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Degradation of mitomycin C in acid phosphate and acetate buffer solutions

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Summary

The degradation of mitomycin C in acid phosphate and acetate buffers has been investigated utilizing high-performance liquid chromatography with ultraviolet detection. Acetylated mitosene degradation products were identified with the use of chromatographic techniques, ultraviolet spectrophotometry and field desorption mass spectrometry. Stereochemical assignments were given using circular dichroism spectroscopy. Rate constants of the degradation as function of pH are presented and some mechanistic aspects of the acid degradation of mitomycin C are discussed.

Introduction

Among the group of clinically useful cytostatics, mitomycin C (MMC) (Fig. 1), takes an interesting position as the drug exerts cytotoxicity only after it has been activated. After the activation step MMC is capable of alkylating DNA and inducing cross-linkages between complementary strands of the nucleic acid (Carter and Crooke, 1979).

Two pathways have been advocated for the in vivo activation of the MMC molecule. The first pathway involves reduction of the quinone to its semiquinone or hydroquinone, inducing cleavage of methanol from C9a and C9 and aziridine ring opening, generating an electrophilic center at C1 which serves as a target for nucleophilic DNA moieties. Successive cleavage of the carbamoyl group might introduce a second alkylating center at C10 (Szybalski and Iyer, 1967; Moore, 1977). Therefore, MMC is often designated as a monofunctional as well as bifunctional bioreductive alkylating agent (Lin et al., 1974; Moore and Czerniak, 1981). The second activation pathway involves acid degradation of MMC and requires no reducing conditions (Lown et al., 1976; Lown and Weir, 1978; Tomasz and Lipman, 1979). Acid hydrolysis of MMC starts with protonation of the

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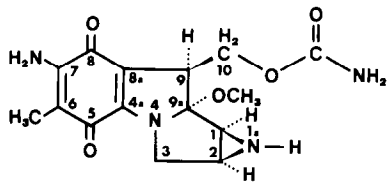

Fig. 1. Structure of mitomycin C.

C9a methoxy function, through which it becomes an excellent leaving group and is expelled as methanol. Next, the C9 hydrogen of the resulting cation is cleaved affording a double-bond between C9 and C9a. The 1,2-fused aziridine ring of the resulting indoloquinone opens after protonation, generating a carbonium ion at C1 (intermediate Im, Fig. 2). Nucleophilic water attack at C1 yields 1,2-*trans*- and 1,2-*cis*-2,7-diamino-1-hydroxymitosene (III and IV, Fig. 2) (Underberg and Lingeman, 1983; Iyengar and Remers 1985; McClelland and Lam, 1985). In the presence of nucleophiles such as phosphate ions and nucleotides nucleophile–mitosene adducts, covalently linked at C1, are generated (Tomasz and Lipman, 1979; Tomasz et al., 1985).

The reduction and acid activation mechanisms show much resemblance, differing actually only in the oxidation level of the reactive intermediate species. However, the acid degradation mechanism does not fit with a second alkylating center. Albeit that the yield is low, it has been found that cross-linking occurs after acid activation (Lown and Weir, 1978). The chemical nature of these DNA cross-linkages formed by acid-activated MMC and the underlying mechanisms have not been unraveled yet. The alkylating capability of MMC under reductive conditions has been investigated in detail using model compounds as incoming nucleophiles (Hornemann et al., 1976, 1979, 1983; Bean and Kohn, 1983, 1985) as well as DNA (Hashimoto et al., 1982, 1983). On the other hand, studies on the alkylating reactivity of MMC at low pH with nucleophilic model compounds are limited and provide no quantitative data of the degradative reactions. The goal of the present study is to provide a contribution to the understanding of the acid activation of MMC, especially the stereochemical features of the aziridine ring opening. The acid degradation of MMC was explored with water, phosphate and acetate ions

