# Solubility and Mass and Nuclear Magnetic Resonance Spectroscopic Studies on Interaction of Cyclosporin A with Dimethyl-α- and -β-Cyclodextrins in Aqueous Solution

KOUZOU MIYAKE, FUMITOSHI HIRAYAMA, AND KANETO UEKAMA*

Contribution from *Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan.*

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**Abstract** □ The interaction of cyclosporin A (CsA) with dimethyl-α- and -β-cyclodextrins (DM-α-CyD and DM-β-CyD) was investigated by the solubility method, electrospray ionization mass spectrometry (ESI-MS) and $^1$H-nuclear magnetic resonance spectroscopy ($^1$H NMR). The extremely low solubility ($1.9 \times 10^{-5}$ M at 25 °C) of CsA in water was significantly improved by the complexation with DM-CyDs: for example, the solubility increased 87-fold in the presence of $5.0 \times 10^{-2}$ M DM-β-CyD. The phase solubility diagram of CsA/DM-CyD systems showed an Ap type and the stability constants (1060 $M^{-1}$ and 1050 $M^{-1}$, respectively) of the 1:1 CsA/DM-α-CyD and CsA/DM-β-CyD complexes were much higher than those of the 1:2 complexes (15 $M^{-1}$ and 21 $M^{-1}$, respectively). In ESI-MS spectra of the CsA/DM-β-CyD system, a new signal emerged at 1268 which corresponds to the 1:1 adduct of the di-ionized guest molecule with the host molecule. This signal intensity was significantly decreased by the addition of chlorpromazine (CPZ) which has a large stability constant (8800 $M^{-1}$) of the DM-β-CyD complex, whereas the signal corresponding to the CPZ/DM-β-CyD complex was little affected by the addition of CsA, indicating a competitive inclusion of CPZ and CsA within the host cavity. CsA gave many new peaks in the $^1$H NMR spectrum when the solvent was changed from chloroform to methanol/water, suggesting conformational diversity of CsA in polar solvents. Inspection of $^1$H-chemical shift changes and the two-dimensional rotating frame nuclear Overhauser effect (ROESY) spectra of the CsA/DM-CyD system suggested that the side chains of amino acids in CsA molecule take part in the inclusion within DM-CyDs, although there is seemingly no preference of particular amino acid residues. All the data obtained here suggested that CsA forms inclusion complexes with DM-α- and -β-CyDs in an aqueous medium and side chains of CsA are mainly involved in the inclusion.

## Introduction

Cyclodextrins (CyDs) are known to form inclusion complexes with various drug molecules in both solution and solid state, and their host/guest interaction has been investigated by using a number of chemical and physical techniques such as spectroscopies, potentiometric titration, kinetics, and the solubility method, etc.[1,2] For example, changes in ultraviolet (UV) and fluorescence spectra of a guest molecule due to the complexation are quantitatively analyzed to obtain the stability constant and stoichiometry of the complex and to gain insight into the inclusion structure in aqueous solution.[3] These physicochemical techniques are applicable on the interaction studies without difficulty when the guest or host is soluble in water and has some chromophores higher than about 210 nm. However, such application is limited when the guest is less soluble or insoluble in water and simultaneously has no chromophores in a molecule, because of difficulty in the preparation of the guest or host solution at suitable concentrations for UV or fluorescence measurements. Cyclosporin A (CsA) employed in this study falls under the category of drugs described above, i.e., the solubility in water being very low ($1.9 \times 10^{-5}$ M at 25 °C) and having no strong chromophore above 210 nm (Figure 1). Recently, mass spectrometry with the use of soft ionization techniques such as electrospray, ionspray, and fast atom bombardment has allowed the study of supramolecular complexes involving CyDs and crown ether,[4] etc., to go forward and has demonstrated capabilities for detecting intact noncovalent bound complexes including peptide/CyD complexes.[5−7] Furthermore, it is possible to use organic solvents such as methanol, acetonitrile, or glycerol as a matrix for solubilization of highly hydrophobic guest molecules.

CsA is an immunosuppressive drug and used in the treatment of autoimmune diseases and prevention of allograft rejection after organ transplantation. However, as has been pointed out, it is a drug exhibiting low oral bioavailability and quite large variability in absorption.[8,9] In a previous paper,[10] we reported that the low oral absorption and large variability of CsA is significantly improved by complexation with CyDs, particularly dimethyl-α- and -β-cyclodextrins (DM-α-CyD and DM-β-CyD, respectively) in rat model. In this continuing study, we investigated the complexation of CsA with DM-α-CyD and DM-β-CyD in water by means of the solubility method and electrospray mass spectrometry, both being useful for the interaction study of guests having low aqueous solubility. Furthermore, $^1$H-nuclear magnetic resonance (NMR) spectroscopic studies of the CsA/CyD system were carried out in order to gain insight into the inclusion mode of CsA, using a mixed solvent of water/methanol.

