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Interaction of mitomycin C with thiol groups of peptides and proteins

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INTERACTION OF MITOMYCIN C WITH THIOL GROUPS OF PEPTIDES AND PROTEINS.

BY

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ABSTRACT

INTERACTION OF MITOMYCIN C WITH THIOL GROUPS OF PEPTIDES AND PROTEINS.

by

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Mitomycin C (MC), an antitumor antibiotic, has been widely used in clinical cancer chemotherapy. Its mode of action is intrinsically related to its ability to covalently bind DNA in both mono-functional and bifunctional manners, resulting in the latter case, in stable cross-links between the complementary strands of the genetic material.

Under physiological conditions the covalent reactivity of MC with DNA requires enzymatic or chemical reduction. In the first portion of this work, a series of different reducing agents were employed for the activation of MC (in the absence of DNA) to compare which reducing agent worked best. Reactivity of mitomycin C has been studied with a variety of thiol type nucleophiles, in order to assess whether MC could bind to peptides and proteins *via* their thiol groups and also whether it could be efficiently detoxified by the glutathione in the cell. Mercaptoethanol and N-acetyl cysteine, both reacted rapidly with MC, forming mono- and bis-adducts with the drug. Under physiological conditions the reactions were strictly dependent upon reductive

activation of MC, by chemical reduction or various flavoreductases. Glutathione gave similar results. The structures of the adducts were determined. Surprisingly, thiol nucleophiles reacted with both alkylating functions (C-1, C-10) of MC under "monofunctional alkylating conditions". A mechanism is proposed to explain this finding.

Effects of glutathione on the crosslinking pattern of oligonucleotides and DNA with MC were also determined. H_2/PtO_2, the enzyme cytochrome c reductase and $Na_2S_2O_4$ were used as MC activators. Extent of modification was determined by HPLC (high performance liquid chromatography) of digests. In presence of excess MC and glutathione there is decreased formation of mono- and bis-adducts of MC linked with guanines at their N^2- positions.

Extent of modification of proteins like protein kinase C and metallothionein which contain sulfhydryl groups from their cysteine residues, was also studied. In presence of MC and a reductive activator metallothionein bound MC with high affinity. Interestingly MC was also found to bind to its reductive activator, the enzyme xanthine oxidase. In addition, binding to the two proteins by the more toxic analog of MC, mitomycin A, was investigated. Results from this study indicated that mitomycin A binds to both the proteins even in the absence of reducing agents. It was not determined whether MC and MA were linked to the proteins at sulfhydryl or other alkylatable groups.

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To the research fellows and colleagues in our laboratory: Roselyn Lipman, Dr. Dondapati Chowdary (presently at University of Medicine and Dentistry, New Jersey), Dr. Shiv Kumar (presently at US Biochemicals in Cleveland, Ohio), Dr. Roland Bizanek (presently at Vanderbilt in Nashville, Tennessee) and Quiao Yun He for their cooperation and encouragement and for being wonderful friends.

To my parents and all family members for their continuous moral support and encouragement over all these years.

I wish to express my deep appreciation and thanks to my dear husband, Sanjiv, for his patience and for being a pillar of strength and support for me and for encouraging me to keep on going even during difficult times.

Finally I wish to dedicate this thesis to my son, Tanuj, who chose to make his appearance in this world, very appropriately, the day after mommy mailed the final draft of her thesis to Dr Tomasz.

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BR	binding ratio; mole of bound drug per mole of mononucleotide unit
CH_3CN	acetonitrile
Cys	cysteine
cpm	counts per minute
DMAP	dimethyl amino pyridine
DMC	10-decarbamoyl mitomycin C
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dT	thymidine
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
EDTA	ethylene diamine N,N,N',N'-tetraacetate
EGTA	ethylene glycol bis(B-aminoethylether)-N,N,N',N'-tetraacetate
GSH	glutathione
H_2/Pd-C	hydrogen/palladium-charcoal
HPLC	high performance liquid chromatography
H_2/PtO_2	hydrogen/platinum oxide
MA	mitomycin A
MC	mitomycin C
MT	metallothionein
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate

NMR	nuclear magnetic resonance
$Na_2S_2O_4$	sodium dithionite
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
PS	phosphatidylserine
SVD	snake venom phosphodiesterase
UV	ultraviolet
X.OX	xanthine oxidase
XTT	2,3-bis [2-methoxy-4 nitro-5 sulfophenyl]- -2H-tetrazolium-5-carboxanilide inner salt

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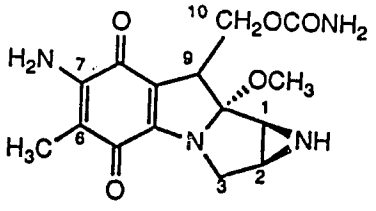
PART I. ACTIVATION OF MC BY DIFFERENT REDUCING AGENTS.

Introduction

The neoplastic cell is eradicated only with great difficulty. Toward this goal, chemotherapy has achieved success in select instances, through careful administration of appropriate chemical agents. Yet despite much effort the number of successful antitumor antibiotics remains small, and for many of these the mechanistic basis of their antitumor activity remains unknown. One possible exception is mitomycin C **(1)** (Figure 1, p.14). Mitomycin C is an alkylating agent that is unmasked *in vivo*. It contains two latent functionalities, a carbamate at C-10 and an aziridine at C-1, which under appropriate circumstances are transformed into electrophilic species. The presumed target of this drug *in vivo* is DNA, and the molecular structure of the resulting covalent adducts has yielded to extensive study [1-6].



(1)

Both of its alkylating functions react exclusively with N^2-positions of guanines. This was demonstrated recently in the Tomasz and Nakanishi laboratories, in collaboration, primarily by isolation and charaterization of mono adducts **2** and **3** and bis adducts **4** and **5** from DNA exposed to MC[2,9,10,45] (Figure 1, p.14). These reactions occur only upon reductive activation of MC both *in vitro* or intracellularly, as proposed originally by Iyer and Szybalski (1964)[7]. Sartorelli and co-workers (Lin et al.,1976)[8], who studied the enzymology of the reductive activation, introduced the term "bioreductive alkylation" for the action of MC. Monofunctional reductive activation of MC is achieved when only C-1 is activated. Bifunctional activation, i.e. activation of both C-1 and C-10, gives rise to the bis adduct **4** when two guanines are suitably close in duplex DNA as substrates for both active functions of one MC molecule. Adduct **3** originates from DNA sequences where this condition is not met, and the 10-position of MC reacts with H_2O instead of guanine (Figure 1, p. 14). The earlier discovery of Iyer and Szybalski (1964)[7] that exposure to MC *in vivo* or *in vitro* induces cross-links between the complementary strands of DNA is now fully explained by the isolation and identification of the bis adduct **4** from such DNA (Tomasz et al.,1987)[9]. In addition to the interstrand cross-link, reductively activated MC forms intrastrand cross-links (**5**, Figure 1, p.14) in DNA between adjacent guanines in significant proportions to the interstrand cross-links. The

crucial characteristic of the intrastrand cross-link adduct **5** which distinguishes it from the interstrand cross-link **4** is the presence of a phosphodiester group between the two guanine residues[10].

Appropriately, considerable attention has therefore been focused on the mechanism of DNA adduct formation, and DNA adduct formation is now recognized to occur under two different circumstances. One is acid activation[3,11], and the other is enzyme-mediated reduction of the mitomycin C quinone [4,12-15]. Evidence for the relevance of the latter pathway to the *in vivo* activity of mitomycin C has been provided by Sartorelli and colleagues, who have observed a selective toxicity of mitomycin C to hypoxic tumor cells[15,16]. Thus , the chemistry associated with reductive activation has relevance and has proven - perhaps not surprisingly - to exhibit rich complexity.

Although the mitomycin C - modified bases have been identified, the mechanism of reductive activation of mitomycin C and the fate of the reaction intermediates were unknown until very recently. An "auto-catalytic pathway" has been put forward by Peterson and Fisher[17] (Figure 2, p.15) which was later confirmed in Dr. Tomasz' laboratory using DNA itself as the nucleophile[18]. Several groups have identified and characterized the various reduction intermediates of this complex process [14-20] named the

"activation cascade" by Danishefsky and his collaborators [19,20].

The principal uncertainties in the reductive activation process rest amid the classic puzzle of mechanistic organic chemistry, the understanding of how intermediates partition. An outline of the present situation for mitomycin C is given in Figure 2 (page 15). Reduction of quinone to the hydroquinone **6** renders the carbinolamine more labile, allowing the loss of methanol with the formation of the electrophilic imine. Although this imine may be trapped by exceptional nucleophiles[12], more commonly intramolecular tautomerization to the indole proceeds rapidly. This new intermediate however is also not stable, and aziridine ring opening ensues, leading to a quinone methide presumed to have structure **7**. Evidence for this quinone methide stems from its ambivalent character, where both electrophilic and nucleophilic characters are expressed in the reactions leading to the ultimate products. Three major products are obtained. At neutral pH *in vitro*, the quinone methide's primary fate (pathway **a**, Figure 2) is a reaction as an electrophile toward water as a nucleophile, leading to the formation of the diastereoisomeric *cis*- and *trans*- 1-hydroxy-2,7-diaminomitosenes **10** (M2) and **11** (M1). This stereoisomeric formation argues against any alternative mechanism, not involving the quinone methide. Direct arizidine cleavage at the hydroquinone level should have

given only a single product stereoisomer. Under slightly more acidic conditions, mitomycin reductive activation gives in addition to **10** and **11** the 2,7-diaminomitosene (**9**, M3), arising from a nucleophilic reaction (pathway **b**) with a solvent-derived proton[4,21]. Apart from the ambivalent quinone methide behavior, an additional distinction between these two pathways is that the electrophilic reaction of the quinone methide yields its products at the hydroquinone oxidation level (pathway **a**) while nucleophilic reaction yields its products at the quinone oxidation level (pathway **b**)[17].

The central focus in the understanding of the anaerobic reductive activation of mitomycin C is the partitioning of this quinone methide between its nucleophilic and electrophilic character. Oxidation of the mitosene hydroquinone derived from nucleophile addition to the quinone methide **7** will be a stabilizing event. Indeed, it appears that in many of the previous studies of nucleophile trapping by **7** the resulting adducts were initially generated at the *quinone* oxidation level. This apparently peculiar circumstance has been noted upon only by Tomasz and Lipman[4] who correctly noted that "the hydroquinone forms of the products are probably reoxidized to quinone during isolation and/or by intermolecular electron transfers between various quinones, semiquinones, and hydroquinones present during the reaction".

Choice of the reducing system for MC activation has a profound influence on whether MC reacts as monofunctional or bifunctional DNA alkylating agent. The distribution of the three adducts (2,3,4; p.14) shows a striking dependence on the conditions of the reductive activation *in vitro*. While $Na_2S_2O_4$ is employed for bifunctional activation, monofunctional activation may be achieved by a number of different reducing agents. Several enzymes have been implicated in this activation pathway including xanthine oxidase, DT diaphorase, NADPH-cytochrome P450 reductase and mitochondrial NADPH reductase [1,22,24].

This part of the project was designed to determine which monofunctional activators are more efficient in reducing mitomycin C. This is considered a preparatory stage of the thesis project, in which published methods were retested in the context of the goals of this thesis. All the activations were performed in the absence of DNA.

EXPERIMENTAL SECTION

MATERIALS

The materials used and their sources are as follows;
Mitomycin C was a generous gift from Dr. D. Vyas of Bristol Myers-Squibb Co. Wallingford, CT. Platinum IV oxide hydrate (PtO_2) was obtained from Aldrich Chemical Co., Palladium

charcoal, NADH, xanthine oxidase (EC 1.1.3.22), xanthine, DT diaphorase (EC 1.8.1.4) (Clostridium kluyveri), NADH-cytochrome c reductase (EC 1.6.99.3) and thymidine were obtained from Sigma. NADPH-cytochrome c reductase (EC 1.6.2.4) was a gift from Dr. Wayne Backes, Lousiana State Medical School, New Orleans, LA. NADPH was obtained from Boehringer Mannheim.

METHODS

All reactions were conducted with 0.45 mM MC and in the presence of 0.45 mM thymidine as the internal HPLC standard. A 12.5 M stock solution of thymidine (extinction coefficient 7000 at 254 nm) was prepared and diluted to 0.45 mM in each reaction. While MC undergoes reduction, thymidine remains inert in all reactions. % MC recovered in each reaction was determined by directly injecting part of the reaction mixture onto HPLC. The percent unreduced MC was calculated from relative areas of the appropriate HPLC peaks; the areas were given by the integrator of the HPLC apparatus.

$$\% \text{ unreduced MC} = \frac{(\text{area MC recovered}/\text{area thymidine}) \times 100}{(\text{area MC}/\text{area thymidine})_{0 \text{ time}}}$$

Activation of MC by catalytic hydrogenation:

(i) with catalyst PtO_2: This was accomplished by deaerating, by bubbling helium, a mixture of thymidine, MC , and PtO_2 (89 ug/umol MC) in 5 ml of 0.015 M Tris buffer, pH 7.4, for 15 minutes. Hydrogen was then bubbled in for 5 minutes followed by helium again for 5 minutes. The reaction was conducted at room temperature. A color change from blue (MC) to deep pink-purple was noticeable. The mixture was exposed to air , filtered and the filtrate was then subjected to HPLC.

(ii) with Pd/charcoal: A 5 ml solution containing thymidine, MC and 162 ug/umol MC of Pd/charcoal, in Tris 0.015 M ,pH 7.4 was subjected to hydrogenation as above. The mixture was then exposed to air. No color change was noticeable as charcoal renders the solution black. Mixture was filtered, a brownish purple filtrate was obtained which was analyzed by HPLC.

Activation of MC by different enzyme systems

(i) Xanthine oxidase / NADH as reducing agent: **(a)** In two separate vessels 2.2 units of xanthine oxidase in 0.2 ml Tris buffer, at 0°C and a mixture of thymidine, MC and NADH (2.9 umol) in 4.8 ml of 15 mM Tris buffer, pH 7.4, at 37°C were deaerated for 5 and 15 min, respectively. Xanthine

oxidase was then transferred to the MC solution (total volume 5.0 ml) and incubation was continued for 20 min under helium atmosphere (color change was noticeable after 4 min). Mixture was then exposed to air.

(b) Same as (a), with a ten fold increase in NADH (6 mM).

(ii) Xanthine oxidase / xanthine as reducing agent: Ingredients used and the procedure were same as above except that 11 umol xanthine was used in place of NADH. Mixture was exposed to air after 20 min incubation at 37°C.

(iii) NADH-Cytochrome c reductase / NADH as reducing agent: MC, thymidine and NADH (2.9 umol) were dissolved in 4.5 ml Tris (15 mM, pH 7.4) and deaerated for 15 min at 37°C. In a separate flask cytochrome c reductase (1 unit/umol MC) in Tris, was deaerated for 5 min at 0°C. Enzyme was then transferred to reaction flask at 37°C and the mixture exposed to air after incubation for 20 min under helium atmosphere.

(iv) NADPH-cytochrome c reductase / NADPH as reducing agent: Same as above but used NADPH-cytochrome c reductase (2.5 units) and NADPH (0.52 mM) instead of NADH.

(v) DT diaphorase / NADH as reducing agent: (a) Ingredients and procedure same as in (iii) but enzyme DT diaphorase (2.2 units) was used in place of cytochrome c reductase.

(b) Same as (a) except 11 units of DT diaphorase was used.

(c) Same as (b) but reaction was conducted under *aerobic* conditions.

HPLC of reaction mixtures

Reaction mixtures were analyzed by HPLC using a reverse phase column (Beckman ultrasphere ODS 0.4 x 25 cm). For peak area quantitation a Beckman Model 427 M integrator was used attached to a Model 265A absorbance detector (set at 254 nm), both as parts of Model 338 HPLC system. Eluant was 2% to 15% CH_3CN/0.03 M ammonium acetate (pH 6.9) over 15 minutes. Flow rate was 1 ml/min.

RESULTS AND DISCUSSION

The reductive capacity of different reducing agents was examined in this study. Results obtained after HPLC of the reaction mixtures, are summarized in Table 1 (p.19). Figure 3b (p. 16) shows the HPLC profile of the reduction of MC in presence of the chemical reducing agent H_2/PtO_2. Reaction in presence of H_2/Pd/charcoal yielded essentially identical product pattern. With H_2/PtO_2 70.0% MC was reduced while 73% MC was reduced with H_2/Pd/charcoal. Three major products (M1, M2, and M3) which have been identified earlier by Tomasz and Lipman [4], were formed (Figure 4, p. 17). The pair of peaks (Figure 3b, p.16) at 32 min and 36 min respectively

are the isomeric products 1,2 -*cis* and -*trans* -2,7 diamino -1 hydroxymitosenes (M2 and M1). The third peak at 38 min is 2,7 -diaminomitosene (M3). MC eluted at 40 min. Thymidine (dT) eluted at 20 min. All products were identified by co-injections with authentic products obtained by the method of Tomasz and Lipman [4].

The reducing capability of NADPH-cytochrome c reductase, xanthine oxidase and DT diaphorase have also been examined. These enzymes are major reductases in biological systems. They can use NADH and/or NADPH as electron donor. The results of this study (summarized in Table 1, p. 19), clearly indicated that the reducing capability of xanthine oxidase using NADH or xanthine as electron donor, is superior to that of cytochrome c reductase or DT diaphorase (for HPLC profiles see Figure 5, p.18). The percent MC reduced in the reaction utilizing xanthine oxidase/NADH (0.6 mM) was 82.0%. With excess NADH reducing capability decreased somewhat. Percent MC reduced in the xanthine oxidase/xanthine system was also 82.0. In the cytochrome c reductase systems the amount of MC reduced was similar to that in the reactions utilzing chemical reducing agents (72.0%). DT diaphorase also proved to be an enzyme that could be used for reduction of MC. No reduction was observed when 1 unit DT diaphorase/umol MC was used . However, with a five fold increase (5 units/umol MC) in the enzyme 63% MC

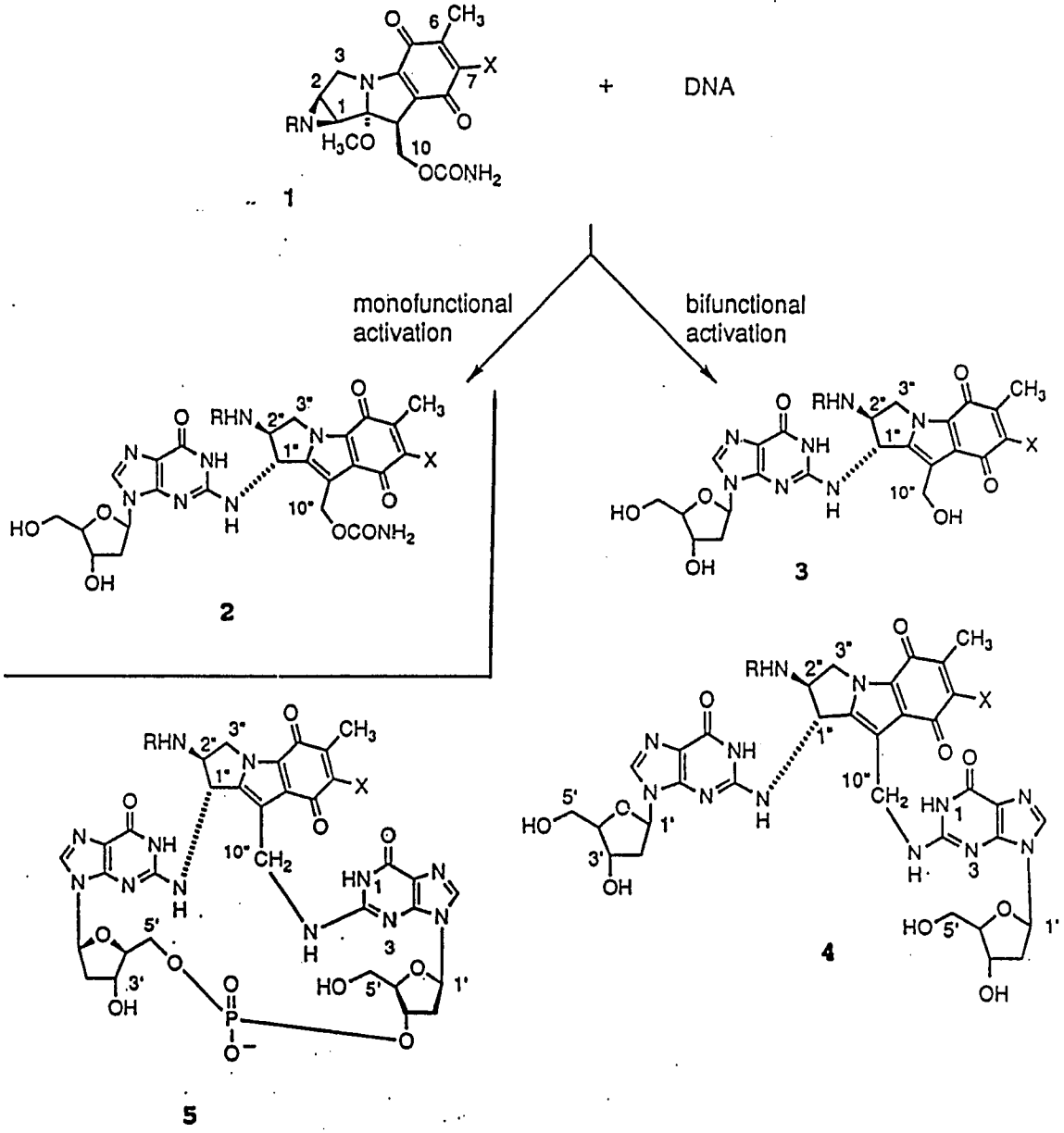
was reduced. No reduction was observed under aerobic conditions.

The results obtained in this study, with NADPH-cytochrome c reductase/NADPH and xanthine oxidase/NADH, are comparable to those obtained by Pan et al.,1984[14]. They conducted the experiments in 0.1 M phosphate buffer at pH 7.4 and approximately 80% MC was reduced in their system.

The xanthine oxidase/xanthine system has been employed to activate MC for the first time. Schreiber et al.,1987[23] used this system for the reduction of daunomycin (anthracycline drug) and concluded that the mode of quinone reduction by xanthine oxidase/xanthine is quite general and can be applied to other biologically relevant quinones.

The role of DT diaphorase in bioreductive activation of MC has been controversial because attempts to demonstrate metabolism of MC by purified DT diaphorase from mammalian cells (rat liver, human kidney, human colon carcinoma, Walker cells) failed. However, Siegel et al.1990[24], finally showed that metabolism of MC by purified mammalian DT diaphorase was pH dependent and that the major metabolite at pH 5.8 and 7 under aerobic and anaerobic conditions was 2,7-diamino mitosene (M3). In the present study, a bacterial DT diaphorase was used and the results indicate that with bacterial DT diaphorase MC can be activated to yield all three products M1, M2, M3 (p.17) under anaerobic conditions.

All the reducing agents examined in this study thus, may be successfully utilized for reducing mitomycin C.



