Interaction of Heptakis (2,3,6-Tri-*O*-methyl)-β-cyclodextrin with Cholesterol in Aqueous Solution

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The interaction of cholesterol with heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin (TOM-β-CyD) was investigated in water using solubility method. It was found that TOM-β-CyD forms two kinds of soluble complexes, with molar ratios of 1 : 1 and 1 : 2 (cholesterol : TOM-β-CyD). The thermodynamic parameters for 1 : 1 and 1 : 2 complex formation of cholesterol with TOM-β-CyD were: $\Delta G^0_{1:1}=-11.0$ kJ/mol at 25 °C ($K_{1:1}=7.70\times10\ \mathrm{M}^{-1}$); $\Delta H^0_{1:1}=-1.28$ kJ/mol; $T\Delta S^0_{1:1}=9.48$ kJ/mol; $\Delta G^0_{1:2}=-27.8$ kJ/mol at 25 °C ($K_{1:2}=7.55\times10^4\ \mathrm{M}^{-1}$); $\Delta H^0_{1:2}=-0.57$ kJ/mol; $T\Delta S^0_{1:1}=27.3$ kJ/mol. The formation of the 1 : 2 complex occurred much more easily than that of the 1 : 1 complex. The driving force for 1 : 1 and 1 : 2 complex formation was suggested to be exclusively hydrophobic interaction. Based on the measurements of proton nuclear magnetic resonance spectra and studies with Corey–Pauling–Koltun atomic models, the probable structures of the 1 : 2 complex were estimated. In addition, the interaction of TOM-β-CyD with cholesterol was compared with that of heptakis (2,6-di-*O*-methyl)-β-CyD (DOM-β-CyD). The interaction of TOM-β-CyD is more hydrophobic than that of DOM-β-CyD, and the life time of the complexed TOM-β-CyD is sufficiently long to give separated signals, at the NMR time scale, which differs from that of complexed DOM-β-CyD.

Key words 2,3,6-tri-*O*-methyl (TOM)-β-cyclodextrin; cholesterol; interaction; solubility method; thermodynamic parameter; ^{1}H-NMR

Heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin (TOM-β-CyD) has recently been used to alter the membrane cholesterol content. For example, using TOM-β-CyD, cholesterol of the myometrial plasma was selectively depleted from the myometrial plasma membrane.[1] TOM-β-CyD has also been used to clarify whether membrane proteins exist at an association with specialized microdomains called lipid rafts by depleting cholesterol contained in them.[2] It is clear that cholesterol forms a soluble complex with TOM-β-CyD in aqueous solution. Although the interactions of cholesterol with TOM-β-CyD are particularly important, to the best of our knowledge, no studies on the thermodynamic parameters of the interaction in aqueous solution have been reported.

We therefore investigated the interactions of cholesterol with TOM-β-CyD in aqueous solution quantitatively using the solubility measurement method now common in the pharmaceutical field, measurement of proton nuclear magnetic resonance (^{1}H-NMR), and Corey–Pauling–Koltum (CPK) atomic models. As cholesterol exists in a buried form in the phospholipid bilayer in biomembranes[3,4] the interactions of cholesterol with phospholipids are suggested to be based on the hydrophobic interaction. On the other hand, TOM-β-CyD has a deeper and more hydrophobic cavity than the parent cyclodextrin.[5] Therefore, it is suggested that the interaction of cholesterol with TOM-β-CyD is based on the hydrophobic interaction in aqueous solution. In a previous paper,[6] we reported that heptakis (2,6-di-*O*-methyl)-β-cyclodextrin (DOM-β-CyD), which like TOM-β-CyD has a deeper and more hydrophobic cavity than the parent cyclodextrin, forms soluble complexes with cholesterol in aqueous solution.

However, the inclusion behaviors of these hydrophobic cyclodextrins differ in general. For example, DOM-β-CyD penetrates the matrix of liposomes and extracts phospholipid from liposomes to form a soluble complex, whereas only a small amount of TOM-β-CyD penetrates the matrix of liposomes to remain there and therefore, TOM-β-CyD has very week ability to form a soluble complex with phospholipids.[7,8] For these reasons, TOM-β-CyD is used to alter membrane cholesterol content as mentioned above, although DOM-β-CyD is not used for this purpose. As another example, the formation constant of the complex between TOM-β-CyD[9] and 8-anilino-1-naphthalenesulfonate (ANS) is smaller than that between DOM-β-CyD[10] and ANS. Furthermore, each incluson mode is different in the TOM-β-CyD-ANS complex and DOM-β-CyD-ANS complex. Therefore the comparison of the interaction between TOM-β-CyD and cholesterol with that between DOM-β-CyD and cholesterol in present paper would be informative and pertinent.

