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# Metabolites and DNA Adduct Formation from Flavoenzyme-Activated Porfiromycin

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**SUMMARY**

Porfiromycin was reductively metabolized by NADPH cytochrome P-450 reductase and xanthine oxidase under anaerobic conditions. The production of metabolites varied with the pH and the contents of the reaction buffer. In Tris buffer, two major metabolites were produced at pH 7.5 and above, whereas one major metabolite was produced at pH 6.5. The three major metabolites were separated and isolated by HPLC. Identification by californium-252 plasma desorption mass spectrometry showed that the two major metabolites from pH 7.5 were (*trans*) and (*cis*)-forms of 7-amino-1-hydroxyl-2-methylaminomitosene and the major metabolite from pH 6.5 was 7-amino-2-methylaminomitosene. All three major metabolites showed substitutions at the C-1 position. DNA was alkylated readily by enzyme-activated porfiromycin. Digestion of porfiromycin-alkylated DNA by DNase, snake venom phosphodiesterase, and alkaline phosphatase resulted in an insoluble nuclease-resistant fraction and a soluble fraction. The nuclease-resistant fraction reflected a high content of cross-linked adducts. Upon HPLC analysis, the solubilized fraction contained two monofunctionally linked porfiromycin adducts and a possibly cross-linked dinucleotide. The major adduct was isolated by HPLC and identified by NMR, as $N^2$-(2′-deoxyguanosyl)-7-amino-2-methylamino-mitosene. The $N^2$ position of deoxyguanosine appeared as the major monofunctional alkylating site for DNA alkylation by porfiromycin. Thus, mitomycin C and porfiromycin (which differs from mitomycin C only by the addition of a methyl group to the aziridine nitrogen) share the same enzymatic activating mechanism that leads to the formation of the same types of metabolites and the same specificity of DNA alkylation.

PFM (Fig. 1), the aziridine *N*-methyl analogue of MC, is a potent antitumor agent. This agent behaves similarly to MC, being a reductive alkylating agent and producing monofunctional and bifunctional covalent bonds with macromolecules such as DNA, RNA, and protein (1–3). Chemically, the difference rendered by the methylation of the aziridine nitrogen is minimal and changes the p$K_a$ value from 1.19 to 0.91 (4). Clinically, PFM is not used as widely as MC although the two agents show similar efficacy and toxicity (5) and the tolerated dose of PFM is higher than that of MC in human trials (5–7). Recently, increasing attention has been focused on these agents due to the discovery that PFM and MC are preferentially more toxic to hypoxic cells (8, 9), a feature of significant clinical interest. The molecular mechanism of MC in terms of its metabolism and specific alkylating sites has been well studied by Tomasz *et al.* (10–13) and Pan *et al.* (14, 15). The current paper will present 1) the preparation of metabolites and PFM-DNA by enzyme-activated PFM, 2) the effects of reaction conditions of the formation of metabolites and DNA adducts, and 3) the isolation and structural analysis of those metabolites and DNA adducts.

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## Materials and Methods

PFM was kindly supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD) and by Dr. J. P. McGovren, Upjohn (Kalamazoo, MI). Bovine milk xanthine oxidase (EC 1.2.3.2) and rat liver NADPH cytochrome P-450 reductase (EC 1.6.2.4) were isolated according to the method of Nelson and Handler (16) and Yasukochi and Masters (17), respectively. Proteins were determined by a modified method of Lowry *et al.* (18). The sources for other enzymes and compounds are as follows: snake venom phosphodiesterase (EC 3.1.4.1), and alkaline phosphatase (EC 3.1.3.1) from Worthington (Freehold, NJ); calf thymus DNA, *Micrococcus lysdeikticus* DNA, and pancreatic DNase (EC 3.1.4.5) from Sigma Chemical Co. (St. Louis, MO); pyridine nucleotides from Boehringer Mannheim Biochemical (Indianapolis, IN). Radioactive PFM ([$^{14}$C]PFM) was synthesized by the reaction of MC with [$^{14}$C]methyl iodide (19) and was purified by HPLC (14).

### Enzymatic Reduction of PFM

The reduction of PFM by NADPH cytochrome P-450 reductase or xanthine oxidase was carried out as reported by Pan *et al.* (14) except that MC was replaced by PFM. Tris-HCl (50 mM) was used to buffer reactions at pH 7.5 or higher, and imidazole·HCl (50 mM) was used to buffer reactions at pH 7.0 or lower.

**ABBREVIATIONS:** PFM, porfiromycin; MC, mitomycin C; PFM-DNA, porfiromycin-alkylated DNA; PFM-adduct, porfiromycin-linked nucleoside; HPLC, high performance liquid chromatography.