R = H

X = NH_2

FIGURE 1

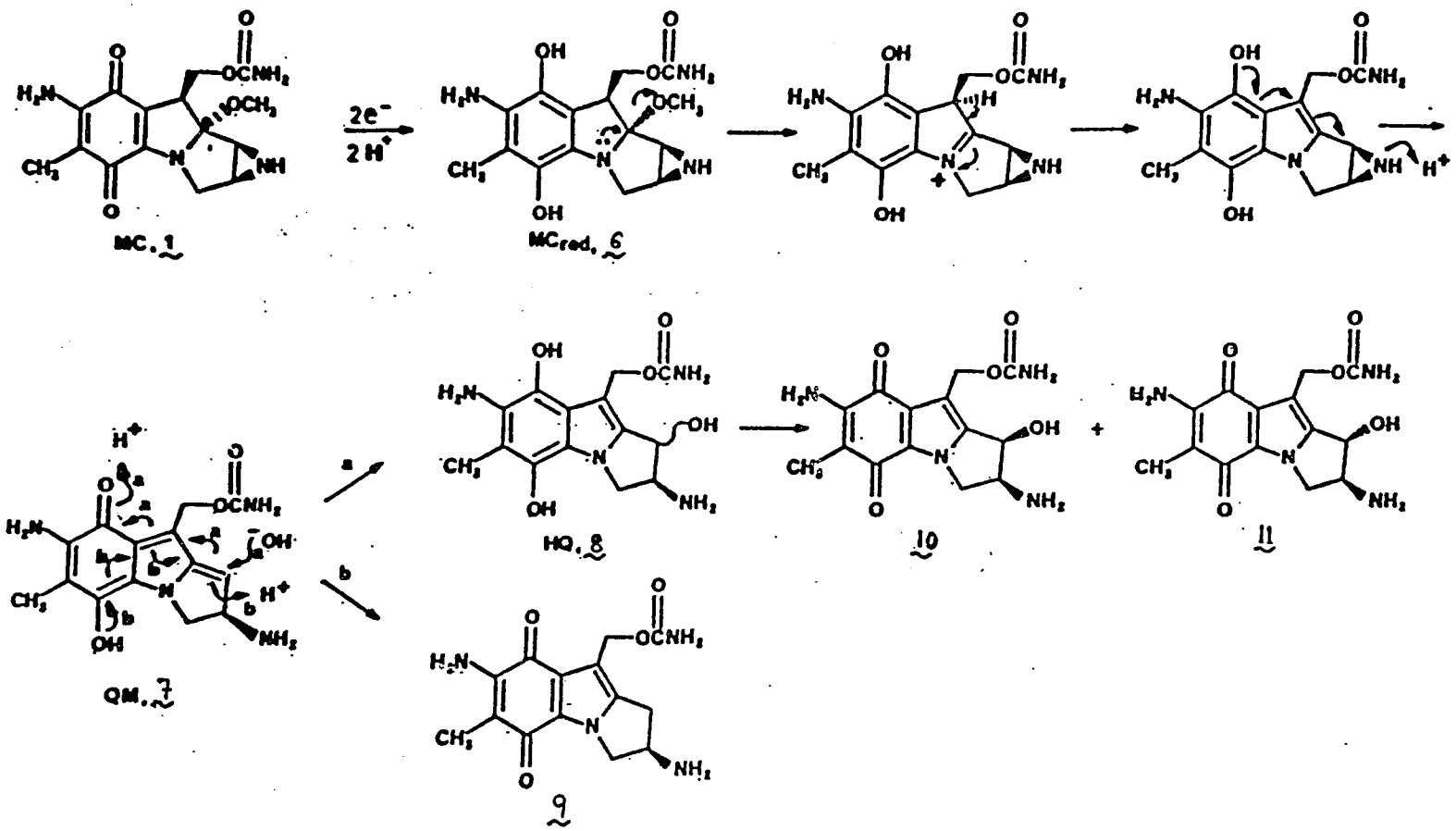


FIGURE 2.

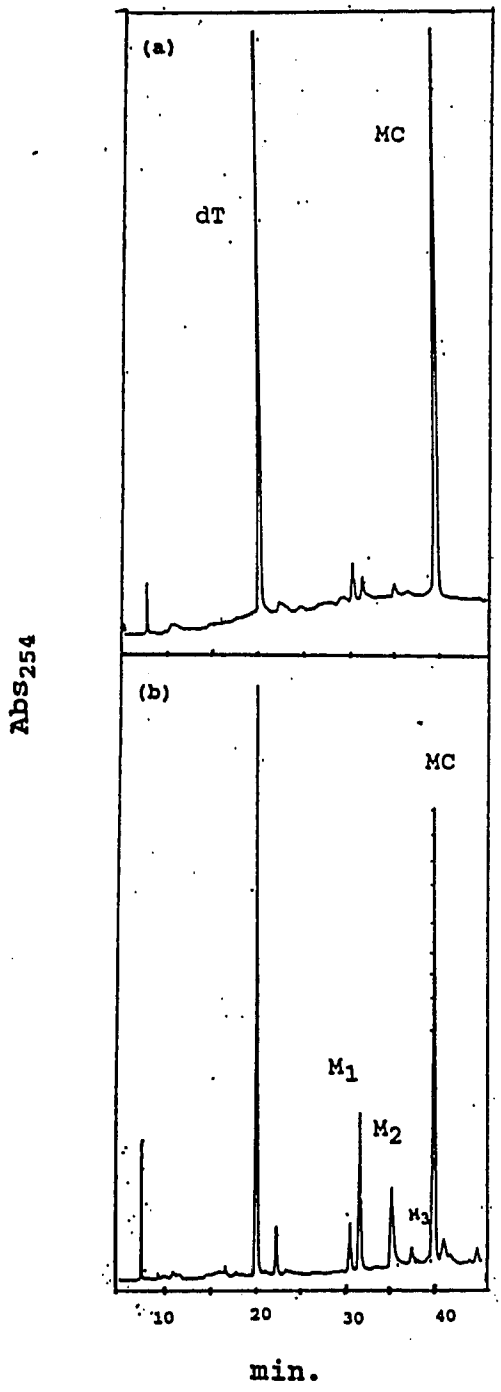


FIGURE 3. HPLC profiles of reaction mixtures;

(a) Control: MC and thymidine (no reducing agent),

(b) $MC/H_2/PtO_2$

MC

M_1 : X = ·····OH

M_2 : X = ▬OH

M_3 : X = H

FIGURE 4.

Structures of **M1** (*trans*-1-hydroxy-2,7,-diaminomitosene),
M2 (*cis*-1-hydroxy-2,7,-diaminomitosene)
and **M3** (2,7-diaminomitosene).

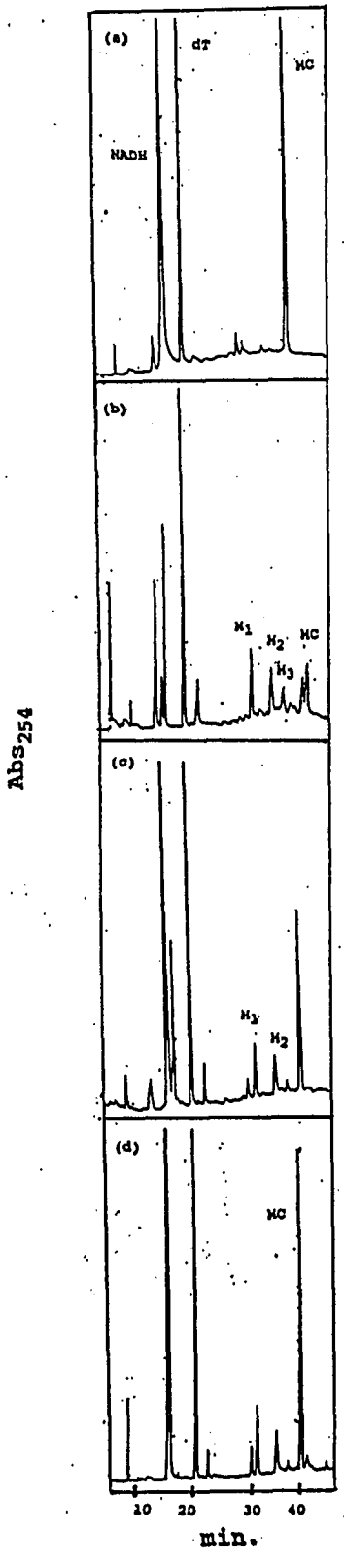


FIGURE 5. HPLC profiles of the following reaction mixtures;

(a) Control: MC,thymidine (dT) and NADH,

(b) MC/xanthine oxidase/NADH,

(c) MC/cytochrome c reductase/NADH and

(d) MC/DT diaphorase/NADH

TABLE 1

REACTION	% MC REDUCED
MC/H_2PtO_2	70
MC/H_2/Pd/charcoal	73
MC/NADH/xanthine oxidase	82
MC/xanthine/xanthine oxidase	82
MC/NADPH cytochrome c reductase/NADH	72
MC/NADPH cytochrome c reductase/NADPH	74
MC/DT diaphorase/NADH	63

PART II. BIOREDUCTIVE ALKYLATION OF THIOLS BY MITOMYCIN C

Introduction

Alkylating agents are a mainstay in the neoplasia therapy of both early and advanced cancers. Unfortunately, the emergence of clinical drug resistance frequently limits the efficacy of these agents. It is therefore important to develop both new alkylating agents and better methods to detect and ultimately, circumvent drug resistance[25].

The mechanisms responsible for chemotherapeutic drug resistance are multifactorial and include altered drug delivery and uptake, decreased drug activation, increased metabolic inactivation, active drug efflux and enhanced repair of DNA[26]. Glutathione (GSH), represents the major intracellular nonprotein thiol. As such, it is notable that elevated GSH levels have been found in tumor cells resistant to alkylating agents[31-34] and that DNA interstrand crosslinking is reduced in alkylating agent - resistant cells that have elevated levels of intracellular non protein thiols[12]. Additionally, experimental cellular GSH depletion with buthionine sulfoximine , which irreversibly binds the enzyme glutamylcysteine synthetase to block GSH synthesis results in enhanced cytotoxicity of a number of alkylating agents[35,36].

Inactivation of alkylating agent type anticancer drugs. by GSH is one of the mechanisms known to be involved in acquired multidrug resistance (MDR) in cancer chemotherapy. This inactivation is closely related to a natural function of GSH : cellular detoxification of xenobiotics by formation of GSH conjugates. Glutathione-S-transferases catalyze the alkylation of the sulfhydryl group of GSH by electrophiles of wide variety. Non-enzymatic alkylation is also significant since GSH is present at high (typically 5 mM) concentration in the cytosol[37].

Since MC acts as a bifunctional alkylating agent upon reductive activation in the cell, it is likely to be subject to reaction with cellular GSH and perhaps other thiols. So far, only indirect evidence exists to indicate that GSH affects various biological activities of MC. Thus, GSH and N-acetyl cysteine inhibited the alkylating activity of MC in cell-free rat hepatic or EMT6 mouse mammary tumor cell nuclei[37]. A multidrug resistant CHO cell line, highly resistant to MC showed overexpression of an α-class glutathione-S-transferase, suggesting that this plays a role in the decreased cytotoxicity of MC. Depletion of cellular GSH or inhibition of glutathione-S-transferase in multidrug resistant mouse leukemia cells increased sensitivity to MC significantly[37]. Although other mechanisms are possible, it is reasonable to hypothesize, based on analogy to other alkylating agents[37], that these modulations of MC activity

are caused (at least partially) by direct interaction of MC with GSH, resulting in non-cytotoxic MC-GSH conjugates. Formation of such products, however has not been studied previously either in cell-free systems or in intact cells. In fact, no reactions of MC with GSH or other thiols have been documented. In order to gain such basic information needed to unravel the role of GSH in the activity of MC we studied reactions between these two substances as well as alkylation of other thiols by MC and its mitosene derivatives. Alkylation of thiols by DNA-linked mitosenes was also studied. Preliminary findings on alkylation of proteins by MC are also reported in the last part of the thesis.

This section describes the alkylation reactions of DNA alkylator, mitomycin C, with glutathione and model thiols such as mercaptoethanol and N-acetyl cysteine. This process is mediated by a bioreductive activation of mitomycin C, similar to its bioreductive alkylating action on DNA.

EXPERIMENTAL SECTION

MATERIALS

MC was generously supplied by Bristol-Myers Squibb Company, Wallingford, CT. Mercaptoethanol, N-acetyl cysteine, GSH, NADH, platinum oxide and NADH-cytochrome c reductase were obtained from Sigma Chemicals. NADPH-cytochrome c reductase was a gift from Dr. Wayne Backes, Lousiana State Medical School, New Orleans, LA. NADPH was from Boehringer Mannheim. Radioactive glutathione [glycine 2-^{3}H] was obtained from NEN Dupont, specific activity 1.0 Ci/mmol. Decarbamoyl MC (DMC) was synthesized as described[38].

Rat liver microsomes and cytosol (male Sprague-Dawley rats, 200-250 g weight; not fasted) were prepared by the method of Cederbaum et al.[39] and were a gift from Dr. Arthur Cederbaum, Mount Sinai School of Medicine, New York. The pelleted microsomal preparation was suspended in 0.125 M KCl and stored at -150^{o}C. The cytosol was dialyzed in 0.1 M potassium phosphate buffer (pH 7.0) and also stored at -150^{o}C. Protein content of the dialyzed cytosol (19.8 mg/ml) and microsomes (13.4 mg/ml) were determined by the Bradford method[40].

DT diaphorase in the dialyzed cytosol was assayed by the method of Benson et al.[41]. All diaphorase activity was inhibited by 10 ug dicumarol in 3 ml of the assay mixture containing 10 ul of the dialyzed cytosol.

SPECTROSCOPIC TECHNIQUES

Proton nuclear magnetic resonance spectra were measured on a G.E.QE-300 NMR spectrometer (300 MHz), in Me_2SO-d_6, MeOD or D_2O. Chemical shifts are reported in ppm. UV spectra were obtained in water, on a Varian Cary 3 spectrophotometer. Chemical ionization and FAB mass spectra were obtained on a VG7070 double focussing mass spectrophotometer, at Rockefeller University.

METHODS

I. Preparation of MC thiol adducts under chemical or enzymatic reductive activation conditions.

Stock solutions of N-acetyl cysteine and glutathione (0.13M) were prepared by dissolving them in 0.015 M Tris (pH 7.4) and then adjusting the pH to 7.4 with 0.1 N NaOH. Mercaptoethanol was used directly from the bottle.

(i) Control incubation of MC with thiol in absence of reducing agent. The following MC : thiol molar ratios were used; MC : mercaptoethanol, 1.0:100; MC : N-acetyl cysteine, 1.0 : 50 ; MC : GSH, 1.0 : 27. The MC concentration was 0.45 mM in 0.015 M Tris-HCl (pH 7.4). Upon addition of thiol the mixture was deaerated for 25 min.

(ii) Reaction in presence of H_2/PtO_2 as reducing agent. PtO_2 (89 ug/umol MC) was added to the above reaction mixture (i), which was deaerated for 15 min. Hydrogen was then

bubbled for 5 min, followed by helium again for 5 min. The reaction mixture was then exposed to air and filtered.

(iii) Reaction in presence of NADPH-cytochrome c reductase.

To the incubation mixtures in (i) NADPH-cytochrome c reductase (0.27 unit/ml) and NADPH (0.52 mM) were added, followed by incubation for 1 hr at 37°C under helium atmosphere.

HPLC separations

HPLC was performed using reverse-phase columns (Beckman Ultrasphere ODS; 1.0 x 25 cm for semi-preparative and 0.4 x 25 cm for analytical purpose). For peak area quantitation a Beckman Model 427M integrator was attached to a Model 265A absorbance detector, set at 254-nm wavelength, both as parts of a Model 338 HPLC system. Flow rate for semi-prep HPLC was 2 ml/min and for analytical HPLC it was 1 ml/min. Eluant was 12% to 20% CH_3CN/0.03 M ammonium acetate, pH 6.9 over 20 minutes for mercaptoethanol reaction products, 8% to 20% CH_3CN/0.03 M ammonium acetate, pH 6.9 over 40 min for N-acetyl cysteine reaction products and 0% to 15% CH_3CN/H_2O over 35 min for the GSH reaction products. The fractions containing UV-absorbing products were collected by hand, then concentrated and lyophilized.

Preparation of MC-[^{3}H]-GSH adducts using chemical reducing agent (H_2/PtO_2) for activation.

MC (0.45 mM), PtO_2 catalyst (89 ug/umol MC) and [^{3}H]-GSH (12 mM, 2 uCi/ml) were mixed in 15 mM Tris-HCl, pH 7.4. Helium gas was bubbled for 10 minutes followed by hydrogen gas for

5 minutes. The reaction mixture was exposed to air after bubbling helium again for 5 minutes.

HPLC separation (as described above)

Counting of radioactivity: The appropriate peaks were collected separately from HPLC and concentrated to dryness by rotary evaporation. Each of these was lyophilized then dissolved in 1 ml 10% CH_3CN in 0.03 M ammonium acetate and their A_{310} was determined. This was followed by the addition of 4 ml of the liquid scintillation fluid Scintiverse LC (Beckman). Scintillation counting of the samples was done by using LS 6800 scintillation counter.

Determination of umol GSH/umol mitosene: For the calculation of umol GSH/umol mitosene, the following conversions were performed; cpm/A_{310} units to cpm/umol mitosene to dpm/umol mitosene to uCi/umol mitosene to umol GSH/umol mitosene. The specific radioactivity of [^{3}H]-GSH was 0.1 uCi/umol.

Acetylation of MC-thiol adducts

Acetylation of individual MC-thiol adducts were carried out at room temperature for 1 hour using 8 equivalents of acetic anhydride and a crystal of DMAP in 2 ml of dry pyridine in vacuo; the product of each reaction was isolated by TLC (Analtech preabsorbent silica gel, 2:1 isopropanol:NH_4OH). Further purification was completed by **semi-preparative HPLC.** Eluant was 20% to 50% CH_3CN/0.03 M ammonium acetate, pH 6.9, for acetylated MC-mercaptoethanol adducts. The adducts eluted between 15 to 45 minutes. For acetylated MC-

N-acetyl cysteine adducts, the gradient used was 15% to 40% over 30 minutes and the adducts eluted between 7 and 20 minutes.

II. Reactions of the aziridine ring-opened mitosenes with GSH under reductive activation.

HPLC purified mitosenes M1, M2 and M3 (Figure 2, p. 48) used in these experiments were obtained as described in the first part of this thesis. The mitosene : thiol molar ratio used was 1:27. Mitosene (M1, M2, or M3 ; 2.2 umol) and the thiol were dissolved in 5 ml of 0.015 M Tris-HCl buffer (pH 7.4) to give 0.44 mM mitosene and 12 mM thiol concentration, and the mixture was deaerated by bubbling helium for 25 minutes, at room temperature. It was then processed as follows:

(i) *Reaction in absence of reducing agent (H_2/PtO_2).*

No reducing agent was added to the reaction mixture. After 25 minutes it was exposed to air and filtered.

(ii) *Reaction in the presence of reducing agent (H_2/PtO_2).*

PtO_2 (89 ug/umol mitosene) was added to the reaction mixture which was then further deaerated by helium for 15 minutes. Hydrogen was bubbled for 5 minutes followed by helium again for 5 minutes. The reaction mixture was exposed to air and filtered.

(iii) *Reaction in absence of thiol.*

Same as (ii) but conducted without addition of thiol.

HPLC separations

The analytical reverse phase column was used for separation of reaction products. Eluant was 0% to 20% CH_3CN/ 0.03 M ammonium acetate, pH 6.9; over 35 min, at flow rate 1 ml/min.

Reactions of decarbamoyl MC (DMC) (Figure 2, p.48) with GSH under reductive activation.

These reactions were conducted analogously to those in **II.**

III. Which thiol works best

Comparative hydrogenation reactions of MC were conducted with three thiols: mercaptoethanol, N-acetyl cysteine and glutathione. Thymidine was added as an internal HPLC standard. The amount of thymidine added is the same as the amount of MC in each reaction (0.45 mM). While MC undergoes reduction , thymidine remains inert in each reaction. % MC recovered was determined by injecting an aliquot of the reaction mixture directly in the HPLC. The percent unreduced MC was calculated from relative areas of the appropriate HPLC peaks. Areas were given by the integrator of the HPLC apparatus.

$$\% \text{ unreduced MC} = \frac{(\text{area MC recovered/area thymidine}) \times 100}{(\text{area MC/area thymidine})_{0 \text{ time}}}$$

The molar ratio of MC:thiol used was 1:27 in each reaction. Reaction mixtures containing MC (0.45 mM), thymidine (0.45 mM), thiol (12 mM) and PtO_2 (89 ug/ umol MC) in Tris buffer,

0.015 M, pH 7.4, were deaerated for 15 minutes. Hydrogen was then bubbled in for 5 minutes followed by helium again for 5 minutes. Reaction mixtures were then exposed to air and filtered. **HPLC** of the three reactions was conducted using the analytical reverse phase column 0.4 x 25 cm . Eluant was 0% to 15% CH_3CN/0.03 M ammonium acetate, pH 6.9, at 1 ml/min.

IV. What GSH concentration works best

Hydrogenation (catalyst H_2/PtO_2, 89 ug/umol MC) reactions of 0.45 mM MC in Tris (0.015 M, pH 7.4) were conducted as in **(III)** in the presence of three different concentrations of glutathione i.e. 0.45 mM, 4.5 mM, and 12 mM. One reaction was conducted in the absence of glutathione to serve as control. Thymidine (0.45 mM) was added to each as the internal HPLC standard. Each reaction mixture was analyzed by HPLC using the analytical reverse phase column. Eluant was 0% to 15% CH_3CN/ammonium acetate (0.03 M, pH 6.9), over 35 min.

V. Kinetics of the reductive activation of MC in absence and presence of glutathione

A reaction flask containing MC (0.45 mM), thymidine (0.45 mM), NADH (0.58 mM) and glutathione (12 mM) for the reaction in presence of glutathione, in Tris-HCl buffer (0.015 M, pH 7.4) was placed in a 37^{o}C water bath and the mixture was deaerated for 15 minutes. Enzyme NADH-cytochrome c reductase

(1 unit/ umol MC) was deaerated in a separate flask, on ice and then transferred to the reaction flask. A 50 ul aliquot was withdrawn from the reaction flask just before the addition of enzyme for zero time reaction. Subsequently, aliquots (50 ul each) were drawn at 1, 2, 5, 25 and 45 minutes and immediately frozen. The remaining reaction mixture was frozen at 60 min.

A reaction in the absence of GSH was conducted in exactly the same way.

HPLC of the aliquots

HPLC column and conditions were the same as in (III). Percent MC recovered was obtained from the ratio of Area MC / Area thymidine, from the equation given on page 27. This was then plotted against time for the reaction in absence and presence of GSH.

VI. Formation and identification of MC-GSH adducts in subcellular systems.

A. Activation of MC in rat liver cytosol

(i) ***Activation of MC under anaerobic conditions : Control.***

In one vessel 500 ul of dialyzed cytosol at $0^{o}C$ and in another vessel a mixture of MC (2 umol), thymidine (internal HPLC standard, 1 umol), NADH (10 umol) in 1.5 ml potassium phosphate buffer (0.5 M, pH 5.8) at room temperature were deaerated by bubbling helium gas for 10 min; then the cytosol was transferred to the MC solution. Helium bubbling deaeration was continued for 4 h after which the mixture

was exposed to air. Aliquots (200 ul) for HPLC analysis were withdrawn at 30 minutes, and at 2 hrs and frozen immediately on acetone and dry ice.

(ii) *Anaerobic activation in presence of glutathione.*

The above reaction was repeated in the presence of 50 mM GSH.

(iii) *Reaction in presence of dicumarol.*

The above reactions were repeated in the presence of 0.074 mM dicumarol (reaction volume was increased to 4 ml by acetone and buffer to dissolve the dicumarol completely).

(iv) *Activation of MC in the cytosol under aerobic conditions*

Reactions (i), (ii) and (iii) above were repeated under aerobic conditions (mixtures were exposed to air all through reaction time).

