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LIQUID CHROMATOGRAPHIC AND MASS SPECTRAL ANALYSIS OF MITOSANE AND MITOSENE DERIVATIVES OF MITOMYCIN C*

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SUMMARY

Mitomycin C (MC), two mitosane, and twelve mitosene derivatives were characterized by reversed-phase high-performance liquid chromatography (HPLC). Compounds were eluted from a Radial-Pak C_{18} cartridge with a 13-min linear gradient of 0–50% methanol in a 0.01 *M* pH 7.0 potassium phosphate buffer. Compounds were detected at both 313 nm and 365 nm. The assay was sensitive to 5 pmol of MC injected. A new compound, 7-amino-2-formamido-1-hydroxymitosene, was identified from the reaction of MC with dimethylformamide at pH 3.0. These compounds were also characterized by electron-ionization mass spectrometry following their acetylation. Common fragmentation pathways were identified. The HPLC and mass spectral methods described offer a specific, sensitive and definitive means of detecting and identifying the numerous bioreduction, chemical reduction and hydrolytic products of MC and MC analogues.

INTRODUCTION

Mitomycin C (MC) is a potent antitumor antibiotic used in the treatment of pulmonory, mammary, gastric, cervical, colonic and head and neck cancers[1]. Since the pioneering studies by Iyer and Szybalski[2] and Schwartz *et al.*[3], MC has been regarded as the sole known bioreductive alkylating agent; and Kennedy *et al.*[4] have suggested that MC may possess especial selectivity in the chemotherapeutic treatment of hypoxic cells in solid tumors. Recently Tomasz and Lipman[5] have succeeded in identifying the major metabolites and alkylation products of MC generated by bioreduction of MC with rat liver microsomes. Their work has provided the first direct evidence of MC's bioreductive alkylating character and has confirmed the suscepti-

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bility of the C-1 position of bioreduced MC to nucleophilic attack. Despite the strong evidence that MC induces DNA interstrand crosslinks in addition to monoalkylated sites on DNA, direct evidence of a second reactive center on MC has not come forth.

Knowledge of the clinical pharmacology of MC and its metabolic fate in humans and animals has been meager. The major factor throttling progress in this area has been the lack of a convenient, sensitive and specific assay for resolving and quantifying MC and potential metabolites. A microbiological assay[6] has been the principal method for garnering pharmacokinetic data.

The separation and isolation of MC metabolites and chemical derivatives has been accomplished by a variety of chromatographic techniques. An abundance of thin-layer chromatographic (TLC) systems have been reported[5,7–9]. Paper chromatography was used to study the hydrolytic degradation of MC[7] and paper electrophoresis has been used to characterize MC and derivatives[5,9]. Sephadex gel column chromatography was exploited by Tomasz and Lipman[5,9] to isolate the numerous MC bioreduction, chemical reduction and hydrolytic products. Although these methods may be adequate for the separation of MC derivatives, they do not provide a facile means of quantifying these species.

For our own interests in the kinetics of the metabolism of MC by bioreductive processes, we sought a method that could rapidly resolve and concomitantly quantify MC derivatives possessing a wide range of polarities. The technique of reversed-phase high-performance liquid chromatography (HPLC) is an ideal solution to this analytical problem. An HPLC method was reported that utilized a paired-ion reversed-phase analysis to determine the stability of MC in solution[10]. A normal-phase HPLC system was briefly described that separated mitomycin congeners and unknown products of the reaction of MC with sodium dithionite[11]. Kono *et al.*[12] reported a reversed-phase HPLC method that could chromatograph MC extracted from human plasma. Most recently Den Hartigh *et al.*[13] have reported the application of a reversed-phase HPLC method to the pharmacokinetic analysis of MC in human plasma and found the appearance of two unknown compounds (presumably MC metabolites) in these samples. A recent report by van Hazel and Kovach[14] also described a reversed-phase HPLC method that was applied to the pharmacokinetic analysis of MC in humans and rabbits. We report here our own development of a reversed-phase HPLC technique for the separation and quantitation of MC. Further, we have synthesized by previously published methods a number of products that could potentially be metabolites of MC, and we systematically characterized their behavior in our HPLC system.

Another factor hampering the understanding of the metabolic fate of MC has been the lack of a convenient and sensitive spectroscopic method that can provide definitive structural information on metabolic products. High-resolution nuclear magnetic resonance (NMR) has been used successfully on many chemical products[5,8,15–17] but is not well suited for use on metabolites that are isolated in microgram quantities. Mass spectrometry (MS) is an obvious choice in this regard, due to both its inherently high sensitivity and the structural data available. The electron-ionization (EI) mass spectra of MC and numerous mitosane analogues was reported by Van Lear[18]. Field desorption MS has been applied to the analysis of some mitosene derivatives[5,16]. We have found EI-MS to be unsuitable for the analysis of intact mitosene compounds. We have, however, developed an EI-MS method for the analy-

sis of acetylated mitosenes. We report here the mass spectra and characterization of the fragmentation pathways of these compounds. This method provides definitive spectroscopic evidence of the structures of MC products generated both by chemical and metabolic means.