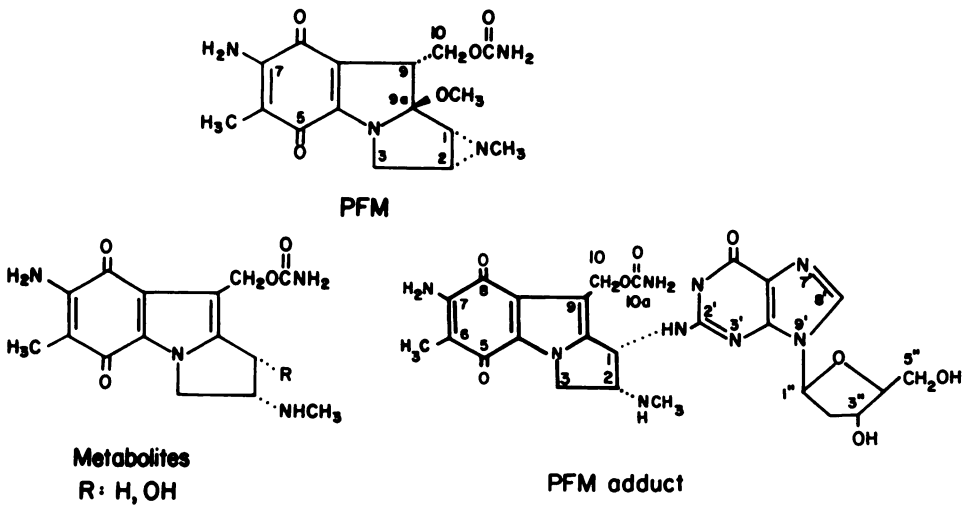


Fig. 1. Structure of 1) PFM; 2) 7-amino-1-hydroxyl-2-methylaminomitosene or 7-amino-2-methylaminomitosene; and 3) PFM nucleoside adduct.

### PFM-DNA Preparation and Digestion

Procedures for the preparation of PFM-DNA by xanthine oxidase-activated PFM were the same as previously described for MC-DNA with some modifications (15). 1) PFM-DNA and [$^{14}$C]PFM-DNA for analytical use were prepared with 1 mg/ml calf thymus DNA and 0.1 mM PFM (specific activity at 22.8 nCi/nmol for [$^{14}$C]PFM). 2) PFM-DNA used for the isolation of PFM-adducts was prepared with 2 mg/ml micrococcal DNA (high GC content) and 2 mM PFM. PFM-modified DNA was precipitated with 2 volumes of cold 95% ethanol. The DNA precipitate was twice dissolved in 50 mM Tris·HCl/100 mM NaCl (pH 7.5) and precipitated by ethanol. The resulting DNA solution was heated at 100° for 15 min and reannealed to remove possible intercalated PFM or metabolites. This final PFM-DNA was reprecipitated and dissolved in 50 mM Tris·HCl/1 mM $MgCl_2$ (pH 7.0) for nuclease digestion. Specifics of the digestion of PFM-DNA by DNase, snake venom phosphodiesterase, and alkaline phosphatase were as described previously for MC-DNA (15). PFM-DNA was also prepared with sodium dithionite-activated PFM, purified, and digested according to the method reported by Tomasz *et al.* (13).

### Estimation of PFM Bound to DNA

Quantitative estimation of the amount of PFM covalently bound to DNA was based on the molar absorptivities of native DNA (7000 at 260 nm), an MC-adduct, and $N^2$-(2′-deoxyguanosyl)-2,7-diaminomitosene (6030 at 312 nm) reported by Tomasz *et al.* (11, 12) and assumed the molar absorptivity of DNA-bound PFM to be close to that of the MC-adduct. Measurements, before nuclease digestion, of PFM-modified DNA at 312 nm reflected the total bound PFM. The PFM-adducts resolved by HPLC were calculated with the purified major MC-adduct, $N^2$-(2′-deoxyguanosyl)-2,7-diaminomitosene as the external standard. Results were presented as number of nmoles of PFM-adducts per μmole of DNA. The amount of total [$^{14}$C]PFM bound to DNA was determined by counting samples of [$^{14}$C]PFM-DNA in a Searle Mark III scintillation counter.

### Detection and Isolation of Metabolites and PFM-Adducts

A reversed phase HPLC method published earlier (14, 15) was used to separate and detect the metabolites in reaction mixtures or PFM-adducts in nuclease-digested DNA. To detect radioactive adducts, HPLC fractions of 0.5 ml were collected with a Pharmacia FRAC-100 fraction collector and the $^{14}$C in each fraction was determined by liquid scintillation counting. Purified nonradioactive adducts and [$^{14}$C]PFM were used as internal and external HPLC standards, respectively. For the isolation of metabolites or adducts, the products were collected upon elution from the column, concentrated, and lyophilized. Comparative analysis of PFM-DNA prepared by enzymatic and dithionite methods was performed with a Hewlett Packard 1090M liquid chromatograph (HP 1090) equipped with a UV/visible diode array absorbance detector (Hewlett Packard, Palo Alto, CA) and a Brownlee RP-18 5 μm, 22 cm × 4.6 mm column. The mobile phase, consisting of a gradient of 0 to 50% methanol in 10 mM phosphate over 25 min, was pumped at 1.2 ml/min.

### Spectroscopic Analysis

**UV and visible absorbance.** Absorbance spectra of PFM metabolites and PFM-adducts were obtained with a Cary 118 spectrophotometer (Varian, Palo Alto, CA). All measurements were made in 0.1 M phosphate buffer (pH 7.5). During HPLC separation of PFM-adducts, absorbance spectra were observed and analyzed with the diode array detector and its software.