B. Activation of MC in a rat liver microsomal system

(i) *Activation of MC under anaerobic conditions: Control.*

In a small flask 200 ul of microsomal preparation was mixed with 300 ul of 0.2 M sodium phosphate buffer (pH 7.1) and was deaerated at 0°C for 10 minutes. In another flask MC (1 umol) was mixed with NADPH (5 umol) in 1.5 ml sodium phosphate buffer and deaerated for 10 minutes at room temperature. The microsomes were then transferred to the mitomycin and deaeration continued for 4 hours, after which the mixture was exposed to air. Aliquots (100 ul each) for HPLC were removed at 0 time (just before the addition of

enzyme to reaction flask), 30 minutes, 2 hours and 4 hours. Aliquots were immediately frozen on acetone and dry ice.

(ii) *Activation in presence of GSH:* Reaction (i) was repeated with 100 umol of GSH.

(iii) *Aerobic reaction in absence of GSH:* Reaction (i) was repeated under aerobic conditions i.e. reaction flask was exposed to air all through the reaction time.

(iv) *Aerobic reaction in presence of GSH:* Reaction (iii) was repeated with 100 umol of GSH.

HPLC separations

A reverse phase column 0.4 X 25 cm was used for the HPLC of all the aliquots. Eluant was CH_3CN/0.03 M ammonium acetate (pH 6.9) 0% to 15% over 35 minutes.

VII. Cytotoxicity assay of MC-glutathione conjugates

In vitro cytotoxicity of MC-glutathione conjugates (**13**, **14** and **15**; p.54) was assayed in HCT 116 human colon carcinoma cells at Bristol-Meyers Squibb Company (Dr. Annamaria Casezza). IC_{50} was determined using XTT dye reduction after 72 hour drug exposure[42].

RESULTS

I. Formation of MC-thiol adducts under chemical or enzymatic reductive activation conditions

In the absence of a reducing agent no interaction was observed between MC and any of the thiols. In presence of a reducing agent (H_2/PtO_2 or enzyme), five products were formed in the reaction with each thiol (mercaptoethanol, products **1-5**, Figure 3,4, p.49,50; N-acetyl cysteine, products **6-10** Figure 5,6, p.51,52; and glutathione, products **11-15**, Figure 7,8, p.53,54).

Comparison of HPLC patterns of chemical and enzymatic reaction mixtures

MC-thiol adducts formed under enzymatic activation (NADPH cytochrome c reductase) yielded patterns (Figure 3c, 5c and 7c,) essentially identical to those obtained using H_2/PtO_2 (Figure 3b, 5b and 7b; p.49, 51 and 53 respectively). The only difference observed was in the distribution (ratio) of adducts.

Identification of MC-thiol adducts

Ultraviolet spectroscopy : UV spectra of all fifteen adducts were obtained in water (Figure 10, p.56). All adducts reveal the characteristic[6] mitosene like spectrum with lambda max at 245-268 nm and another one around 313 nm, indicating the presence of mitosene moiety in each adduct.

^{1}H-NMR spectroscopy : The ^{1}H-NMR spectra (Figures 11-16; p.57-62; for assigned chemical shifts see Tables 1,2 and 3, p.66-68) were analyzed in comparison to the known NMR spectra of 1-α hydroxy-2β,7-diaminomitosene (M1), 1β-hydroxy-2β,7-diaminomitosene (M2) and 2,7-diaminomitosene (M3) and their acetylated derivatives[4,11]. ^{1}H NMR spectra of mercaptoethanol, N-acetyl cysteine, and glutathione (Figure 11, p.57) were also obtained for direct comparison. The main supporting features for the structures by NMR of all adducts are the following : **(a)** Loss of carbamate ($OCONH_2$) indicated by the disappearance of C_{10a}-NH_2 signal (singlet) at 6.47 ppm. **(b)** Of particular note in the NMR spectra of all the adducts is the upfield shift of the typical AB quartet of the mitosene 10-CH_2 protons from 5.0 ppm to approximately 3.7 - 3.9 ppm, consistent with the CH_2-O-CO-NH_2 ------> CH_2-S- change in all the adducts. In addition, assignment of this upfield shift to the presence of a C-10-sulfur linkage is in agreement with the NMR data published for other C-10-sulfur-substituted mitosenes [13,43,44].

As further proof NMR spectra of the acetylated derivatives of all the adducts were obtained (structures: Figure 4,6,8, p.50,52,54). Acetylation of these mitosene-thiol adducts furnish derivatives that exihibit superior spectral quality to the original underivatized adducts as usual in the mitosene series[1,2,52]. NMR spectrum of the acetylated derivatives (e.g.Figure 12,13,16; p.58,59,61,62) show the

acetyl groups (one, two or three) between 1-2 ppm and a low-field doublet (8.4 ppm) observed for the -NH-proton in C_2-N-acetyl mitosenes. These and all other features of the spectra are fully consistent with the structures assigned to the fourteen adducts **1-15**; with the exception of **10** which was not identified.

Mass spectroscopy : Mass spectra could be obtained only for MC-mercaptoethanol adducts (Table 1, **1-5**, p.66) and MC-N-acetyl cysteine adducts (Table 2, adducts **6-9**, p.67). Mass spectra for MC-mercaptoethanol adducts (**1-5**) were obtained by chemical ionization (Figure 17,18, p.63,64) and those for MC-N-acetyl cysteine adducts by FAB (Figure 19, p.65).

Use of radiolabel for characterization of MC-[^{3}H]-GSH adducts.

Since mass spectra of MC-GSH adducts could not be obtained, MC-GSH adducts radiolabelled in GSH were prepared. HPLC of the reaction mixture indicated the formation of five adducts (**11-15**, Figure 8, p.54). These five peaks and the void volume where glutathione elutes, were collected separately and the umol GSH/umol mitosene ratio was determined (see Methods) which indicated the presence of two glutathione moieties per mitosene moiety in adducts **11** and **12**, and the presence of one glutathione moiety in adducts **13**, **14** and **15** (see Table 3, p.68).

Further test of structures: The GSH-adducts **11-15** were each tested quantitatively, for the presence of free thiol group (-SH) by the DTNB assay[66]. These experiments were carried out by Roselyn Lipman of this laboratory. None of the adducts tested positive for the presence of free thiol, consistent with the assigned structures.

II. Confirmation of the assigned structures 13-15 (GSH-MC monoadducts; p.54) by comparison with the products of reaction between GSH and aziridine-ring-opened mitosenes M1,M2 and M3 and decarbamoyl MC (DMC) (p.74-76; Figures 24-26).

HPLC analysis of the hydrogenation reaction mixtures (M1+GSH+H_2/PtO_2, M2+GSH+H_2/PtO_2, M3+GSH+H_2/PtO_2) indicated the formation of single products with GSH (Figure 25, p.75). The adducts were identified as **13**, **14** and **15**, respectively, by direct comparison of the HPLC retention times and by co-injection with the authentic compounds, formed also from MC itself as a mixture (see above; Figure 25 b,e,h).

Interestingly, the reaction with decarbamoyl MC in presence of GSH and H_2/PtO_2 gave no GSH adducts; only decarbamoyl M1, decarbamoyl M2 and decarbamoyl M3 were formed (Figure 26, p.76) (the hump in decarbamoyl M1 peak (Figure 26c) could be a minimal amount of adduct with GSH at C1). Decarbamoyl M1, decarbamoyl M2 and decarbamoyl M3 are known compounds[9] and were used for identification of the products.

III. Which thiol works best

HPLC profiles (Figure 20, p.70) of the three hydrogenation reactions of MC in presence of thiols mercaptoethanol, N-acetyl cysteine and glutathione, showed qualitatively that the reaction with glutathione was the fastest. By calculation (Methods) the ratio of MC/thymidine was 0.99 (0 time), 0.22, 0.077 and 0.033, respectively. These ratios indicated that 78% , 92% and 97% MC reacted with mercaptoethanol, N-acetyl cysteine and GSH, respectively, under the experimental conditions.

IV. What GSH concentration works best

HPLC analysis of the three hydrogenation reactions of MC in presence of GSH using three different concentrations of GSH, indicated faster consumption of MC with increasing concentration of GSH. At 0.45 mM GSH (1:1 MC:GSH), 31% of MC was recovered and there was formation of only M1, M2 and M3 (no MC-GSH adducts were formed). At 4.5 mM GSH (1:10 MC:GSH), 24% MC was recovered and there was formation of MC-GSH adducts. However, at 12 mM GSH (1:27 MC:GSH) only 7% MC was recovered and mostly the MC-GSH adducts were formed (Figure 21,22, p.71,72).

V. Kinetics of the reductive activation of MC in the absence and presence of GSH.

Percent MC recovered was obtained from the MC/thymidine ratio after HPLC of each aliquot of the kinetic experiment

(Methods). % MC was plotted against time (Figure 23, p.73). Disappearance of MC in the reaction in presence of thiol is remarkably fast compared to no thiol present. At 2 minutes only 12% MC is recovered as compared to 65% and at the completion of reaction only 6.5% MC is recovered.

VI. Formation of MC-GSH adducts in subcellular systems from rat liver.

A. Activation of MC in the cytosol.

Reactions under anaerobic conditions : HPLC analysis of the reaction products of MC formed in dialyzed cytosol upon addition of NADH shows the formation of M3 (2,7-diaminomitosene) eluting at 35 minutes (Figure 27a, b; p.77) and the formation of M3 and the GSH adduct **15** eluting at 32 minutes, in the presence of GSH (Figure 27c,d). Both adducts were identified by co-injections with the authentic compounds. Dicumarol completely inhibited any transformation of MC (Figure 27e). *Reactions under aerobic conditions* yielded essentially the same results (Figure 28, p.78).

B. Activation of MC by rat liver microsomes and added NADPH.

Anaerobic reaction in absence of GSH : The HPLC pattern (Figure 29a,b; p.79) indicates the complete reduction of MC in 30 min resulting in the formation of M1, M2 and M3, identified by co-injections with authentic compounds.

Anaerobic reaction in presence of GSH : The reaction in presence of GSH shows the formation of three products. These were identified as **13**, **14** and **15** by co-injections with the authentic samples (Figure 29c,d,e; p.79).

Under *aerobic conditions*, MC was not reduced under any of the above conditions.

VII. Cytotoxicity of the MC-GSH adducts.

IC_{50} (uM) values obtained for MC-glutathione metabolites were as follows:

Compound	**IC_{50} (uM)**	
	HCT 116	**HCT/V**
MC	0.194	0.146
M1-GSH (**13**)	519	496
M2-GSH (**14**)	>443	>443
M3-GSH (**15**)	>910	>910

The IC_{50} uM values obtained for the adducts 13-15 are very high as compared to those of MC. These high values reflect the non-cytotoxicity of these adducts (HCT 116 is a human colon tumor cell line and HCT/V is a mutant resistant to etoposide).

DISCUSSION

These results demonstrate unambiguously that mercaptoethanol, N-acetyl cysteine and glutathione (Fig.1, p.47) do not react with MC under physiological conditions. Glutathione and other thiols are alkylated by MC only in the presence of reducing agents capable of activating MC. Five different MC-thiol adducts having one or two thiol residues per MC were isolated from the reaction of activated MC with each thiol (adducts **1-15**, p.50,52,54). MC reacts exclusively as a bifunctional alkylating agent in the presence of thiols. The adducts **1** to **15** (except **10**) have been assigned the respective structures on the basis of spectroscopic proof, by combination of UV, MS and ^{1}H-NMR data, as follows:

The UV spectra of the two classes, 7-amino mitosenes exemplified by M1,M2 and M3 (p.48) and 7-amino mitosanes exemplified by MC, are highly distinguishable. The absorption spectrum of MC is characterized by maxima at 216 nm and 360 nm. The UV absorption spectra of 2,7-diamino mitosenes (with or without substitution at C-1) show maxima at 248 nm and 310 nm. The UV spectra of adducts **1-15** all revealed the 7-amino mitosene spectrum (Figure 10, p.56).

The resonances exhibited in the NMR by most of these adducts were quite broad as has been seen for all of the underivatized MC-deoxyguanosine adducts so far

isolated[2,9,13,43,45,52]. However acetylation of the adducts resulted in sharpening of most of the signals. A notable feature in the NMR spectra of all the adducts was the upfield shift of the typical AB quartet of the mitosene 10-CH_2 protons, to approximately 3.7 - 3.9 ppm. Decarbamoyl mitosenes exhibit similar upfield shifts[45]. In addition, assignment of this upfield shift to the presence of a C_{10} - sulfur linkage is in agreement with the NMR data published for a series of 10-(ethyl xanthyl) mitosenes [13,43] and for a C-10-bisulfite mitosene isolated in our laboratory[44].
Further, the C_{10} protons of adducts **3**, **9** and **15**, form a sharp singlet rather than an AB quartet. This indicates that the asymmetry of C_1, responsible for the AB quartet type splitting[4], is not present in these adducts. The C_1 protons of these adducts also shifted far upfield, from 4.5 ppm to 3.3 and 2.9, as two double doublets, consistent with the C(OH)H ---> CH_2 change[4]. The position of C-1 protons also indicates the α or β stereochemistry of substituents (e.g. -OH) at C-1. When -OH is α, C-1 proton appears as a singlet at 4.60 ppm and when -OH is β it appears as a doublet at 4.67 ppm. Further, when these -OH groups are acetylated, there is an downfield shift of C-1 protons. For an α -$OCOCH_3$, C-1 protons appear at 6.07 ppm as a doublet and for β -$OCOCH_3$ a singlet appears at 5.94 ppm[4].

The structures of compounds **1-9** (p.50,52) were confirmed by mass spectroscopy (Tables 1 and 2, p.66,67). Mass spectra of

compounds **11-15** (p.54) could not be obtained presumably because they are very polar and non-volatile. However, mol GSH/mol mitosene values (Table 3) obtained from the specific radioactivity of MC-[^{3}H]GSH adducts clearly indicated the presence of two (in adducts **11** and **12**) and one (in adducts **13**, **14** and **15**) GSH moiety per mitosene chromophore. The structures, including the C-1 stereochemistry, of the GSH adducts **13**, **14** and **15** were independently and conclusively confirmed by the reactions of the three mitosenes M1, M2 and M3 with GSH upon reduction (p.74). The finding that the known compounds M1, M2 and M3 yielded the single products **13**, **14** and **15** respectively, defines the stereochemistry of **13** and **14** at C-1; furthermore, together with the other structural data, confirm the assigned structures **13**, **14** and **15**.

The formation of bifunctional alkylation products with thiols is in contrast to the exclusively monofunctional alkylating activity of MC in the presence of other previously studied nucleophiles, such as H_2O, phosphate ions, deoxyguanosine[4,52] observed under the same reductive activating conditions as those used with the thiols here. As another surprising finding thiols alone do not reduce MC but they apparently increase the reductive activation rate of MC by other reducing agents, as seen by the lack of recovery of any MC after reduction in the presence of thiols. In contrast, in the absence of thiols large amount of unreacted

MC is recovered under the same reduction conditions (Figure 9, p.55).

The mechanism of interaction of MC with thiols thus differs from the known mechanism of MC alkylation with DNA. In order to explain the induction of bifunctional alkylating activity of MC in the presence of thiols, further study involving reduction of mitomycin C derivatives M1, M2 and M3 in presence of GSH was conducted. In addition, the activation of decarbamoyl MC was also studied in the presence of GSH. The reduction of M1, M2 and M3 in presence of GSH led to displacement of the carbamate by GSH at C-10 (**13**, **14**, and **15**, p.74). No displacement by water was observed in the absence of GSH. Further, no DMC-GSH adducts were obtained in the DMC reaction (p.28). DMC has an -OH at C-10 - a bad leaving group. Thus it appears that -SH of GSH (and other thiols, pKa of GSH = 9.2) which is an excellent nucleophile displaces the carbamate directly by an S_N2 mechanism. [The nucleophilicity **n** (derived from the Swain-Scott equation) of H_2O = 0.0, OH^- = 4.2, RNH_2 = 4.49 (where R is phenyl), SH^- = 5.1 and RS^- = greater than 5.1 depending on R]. This is in contrast to the S_N1 substitution of the 10-carbamate via the "iminum" intermediate observed in the absence of thiols[9]. On the basis of these results, a proposed mechanism of the reaction of MC with thiols is outlined in Figure 30 (p.80) . Upto product **E**, the steps are identical with the well established reductive transformations of MC in aqueous

medium (see part I; also see review by Franck and Tomasz[46]). Reduction of the quinone to hydroquinone renders the carbinolamine more labile, allowing the loss of methanol. The intermediate formed, not being stable ensues opening of the aziridine, leading to a quinone methide presumed to have structure **B**. Evidence for the quinone methide stems from its ambident character, where both electrophilic and nucleophilic characters are expressed in the reactions leading to the ultimate products. Initial attack at C-1 by thiol, water and solvent derived proton, leads to both *trans* and *cis* substituted adducts of **C** and **D**, and **E**. The ratio of 1,2-*trans* and -*cis* products in **C** and **D** is approximately 1:1. The relative amount of **C** (with thiol at C-1) compared to **D** (with -OH at C-1) and **E** (proton attack at C-1) appears to depend on the size of the thiol. C-1 is probably sterically hindered, favoring attack by smaller thiols to attack by bigger thiols, as indicated by different yields of the bis-thiol in MC-mercaptoethanol and MC-glutathione adducts. The ratio of bis-thiol adducts to mono-thiol adducts in MC-mercaptoethanol reaction is higher than in the MC-GSH or MC-N-acetyl cysteine reaction (p.69; Table 4). The second alkylating step of MC is where the mechanism with thiols differs. The second step in case of thiols is very <u>fast</u> in contrast to the slow step in case of other nucleophiles (H_2O,-NH_2); this is because sulfhydryl being a stronger nucleophile is able to cause an S_N2 displacement of the carbamate. Thus the mechanism of the second alkylating step

of MC is the S_N2 displacement of carbamate resulting in the formation of thiol bis-adducts **F** (*cis* and *trans*) and thiol monofunctional adducts **G** (*cis* and *trans*) and **H**. Displacement of the C-10 leaving group is the critical step toward bifunctional activation. Apparently, the C-10 site is not sterically hindered enabling thiol to displace the carbamate rapidly. It is important to note that displacement of carbamate by water when any mitosene was reduced with H_2/PtO_2 in absence of GSH was negligible. These results therefore, indicate that thiols enhance the bifunctional alkylation by MC. In particular, they react with the second active site of MC, the C-10 carbamate position by S_N2 attack. This results in alkylation of only the thiol, however. Other nucleophiles present will not be alkylated by the C_{10} function of MC. All this taken together, we conclude that glutathione and other thiols react <u>faster</u> with MC than other nucleophiles present. They seem to act as "scavengers" of activated MC under the model conditions reported here. Of all the thiols studied, glutathione was found to react faster than the others.

The observed reactivity of GSH with activated MC under phisiological conditions suggests that such reactions occur intracellularly. This was proved to be correct from the results of the experiments with subcellular systems (activation of MC in rat liver cytosol and microsomes in absence and presence of GSH). In the cytosol reactions MC

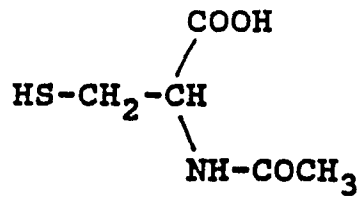
was metabolized by DT diaphorase in the cytosol. This is a low pH-dependent reaction[47]. At pH 7.8 reduction of MC by DT diaphorase leads to inhibition of the enzyme. The major metabolite detected during metabolism of MC by DT diaphorase at pH values between 5.8 and 7, under either aerobic or anaerobic conditions was 2,7 -diaminomitosene (M3, p.48)[47]. This metabolite was formed in the reactions conducted in this study also. In addition, adduct **15** (p.54) was also obtained in the reactions (aerobic and anaerobic) in presence of GSH. Further, MC was rapidly metabolized in the presence of rat liver microsomes. In presence of GSH, metabolites **13**, **14** and **15** were obtained. The possibility of formation of **11** and **12** is not excluded (p.54). These bis-adducts elute better when CH_3CN/H_2O is used as an eluant, however the presence of cytosol and microsomes in the HPLC mixtures made it unsuitable to use this eluant system.

These data provide unequivocal evidence that MC is activated to an alkylating agent by enzymatic reduction and that a variety of reductases can activate mitomycin C to reactive species which can form adducts with GSH. GSH being a detoxifying agent, thereby renders MC non-toxic. The cytotoxicity assay confirmed the non-toxic nature of metabolites **13**, **14** and **15**. Thus changes in intracellular glutathione levels may modulate the toxicity and antitumor activities of mitomycin C by the reactions described herein.

Figure 1. Structures of the thiols.

Mercaptoethanol $HS-CH_2-CH_2-OH$

N-acetyl cysteine



Glutathione

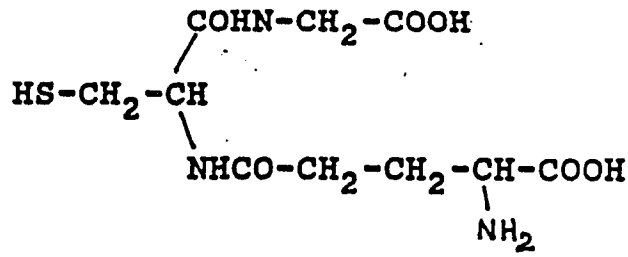
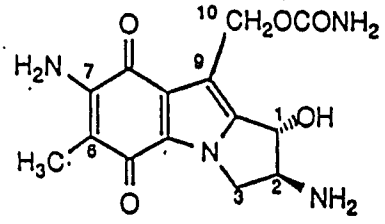
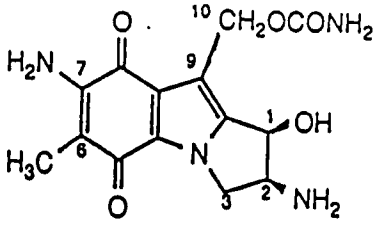


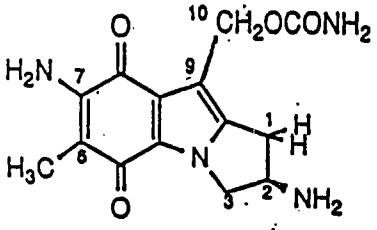
FIGURE 2. STRUCTURES OF M1, M2, M3 AND DECARBAMOYL MC



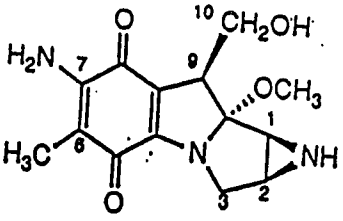
M1



M2



M3



DECARBAMOYL MC

(DMC)

Reaction of MC with mercaptoethanol : isolation of five reductive alkylation products by HPLC

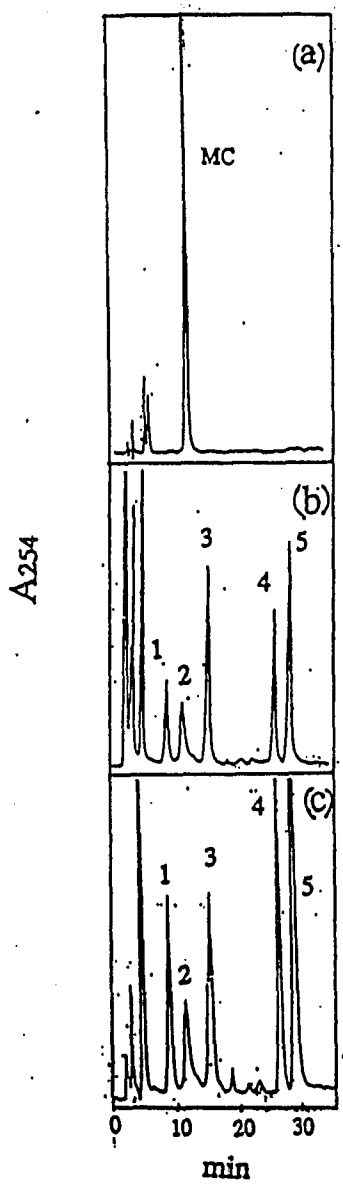
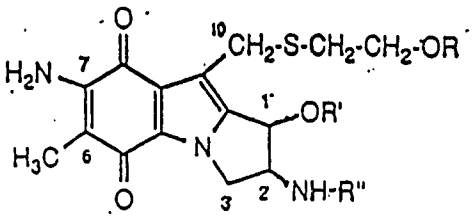


Figure 3. HPLC of the reaction mixture of MC and mercaptoethanol under (a) no reducing agent (b) H_2/PtO_2 reducing conditions (c) NADPH cytochrome c reductase/NADPH reducing conditions.

