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A Systematic Study on the Chemical Stability of Mitomycin A and Mitomycin B

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The kinetics and mechanisms of the degradation of mitomycin A and mitomycin B in aqueous solution were investigated by means of ultraviolet (UV) spectrophotometry and high-performance liquid chromatography (HPLC). The influences of pH, buffers and temperature on the degradation were quantified. Degradation products were isolated and characterized by mass spectrometry (MS), UV-VIS spectrophotometry, chromatography and circular dichroism (CD) spectroscopy. The degradation reactions follow (pseudo) first-order kinetics. The collected kinetic data allowed an accurate determination of the pK_a value of the aziridine moiety of the mitomycins by using computerized curve-fitting models.

Keywords——mitomycin A; mitomycin B; degradation product; 7-methoxymitosene; 7-hydroxymitosane; degradation kinetics; degradation mechanism; kinetic pK_a determination

Introduction

The mitomycins are a group of bioreductive alkylating antibiotics, produced by different *Streptomyces* cultures.[1] The compounds were discovered in Japan some thirty years ago. Mitomycin C (MMC) is nowadays an accepted drug in clinical practice, exerting antitumor action against, for instance, gastric tumors and superficial bladder cancer.[2]

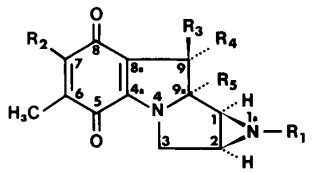
The 7-substituted mitomycins, particularly the 7-alkoxymitomycins, are currently receiving increasing attention because of their strong biological activity.[3-6] Interest in these compounds increased when it appeared that a correlation exists between the antibacterial/antitumor activity and the polarographic half-wave potential of the quinoid chromophore, which can be manipulated by changing the nature of the 7-substituent.[7-9] Moreover, evidence in favor of the importance of lipophilicity (due to the 7-substituent), in connection with the ease of diffusion into the tumor cell, has also been presented.[10]

Among the naturally occurring 7-alkoxymitomycins, mitomycin A (MMA) and mitomycin B (MMB) are the best known representatives and these compounds have been the subjects of several studies.[11,12] These substances, as well as MMC and 1a-*N*-methyl-MMC (porfiromycin; PM), are not stable in aqueous solutions. While the hydrolytic degradation processes of MMC and PM have been investigated thoroughly,[13-18] there is a lack of systematic kinetic studies on the degradation of MMA and MMB. Such a study would be of interest as MMA and MMB differ structurally from MMC in several ways (*e.g.*, in the presence of the 7-methoxy group *versus* the 7-amino function in MMC). Furthermore, MMB uniquely possesses an opposite stereochemistry at the C9 asymmetric center in comparison to MMA, MMC and PM. MMB differs from the other mitomycins in the presence of a C9a hydroxyl group *versus* a C9a methoxy function. These structural differences may have

pronounced effects on the chemical stability of MMA and MMB, especially in quantitative terms.

The primary objective of the present study was to explore the qualitative and quantitative aspects of the degradation of MMA and MMB in a systematic way and to compare the results with previous data obtained for MMC and PM.[13-18] Moreover, the stability data may shed more light on the complex degradation mechanisms of the mitomycins which are still not satisfactorily elucidated. In particular, knowledge on the acid degradation is of considerable interest because the acid-catalyzed degradation may be an activation step of mitomycins in the process of binding to deoxyribonucleic acid (DNA),[11,19] next to the bioreductive activation.[20] In a recent report, Kennedy *et al.*[21] clearly demonstrated that MMC-induced interstrand cross-linking of DNA is highly dependent upon the cellular micro-environmental pH; a lower pH enhances the alkylation.

The structures of various mitomycins are depicted in Fig. 1. The absolute stereochemical configurations of the mitomycins were recently reassigned on the basis of X-ray crystallography[22] and circular dichroism (CD) studies.[23]



	R_1	R_2	R_3	R_4	R_5
mitomycin A	H	H_3CO	CH_2OCONH_2	H	OCH_3
mitomycin B	CH_3	H_3CO	H	CH_2OCONH_2	OH
mitomycin C	H	H_2N	CH_2OCONH_2	H	OCH_3
porfiromycin	CH_3	H_2N	CH_2OCONH_2	H	OCH_3
1a-acetylmitomycin C	$COCH_3$	H_2N	CH_2OCONH_2	H	OCH_3

Fig. 1. Structures of Mitomycins

Experimental

Materials——MMC was kindly provided by Bristol Myers, Weesp, The Netherlands. MMA and MMB were generous gifts from Dr. K. Shirahata, Kyowa Hakko Kogyo Chemical Co., Tokyo, Japan. PM was supplied by Cyanamid, Pearl River, NY, U.S.A. The compounds were used as obtained. 1a-Acetyl-MMC (AcMMC) was prepared according to the procedure described by Stevens and co-workers.[15] All chemicals used were of analytical grade, and deionized water was used throughout.

Buffer Solutions——For the kinetic studies, the following aqueous buffer solutions were used: H_0/pH −2—2, perchloric acid; pH 2—6, acetate; pH 6—8, phosphate; pH 8—10, borate; pH 10—11.5, phosphate; pH > 11.5, sodium hydroxide. The pH values between 1 and 12 were measured at 25 °C with an Ingold LOT-401 combined glass-reference electrode and a Radiometer PHM 64 research pH meter (Copenhagen, Denmark). Extension of the acidity scale below 1 was accomplished with the Hammett acidity function.[24] pH values between 12 and 13 were calculated using the Debye–Hückel equation.[24]

Kinetic Experiments——All kinetic studies were conducted at 25 °C unless otherwise stated. The degradation of MMC, PM and AcMMC at pH < 4 was quantified spectrophotometrically at 360 nm as described earlier.[13] For the kinetic studies of MMA and MMB degradation, 2.5 ml of a buffer solution was spiked with 10 μl of a stock solution of the appropriate compound ($c = 5.8 \times 10^{-3}$ M) to give an initial concentration of 2.3×10^{-5} M. The degradations of MMA and MMB were followed by monitoring the increase of absorbance at 280 nm (pH < 5) and 360 nm (pH > 9). The 1 cm quartz cells were thermostated at 25 ± 0.2 °C. Between pH 5 and 9 the degradation was followed by using a stability-indicating HPLC assay. These experiments were initiated in the same way as the spectrophotometric measurements. The reaction solutions were kept in screw capped test tubes at 25 ± 0.2 °C in the dark. Periodically, samples were removed and immediately analyzed for undegraded mitomycin by the HPLC method. Degradation studies of MMC, PM and AcMMC at pH = 11.0 were performed by means of the previously reported HPLC method.[17]