**Mass spectrometry.** Mass spectra were obtained with a californium-252 plasma desorption mass spectrometer (built by Dr. R. MacFarlane at Texas A & M University, College Station, TX). Samples were applied by electrospraying. Data were accumulated for 30 min each at accelerating voltage of 10 kV and were processed with a Perkin-Elmer 3220 data system. (Mass spectra were kindly provided by Dr. Henry M. Fales of the National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD.)

**NMR spectra.** Proton NMR spectra were obtained with a Varian XL-200 NMR spectrometer. Samples of 300 μg were lyophilized twice, redissolved in 0.5 ml of deuterated dimethyl sulfoxide, and scanned with tetramethylsilane as standard. Data were accumulated over 512 scans. (NMR spectra were kindly provided by Dr. Robert Highet of the National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD.)

## Results

### Reduction of PFM

Anaerobic reduction of PFM by either NADPH cytochrome P-450 reductase or xanthine oxidase generated several metabolites. HPLC analyses of reaction mixtures revealed different profiles of metabolites depending on the pH and the contents of the reaction mixture. At pH 6.5 to 7.0 in imidazole·HCl buffer (Fig. 2a), there were one major metabolite ($k'$ = 20.5) and four less prominent metabolites ($k'$ = 14.9, 17.6, 18.2, and 22.0) with the high absorbance at 313 nm that is typical of mitosene compounds. In Tris·HCl buffer, at pH 7.5 to 8.5 (Fig. 2b) two of the minor metabolites produced at pH 6.5 ($k'$ = 14.9 and 17.6) became the dominant products. Also, at these more alkaline conditions, a number of minor metabolites ($k'$ = 7.1, 8.6, 11.6, 18.2, 19.3, and 19.9) were detected that were not formed at the lower pH. The amount of product with $k'$ of 20.5

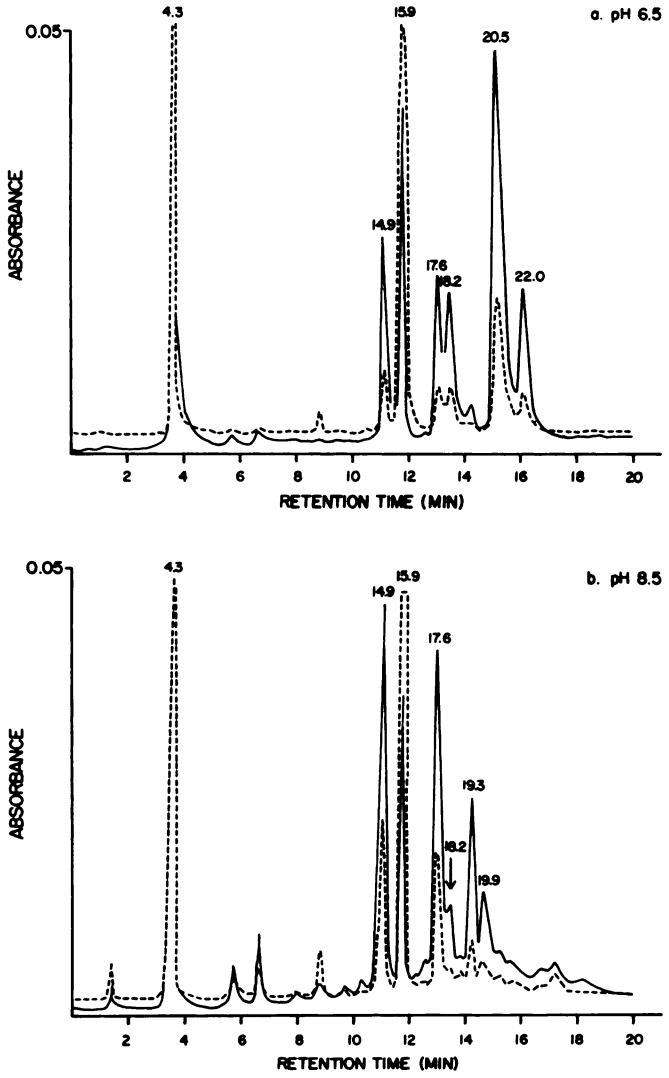


**Fig. 2.** HPLC analysis of the metabolites of PFM catalyzed by xanthine oxidase. The reaction and HPLC were carried out as described in text (a) in 0.1 M imidiazole·HCl at pH 6.5; (b) in 0.1 M Tris·HCl at pH 8.5. Detector settings were 0.05 absorbance units full scale. ——, 313 nm; – – –, 365 nm.

decreased as the pH of the reaction increased. At pH 8.5, metabolite 20.5 was not detectable. Unreacted PFM with high absorbance at 365 nm was detected at $k' = 15.9$. If reactions were carried out in phosphate buffers, the metabolites produced at pH 6.5 were essentially the same as those produced in the imidazole·HCl buffer at pH 6.5. But at pH 7.5 and higher, two more prominent metabolites not observed in Tris·HCl buffer ($k' = 9.6$, 11.8) were detected (data not shown).