MATERIALS AND METHODS

MC was obtained from the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD, U.S.A.). Samples of 1,2-*cis*- and -*trans*-2,7-diamino-1-hydroxymitosene (2d) were prepared by hydrolysis of MC in 0.1 *M* hydrochloric acid for 30 min as previously described[7], 1,2-*cis*- and *trans*-2-amino-1,7-dihydroxymitosene (2f) were prepared by hydrolysis of MC in 0.1 *M* hydrochloric acid for 20 h at room temperature[7] and 1,2-*cis*- and *trans*-2-amino-10-decarbamoyl-1,7-dihydroxymitosene (3a) were prepared by hydrolysis of MC in 2.0 *M* hydrochloric acid for 2.5 h at 65°C[7]. 2,7-Diaminomitosene was prepared by catalytic hydrogenation of MC with platinum oxide[5]. 10-Decarbamoylmitomycin C was prepared by treatment of MC with sodium methoxide[19], 1,2-*cis*- and *trans*-10-decarbamoyl-2,7-diamino-1-hydroxymitosene were prepared by hydrolysis of 10-decarbamoyl-MC in 0.1 *M* hydrochloric acid for 30 min at room temperature[5] and 1,2-*cis*- and *trans*-2,7-diamino-1-phosphomitosene were prepared by adding MC to 0.34 *M* NaH_2PO_4 (pH 3.0)[9]. N-Methyl-MC (porfiromycin) was prepared by reaction of MC with methyl iodide[7]. The *cis* and *trans* isomers of the above mitosene compounds were separated and purified by semi-preparative HPLC.

Alkaline phosphatase (E.C. 3.1.3.1) was obtained from Sigma (St. Louis, MO, U.S.A.). Incubations were run in 1.5 *M* Tris–HCl buffer at pH 8.0.

MC was quantified spectrally at 367 nm (molar absorption coefficient of 21,800 $l\ mol^{-1}\ cm^{-1}$). Compound 2d was quantified at 313 nm (molar absorption coefficient of 11,400 $l\ mol^{-1}\ cm^{-1}$). Absorbance spectra were recorded on a Cary 118 spectrophotometer (Varian, Palo Alto, CA, U.S.A.).

Acetic anhydride (Supelco, Bellafonte, PA, U.S.A.) and pyridine (Pierce, Rockford, IL, U.S.A.) were silylation grade. Octylamine hydrochloride was obtained from Kodak (Rochester, NY, U.S.A.) and tetraammonium EDTA was made as previously described[20]. All other chemicals were reagent grade.

Analytical HPLC

We used an HPLC apparatus consisting of the following Waters units (Waters Assoc., Milford, MA, U.S.A.): 6000 A pump, M-45 pump, Model 660 solvent programmer, Model 440 dual-wavelength detector, U6K injector, Model 730 data module and RCM-100 radial compression module. A 10 cm × 8.0 mm Radial-Pak C_{18} (10-μm particle size) column was used. A 10 cm × 8.0 mm Radial-Pak C_8 column was also tried. The RCM-100 was fitted with a 7 cm × 2.1 mm guard column (Whatman, Clifton, NJ, U.S.A.) packed with Co:Pell ODS (Whatman, particle size 25-40 μm). Potassium phosphate buffer (0.01 *M*, pH 7.0) for the mobile phase was prepared from de-ionized water (Millipore, New Bedford, MA, U.S.A.) and was filtered (Millipore HA, 0.45 μm) and degassed. Methanol or acetonitrile (Burdick and Jackson Lab., Muskegon, MI, U.S.A.) was filtered (Millipore FH, 0.45 μm) and degassed prior to mixing with phosphate buffer. MC and mitosene derivatives were eluted with

a linear gradient from 100% A (phosphate buffer), 0% B (phosphate buffer–methanol, 1:1) to 0% A, 100% B in 13 min. We used a flow-rate of 3.0 ml/min and detected peaks at 313 and 365 nm.

Semi-preparative HPLC

The same system was used for semi-preparative HPLC as for the analytical separations except that the RCM-100 module was replaced with a 50 cm × 9.4 mm M9 ODS-3 semi-preparative column (Whatman, particle size 10 μm). Instead of phosphate buffer for the mobile phase, we used ammonium bicarbonate (0.01 *M*) adjusted to pH 7.0 with 1.0 *M* hydrochloric acid. The same gradient conditions were used as for the analytical separation and the flow-rate was 4.0 ml/min.

Derivatization

Compounds were purified by semi-preparative HPLC and derivatized with acetic anhydride–pyridine (5:1) at room temperature for 1.0 h. Solvents were then evaporated with a nitrogen stream. Toluene was added to the vial and the solution evaporated again to remove traces of pyridine. Alternatively some compounds were loaded as methanol–water solutions onto Sep-Pak C_{18} cartridges (Waters Assoc.) that had been primed with methanol and then water. The loaded cartridge was flushed first with water to remove pyridine and then with methanol which eluted the purple band. Compound 2d in particular was handled in this manner as it proved especially difficult to remove pyridine traces from this compound by azeotrope formation with toluene.

Mass spectrometry

MS was performed by direct probe analysis on a VG Micromass 30F mass spectrometer (VG Analytical, Altrincham, Great Britain) operated under VG Datasystems 2040 computer control. Source conditions for EI analysis were: 200°C source temperature, 70 eV electron energy, 4kV accelerating voltage and 170 μA trap current. The source conditions for isobutane chemical-ionization (CI) analysis were: 200°C, 52 eV, 4kV and 170 μA emission current. Mass spectra obtained by in-beam techniques[21] used a Vespel rod probe tip that rested 3 mm from the center of the electron beam in the source.

RESULTS AND DISCUSSION

Previous reports by several workers on the HPLC analysis of MC[10–14] have shown satisfactory chromatography of MC. None of these methods, however, attempted to chromatograph or resolve from MC any other mitosane or mitosene derivatives that might be metabolic products. Our approach was to synthesize systematically known or suspected metabolites of MC and to characterize their HPLC retention. Following the optimization of mobile phase, column and gradient conditions for the best resolution of these compounds, we could then apply this method to the rational analysis of *in vitro* or *in vivo* MC metabolites.

MC is unstable in acid or base. To prevent degradation of MC during analysis, we chose to buffer the mobile phase at pH 7.0 with 0.01 *M* potassium phosphate. Acid treatment of MC causes a spontaneous loss of methanol to form the indolo-

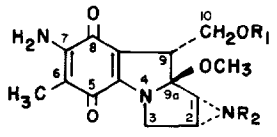
quinone (or mitosene) moiety. This loss causes a shift of the absorbance maximum from 363 to 313 nm in the mitosene compound (Table I). Thus, the use of dual-wavelength detection at 313 nm and 365 nm to analyze MC and its products was imperative.

