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The effect of heptakis (2,6-di-*O*-methyl)-γ-cyclodextrin on mitomycin C stability in aqueous solution

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Summary

The inclusion of mitomycin C in γ-cyclodextrin and two of its methylated derivatives has been investigated as well as the effect of the process on the stability of the guest molecule.

Inclusion of mitomycin C in heptakis (2,6-di-*O*-methyl)-γ-cyclodextrin has the greatest effect, whilst in γ-cyclodextrin it is less pronounced and in the case of heptakis (2,3,6-tri-*O*-methyl)-γ-cyclodextrin activity was undetectable.

On complexation with cyclodextrins, the degradation of mitomycin C proceeds at a slower rate, in both acidic and alkaline media. The effect of heptakis (2,6-di-*O*-methyl)-γ-cyclodextrin is also greater in this respect and is apparently correlated with the stability of the complex.

Using CD and ^{1}H NMR spectroscopy some insight has been gained into the structure of mitomycin C-cyclodextrin complexes.

Introduction

Mitomycin C (MMC) is a clinically significant antineoplastic antibiotic (Comis and Carter, 1974; Samson et al., 1978). Recent studies have shown that during the process of bioreductive alkylation, believed to evote cytotoxic activity in MMC, both carbon atoms C_1 and C_{10} are likely to be DNA-binding sites (Moore, 1977; Moore and Czerniak, 1981; Hashimoto et al., 1983; Hornemann et al., 1983; Tomasz et al., 1983, 1984; Bean and Kohn, 1985; Danishefsky and Egbertson, 1986).

From a pharmaceutical point of view, this drug attracts a great deal of attention because of its chemical instability. This aspect demands special precautions in the preparation and storage of MMC formulations as degradation is accompanied by a loss of antitumor activity.

Cyclodextrins (CyDs) have been used extensively to improve various physicochemical properties of drug molecules (Bender and Komiyama, 1978; Szejtli, 1982; Uekama and Otagiri, 1987) by

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forming inclusion complexes in which the drug molecules are included in the relatively hydrophobic cavity of the CyDs (Saenger, 1980).

The mechanisms of degradation of MMC in acidic and alkaline solutions have been described previously (Beijnen et al., 1985, 1986a; Beijnen and Underberg, 1985; Underberg and Beijnen, 1987). Furthermore, recent investigations at our laboratories indicate that degradation of MMC is inhibited by CyDs, especially γ-cyclodextrin (γ-CyD). In this series of continuing studies, the effects of heptakis (2,6-di-*O*-methyl)-γ-cyclodextrin (DM-γ-CyD) and heptakis (2,3,6-tri-*O*-methyl)-γ-cyclodextrin (TM-γ-CyD) on MMC degradation are examined and compared to those of the parent γ-CyD.

Experimental

Materials

MMC was kindly provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo). α-, β-, γ- and 2-hydroxypropyl-γ-CyD (HP-γ-CyD) were donated by Nihon Shokuhin Kako Co., Ltd. (Tokyo), and recrystallized from water. Heptakis (2,6-di-*O*-methyl)-β-CyD (DM-β-CyD) and TM-γ-CyD were supplied by Toshin Chemical Co., Ltd. (Tokyo). All other materials were of special reagent grade, and deionized and distilled water was used throughout.

For the synthesis of DM-γ-CyD, 3.0 g of γ-CyD was dissolved in 50 ml DMF, then 4.5 g Bao and 2.3 g $Ba(OH)_2 \cdot 8H_2O$ were added, the solution cooled on ice and 3.4 ml CH_3I added with the temperature being maintained below 5 °C. The mixture was stirred for 3 h at 20 °C, then diluted with chloroform and filtered through Celite. The filtrate was washed with $Na_2S_2O_3$ solution and water, then concentrated. The residue was first pre-fractionated on a Lobar LiChroprep Si 60 column with benzene-acetone as eluent. Fractions were checked by TLC and then DM-γ-CyD was isolated from appropriate fractions by HPLC on an ODS column with 1-propanol-water.

Full details of the procedure for synthesis are given elsewhere (Tanimoto et al., 1989).

Buffer solutions

For the kinetic studies with CyD, various amounts of CyD were added to 0.001 M acetate buffers (ranging from pH 2.0 to 5.0) or 0.05 M borate buffers (pH range 9.0–12.0), with the pH being adjusted to the desired value using $HClO_4$ or NaOH. These solutions were freshly prepared before use.