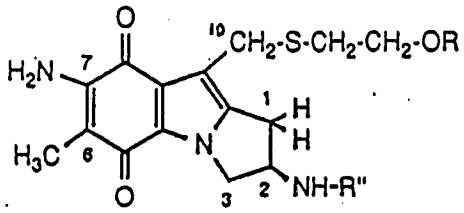
FIGURE 4. STRUCTURES OF MC - MERCAPTOETHANOL ADDUCTS



1 : 1-α-OR′; **2** : 1-β-OR′

1, 2 R, R′, R" = H

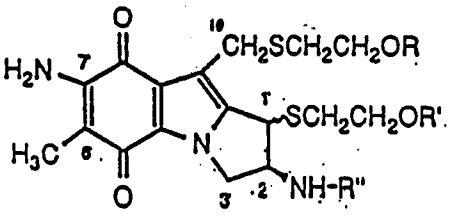
1a and **2a** R, R′, R" = Ac ($COCH_3$)



3

3 R, R" = H

3a R, R" = Ac



4 : 1-α-SCH_2CH_2OR'

5 : 1-β-SCH_2CH_2OR'

4, 5 R, R′, R" = H

4a and **5a** R, R′, R" = Ac ($COCH_3$)

Reaction of MC with N-acetyl cysteine : isolation of five products by HPLC

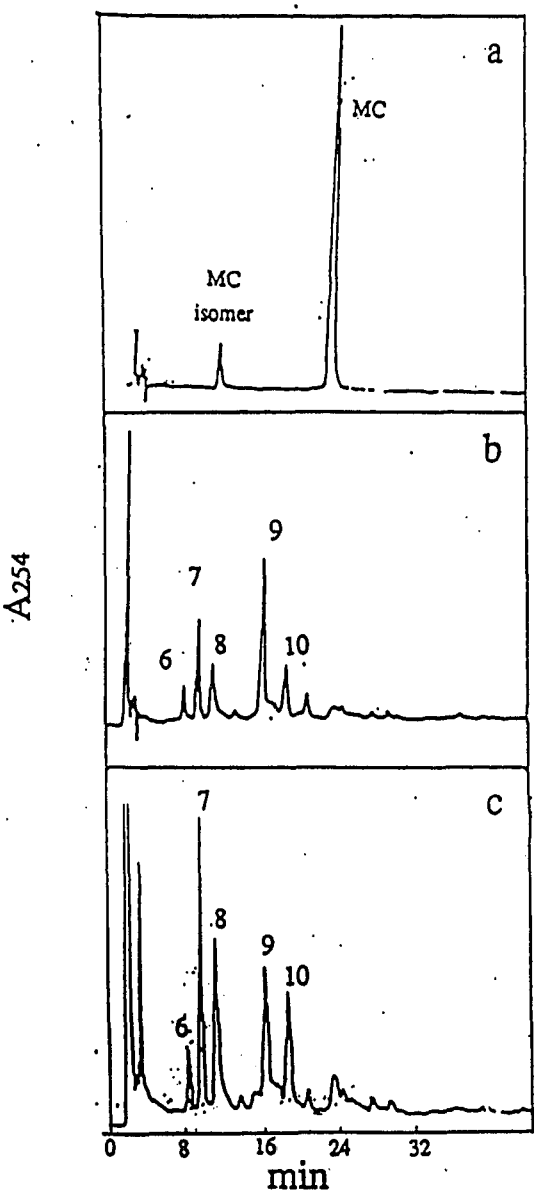
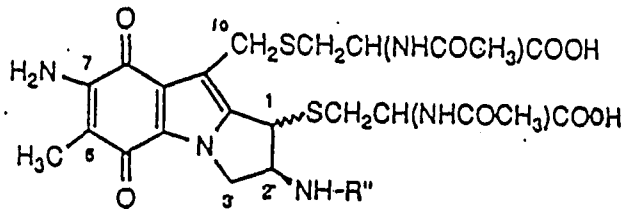


Figure 5. HPLC of the reaction mixture of MC and N-acetyl cysteine under **(a)** no reducing agent **(b)** H_2/PtO_2 reducing conditions **(c)** NADPH cytochrome c reductase/NADPH reducing conditions.

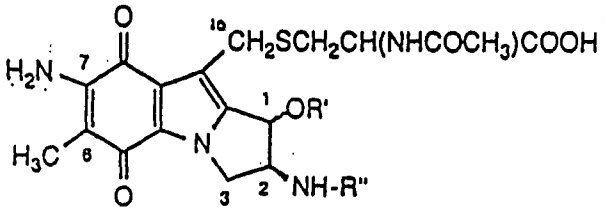
FIGURE 6. STRUCTURES OF MC-N-ACETYL CYSTEINE ADDUCTS



6

6 R′ = H

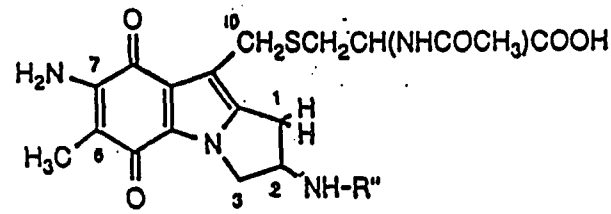
6a R′ = Ac



7, 8 R′,R" = H

7a and 8a R′,R" = Ac

7 : 1-α-OR′; 8 : 1-β-OR′



9

9 R" = H

9a R" = Ac

Reaction of MC with glutathione : isolation of five products by HPLC

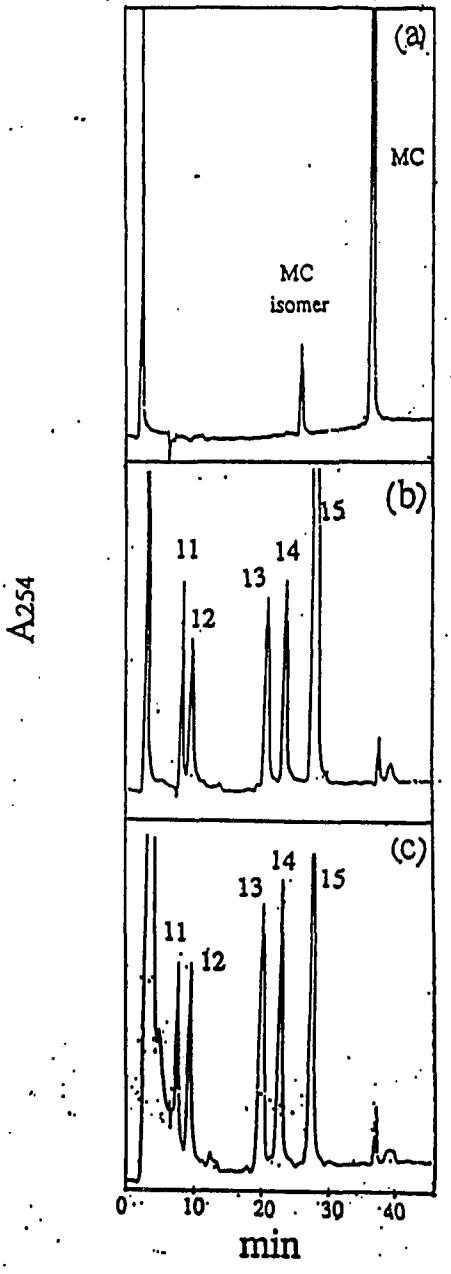
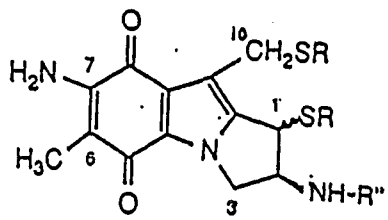


Figure 7. HPLC of the reaction mixture of MC and N-acetyl cysteine under (a) no reducing agent (b) H_2/PtO_2 reducing conditions (c) NADPH cytochrome c reductase/NADPH reducing conditions.

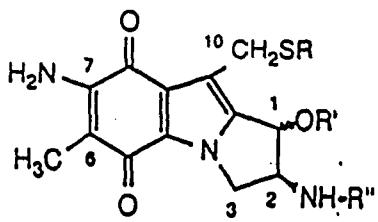
FIGURE 8. STRUCTURES OF MC-GLUTATHIONE ADDUCTS

R = $-CH_2CH(CONHCH_2COOH)NHCOCH_2CH_2CH(NH_2)COOH$



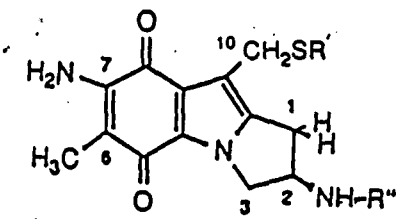
11, 12	R', R" = H
11a and 12a	R', R" = Ac

11 : 1-α-SR; 12 : 1-β-SR



13, 14	R', R" = H
13a and 14a	R', R" = Ac

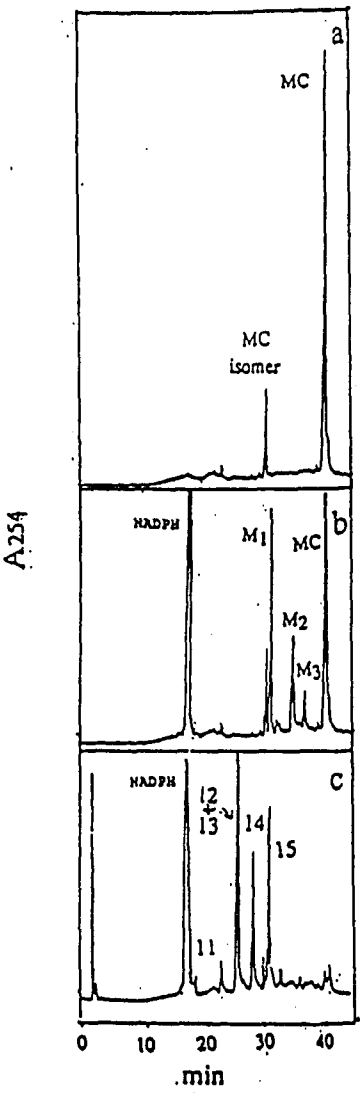
13 : 1-α-OR'; 14 : 1-β-OR'



15	R',R" = H
15a	R', R" = Ac

15

Reductive activation of MC is accelerated in the presence of thiols.



CONTROL (a) : MC + RSH

RESULT : No reducing agent

CONTROL (b) : MC + Reductase (no thiol)

RESULT : Reaction products with water are formed. Much MC recovered.

COMPLETE

REACTION (c) : MC + Reductase + RSH

RESULT : Reaction products with RSH. No MC recovered.

Figure 9. Comparison of the products of reduction of MC in aqueous buffer in the absence and presence of glutathione (RSH). Eluant is CH_3CN/0.03 M ammonium acetate, pH 6.9, 0% to 15% over 35 minutes, since M1, M2 and M3 do not elute properly with CH_3CN/H_2O. (Same retention time for 12 and 13 with this eluant).

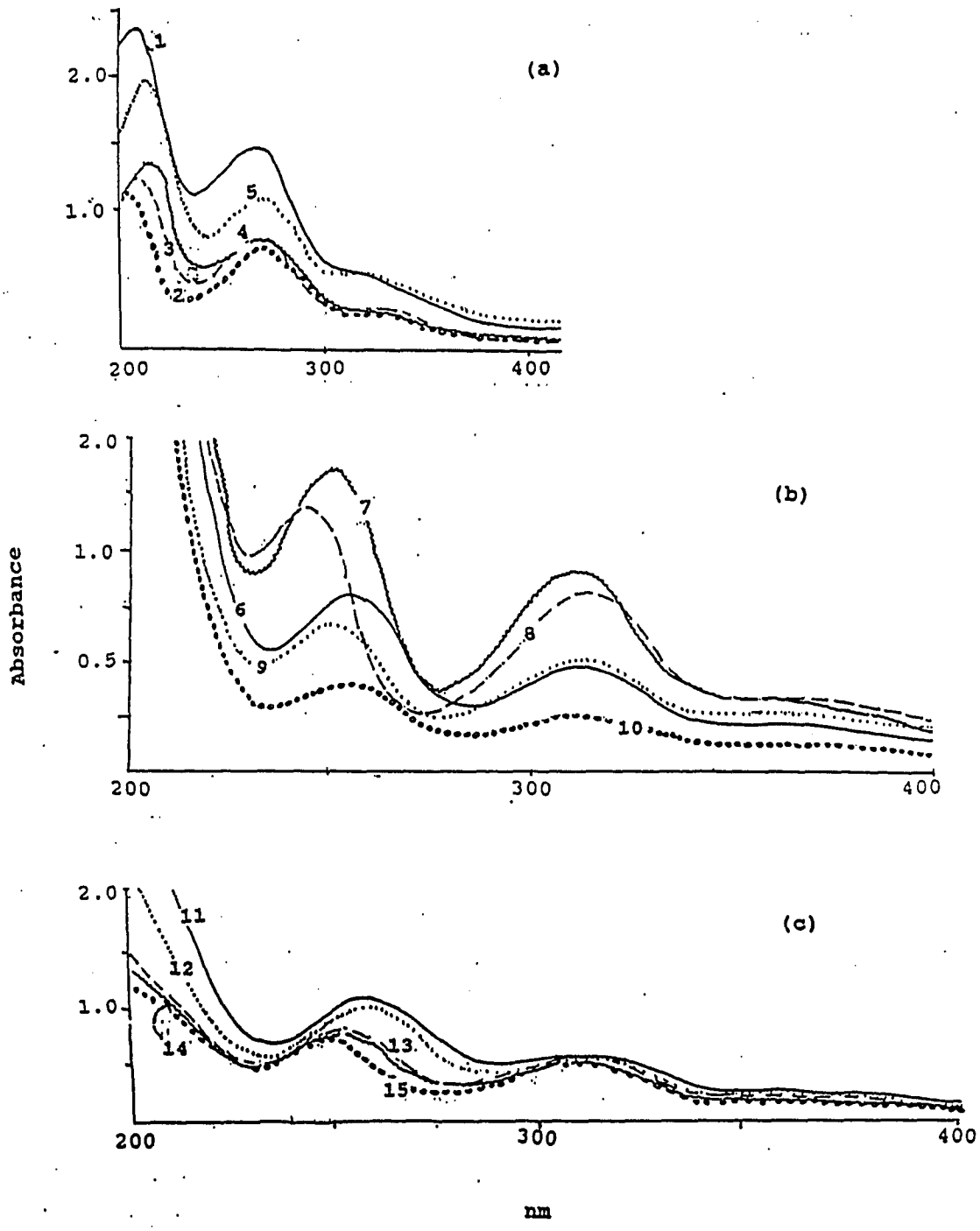


Figure 10. (a) UV of MC-mercaptoethanol adducts (1-5).
(b) UV of MC-N-acetyl cysteine adducts (6-10).
(c) UV of MC-glutathione adducts (11-15).

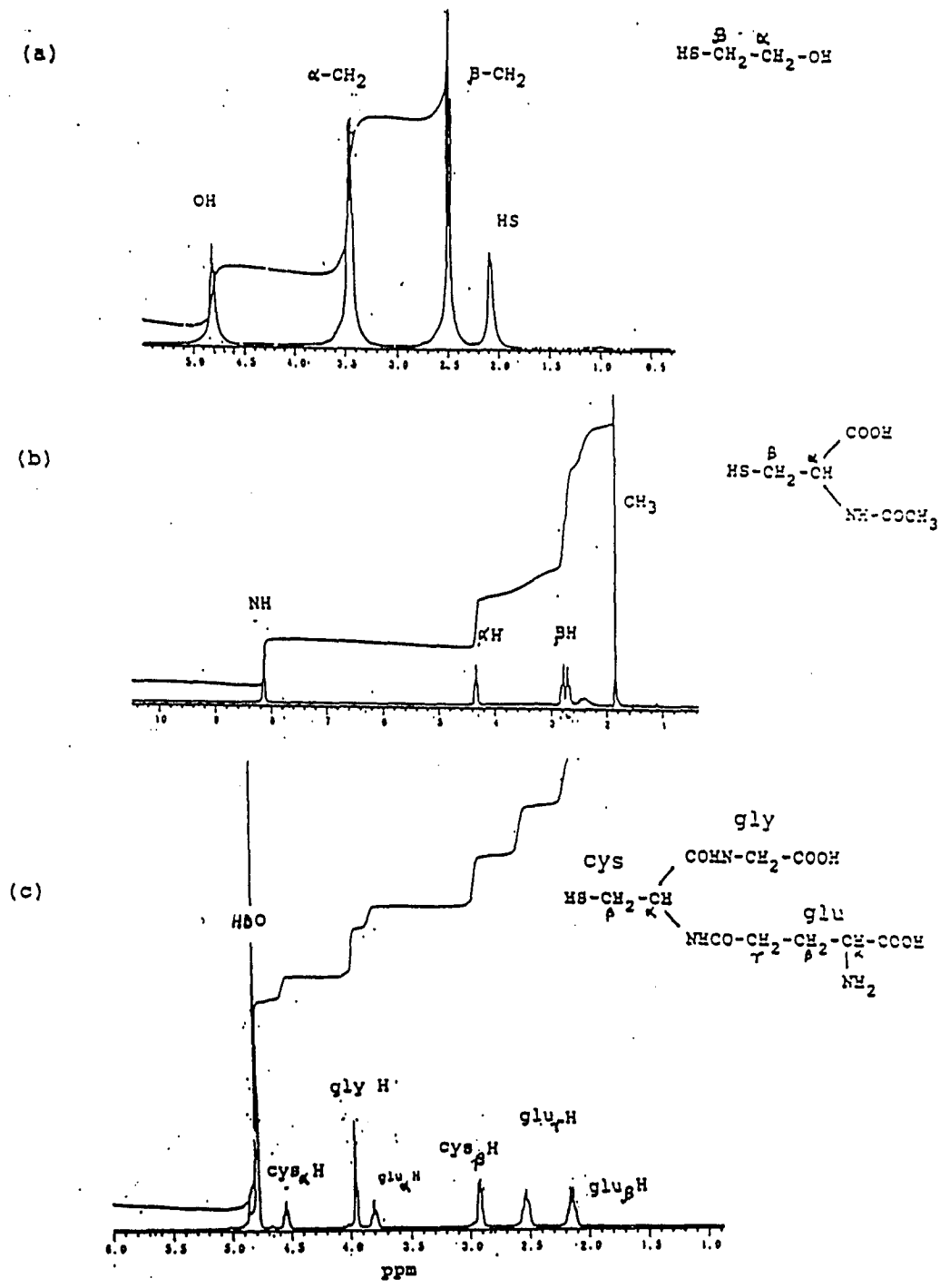


Figure 11. Proton NMR spectra of (a) Mercaptoethanol in Me_2SO-d_6, (b) N-acetyl cysteine in MeOD and (c) Glutathione in D_2O.

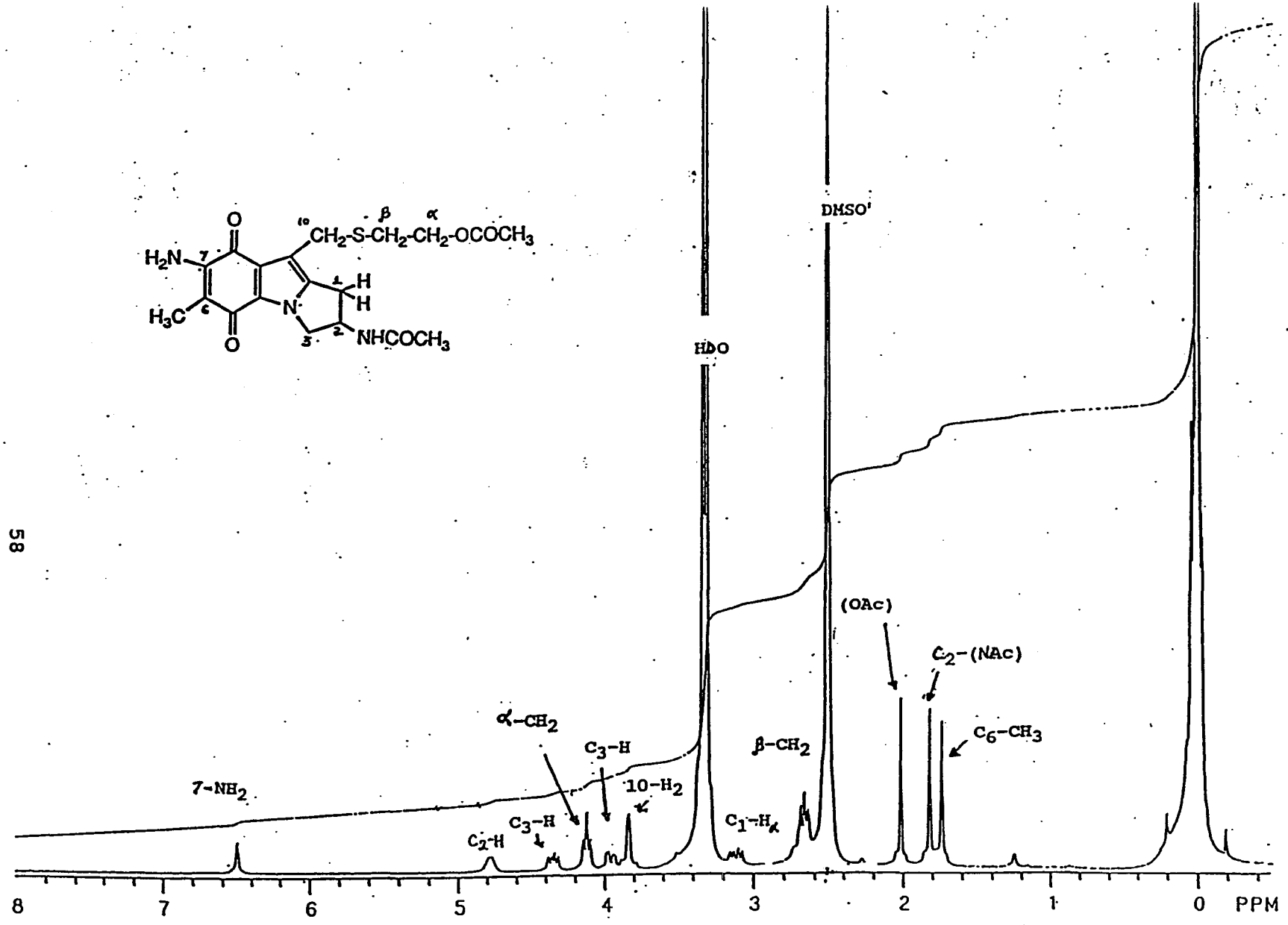


Figure 12. Proton NMR of acetylated MC-mercaptoethanol adduct 3, (in Me_2SO-d).