Experimental

Materials TOM-β-CyD purchased from Nacalai Tesque Co. (Kyoto, Japan) was used after recrystallization from water and dried for 12 h at 110 °C in a vacuum before use. Cholesterol purchased from Sigma (St. Louis, MO, U.S.A.) was used without further purification. Water purified with Milli-Q Labo (>18 MΩ·cm) was used throughout the experiments.

Solubility Method: Solubility of Cholesterol in the Presence of TOM-β-CyD Aliquots (5.0 ml) of TOM-β-CyD aqueous solution at the appropriate concentration and excess cholesterol were placed in 20 ml L-type test tubes and the tubes were sealed. The test tubes were kept at 10, 25, 37 and 45±0.05 °C, respectively, with shaking, for 1 week until solubility equilibrium was achieved. Then the solution was filtered through a membrane filter (0.45 μm, Steradisc 13, KURABO) (Osaka, Japan) and cholesterol in the filtrate was determined using the Free Cholesterol E-Test Wako supplied by Wako Pure Chemical Industries, Ltd. (Osaka , Japan).

^{1}H-NMR Spectra ^{1}H-NMR measurements were recorded on a Varian VXR-500 spectrometer and INOVA-Unity 500 in deuterium oxide. Tetramethylsilane (TMS) was used as an external reference in deuterium oxide. Two-dimensional rotating frame nuclear overhauser effect spectroscopy (ROESY) experiments were performed in the phase-sensitive mode using the State–Haberkorn method. A spinlock mixing pulse of 200 ms was used.

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Results

Since soluble complex formation between cholesterol and TOM-β-CyD was strongly suspected, the interaction between them was investigated by making a phase solubility diagram, based on the solubility method. The results at 10 and 37 °C are shown in Fig. 1. Phase solubility diagrams of cholesterol with TOM-β-CyD are of the Ap type. Therefore it is presumed that cholesterol forms two types of complex with TOM-β-CyD, with molar ratios of 1 : 1 and 1 : 2, respectively, in aqueous solution. It was also found that the concentration of cholesterol solubilized by TOM-β-CyD increases with increasing temperature. These phase solubility diagrams are very similar to that of DOM-β-CyD reported previously.[6)]

The formation constants $K_{1:1}$ and $K_{1:2}$ defined by following Eqs. 3 and 4 were determined:

$$\text{Cho}+\text{CD}\rightleftarrows\text{Cho-CD} \tag{1}$$

$$\text{Cho-CD}+\text{CD}\rightleftarrows\text{Cho-CD}_2 \tag{2}$$

where [Cho] and [CD] are concentrations of free cholesterol and TOM-β-CyD, respectively, and [Cho-CD] and [Cho-CD_2] are concentrations of complexes with

$$K_{1:1}=\frac{[\text{Cho-CD}]}{[\text{Cho}][\text{CD}]} \tag{3}$$

$$K_{1:2}=\frac{[\text{Cho-CD}_2]}{[\text{Cho-CD}][\text{CD}]} \tag{4}$$

$$[\text{Cho}]_t=[\text{Cho}]+[\text{Cho-CD}]+[\text{Cho-CD}_2] \tag{5}$$

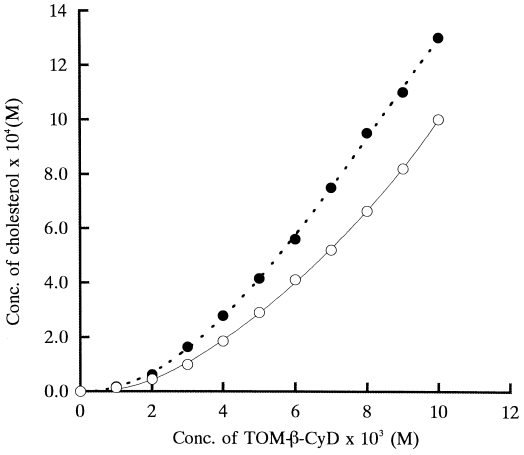

Fig. 1. Phase Solubility Diagrams of Cholesterol with TOM-β-CyD in Water

●: at 37 °C, ○: at 10 °C.

$$[\text{CD}]_t=[\text{CD}]+[\text{Cho-CD}]+2[\text{Cho-CD}_2] \tag{6}$$

molar ratios of 1 : 1 and 1 : 2, respectively. $[\text{Cho}]_t$ and $[\text{CD}]_t$ represent total concentrations of cholesterol and TOM-β-CD, respectively.