## Experimental Section

**Materials**—CsA was donated by Research Center of Shiseido Ltd. (Yokohama, Japan). DM-α-CyD and DM-β-CyD were supplied from Japan Maize Co. (Tokyo, Japan) and contained hexakis(2,6-di-*O*-methyl)- and heptakis(2,6-di-*O*-methyl)cyclodextrins (degrees of substitution of methyl group (DS) = 12 and 14, respectively) as a major component and the over-methylated homologue, 2,6-per-*O*-methyl-3$^A$-*O*-methyl-α- and -β-CyDs (DS = 13 and 15, respectively) as a minor component.[11] Because the separation of the minor component by recrystallization and column chromatography was significantly difficult, the DM-CyDs were used after recrystallization from methanol/water. The composition of the pure dimethyl derivative and the over-methylated homologue was determined to be 3:1 by fast atom bombardment mass spectrometry. All other chemicals and solvents were of analytical reagent grade, and deionized double-distilled water was used throughout the study.

* Corresponding author Tel and Fax: (+81-96)-371-4160; E-mail: uekama@gpo.kumamoto-u.ac. jp.



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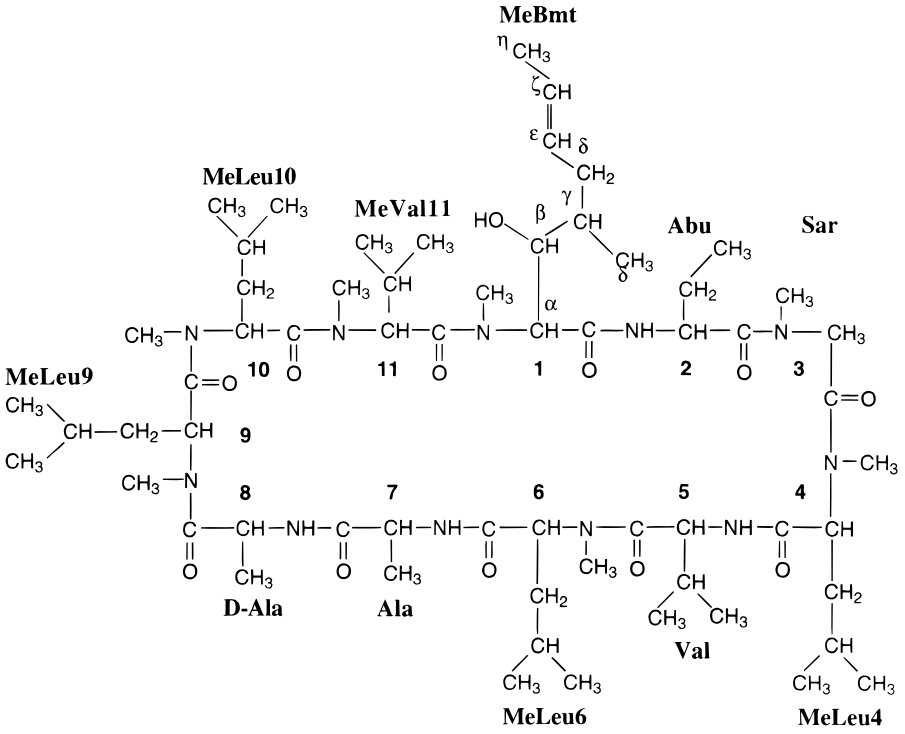


**Figure 1**—Structure of CsA.

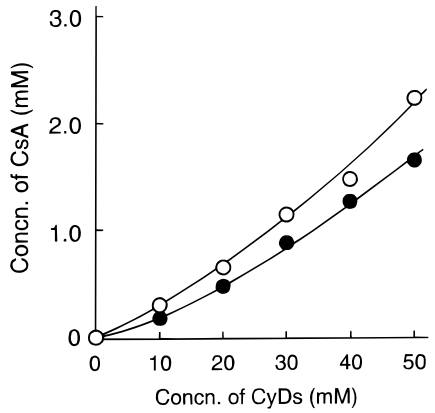


**Figure 2**—Phase solubility diagrams of CsA/DM-CyD systems in water at 25 °C. (○) DM-α-CyD, (●) DM-β-CyD.

**Solubility Measurements**—The solubility method was carried out according to the method of Higuchi and Connors.[12] The screw-capped vials containing CsA (5.0 mg) in excess amount in aqueous CyD solutions (5.0 mL) at various concentrations ($1.0-5.0 \times 10^{-2}$ M) were shaken at 25 °C. After equilibrium was attained (about 4 days), the solution was centrifuged at 800*g* force for 5 min, and the supernatant was filtered through a membrane filter (cellulose acetate membrane filter, ADVANTEC DISMIC 3CP045 (TOYO-Roshi), Tokyo, Japan) and analyzed for CsA by high-performance liquid chromatography (HPLC) under the following condition: a Hitachi L-6000 pump and an L-4000 UV detector (Tokyo, Japan) at 214 nm; a YMC ODS AM-312 column (6.0 mm × 150 mm i.d., Kyoto, Japan); a mobile phase of acetonitrile/methanol/water (60:30:10 v/v); a flow rate of 1.0 mL/min and a column temperature of 50 °C.

**Mass Spectrometries**—Electrospray ionization mass spectrometry (ESI-MS) was carried out on a Hitachi M-1200H LC/MS system (Tokyo, Japan) equipped with an electrospray ionization source. Ionization was achieved by applying 3.0 kV to the spray needle. The orifice potential was maintained at 70 V, and the spectra were recorded over a mass range of 300−2000 units in the positive-ion detection mode. Mass scale calibration was conducted using poly(ethylene glycol) 400, 600, and 1000, and agreement between observed and calculated *m/z* values was within 0.4. CsA or chlorpromazine (CPZ) was dissolved in water/methanol/acetic acid (47:47:6 v/v, pH ≈ 3) in the absence or presence of DM-CyDs. The final concentrations of CsA, CPZ, and DM-CyDs were $1.0 \times 10^{-3}$ M, $1.0 \times 10^{-3}$ M, and $2.0 \times 10^{-3}$ M, respectively. An aliquot (2.0 μL) of the solution was injected into a flow (0.1 mL/min) of water/methanol/acetic acid (47:47:6 v/v) and transferred to the electrospray source.