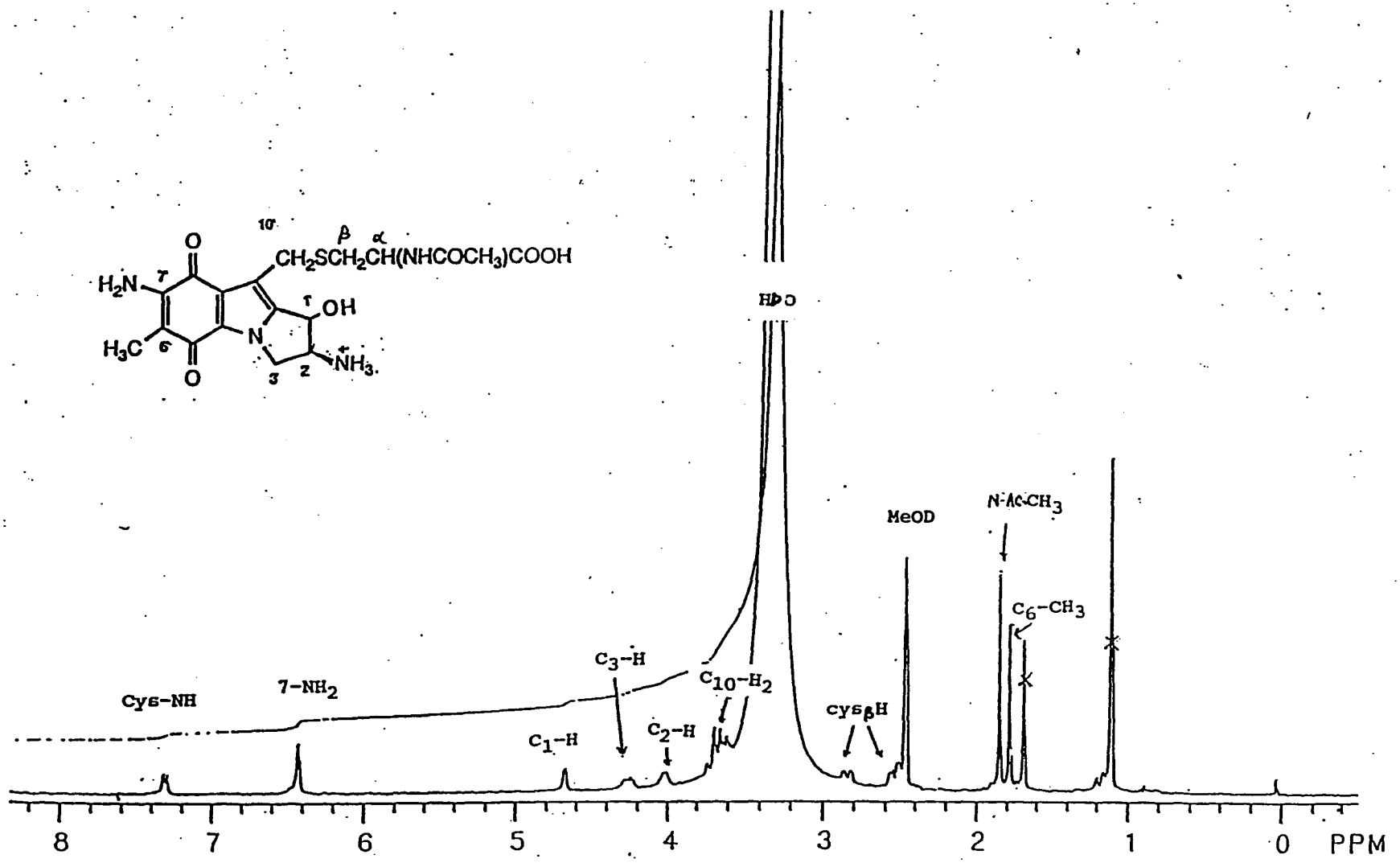


Figure 13. Proton NMR of MC-N-acetyl cysteine adduct 7 (in MeOD).

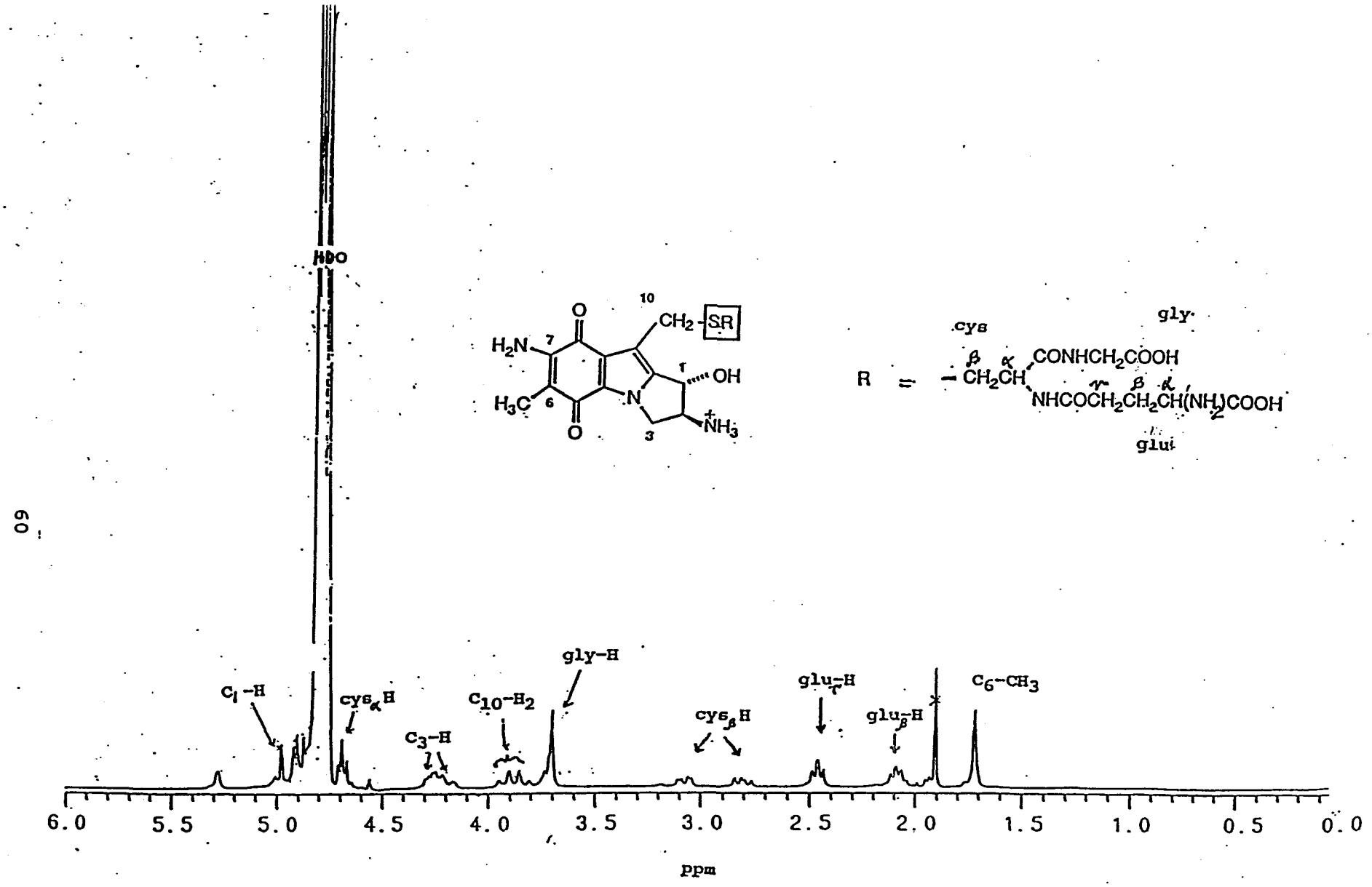


Figure 14. Proton NMR of MC-glutathione adduct 13 (in D_2O).

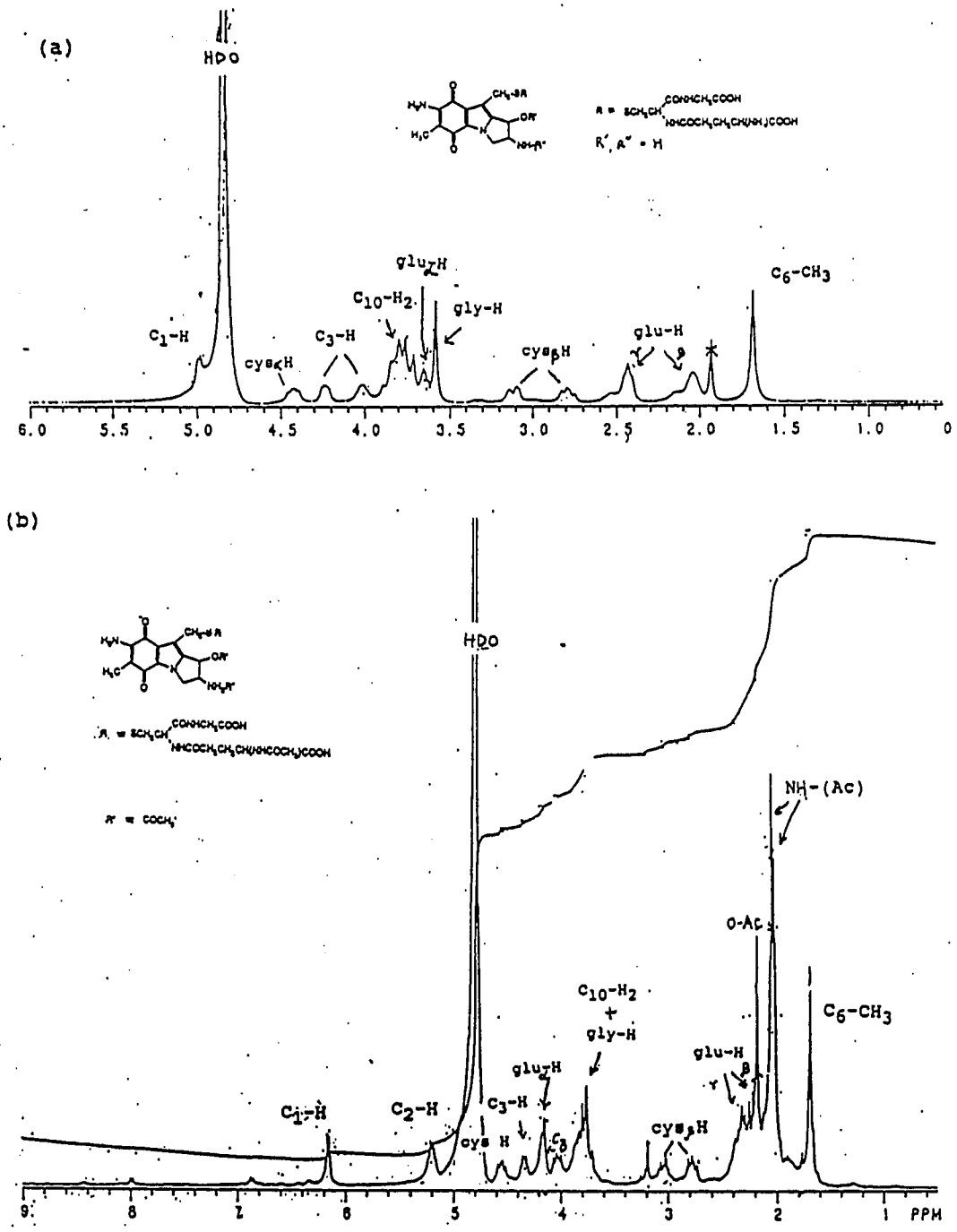


Figure 15. Proton NMR of MC-glutathione adducts (in D_2O), (a) adduct 14 and (b) acetylated adduct 14.

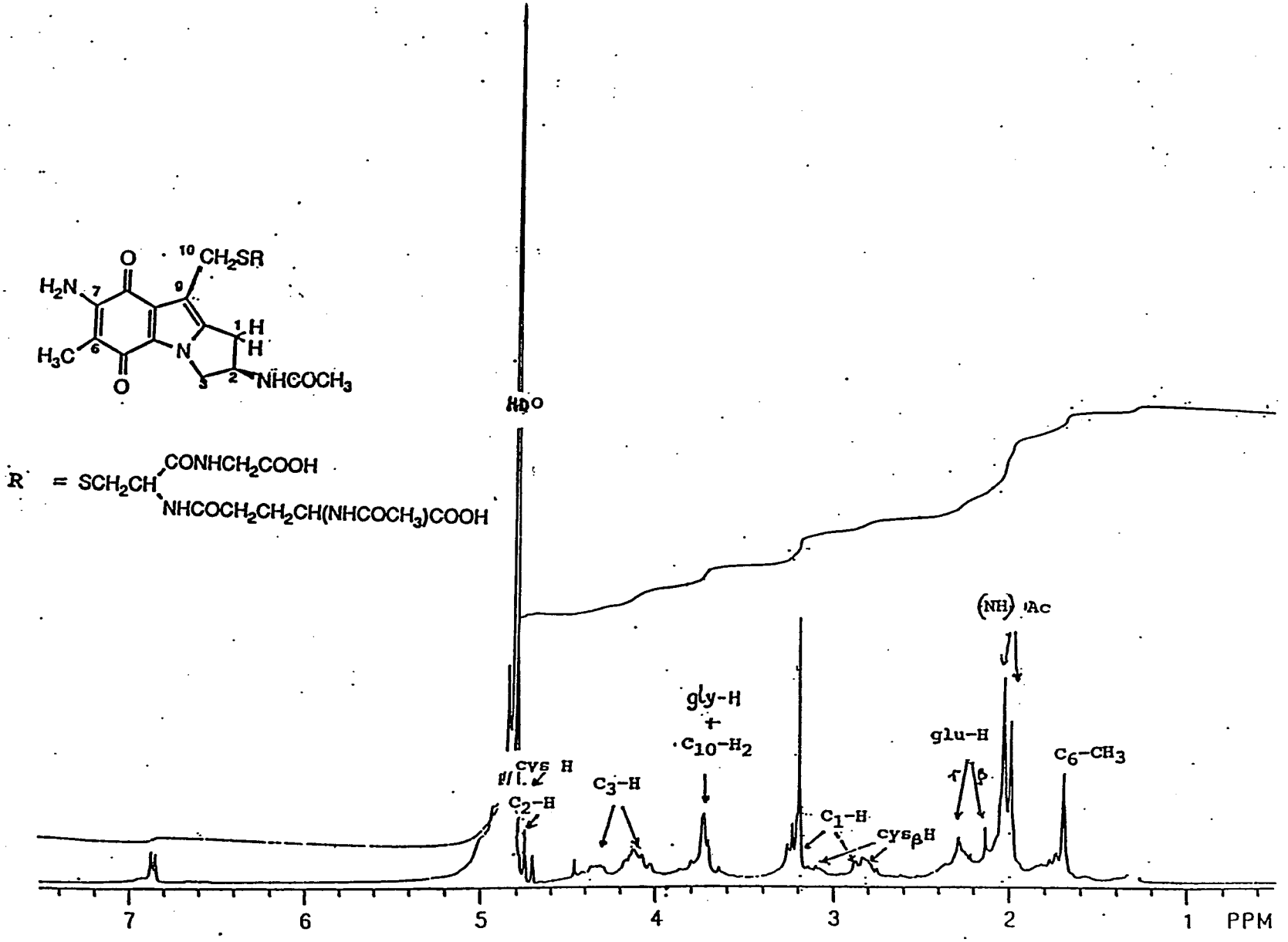


Figure 16. Proton NMR of acetylated MC-glutathione adduct **15**, (in D_2O).

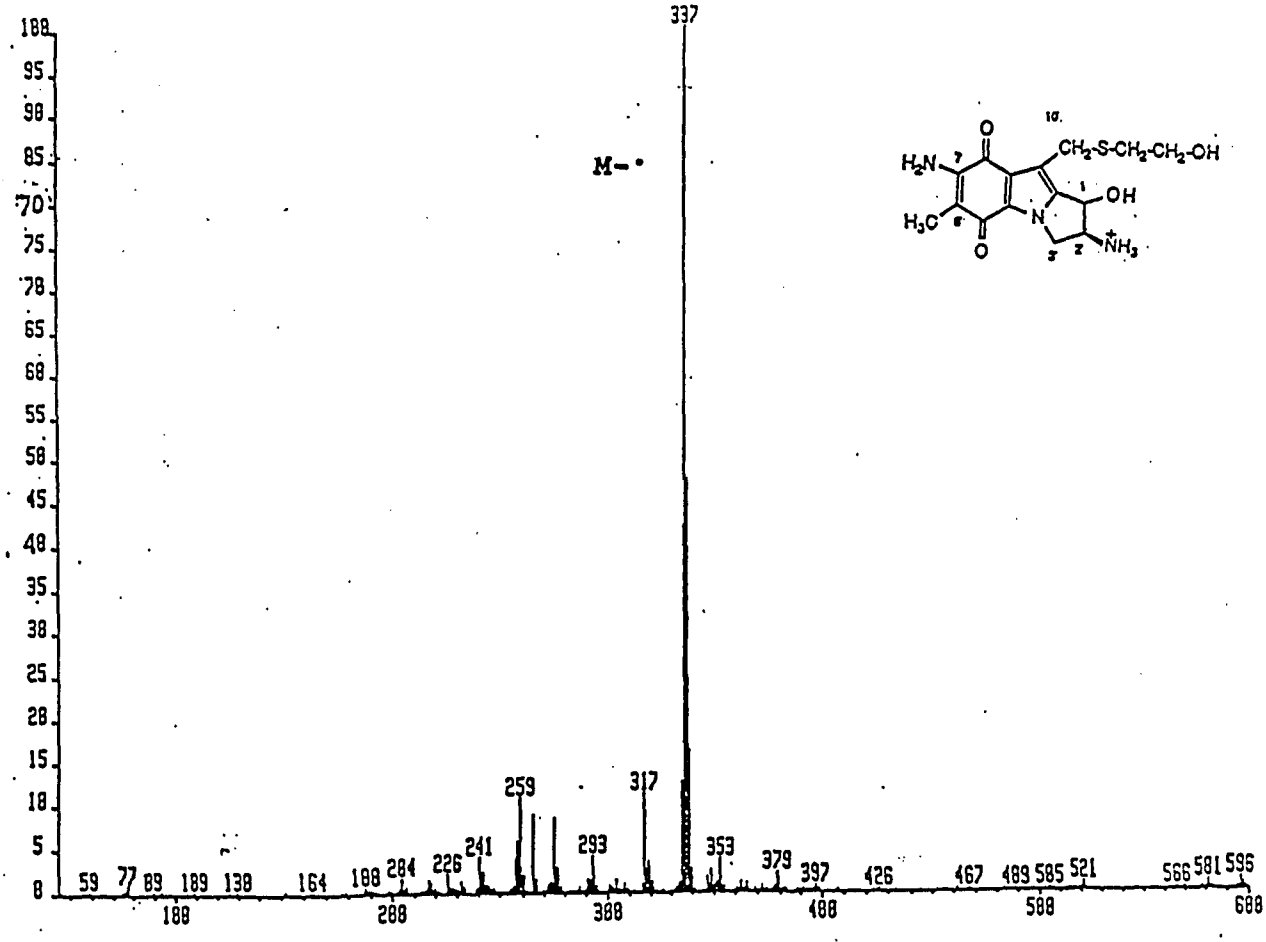


Figure 17. Mass spectrum (CI) of MC-mercaptoethanol adduct 2 (adduct 1 gave similar spectrum).

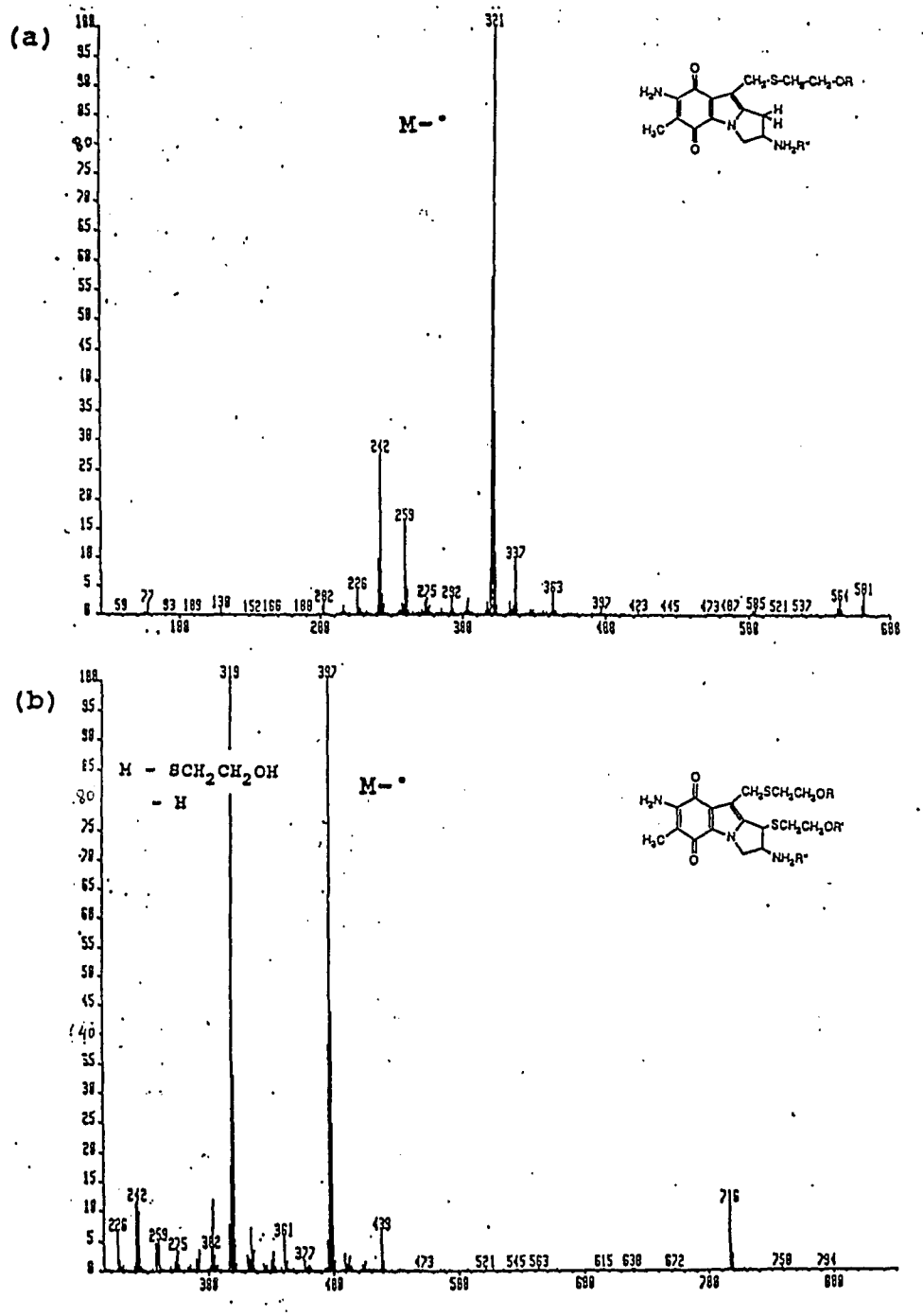


Figure 18. Mass spectrum (CI) of **(a)** MC-mercaptoethanol adduct 3, **(b)** MC-mercaptoethanol adduct 5 (adduct 4 gave the same spectrum.