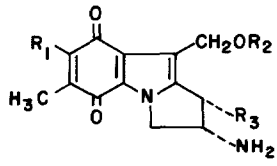
Known metabolites of MC possess a wide range of polarities, from the polar mitosene phosphates to the non-polar 2,7-diaminomitosene (Fig. 1). To obtain adequate retention and resolution of these diverse compounds, the use of gradient elution was necessary. We found a linear gradient of 0–50% methanol in 13 min gave the optimal analysis time and resolution (Fig. 2). Acetonitrile–phosphate buffer (1:1) was also tried as the B solution with an identical gradient. The acetonitrile caused earlier elution of peaks (capacity factor, $k' = 8.50$ for MC) and sharper peaks; but also caused fusion of the MC peak with the *trans*-2d peak (separation factor, $\alpha = 1.06$ *versus* $\alpha = 1.11$ with methanol). A Radial-Pak C_8 cartridge was compared to the Radial-Pak C_{18}. The Radial-Pak C_8 gave similar efficiency (plate number, $N = 23{,}000$), resolution and k' values as the C_{18} cartridge.

The envisioned goal of this method was to provide a means of analyzing the metabolism of MC by microsomal enzymes and eventually the pharmacology and metabolism of MC in humans. Either of these applications would require injection of biologically contaminated samples. We chose to protect our column from contamination by fitting the radial compression module with a guard column. Remarkably, the guard column had little effect on the retention of MC and derivatives; however, the efficiency (N) of the separation for MC was 19,000 plates with the guard column and 23,000 plates without the guard column.

Mild acid hydrolysis of MC (0.1 M hydrochloric acid) generated two products at $k' = 10.96$ and 14.42. These were identified as *trans*- and *cis*-2d, respectively, by comparison of their R_F values on TLC to literature values[8]. These compounds were purified in milligram quantities by semi-preparative HPLC.

Prolonged hydrolysis of MC produced two products at $k' = 6.53$ and 8.52.





Mitosane	R_1	R_2	Mitosene	R_1	R_2	R_3
mitomycin C	$CONH_2$	H	2d	NH_2	$CONH_2$	OH
10-decarbamoyl-mitomycin C	H	H	2f	OH	$CONH_2$	OH
porfiromycin	$CONH_2$	CH_3	3a	OH	H	OH
			2d-phosphate	NH_2	$CONH_2$	O-P-OH (O, OH)
			2,7-diaminomitosene	NH_2	$CONH_2$	H
			10-decarbamoyl-2d	NH_2	H	OH

Fig. 1. Structures of mitosane and mitosene compounds characterized by HPLC and mass spectrometry. Only the 1,2-*cis*-mitosene structures are depicted.

TABLE I

CAPACITY FACTORS AND ABSORBANCE MAXIMA OF MITOMYCIN C AND DERIVATIVES

M9 = Whatman semi-preparative column.

Compound	*k' on Radial-Pak* C_{18}*		*k' on M9 ODS-3***	*λ_{max} (nm)*	
	Non-acetylated	*Acetylated*		*0.1 M hydrochloric acid*	*0.1 M sodium hydroxide*
trans-2d-Phosphate	6.67	–	2.15	–	250, 310
cis-2d-Phosphate	8.60	–	2.53	–	250, 310
trans-3a Phosphate	6.17	20.36	2.10	236, 295, 344	253, 310
cis-3a Phosphate	8.14	19.64	2.35	236, 295, 344	253, 310
trans-2f Phosphate	6.53	18.43	2.31	236, 294, 342	255, 310
cis-2f Phosphate	8.52	17.46	2.66	236, 294, 342	255, 310
trans-2d Phosphate	10.96	15.81	3.89	250, 308	248, 311
cis-2d Phosphate	14.42	14.81	4.39	250, 308	248, 311
10-Decarbamoyl MC	11.86	15.86	4.05	247, 357	298, 366
MC	12.50	13.95	4.10	246, 356	294, 363
trans-10-Decarbamoyl-2d	10.36	17.64	–	250, 308	250, 314
cis-10-Decarbamoyl-2d	14.42	17.07	–	250, 308	250, 314
Porfiromycin	13.65	–	–	–	–
2,7-Diaminomitosene	15.79	14.03	4.43	243, 312	243, 316
N^2-Formyl-2d	12.89	12.09	4.23	248, 310	246, 310

* Retention time of unretained compound, t_0 = 0.70 min.

** t_0 = 3.55 min.

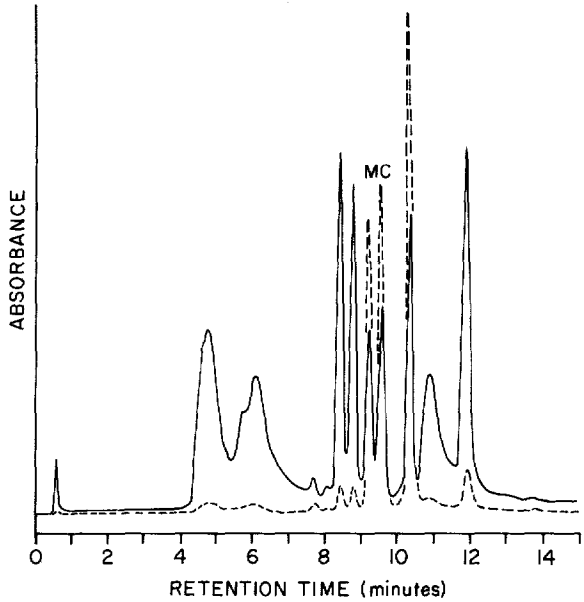


Fig. 2. Chromatogram of a mixture of standards (3a, 2f, 2d, decarbamoyl-2d, decarbamoyl-MC, MC, porfiromycin, 2,7-diaminomitosene). Compounds were eluted with a 13-min linear gradient of 0–50% methanol in 0.01 *M* pH 7.0 potassium phosphate buffer. The flow-rate was 3.0 ml/min and compounds were detected at 313 nm (0.05 a.u.f.s., ———) and 365 nm (0.2 a.u.f.s., – – –). MC peak corresponds to 3.8 nmol.