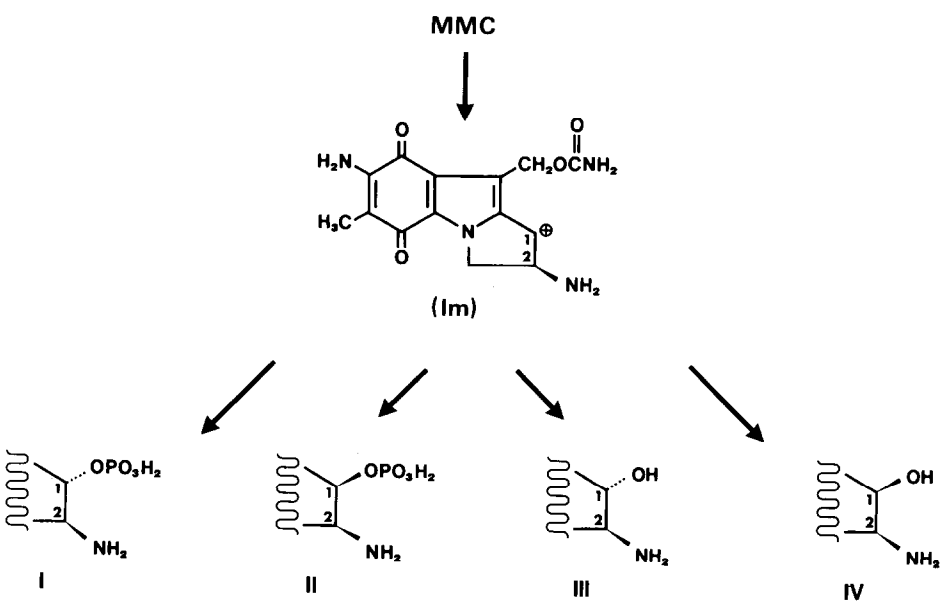

Fig. 2. Degradation scheme of MMC in 1.0 M phosphate buffers (pH < 6).

as entering nucleophiles. An isocratic reversed-phase high-performance liquid chromatographic (HPLC) assay has been devised in which MMC and eight mitosene degradation products can be separated, quantified simultaneously and isolated. The mitosene products were identified and rate constants are given. Furthermore some remarks on the acid-catalyzed alkylation scenario are presented.

This project is a sequel to earlier studies in which the mechanism of the conversion of MMC into 1,2-*trans*- and 1,2-*cis*-2,7-diamino-1-hydroxymitosene was investigated (Underberg and Lingeman, 1983; Beijnen and Underberg, 1985).

Experimental

Materials

MMC was kindly provided by Bristol Myers (Weesp, The Netherlands). All other chemicals were of analytical grade and deionized water was used throughout.

Buffer solutions

1.0 M phosphate and 1.0 M acetate buffers were prepared by dissolving the appropriate sodium salt and the pH values of these solutions (ranging from 1.0 to 6.0) were adjusted with perchloric acid and sodium hydroxide. pH measurements were performed at 25°C using a glass-reference electrode and pH meter (Metrohm, E516 Titriskop, Herisau, Switzerland).

Kinetic measurements

The buffer solutions, kept in stoppered glass test-tubes, were allowed to equilibrate to the temperature of study (25 ± 0.2°C), controlled by a thermostatic water bath. The degradation was initiated by spiking 3 ml of a buffer solution with 15 μl of a stock solution of MMC in methanol (3×10^{-2} M). At appropriate intervals 15 μl aliquots of these solutions were taken and injected immediately onto the HPLC column. All analyses were performed in duplicate.

Apparatus and analytical procedures

The conversion of MMC into the mitosene degradation products was monitored by reversed-phase HPLC. For the analysis of the decomposition mixtures in the phosphate buffers, the chromatographic system, developed by Andrews et al. (1983) was used. This system was realized with two model 510 pumps, a model 680 Automated Gradient Controller, a Radial-Pak C_{18} cartridge (10 μm) (10 cm × 8 mm, i.d.) in combination with a RCM-100 Radial Compression Module and a model 440 dual wavelength UV detector (all from Waters Assoc., Milford, MA, U.S.A.) operating at 254 and 313 nm. MMC and the mitosenes were eluted against a linear gradient from 100% mobile phase A (aqueous phosphate (1×10^{-3} M) buffer pH 7) to 100% mobile phase B (methanol + aqueous phosphate (1×10^{-3} M) buffer pH 7; 50 + 50 v/v) in 13 min. The flow rate was 3.0 ml/min. For the analysis of MMC and the mitosenes arising in the acetate buffer solutions, an isocratic HPLC system was developed. It consisted of a model M-45 solvent delivery system (Waters Assoc.), a Lichrosorb RP8 (5 μm) (12.5 cm × 4 mm, i.d.) column (Merck, Amsterdam, The Netherlands) and a model 440 dual wavelength detector (Waters Assoc.) with fixed wavelength filters for detection at 254 and 313 nm. The eluent comprised acetonitrile + water (15 + 85, w/w). The aqueous phase contained 5.88 g glacial acetic acid per kg and the pH was adjusted to 3.1. The flow rate was 1.0 ml/min. Quantitation of MMC and the degradation products was based on peak area measurements using a SP 4270 integrator (Spectra Physics, Santa Clara, CA, U.S.A.). Calibration curves of standard solutions of MMC in methanol were linear in the concentration range of interest (1.5×10^{-4} M to 2×10^{-6} M).

UV spectra of the mitosenes were recorded on line with the use of a PU 4021 photo diode array detector (Pye Unicam, Cambridge, U.K.). Circular dichroism (CD) spectra of the degradation products were obtained in methanol solution with a Dichrograph III (Jobin Yvon, Longjumeau, France). The conditions and equipment utilized for field desorption mass spectrometry (FD-MS) were reported earlier (Beijnen and Underberg, 1985).