High-Performance Liquid Chromatography (HPLC)——The chromatographic system consisted of a 6000 A solvent delivery system equipped with a UV 440 dual-wavelength detector (both from Waters Assoc., Milford, MA, U.S.A.) operating at 313 and 280 nm for the analysis of MMA and MMB. A stainless steel column (30 cm × 3.9 mm

i.d.) was packed by us with Lichrosorb RP18 (10 μm) packing material (Merck, Darmstadt, FRG). Two mobile phases were used. Mobile phase I, for MMA analysis, was methanol–water (50 : 50, w/w) to which 1% (v/w) 0.5 M sodium phosphate buffer (pH 7.0) was added. For the analysis of MMB decomposition mixtures mobile phase II was used, differing from mobile phase I only in the methanol–water ratio (35 : 65, w/w). The flow rate was 1.0 ml/min, and 20 μl samples were injected into the liquid chromatograph. Peak heights were measured to quantitate residual MMA and MMB. The peak height *versus* concentration plots of standard solutions of MMA and MMB were linear ($r > 0.999$) in the working concentration range (2.8×10^{-5}—1.4×10^{-6} M). Relative standard deviations of 0.6 and 2.8% were obtained for replicate injections ($n = 8$) of the 2.8×10^{-5} and 1.4×10^{-6} M solutions, respectively.

Thin-Layer Chromatography (TLC)——TLC was carried out on glass plates precoated with a 0.25 mm layer of Silicagel $60F_{254}$ (Merck, Darmstadt, FRG), using methanol as the mobile phase.

Ultraviolet-Visible (UV-VIS) Spectrophotometry——Absorbance measurements were performed with a Shimadzu UV-140 double beam spectrophotometer equipped with an ACP-140 Cell Positioner. UV-VIS spectra were recorded on a Shimadzu UV-200 double beam spectrophotometer.

Mass Spectrometry (MS)——Methane chemical ionization (CI) and field desorption (FD)-MS of the mitomycin degradation products were recorded; the experimental conditions and equipment were reported earlier.[25]

CD Spectroscopy——The CD spectra of the compounds were obtained at 25 °C in 1 cm quartz cells with a Jobin-Yvon dichrograph III.

Isolation of Degradation Products——The 7-hydroxymitosane degradation products of MMA and MMB were prepared, isolated and derivatized with propionic anhydride–pyridine prior to MS analysis as described earlier for MMA.[25] For the preparation and isolation of the mitosene degradation products, about 2 mg of MMA or MMB was dissolved in 2 ml of perchloric acid solution, pH 2. The solutions were allowed to stand at 25 °C in the dark for 1.5 h to ensure almost complete degradation. Subsequently, sodium phosphate was added to adjust the pH to 7. The aqueous solution was then evaporated to dryness under nitrogen and the residue was dissolved in methanol and subjected (as a band) to TLC. After development of the chromatogram, the zones corresponding to the mitosenes were scraped off and eluted with methanol. The eluates were subjected to HPLC, UV-VIS spectrophotometry, CD and FD-MS analysis.

Results

Degradation Products

An overall degradation scheme for MMA, MMB, MMC and PM is given in Fig. 2. The structure elucidation of the various degradation products was based on several analytical techniques.

7-Hydroxymitosanes——In alkali, the 7-substituent is replaced by a hydroxyl group. The pK_a of the resulting 7-enolic function, being 4.2 for the 7-hydroxymitosanes originating from both MMA and MMB, was determined titrimetrically by monitoring the absorbance at 365 nm as a function of acidity.[25] As the compounds only emerge at pH values over 6, they

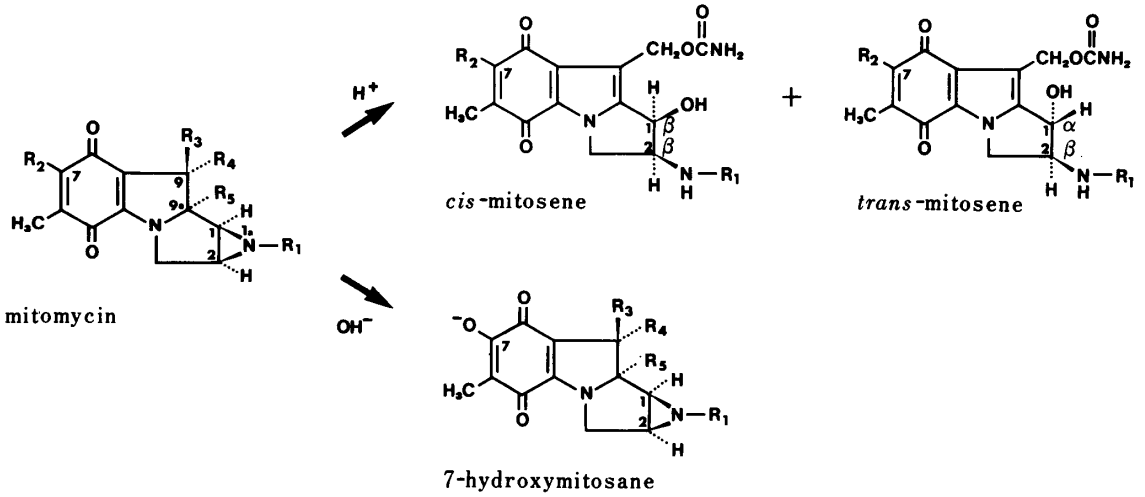


Fig. 2. Overall Degradation Scheme for MMA, MMB, MMC and PM in Acidic and Alkaline Solutions

See Fig. 1 for R_1, R_2, R_3, R_4 and R_5 definitions.

