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Degradation kinetics of 7-*N*-(*p*-hydroxyphenyl)mitomycin C (M-83) in aqueous solution

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Summary

The degradation kinetics of a new mitomycin derivative, 7-*N*-(*p*-hydroxyphenyl)mitomycin C (M-83), have been investigated over the H_0/pH region −1 to 13. Degradation mixtures were analyzed by means of UV-vis spectrophotometry and high-performance liquid chromatography with UV detection. A pH–rate profile was constructed after corrections for buffer influences. The degradation kinetics have been modelled using a non-linear curve-fitting computer program. Degradation mechanisms are discussed.

Introduction

As long as the use of currently available anticancer drugs is hampered by the occurrence of serious side-effects and low efficacy, the need will exist and the search will continue for new cytotoxic agents possessing a more favourable therapeutic index. In anticancer drug development two strategies are usually followed. The first is the development of analogues of compounds of proven usefulness and the second is the investigation of new structures obtained by chemical synthesis or by isolation from natural sources. As a result of intensive research in the field of mitomycin analogue development, 7-*N*-(*p*-hydroxyphenyl)mitomycin C (KW 2083; M-83) was selected for further development. M-83 has been reported to have excellent antitumour activity in several in vitro and in vivo experimental tumour systems (Asanuma et al., 1983; Asanuma, 1985; Imai et al., 1980, 1981, 1982; Kanzawa et al., 1985; Shimizu et al., 1983), being comparable, or even greater in some cases, to that of mitomycin C (MMC). In particular, M-83 attracts attention because it shows a significantly lower hematological toxicity than MMC when compared at equivalent doses (Kobayashi et al., 1981).

A review on M-83 has been published by Sakurai (1983) and pharmacokinetic and clinical studies have been conducted (Shinkai et al., 1983; Meguro et al., 1984; Fujita, 1985). The mechanism of action of the new 7-substituted mitomycin de-

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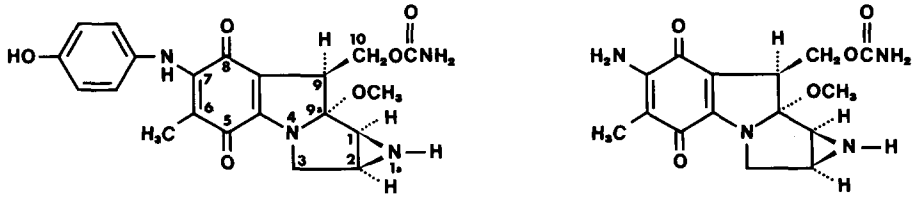

M-83 MMC

Fig. 1. Structures of 7-*N*-(*p*-hydroxyphenyl)-mitomycin C (M-83) and mitomycin C (MMC).

rivative has been investigated by Hashimoto et al. (1982) and Okada et al. (1985).

For MMC, extensive degradation studies have been completed (Beijnen et al., 1983, 1985; Beijnen and Underberg, 1985; Beijnen et al., 1986a, b and c) but stability data of M-83, a prerequisite for the proper handling of the drug, have not been published hitherto.

Structurally, M-83 differs from MMC by the presence of a *p*-hydroxyphenyl group at N-7 (Fig. 1) which can be expected to have a great impact on its chemical stability in both qualitative and quantitative respect. The objective of this study was to extend the insight into the degradation kinetics of M-83 and to compare the results with those obtained earlier for MMC and related mitomycins (Beijnen et al., 1986a, b and c).

Experimental

Chemicals

7-*N*-(*p*-Hydroxyphenyl)mitomycin C (M-83) was a generous gift from Dr. M. Morimoto, Kyowa Hakko Kogyo Co., Mishima, Japan. Mitomycin C was provided by Bristol Myers BV, Weesp, The Netherlands. The compounds were used as obtained. All other 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 −1 to 3: perchloric acid; pH 3–6; acetate; pH 6–9: phosphate; pH 9–10.5: carbonate; pH 10.5–12: phosphate; pH over 12: 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 (Bates, 1973).

Kinetic experiments

The kinetic studies were conducted at 25 °C. The reaction were initiated by spiking 2 ml of a buffer solution with 50 µl of a M-83 stock solution in methanol to give an initial concentration of 18 µg/ml (4.2×10^{-5} M). Degradation studies at pH values lower than 6 were followed spectrophotometrically by continuously monitoring the absorbance at 385 nm. These solutions were also analyzed by HPLC, for which purpose periodically 20 µl samples were withdrawn and injected into the chromatograph. Degradation studies between pH 6 and 11 were exclusively followed by HPLC. M-83 degradation at pH values over 11 was followed spectrophotometrically by continuously monitoring the decrease in absorbance at 465 nm.