The combination of Eqs. 3—6 gave Eq. 7.

$$[\text{Cho}]_t=\{4K_{1:1}K_{1:2}[\text{Cho}][\text{CD}]_t-K_{1:1}^2[\text{Cho}]^2+1\}/\{8K_{1:1}K_{1:2}[\text{Cho}]\}-\{8K_{1:1}^3K_{1:2}[\text{CD}]_t[\text{Cho}]^3+8K_{1:1}K_{1:2}[\text{Cho}][\text{CD}]_t+K_{1:1}^4[\text{Cho}]^4-2K_{1:1}^2[\text{Cho}]^2+1-16K_{1:1}^2K_{1:2}[\text{Cho}]^2[\text{Cho}]_t\}^{1/2}/\{8K_{1:1}K_{1:2}[\text{Cho}]\} \tag{7}$$

[Cho], the free concentration of cholesterol equal to the solubility of cholesterol in water is required for the application of Eq. 7 and is available from our previous paper.[6)] The solubilities of cholesterol in water are: 2.6×10^{-6} M, 3.4×10^{-6} M, 4.5×10^{-6} M, and 5.7×10^{-6} M, at 10, 25, 37, and 45 °C, respectively. Formation constants $K_{1:1}$ and $K_{1:2}$ can be estimated from Eq. 7 using the nonlinear least-squares program MULTI.[11)] The values obtained at 10, 25, 37, and 45 °C are shown in Table 1. AIC values were between −214.1 and −214.3. Formation constant $K_{1:1}$ of the 1 : 1 complex was 77.0 M^{-1} and formation constant $K_{1:2}$ of the 1 : 2 complex was 7.55×10^4 M^{-1} at 25 °C, respectively. Thus the $K_{1:1}$ of TOM-β-CyD is smaller than that of DOM-β-CyD, but $K_{1:2}$ of TOM-β-CyD is more larger than that of DOM-β-CyD, resulting in closely similar solubility diagrams.

It was found that the complex with a molar ratio of 1 : 2 (cholesterol : CyD) is formed more easily than that with molar ratio of 1 : 1. The van't Hoff plots for the 1 : 1 and 1 : 2 complexes obtained by plotting $\log K$ against the reciprocal of the absolute temperature are shown in Fig. 2. The change in enthalpy (ΔH^0) accompanying the complexation was determined from the slope of the straight line obtained. The change in entropy (ΔS^0) also was obtained in the usual way. Each value obtained is given in Table 1. The change in enthalpy $\Delta H^0_{1:1}$ of the 1 : 1 complex and $\Delta H^0_{1:2}$ of the 1 : 2 complex were −1.28 kJ/mol and −0.57 kJ/mol, respectively. On the other hand, the changes in entropy $\Delta S^0_{1:1}$ and $\Delta S^0_{1:2}$ at 25 °C were 31.8 J/(mol·K) and 91.6 J/(mol·K), respectively.

^{1}H-NMR spectra and CPK atomic models were used to estimate the structure of the 1 : 2 complex (cholesterol : TOM-β-CyD = 1 : 2). The molecular size and structure of the guest molecule and cavity of the host molecule can be estimated using CPK atomic models. Figure 3a shows the ^{1}H-NMR spectrum of the 1.0×10^{-2} M TOM-β-CyD in deuterium oxide at 25 °C. The assignments of the proton signals of TOM-β-CyD have been reported previously.[12)] In the presence of 1.2×10^{-3} M cholesterol, the proton signals of TOM-β-CyD were observed (Fig. 3b). This sample was prepared in deuterium oxide in a manner based on Fig. 1. The structural

Table 1. Thermodynamic Parameters for Inclusion Complex Formation of TOM-β-CyD with Cholesterol[a)]