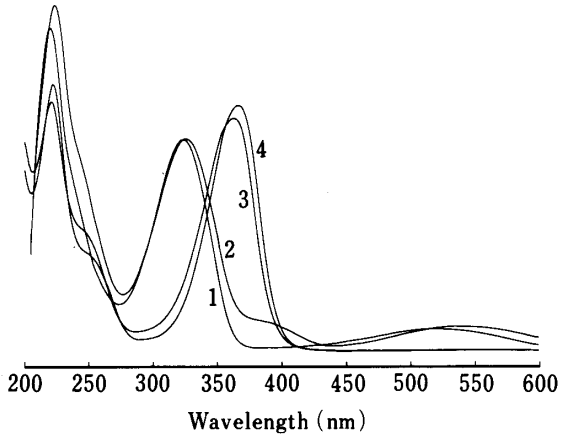


Fig. 3. UV-VIS Spectra of MMA (1) and MMB (2) in Methanol and of the 7-Hydroxymitosane Originating from MMA (3) and MMB (4) in 0.01 M Borate Buffer pH 9.5, Temperature 25 °C

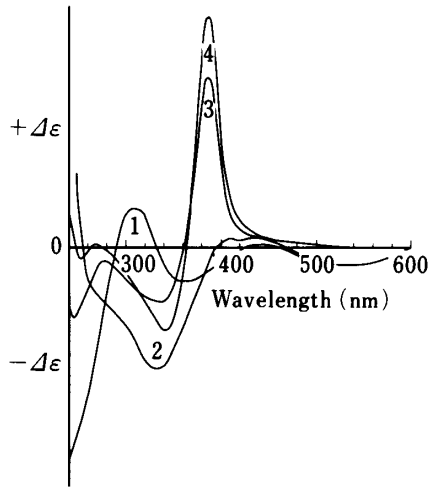


Fig. 4. CD Spectra of MMA (1), MMB (2) in Methanol and of the 7-Hydroxymitosanes Originating from MMA (3) and MMB (4) in 0.01 M Borate Buffer, pH 9.5

The ellipticity is plotted in arbitrary units; time constant 10 s; scan speed 2×10^{-1} nm/s. Temperature 25 °C.

are actually present in decomposition mixtures as anions. Moreover, as a consequence of their anionic nature at pH 7, the 7-hydroxymitosanes elute with the solvent front using mobile phase I or II. Retention can be achieved when a quaternary ammonium pairing ion is added to the eluent.[25] The UV-VIS spectra of the deprotonated 7-hydroxymitosanes derived from MMA and MMB show marked similarity, but are not identical (Fig. 3). This is also the case for the spectra of their mitosane precursors (Fig. 3). Compared with MMA and its 7-hydroxy analogue, the spectra of MMB and its 7-hydroxymitosane derivative, respectively, show a small but significant bathochromic shift (Fig. 3). The CD spectra of MMA and MMB display distinct differences in the region of 240—460 nm (Fig. 4). On the other hand, the CD spectra of their 7-hydroxy derivatives are only slightly different (Fig. 4). After propionylation of the 7-enolic group the difference in chirality between the compounds becomes obvious in the 300 nm region, analogous with the CD behavior of their mitosane precursors MMA and MMB at 300 nm. The CD and UV-VIS spectra of the 7-hydroxymitosanes and their propionylated derivatives, originating from MMA and MMC, are identical. By propionylation of the 7-enolic function, as well as the aziridine secondary nitrogen of the MMA 7-hydroxy degradation product, the compounds can be made sufficiently volatile for MS analysis. The most intense ion in the CI-MS spectrum of the propionylated 7-hydroxymitosane originating from MMA was *m/e* 355 ($M^+ - H_3COH$, O_2CNH_2; 100%). Other fragments were: *m/e* 415 ($M^+ - H_3COH$; 20%) and *m/e* 387 ($M^+ - O_2CNH_2$; 12%). The protonated molecular ion *m/e* 448 (MH^+; 10%) shows a low relative abundance. The propionylated 7-hydroxymitosane derivative from MMB fragments in an analogous pattern: *m/e* 392 (MH^+; 20%), *m/e* 373 ($M^+ - H_2O$; 10%) and *m/e* 313 ($M^+ - H_2O$, O_2CNH_2; 100%). FD-MS provided M^+ and MH^+ ions that are in agreement with the assigned structures.

Mitosenes——The diastereomeric mitosene degradation products of MMA and MMB can be very effectively separated and isolated by the use of TLC and HPLC. The chromatographic properties of the compounds are listed in Table I. The four mitosene degradation products show identical UV-VIS spectra in methanol with absorption maxima (λ_{max}: 235, 285, 345, 435 nm) characteristic for the 7-methoxymitosene chromophore. CD analysis of the *cis*- and *trans*-mitosenes showed pronounced differences. Based on 1,2-*cis*-2-

TABLE I. Chromatographic Properties of MMA and MMB and Their Mitosene Degradation Products

	Rf [a]	k' [b]
Mitomycin A	—	2.86 (I)
1,2-*trans*-2-Amino-1-hydroxy-7-methoxymitosene	0.47	1.08 (I)
1,2-*cis*-2-Amino-1-hydroxy-7-methoxymitosene	0.35	1.84 (I)
Mitomycin B	—	1.89 (II)
1,2-*trans*-2-Methylamino-1-hydroxy-7-methoxymitosene	0.54	2.75 (II)
1,2-*cis*-2-Methylamino-1-hydroxy-7-methoxymitosene	0.42	5.9 (II)

a) Stationary phase, silica gel; mobile phase, methanol. *b*) Column, Lichrosorb 10RP18; mobile phase I or II, flow rate 1.0 ml/min.

amino-1-hydroxy-7-methoxymitosene and 1,2 *trans*-2-amino-1-hydroxy-7-methoxymitosene as reference compounds,[26] it appears that the sign of the 465 nm CD Cotton effect correlates with the C1 configuration, in the same manner and in analogy with the 2,7-diamino-1-hydroxymitosenes orginating from MMC.[27] Conversion of the 7-methoxymitosenes into 7-hydroxymitosenes, similar to the MMA and MMB conversion into their 7-hydroxymitosane analogues, provided compounds with the $\Delta\varepsilon$ (540 nm) values reported earlier.[27] These experiments further support our assignments (1,2-*cis* or 1,2-*trans*) for the 7-methoxymitosenes. The sign of the CD signal at 445 nm is associated with the 1,2-stereochemistry of the mitosenes derived from MMB in the same manner as for those derived from MMA.