These were identified as the 2f isomers from a previous report[7] and from MS (see below). These peaks were identified as the *trans-* and *cis* isomers, respectively, by prolonged hydrolysis of purified *trans-* or *cis*-2d in 0.1 *M* hydrochloric acid. Hydrolysis of *cis*-2d gave only the peak at $k' = 8.52$ and hydrolysis of *trans*-2d gave only the peak at $k' = 6.53$.

Hydrolysis of MC with 2 *M* hydrochloric acid for 25 h at 65°C gave two peaks at $k' = 6.17$ and 8.14. These were identified as *trans-* and *cis*-3a, respectively, from a previous report[7], from MS (see below) and from hydrolysis of purified *trans-* or *cis*-2f in 2 *M* hydrochloric acid at 65°C for 2 h.

Many workers have speculated that, besides the reactive center at C-1, the second reactive center on MC responsible for its cross-linking effects on DNA is at C-10[2,18,22–24]. If instead of DNA, water was the nucleophile reacting with the activated MC, one would expect to obtain 10-decarbamoyl-MC or, more likely, the 10-decarbamoyl-2d isomers. Characterization of the chromatographic properties of these compounds was thus an important goal of our methods development. 10-Decarbamoyl-MC was eluted in front of MC at $k' = 11.86$ and was well resolved from *trans*-2d and MC. Mild acid hydrolysis of 10-decarbomoyl-MC gave *trans-* and *cis*-10-decarbamoyl-2d at $k' = 10.36$ and 14.42, respectively. *trans*-10-Decarbamoyl-2d was resolved from *trans*-2d. *cis*-10-Decarbamoyl-2d, however, co-chromatographed with *cis*-2d. The interpretation of an HPLC analysis of a biological reaction or *in vivo* extract of MC by our method must, then, be tempered by the realization that

retention time alone cannot verify the identity of metabolites. In addition to these two products, one must be aware that there may be, as yet unreported, metabolites that could co-chromatograph with the other known products. Our development of a mass spectrometric procedure for the subsequent characterization of MC and isolated mitosene products thus became an important aspect of our method.

2,7-Diaminomitosene has recently been reported as a novel metabolite of MC produced by microsomal incubations[5]. 2,7-Diaminomitosene generated by catalytic hydrogenation of MC has a k' of 15.79. This compound was found to be the most non-polar of all the products characterized. Its identity was confirmed by MS (see below).

Tomasz and Lipman[5] have reported that inorganic phosphate can act as a nucleophile and attack the C-1 position of microsomally activated MC to yield *trans*- and *cis*-2,7-diaminomitosene-1-phosphates (2d-phosphate). These metabolites were generated by hydrolysis of MC in high concentrations of inorganic phosphate (0.34 *M*) and gave peaks at $k' = 6.67$ and 8.60 for the two isomers (Fig. 3). Mild acid hydrolysis (0.1 *M* hydrochloric acid) of either compound purified by semi-preparative HPLC gave peaks at both *cis*- and *trans*-2d from both isomers. Incubation of the purified phosphates with alkaline phosphatase, however, gave one peak at either *cis*- or *trans*-2d. The stereochemistry of the 2d-phosphates was thus assigned on the assumption that the enzymatic phosphate cleavage proceeded with retention of configuration at C-1. The peak at $k' = 6.67$ was assigned as the *trans* and $k' = 8.60$ as the *cis* isomer. Further support was given by analogy to the other pairs of configurational isomers in which the *trans* compound was always eluted before the *cis* compound and was always obtained in lower yield.

MC was originally added to the 0.34 *M* (pH 3.0) sodium phosphate buffer as a concentrated solution in dimethylformamide (DMF). In these reactions a peak at $k' = 12.89$ was obtained. This compound was isolated by semi-preparative HPLC

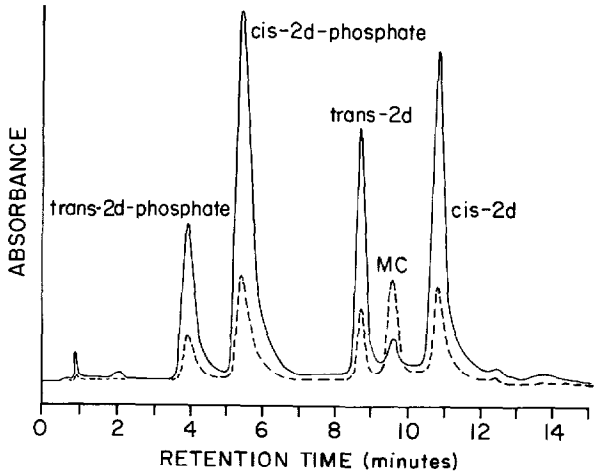


Fig. 3. Chromatogram of products obtained by adding MC to 0.34 *M* pH 3.0 sodium phosphate buffer. HPLC conditions as in Fig. 2. Initial MC concentration was 2.7 m*M* and an injection of 20 μl was made after 120 min. Detector settings were 0.1 a.u.f.s.; 313 nm (———), 365 nm (– – –).