### Structural Analysis of Metabolites

**Absorbance spectra.** All three major metabolites ($k' = 14.9$, 17.6, and 20.5) had maximum absorbance at 312 nm, which is characteristic of 7-aminomitosene compounds (19).

**Mass spectra.** The plasma desorption mass spectrum of PFM gave a molecular ion at $m/z$ 348, and a sodium adduct ion at $m/z$ 371 (Table 1). Loss of 32 (—$CH_3OH$) and 60 (—$OCONH_2$), an indicative fragmentation for PFM, was found at $m/z$ 256. The plasma desorption mass spectra of metabolites $k' = 14.9$ and 17.6 were similar. Each demonstrated a molecular ion at $m/z$ 334 and a sodium adduct ion at $m/z$ 357. Major

TABLE 1

**Proposed fragmentation of plasma desorption mass spectra of PFM and metabolites**

Spectra were obtained with a californium-252 spectrometer. Samples were applied by electrospraying. Data were accumulated for 30 min each at accelerating voltage of 10 kV.

| PFM and metabolites[a] | Ions | | | | |
|---|---|---|---|---|---|
| | M | (M + Na) | M – ($OCONH_2$) | (M + Na) – ($OCOCH_3$) | M – ($OCONH_2$, $CH_3OH$) |
| | *m/z* | | | | |
| PFM | 348 | 371 | | | 256 |
| 14.9 | 334 | 357 | 274 | 297 | |
| 17.6 | 334 | 357 | 274 | 297 | |
| 20.5 | 318 | 341 | 258 | 281 | |

[a] Expressed by $k'$ value of HPLC.

fragment ions of these two metabolites were observed at $m/z$ 274 and 297, corresponding to loss of 60 (—$OCONH_2$) from the parent compound and the sodium adduct, respectively. The plasma desorption mass spectrum of metabolite $k' = 20.5$ revealed a molecular ion at $m/z$ 318 and a sodium adduct ion at $m/z$ 341. This metabolite had analogous fragment ions at $m/z$ 258 and 281 consistent with loss of 60 (—$OCONH_2$) from the parent compound and sodium adduct, respectively. Loss of methanol, a fragmentation that was typical for PFM, was not found in the spectrum of any metabolite. The two metabolites ($k' = 14.9$ and 17.6) with similar mass spectra were designated as (*trans*)- and (*cis*)-7-amino-1-hydroxyl-2-methylaminomitosene, respectively, and metabolite $k' = 20.5$ was designated as 7-amino-2-methylaminomitosene (Fig. 1).

### DNA Alkylation

Enzyme-activated PFM reacted with DNA to produce a DNA product with the high absorbance at 312 nm that is characteristic of mitosene. Total PFM bound to DNA was dependent on 1) reaction pH, 2) concentrations of PFM and DNA, and 3) reaction time (data not shown). Nuclease digestions solubilized this PFM-DNA partially to give an aqueous-insoluble fraction and a solubilized fraction, with both fractions containing covalently bound mitosenes. Nuclease $S_1$, nuclease $P_1$, and micrococcal nuclease (EC 3.1.30.1) were also used alone or in combination, but the resistant fraction persisted. HPLC analysis of the solubilized fraction revealed PFM-adducts. The nuclease-resistant fraction was determined as the total bound PFM minus the HPLC-detected PFM-adducts. The PFM-alkylated DNA products differed in composition when the pH of the reaction mixture was varied from 6.5 to 8.5 (Fig. 3). With similar amounts of total PFM bound to DNA, PFM-DNA prepared at lower pH contained less HPLC-detectable adducts and was more resistant to nuclease digestion than was PFM-DNA prepared at higher pH. We confirmed the observation by using [$^{14}$C]PFM to alkylate DNA. Radioactive adducts detected in the nuclease-solubilized fraction increased with increasing pH (Table 2). The nuclease-resistant fraction represented a large portion of the total bound PFM of all PFM-DNA prepared by enzymatic reduction. PFM-DNA produced by the dithionite method, a complex with a binding ratio (mol of PFM/1 mol of mononucleotide) of 0.05 to 0.07 showed little resistance to nuclease digestion. Greater than 95% of the alkylated products were recovered in soluble form. With the same buffer and the same concentrations of PFM and DNA, enzymatic reduction