In summary, a positive Cotton effect around 540 nm for the 7-hydroxymitosenes[27] and around 450 nm for the 7-methoxymitosenes correlates to the C1β configuration while negative signs at these wavelengths are associated with the C1α configuration. The C2-amino group always has the β configuration, a position similar to the precursor aziridine ring. Epimerization at C2 during aziridine ring opening has never been observed and is not very likely considering the proposed degradation mechanisms.[13] CI-MS analysis of the mitosene compounds was troublesome as only very weak signals in the low mass region were obtained. However, FD-MS provided parent masses (M^+; MH^+) consistent with the assigned structures. In some cases a fragment ($M^+ - 43$) was observed apart from the molecular ion. This might be explained by loss of the C10-carbamate by elimination of isocyanic acid (HNCO).[28]

Degradation Kinetics

Analytical Procedures——The spectral changes due to MMB degradation in acidic and alkaline medium are shown in Figs. 5 and 6, respectively. MMA exhibits similar behavior. The changes are pronounced and justify the use of UV spectrophotometry to quantitate the degradation. The diastereomeric mitosenes are spectrally equivalent. This enables us to represent spectrophotometrically the conversion of a mitomycin into its mitosenes as the conversion into a single compound instead of the formation of two degradation products. The presence of isosbestic points is in complete agreement with this model. At 280 nm for the acid degradation and at 360 nm for the alkaline degradation, the absorbance on degradation (A_t) is the sum of the absorbances of the degradation product(s) (A_t2) and the absorbance of the mitosane precursor (A_t1); so

$$A_t = A_t1 + A_t2 . \tag{1}$$

The disappearance of the precursor can be represented by

$$A_t1 = A_0 \cdot e^{-k_{obs} \cdot t} \tag{2}$$

where A_0 is the absorptivity at $t=0$, and k_{obs} the observed pseudo first-order rate constant.

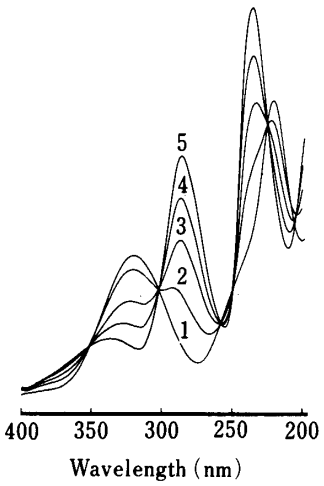


Fig. 5. UV Spectra of MMB

$t=0$ min (1), $t=5$ min (2), $t=15$ min (3), $t=30$ min (4) and $t=60$ min (5) after the start of degradation at pH 3.0. Temperature 25 °C.

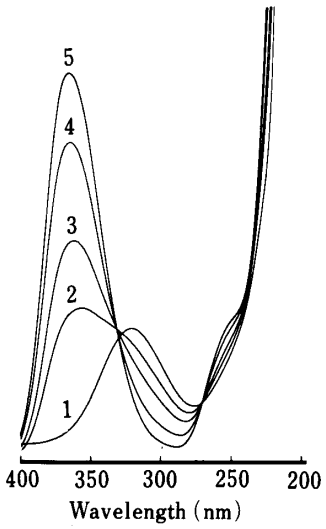


Fig. 6. UV Spectra of MMB

$t=0$ min (1), $t=20$ min (2), $t=36$ min (3), $t=75$ min (4) and $t=170$ min (5) after the start of degradation in 0.1 M phosphate buffer, pH 11.0. Temperature 25 °C.

The appearance of the degradation product(s) is represented by

$$A_t 2 = A_{max} \cdot (1 - e^{-k_{obs} \cdot t}) \tag{3}$$

where A_{max} is the absorptivity at complete conversion of the mitomycin into the degradation product(s). Consecutive degradative reactions can be neglected here. Combination of Eqs. 1, 2 and 3 yields

$$A_t - A_{max} = (A_0 - A_{max}) \cdot e^{-k_{obs} \cdot t} \tag{4}$$

or after rearrangement

$$\ln \left(\frac{A_t - A_{max}}{A_0 - A_{max}} \right) = -k_{obs} \cdot t \tag{5}$$

From Eq. 5, k_{obs} can be extracted graphically. At pH < 5 only mitosenes occur and at pH > 9 the 7-hydroxymitosane is the only degradation product. In the region of 5 < pH < 9 a mixture of these compounds is formed. Therefore, HPLC methods were preferred for degradation studies in that pH region. With the use of the presented HPLC assays the 7-hydroxymitosanes elute with the solvent front and the mitosenes are well separated from their mitosane precursor (Table I).

Order of Reaction——On degradation in buffers, a linear relationship exists between the natural logarithm of residual 7-methoxymitomycin concentration and time, from which the degradation reactions appear to be (pseudo) first-order. This is in agreement with literature data on the degradation kinetics of 7-aminomycins.[13,14,16-18]

Standard Deviation in k_{obs}——The standard deviation (S.D.) in k_{obs} was determined for the degradation of MMB using the UV spectrophotometrical and HPLC assays. These experiments were performed at pH 11.0 and 0.01 M phosphate buffer concentration. The values of $k_{obs} \pm$S.D. were $2.30 \pm 0.01 \times 10^{-4}$ s^{-1} ($n=6$) for the spectrophotometric method and $2.24 \pm 0.04 \times 10^{-4}$ s^{-1} ($n=6$) for the HPLC method. The two techniques can be considered as equivalent for the purpose of quantitation of the degradation of mitomycins. Other rate constants are mean values of duplicate determinations.