Apparatus and analytical procedures

The HPLC analysis was carried out with a chromatographic system consisting of a Model M-6000 pump equipped with a Model 440 dual wavelength detector operating at 254 and 280 nm. The 125 × 4 mm i.d. analytical column was packed with Lichrosorb RP-8 material (particle size 5 µm) (Merck, Darmstadt, F.R.G.). The eluent comprised of methanol–water (35 : 65, m/m). To this mixture 1% v/m of a 0.5 M sodium phosphate buffer solution (pH 7.0) was added. The flow was 1.0 ml/min and the column was used at ambient temperature. Peak area measurements were done with a SP 4270 integrator (Spectra Physics, Santa Clara, CA, U.S.A.) and were used to quantitate

undegraded M-83. Standard curves exhibited linear responses ($r > 0.999$) in the concentration range of interest: 20–1.5 μg/ml. Relative standard deviations of 0,9–3.2% were obtained for replicate injections ($n = 6$) of the upper and lower concentrations of the standard solutions, respectively. UV-spectra of the degradation products were recorded on line with a PU 4021 diode array detector (Pye Unicam, Cambridge, U.K.).

UV-vis spectra were recorded with a Hitachi model 100-60 double-beam spectrophotometer equipped with a Kipp BD 40 Recorder. Kinetic studies were performed spectrophotometrically with a Shimadzu UV-140 double-beam absorption spectrophotometer, equipped with ACP-140 Cell Positioner and thermostated cell compartments, operating at 385 nm for degradation studies at pH under 6 and at 465 nm for experiments at pH values over 11; 1 cm quartz cells were used.

The conditions and equipment utilized for field desorption mass spectrometry (FD-MS) were reported earlier (Beijnen and Underberg, 1985).

Preparation of the degradation products I and II for FD-MS

About 2 mg M-83 was dissolved in 2 ml of a perchloric solution pH 2.0 and this solution was allowed to stand for 1.5 h at room temperature in the dark. Next, solid sodium phosphate was added in order to increase the pH of the mixture to 9. This aqueous solution was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 100 μl methanol. The methanolic solution was subjected to FD-MS analysis. HPLC analysis revealed that M-83 was quantitatively converted into the products I and II.

Results and Discussion

Analytical procedures

Kinetic experiments at pH < 2 and pH > 11 were solely performed with UV spectrophotometry because the degradation rates in those pH regions were too fast to establish with HPLC.

Fig. 2 represents the spectra of undegraded M-83 (curve 1) and of partly degraded samples (curves 2–4) at pH 2.35. The spectral changes are

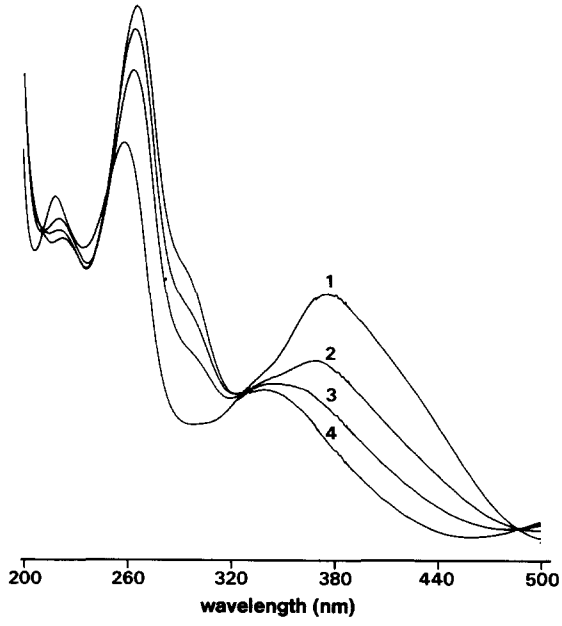

Fig. 2. Ultraviolet spectra of M-83 (initial concentration = 18 μg/ml) at $t = 0$ min. (1), $t = 6$ min; (2), $t = 12.5$ min; (3) $t = 25$ min; (4) after the start of the degradation at pH 2.35 and 25 °C.

pronounced and justify the use of spectrophotometry to quantitate the degradation of M-83 in acidic medium. The presence of isosbestic points indicates that the degradation involves the conversion of one compound into another compound (or more compounds with spectrally equivalent characteristics). The spectral changes during degradation at pH values > 11 were also sufficient for monitoring the degradation spectrophotometrically. The rate constants were calculated from the absorbance measurements by using earlier published equations (Underberg and Lingeman, 1983a and b).