Results and Discussion

Degradation of MMC in phosphate buffers

Degradation products

Degradation of MMC in 1.0 M phosphate buffers in the pH range 1–6 resulted in four degradation products I–IV (Fig. 2). The retention times of the compounds are: I, 3.9 min; II, 5.6 min; III, 8.4 min; MMC, 9.1 min, and IV, 10.5 min. The identities of (I) 1,2-*trans*- and (II) 1,2-*cis*-2,7-diaminomitosene-1-phosphate are confirmed by comparing elution volumes with those of I and II references prepared according to earlier reported procedures (Tomasz and Lipman, 1979; Andrews et al., 1983). Furthermore, the UV spectra of I and II are identical and exhibit maxima at 309 nm and 248 nm and a minimum at 275 nm, characteristic for the 7-aminomitosene chromophore. The phosphate dependency of I and II appears from the absence of these compounds when MMC degrades in a phosphate-free acidic medium. On account of the presence of an acidic 1-phosphate group and a basic 2-amino group the mitosenes I and II may exist as zwitterions in the reaction mixtures.

III and IV were isolated and demonstrated the same R_f values and elution volumes in several systems as authentic samples of III and IV, prepared and separated as described earlier (Stevens et al., 1965; Taylor and Remers, 1975). FD-MS for both compounds revealed m/z values 321 $[M + H]^+$ and 320 $[M]^{+\cdot}$. The 1,2-stereochemistry was determined using CD spectroscopy. A negative sign of the CD Cotton effect at 520 nm for III points to its 1,2-*trans* stereochemistry and the positive sign at 520 nm for IV correlates with 1,2-*cis*-stereochemistry (Tomasz et al., 1983, 1984). Recently, Tomasz et al. have outlined the general applicability of the use of the sign of the CD Cotton effect for the assignment of the 1,2 stereochemistry of mitosenes. The sign of the CD signal in the longer wavelength region is only dependent on the C1 configuration and is unaffected by the nature of C1, C2, C7 and C10 substituents (Tomasz et al., 1983, 1984). Under protracted acid conditions, 7-aminomitosenes (7-AM) hydrolyze to 7-hydroxymitosenes (7-OHM) (Stevens et al., 1965; Garrett. 1963). These degradation products are not analyzed in the assay.

Phosphate was found to be a highly reactive nucleophilic species. When MMC degrades in 1.0 M sulphate or 1.0 M perchlorate solutions pH 3 only III and IV were found. This indicates that not every nucleophile is equally active. Hornemann et al. (1983) demonstrated that MMC also alkylates a limited number of nucleophiles under reductive conditions.

Kinetics

At constant pH, in 1.0 M phosphate buffers, the overall loss of MMC is pseudo-first-order over at least 4 half-lives. The observed rate constants (k_{obs}) are calculated by least-squares linear regression analysis from the slopes of plots of the natural logarithm of the concentration of MMC remaining ($[MMC]_t$) versus time as depicted by Eqn. 1.

$$\ln[MMC]_t = \ln[MMC]_0 - k_{obs}t \qquad (1)$$

where $[MMC]_0$ is the initial MMC concentration. The rate constants for the appearance (k_1) and the disappearance (k_2) of the mitosenes, caused by progressive degradation, were calculated using a non-linear curve fitting program and Eqn. 2, applying to the reaction scheme:

$$\text{MMC} \xrightarrow{k_1} \text{7-AM} \xrightarrow{k_2} \text{7-OHM}$$

Eqn. 2 is defined by

$$[\text{7-AM}]_t = [MMC]_t \frac{k_1}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}) \qquad (2)$$

For $[\text{7-AM}]_t$ the peak area of the appropriate 7-aminomitosene at time t is taken and $[MMC]_0$ is the concentration of MMC, in terms of integrator peak area units, at $t = 0$. In Table 1, k_{obs}, k_1 and k_2 values of MMC, II and IV as a function of acidity are presented. The k_1 and k_2 values of I and III do not differ substantially. From Table 1 it appears that k_{obs} at a fixed pH is of the same order of magnitude as k_1 of each individual mitosene. In an earlier report (Underberg and Lingeman, 1983) we proposed that the degradation of

TABLE 1

OBSERVED RATE CONSTANTS (k_{obs}) FOR THE DEGRADATION OF MMC IN PHOSPHATE BUFFERS AND THE APPEARANCE (k_1) AND DISAPPEARANCE (k_2) OF II AND IV AS FUNCTION OF pH AT 25°C

pH	MMC	II		IV	
	k_{obs}	k_1	k_2	k_1	k_2
1.0	2.7×10^{-3}	3.3×10^{-3}	7×10^{-4}	1.8×10^{-3}	8×10^{-5}
1.5	2.3×10^{-3}	2.0×10^{-3}	1×10^{-4}	1.9×10^{-3}	4×10^{-5}
2.0	2.0×10^{-3}	2.5×10^{-3}	4×10^{-5}	1.9×10^{-3}	2×10^{-5}
2.5	1.7×10^{-3}	1.8×10^{-3}	1×10^{-5}	1.5×10^{-3}	1×10^{-5}
3.0	1.2×10^{-3}	1.0×10^{-3}	9×10^{-6}	9.9×10^{-4}	4×10^{-6}
3.5	5.8×10^{-4}	5.3×10^{-4}	3×10^{-6}	5.7×10^{-4}	2×10^{-6}
4.0	2.1×10^{-4}	2.0×10^{-4}	1×10^{-6}	1.4×10^{-4}	1×10^{-6}
4.5	7.7×10^{-5}	8.0×10^{-5}	9×10^{-7}	6.5×10^{-5}	9×10^{-7}
5.0	2.5×10^{-5}	2.2×10^{-5}	5×10^{-7}	3.0×10^{-5}	2×10^{-7}
5.5	9.8×10^{-6}	8.0×10^{-6}	4×10^{-7}	8.5×10^{-5}	2×10^{-7}
6.0	3.9×10^{-6}	2.4×10^{-6}	5×10^{-7}	3.5×10^{-6}	3×10^{-7}