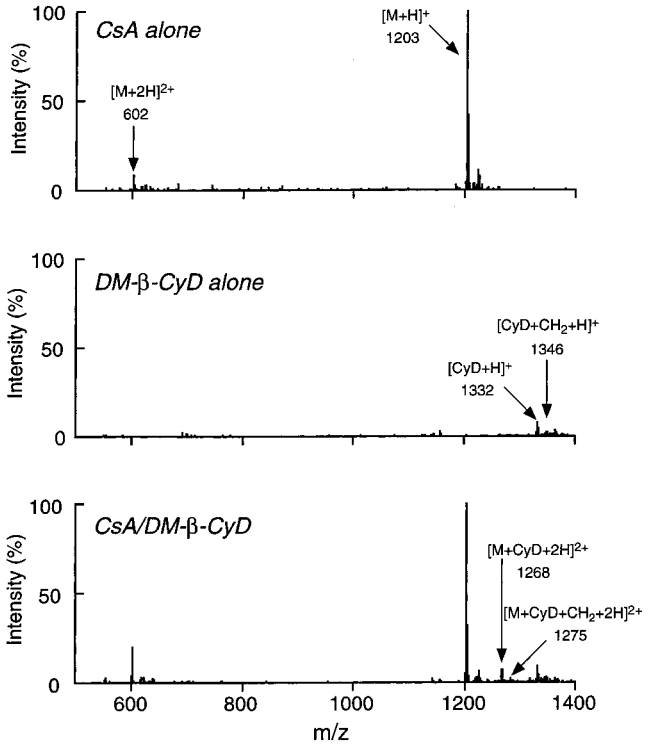


**Figure 3**—ESI mass spectra of CsA (1 mM) in positive ion mode in the absence and presence of DM-β-CyD (2 mM) in water/methanol/acetic acid (47/47/6 v/v).

**$^1$H NMR Spectroscopies**—$^1$H NMR spectra were obtained with a JEOL JNM-α 500 instrument (Tokyo, Japan) with a 5 mm inverse broad band probe, operating at 500 MHz and a sweep width of 10000 Hz, at 25 °C. Chemical shifts are given as parts