Temp (°C)	K (M^{-1})		ΔG^0 (kJ·mol^{-1})		ΔH^0 (kJ·mol^{-1})		ΔS^0 (J·mol^{-1}K^{-1})		$T\Delta S^0$ (kJ·mol^{-1})	
	$K_{1:1}$	$K_{1:2}$	$\Delta G^0_{1:1}$	$\Delta G^0_{1:2}$	$\Delta H^0_{1:1}$	$\Delta H^0_{1:2}$	$\Delta S^0_{1:1}$	$\Delta S^0_{1:2}$	$T\Delta S^0_{1:1}$	$T\Delta S^0_{1:2}$
10	78.1	76000	−10.3	−26.5			31.7±2.5	91.4±7.3	8.98	25.9
25	77.0	75500	−11.0	−27.8	−1.28±0.10	−0.57±0.05	31.8±2.5	91.6±7.3	9.48	27.3
37	73.9	74900	−11.1	−28.9			31.7±2.5	91.5±7.3	9.83	28.4
45	72.1	74500	−11.3	−29.7			31.5±2.5	91.5±7.3	10.02	29.1

a) Formation constant ($K_{1:1}$, $K_{1:2}$); average probable errors ±4%.

assignment of these complexes was made from their ^{1}H–^{1}H COSY and ^{1}H–^{1}H ROESY. New signals for TOM-β-CyD (1-H′, 2-*O*-Me′, 3-*O*-Me′, 3-H′, 2-H′) appeared in the presence of cholesterol at a higher field with respect to those of free species. The new signal 3-H′ appeared at significantly higher field in the presence of cholesterol. These new signals were attributed to complexed species. The 5-H, 6-H$_a$, and 6-H$_b$ signals broadened with the deformation of signals in the presence of cholesterol, although distinct new signals for these could not be detected. These observations show that the life time of the complexed species is sufficiently long to give separated signals distinct from the uncomplexed one or other complexed ones, at the NMR time scale.

The life time of the complexed species was approximately calculated using following Eqs. (8) and (9)[13]:

$$\frac{1}{\tau} = k = \pi(\Delta_e - \Delta_0) \tag{8}$$

$$\tau_{com.} = \frac{\tau}{(1 - p_{com.})} \tag{9}$$

where Δ_e and Δ_0 represent the widths at half-height (Hz) of the signal in the presence of exchange and in the absence of exchange, respectively, and τ and k represent mean life time and mean exchange rate constant, respectively. Also, $\tau_{com.}$ and $p_{com.}$ respresent the life time of the complexed species and mole fraction of the complexed species, respectively. The life time of the complexed species was about 0.078 s at 25 °C in the calculation using 3-OMe signal of TOM-β-CyD.

In contrast to the interaction of TOM-β-CyD, these phenomena were not all observed in the interaction between DOM-β-CyD and cholesterol, as reported previously.[6] Therefore, at the NMR time scale, the exchange rate of DOM-β-CyD is sufficiently fast between complexed species and free one, which differs from the case of TOM-β-CyD. When the exchange rate of TOM-β-CyD between complexed species and the free one is slow at low temperature, at NMR time scale, each signal of TOM-β-CyD is observed. To ensure that the lifetime of complexed species is sufficiently long on NMR time scale, the 1-H signal of TOM-β-CyD in the presence of cholesterol was observed at higher temperatures (Fig. 4). When the temperature was increased to more

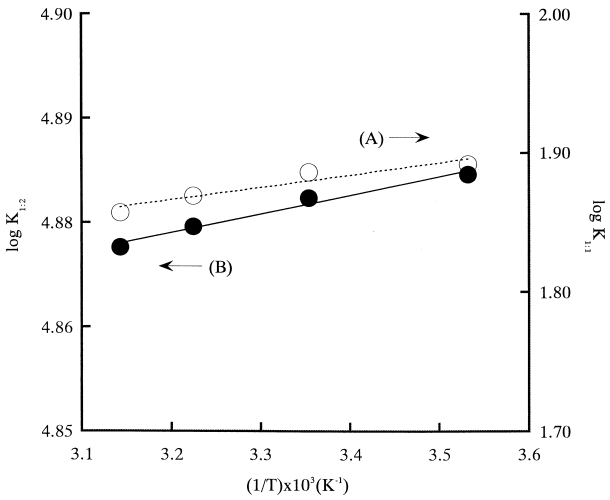

Fig. 2. The van't Hoff Plot of the Data in Table 1
(A) ○ for $K_{1:1}$, (B) ● for $K_{1:2}$.