Kinetic measurements

The buffer solutions, stored in stoppered glass test-tubes, were equilibrated to the temperature of study in a thermostatted water bath. Degradation was initiated by the addition of 30 μl of a stock solution of MMC in methanol (3×10^{-3} M) to 3 ml buffer, yielding an initial concentration of 3×10^{-5} M MMC. For evaluation of K_s and k_{cat}, the γ-CyD or DM-γ-CyD concentrations were varied from 0–1.2×10^{-2} to 0–6.0×10^{-3} M, respectively. For constructing log k_{obs} vs pH profiles, their concentrations were maintained at 1×10^{-2} and 2×10^{-3} M, respectively.

In acidic media, degradation was followed spectrophotometrically by continuous monitoring of the decrease in absorbance at 363 nm. In the case of alkaline solutions, aliquots were withdrawn at appropriate time points and analyzed for undegraded MMC using the HPLC assay described below.

Apparatus and analytical procedures

High-performance liquid chromatography (HPLC) procedures for monitoring degradation of MMC in acidic and alkaline solutions have been developed previously (Beijnen et al., 1985; Beijnen and Underberg, 1985) and were used in the present study. Chromatography was performed using an L-6000 pump and a 655A detector (both from Hitachi, Tokyo). Peak areas were used to quantitate the amount of undegraded MMC. Standard curves from MMC showed good linearity ($r >$ 0.999) over the concentration range of interest.

UV-visible absorbance spectra were recorded on a UV-240 spectrophotometer (Shimazu, Kyoto). Within the concentration range of interest the UV absorbance of MMC at 363 nm obeyed Beer's Law.

400 MHz ^{1}H NMR spectra were registered in D_2O at 30 °C using a JNM-GX 400 spectrometer (JEOL, Tokyo). An average of 200 accumulations with 32768 data points were made at a sweep width of 6000 Hz. The ^{1}H chemical shifts were assigned values based on an external standard of sodium 2,2-dimethyl-2-silapentane-5-sulfonate to an accuracy of ± 0.0015 ppm.

Circular dichroism (CD) spectra were taken on a J-50A recording spectropolarimeter (Jasco, Tokyo), and are expressed as ellipticities in degrees.

Results and Discussion

Degradation mechanism and kinetics

The overall pathway of degradation of MMC in acidic and alkaline medium is depicted in Fig. 1.

In acidic solution, degradation of MMC yields a pair of 1,2-disubstituted mitosenes (**I, II**), in which the 9*a*-methoxy group has been cleaved, resulting in the formation of a double bond between C_9 and C_{9a}, and the opening of the 1,2-fused aziridine ring. Underberg and Lingeman (1983a) have proposed a mechanism for this initial MMC degradation step on the basis of kinetic data. Garrett (1963) postulated that mild alkaline conditions should lead to substitution of the 7-amino group by a hydroxyl group to yield compound **X**. This postulate has been verified by Beijnen et al. (1985).

The course of the degradation follows the usual pseudo-first order kinetic pathway. The rate equation for MMC can be written as

$$\frac{-\mathrm{d}[\mathrm{MMC}]}{\mathrm{d}t} = k_{\mathrm{obs}}[\mathrm{MMC}] \qquad (1)$$

or

$$\ln[\mathrm{MMC}]_t = \ln[\mathrm{MMC}]_0 - k_{\mathrm{obs}}t \qquad (2)$$

where $[\mathrm{MMC}]_t$ represents the concentration of MMC at time t, $[\mathrm{MMC}]_0$ its initial level and k_{obs} the observed rate constant for degradation of MMC. The order of the reaction is indicated by the linearity ($r > 0.999$) in the $\ln[\mathrm{MMC}]_t$ vs time plots.