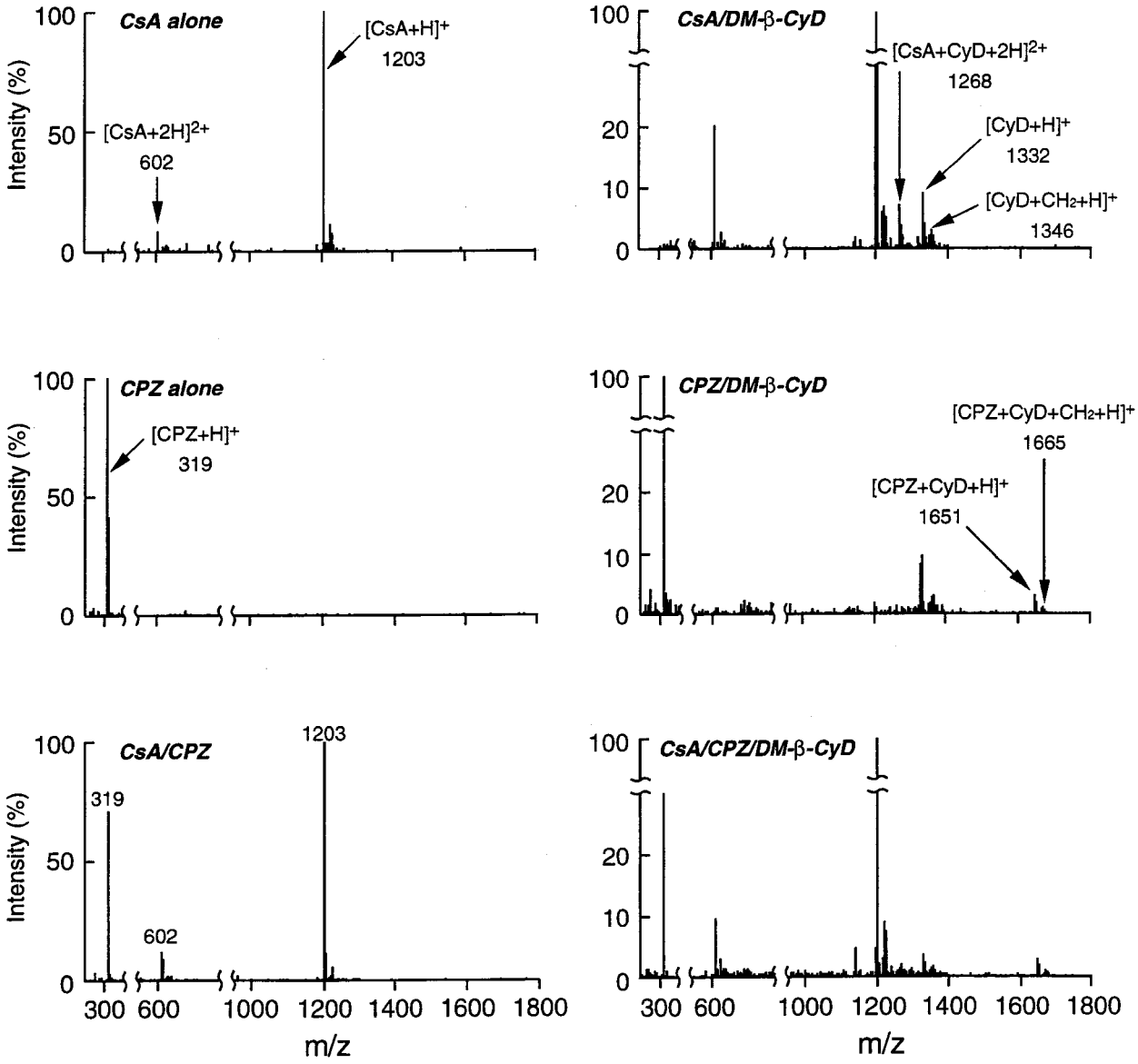


**Figure 4**—Effect of CPZ (1 mM) on ESI mass spectra of CsA (1 mM) in positive ion mode in the absence and presence of DM-$\beta$-CyD (2 mM) in water/methanol/acetic acid (47/47/6 v/v).

per million (ppm) downfield from that of tetramethylsilane, with an accuracy of 0.005 ppm. Phase-sensitive ROESY spectra were measured under the following conditions: sweep width, 5000 Hz; carrier frequency, 2.95 ppm; spin-lock field, 4 kHz; mixing time, 200 ms; 32 scans for each $t_1$ point with a pulse delay of 1.5 s; data matrix, 2 × 256 × 1K. CsA and DM-CyDs were dissolved at concentrations of $4.0 \times 10^{-3}$ M and $3.0 \times 10^{-2}$ M, respectively, in 50% v/v deuterated methanol ($CD_3OD$)/deuterium oxide ($D_2O$).

## Results and Discussion

The solubility method is useful for investigating inclusion complexation of poorly water-soluble drugs with CyDs in water, because it gives not only the solubilizing ability of host molecules but also the stability constant of complexes by analyzing the solubility curve.[2] Figure 2 shows the phase solubility diagrams of CsA for the DM-α-CyD and DM-$\beta$-CyD complexes in water. The extremely low solubility ($1.9 \times 10^{-5}$ M at 25 °C) of CsA was markedly increased by the complexation with DM-CyDs: i.e., the solubility of CsA increased 87-fold in the presence of $5.0 \times 10^{-2}$ M DM-$\beta$-CyD. The phase solubility diagrams showed an Ap type, as defined by Higuchi and Connors,[12] indicating the formation of high order complexes. Therefore, these upward curvatures were analyzed, by the method of Kristiansen and Higuchi using the iteration method,[13] to obtain the stability constants ($K_{1:1}$ and $K_{1:2}$) of 1:1 and 1:2 (guest:host) complexes. The $K_{1:1}$ and $K_{1:2}$ were determined to be 1060 $M^{-1}$ and 15 $M^{-1}$ for the DM-α-CyD complexes and 1050 $M^{-1}$ and 21 $M^{-1}$ for the DM-$\beta$-CyD complexes, respectively. The $K_{1:1}$ values were much higher than the $K_{1:2}$ values, suggesting that CsA forms predominantly the 1:1 complex with DM-CyDs in water.

As described in the Introduction section, the extremely low solubility of CsA together with the absence of chromophores above 210 nm in the molecule make it difficult to utilize UV, circular dichroism and fluorescence spectroscopic techniques for the investigation of CyD interaction with CsA in water. On the other hand, mass spectrometry has proved to be useful as one of the promising tools for detecting CyD complexes with peptides such as bradykinin (consisting of 9 amino acids),[5] synthetic Alzheimer amyloid (consisting of 40 amino acids),[6] and insulin (consisting of 51 amino acids),[7] etc. Therefore, the complexation of CsA (consisting of 11 amino acids) with DM-$\beta$-CyD was investigated by ESI-MS, using a water/organic solvent. Figure