Rate constants in s^{-1}.

MMC is governed by a rate-determining step, probably the protonation and cleavage of the C9a methoxy function after which aziridine ring opening yields the key intermediate Im cation.

In Fig. 3 the summation of the molar percentages of the phosphorylated mitosenes (I + II) and the 1-hydroxymitosenes (III + IV) as function of pH, at the stage of 50% MMC decomposition, are given. Over the entire pH region studied the yield of phosphorylated mitosenes prevails, demonstrating the high reactivity of phosphate in comparison with water molecules to attack the intermediate cation (Fig. 2) in spite of the lower total phosphate concentration ([total phosphate] = 1.0 M; $[H_2O] = 55$ M). The yield of phosphorylated mitosenes declines at higher acidities which indicates the high effectiveness of $H_2PO_4^-$ ions, rather than H_3PO_4 molecules ($pK_{a1} = 2.15$), in attacking the intermediate. This can be explained by the strong electrostatic attraction between the unlike charged $H_2PO_4^-$ ion and the intermediate. On account of the chromophoric similarities, the assumption can be made that the molar absorptivities of all 7-aminomitosenes are of the same order of magnitude. An indication that this assumption is justified was obtained from experiments where MMC (4.5×10^{-5} M) was degraded in 0.001 M perchloric acid (pH 3.0), in 1.0 M acetic acid (pH 3.0) and in 1.0 M phosphate solutions (pH 3.0). After complete degradation, verified with HPLC and UV spectrophotometry, the absorptivities at 310 nm of the three solutions were equal; in the first case the absorption is due to III and IV, and in the other cases the absorptivity is due to the presence of mixtures of phosphorylated or acetylated mitosenes as well as III and IV. In a previous report the degradation of MMC in 0.001 M acetate buffers (pH < 6), in which only III and IV are formed, was described

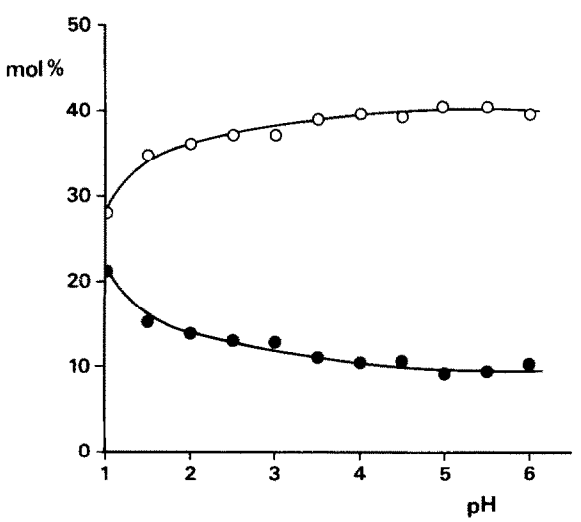

Fig. 3. Molar percentages of the phosphorylated mitosenes (I + II) (○) and the 1-hydroxymitosenes (III + IV) (●) as function of pH at the stage of 50% decomposition of MMC.