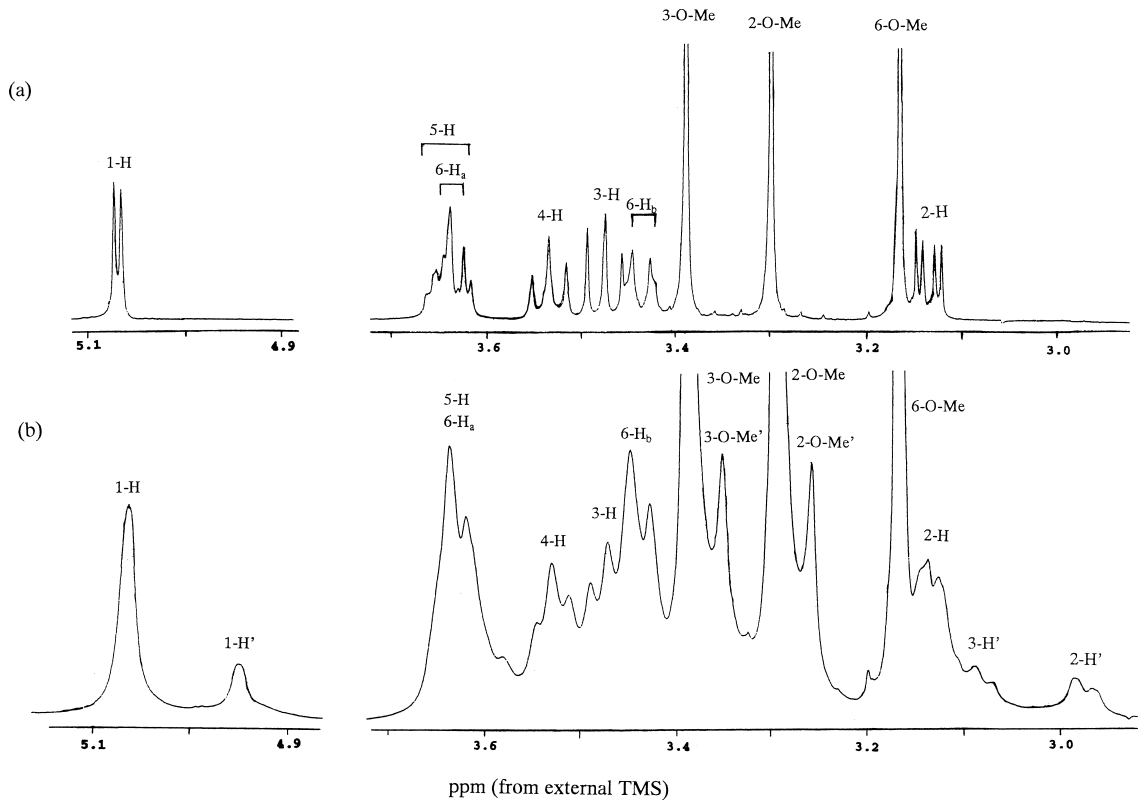

Fig. 3. ^{1}H-NMR Spectra of TOM-β-CyD in the Presence of Cholesterol in Deuterium Oxide at 25 °C
(a) TOM-β-CyD alone (1.0×10^{-2} M), (b) TOM-β-CyD (1.0×10^{-2} M)+cholesterol (1.2×10^{-3} M).

than 63 °C, this 1-H signal coalesced because the exchange rate of TOM-β-CyD between the complexed species and uncomplexed one increased. The other new proton signals coalesced along with 1-H signal at temperature higher than 63 °C. Similar observations were made for the complexation of α-CyD with α,ω-alkanedicarboxylate anion and polymethylene compounds with pyridinium groups as bulky head groups at both terminals, although the signals of uncomplexed speceis were observed without broadening and deformation.[14,15]

To obtain information on the structure of the 1 : 2 complex (cholesterol : TOM-β-CyD), the ROESY spectrum was measured (Fig. 5). In the ROESY spectrum of the solution containing cholesterol (1.2×10^{-3} M) and TOM-β-CyD (1.0×10^{-2} M) , cross peaks connecting the 18-Me of cholesterol to 3-H, 6-H_b, 3-*O*-Me, 3-*O*-Me′, and 3-H′ of TOM-β-CyD were observed. Cross peaks connecting the 26-Me and 27-Me of cholesterol to 6-H_b and 6-*O*-Me of TOM-β-CyD were observed and those connecting the 21-Me of cholesterol to 5-H, 6-H_a, 3-H, 6-H_b, and 3-H′ of TOM-β-CyD were also observed along with cross peaks between 19-Me of cholesterol and 5-H, 3-H, 3-*O*-Me, 3-*O*-Me′, and 3-H′ of TOM-β-CyD. Therefore it is reasonable to assume that the broad deformed signals which appears at almost same field as free TOM-β-CyD consist of complexed species and an uncomplexed one.