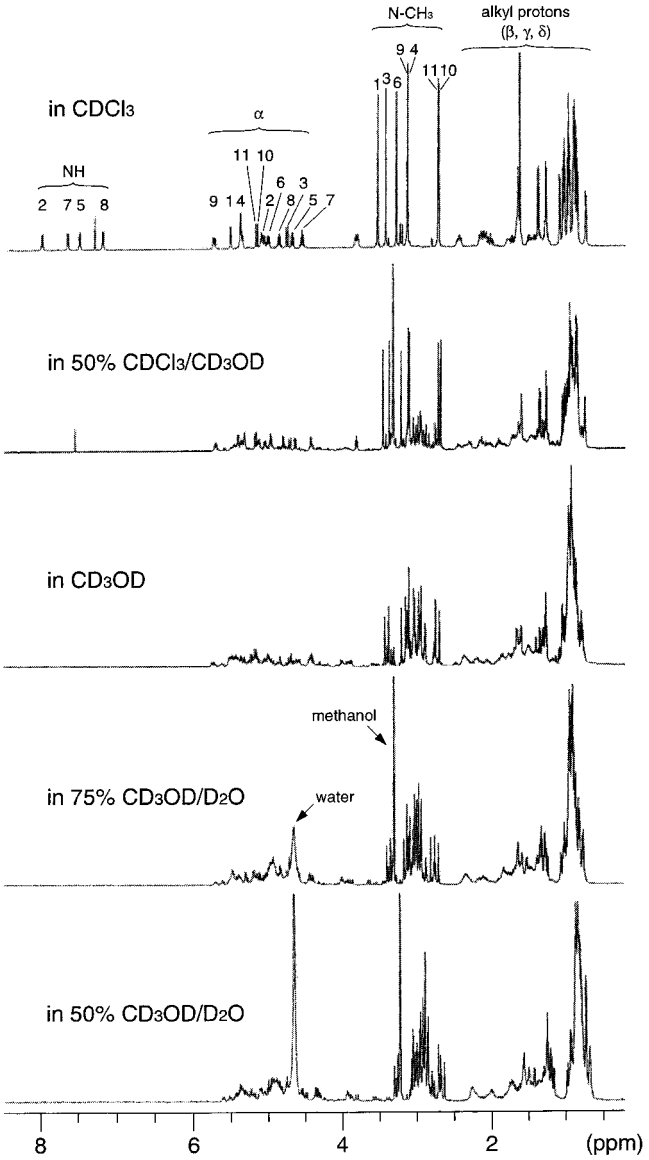


**Figure 5**—$^1$H NMR spectra of CsA (4 mM) in $CDCl_3/CD_3OD/D_2O$ solutions.

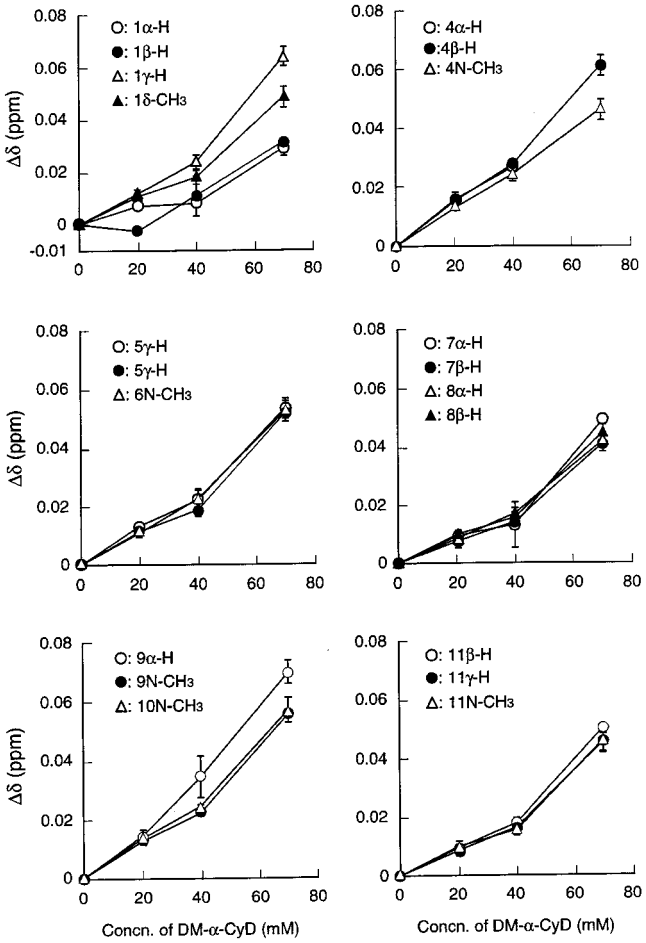


**Figure 6**—$^1$H NMR chemical shift displacements ($\Delta\delta$) of CsA (4 mM) as a function of DM-α-CyD concentrations in 50% v/v $CD_3OD/D_2O$. $\Delta\delta = \delta_{\text{in the presence of CyD}} - \delta_{\text{in the absence of CyD}}$.

3 shows ESI mass spectra of CsA alone, DM-$\beta$-CyD alone and the CsA/DM-$\beta$-CyD mixture in a molar ratio of 1:2 which were dissolved in water/methanol/acetic acid (47:47:6 v/v). CsA gave two signals at 1203 and 602 corresponding to $[M + H]^+$ and $[M + 2H]^{2+}$ ions and DM-$\beta$-CyD gave two signals at 1332 and 1346 corresponding to $[CyD + H]^+$ and $[CyD + CH_2 + H]^+$ of the pure DM-$\beta$-CyD and the over-methylated derivative, respectively, in a peak ratio of about 3:1. By the addition of DM-$\beta$-CyD (Figure 3), a new signal was observed at 1268 which corresponds to the 1:1 adduct of di-ionized CsA with DM-$\beta$-CyD, $[M + CyD + 2H]^{2+}$, whereas no signal corresponding to the adducts was observed by the addition of glucose and maltose. The peak of the 1:1 adduct of CsA with the over-methylated homologue was also observed at 1275, although its intensity was significantly weak and fell within a noise range. These results suggest that CsA interacts with DM-$\beta$-CyD, presumably involving hydrophobic groups of CsA amino acids in the interaction.