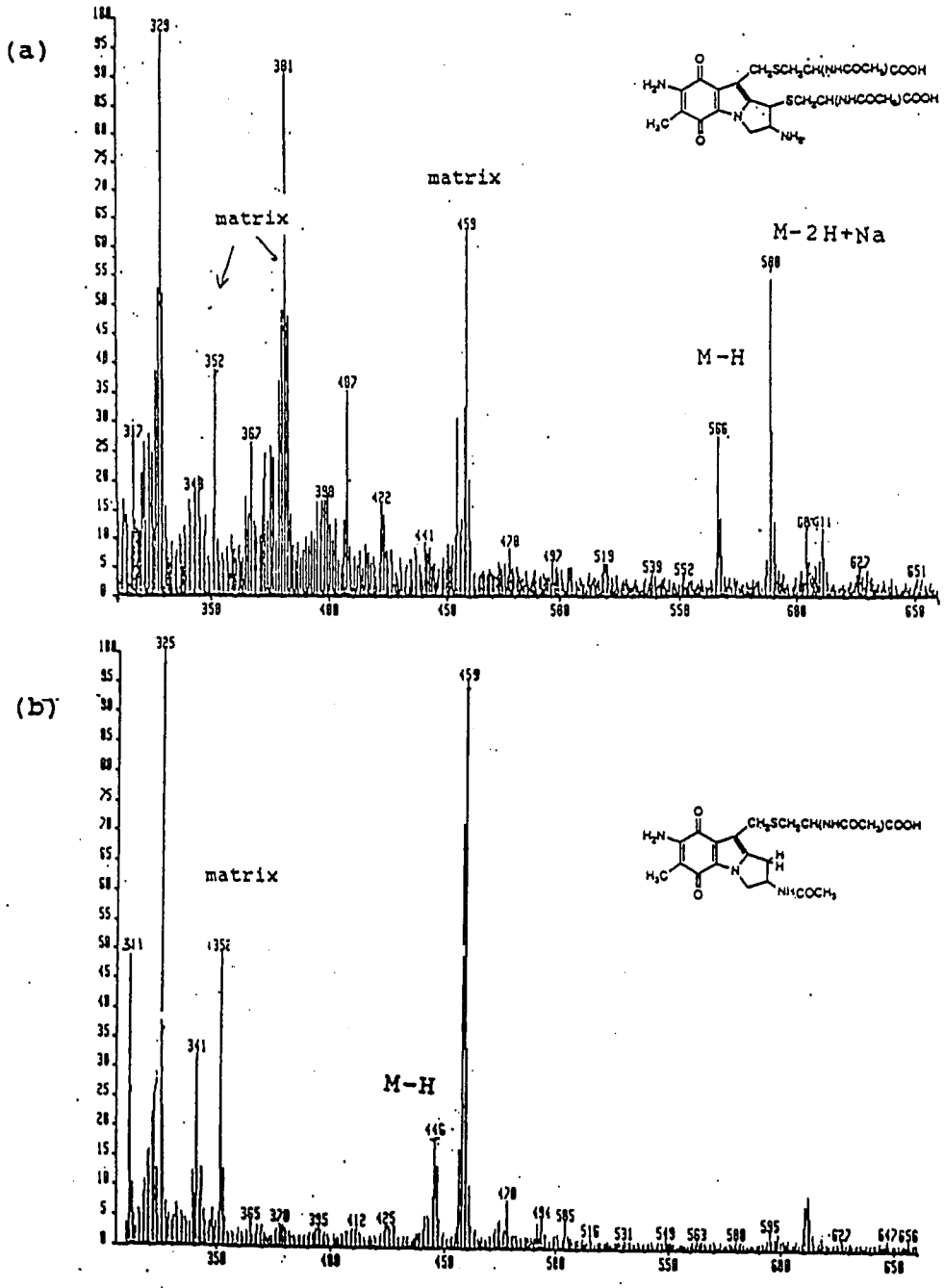


Figure 19. Mass spectrum (neg. FAB) of MC-N-acetyl cysteine adducts **(a)** adduct **6** **(b)** acetylated adduct **9**.

Table 1. Data for structure determination of MC-mercaptoethanol compounds 1 - 5 (p. 50).

COMPOUND	UV Max nm	m/z CI-	Selected ^{1}H-NMR shifts ppm (in Me_2SO-d_6)
1	267,312	337 (M-•)	4.6,s,C_1H; 3.8,q,$C_{10}H$ 3.45,t,α-CH_2(OH)
1a	267,312		5.8,s,C_1H; 3.8,q,$C_{10}H$ 4.1,t, α-CH_2(OAc)
2	267,313	337 (M-•)	4.6,s,C_1H; 3.8,q,$C_{10}H$
2a	267,313		6.02,d,C_1H; 3.8,q,$C_{10}H$
3	268,314	320 (M-•)	2.9,dd,C_1H; 3.8,s,$C_{10}H$
3a	268,314		3.1,dd,C_1H; 3.8,s,$C_{10}H$
4	268,313	397 (M-•)	4.7,C_1H; 3.8,q,$C_{10}H$
4a	268,313		5.0,C_1H; 3.8,q,$C_{10}H$ 4.1,m, α-CH_2(OAc)
5	268,313	397 (M-•)	3.48,m, α-CH_2(OH)
5a	268,313		4.7,C_1H; 4.1,m, α-CH_2(OAc)

Table 2. Data for structure determination of MC-N-acetyl cysteine compounds 6 - 10 (p. 52).

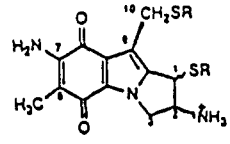
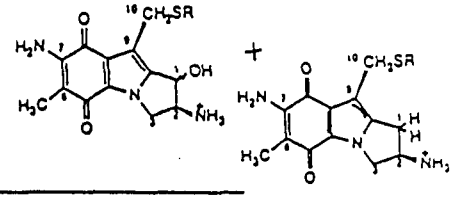
COMPOUND	UV Max nm	m/z neg.FAB	Selected ^{1}H-NMR shifts ppm (in MeOD)
6	256,313	566 $(M-H)^-$	4.55,C_1H; 3.8,q,C_{10}H 4.3,m,cys$_\alpha$H; 2.7,m,cys$_\beta$H
6a	256,313		5.8,s,C_1H; 3.8,q,C_{10}H
7	251,312	421 $(M-H)^-$	4.55, b*,C_1H; 3.8,b,C_{10}H
7a	251,312	467 [M-2($COCH_3$)+2Na]	5.84,s,C_1H; 3.7,q,C_{10}H
8	251,312	no ions	3.8,b,C_{10}H
8a	251,312		6.0,d,C_1H; 3.8,q,C_{10}H
9	245,313	405 $(M-H)^-$	2.9,b,C_1H; 2.5,b,C_1H$_\beta$ 3.8,b,C_{10}H
9a	245,313	447 (M-H)-	3.6,m,C_1H; 3.1,dd,C_1H$_\beta$
10	254,308	no ions	not identified
10a	254,308		not identified

b* = broad

Table 3. Data for structure determination of glutathione-MC adducts 11 - 15 (p. 54).

COMPOUND	UV Max nm	umol GSH per umol mitosene from radiolabel, using [^{3}H]-GSH	Selected ^{1}H-NMR shifts ppm (in D_2O)
11	257,315	2.3/1	4.7,b*,C_1H; 3.9,b,C_{10}H
11a	257,315		4.7,b,C_1H; 3.8,m,glu$_\alpha$H 2.1,m,glu$_\beta$H ; 2.5,m,H$_\gamma$
12	257,314	1.85/1	4.7 b,C_1H; 3.9,b,C_{10}H
12a	257,314		3.8,m,glu$_\alpha$H; 2.1,m,glu$_\beta$H ; 2.5,m,glu$_\gamma$H
13	251,313	0.840/1	4.5,b,C_1H; 3.9,b,C_{10}H
13a	251,313		6.04,s,C_1H; 3.9,q,C_{10}H
14	252,313	1.03/1	4.6 b,C_1H; 3.9,b,C_{10}H
14a	252,313		6.14,d,C_1H; 3.9,q,C_{10}H
15	245,314	1.05/1	3.3,m,C_1H_α; 2.9,m,C_1H
15a	245,313		3.8,s,C_{10}H

Table 4. Ratio of Bis-thiol adducts to mono-thiol adducts obtained in the MC-mercaptoethanol, MC-N-acetyl cysteine and MC-glutathione reactions

REACTION	REDUCING AGENT	BIS-THIOL ADDUCTS 	:	MONO-THIOL ADDUCTS 
MC-mercapto-ethanol	H_2/PtO_2	5.8	:	4
	Enzyme/NADPH	4.1	:	4.4
MC-N-acetyl cysteine	H_2/PtO_2	1	:	12.4
	Enzyme/NADPH	1	:	15.2
MC-GSH	H_2/PtO_2	2	:	8
	Enzyme/NADPH	2	:	5.1

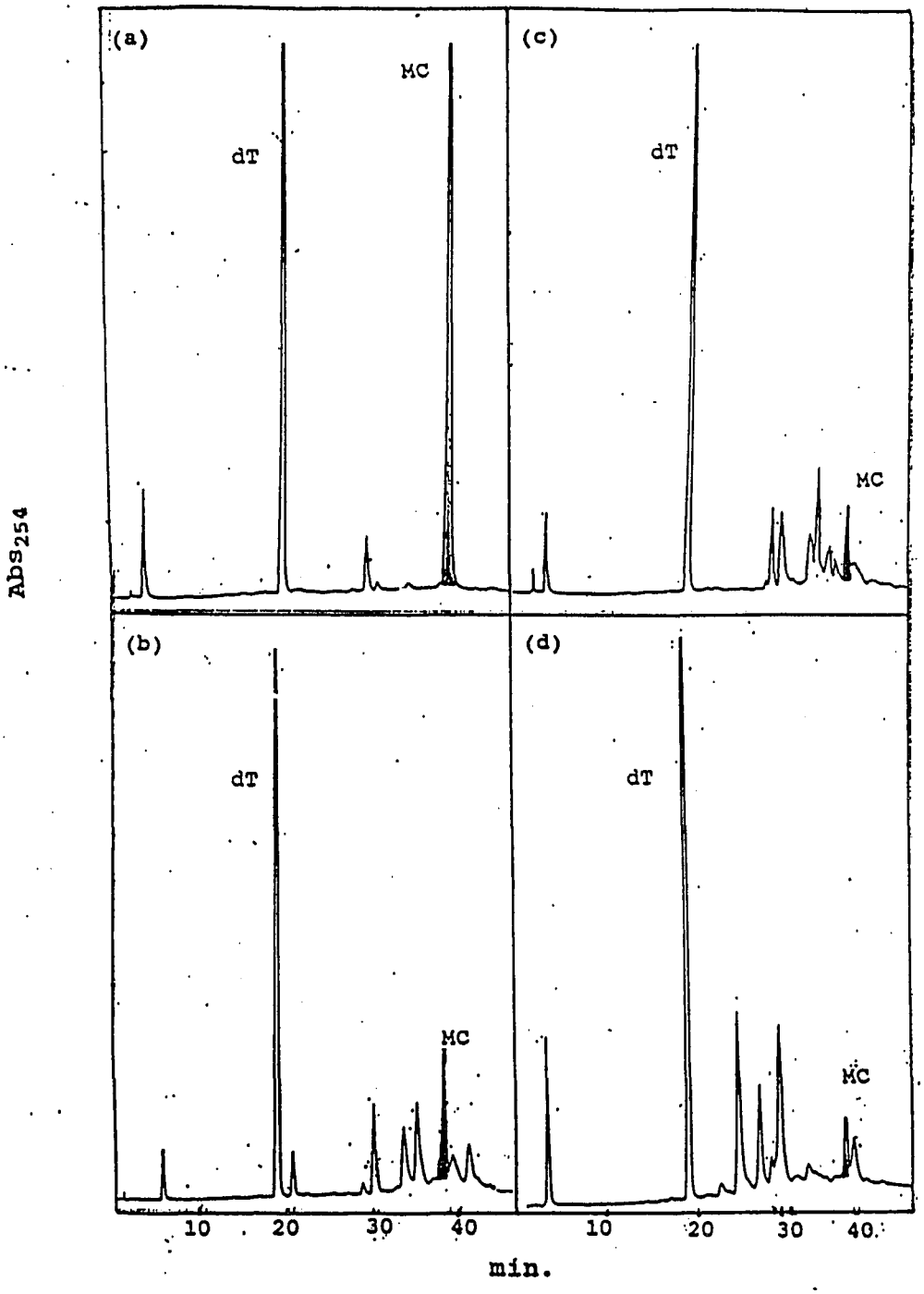


Figure 20. HPLC profiles - which thiol works best

(a) Control : MC + dT (thymidine), (b) MC + dT + H_2/PtO_2 + mercaptoethanol, (c) MC + dT + H_2/PtO_2 + N-acetyl cysteine, (d) MC + dT + H_2/PtO_2 + glutathione.

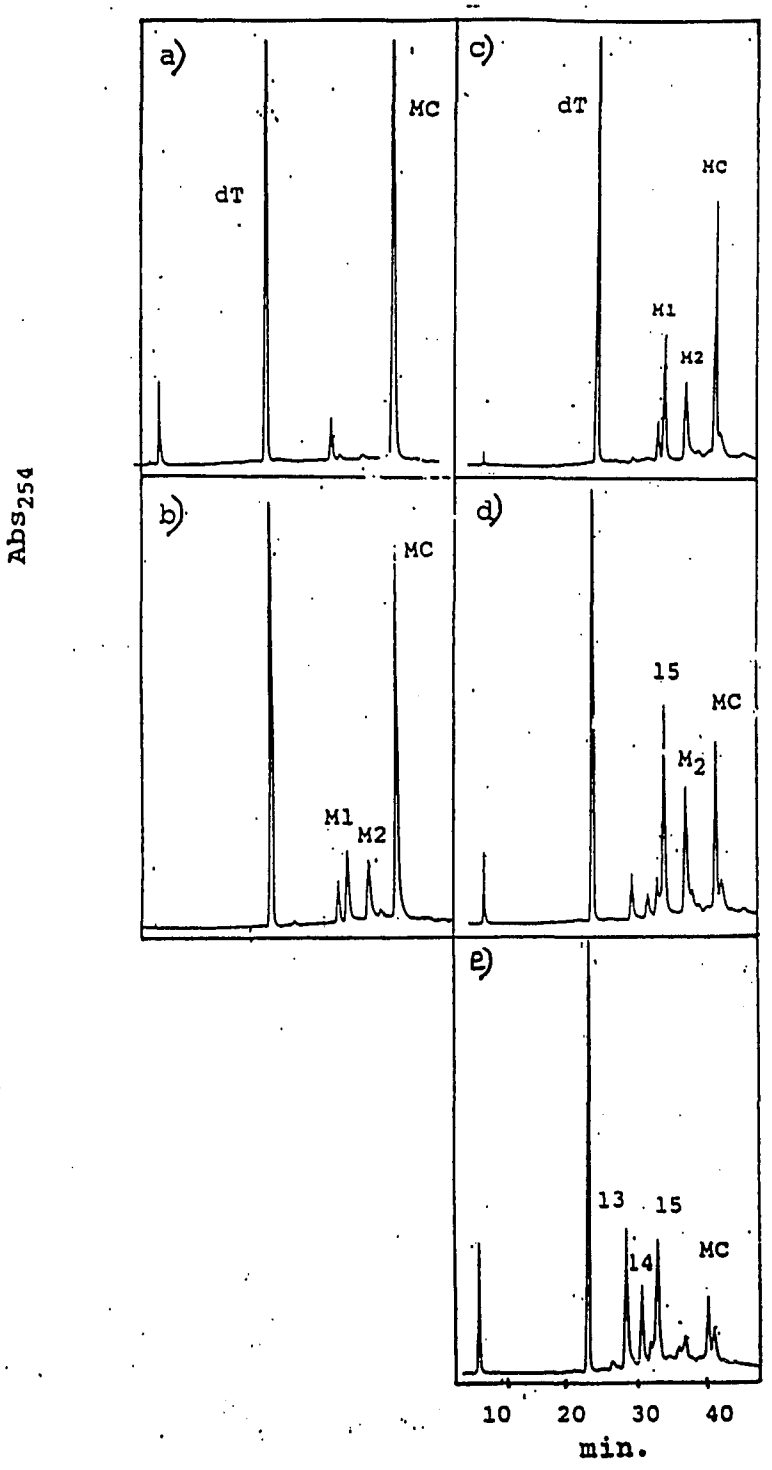


Figure 21. HPLC profiles - what concentration works best. **(a)** Control : MC + dT (thymidine), **(b)** Control MC + dT + H_2/PtO_2 **(c)** MC + dT + H_2/PtO_2 + GSH, MC : GSH = 1:1 **(d)** MC : GSH = 1:10 **(e)** MC : GSH = 1:27.

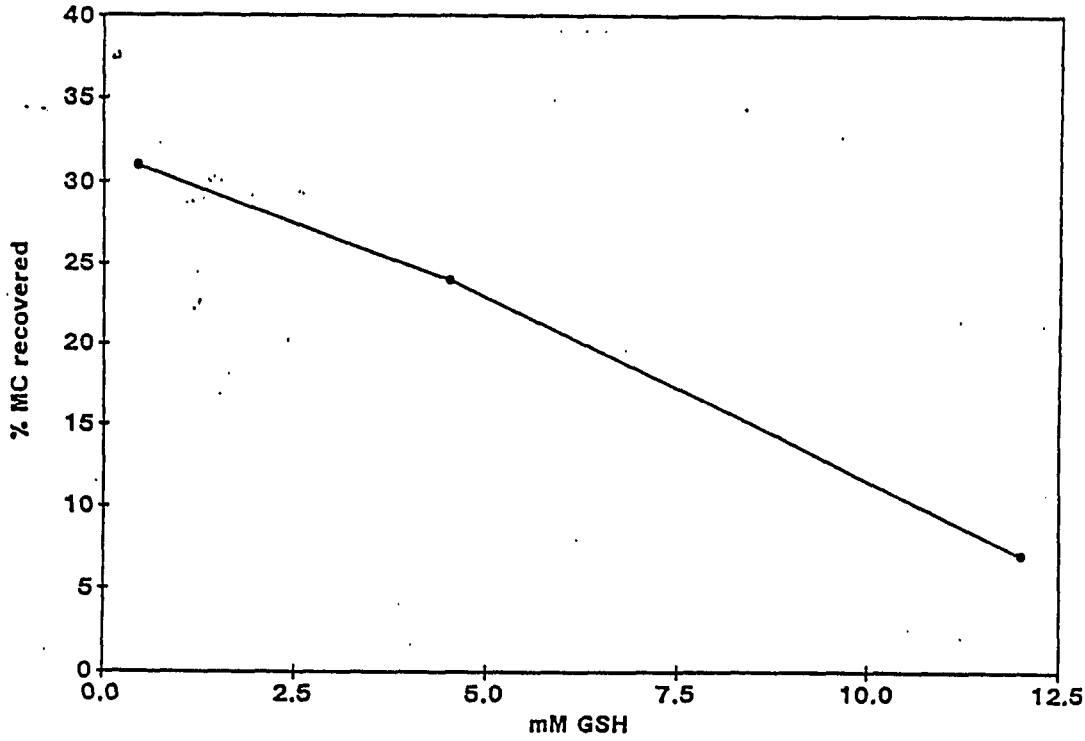


Figure 22. Plot of % MC recovered vèrsus mM GSH, indicating faster consumption of MC with increasing concentration of GSH.

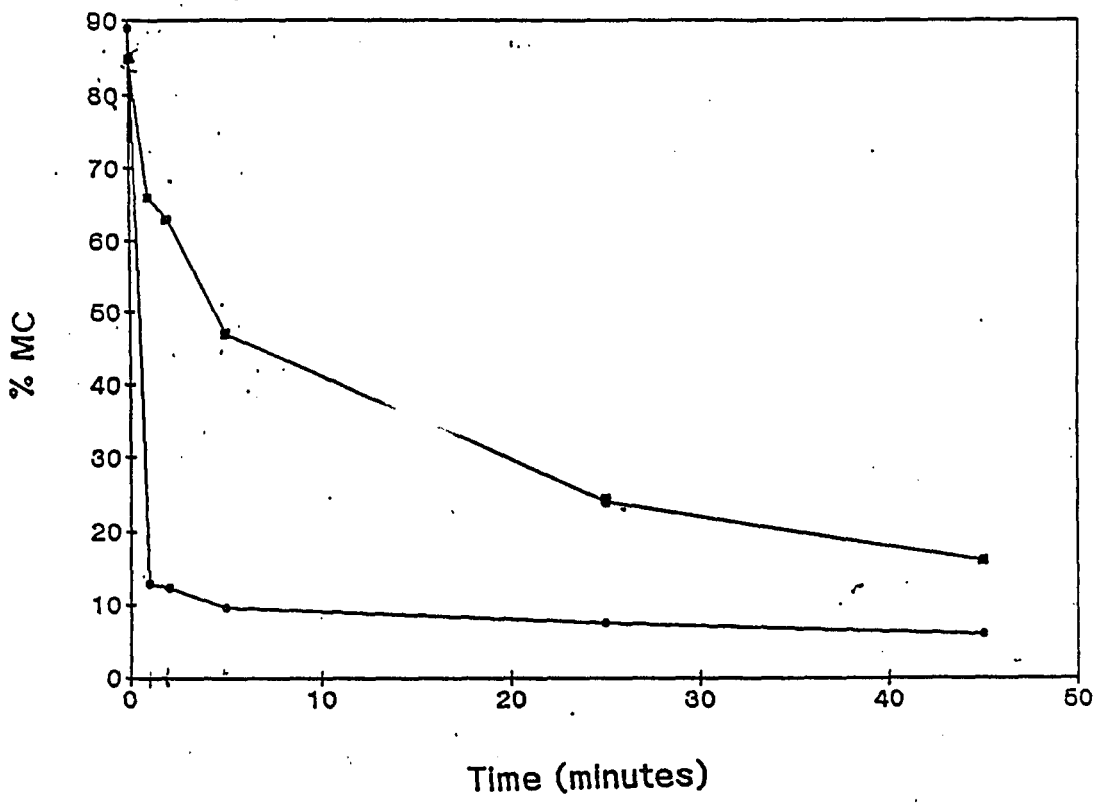
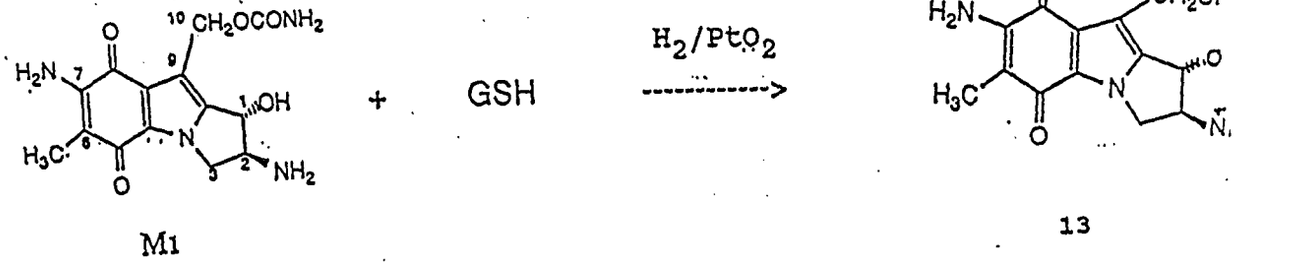
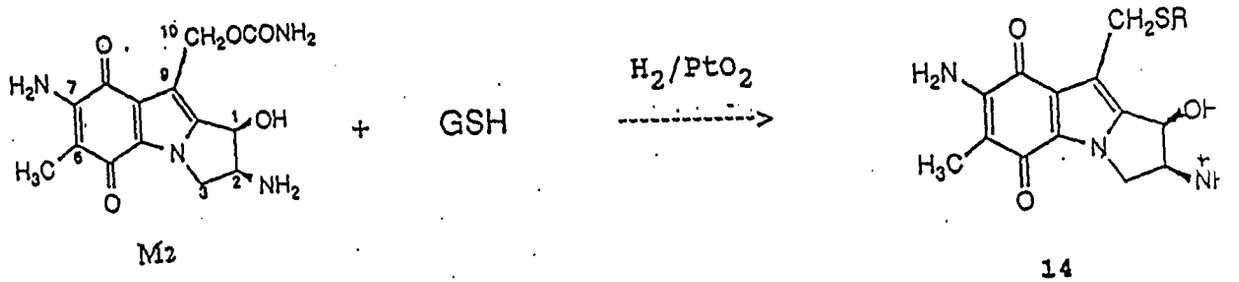


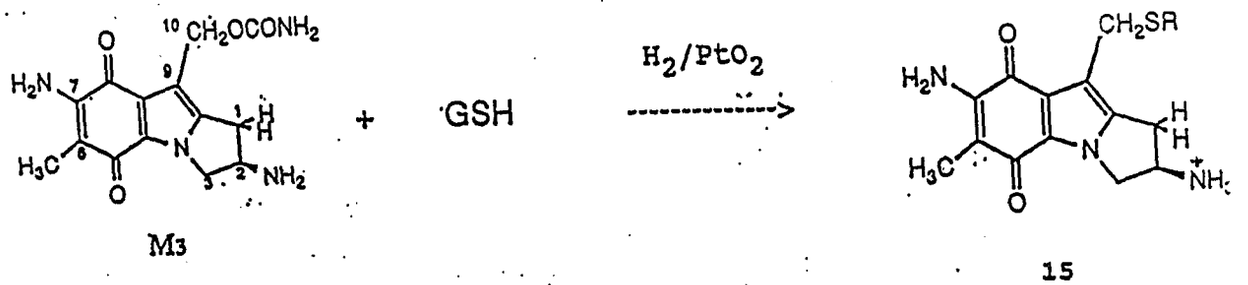
Figure 23. Kinetics of reductive degradation of MC using cytochrome c reductase / NADH as reducing agent.