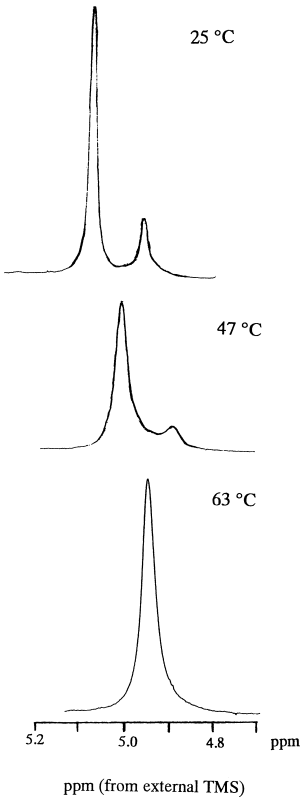

Fig. 4. ^{1}H-NMR Spectra of the 1-H of TOM-β-CyD at Various Temperatures in the Presence of Cholesterol

Concentrations of TOM-β-CyD and cholesterol are 1.0×10^{-2} M and 1.2×10^{-3} M, respectively.

Discussion

Based on the results of ^{1}H-NMR spectra and the investigation using CPK atomic models, three possible structures for the inclusion complex of cholesterol with TOM-β-CyD (cholesterol : TOM-β-CyD=1 : 2) were estimated, as shown in