The inclusion of a guest molecule within the CyD cavity is inhibited by the addition of a second guest molecule, the extent of the inhibition being dependent on the stability constant of each complex. This competition is a characteristic feature of host/guest inclusion complexations involving CyDs and crown ethers, etc. Therefore, the competitive inclusion studies were conducted by ESI-MS in order to confirm the complexation of CsA with DM-$\beta$-CyD. CPZ was chosen as a competitive guest molecule because of the larger stability constant (8800 $M^{-1}$) of its DM-$\beta$-CyD complex,[14] compared with that (1050 $M^{-1}$) of the 1:1 CsA/DM-$\beta$-CyD complex. The relative magnitude of the constants was assumed not to change in the mixed solvent.[15] Figure 4 shows the effect of CPZ on the ESI mass spectra of CsA/DM-$\beta$-CyD complex. The CPZ alone gave a peak at 319 due to $[M + H]^+$, and the mass spectrum of the CsA/CPZ system was simply the superposition of each component, indicating no interaction between CsA and CPZ. The CPZ/DM-$\beta$-CyD system gave two new peaks at 1651 and 1665 corresponding to the 1:1 adducts of CPZ/DM-$\beta$-CyD, $[CPZ + CyD + H]^+$, and CPZ/the over-methylated homologue, $[CPZ + CyD + CH_2 + H]^+$, respectively, in a peak ratio of about 3:1. In the case of the ternary CsA/CPZ/DM-$\beta$-CyD system, the peak intensity of 1268 corresponding to the CsA/DM-$\beta$-CyD markedly decreased, whereas the intensity of the 1651 and 1665 peaks corresponding to CPZ/DM-$\beta$-CyD complex was almost constant. For example, the peak ratio of the CsA complex to the free drug ($I_{\text{complex}}/I_{\text{drug}}$) decreased from 0.064 (binary CsA/DM-$\beta$-CyD system) to 0.027 (ternary CsA/CPZ/DM-$\beta$-CyD system), whereas that of the CPZ complex was almost constant (0.039 and 0.040 for the binary and ternary systems, respectively). These results apparently indicate that CsA forms the complex with DM-$\beta$-CyD and the equilibrium is competitively inhibited by the addition of CPZ.

To gain insight into the inclusion mode of CsA with DM-CyDs, $^1$H NMR spectroscopic studies were carried out.

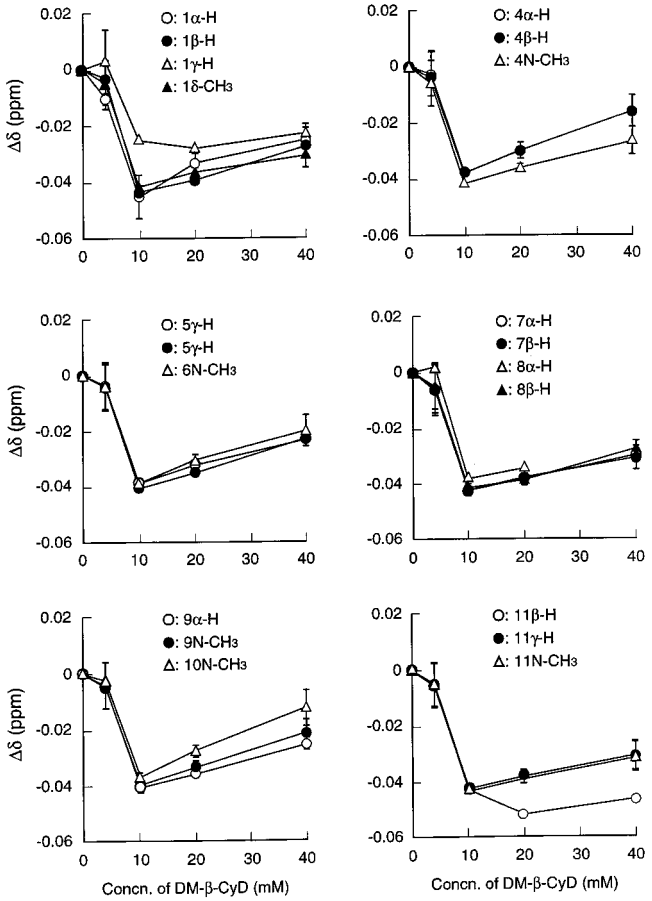


**Figure 7**—$^1$H NMR chemical shift displacements ($\Delta\delta$) of CsA (4 mM) as a function of DM-$\beta$-CyD concentrations in 50% v/v $CD_3OD/D_2O$. $\Delta\delta = \delta_{\text{in the presence of CyD}} - \delta_{\text{in the absence of CyD}}$.