(Beijnen and Underberg, 1985). The ratio (*cis/trans*) of these mitosenes appeared to be strongly dependent on the pH, varying from 4.1 at pH 1 to 1.2 at pH 6. This phenomenon was ascribed to the fact that water molecules are directed to attack the carbonium ion C1 of Im from the *cis* side when the 2-amino function is protonated (ImH^+) and from both *cis* and *trans* side with roughly equal chances when the 2-amino function is not charged. The pK_a of ImH^+ was estimated in the order of 2.8. Again, the degree of protonation of the 2-amino group in Im seems to have a great impact in directing incoming phosphate species. At a fixed pH the molar ratios between IV and III as well as between II and I remain constant during the MMC degradation process. However, the ratios vary as function of pH. The molar ratios IV/III vary from 4.1 at pH 1 to 1.1 at pH 6 from which the pK_a of ImH^+ (2.8) could be deduced which is consistent with earlier observations (Beijnen and Underberg, 1985). It should be noted that this determination is less accurate because the yield of III and IV is low. The *cis/trans* ratio of the phosphorylated mitosenes (II/I) varies from 9.9 at pH 1 to 2.2 at pH 6 (Fig. 4). The phosphorylated mitosenes arising at $pH < 1.5$ are the result of the reaction of $H_2PO_4^-$ and ImH^+ and the prevalence of *cis* stereochemistry may be caused by the directing power of the positively charged 2-amino function in ImH^+ forcing $H_2PO_4^-$ nucleophiles to approach the carbonium C1 in ImH^+ from the *cis* side. In the region $1.5 < pH < 3.0$ the protonation degree of ImH^+ decreases and, accordingly, the preferential tendency of $H_2PO_4^-$ to enter from the *cis* side decreases. The leveling effect of the curve (Fig. 4) in the pH range 3–4.5 may be due to an increase of $[HPO_4^{2-}]$. Although the concentration of this ion at these pH values is low ($[HPO_4^{2-}] = 6.3 \times 10^{-4}$ M at pH = 3; $[HPO_4^{2-}] = 2.0 \times 10^{-3}$ M at pH = 4.5) it can be expected that these phosphate ions (charge: 2 −) possess a high reactivity towards ImH^+ (charge: 2 +), whereas substituent orientation with *cis* predominance still occurs, due to the presence of a protonated amino function in ImH^+. The decrease of the protonation degree of ImH^+, resulting in a decrease in *cis* orientation of the nucleophilic substituent, is opposed by the other effect mentioned. In the region

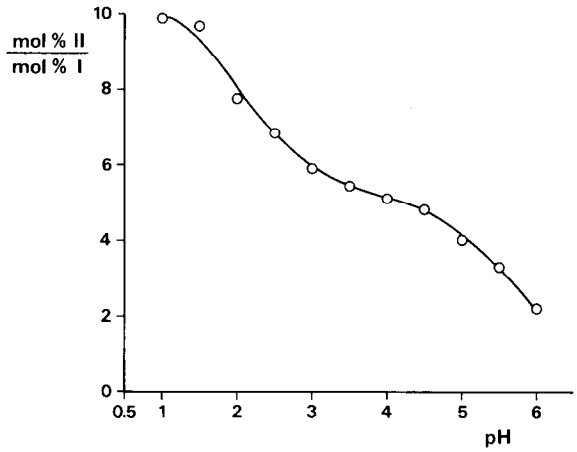

Fig. 4. pH dependence of the ratio between the mole fractions 1,2-*cis*-2,7-diaminomitosene-1-phosphate (II) and 1,2-*trans*-2,7 diaminomitosene-1-phosphate (I).

pH 4.5–6 $[HPO_4^{2-}]$ increases while the *cis/trans* ratio of the phosphorylated mitosenes falls. This might be explained by the fact that the reaction between Im and HPO_4^{2-} gains importance over the reaction between ImH^+ and HPO_4^{2-} because the fraction of ImH^+ strongly declines at higher pH values (pH 4.5: $[ImH^+]/[Im] = 2.0 \times 10^{-2}$; pH 6.0: $[ImH^+]/[Im] = 6.3 \times 10^{-4}$). HPO_4^{2-} ions might show a tendency to approach C1 of Im from the *trans* side because of electrostatic repulsion between the phosphate ion and the lone pair of electrons on the 2-amino function in Im.

Degradation of MMC in acetic acid–acetate buffers

Chromatography

A typical HPLC chromatogram of a decomposition mixture of MMC is depicted in Fig. 5 and in Fig. 6 the structures of the mitosene degradation products are represented. The development of the assay was ruled by two factors. First, the pH of the eluent should not be too low in order to prevent on-column degradation of MMC. On the other hand a $pH > 4$ results in a rapid conversion of IX into VI in the eluent. Maximal stability of IX is at pH 2.5 but this value is too low in view of MMC stability. A compromise of pH 3.1 for the aqueous component of the mobile phase was found. It must be emphasized that this

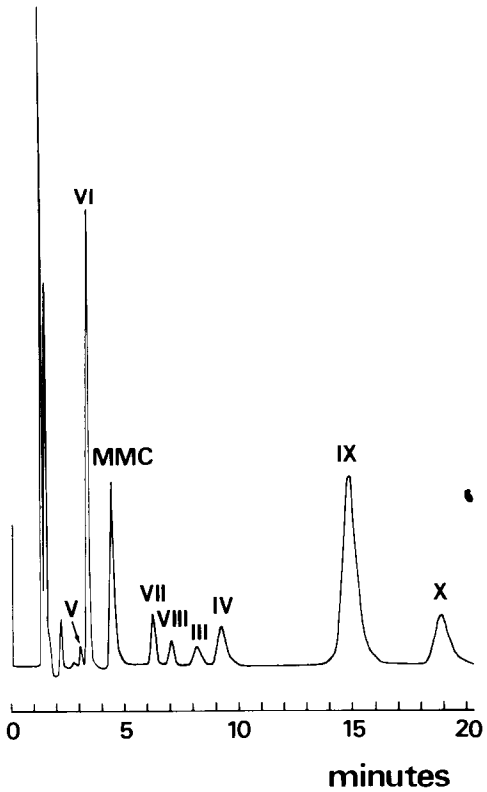

Fig. 5. HPLC chromatogram of a decomposition mixture of MMC in 1.0 M acetic acid–acetate buffer pH 4.0. Detection at 254 nm.

pH value of the eluent is not optimal for analyzing solely MMC as some on column degradation still occurs. However, the MMC degradation does not interfere significantly in the assay.