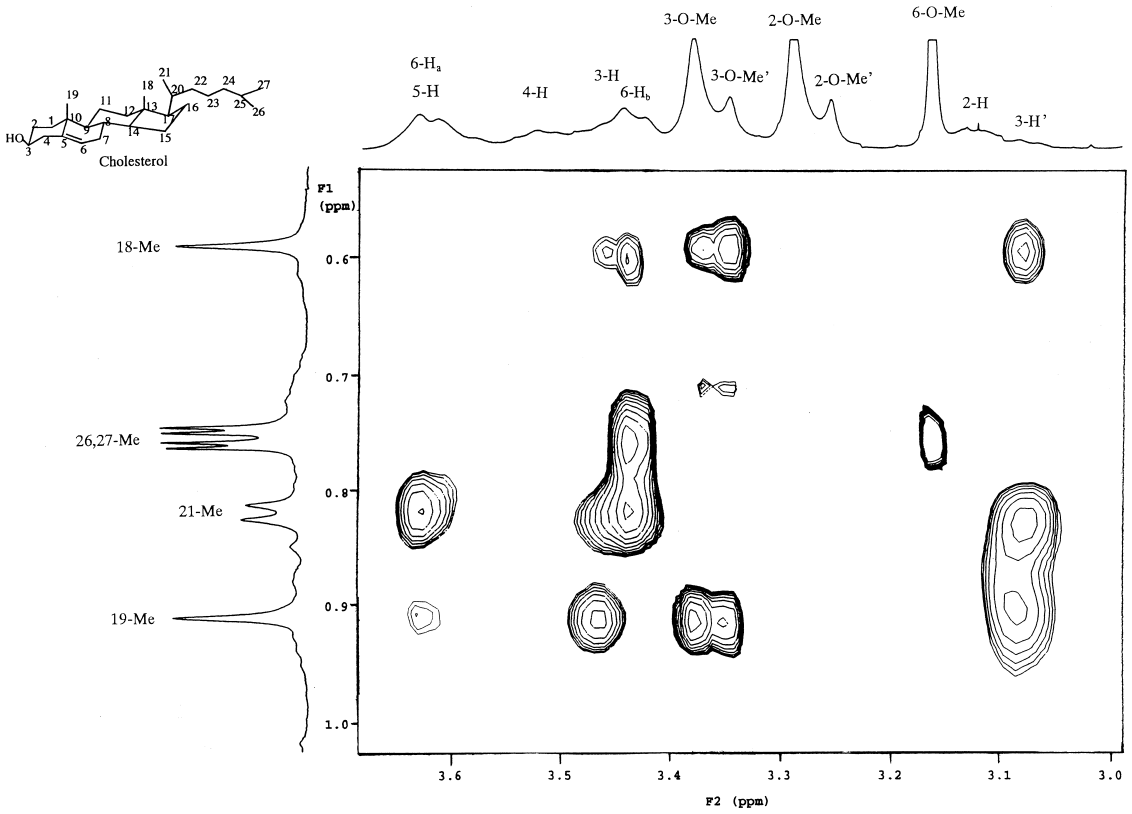

Fig. 5. ROESY Spectrum of the Solution Containing TOM-β-CyD (1.0×10^{-2} M) and Cholesterol (1.2×10^{-3} M)

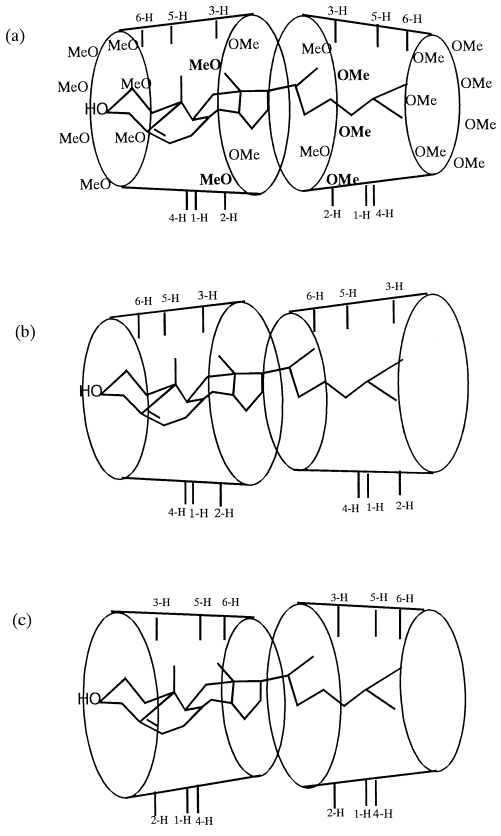

Fig. 6. Possible Structures of the 1 : 2 Complex of Cholesterol with TOM-β-CyD

Some –OMe groups (a) and all of them (b, c) were deleted for easier understanding of the figure. OMe in bold letter represents 2-*O*-Me.

Fig. 6.

In Fig. 6a, cholesterol is enclosed in the cavity of TOM-β-CyD from the methylated secondary hydroxyl group side at the head of the hydroxyl group of cholesterol, followed by the residual moiety of cholesterol being enclosed in the cavity of another TOM-β-CD from the same side. The structure in Fig. 6a is supported by the cross peaks connecting 18-Me of cholesterol to 3-H, 3-*O*-Me of TOM-β-CyD and the cross peaks connecting 21-Me of cholesterol to 3-H of TOM-β-CyD. In Fig. 6b, cholesterol is enclosed in the cavity of TOM-β-CyD from the methylated secondary hydroxyl group side at the head of hydroxyl group of cholesterol, followed by the residual moiety of cholesterol being enclosed in the cavity of another TOM-β-CyD from the methylated primary hydroxyl group side. The structure in Fig. 6b is supported on the basis of the cross peaks connecting 21-Me of cholesterol to 5-H, 6-H_a, and 6-H_b of TOM-β-CyD. Although no cross peaks connecting 26-Me and 27-Me of cholesterol to 3-H of TOM-β-CyD, which would be convenient for the structure in Fig. 6b were observed, this facts might be because the cavity of methylated secondary hydroxyl group side is too extended to produce the cross peaks between them. In Fig. 6c, cholesterol is enclosed in the cavity of TOM-β-CyD from the methylated secondary hydroxyl group side at the head of 26-Me and 27-Me groups of cholesterol, followed by the residual moiety of cholesterol being enclosed in the cavity of another TOM-β-CyD from methylated primary hydroxyl side. This structure is supported on the basis of the cross peaks connecting 19-Me of cholesterol to 3-H, 3-*O*-Me and 3-H′ and the cross peaks connecting 18-Me of cholesterol to 6-H_b of TOM-β-CyD. It was particularly surprising that the three inclusion modes of TOM-β-CyD complexes closely resemble those of DOM-β-CyD, because the chemical and steric structures of TOM-β-CyD are different from those of DOM-β-CyD, and the interaction modes of both CyDs with guest molecule are likely to be different, as mentioned above. These results can be convinced, when considered as follows; cholesterol is bulky for the cavity of CyDs and therefore, when one molecule of cholesterol is included by two molecules of the CyDs, the inclusion modes of the CyDs may be restricted spontaneously.