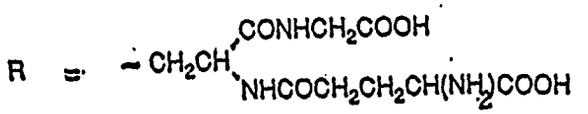
(—•— reaction in absence of GSH ; —•— in presence of GSH)

Figure 24. Reductive activation of mitosenes in presence of GSH.









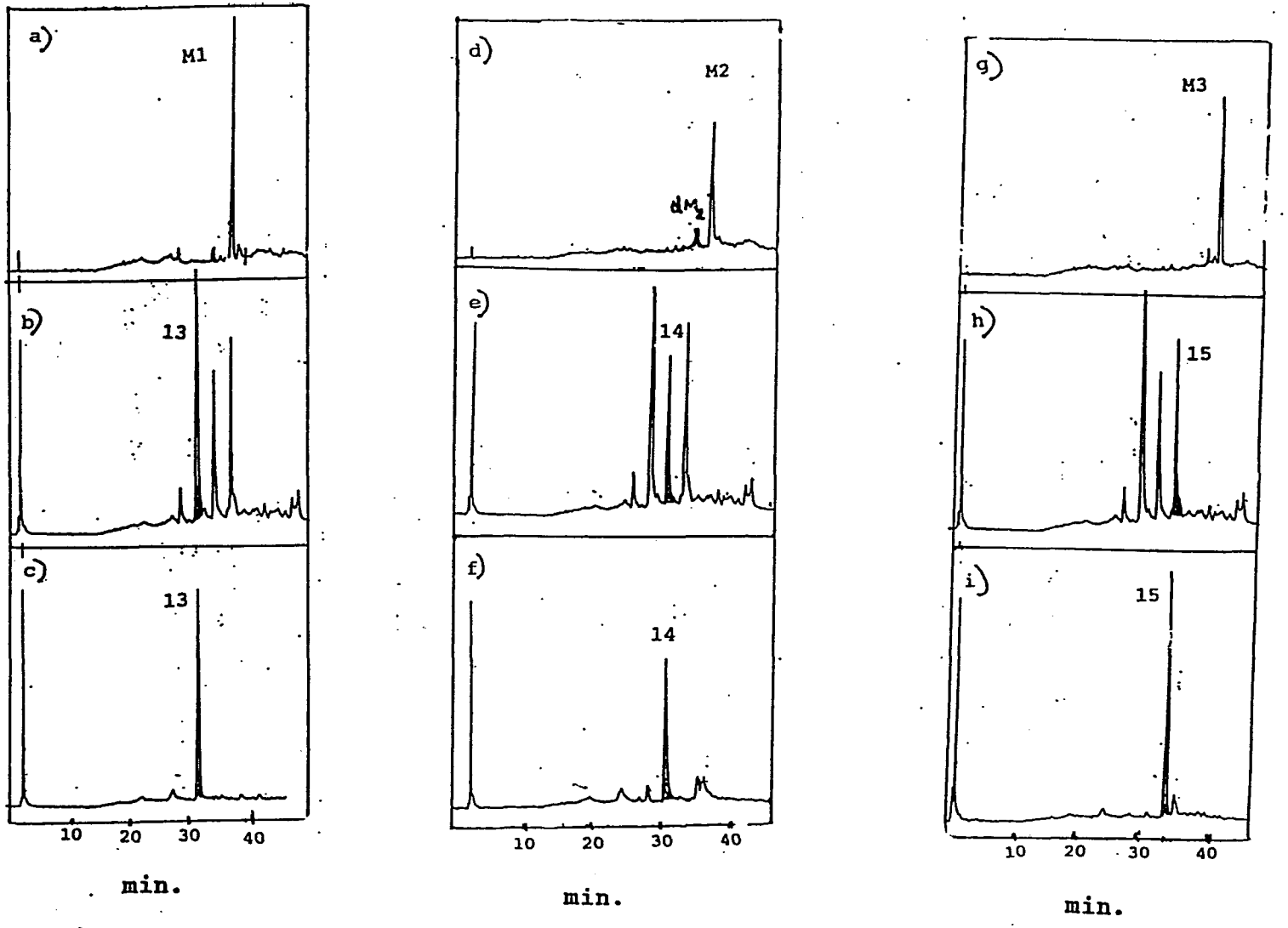


Figure 25. Reductive activation of mitosenes M1, M2 and M3.

(a) M1 + H_2/PtO_2 **(d)** M2 + H_2/PtO_2 **(g)** M3 +H_2/PtO_2

(b) MC + H_2/PtO_2 + GSH **(e)** MC + H_2/PtO_2 + GSH **(h)** MC + H_2/PtO_2 + GSH

(c) M1 + H_2/PtO_2 + GSH **(f)** M2 + H_2/PtO_2 + GSH **(i)** M3 + H_2/PtO_2 + GSH

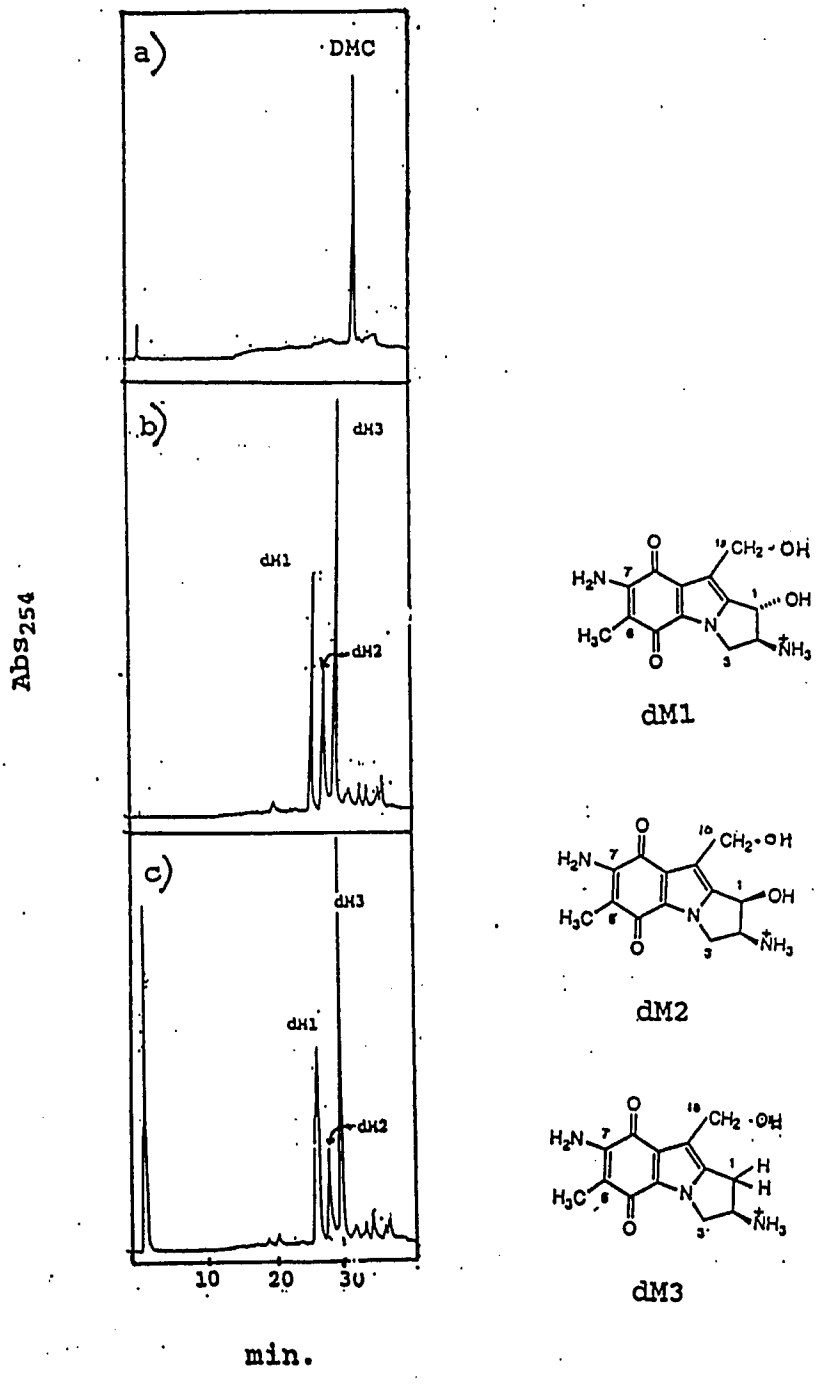


Figure 26. HPLC profiles - Reductive activation of DMC. (a) DMC (b) DMC + H_2/PtO_2 (c) DMC + H_2/PtO_2 + GSH (dM1, dM2 and dM3 = decarbamoyl mitosenes).

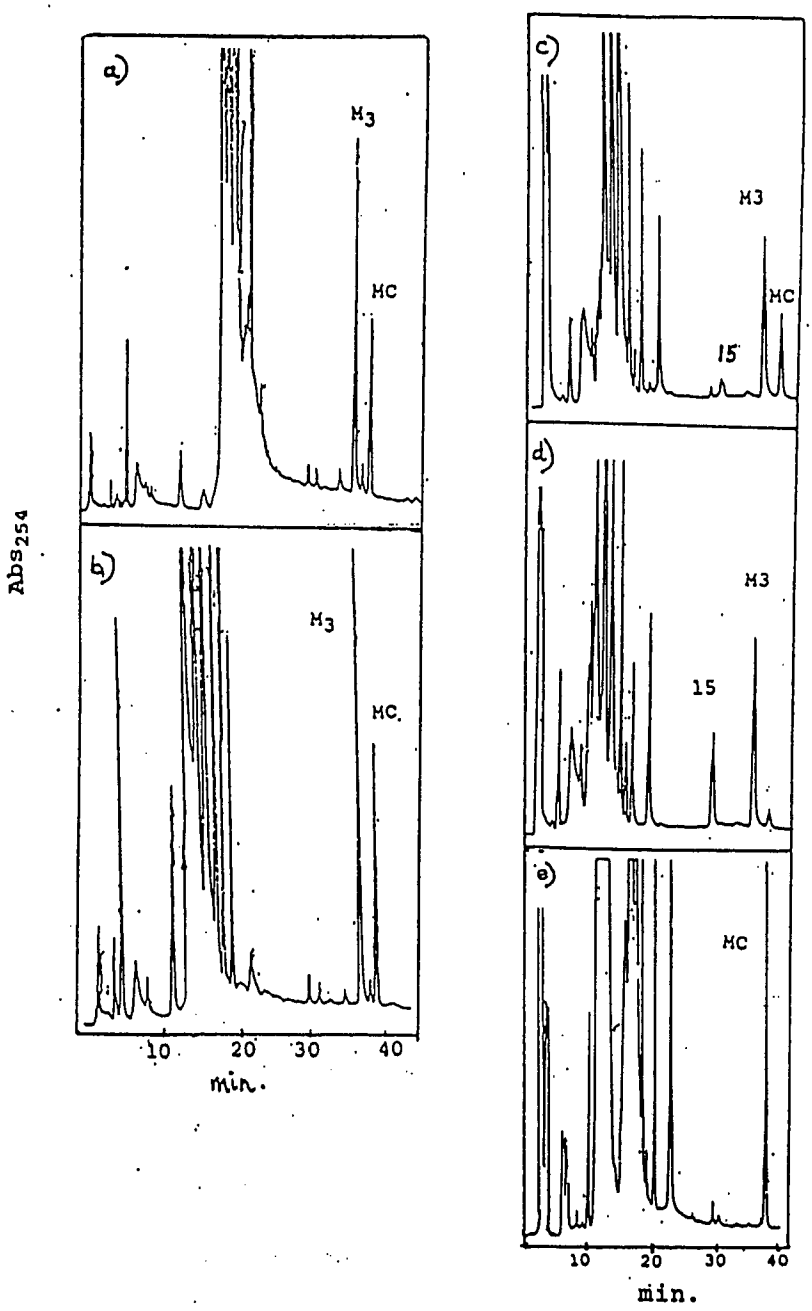


Figure 27. HPLC profiles of MC activation with cytosol under anaerobic conditions.

(a) No GSH added, 30 minute reaction; **(b)** no GSH added, 2 hours; **(c)** GSH added, 30 minute reaction; **(d)** GSH added, 2 hours; **(e)** dicumarol added (after 4 hours, + GSH and -GSH: same result).

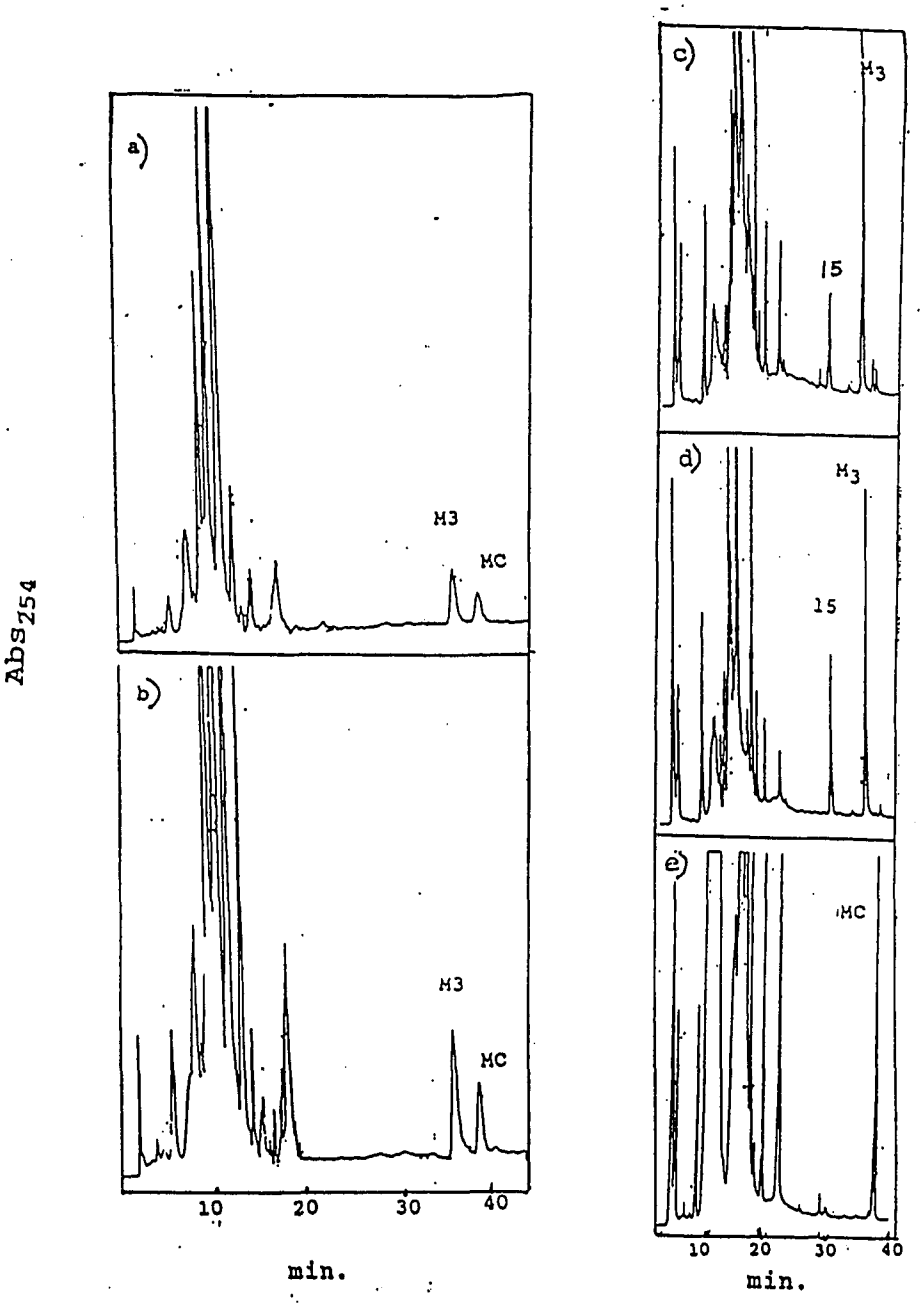


Figure 28. HPLC profiles of MC activation with cytosol under aerobic conditions.

(a) No GSH added, reaction after 2 hours; **(b)** no GSH added, 4 hours; **(c)** GSH added, 2 hours; **(d)** GSH added, 4 hours; **(e)** dicumarol added (after 4 hours, + GSH and -GSH: same result).

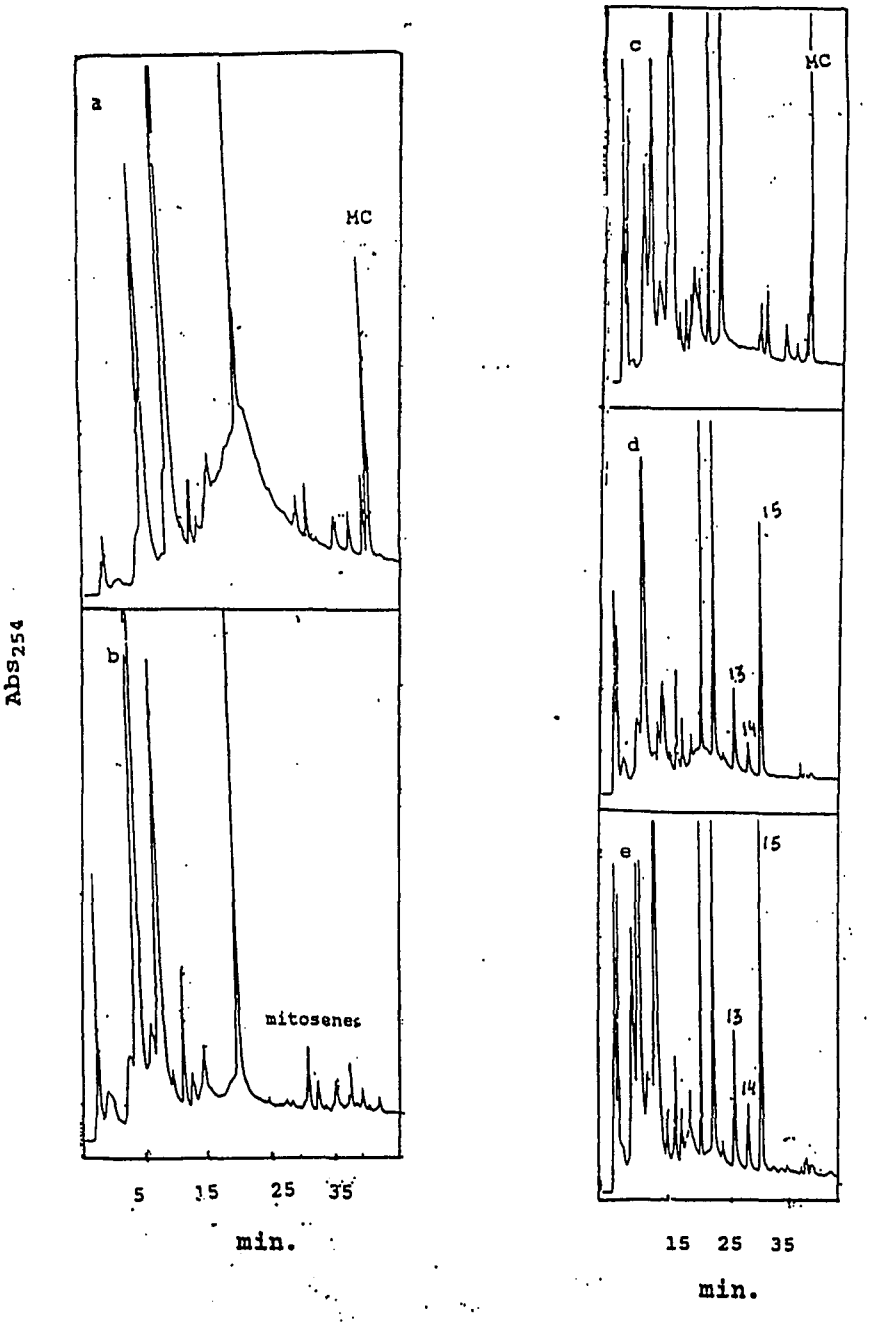


Figure 29. HPLC profiles of MC activation by microsomes. **(a)** No GSH added, 0 min reaction; **(b)** 30 min (reaction at 2 hours and 4 hours same as 30 min). **(c)** GSH added, 0 min **(d)** GSH added, 30 min; **(e)** 2 hours (same at 4 hours).