Variation of the pH of the eluent or using a propionate buffer instead of an acetate buffer, while keeping the water/acetonitrile ratio constant, results in changes in the capacity factors of the mitosenes carrying a primary amino group at C2. The chromatographic behaviour of MMC and 2-acetamidomitosenes was unaffected by these procedures. These experiments support the assumption that ion pair chromatography contributes to an important extent to the chromatography of the mitosenes, possessing a primary aliphatic amino group at C2 (pK_a 7; Stevens et al., 1965), which are protonated at the pH of the mobile phase and are able to form ion pairs with acetate ions. Upon acylation of the 2-amino function, the basicity of this function disappears and, consequently, the chromatography is not influenced by pH variation anymore. Conclusively, the chromatographic behaviour of the mitosenes, on variation of the pH or buffer species of the mobile phase, gives an indication of the status of the 2-amino function. The 7-hydroxymitosene degradation products, having almost no absorptivity

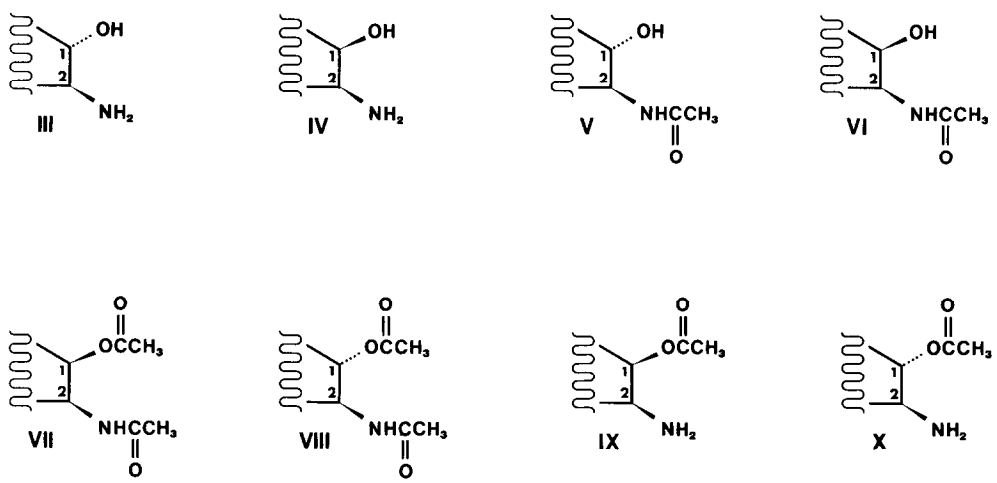

Fig. 6. Structures of mitosene degradation products arising in acetic acid–acetate buffers (pH < 6).

at 254 and 313 nm at pH 3 (Garrett, 1963), are not detected in the assay.

Degradation products

After degradation of MMC in 1.0 M acetic acid–acetate buffers eight mitosene products could be traced, the structures of which are represented in Fig. 6. In Fig. 7 the yield of the degradation products at the stage of 50% MMC decomposition is illustrated.

All compounds have identical UV spectra, recorded on line in the eluent using the photo diode array detector, showing the characteristics of the 7-aminomitosene chromophore (λ_{max} 248 nm, 309 nm; λ_{min} 275 nm). Furthermore it was established that the generation of compounds V, VI, VII, VIII, IX and X is acetate-dependent.

Compounds III and IV. The identification of (III) 1,2-*trans*- and (IV) 1,2-*cis*-2,7-diamino-1-hydroxymitosene is discussed in an earlier section of the paper (Degradation of MMC in phosphate buffers).

Compounds VII and VIII. Acetylation of III and IV with acetic anhydride–pyridine (5 : 1, 1 h, 25 °C) afforded compounds with the same retention times as VIII and VII, respectively. FD-MS analysis of VII and VIII gave m/z values 427 $[M + Na]^+$, 405 $[M + H]^+$, 404 $[M]^+$, 361 $[M\text{-}COCH_3]^+$, 319 $[(M + H)\text{-}2COCH_3]^+$ and 301 $\{[(M + H)\text{-}2COCH_3]\text{-}H_2O\}^+$, consistent with their 1-acetyl-2-acetamido-7-aminomitosene structure. Conclusive evidence about the 1,2-stereochemistry was provided by CD spectropolarimetry in analogy with III and IV.