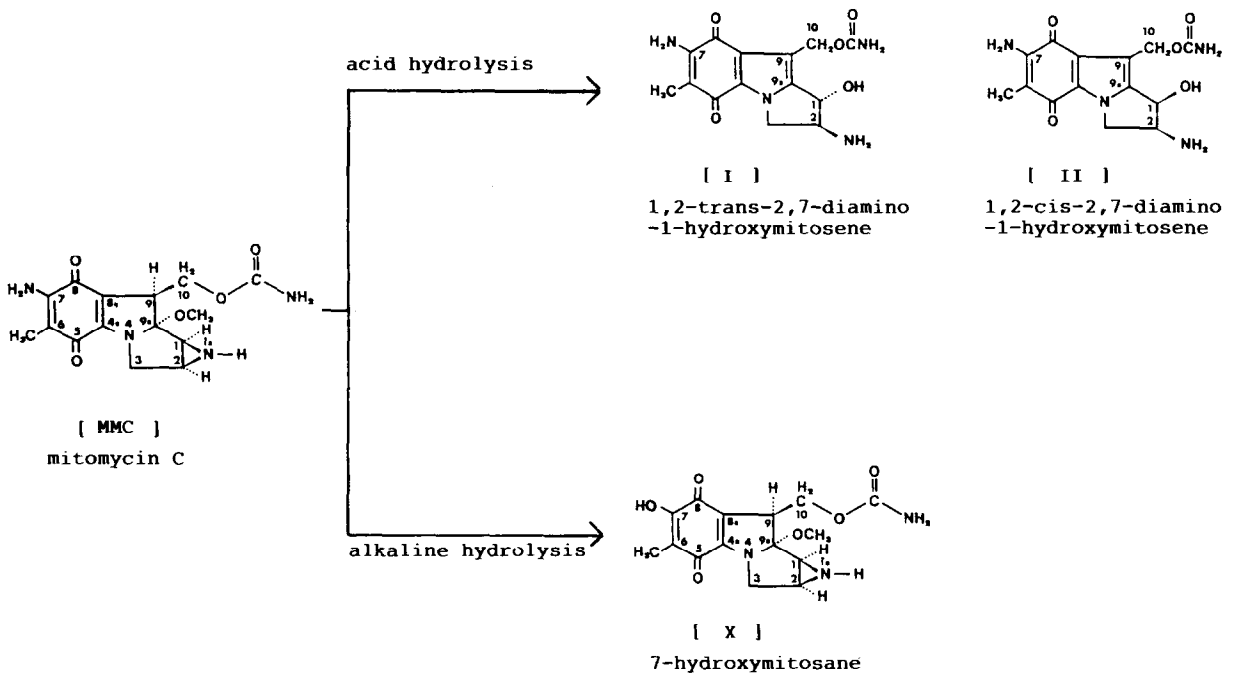


Fig. 1. Overall pathway for degradation of MMC in acidic and alkaline solutions.

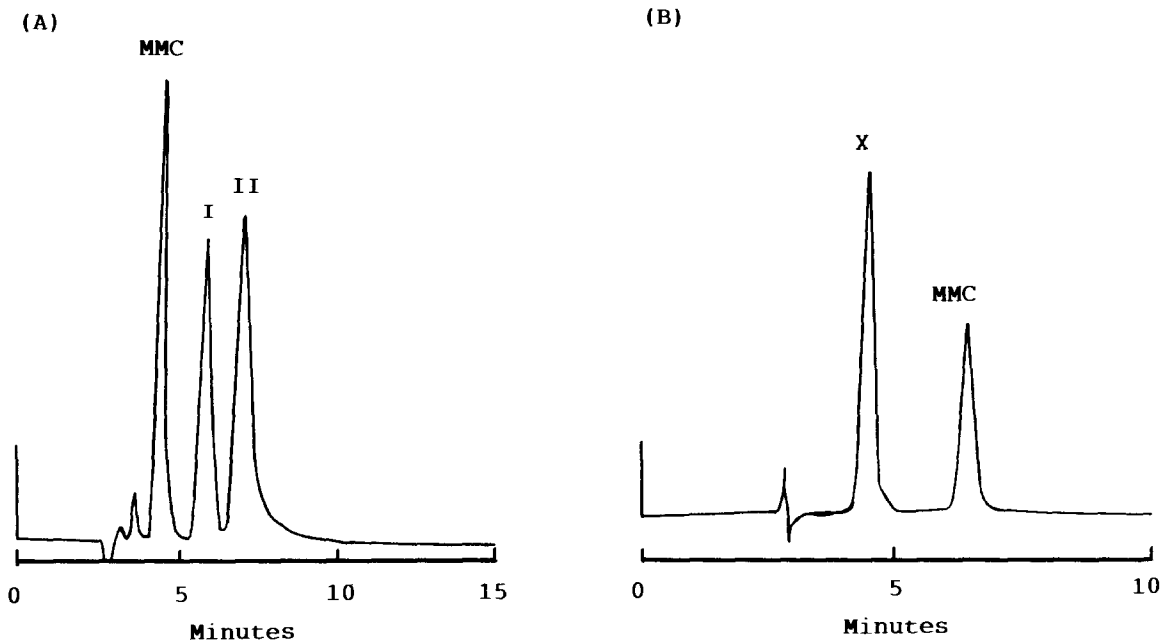


Fig. 2. HPLC chromatograms of decomposition mixtures of MMC in 0.001 M acetate buffer solution (pH 4.8) (A) and 0.05 M borate buffer solution (pH 9.0) (B) in the presence of DM-γ-CyD.