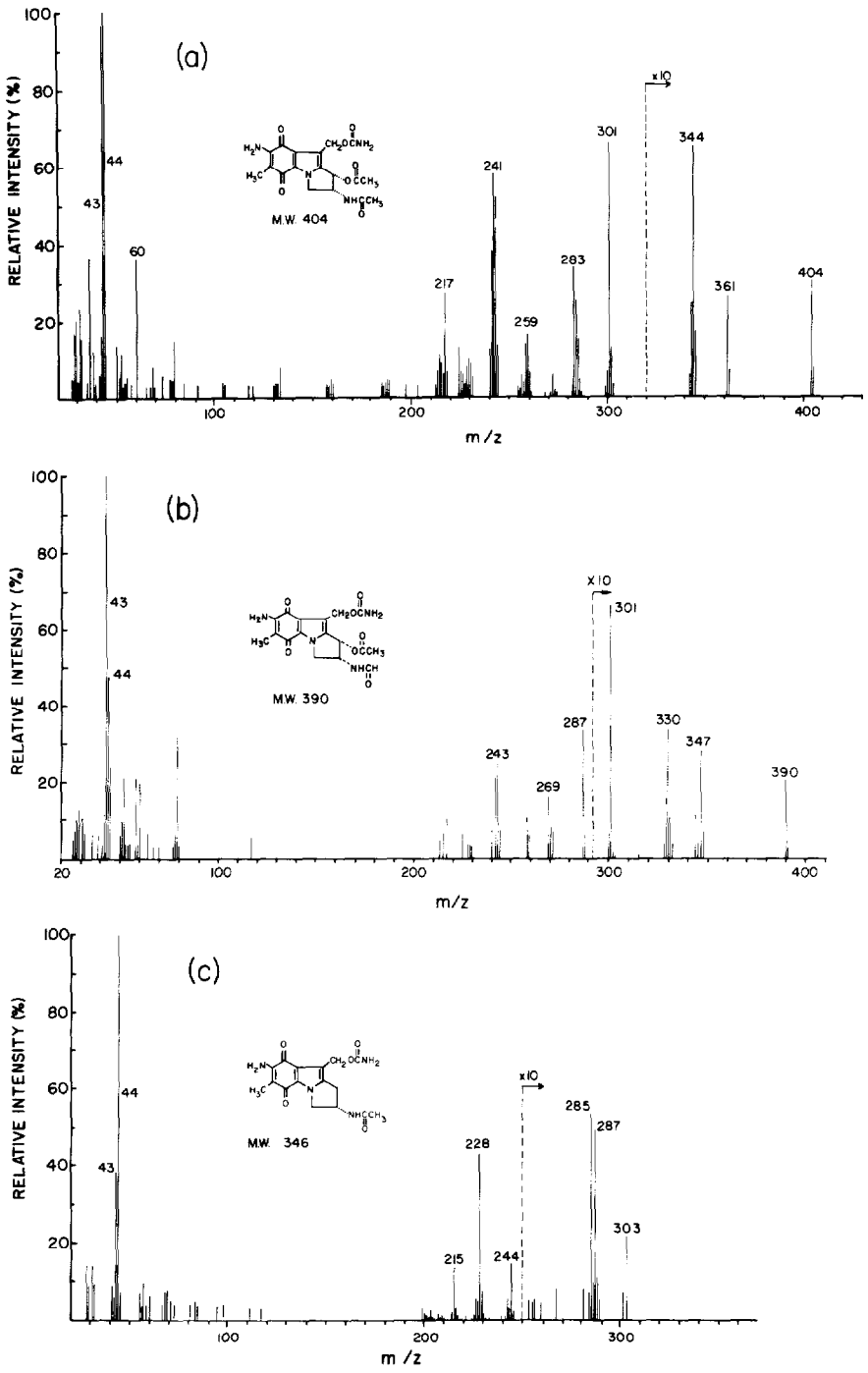


Fig. 4. EI mass spectra of acetylated mitosene compounds. Compounds were analyzed by direct probe insertion on a Vespel rod probe tip at 200°C and 70eV. Compounds are (a) acetylated *cis*-2d, (b) acetylated N^2-formyl-2d and (c) acetylated 2,7-diaminomitosene. Only peaks greater than 3% relative intensity are shown below *m/z* 250.

and had an identical absorbance spectrum to 2d (Table I). Acetylation and mass spectral analysis indicated this compound had a molecular weight fourteen mass units less than *cis*-2d (Fig. 4b). The yield of this compound was dependent on the DMF concentration. In reactions where MC was added from a stock solution in water, or in dimethyl sulfoxide, the peak was absent. The evidence indicates that this compound is 7-amino-2-formamido-1-hydroxymitosene (N^2-formyl-2d). The presence of a molecular ion and high mass fragments in the mass spectrum indicates that this was the *cis* isomer (see *Mass spectrometry*). The appearance of a separate peak that might correspond to the *trans* isomer was not observed. The *trans* isomer may have co-chromatographed with *trans*-2d as the ratio of *trans*- to *cis*-2d increased from 0.56:1.0 with 0% DMF to 0.84:1.0 with 33% DMF in the phosphate buffer. Another possibility was that the formylation–ring cleavage reaction was stereospecific and produced only the *cis* isomer. Both *cis*- and *trans*-2d in 0.34 *M* NaH_2PO_4 (pH 3.0)–10% DMF gave no reaction as analyzed by HPLC and MS. This indicates that the formylation reaction requires the intact aziridine ring. Under acidic conditions, then, the aziridinyl nitrogen must attack the DMF resulting in formylation of the 1a-nitrogen. Simultaneous or subsequent nucleophilic attack by water at C-1 results in the opening of the aziridine ring. Although the mechanism is unclear, this reaction is noteworthy since MC is known to induce DNA interstrand cross-links. These results indicate the 1a-position (aziridinyl nitrogen) can react to form a covalent bond with a suitable substrate in addition to the alkylation reaction that occurs at C-1 at acid pH. If activated MC is juxtaposed in a reactive position on the DNA strands, perhaps a similar reaction could ensue that results in cross-link formation. It remains to be seen if the same formylation reaction can occur when MC is activated by bioreduction rather than acid. In any case DMF was avoided as a solvent in subsequent work.