Typical HPLC chromatograms of acidic and alkaline decomposition mixtures are shown in Figs. 3 and 4, respectively. The stability indicating properties of the HPLC assay is demonstrated by the fact that the ratio between the UV detector signals (at 254 and 280 nm) remained constant during the disappearance of the M-83 peak. This procedure is justified for checking the stability-indicting capability of the assay as the degradation of M-83 is accompanied with pronounced spectral changes.

Degradation products

When M-83 degrades in acidic medium, two degradation products are observed (I and II, Fig. 3). FD-MS analysis of a mixture of these compounds yielded two peaks: $m/z = 413$ $(M + H)^+$ and $m/z = 435$ $(M + Na)^+$, M-83 reference compound gives only one peak, $m/z = 426$ $(M)^+$, in the FD mass spectrum. The on-line UV-spectra of I and II were identical. These preliminary data are in agreement with the hypothesis that M-83 in acidic solution degrades in a similar way as MMC, involving cleavage of the C9a methoxy group, introduction of a double bond between C9 and C9a, followed by aziridine ring opening. Aziridine ring opening occurs with configurational retention at C2 and water attachment at C1 with inversion as well as retention of the C1 stereochemistry yielding 1,2-trans- and 1,2-cis-1-hydroxy-2-amino-mitosenes, respectively.

For several types of mitosenses it has been noticed that the 1,2-trans diastereomer gives a sharp peak while the 1,2-cis diastereomer elutes as a tailing peak in reversed phase HPLC systems (Beijnen et al., 1986b). Furthermore, the abundant formation of II at low pH values indicates that II is 1, 2-cis-1-hydroxy-2-amino-7-*N*-(*p*-hydroxyphenyl)mitosene and I 1,2-trans-1-hydroxy-2-amino-7-*N*-(*p*-hydroxyphenyl)mitosene (Beijnen and Underberg, 1985), Circular dichroism spectroscopic experiments are in progress to prove this. At pH values > 10 M-83 degrades into only one product (Fig. 4). The UV spectrum, recorded on line, and the chromatographic behaviour of this compound are identical to that of MMC reference compound. The proposed overall degradation scheme of M-83 is depicted in Fig. 5.

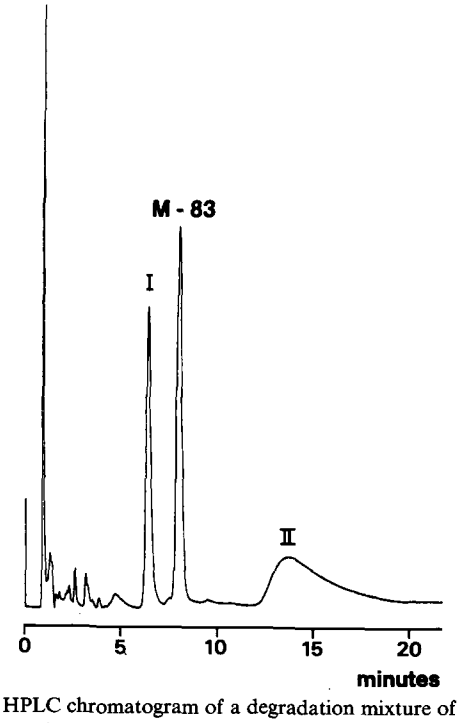

Fig. 3. HPLC chromatogram of a degradation mixture of M-83 (pH 2.35); detection 254 nm. For chromatographic conditions: see text.

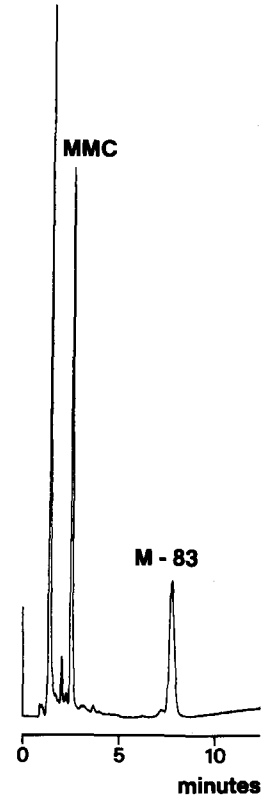

Fig. 4. HPLC chromatogram of a degradation mixture of M83 (pH 10.0); detection 254 nm. For chromatographic conditions: see text.