The presence of CyD does not change this kinetic behavior. This is indicated by the linearity of ln[MMC]$_t$ vs time plots being maintained in the presence of various CyDs as well as on the basis of the formation of identical degradation products, as shown by HPLC (Fig. 2). The presence of CyD has no effect on the capacity factor of the degradation products.

CD and UV spectra

Fig. 3 shows the CD and UV spectra of MMC in the absence and presence of γ-CyD and DM-γ-CyD in aqueous solution at pH 7.0. DM-γ-CyD alters the shape of the CD curve accompanying a slight decrease in UV absorbance, while the UV and CD spectra of MMC are scarcely affected by γ-CyD. Such small differences in the spectra observed for the two systems may be attributed to a slightly different orientation and/or disposition of the guest molecule within the CyD cavity.

Influence of CyD structure

The influence of various CyDs on the degradation of MMC has been studied at pH 2.8 and 20 °C as well as at pH 10.0 and 50 °C. Table 1 summarizes the k_{obs} values. It is clear that α-, β-, DM-β- and TM-γ-CyD exert virtually no stabilizing effect on MMC degradation, while γ- and HP-γ-CyD result in a significant positive influence on the stability of MMC. The strongest stabilizing effect is exerted by DM-γ-CyD. Apparently, the lipophilic moiety in MMC fits best within the larger γ-CyD cavity, whereas the hydrophobic interactions between this moiety and the DM-γ-CyD cavity are the most intense. All other experiments were therefore established by comparison of γ-CyD and DM-γ-CyD.

The complexation of MMC with DM-γ-CyD, as an example, as well as the degradative reaction of the free drug and guest molecule in the inclusion complex is represented schematically in Fig. 4. In this scheme, K_s denotes the formation constant of the inclusion complex, with k_0 and k_{cat} being the respective rate constants for degradation of the free drug and guest molecule in the complex. The values of these constants can be extracted from Eqn. 3 (Colter et al., 1964) in Fig. 4. By plotting $[CyD]/k_0 - k_{obs}$ vs [CyD], Line-

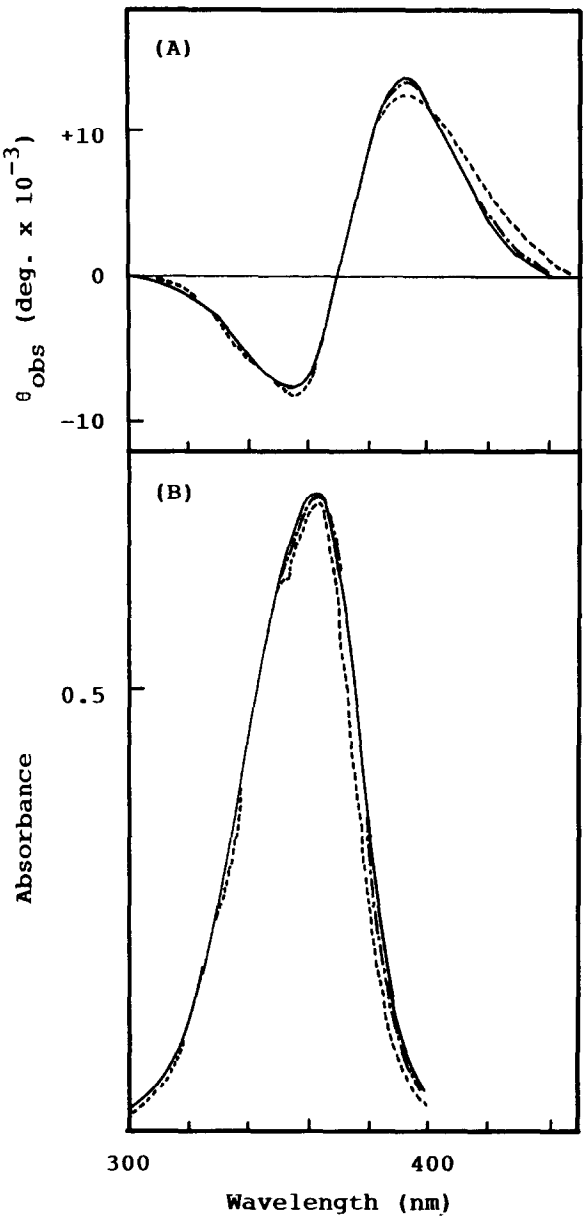


Fig. 3. CD (A) and UV (B) spectra of MMC in the absence and presence of γ-CyD or DM-γ-CyD in 0.03 M phosphate buffer solution (pH 7.0) at 20 °C. (———) MMC alone (1×10^{-4} M), (- - - - -) with DM-γ-CyD (2×10^{-3} M), (— - —) with γ-CyD (2×10^{-3} M).