The HPLC assay was found to be quite sensitive. The setting of 0.1 a.u.f.s. was routinely used and MC could be detected (0.24 mV) down to 100 pmol injected using the 365 nm filter. At the most sensitive setting of 0.005 we were able to detect as little as 5 pmol which compares well with the previous reports of 3.0 pmol[13] and 15 pmol[14]. The sensitivity of *cis*-2d was not as high due to its lower absorbtivity and broader peak shape. At 0.1 a.u.f.s. we were able to detect as low as 600 pmol at 313 nm. The 0.005 a.u.f.s. setting allowed detection of 30 pmol.

The quantitation of MC and metabolites in plasma, bile or tissue requires the use of an internal standard for accurate results. Porfiromycin, in which the aziridinyl nitrogen is methylated, is a likely candidate. Porfiromycin had a capacity factor (k' = 13.65) such that it was eluted very close to *cis*-2d and decarbamoyl-2d. Porfiromycin, then, seems to be an adequate choice as an internal standard but may cause problems in the quantitation of low amounts of *cis*-2d.

The peak shapes of the 3a and 2f isomers were broad. The peak shape of *cis*-2d was particularly broad and tailed on new columns but the asymmetry gradually improved with use. In an attempt to improve the symmetry of these peaks, the mobile phase was modified by adding octylamine hydrochloride (0.005 *M*) or tetraammonium EDTA (0.001 *M*). Neither of these modifiers gave noticeable improvement in the chromatography of these compounds.

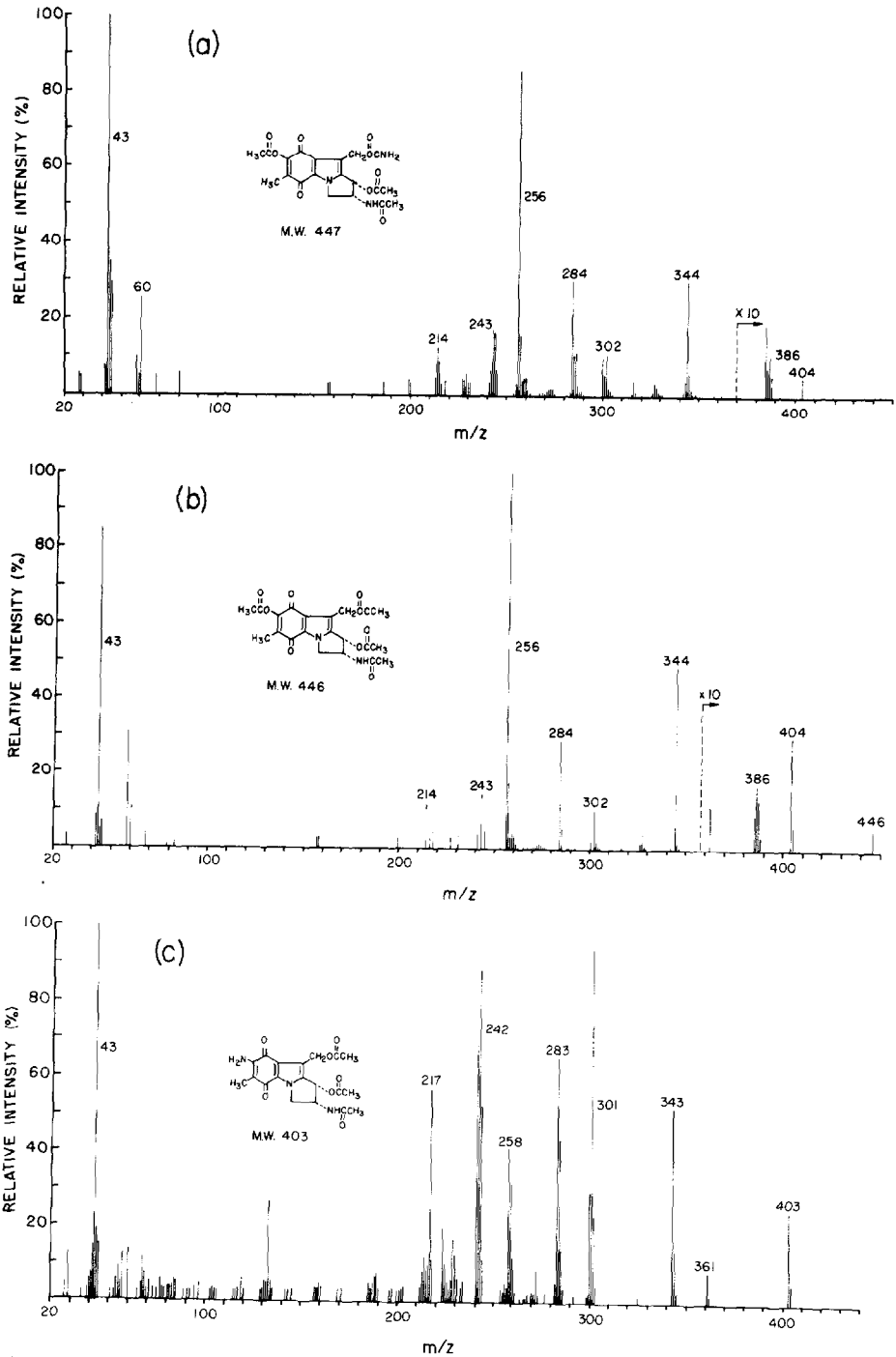


Fig. 5. EI mass spectra of acetylated mitosene compounds. Compounds are (a) acetylated *cis*-2f, (b) acetylated *cis*-3a and (c) acetylated *cis*-decarbamoyl-2d. Only peaks greater than 3% relative intensity are shown below *m/z* 250.