Degradation kinetics

Order of reaction. The degradation of M-83 in both acidic and alkaline buffers shows a linear

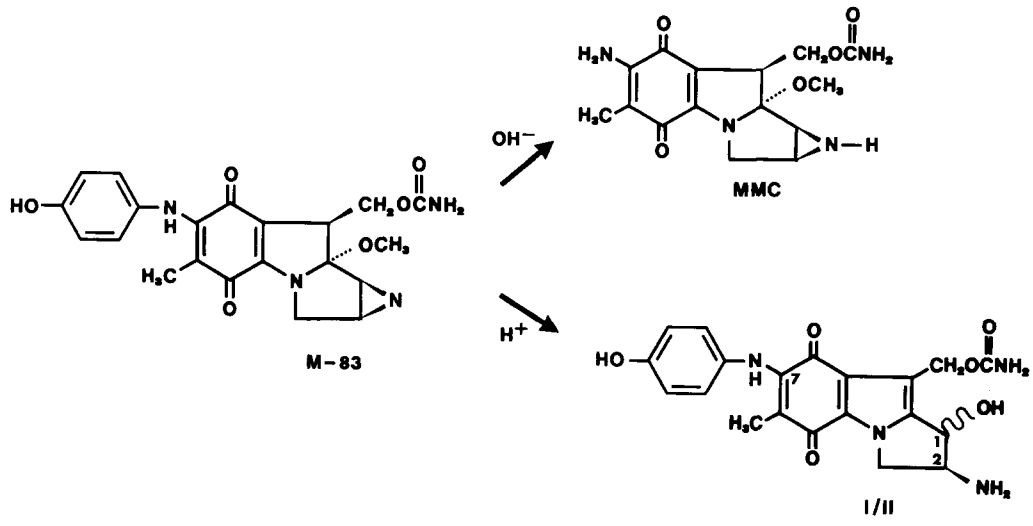

Fig. 5. Proposed overall degradation scheme for M-83 in acid and alkaline medium.

relationship between the natural logarithm of residual M-83 concentration and time, indicating that the degradation reactions follow (pseudo) first-order kinetics.

Standard deviation in k_{obs}. The standard deviation (S.D.) in the observed (pseudo) first-order rate constants, k_{obs}, was determined for the UV spectrophotometric assay and the HPLC assay at pH = 3.0. The values of $k_{obs} \pm$ S.D. were $5.96 \pm 0.09 \times 10^{-4}$ s^{-1} ($n = 6$) for the spectrophotometric method and $6.10 \pm 0.15 \times 10^{-4}$ s^{-1} ($n = 6$) for the HPLC method. Other rate constants are mean values of duplicate determinations.

Influence of pH. It is necessary to keep the pH constant with buffers since decomposition of M-83 leads to the formation of (basic) mitosenes. However, the catalytic effects of buffer components must be taken into account for the evaluation of the pH–rate profile. Corrections for these catalytic effects were carried out according to earlier reported procedures (Underberg and Lingeman, 1983a; Beijnen et al., 1986c). The corrected rate constants were used for the construction of the pH–rate profile (Fig. 6). Representative kinetic data of M-83 degradation in buffers are listed in Table 1.

From the inflection points of the pH–rate profile it can be deducted that M-83 is involved in at least two prototropic equilibria. The first pK_a is, at first sight, approximately 2.5, the second pK_a is approximately 10. However, closer examination of the curve reveals that the first derivative of the logarithm of k' between pH 8 and 9 is approximately 2, between pH 9 and 10 it is less than 1 and between pH 10 and 11 it is approximately 1. These changes in slope can be explained assuming that between 8 and 12 two protolytic equilibria occur. These observations correspond well with

TABLE 1

k_{obs} values (in s^{-1}) for the degradation of M-83 in various buffer solutions (in M) [a]

pH	[buffer]	k_{obs}	pH	[buffer]	k_{obs}
3.8	0.01	1.6×10^{-4}	9.1	0.01	9.7×10^{-6}
	0.05	1.8×10^{-4}		0.05	1.1×10^{-5}
	0.10	2.0×10^{-4}		0.10	1.4×10^{-5}
	0.20	2.4×10^{-4}		0.20	2.0×10^{-5}
5.5	0.01	2.1×10^{-6}	10.5	0.01	7.7×10^{-4}
	0.05	2.5×10^{-6}		0.05	1.1×10^{-3}
	0.10	2.9×10^{-6}		0.10	1.2×10^{-3}
	0.20	3.5×10^{-6}		0.20	1.8×10^{-3}