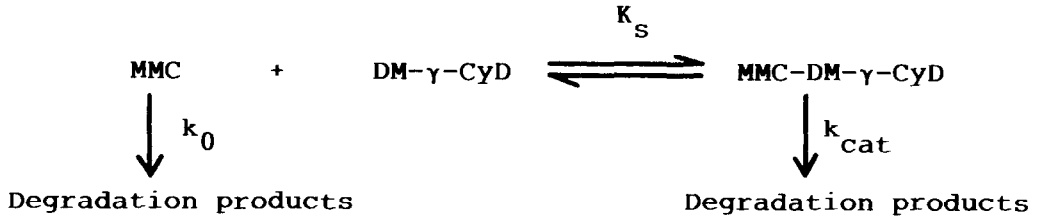
weaver-Burk plots are obtained. If k_0 is known, then k_{cat} can be calculated from the slope and K_s from the intercept of the straight line. However, Lineweaver-Burk plots are only valid under certain conditions. An important assumption is that only one species of drug is present in the solution, which then becomes partially included in the CyD cavity. However, MMC in solution has two prototropic functions, with pK_a values of 2.8 and 12.4, respectively (Underberg and Lingeman, 1983b). Theoretically, the Lineweaver-Burk equation can therefore only be used within the range pH 4.8–10.4, where virtually all MMC is present as the neutral form, to obtain the true values of K_s and k_{cat}. At pH < 4.8 and > 10.4, significant amounts of protonated and deprotonated MMC are present, respectively. These ions undergo degradation at different velocities and may or may not be included in CyD. However, the Lineweaver-Burk plots obtained at pH 2.8, 11.0 and 12.0 were also straight lines, indicating that either full complexation of the charged MMC species occurs or none at all, whereas the pK_a values of the prototropic functions are scarcely affected or drastically altered. Hence, the K_s and k_{cat} values obtained at these pH values must be considered as referring to 'apparent' constants, the designations of which are K_s^* and k_{cat}^*. At pH 11.0 only 3.5% of the total MMC concentration is deprotonated, therefore, at this pH value K_s^* and k_{cat}^* approach the real values for K_s and k_{cat}. In Table 2 the kinetic parameters characterizing the MMC-γ-CyD and MMC-DM-γ-CyD complexes have been summarized. In acidic as well as in alkaline medium, MMC included in CyD appears to undergo degradation at a slower rate compared to the free

TABLE 1

Observed rate constants (k_{obs}) for MMC degradation in the presence of various CyDs

Type of CyD	k_{obs} (s^{-1})	
	pH 2.8, 20 °C	pH 10.0, 50 °C
–	4.6×10^{-4}	2.3×10^{-5}
α	3.9×10^{-4}	2.4×10^{-5}
β	3.9×10^{-4}	2.4×10^{-5}
DM-β	3.7×10^{-4}	2.0×10^{-5}
γ	2.6×10^{-4}	1.3×10^{-5}
TM-γ	4.2×10^{-4}	2.2×10^{-5}
HP-γ	2.4×10^{-4}	1.2×10^{-5}
DM-γ	1.2×10^{-4}	4.8×10^{-6}

[CyDs] = 1×10^{-2} M, [MMC] = 3×10^{-5} M.



$$\frac{[CyD]}{k_0 - k_{obs}} = \frac{1}{k_0 - k_{cat}}[CyD] + \frac{1}{K_s^*(k_0 - k_{cat})} \qquad \text{(eq.3)}$$

Fig. 4. Complexation of MMC with DM-γ-CyD (1:1) and the degradation reactions of the free drug and guest molecule in the inclusion complex.

drug. K_s^* for MMC-DM-γ-CyD is larger compared to that of MMC-γ-CyD, especially at pH 12.0.

Influence of pH

Fig. 5 demonstrates a comparison of the log k_{obs} vs pH profiles of free MMC and MMC included in both γ-CyD and DM-γ-CyD. The shapes of the curves are similar except for the regions in which pH < 1 and > 12, where they tend to coincide. Within the range pH 4–11, the effect of both CyDs is independent of pH.