Figure 5 shows $^1$H NMR spectra of CsA in chloroform, 50% v/v methanol/chloroform, methanol, 75% v/v methanol/water, and 50% v/v methanol/water. It was difficult to obtain high-resolution spectra of CsA below 50% methanol concentration, because of precipitation of the substrate. The $^1$H NMR signals of CsA in chloroform were successfully assigned according to the report of Kessler et al.[16] However, many new peaks appeared with severe splitting in methanol/chloroform solution, and this tendency became significant when polarity of solvents increased. For example, by changing the solvent from chloroform to 50% v/v methanol/water, the quintet (4.52 ppm) of the α proton of Ala7 (7α-H, Figure 1) changed to the multiplet (4.41 ppm), and the double-doublet (5.70 ppm) of the α proton of MeLeu9 (9α-H) changed to at least two double-doublets (5.60 and 5.52 ppm). Furthermore, the peak area of 5.60 ppm increased compared with that of 5.52 ppm. These results were in agreement of those reported by Kessler et al.,[16] Ko et al.[17] and Hasumi et al.,[18] suggesting that several conformational isomers of CsA may exist in polar solvents and they exchange slowly on the NMR time scale, whereas in chloroform CsA exists in one conformation. Because of the conformational diversity of CsA in polar solvents, it was difficult to assign unambiguously all $^1$H-signals of CsA protons, but some of them in 50% v/v methanol/water solution could be assigned as follows: [1α-H (5.30 ppm), 1$\beta$-H (4.02 ppm), 1$\gamma$-H (2.33 ppm), and 1$\delta$-$CH_3$ (0.76 ppm) for MeBmt], [4α-H (5.18 ppm), 4$\beta$-H (1.64 ppm), and 4N-$CH_3$ (3.08 ppm) for MeLeu4], [5$\gamma$-H (1.01 and 0.81 ppm) for Val5], [6N-$CH_3$ (3.15 ppm) for MeLeu6], [7α-H (4.41 ppm) and 7$\beta$-H (1.30 ppm) for Ala7], [8α-H (4.83 ppm) and 8$\beta$-H (1.28 ppm) for D-Ala8], [9α-H (5.60 ppm) and 9N-$CH_3$ (3.13 ppm) for MeLeu9], [10N-$CH_3$ (2.71 ppm) for MeLeu10]

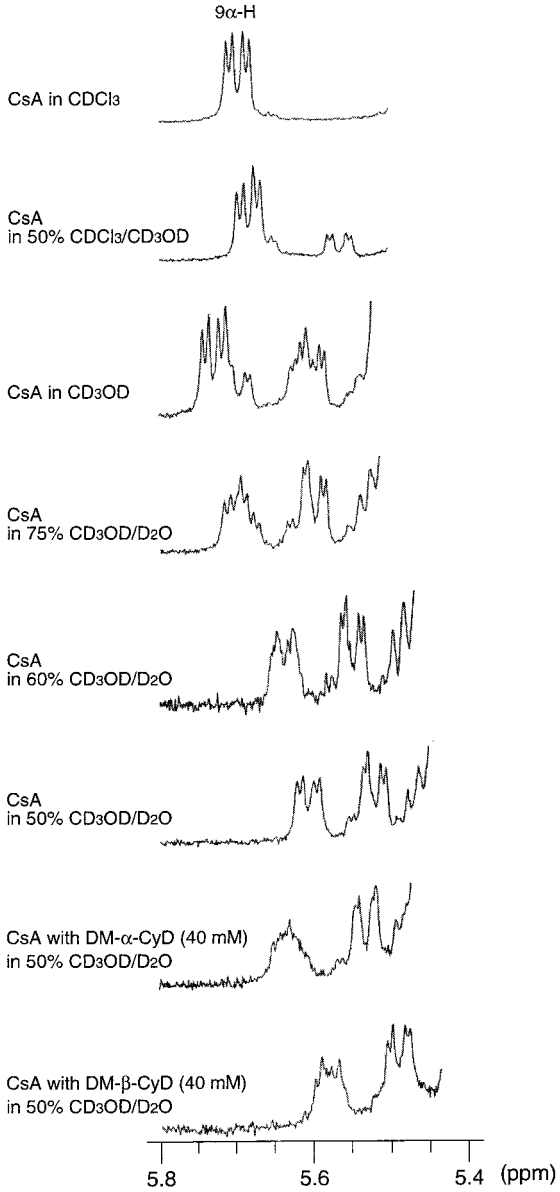


**Figure 8**—Changes of α proton peaks of MeLeu9 by the addition of DM-CyDs (40 mM) in 50% v/v $CD_3OD/D_2O$.

and [11$\beta$-H (2.11 ppm), 11$\gamma$-H (1.24 ppm) and 11N-$CH_3$ (2.76 ppm) for MeVal11].

Figures 6 and 7 show the effect of DM-α-CyD and DM-$\beta$-CyD, respectively, on the chemical shift of the assigned protons of CsA in 50% v/v methanol/water solution. The chemical shift of CsA protons shifted downfield by the addition of DM-α-CyD and magnitude of the change decreased generally in the order of (MeBmt ≈ MeLeu9 ≈ MeLeu10 ≈ MeLeu4) > (MeLeu6 ≈ Val5) > (MeVal11 ≈ Ala7 ≈ Ala8). These results suggest that DM-α-CyD includes preferably the hydrophobic side chains of amino acids such as the butenylthreonine and leucine, although there is seemingly no preference of the inclusion between MeBmt and four leucine residues in CsA molecule. On the other hand, DM-$\beta$-CyD system showed the upfield shift of CsA protons at lower concentrations of the host, while the shift was directed gradually toward downfield at higher concentrations, suggesting high order complexation. Similar biphasic change of $^1$H NMR chemical shift was observed for the interaction of an antiulcer chalcone derivative with $\beta$-CyD.[19] The DM-$\beta$-CyD-induced shifts were uniform for all protons of CsA, indicating that the host having the larger cavity includes loosely a side chain of CsA without