Compound X. Compound X was isolated and acetylated with acetic anhydride-pyridine. The resulting reaction product has the same capacity factor (k′) as VIII. The FD mass spectrum of X shows two major peaks: m/z 362 $[M]^+$ and m/z 319 $[M\text{-}COCH_3]^+$ which is in accordance with the assigned structure 1-acetyl-2,7-diaminomitosene. The 1,2-*trans* stereochemistry could be assigned on the basis of the observed negative sign of the CD Cotton effect at 520 nm. The absence of an acetyl substituent on the 2-amino function of X appears from the changing retention times at various pH and with different pairing ions in the eluting solvent.

Compounds IX and VI. IX is a labile compound and converts rapidly into VI by O → N acetyl migration (Fig. 8) which prevented effective isolation. A non-acetylated amino function in IX could be deduced from its chromatographic behaviour when pH and pairing ion reactant were changed. The capacity factor of VI was unaffected by these manipulations indicating that the basicity of VI is masked. Indications for 1,2-*cis* stereochemistry of IX were obtained from experiments where MMC is degraded at pH 3.5 and CD spectra are recorded as function of time. At pH 3.5 MMC is mainly converted into IX (Fig. 7). The occurrence of a positive CD Cotton effect at 520 nm confirms the 1,2-*cis* stereochemistry of IX. Acetylation of VI yielded a product co-eluting

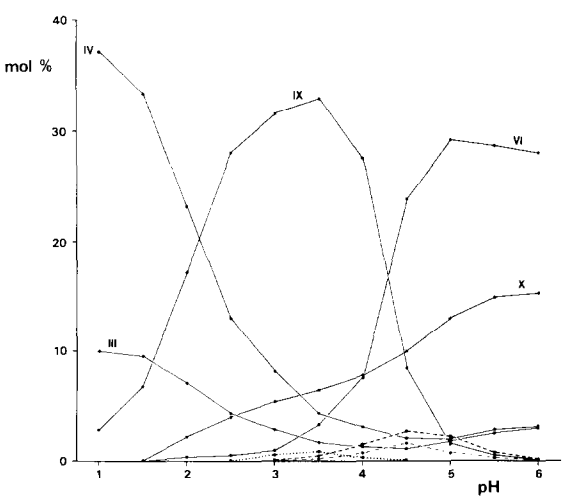

Fig. 7. The molar percentages of mitosene degradation products III, IV, V (· · · · · ·), VI, VIII (- - - -), VII (-·-·-), IX and X, arising in 1.0 M acetate buffers, as function of pH at the stage of 50% decomposition of MMC.

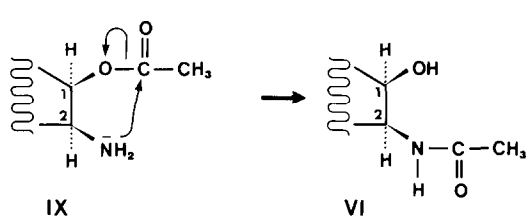

Fig. 8. Intramolecular O → N acylation.

with VII, indicating 1,2-*cis* stereochemistry in VI which was further corroborated by CD. The most abundant peak in the FD mass spectrum of VI was m/z 362 $[M]^{+\cdot}$.

Compound V. V only occurred in traces during MMC degradation in acetate media. The amount of isolated material was insufficient for FD-MS and CD analysis. However, evidence in favour for the assigned structure is provided by the fact that after acetylation V is converted into VIII while its chromatographic behaviour suggest that the 2-amino function bears an acetyl function. Furthermore, it appears that on storage VII hydrolyzes to V. The above-mentioned observations support the identity of V as 1,2-*trans*-2-acetamido-1-hydroxy-7-aminomitosene.

Kinetics

At each pH the degradation of MMC in 1.0 M acetic acid–acetate buffers can be described with Eqn. 1 indicating that (pseudo) first-order kinetics apply. The log k_{obs}–pH profile of MMC is shown in Fig. 9. The rate constants for the formation (k_1) and degradation (k_2) of the mitosenes III, IV, IX and X were calculated with Eqn. 2. The results are plotted in Fig. 9 and support the concept of a rate-limiting unimolecular reaction in the degradation process governing the degradation rate. After Im is generated, water and acetate molecules compete in attacking the C1 carbonium ion of Im yielding III, IV, IX and X. At first sight the formation of VI (2-acetamido-1-hydroxy-7-aminomitosene) does not fit with this reaction scenario. However, the reaction rate constants reveal that VI is not directly formed out of MMC but from IX (through O → N acylation) (Fig. 8), as a secondary degradation product. The pH–rate profile for the degradation of IX is included in Fig. 9 (k_2(IX)). At pH < 2 IX is not converted into VI but continues to degrade, in analogy with the other mitosenes, whereby the 7-amino group is replaced by a hydroxyl group (Garrett, 1963; Stevens et al., 1965). At pH > 2.0 the O → N acetyl migration occurs. This process is only possible when the 2-amino function in IX is not protonated, having the lone pair of electrons available for attacking the 1-acetyl group (Fig. 8). In the region pH 4.5–6.0 the formation rate constant of