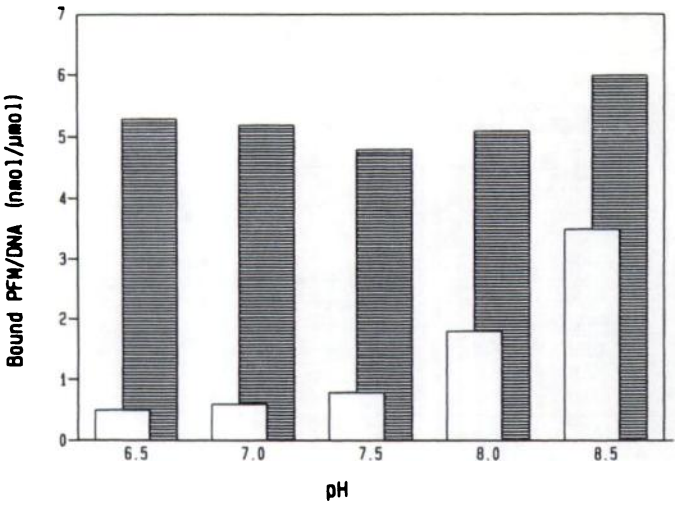


**Fig. 3.** Effect of buffer pH on PFM alkylation of DNA. Xanthine oxidase reductase was the activating enzyme. Incubation time was 45 min. Initial PFM/DNA concentration ratio was 0.1 mM/2 mM. Preparation, nuclease digestion of PFM-DNA, and the HPLC method are as described earlier (14, 15). Total amount of PFM bound in PFM-DNA was measured by absorbance at 312 nm, taken before nuclease digestion as total alkylation (■). HPLC-detected PFM-adducts (□) were monitored by absorbance at 313 nm during HPLC separation using purified $N^2$-(-2′-deoxyguanosyl)-2,7-diaminomitosene as external standard. Calculation was based on the molar absorptivity of $N^2$-(-2′-deoxyguanosyl)-2,7-diaminomitosene (6030 at 312 nm). DNA was estimated by absorbance at 260 nm (molar absorptivity, 7000).

TABLE 2

**Distribution of radioactivity of [$^{14}$C]PFM-DNA**

Preparation of [$^{14}$C]PFM-DNA, DNA digestion by nucleases, and the detection of radioactivity were as described in text.

| pH | Radioactivity | |
|---|---|---|
| | Total | Soluble adducts |
| | *dpm* | |
| 6.5 | 9,650 | 1,850 (19.1)[a] |
| 7.5 | 11,200 | 2,780 (24.8) |
| 8.5 | 12,650 | 6,300 (49.8) |

[a] Percentage of total.

yielded a complex with a binding ratio of 0.2 to 0.3, but less than 40% of the total was solubilized by nucleases.

### Identification of PFM-Adducts

**HPLC analysis.** HPLC analysis of PFM-DNA generated by the enzymatic method revealed four adduct peaks (I, II, III, and IV) at 19.37, 19.64, 20.54, and 21.26 min, respectively, having high absorbance at both 313 and 254 nm (Fig. 4). Other materials separated had high absorbance at 313 nm but not at 254 nm. HPLC analysis of PFM-DNA generated by the dithionite method had peaks at 19.64, 20.54, and 21.26 min, which co-chromatographed with the adduct peaks II, III, and IV produced by the enzymatic method (Fig. 4). Unalkylated nucleosides were eluted during the first 8 min of the gradient (not shown in the graph). Peak I was the major adduct produced by the enzymatic method, containing up to 90% of the total soluble adducts. The PFM-DNA produced by the dithionite method had no detectable peak I, whereas peaks II and III were produced as major adducts, containing about 50 and 40% of the total adducts, respectively.

**UV and visible absorbance.** The absorbance spectra, obtained by diode array detector during HPLC separation, for

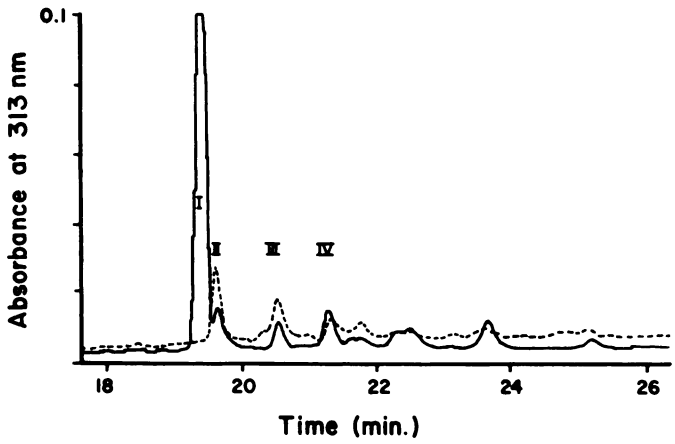


**Fig. 4.** HPLC profile of PFM-DNA after digestion by nucleases. HPLC analysis with HP1090 equipped with diode array detector was carried out as described in text. Chromatograph with absorbance at 313 nm is shown between 18 to 26 min. Detector setting was 0.1 absorbance units full scale. DNA alkylation was performed in 15 mM Tris·HCl at pH 7.4 with 0.335 mM PFM and 0.07 mM DNA and xanthine oxidase as PFM-activating enzyme (——), or sodium dithionite as reducing agent (1.5 mol/mol of DNA) as described by Tomasz *et al.* (13) (– – –). Methods for PFM-DNA digestion by DNase, snake venom phosphodiesterase, and alkaline phosphatase were described earlier (15).