The close coincidence between the curves at the pH extremes indicates that the ionic species of MMC do not form complexes with either CyD. Only the neutral form of MMC, existing mainly in the region pH 4–11, becomes included and, consequently, stabilized. The stabilizing effect of DM-γ-CyD is greater than that of γ-CyD, in both acidic and alkaline media. This is illustrated on inspection of the ratios k_0/k_{cat}^* at pH 2.8 and 12.0 (Table 2). Apparently, the more stable complex formed with DM-γ-CyD (see K_s^* values in Table 2) accounts for this effect.

Influence of microsolvent

Fig. 6 illustrates influence of the concentration of dioxane on k_{obs} for the case of MMC degradation in a water-dioxane mixture at pH 2.8. One can observe that for the increasing concentration of dioxane which gives rise to greater hydrophobicity of the medium, the values of k_{obs} diminish. This effect is similar to that of CyD on k_{obs} for the degradation of MMC. Probably, the

TABLE 2

Influence of γ-CyD and DM-γ-CyD on rate constants (k_{cat}) and stability constants (K_s) for the degradation of MMC at various pH values and at 20°C

pH	γ-CyD				DM-γ-CyD			
	k_0 (s^{-1})	k_{cat}^* (s^{-1})	K_s^* (M^{-1})	k_0/k_{cat}^*	k_0 (s^{-1})	k_{cat}^* (s^{-1})	K_s^* (M^{-1})	k_0/k_{cat}^*
2.8	4.6×10^{-4}	1.1×10^{-4}	115	4.2	5.1×10^{-4}	0.7×10^{-4}	350	7.2
4.8	8.6×10^{-6}	1.8×10^{-6}	300	4.8	–	–	–	–
11.0	4.9×10^{-6}	2.7×10^{-6}	500	1.8	–	–	–	–
12.0	8.8×10^{-5}	2.8×10^{-5}	155	3.2	6.5×10^{-5}	1.0×10^{-5}	1700	6.3

–, not determined.

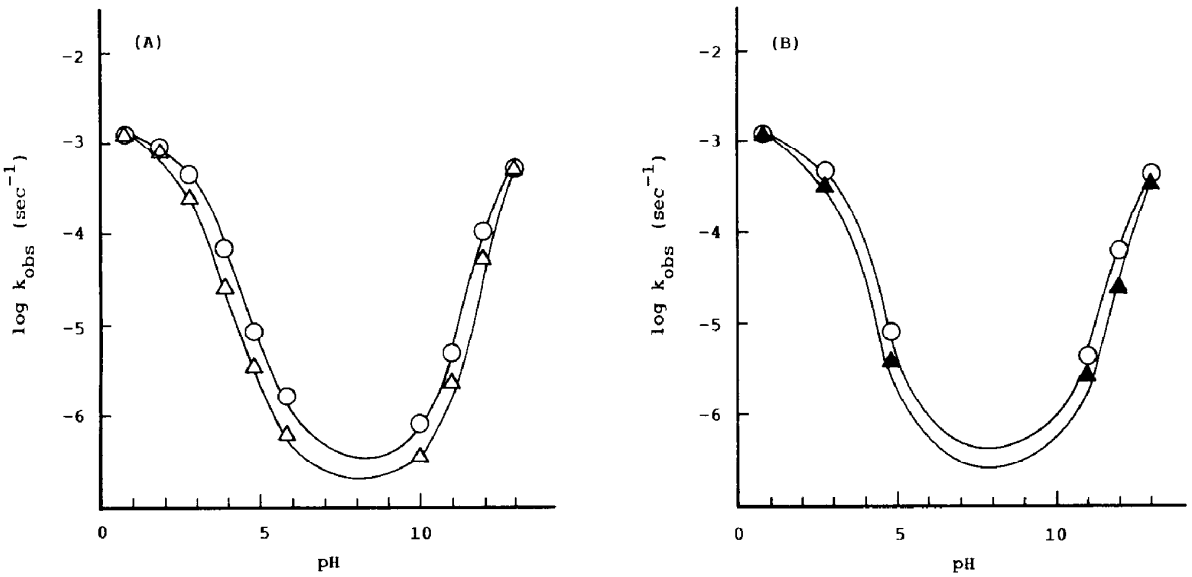


Fig. 5. Variation in rate of degradation of MMC as a function of pH in the absence and presence of γ-CyD (A) or DM-γ-CyD (B) at 20 °C. (○) MMC alone, (△) with γ-CyD (1×10^{-2} M), (▲) with DM-γ-CyD (2×10^{-3} M).

hydrophobic environment of the CyD cavity is responsible for a conformational rearrangement in the MMC molecule which retards the rate of reaction.