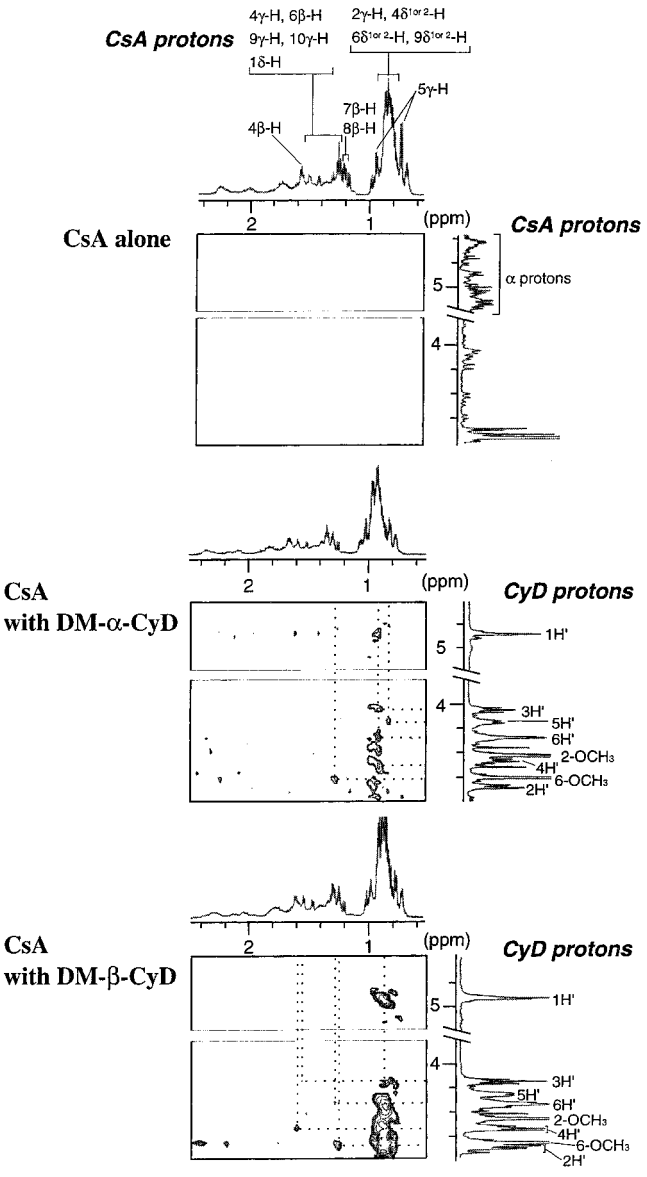


**Figure 9**—Partial contour plots of ROESY spectra of CsA (4 mM)/DM-CyDs (30 mM) systems in 50% v/v $CD_3OD/D_2O$.

a preferential inclusion of one particular amino acid. Figure 8 shows the changes of the α proton peaks of MeLeu9 (9α-H) due to the addition of DM-CyDs. As described above, the double-doublet (5.70 ppm) of 9α-H proton changed to at least two double-doublets (5.60 and 5.52 ppm), and the ratio of peak areas ($A_{5.52}/A_{5.60}$) at 5.60 and 5.52 ppm increased from 0 to 0.20, 0.69, 0.93, 0.95, and 1.00 by changing the solvent from chloroform to 50% v/v chloroform/methanol, methanol, and 75%, 60% and 50% v/v methanol/water solutions, respectively. The ratio of the peak area in 50% v/v methanol/water solution was insignificantly altered by the addition of DM-CyDs (0.91 and 1.06 in the presence of $4.0 \times 10^{-2}$ M DM-α-CyD and $4.0 \times 10^{-2}$ M DM-β-CyD, respectively). These results suggest that DM-CyDs do not interact so strongly with CsA as to alter the backbone structure of the guest, while they interact rather weakly with the side chains of CsA. The partial inclusion mode was further confirmed by ROESY spectra,[20,21] as described below. Figure 9 shows ROESY spectra of CsA in the absence and presence of DM-CyDs in 50% v/v methanol/water solution. DM-CyDs gave one $^1$H-signal at 5.1 ppm and other signals between 3−4 ppm, and all signals were assigned according to the report of Chujo et al.[22] The signals of CsA can be generally classified to three groups, i.e., 4−6 ppm for α protons of amino acids, 2.5−3.5 ppm for *N*-methyl protons, and 0−2.4 ppm for protons of alkyl side chains. By the addition of DM-CyDs, new ROESY cross-peaks emerged between CyD protons and CsA alkyl side chain protons, whereas no cross-peaks were observed for *N*-methyl protons and α protons of CsA amino acids. These cross-peaks between DM-CyDs and the alkyl side chains were not observed in the absence of DM-CyDs. Furthermore, not only the inside protons of CyD (H3 and H5) but also the outside protons (H2 and H4) were involved in the cross-peaks. These results suggest that a hydrophobic side chain of CsA is included within the DM-CyD cavity, and other side chains interact with or fold on the outside of CyDs.

In conclusion, the data obtained from the solubility method and mass and NMR spectroscopic studies indicate that CsA interacts with DM-CyDs in aqueous solution, and the side chains of CsA may be involved in the inclusion. Furthermore, there is seemingly no preference of the inclusion of a particular side chain of CsA amino acid, but several complexes with different inclusion structures may exist simultaneously in solution, i.e., the so-called multimodal inclusion occurs in the interaction of CsA with DM-CyDs.

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