TABLE II

RELATIVE INTENSITIES (%) OF MAJOR FRAGMENTS IN MASS SPECTRA OF MITOSANE COMPOUNDS

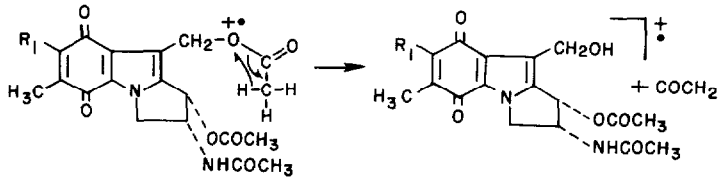
Compound	*Mol.wt.*	$M^{+\cdot}$	*M − 32*	*M − 61*	*M − 43*	*M − 92*	*M − 75*	*M − 76*	*M − 104*	*m/z 43*	*m/z 44*	*Other m/z (%)*
1a-Acetyl-MC	376	0.0	7.1	82.0	1.3	13.9	24.8	–	11.9	47.5	15.1	242 (100.0), 258 (29.9)
10-Decarbamoyl-1a,10-diacetyl-MC	375	1.5	12.2	78.8*	1.4	14.5**	25.0	1.4	11.6***	81.3	5.4	242 (100.0), 258 (39.4)
MC	334	0.8	11.7	64.7	8.5	100.0	29.1	27.5	44.9	16.3	17.6	
10-Decarbamoyl-MC	291	23.2	15.3	100.0	–	–	6.2	6.3	–	43.6	11.3	261 (69.0), 207 (31.7)
Porfiromycin	348	1.0	31.4	83.4	5.3	91.0	47.9	100.0	56.7	15.1	64.6	31 (45.8), 275 (31.3)

* Intensity of ion at $[M - 60]^{+\cdot}$.
** Intensity of ion at $[M - 91]^{+}$.
*** Intensity of ion at $[M - 103]^{+}$.

Mass spectrometry

The MS behavior of mitomycin C and other mitosane analogues has been studied previously by Van Lear[18]. EI-MS of these compounds produced weak molecular ions and extensive but informative fragmentation. The mass spectrum of MC and porfiromycin obtained by us (Table II) was very similar to these previously reported spectra. We could also obtain a mass spectrum of the other mitosane compound, 10-decarbamoyl-MC (Table II). The mitosene compounds, however, where methanol has been eliminated and the aziridine ring cleaved, gave spectra virtually devoid of high mass fragments. The inferior results we obtained with these compounds was suspected to be due to their low volatility caused by intermolecular hydrogen bonding. In an effort to improve their volatility we decided to acetylate these compounds prior to EI-MS analysis. Acetylation of each compound gave near quantitative conversion to the product. An interesting change in the HPLC retention of the isomeric pairs was observed in which the order of elution was reversed compared to the non-acetylated compounds, *i.e.* the acetylated *cis* isomer was eluted before the acetylated *trans* isomer (Table I). The peak shapes of 2f, 3a and *cis*-2d also improved to sharp symmetrical peaks after acetylation. Acetylation of these compounds did indeed improve their EI-MS analysis, giving spectra with molecular ions and extensive fragmentation. The molecular ions, though, were consistently of low intensity relative (less than 1.0%) to the base peak. To improve further the volatilization/desorption of these compounds, we applied them as methanol solutions to a Vespel rod probe tip instead of the customary glass capillary sample tube. A noticeable improvement was seen in the mass spectra, *i.e.* the molecular ion and the major fragments all increased in intensity relative to the base peak (Figs. 4 and 5). The Vespel tip was thus used routinely for the analysis of acetylated mitosene compounds.

The mass spectra of the *cis* isomers all had molecular ions except for *cis*-2f (Fig. 5a). The compounds also had common fragmentation pathways. Loss of an acetyl group (43 a.m.u.) was prominent in all spectra except *cis*-3a (Fig. 5b) and *cis*-10-decarbamoyl-2d (Fig. 5c). These two compounds, instead, gave loss of ketene (42 a.m.u., $COCH_2$). The major difference between these two compounds and the other mitosenes was the presence of an acetoxy group at C-10 instead of a carbamate. It seems likely, then, that the ketene was lost from the C-10 side chain. We suggest the following fragmentation mechanism to explain these results:



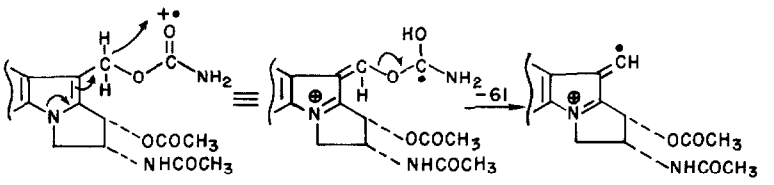
Other prominent ions were loss of 60 (CH_3COOH) and 61 (NH_2COOH) mass units. The loss of carbamic acid can be explained by a mechanism similar to the one proposed by Van Lear[18] for the mitosanes but involving a C-10 hydrogen in a five-center fragmentation instead of a C-9 hydrogen:

TABLE III

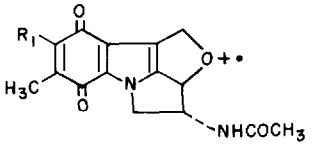
RELATIVE INTENSITIES (%) OF MAJOR FRAGMENTS IN MASS SPECTRA OF *trans* MITOSENE COMPOUNDS

Compound	*Mol.wt.*	$M^{+\cdot}$	*M − 43*	*M − 60*	*M − 61*	*M − 103*	*M − 120*	*M − 162*	*M − 190*	*m/z 43*	*m/z 44*	*Other m/z (%)*
2d	404	–	–	10.8	6.7	57.6	11.5	50.9	8.4	100.0	30.6	–
2f	447	–	–	0.6	1.3	2.7	–	10.1	13.6	100.0	78.2	245 (14.9)
3a	446	–	0.7*	9.4	1.9	38.5**	4.8***	34.4	62.3	100.0	4.6	302 (66.2), 243 (48.5) 215 (26.0)
10-Decarbamol-2d	403	–	–	14.4	–	27.0**	28.6***	10.9	–	100.0	27.3	258 (8.1)

* Intensity of ion at $[M - 42]^+$.
** Intensity of ion at $[M - 102]^+$.
*** Intensity of ion at $[M - 119]^+$.