Influence of pH——On degradation of the mitomycins in acidic medium the mitosenes,

which have basic properties, arise, while in alkali an acidic degradation product is formed. This leads to a gradual change of pH on degradation in an unbuffered solution. Therefore, the kinetic studies were performed in buffers. From experiments at constant pH and various buffer concentrations it appeared that buffer ions influence the degradation rate. Therefore the kinetic studies at each pH were performed at several buffer concentrations and the observed rate constants (k_{obs}) were corrected for buffer influences by extrapolation to zero buffer concentration to give the rate constant (k') for that pH, incorporating only H^+, OH^- and H_2O catalysis. A linear relationship exists at each pH between k_{obs} and the concentration of phosphate (up to 0.25 M) or borate (up to 0.05 M) or acetate (up to 0.1 M). The obtained k' is defined by Eq. 6

$$k' = k_0 + k_H \cdot [H^+] + k_{OH} \cdot [OH^-] \quad (6)$$

where k_0 is the pseudo first-order rate constant for degradation in water only, while k_{OH} and k_H are the second-order rate constants for hydroxyl ion- and proton-catalyzed degradations, respectively. If the substrate studied occurs in protonated and deprotonated forms, each of these forms can undergo solvent-, proton- and hydroxyl ion-catalyzed degradation. These reactions are influenced by the pH in different ways. At pH values $\ll pK_a$ the mitomycins are virtually exclusively present in the protonated form; the concentration of this form is then constant. The concentration of the deprotonated neutral form in this pH region is inversely proportional to the proton concentration. At pH values $\gg pK_a$ the concentration of the deprotonated form is constant. The concentration of the protonated form in this pH region is proportional to the proton concentration. The degradation rates of the six degradation reactions are all proportional to either the concentration of the protonated or the deprotonated form. They therefore exhibit an abrupt change at the pK_a value of the mitomycin

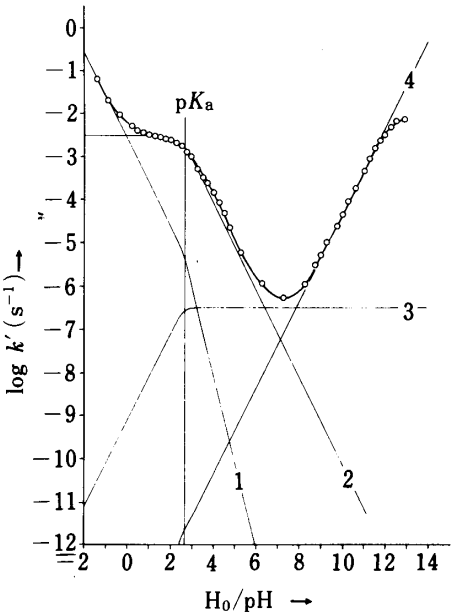


Fig. 7. pH–Rate Profile for MMA and Theoretical pH Dependence of the Individual Degradation Reactions

1. proton-catalyzed degradation of the protonated form. 2. proton-catalyzed degradation of the deprotonated form; solvent-catalyzed degradation of the protonated form. 3. solvent-catalyzed degradation of the deprotonated form; hydroxyl ion-catalyzed degradation of the protonated form. 4. hydroxyl ion-catalyzed degradation of the deprotonated form.

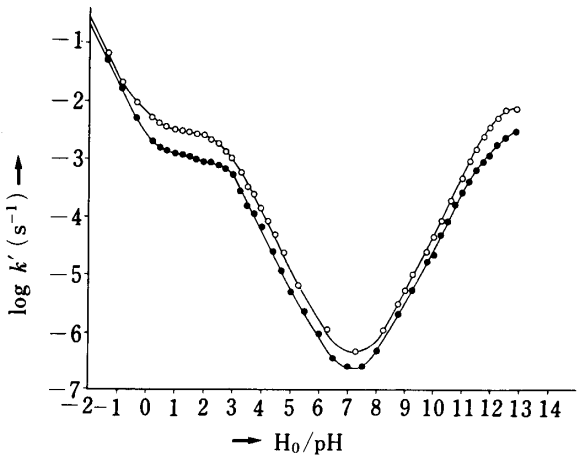


Fig. 8. H_0/pH–Rate Constant Profiles for MMA (○) and MMB (●) Degradation at 25 °C

involved. The pH dependencies of the degradations by the individual reactions are determined by both the pH dependence of the concentration of the substrate and that of the reactant (protons, solvent or hydroxyl ions). Combining the influence of the pH on the concentrations of the substrate and the reactants indicates that the pH dependence of the six reactions can be categorized into four types. These are illustrated in Fig. 7. Type 1 is caused by the proton-catalyzed degradation of the protonated mitomycin form. Type 4 is caused by the hydroxyl ion- catalyzed degradation of the deprotonated form. The proton-catalyzed degradation of the deprotonated form and the solvent-catalyzed degradation of the protonated form both show the pH dependence of type 2. As it is impossible to deduce from the pH dependence which reaction occurs, these reactions are called kinetically indistinguishable. The solvent catalyzed degradation of the deprotonated substrate and the hydroxyl ion-catalyzed degradation of the protonated substrate are also kinetically equivalent reactions. They show the type 3 pH dependence. The overall pH–rate profile is obtained by summation of the profiles of the four types, as shown for MMA in Fig. 7. Without further mathematical analysis of the plotted data, the occurrence of degradation reactions of types 1, 2 and 4 can be deduced from this figure. The pH profiles of both MMA and MMB are shown in Fig. 8 and can be described in the pH region −2—12, as:

$$k' = (k_0^+ + k_H^+ \cdot [H^+] + k_{OH}^+ \cdot [OH^-])f + (k_0 + k_H \cdot [H^+] + k_{OH} \cdot [OH^-])(1-f) \qquad (7)$$

where the superscript + refers to the protonated mitomycin species; when no superscript is used it refers to the neutral, deprotonated mitomycin form. The subscripts 0, H and OH refer to the solvent-, proton- and hydroxyl ion-catalyzed degradation reactions, and f is the mole fraction of the protonated mitomycin species (consequently $(1-f)$ is the mole fraction of the neutral, deprotonated species). The K_a value of the mitomycins is defined by:

$$K_a = \frac{[H^+] \cdot (1-f)}{f} \qquad (8)$$

Combination and rearrangement of Eqs. 7 and 8 and insertion of $[OH^-] = \frac{K_w}{[H^+]}$ leads to:

$$k' = \frac{k_H^+ \cdot [H^+]^2 + (k_0^+ + k_H \cdot K_a)[H^+] + (k_{OH} \cdot K_w \cdot K_a)\frac{1}{[H^+]} + (k_{OH}^+ \cdot K_w + k_0 \cdot K_a)}{[H^+] + K_a} \qquad (9)$$