It was found that TOM-β-CyD forms a 1 : 2 complex (cholesterol : TOM-β-CyD) with cholesterol more easily than 1 : 1 complex. In addition, 1 : 2 and 1 : 1 complex formations were accompanied by a less negative change in enthalpy and more positive change in entropy. From the thermodynamic parameters shown in Table 1 for the formation of the 1 : 2 complex at 25 °C, the entropy term ($T\Delta S^0_{1:2}$=27.3 kJ/mol) contributes more extensively to the standard Gibbs free energy change ($\Delta G^0_{1:2}$=−27.8 kJ/mol) than that of the enthalpy term ($\Delta H^0_{1:2}$=−0.57 kJ/mol). The contribution of the entropy term was about 98%. Based on these results, it is apparent that the driving force for 1 : 2 complex formation is hydrophobic interaction, as expected. In 1 : 1 complex formation at 25 °C, the entropy term ($T\Delta S^0_{1:1}$=9.48 kJ/mol) also contributes much more to standard Gibbs free energy change ($\Delta G^0_{1:1}$=−11.0 kJ/mol) than does the enthalpy term ($\Delta H^0_{1:1}$=−1.28 kJ/mol). The contribution of the entropy term was 86%. The main driving force for 1 : 1 complex formation was found to be a hydrophobic interaction, although the rate of contribution of the entropy term is less than that for 1 : 2 complex formation. The contributions of entropy terms for standard Gibbs free energy changes are larger in the complexes of TOM-β-CyD than in the complexes of DOM-β-CD, although these thermodynamic parameters are similar to those for complex formation of DOM-β-CyD with cholesterol.[6)]

TOM-β-CyD has a chemical structure in which three hydroxyl groups of β-CyD are permethylated. Therefore it has a deep cavity and is more hydrophobic than other CyDs. This might enable TOM-β-CyD to form inclusion complexes with the much more hydrophobic compound cholesterol through hydrophobic interaction. It is reported that the crystal structure of TOM-β-CyD with *p*-iodophenol complex is in the shape of an elliptically-distorted and truncated cone and the macrocyclic ring is markedly distorted from a regular heptagonal structure,[16)] which has a wider cavity on the side of the methylated secondary hydroxyl groups, although the entrance to the cavity on the methylated primary hydroxyl group is narrower. From the investigation using CPK atomic models, it was confirmed that if a larger molecule such as cholesterol is inserted into the TOM-β-CyD ring, the macrocyclic ring is much more distorted. The distortion of the macrocyclic ring is readily confirmed by the absence of the cross peak between 2-*O*-Me groups and cholesterol is not observed in the ROESY spectrum in Fig. 5. It is assumed that the 2-*O*-Me groups toward the outside and the 3-*O*-Me groups toward the inside of the cavity. It was also found that

the cholesterol is tightly included near the 5-H of TOM-β-CyD and is loosely included near the methylated secondary hydroxyl group. The nearly zero change in enthalpy, particularly $\Delta H^0_{1:2}$ for the complex formation in spite of the accompanying tight inclusion might be because the energy is spent on structural change of the macrocyclic ring for complex formation. The rate at which the cholesterol trapped tightly in the two cavities of TOM-β-CD with distorted structure leaves the cavities is slow on NMR time scale, resulting in appearance of new signals separate from the peaks of free species.

Conclusion

Since the phase solubility diagram of cholesterol with TOM-β-CyD and that of cholesterol with DOM-β-CyD, and possible structures of the complexes are similar, the interactions of both CyDs with cholesterol are similar on the macroscopic scale. However, the interaction of TOM-β-CyD with cholesterol is more hydrophobic than that of DOM-β-CyD and the exchage rate of TOM-β-CyD between complexed species and free one is slower than that of DOM-β-CyD on NMR time scale. Thus, there are the differences on the microscopic scale between the interaction of TOM-β-CyD with cholesterol and that of DOM-β-CyD with cholesterol.

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