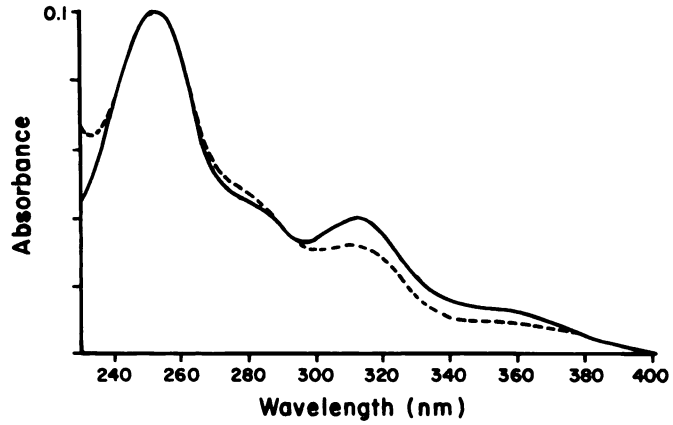


**Fig. 5.** UV and visible absorbance spectra of PFM-adduct peaks during HPLC separations. Spectra were recorded by UV/visible diode array detector. Spectra were present after normalization at 254 nm. ——, peak I; – – –, peak III.

adduct peaks I and III are presented after normalization of each spectrum at 254 nm (Fig. 5). Peaks I, II, and IV from both sources showed similar spectra whereas peak III showed substantially less absorbance at 312 nm. For peak III, the ratio of absorbance at 254 nm to absorbance at 312 nm was between 2.8 and 3.1 whereas this ratio for peaks I, II, and IV was between 2.3 and 2.5, indicating that peak III contained two nucleoside units. Subsequently, the main nucleoside adduct (peak I) was isolated and its absorption spectrum determined. This spectrum, with maximum absorption at 312 and 252 nm and with shoulders at 360 and 284 nm, was a combination of the spectra of nucleoside and mitosene.

**NMR spectra.** The proton NMR spectrum of the major nucleoside adduct (peak I) was obtained in deuterated dimethyl sulfoxide (Fig. 6). For the mitosene portion, it showed the 6-methyl peak at $\delta$1.75 and the 2-*N* methyl at $\delta$2.43. The 10-methylene protons were seen at $\delta$4.95. The mitosene 1-proton was overlapped by 3″-H of deoxyribose at $\delta$5.15. The 3-protons

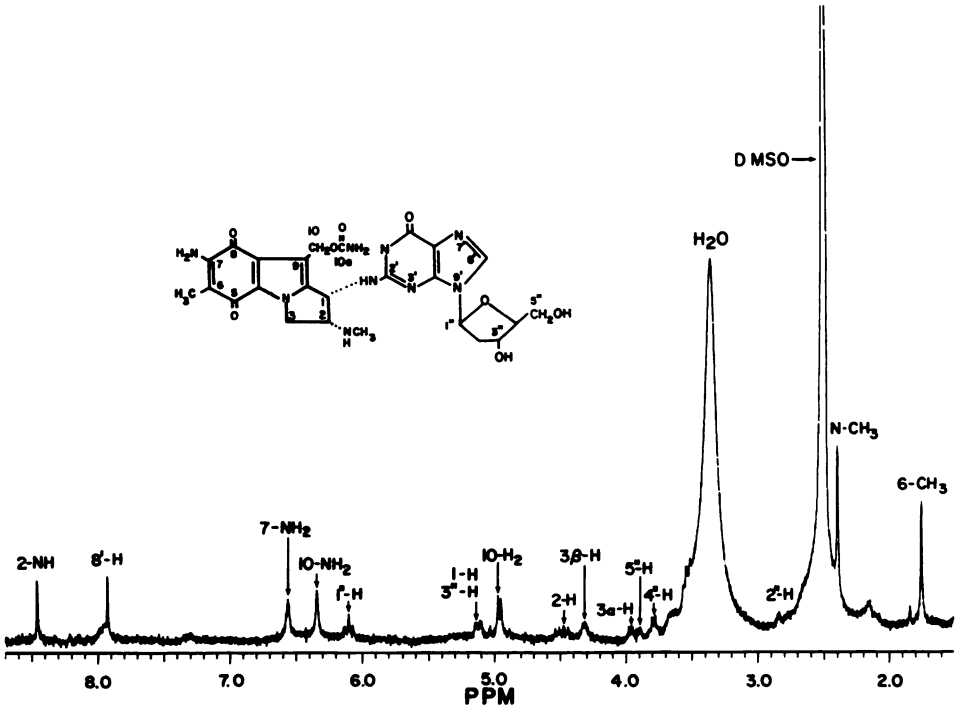


**Fig. 6.** Proton NMR spectrum of the major PFM-adduct. NMR spectrum was obtained with a Varian XL-200 NMR spectrometer. Sample of 300 μg was lyophilized twice then dissolved in 0.5 ml of deuterated dimethyl sulfoxide. Accumulation of 512 scans is presented.

were shown at δ3.95 and δ4.33. The multiplet around δ4.5 was probably the 2-proton. The 7-amino and 10a-amino protons were shown as singlets at δ6.35 and δ6.56. A downfield proton signal at δ8.45 was assigned to 2-NH. As for the deoxyribose portion, a triplet at δ6.10 was assigned to the 1″ proton and multiplets at δ2.20 and δ2.85 were assigned to 2″ protons. Anomeric 4″ and 5″ protons were detected at δ3.8 and δ3.9, respectively. The singlet at δ7.95 were assigned to the 8′-H proton of the purine. We were unable to obtain enough of the minor adducts for NMR analysis.