[a] pH 3.8: acetate; pH 5.5: acetate; pH 9.1: phosphate; pH 10.5; phosphate.

the results obtained with regression analysis. Using a two-pK_a model (eqn. 1)

$$k' = \frac{M_1 \cdot [H^+] + M_2 + \frac{M_3}{[H^+]} + \frac{M_4}{[H^+]^2} + \frac{M_5}{[H^+]^3}}{1 + \frac{K_1}{[H^+]} + \frac{K_1 \cdot K_2}{[H^+]^2}} \quad (1)$$

no satisfactory results were obtained.

Using a three-pK_a model (eqn. 2)

$$k' = \left\{ M_1[H^+] + M_2 + \frac{M_3}{[H^+]} + \frac{M_4}{[H^+]^2} + \frac{M_5}{[H^+]^3} + \frac{M_6}{[H^+]^4} \right\} \times \left\{ 1 + \frac{K_1}{[H^+]} + \frac{K_1 \cdot K_2}{[H^+]^2} + \frac{K_1 \cdot K_2 \cdot K_3}{[H^+]^3} \right\}^{-1} \quad (2)$$

a good correlation was found between the model and the measured values. The constants M_1, M_2, M_3, M_4, M_5 and M_6 are the macro reaction constants (Van der Houwen et al., 1988), while K_1, K_2 and K_3 are the protolytic constants. The calculated macro reaction constants and pK_a values are given in Table 2. The first pK_a can be assigned to the conjugated acid of the aziridine nitrogen base. Its value is in reasonable agreement with the value for MMC (2.74) (Beijnen et al., 1986c). The second and third deprotonation step can be explained by dissociation of the phenolic hydroxyl function and the 7-amino group, probably after keto–enol tautomerization in analogy with MMC (Underberg and Lingeman, 1983b). According to the pK_a values calculated M-83 is present in solution as different species: M-83$^+$, M-83, M-83$^-$ (two different forms) and M-83^{2-}. The two different forms of M-83$^-$ result from the fact that the difference between the second and the third pK_a value is relatively small which indicates that the two protolytic equilibria overlap. The pK_a values are therefore macro pK_a values that cannot be associated with a particular acidic function. Assuming that each species is subject to proton-, solvent- and hydroxyl-catalyzed degradation reactions, the meaning of the constants

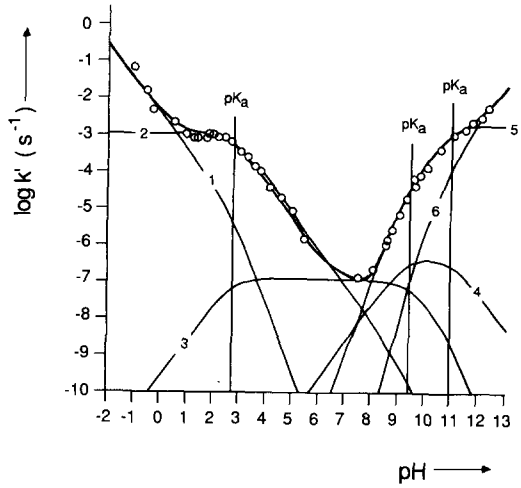

Fig. 6. Log k′–pH profile for the degradation of M-83 at 25 °C.
(1) proton-catalyzed degradation of M-83$^+$.
(2) proton-catalyzed degradation of M-83^0.
solvent-catalyzed degradation of M-83$^+$.
(3) hydroxyl-catalyzed degradation of M-83$^+$.
solvent-catalyzed degradation of M-83^0.
proton-catalyzed degradation of M-83$^-$.
(4) solvent-catalyzed degradation of M-83$^-$.
hydroxyl-catalyzed degradation of M-83^0.
proton-catalyzed degradation of M-83^{2-}.
(5) solvent-catalyzed degradation of M-83^{2-}.
hydroxyl-catalyzed degradation of M-83$^-$.
(6) hydroxyl-catalyzed degradation of M-83^{2-}.