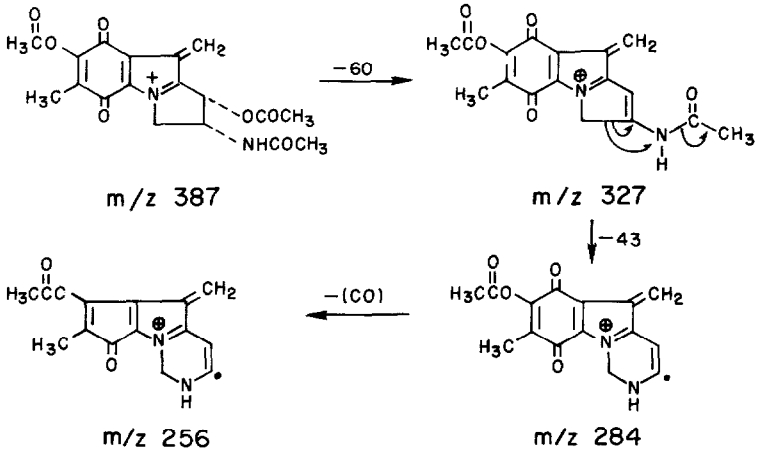


A common fragment was observed at $]M - 103]^{+\cdot}$ in the spectra of acetylated 2d, 2f and N^2-formyl-2d. This fragment ion corresponds to loss of 43 ($-COCH_3$) and 60 ($-OCONH_2$). Acetylated 3a and 10-decarbamoyl-2d had an analogous fragment ion at $]M - 102]^{+\cdot}$ from loss of 43 ($-COCH_3$) and 59 ($-OCOCH_3$). An attractive explanation for the prominence of this peak is the formation of the following fragment ion:



The absence of this fragment ion in the spectrum of acetylated 2,7-diaminomitosene lends support for this proposed structure.

Both acetylated 2f and 3a had intense fragment ions at *m/z* 284 and 256. These ions could conceivably arise from the following fragmentation pathway:



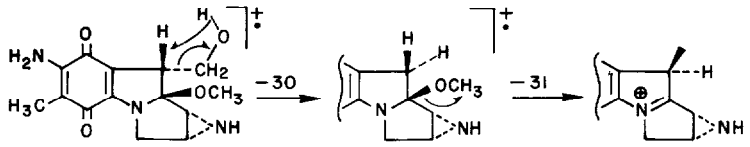
The spectra of acetylated *cis*-2f and *cis*-3a were very similar (Fig. 5a,b). The features that would allow these two compounds to be distinguished (other than by HPLC retention time) are that *cis*-3a has a molecular ion at *m/z* 446 and a lack of an intense ion at *m/z* 44, which is the carbamylium ion. This ion was also very useful in distinguishing between *cis*-2d and *cis*-10-decarbamoyl-2d which had very similar spectra also (Fig. 4a,b).

Other than *cis*-2f the only compound that did not yield a molecular ion was 2,7-diaminomitosene (Fig. 4c). To confirm the structure of the acetylated compound,

it was subjected to field desorption MS at the Middle Atlantic Mass Spectrometry Laboratory. The spectrum obtained was consistent with N^2-acetyl-2,7-diaminomitosene (*m/z* 347 $[MH]^+$, 330 $[MH - NH_3]^+$).

The spectra of the *trans* isomers are shown in Table III. None of the acetylated *trans* compounds gave a molecular ion. The $[M - 43]^+$ ion was usually absent and all the fragments had diminished intensity relative to the base peak. The *trans* mitosenes are thus, either more difficult to volatilize due to more stable crystal structures or are inherently more unstable following ionization than the corresponding *cis* isomers.

Since isolation and acetylation of an unknown peak could conceivably yield a mitosane compound (although an increased response at 365 nm *vs.* 313 nm by HPLC would indicate a mitosane), we acetylated MC and 10-decarbamoyl-MC and characterized their mass spectra (Table II). Interestingly, 1a-acetyl-MC did not give a molecular ion, although acetylated 10-decarbamoyl-MC and porfiromycin did. As mentioned above, underivatized MC and porfiromycin gave spectra similar to those of Van Lear[18]. The spectrum of acetylated 10-decarbamoyl-MC had a fragment ion at $[M - 61]^+$ which usually arises from loss of carbamic acid. The spectrum also had a prominent fragment ion at $[M - 30]^{+\cdot}$. An explanation for these ions in 10-decarbamoyl-MC is proposed in the following fragmentation pathway:



The isobutane CI-MS properties of the acetylated mitosenes were also investigated. Protonated molecular ions, $[MH]^+$, did not appear but instead weak molecular ions, $[M]^{+\cdot}$, were sometimes seen. Clusters of common fragment ions appeared at $[MH - 60]^+$, $[MH - 120]^+$ and $[MH - 162]^+$. CI-MS offered no advantage over the EI mode of operation.

In conclusion, we have examined the properties of a diverse series of mitosane and mitosene derivatives of MC in a reversed-phase HPLC system. We have characterized the retention of these compounds, many of which are potential or known products of the microsomal metabolism of MC. We have also developed a novel EI-MS method for these compounds that volatilizes the acetylated mitosane or mitosene from a Vespel rod probe tip. This technique will assist in the identification of trace amounts of *in vitro* or *in vivo* metabolites, or degradation products of MC. We have used these techniques to isolate and identify a new compound, N^2-formyl-2d, obtained from the reaction of MC with DMF of pH 3.0. This product implicates a new mechanism for the cross-linking ability of MC on DNA.

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