## Discussion

Three major metabolites of PFM were identified as 7-amino-2-methylaminomitosene and the two isomeric forms of 7-amino-1-hydroxyl-2-methylaminomitosene. The relative production of these three metabolites is directly dependent on reaction conditions. These results confirm our earlier proposal for the reaction mechanism of MC. Only the initial step of MC activation is enzyme catalyzed and the subsequent production of primary metabolites is dependent on reaction conditions (14). We believe that, in a similar fashion, PFM is activated to the PFM free radical anion, followed by the loss of methanol between C-9 and C-9a. Conversion to metabolites at this point would depend on reaction conditions. Nucleophilic attack by water would give rise to the isomers of 7-amino-1-hydroxyl-2-methylaminomitosene, and H-abstraction would result in the formation of 7-amino-2-methylaminomitosene. Two of the extra metabolites formed in phosphate buffer ($k' = 9.6$ and 11.8) are probably the isomers of 7-amino-2-methylamino-1-phosphomitosene, which result from nucleophilic attack by phosphate. These primary metabolites argue for the C-1 position of PFM as the center of action for monofunctional binding. The primary metabolites do not give any information about the two sites on the PFM molecule required for bifunctional alkylation (cross-linking). It is possible that some of the less abundant metabolites contain this information. It is also possible that metabolites that would provide information about cross-linking have different absorbance maximum and we failed to detect them.

PFM-DNA produced enzymatically contains one major adduct (peak I) that is not produced by the dithionite method. Our structural identification of this major PFM-DNA adduct is based primarily on NMR data (Fig. 6). Of the possible alkylating sites on the mitosene moiety, e.g., C-10 carbamate, C-7 amino, C-6 methyl, 2-*N*-methyl, 2-amino, and 1-proton, all except the C-1 position are accounted for as unsubstituted positions. Therefore, we feel that the C-1 position is where mitosene is linked to nucleosides. As for the position on the deoxyribose that might be linked to PFM, 3″-OH and 5″-OH should be unsubstituted, inasmuch as both positions are linked to phosphate in the DNA skeleton and the removal of phosphate from nucleotide by alkaline phosphatase did not have any effect on the mitosene linkage. Anomeric protons at 1″, 2″, 3″, 4″, and 5″ were seen in the NMR spectrum, which means they are unsubstituted. Therefore, mitosene is not likely to be attached to deoxyribose and the linkage must be on the purine moiety. The 8′-H proton signal indicates no substitution at this position. Position 7′ is ruled out due to the fact that the signal of the 8′-H proton is unshifted. The remaining possible positions are N-1, $N^2$, N-3, and $O^6$. The N-3 position is not likely because products alkylated at this position are usually unstable. The linkage of mitosene to nucleoside is, therefore, likely to be at either the $O^6$ or $N^2$ positions of guanine. Tomasz *et al.* (11) identified a major MC-deoxyguanosine adduct as $O^6$-deoxyguanosyl-2,7-diaminomitosene, and later reassigned it to be $N^2$-deoxyguanosyl-2,7-diaminomitosene (12). They have suggested that a free 2′-$NH_2$ group of the guanosine moiety would be expected to exhibit proton resonances in the region around 6–7 ppm. If indeed the 2′-$NH_2$ is free, then the three amino groups, including the 7-amino and 10a-amino protons of

mitosene, should allow six protons to be observed. Our present NMR spectrum only indicates four protons, compatible with two amino groups in this region, which suggests that the 2′-$NH_2$ group of guanosine is not free. We feel that our major PFM-adduct has an $N^2$-deoxyguanosine linkage similar to the MC-adduct described by Tomasz *et al.* (12). This adduct is probably $N^2$-(-2′-deoxyguanosyl)-7-amino-2-methylaminomitosene.

For PFM-DNA synthesized by both the enzymatic and the chemical methods, only one adduct, peak III, was probably a cross-linked adduct or an adduct with two nucleosides, as indicated by the higher ratio of absorbance at 254 to 312 nm. Although peak III represented only a small fraction of the total soluble adducts in enzymatically produced PFM-DNA, the variation with pH in the amounts of nuclease-resistant fractions is probably related to the degree and the complexity of DNA cross-linking. Presently, the precise nature of this nuclease resistant fraction is not clear. Most recently Tomasz *et al.* (13) reported the isolation and structural determination of a covalent cross-link adduct between MC and DNA. In this adduct, the $N^2$ positions on each of two deoxyguanosines are linked by the C-1 and C-10 positions of MC. These workers also found that reducing systems influence whether MC reacts to produce monofunctional or bifunctional DNA adducts. Under their conditions, use of flavoenzymes or $H_2/PtO_2$ as the reductive activating agent resulted in the production mainly of monofunctional adducts and small amounts of the bifunctional DNA-adduct, whereas use of dithionite as the reductive activating agent resulted in the production of predominantly one monofunctional and one bifunctional adduct. They felt that flavoenzymes behaved similarly to the $H_2/PtO_2$ system but not the dithionite system. Their suggestion is that the factor controlling monofunctional versus bifunctional activation is the reduction kinetics rather than reduction thermodynamics of these different reducing systems. Cross-linking is formed by a secondary reaction of activated monoadduct. In the case of flavoenzymes and $H_2/PtO_2$ activation, autocatalytic reaction of MC leads the activated monoadducts to nonactive species; therefore, fewer cross-links are formed. It is not certain whether other factors are also involved with the enzyme activation, because a large fraction of nuclease-resistant adducts is present in our preparations and their nature remains to be identified.