TABLE 2

Micro reaction constants for the degradation of M-83 and acid-dissociation constants at 25 °C

M_1	5.3×10^{-3} $M^{-1}\cdot s^{-1}$		
M_2	1.5×10^{-3} s^{-1}	K_1	1.75×10^{-3} (pK 2.76)
M_3	2.3×10^{-10} $M\cdot s^{-1}$	K_2	3.9×10^{-10} (pK 9.41)
M_4	4.3×10^{-19} $M^2\cdot s^{-1}$	K_3	1.16×10^{-11} (pK 10.9)
M_5	2.2×10^{-26} $M^3\cdot s^{-1}$		
M_6	2.7×10^{-38} $M^4\cdot s^{-1}$		

M_1–M_6 is given by the following equations (Van der Houwen et al., 1988).

$$M_1 = k_{0,H}$$

$$M_2 = k_{1,H} \cdot K_1 + K_{0,S}$$

$$M_3 = k^{I}_{2,H} \cdot K_1 \cdot K_I + k^{II}_{2,H} \cdot K_1 \cdot K_{II} + k_{1,S} \cdot K_1 + k_{0,OH} \cdot K_w$$

$$M_4 = K_1 \cdot (k_{3,H} \cdot K_2 \cdot K_3 + k^{I}_{2,S} \cdot K_1 + k^{II}_{2,S} \cdot K_{II} + k_{1,OH} \cdot K_w)$$

$$M_5 = K_1 \cdot (k_{3,S} \cdot K_2 \cdot K_3 + k^{I}_{2,OH} \cdot K_I \cdot K_w + k^{II}_{2,OH} \cdot K_{II} \cdot K_w)$$

$$M_6 = K_1 \cdot K_2 \cdot K_3 \cdot k_{3,OH} \cdot K_w$$

The rate constants of the two species of M-83$^-$ are indicated with the superscript ^{I} and ^{II}, respectively. The corresponding micro protolytic dissociation constants are indicated by K_I and K_{II}. The reaction constants combined in a single macro reaction constant are kinetically indistinguishable. It is therefore impossible to determine their individual contribution to the pH profile. The contribution of the individual macro reaction constants to k_{obs} is included in the graph of the pH profile. From this plot it can be seen that M_2 and M_5 largely determine the pH profile. M_1, M_3 and M_6 contribute to a lesser extent, while the contribution of M_4 is negligible. This correlates with the precision with which these constants have been estimated.

Influence of buffers. Buffer ions catalyze the degradation of M-83 in acidic and alkaline medium. The catalytic effect was demonstrated by measurements of the decomposition rate at constant pH and temperature, but at different buffer concentrations. Representative results from these experiments are listed in Table 2. General acid–base catalysis has also been demonstrated to occur with related mitomycins (Beijnen et al., 1986c).

Degradation mechanism

The M-83 degradation in acidic medium, with the formation of the 1,2-cis- and 1,2-trans-1-hydroxy-2-amino-7-*N*-(*p*-hydroxyphenyl)mitosenes, is likely to occur through the same mechanism as proposed for MMC (Beijnen et al., 1986b).

When the degradation of M-83 took place in buffer solutions more degradation products were observed in the chromatograms. The presence of these compounds were dependent upon pH, buffer composition and molarity. This in complete agreement with our earlier reports about MMC degradation in acid phosphate and acetate buffer solutions where, apart from 1-hydroxy-2,7-diaminomitosenes, acetylated and phosphorylated mitosene products were found (Beijnen et al., 1986a). This indicates that M-83, like MMC, degrades in acidic solution through the formation of an intermediate possessing a reactive center at C1, acting as target for incoming nucleophiles, among which water molecules, acetate and phosphate ions. The 7-*N*-(*p*-hydroxyphenyl) group of M-83 remains intact in acidic medium, as far as the first degradation step is involved.

In alkaline medium, the initial degradation step of M-83 appears to be cleavage of the 7-*N*-substituent yielding MMC. Imai and collaborators (1982) investigated the stability of M-83 in mouse liver homogenate preparations. MMC could not be detected in these reations mixtures incubated with M-83. However, in our study, we established that M-83 can be converted into MMC by simple storage of M-83 at pH > 7 at room temperature. During the course of M-83 degradation in alkaline medium we noticed that a peak, which elutes near the solvent fronts, also increases. This may be due to the formation of a product originating from the cleaved 7-*N*-(*p*-hydroxyphenyl) group of M-83. The structure of this degradation product is not elucidated yet. However, for the establishment of the mechanism for the conversion of M-83 into MMC identification of this product is a prerequisite.

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