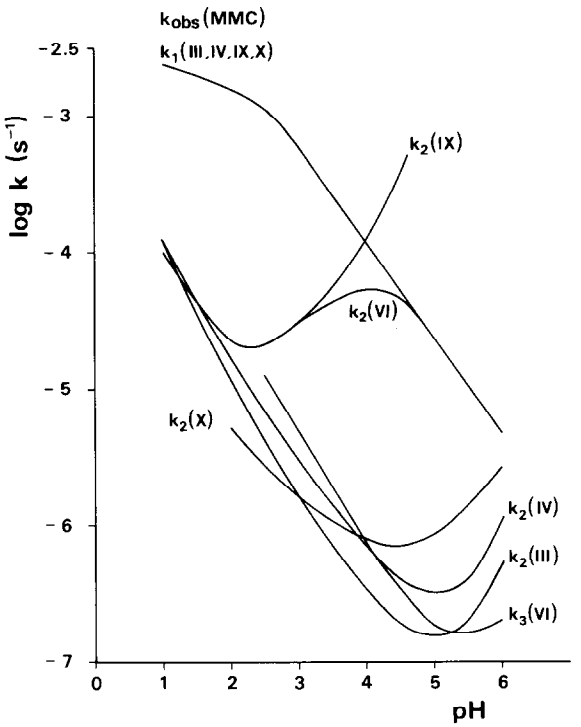

Fig. 9. pH–rate constant profiles for the degradation of MMC (k_{obs}), the appearances (k_1, k_2 in the case of VI) and disappearances (k_2, k_3 in the case of VI) of III, IV, IX and X.

VI (k_2(VI)) equals k_{obs}(MMC) because in this region the conversion MMC → IX → VI is determined by the rate-limiting MMC → IX conversion. The course of the concentration of IX with time could be described adequately with Eqn. 2. The time course of VI, which is also liable to consecutive degradation, could be effectively described by Eqn. 3

$$(VI)_t = (MMC)_0 \left\{ \left(\frac{k_1 k_2}{(k_2 - k_1)(k_3 - k_1)} \right) e^{-k_1 t} + \left(\frac{k_1 k_2}{(k_1 - k_2)(k_3 - k_2)} \right) e^{-k_2 t} + \left(\frac{k_1 k_2}{(k_1 - k_3)(k_2 - k_3)} \right) e^{-k_3 t} \right\} \quad (3)$$

associated with the scheme:

$$MMC \xrightarrow{k_1} IX \xrightarrow{k_2} VI \xrightarrow{k_3} 7\text{-OHM}$$

Plots of the summation of the molar percentages III and IV and of the mitosenes, arisen from acetate attack, show a clear pH dependency and indicate that acetate is the actual incoming species, rather than acetic acid (Fig. 10). Acetate ions entering from the *cis* side yield IX (and indirectly VI) and those entering from the *trans* side yield X. A plot of the ratio of the molar percentages of (IX + VI) and X is S-shaped and declines from 8.0 at pH 2.0 to 1.9 at pH 6.0. The protonation degree of the 2-amino function in the key intermediate (Im) may again account for these observations.

The origin of V, VII and VIII, formed in low amounts, is not yet clear. The rate constants of the formation of these compounds were calculated using Eqn. 2 and the results reveal that these mitosenes do not arise directly from MMC such as III, IV, IX and X do.

From this study no indications were obtained for nucleophilic substitution of the C10 carbamate, proposed as the second alkylating center in the reductive activation mechanism of MMC (Moore, 1977). The formation of DNA cross-links after acid MMC activation has been reported; however, the yield of bisadducts was very low (Lown et al., 1976; Lown and Weir 1978). Although speculative, the possibility for the occurrence of bisadducts may exist, due to the capacity of the aziridinyl nitrogen to alkylate carboxylic functions after acid activation of MMC. The existence of two reactive sites (C1 and N1a) is suggested in this study with the identification of products V, VII and VIII.

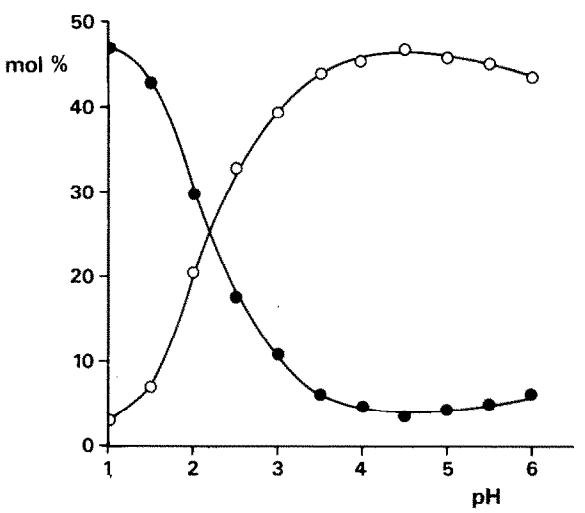

Fig. 10. The solar percentages of the acetylated mitosenes (V + VI + VII + VIII + IX + X) (○) and 1-hydroxymitosenes (III + IV) (●) as function of pH at the stage of 50% decomposition of MMC.

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