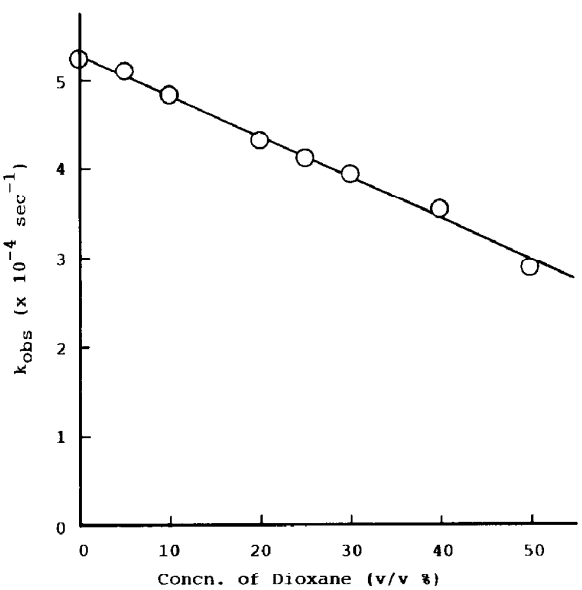


Fig. 6. Effect of dioxane concentration on the rate of degradation of MMC in acetate buffer solution (pH 2.8) at 20 °C.

Influence of temperature

The temperature dependence of MMC degradation in the presence of CyD was studied and compared to the free drug, with the experiments being performed at pH 2.8 and 11.0. Arrhenius plots were constructed and the thermodynamic parameters calculated.

The results obtained for the free drug are consistent with literature values (Beijnen et al., 1985, 1986b). The parameters showed no significant change on complexation MMC with γ-CyD or DM-γ-CyD.

Structure of the inclusion complex

^{1}H NMR spectra were examined in order to elucidate the mode of inclusion of MMC within the CyD cavity in aqueous solution. Assignment of the ^{1}H NMR signals of both MMC and CyD was straightforward and was carried out according to Lown and Begleiter (1974) and Wood et al. (1977). Figs. 7 and 8 show the effects of γ-CyD and DM-γ-CyD, respectively, on the ^{1}H chemical shifts of MMC. The other proton signals, under the present conditions, were too weak to permit quantitative analysis. Inclusion of MMC in γ-CyD leads to chemical shifts for C_{10}-H, C_{10}-H′, C_{9a}-

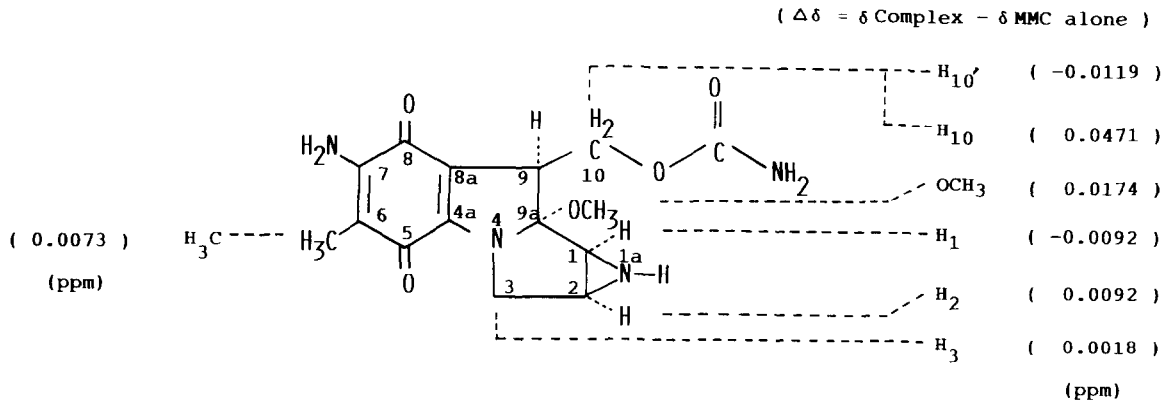


Fig. 7. Influence of γ-CyD on the ^1H chemical shifts of MMC (pH 6.3).