The $(k_0^+ + k_H \cdot K_a)$ term and $(k_{OH}^+ \cdot K_w + k_0 \cdot K_a)$ term are designated as k_I and k_{II}, respectively. Using a nonlinear curve fitting program, the specific rate constants k_H^+ and k_{OH}, the "coupled rate constants" in terms of k_I and k_{II} and the dissociation constant K_a for MMA and MMB were calculated. The values are listed in Tables II and III. The rate constants k_H^+ and k_{OH} can also be calculated from the linear parts of the pH profile that correspond with the type 1 and 4 pH dependences by linear regression analysis using a least-squares method. The pK_a value can be estimated graphically from the inflection point of the type 2 part of the pH profile. The

TABLE II. Rate Constants[a] for Catalyzed Degradation Reactions of Mitomycins at 25 °C

Rate constant	MMA	MMB	MMC	PM
k_H^+	2.6×10^{-3}	1.8×10^{-3}	1.8×10^{-3}	9.2×10^{-4}
k_I	3.1×10^{-3}	1.1×10^{-3}	1.8×10^{-3}	3.0×10^{-3}
k_{II}	3.3×10^{-7}	2.7×10^{-10}	n.d.	n.d.
k_{OH}	0.465	0.259	1.2×10^{-2} [17]	n.d.

a) k_H^+ and k_{OH} in $mol^{-1}\,s^{-1}$; k_I in s^{-1}; k_{II} in $mol\,s^{-1}$; n.d. = not determined.

TABLE III. Aziridine pK_a Values of Several Mitomycins at 25 °C

MMA	2.62	PM	2.40
MMB	2.80	AcMMC	<0
MMC	2.74		

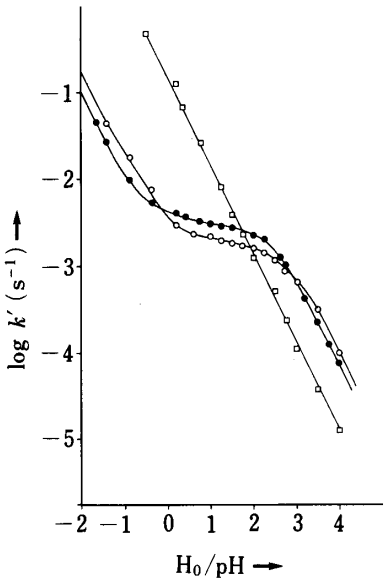


Fig. 9. H_0/pH–Rate Constant Profiles for MMC (○), PM (●) and AcMMC (□) Degradation at 25 °C

constants thus obtained correspond well with those calculated with the nonlinear curve fitting program. To study the effect of the pK_a on the shape of the pH profile the observed rate constants of MMC, PM and AcMMC were measured between pH −2 and 4. The results are shown in Fig. 9. From the graphs it is clear that MMC and PM exhibit a pH-dependent degradation involving a combination of type 1 and 2 processes, which corresponds to the pH profiles observed for MMA and MMB in this pH region. Using the nonlinear curve-fitting program with a reduced model equation (Eq. 10). the k_H^+, k_I and K_a values have been calculated for MMC and PM.

$$k' = \frac{k_H^+ \cdot [H^+]^2 + k_I \cdot [H^+]}{[H^+] + K_a} \tag{10}$$

The results are listed in Tables II and III. The pH–rate profile of AcMMC differs from that of MMA, MMB, MMC and PM in that there is no indication of a type 2 pH dependence. This can be explained by the loss of the basic character of the aziridine group attributable to the electron withdrawing properties of the acetyl group. The pH profile of this substance should therefore be described by a combination of the proton-, solvent- and hydroxyl ion-catalyzed degradations of a nonprotonated species. The overall reaction constant is then given by:

$$k' = k_H \cdot [H^+] + k_0 + \frac{k_{OH} \cdot K_w}{[H^+]} \tag{11}$$

From the data obtained it can be seen that for pH values between −0.5 and 4 the degradation rate is determined by a proton catalyzed decomposition reaction. Linear regression analysis of the data yields a value for k_H of $0.14\,mol^{-1}\,s^{-1}$.

The slopes in the pH profiles (Figs. 8, 9) of MMA, MMB, MMC and PM at pH<0 are −1, revealing specific proton catalysis for the degradation of the protonated species. In the region of 3<pH<6 the slope is −1, demonstrating that the degradation in that pH region is first-order with respect to (H^+) and/or the concentration of protonated mitomycin species.

TABLE IV. Rate Constants[a] for Buffer Catalyzed Degradation Reactions of MMA and MMB at 25 °C

Rate constant	MMA	MMB
k_{Ac}^{+}	0.39	0.05
k_{Ac}; k_{HAc}^{+}; k_{HAc}	0	0
$k_{H_2PO_4}$	2×10^{-5}	4×10^{-5}
k_{HPO_4}	5×10^{-5}	8×10^{-5}
k_{BO_2}	3.4×10^{-4}	7.4×10^{-4}
$k_{H_3BO_2}$	0	0
k_{Cl}	0	0

a) Rate constants in $mol^{-1}s^{-1}$.

The $\log k'$–pH plot for AcMMC has a slope equal to -1, indicating that the degradative process of this compound involves specific acid catalysis. In the region of $8<pH<11.5$, a positive slope equal to unity emerges (Fig. 8) indicating specific base catalysis for the degradation of neutral MMA and MMB species. Maximum stability for MMA and MMB occurs at pH 7.2, the half lives ($t_{1/2}$) being 470 and 812 h, respectively.