In comparing the characteristics of PFM with MC in terms of metabolite formation and adduct formation, the addition of a methyl group on the aziridine nitrogen for PFM did not have any effect. The two compounds generated similar patterns of metabolites and the same pattern of DNA adducts after enzymatic and chemical activation. Although the precise structure of cross-linking for PFM remains to be analyzed, our belief is that the soluble PFM cross-linked adduct (peak III) has a structure similar to that of the MC cross-linked adduct identified by Tomasz *et al.* (13).

**Acknowledgments**

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**References**

1. Weissbach A., and A. Lisio. Alkylation of nucleic acids by mitomycin C and porfiromycin. *Biochemistry* **4**:196–200 (1965).
2. Iyer, V. N., and W. Szybalski. Mitomycins and porfiromycin: chemical mechanism of activation and cross-linking of DNA. *Science (Wash. D. C.)* **145**:55–58 (1964).
3. Iyer, V. N., and W. Szybalski. A molecular mechanism of mitomycin action: linking of complementary DNA strands. *Proc. Natl. Acad. Sci. USA* **50**:355–362 (1985).
4. Underberg, W. J. M., and H. Lingeman. Aspects of the chemical stability of mitomycin and porfiromycin in acid solution. *J. Pharm. Sci.* **72**:549 (1983).
5. Baker, L. H., R. M. Izbicki, and V. K. Vaitkevicius. Phase II study of porfiromycin vs. mitomycin C utilizing acute intermittent schedules. *Med. Pediatr. Oncol.* **2**:207–213 (1976).
6. Foley, T. H., B. I. Schnider, L. G. Gold, P. I. McTias, J. Colsky, and S. P. Miller. Phase I studies of porfiromycin (NSC-56410). *Cancer Chemother. Rep.* **51**:283–293 (1967).
7. Grage, T. B., A. J. Weiss, W. Wilson, and V. Reynolds. Phase I studies of porfiromycin (NSC-56410) in solid tumors. *J. Surg. Oncol.* **7**:415–410 (1975).
8. Kennedy, K. A., S. Rockwell, and A. C. Sartorelli. A preferential activation of mitomycin C to cytotoxic metabolites by hypoxic tumor cells. *Cancer Res.* **40**:2356–2360 (1980).
9. Keyes, S. R., S. Rockwell, and A. C. Sartorelli. Porfiromycin as a bioreductive alkylating agent with selective toxicity to hypoxic EMT6 tumor cells *in vivo* and *in vitro*. *Cancer Res.* **45**:3642–3645 (1985).
10. Tomasz, M., and R. Lipman. Reductive metabolism and alkylating activity of mitomycin C induced by rat liver microsomes. *Biochemistry* **20**:5056–5061 (1981).
11. Tomasz, M., R. Lipman, J. K. Snyder, and K. Nakanishi. Full structure of a mitomycin C dinucleoside phosphate adduct: use of differential FT-IR spectroscopy in microscale structural studies. *J. Am. Chem. Soc.* **105**:2059–2063 (1983).
12. Tomasz, M., R. Lipman, G. L. Verdine, and K. Nakanishi. Reassignment of the guanine-binding mode of reduced mitomycin C. *Biochemistry* **25**:4337–4344 (1986).
13. Tomasz, M., R. Lipman, D. Chowdary, J. Pawlak, G. L. Verdine, and K. Nakanishi. Isolation and structure of a covalent cross-link adduct between mitomycin C and DNA. *Science (Wash. D. C.)* **235**:1204–1208 (1987).
14. Pan, S., P. A. Andrews, C. J. Glover, and N. R. Bachur. Reductive activation of mitomycin C and mitomycin C metabolites catalyzed by NADPH-cytochrome P-450 reductase and xanthine oxidase. *J. Biol. Chem.* **259**:959–966 (1984).
15. Pan, S., T. Iracki, and N. R. Bachur. DNA alkylation by enzyme activated mitomycin C. *Mol. Pharmacol.* **29**:622–628 (1986).
16. Nelson, C. A., and P. Handler. Preparation of bovine xanthine oxidase and the subunit structures of some iron flavoproteins. *J. Biol. Chem.* **234**:5368–5373 (1968).
17. Yasukochi, Y., and B. S. S. Masters. Some properties of a detergent-solubilized NADPH-cytochrome *C* (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J. Biol. Chem.* **251**:5337–5344 (1976).
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
19. Steven, C. L., K. G. Taylor, M. E. Munk, W. S. Marshall, K. Noll, G. D. Shah, L. G. Shah, and K. Uzu. Chemistry and structure of mitomycin C. *J. Med. Chem.* **8**:1–10 (1964).

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