OCH_3, C_1-H and C_2-H that are significantly larger than others. Complexation with DM-γ-CyD causes more pronounced shifts, but the general pattern is similar to that of the MMC-γ-CyD complex. In the latter case, the C_{10}-H and C_{9a}-OCH_3 peaks are shifted downfield, whereas the C_{10}-H′ and C_1-H signals show an upfield shift. This leads to the suggestion that the aziridine ring and the C_9-urethane substituent are involved in the process of inclusion into the CyD cavity. As yet, the nature of participation of the benzoquinone moiety in inclusion remains unclear. However, since a marked difference is evident in the K_s* values at pH 2.8 and 12.0 (Table 2), variations in the mode of complexation may occur in acidic and alkaline media and the benzoquinone structure could make a larger contribution to the process of inclusion at high pH values. An indication that this phenomenon could occur is provided by the situation for MMC degradation in alkaline medium, in which another part of the molecule is involved, as compared to acidic conditions, which is also retarded on complexation with CyD. Further research may cast more light on this matter.

The increase in magnitude of the chemical shifts on inclusion of MMC in DM-γ-CyD in comparison with γ-CyD may be attributed to the more bulky structure of DM-γ-CyD.

Table 3 lists the effects of MMC on the ^1H chemical shifts of γ- and DM-γ-CyDs. On binding to MMC, H_1, H_3, H_5 and H_6 showed upfield shifts and significant shifts were observed for H_3 and H_5, protons located around the interior of the γ-CyD. In the case of DM-γ-CyD, an appreciable upfield shift was observed for H_3 while H_5, another proton located around the interior of CyD showed

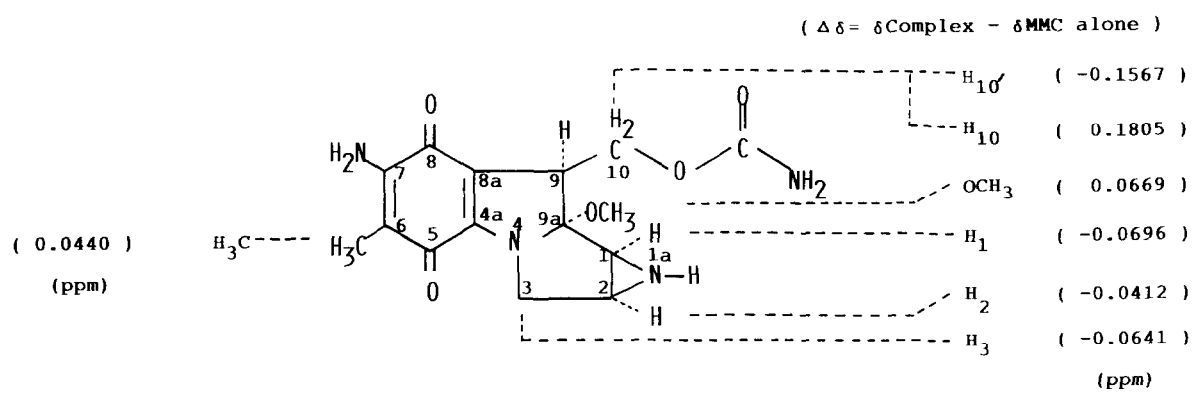


Fig. 8. Influence of DM-γ-CyD on the ^1H chemical shifts of MMC (pH 6.8).

TABLE 3

MMC-induced ^1H chemical shifts of γ-CyD and DM-γ-CyD

Proton	δ (ppm)	
	γ-CyD	DM-γ-CyD
H_1	−0.0032	−0.0408
H_2	0.0018	0.0158
H_3	−0.0104	−0.0180
H_4	0.0031	[a]
H_5	−0.0133	0.0110
H_6	−0.0092	0.0197
C_2-OCH_3		0.0028
C_6-OCH_3		−0.0156

[a] Could not be determined due to overlapping MMC signal.

a downfield shift. These limited data suggest that the MMC molecule preferentially interacts at the larger entrance side of the cavity of DM-γ-CyD and could penetrate further into the γ-CyD cavity (Yamamoto et al., 1988).

Conclusion

MMC is included to a significant extent into the internal cavity of DM-γ-CyD. Inclusion in γ-CyD, is, however, less pronounced, whereas TM-γ-CyD is inactive.

Inclusion in CyD results in appreciable stabilization of MMC, with DM-γ-CyD exerting the greatest effect.

The results of CD spectroscopic analysis, the effect of dioxane addition to the solvent and ^1H NMR experiments indicate that inclusion of the aziridine and urethane moieties of MMC in the cavity of CyD places the unstable part of the guest molecule in a hydrophobic microenvironment that is less favorable for the degradation reaction to proceed.

The stabilizing effect of CyD on MMC in alkaline medium and the large change in K_s* for the MMC-DM-γ-CyD complex on changing from pH 2.8 to 12.0 have yet to be explained.

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