or G_2-βCD, and the cytotoxicity of $(G_2)_3$-βCD in Caco-2 cells is negligible.

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