Influence of Buffer Ions——Buffer ions catalyze the degradation of MMC and PM,[13,17,18] as was also ascertained form MMA and MMB. Linear relationships between k_{obs} and total buffer concentration indicate the occurrence of general acid-base catalysis. The quantitation of the influences of phosphate and borate was accomplished in the same way as reported earlier.[17] The results are included in Table IV. For acetate–acetic acid buffers the catalysis was quantified as follows. The pK_a of acetic acid is 4.76, which implies that at pH 2.5, virtually all acetate is present as (protonated) acetic acid. At pH 2.5 MMA and MMB exist as both neutral and protonated species and therefore, in the presence of HAc, equation 7 must be extended to:

$$k_{obs}=(k_0^{+}+k_H^{+}\cdot[H^+]+k_{OH}^{+}\cdot[HO^-])f+(k_0+k_H\cdot[H^+]+k_{OH}\cdot[OH^-])(1-f)+(k_{HAc}^{+}\cdot f+k_{HAc}\cdot(1-f))[HAc] \quad (12)$$

where f represents the mole fraction of the protonated mitomycin species to the total mitomycin concentration and consequently $(1-f)$ is the mole fraction of the neutral mitomycin species. The values can be calculated by using Eq. 8. Experiments at pH 2.5 and increasing (HAc) (up to 0.5 M) revealed no change in the degradation rate due to the presence of acetic acid. Consequently the summation $(k_{HAc}^{+}\cdot f+k_{HAc}\cdot(1-f))$ and, obviously, k_{HAc}^{+} and k_{HAc} are zero. In the pH region near the dissociation constant of acetic acid ($pK_a=4.76$), the expression for k_{obs} is

$$k_{obs}=(k_0^{+}+k_H^{+}[H^+]+k_{OH}^{+}[OH^-])f+(k_0+k_H[H^+]+k_{OH}[OH^-])(1-f)+(k_{Ac}^{+}\cdot f+k_{Ac}(1-f))[Ac^-] \quad (13)$$

A plot of k_{obs} *versus* $[Ac^-]$ at a fixed pH yields a straight line with slope $(k_{Ac}^{+}\cdot f+k_{Ac}(1-f))$. By repeating this procedure at other pH values in the region around the pK_a of acetic acid, a plot can be made of the obtained slopes *versus* f. This resulted in a straight line with slope $(k_{Ac}^{+}-k_{Ac})$ and intercept k_{Ac}. The calculated values are listed in Table IV.

Influence of Additives——The influence of chloride and acetate ions on the hydroxyl ion-catalyzed degradation of MMA and MMB has been established by adding various concentrations of the appropriate sodium salts of solutions of the mitomycins at pH 10.5 while the total buffer concentration was kept constant. No significant alterations in k_{obs} were observed even when the salt level was increased to 1.0 M. Consequently the specific rate

Table V. Activation Energies (E_a) and Frequency Factors (A) for the Degradation of MMA and MMB at Various pH Values

H_0/pH	MMA		MMB	
	E_a [a]	A [b]	E_a [a]	A [b]
−1.00	84	1×10^{13}	75	2×10^{11}
0.35	88	2×10^{13}	83	1×10^{12}
1.12	84	2×10^{12}	73	8×10^{9}
2.00	92	3×10^{13}	85	1×10^{12}
2.98	84	6×10^{11}	77	2×10^{10}
3.98	77	4×10^{9}	72	4×10^{8}
5.04	72	5×10^{7}	75	8×10^{7}
6.03	81	2×10^{8}	76	2×10^{7}
7.04	80	5×10^{7}	82	9×10^{7}
8.02	96	5×10^{10}	103	2×10^{12}
8.50	84	8×10^{8}	94	3×10^{10}
9.03	102	3×10^{12}	98	2×10^{11}
9.50	76	1×10^{8}	87	5×10^{9}
9.97	56	3×10^{5}	56	2×10^{5}
11.04	63	7×10^{7}	66	2×10^{8}
12.11	61	2×10^{8}	71	4×10^{9}
12.93	93	1×10^{14}	91	3×10^{13}

a) E_a in kJ mol^{-1}; *b*) A in s^{-1}.

Table VI. Pseudo-First Order Rate Constants for the Degradation of Mitomycins in 0.01 M Phosphate Buffer pH 11.0 at 25 °C

	k_{obs} [a]
MMA	4.55×10^{-4}
MMB	2.24×10^{-4}
MMC	1.19×10^{-5}
PM	1.42×10^{-5}
AcMMC	4.25×10^{-4}

a) k_{obs} in s^{-1}.

constants for the chloride (k_{Cl}) and acetate (k_{Ac}) ions are zero. The same applies to MMC.[17]

Influence of Temperature——The influence of temperature on the degradation of MMA and MMB was investigated in 0.01 M buffers, perchloric acid and sodium hydroxide solutions. The temperature range was 20—50 °C. At each pH a linear relationship was found between the reciprocal of absolute temperature ($1/T$) and the natural logarithm of the observed rate constant ($\ln k_{obs}$), so the Arrhenius equation is obeyed. The intercept ($\ln A$) and the slope ($-E_a/R$) of the straight line allows A (the frequency factor) and E_a (the Arrhenius activation energy) to be evaluated. The values are listed in Table V.

Chemical Stability of the Mitomycins at pH 11.0——The degradation of the four naturally occurring mitomycins at pH 11.0 involves hydrolysis of the 7-substituent, yielding the corresponding 7-hydroxymitosane. AcMMC is first converted to MMC. The k_{obs} values for the degradation of the compounds were determined in 0.01 M phosphate buffer, pH 11.0, and are listed in Table VI.

Discussion

Considering the similarity between the chromophoric parts of MMA and MMB, the UV-VIS spectra were expected to be identical. However, significant differences were observed for MMA and MMB as well for their 7-hydroxymitosane derivatives (Fig. 3). Possible explanations are a) influence of the C9a function on the electron density at N4 and, thus, on the chromophore, b) interaction between the C9 side chain and the chromophore. As the UV-VIS spectra of PM and MMC are identical, the N1a-methyl function appears to have no influence.

The CD spectra of MMA and MMB show pronounced qualitative differences (Fig. 4). Such mutual differences were not found for the 7-hydroxymitosanes. After propionylation of the 7-enolic function the difference in chirality could be proven. From this study is appears that a positive CD Cotton effect in the 450 nm region is associated with a C1β configuration,

and a negative Cotton effect is associated with a C1α configuration of the 2-amino- and 2-methylamino-7-methoxy-1-hydroxymitosenes. This CD signal correlates with the weak absorption of the compounds at 435 nm. This is in complete analogy with the 2,7-diamino-1-hydroxy and 2-amino-1,7-dihydroxymitosenes originating from MMC. Tomasz and associates[27,29] recently described the use of the sign of the weak CD Cotton effect in the longer wavelength region to assign the configuration at C1 in the mitosene degradation products of MMC, PM and 10-decarbamoyl-MMC.

This study demonstrates that the inflexion in the pH profile of the mitomycins is unambiguously associated with the protonation of the aziridine nitrogen. In an earlier study[13] on the degradation of MMC and PM at 40 °C the kinetic data were erroneously used for the calculation of the aziridine pK_a. A correction is given in this report, based on the use of a curve-fitting program. In the literature, several pK_a's have been reported (MMC in 50% methanol, 3.2[15]; MMB, 4.3[11]). These values were determined by potentiometric methods, and degradation would inevitably have interfered. On degradation the mitosenes, with moderately basic properties (pK_a *ca.* 7[16]), appear and this may account for the fact that the values are higher than the kinetically determined pK_a values presented in this report (Table III). We consider the present pK_a values to be more accurate and reliable. pK_a differences as those found for MMC and PM are usually encountered with secondary and tertiary amines (compare dimethylamine, 10.77 and trimethylamine, 9.80). The lower pK_a of PM should be explained by a lesser degree of hydration and, therefore, lesser stabilization of the cation. This explanation is not applicable for the pK_a difference between MMA and MMB. The C9a hydroxyl function (or even the steric configuration of the C9 urethane side chain) in MMB may be contributing to an increase in the hydration possibilities and therefore to the stabilization of the protonated molecule. Above all it is striking that the aziridine pK_a values are very low compared with other aziridines.[30] Hypotheses for this unusually weak basicity have been proposed in the literature[18,31] but seem unsatisfactory.

Mitosenes are the main degradation products at pH values <5. This indicates that mitosenes are formed both by the proton-catalyzed degradation of the protonated form and by the reaction(s) resulting in the coupled constant k_I. The hydroxyl ion-catalyzed degradation of the deprotonated form results in the formation of 7-hydroxymitosanes. The nature of the reaction products formed by the reaction(s) which result in the coupled constant k_{II} is not known. Elucidation of their nature is rather difficult due to the fact that these coupled reactions only contribute to the overall pH profile significantly at the pH region of approximately 7, where the stability of the mitomycins is maximal. Furthermore, at this pH the degradation reactions of types 2 and 4 still contribute significantly to the overall degradation.

The mechanism of the acid-catalyzed degradation of mitomycins has intrigued many investigators.[13,18,31–34] From this and other reports[34] it appears that the 7-methoxymitomycins MMA and MMB are degraded analogously with 7-aminomitomycins MMC and PM. In acid, the labile hydroxyl (for MMB) or methoxyl group (for MMA, MMC and PM) leaves after protonation as water or methanol, respectively, resulting in a C9–9a double bond and subsequent cleavage of the aziridine moiety. This degradative pathway was proposed for MMC and PM[13] and was recently supported by deuterium incorporation experiments done by Iyengar and Remers.[32] The mechanism also seems applicable to the 7-methoxymitomycins.

The temperature effects on the degradation of MMA and MMB show no clear trend, and no conclusions can be drawn from Table V. The data should therefore only be used for the calculation of k_{obs} values at different temperatures.

From the data in Table VI, it appears that the 7-methoxy function is more susceptible to alkali than a 7-amino group. This may be due to a relatively low electron density at C7 in the

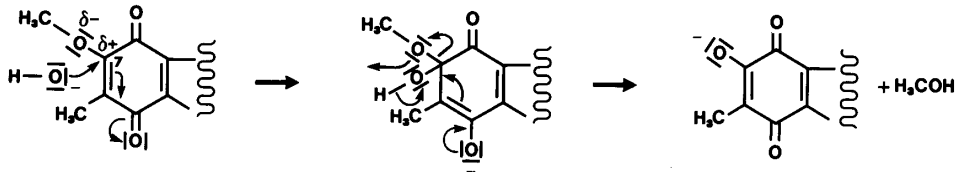


Fig. 10. Mechanism for the Initial Degradation of 7-Methoxymitomycins in Alkaline Medium

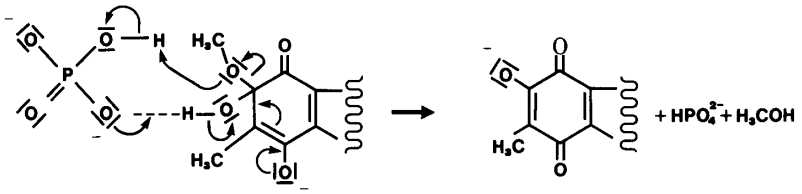


Fig. 11. Phosphate Catalysis of the Initial Degradation of 7-Methoxymitomycins in Alkaline Medium

case of MMA and MMB in which C7 is attacked more easily by hydroxyl ions. The reaction scenario is represented in Fig. 10. The proton transfer in the mechanism depicted in Fig. 10 is facilitated by the intervention of water, hydroxyl ions or buffer ions. Figure 11 illustrates the action of dibasic phosphate as a proton conductor. An analogous mechanism can be written for the hydrolysis of the 7-amino group of MMC and PM, with ammonia as the leaving group. The instability of AcMMC st pH 11.0 can be explained by the base susceptibility of the N1a-amide linkage.

MMB is more stable than MMA over the entire pH region (Fig. 8), independent of the type of degradation reaction. This may suggest an overall favorable solvatation of MMB in aqueous solution, whereas the solute is less rapidly attacked by protons or hydroxyl ions. Unfortunately evidence in favor of this hypothesis can not be presented as yet. Another feature that remains to be elucidated is the curvature of the pH profiles of MMA and MMB at pH >11—12. An inflection at these high pH values could be explained in the case of MMC by the occurrence of keto-enol tautomerism and deprotonation of the 7-aminoquinoid chromophore.[17] Such an explanation can not hold in the case of the 7-methoxymitosanes. Further research may cast more light on this matter.

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