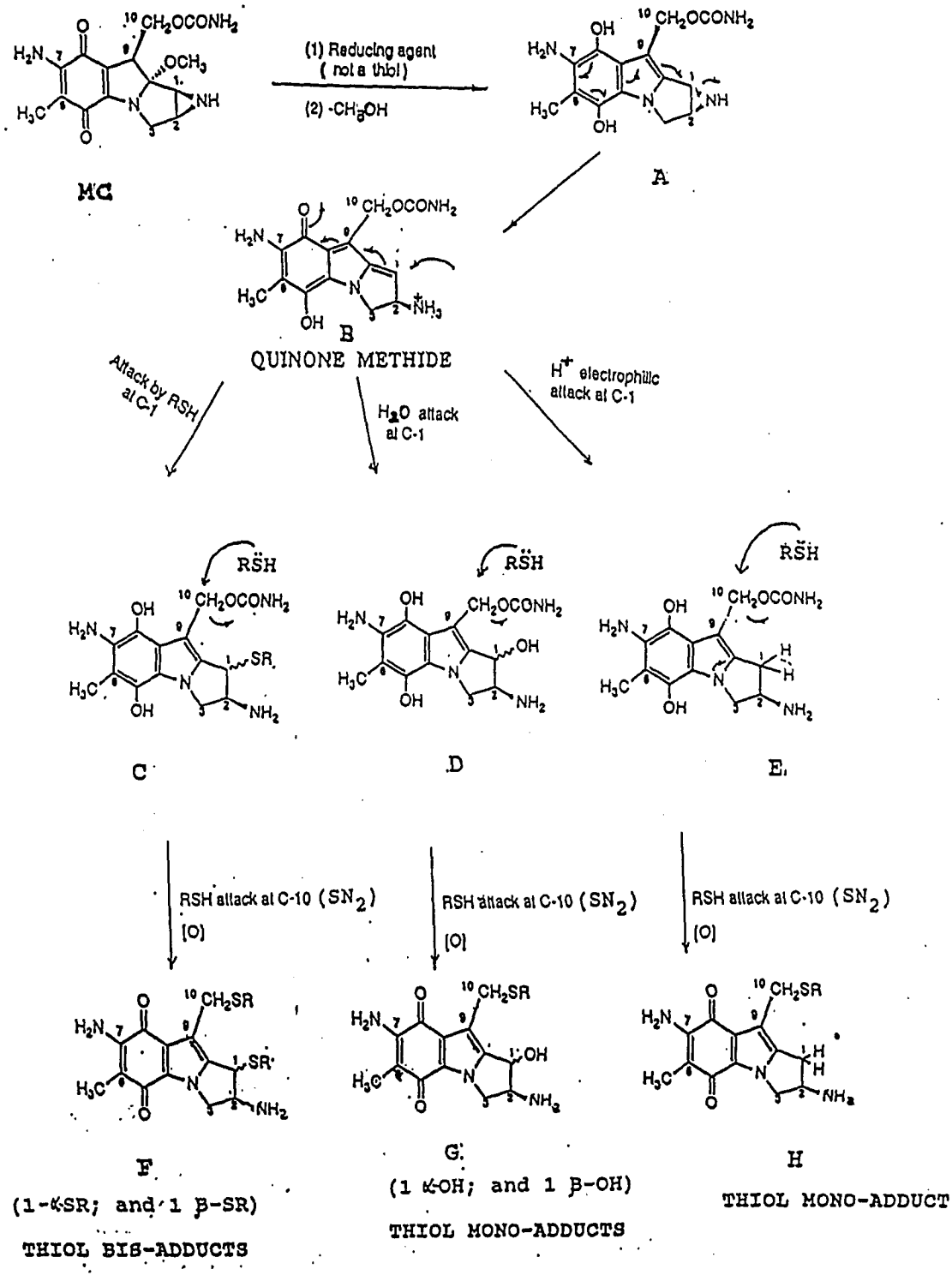

(1) Reducing agent
(not a thiol)
(2) -CH3OH
MC
A
B
QUINONE METHIDE
Attack by RSH at C-1
H2O attack at C-1
H+ electrophilic attack at C-1
C
D
E
RSH attack at C-10 (SN2)
[O]
RSH attack at C-10 (SN2)
[O]
RSH attack at C-10 (SN2)
[O]
F
(1-α-SR; and 1 β-SR)
THIOL BIS-ADDUCTS
G
(1 α-OH; and 1 β-OH)
THIOL MONO-ADDUCTS
H
THIOL MONO-ADDUCT

FIGURE 30

PART III. DNA ALKYLATION BY MC IN PRESENCE OF THIOL NUCLEOPHILES

Introduction

Drugs, acting as covalent DNA-damaging agents, are usually selective in their reactions with DNA with respect to their precise covalent target among the four mononucleotide units of DNA. Reduced MC, for example, is highly selective to guanine N^2 positions. It is increasingly evident, however, that often a higher order of selectivity is imposed by the DNA sequence surrounding the primary target; that is, some target nucleotides are more reactive than others in the same DNA. A wide variety of underlying causes have been proposed, usually unique to the structure and reactivity of the particular drug[48]. It is most likely that such phenomena contribute to the biological mode of action of a drug. In addition, they can reveal new information about the mechanism of the chemical reaction between drug and DNA[10].

It has been reported earlier[72], that MC cross-link formation in an *in vitro* system occurred selectively between two guanines in the CG.CG sequence of a hexanucleotide duplex, as opposed to those in the GC.GC sequence of another hexamer, having otherwise identical sequence, suggesting a general specificity of the cross-link for CG.CG sequences. The generality of this phenomenon was confirmed, showing,

furthermore that the CG.CG sequence is absolutely required for DNA cross-link formation by MC. Superimposed upon the CG.CG requirement , an additional four-base-pair sequence preference was also reported[57].

Our earlier work described in this thesis (part II), suggested that GSH was involved in the overall metabolism of mitomycin C to non-toxic metabolites. We wanted to see whether the presence of GSH could modulate the alkylation of DNA by MC. As an interesting precedent, Guengerich and co-workers[45,50], have reported the formation of mixed GSH-DNA adduct in case of the potent bifunctional alkylating carcinogen 1,2-dibromoethane (ethylene dibromide). Activation of this agent by conjugation with GSH was reported to be necessary for *in vitro* mutation and DNA alkylation. The major DNA adduct is S-[2-(N^7-guanyl) ethyl] GSH, which accounts for >95% of the adducts formed *in vitro* or *in vivo*. Several lines of evidence suggest that the major S-[2-(N^7-guanyl) ethyl] GSH adduct may be mutagenic, at least in bacterial systems, and that the structural alterations of the GSH moeity can have dramatic influences on the mutagenicity of such adducts[50].

It is also possible that GSH competes with DNA alkylation by MC by its "MC-scavenging" action, described in the preceding section (part II). This phenomenon could be involved in the mechanism of MC-resistance in tumor cells which have

elevated levels of GSH and/or glutathione-S-transferase [37,51].

Therefore in this study model reactions were conducted with oligonucleotides and DNA to understand the chemistry of interactions among the components involved in these hypotheses. *In vitro* experiments were conducted , in which MC was reduced by H_2/PtO_2, $Na_2S_2O_4$ and enzymes NADH-cytochrome c reductase and NADPH-cytochrome c reductase, in presence of GSH and oligonucleotides or DNA, to see if mixed bifunctional adduct GS-MC-DNA was formed and how the DNA cross-linking caused by MC would be effected by the presence of GSH. Oligonucleotides possesing a central CG.CG sequence were chosen for this study. *Micrococcus luteus* DNA, also used in this study, contains a lot of CG.CG sequences.

EXPERIMENTAL PROCEDURES

Materials

L-Cysteine, glutathione, cytochrome c reductase, NADH, $Na_2S_2O_4$, and PtO_2 were obtained from Sigma.
Stock solutions of cysteine and glutathione (0.13 M) were prepared by dissolving them in 0.015 M Tris (pH 7.4) and then adjusting the pH to 7.4 with 0.1 N NAOH.

Micrococcus luteus DNA was obtained from ICN Biomedicals, Costa Mesa, CA. DNA was sonicated before use.

The complementary oligonucleotides 8-mer and 9-mer were particularly chosen for this study because the parents and

Oligonucleotides	5′- TIACGTIT -3′	8-mer
	3′- TACTGCACA -5′	9-mer

their MC-modified derivatives can be well separated by HPLC. Oligonucleotides 8-mer and 9-mer were synthesized by a DNA synthesizer, Model 380B, Applied Biosystems, Inc., using the phosphoramidite method. All reagents were purchased from Applied Biosystems, Inc., Foster City, CA. The crude products (1-10 umol scale) were purified by HPLC, both at the "trityl-on" stage and after removal of the trityl group, according to the manufacturers' protocol (Users Bulletin, No.13, Revised, April 1, 1987). The base composition of purified oligonucleotides was routinely checked by nucleotide analysis (as below).

Enzymes used and their sources were as follows: snake venom diesterase (Crotalus adamanteus: phosphodiesterase I), Cooper Biochemicals; Escherichia coli alkaline phosphatase (type III-R), Worthington.

Mitomycin C ("bulk") was supplied by Bristol Laboratories, Syracuse, NY.

METHODS

I. Preparation of cysteine-M-dG mixed adduct (Scheme I, p.102).

M-dG (**2b**; Figure 1, p.101) (0.45 mM), PtO_2 (89 ug/umol M-dG) and L-cysteine (13 mM) were dissolved in Tris 0.015 M, pH 7.4. The mixture was deaerated for 15 minutes. Hydrogen was then bubbled in for 5 minutes, followed by helium again for 5 minutes. Mixture was exposed to air and filtered.

II. Preparation of GS-M-dG mixed adduct (Scheme I, p.102).

Reaction **I** was repeated with glutathione in place of cysteine.

HPLC analysis

HPLC of the above reaction mixtures was performed using reverse-phase column 4.6 x 250 mm (Beckman Ultrasphere ODS). CH_3CN/0.03 M KH_2PO_4, pH 5.0 was used as the eluant. Flow rate was 1 ml/min. Gradient employed for reaction **I** was 3% to 18% over 70 minutes, while for reaction **II**, the gradient was 6% to 18% over 30 minutes.

Identification of the mixed adducts

Identification of the cysteine-M-dG (**4**) and GS-M-dG (**5**) adducts was based on their UV spectra. GS-M-dG was further identified by Electron Spray mass spectroscopy (MW = 814.6).

III. Treatment of synthetic oligonucleotides 8-mer and 9-mer (p.84) with MC under reductive activation conditions, in absence and presence of cysteine.

(a) Reaction in presence of reducing agent H_2/PtO_2.

Reaction in absence of cysteine: A solution of the 8-mer and the 9-mer oligonucleotides (1:1 molar ratio ; 10 A_{260} units), 8.0 mM MC and PtO_2 (89ug/umol MC) in 0.015 M Tris, pH 7.4, buffer was placed in ice and deaerated by purging with argon for 10 minutes. Hydrogen was then bubbled for 45 minutes followed by deaeration again for 5 minutes. Mixture was then exposed to air and filtered.

Reaction in presence of cysteine : The above reaction was repeated in presence of 216 mM cysteine.

(b) Reaction in presence of reducing agent $Na_2S_2O_4$.

Reaction in absence of cysteine: Oligonucleotides (8-mer and 9-mer, 1:1 molar mixture of complementary strands , 10 A_{260} units) were mixed with 0.45 mM MC in 0.015 M Tris buffer, pH 7.4, and the solution was deaerated under helium at 5°C. $Na_2S_2O_4$ (6 umol; 0.124 ml of freshly made anaerobic solution in the same buffer; 0.052 M) was added in 10 ul increments at 10-minute intervals to 12 mM concentration. The reaction was terminated at 60 minutes by exposure to air.

Reaction in presence of cysteine: The above reaction was repeated in the presence of 216 mM cysteine.

(c) Reaction in presence of reducing agent NADH-cytochrome c reductase/NADH.

In absence of cysteine: 10 A_{260} units of oligonucleotides (8-mer and 9-mer, 1:1 molar mixture of complementary strands) were mixed with 8.0 mM MC and 10.2 mM NADH in 0.015

M Tris buffer, pH 7.4. The mixture was deaerated under helium (at 5°C). In a separate flask 3.6 units (approximately 1 unit / umol MC) of enzyme NADH-cytochrome c reductase in a small volume of Tris buffer was also deaerated at 0°C. The enzyme was transferred to the reaction flask after 10 minutes. Reaction was terminated after 60 minutes by exposure to air.

In presence of cysteine: The reaction above was repeated in presence of 216 mM cysteine.

IV. Treatment of synthetic oligonucleotides 8-mer and 9-mer (p.84) with MC under reductive activation conditions in absence and presence of glutathione (Scheme II, p.115).

Reactions **III (a)** to **(c)** were repeated using 216 mM glutathione in place of cysteine.

Sephadex chromatography of reaction mixtures

All reaction mixtures (from **III** and **IV**) were chromatographed over a Sephadex G-25 column with 0.02 M NH_4HCO_3 buffer as eluant. The oligonucleotides and their reaction products eluted in fractions of the void volume, which were pooled and lyophilized.

V. Enzymatic digestion of MC and GSH-oligonucleotide complexes; nucleoside , MC-nucleoside and GS-MC-nucleoside analysis by HPLC.

The mixtures of unreacted oligonucleotides and their reaction products prepared by the method described above were digested to the nucleoside level by the following protocol. (Digestions were carried out on 0.2 to 2.0 A_{260} of oligonucleotides). Snake venom diesterase (SVD) (2 units / A_{260} unit of complex) and alkaline phosphatase (0.78 units / A_{260} unit of complex) were added to the complex, in 0.1 M Tris and 2 mM $MgCl_2$ buffer, pH 8.2, and incubated at 45°C for 4.5 hrs. The resulting nucleosides were analyzed by HPLC.

HPLC for nucleoside and modified nucleoside adduct analysis was carried out by using reverse-phase columns of small pore size (Beckman, ODS Ultrasphere, 4.6 x 250 mm). For peak area quantitation a Beckman Model 427 M integrator was used attached to a Model 265 A absorbance detector (set to 254-nm wavelength), both as parts of a Model 338 HPLC system. In all cases a mixture of 0.03 M KH_2PO_4 buffer, pH 5.0, and acetonitrile was used as eluant in a linear gradient of 3% CH_3CN to 18% over 70 minutes for cysteine-MC-Oligo complexes and 6% to 18% over 30 minutes for GS-MC-Oligo complexes, at flow rate 1 ml/min. Elution times of dC, dI, dG, dT, and dA (with 3% to 18% over 70 min gradient) were 5.2, 9.7, 10.5, 12.7, 17.7 min. **2b**, **2c**, **3**, and **4** (p.101) eluted at 31.0, 25.0, 33.2 and 21.3 minutes respectively. Elution times (with 6% to 18% gradient) were 3.5 (dC), 5.0 (dI & dG), 6.2 (dT) and 8.1 (dA). **2a**, **2b**, **2c**, **3** and **5** eluted at 13.5, 14.5, 11.3, 15.0 and 10.5 min respectively.

Identification of known adducts[2,9,44,52] and nucleosides in the HPLC patterns was based upon direct comparison of elution times with those of authentic standards, chromatographed just before or after running the sample; mixed runs were carried out in cases of ambiguity.

Quantitative analysis of oligonucleotides and cross-linked oligonucleotide-MC and olgonucleotide-MC-thiol complexes was based on absorbance measurements in 0.015 M Tris, pH 7.4 buffer. The molar extinction coefficients E_{260} of single-stranded oligonucleotides were calculated as equal to (number of purines)(14000) + (number of pyrimidines)(7000)[53]. For calculating the E_{260} of MC-modified oligonucleotides, the E_{260} of bound MC was assumed to be 12000[54] and added to E_{260} of the oligonucleotide.

*Molar ratios of nucleosides and MC adducts (***2a, 2b, 2c, 3, 4, and 5***) in enzymatic digests of oligonucleotides alkylated by MC*. Molar ratios of each individual component of the HPLC pattern were calculated by dividing a peak area by E_{254} of the corresponding nucleoside or nucleoside-MC and/or GSH adduct [dC,6300; dG, 13000; dT, 6600; dA, 13300; adducts **2a**, **2b**, **2c**, **4** and **5**, 24000; adduct **3**, 30000[52].

Calculation of mole percent yield of adducts **2a, 2b, 2c, 3, 4,** *and* **5** *per mole of duplex oligonucleotide*. From the molar ratio of dT, above, and the known nucleoside composition of the oligonucleotide, the molar ratio of the original duplex oligonucleotide was calculated as equal to (molar ratio of dT)/(number of dT per duplex oligonucleotide). In turn, mole

% yield of any nucleoside-MC or nucleoside-MC-thiol adduct = (molar ratio of adduct/molar ratio of duplex oligonucleotide) x 100 = (molar ratio of adduct/ molar ratio of dT/number of dT per duplex oligonucleotide) x 100 [55].

VI. Preparation of MC-DNA and GS-MC-DNA complexes under various reductive activating conditions.

(a) 2.86 umol MC / 1 umol DNA ; 27 umol GSH / 1 umol MC.

MC (8 mM), *M. luteus* DNA (2.8 mM mononucleotide units) and 216 mM GSH were reacted the same way as in **III(a) -(c)**. The reactions in presence of reducing agent H_2/PtO_2 and $Na_2S_2O_4$ were conducted at room temperature and the one in presence of cytochrome c reductase at 37°C.

(b) 0.5 umol MC / 1 umol DNA; 3 or 15 umol GSH / umol MC. Reducing agent H_2PtO_2.

Control - in absence of GSH : 0.33 mM MC was mixed with 0.66 mM *M.luteus* DNA and PtO_2 (89 ug / umol MC) in 0.015 M Tris buffer , pH 7.4. Hydrogenation was conducted at room temperature, as in **III (a)**. Reaction mixture was exposed to air and filtered.

In presence of GSH (1 mM and 5 mM) : The above reaction was repeated in presence of 1.0 mM GSH and also in presence of 5.0 mM GSH.

(c) 0.5 umol MC / 1 umol DNA; 3 or 15 umol GSH / umol MC. Reducing agent $Na_2S_2O_4$.

Control-in absence of GSH : A solution of 0.33 mM MC and 0.66 mM DNA in 0.015 M Tris, pH 7.4 was deaerated under helium, at room temperature, for 10 minutes. $Na_2S_2O_4$ (0.5 mM; 40 ul of freshly made anaerobic solution in the same buffer) was added in 10 ul increments at 10-minute intervals. The reaction was terminated at 60 minutes by exposure to air.

In presence of GSH (1 mM and 5mM) : The above reaction was repeated in presence of 1 mM GSH and also in presence of 5 mM GSH.

(d) 0.5 umol MC / 1 umol DNA; 3 or 15 umol GSH / umol MC. Reducing agent NADPH-cytochrome c reductase/NADPH.

Control - in absence of GSH : A solution of 0.33 mM MC, 0.66 mM DNA and 0.40 mM NADPH, in 0.015 M Tris buffer, pH 7.4 was placed in a water bath at 37°C and deaerated under helium, for 10 minutes. In a separate flask NADPH-cytochrome c reductase (1 unit / umol MC) , in a small volume of Tris buffer , was deaerated, at 0°C. After 10 minutes the enzyme was transferred to the reaction flask and the reaction was allowed to proceed for 60 minutes.

In presence of GSH (1 mM and 5 mM) : The above reaction was repeated in presence of 1 mM GSH and also in presence of 5 mM GSH.

Sephadex chromatography

Reaction mixtures were chromatographed over a G-100 column with 0.02 M NH_4HCO_3 buffer as eluant. Complexed and uncomplexed DNA eluted in fractions of the void volume, which were pooled and lyophilized.

Enzymatic digestion of MC-DNA and GS-MC-DNA complexes by DNase I / SVD / Alkaline phosphatase :

MC-DNA and GS-MC-DNA complexes in 0.005 M Tris-HCl/0.001 M $MgCl_2$, pH 7.0 (3 A_{260} units/ml) was digested at 37°C with enzymes according to the following protocol : DNase I (16 units/A_{260} unit) at 0 hr and 1 hr; SVD (1.25 units/A_{260} unit, pH increased to 8.2) at 2 hr and 5 hr; alkaline phosphatase (0.5 unit/A_{260} unit) at 7 hr, incubation continuing until 24 hrs.

HPLC separation

HPLC analysis was conducted as in **V.** Binding ratio (BR) = mol MC bound to dG/mol total dG of DNA was determined as;

$$BR = \frac{\text{area (adducts)}}{\text{area (dG + adducts)}}$$

where dG = deoxyguanosine

and adducts = M-dG (**2b**), M-$(dG)_2$ (**3**), and GS-M-dG (**5**) (p.101,102).

VII. Treatment of duplex oligonucleotides (8-mer + 9-mer) with MC under aerobic $Na_2S_2O_4$ activation (monofunctional

activation) and isolation of monoadducts 6 and 7, on preparative scale.

Oligonucleotides

5′- TIACGTIT -3′ 8-mer

3′- TACTGCACA -5′ 9-mer

Monoadducts

6 = 5′- TIACGTIT -3′
MC

7 = 3′- TACTGCACA -5′
MC

8-mer and 9-mer oligonucleotides (1:1 molar mixture of complementary strands; 104 OD_{260} corresponding to 10.4 umol) were mixed with 8 mM MC in 0.1 M sodium phosphate buffer pH 7.5 (5.2 ml). Reaction flask was placed in ice; exposed to air (no deaeration as reaction was conducted under aerobic conditions). After 10 minutes, a one time addition of 2 mM $Na_2S_2O_4$ (freshly prepared , anaerobically, 0.052 M in the same buffer) was made.

After 1 hr, the reaction mixture was chromatographed over a 5 x 56 cm Sephadex G-50 column with 0.02 M NH_4HCO_3 buffer as eluant. Parent and complexed oligonucleotides eluted in fractions of the void volume which were pooled and lyophilized. Final or standard scale purification was achieved by HPLC (semi preparative on a Rainin C-4 column, 10 x 250 mm). Eluant was a mixture of 0.1 M TEA buffer , pH 7.0 and acetonitrile, in a linear gradient of 4.5% acetonitrile to 15% over 300 minutes. Flow rate was 4 ml/min. Monoadduct of 8-mer (6) eluted at 93 minutes and

that of 9-mer (7) at 108 minutes. The products were characterized by ultraviolet spectra and nucleoside and MC-nucleoside adduct content as described before.

IX. Conversion of monoadducted 8-mer (6) oligonucleotide to GS-MC-Oligonucleotide ternary complex, in absence and presence of complementary (unadducted) strand (9-mer).

By H_2PtO_2 treatment :

(i) *In absence of complementary strand :* Monoadducted oligo **6** (0.7 OD_{260}, 0.035 mM) was mixed with PtO_2 (89 ug/umol bound mitosene[73] and 2.7 mM GSH in 0.1 M Tris, pH 7.4. The mixture was placed in ice, deaerated for 10 minutes, hydrogenated for 40 minutes followed by deaeration again for 10 minutes.

(ii) *In presence of complementary strand :* The above reaction was repeated in presence of 1.4 OD_{260} (0.07 mM) complementary strand (9-mer).

(iii) Both reaction **(i)** and **(ii)** were repeated in absence of GSH to serve as controls.

By $Na_2S_2O_4$ treatment

(iv) *In absence of complementary strand :* A solution of 0.7 OD_{260} (0.035 mM) of **6** was mixed with GSH (2.7 mM) in 0.1 M Tris, pH 7.4; was placed in ice and deaerated for 10 minutes. 0.2 mM of freshly prepared $Na_2S_2O_4$ (0.05 M) in the

same buffer, was added to the mixture. Reaction was terminated after 1 hr by exposure to air.

(v) *In presence of complementary strand* : The above reaction was repeated in presence of 1.4 OD_{260} (0.07 mM) complementary strand (9-mer).

(vi) Both reactions **(iv)** and **(v)** were repeated in absence of GSH to serve as controls.

Sephadex chromatography

All reaction mixtures were chromatographed on a G-25 column. The oligo fractions eluting in the void volume were pooled and lyophilized.

Enzymatic digestion of complexed oligonucleotides and HPLC analysis

Enzymatic digestion with SVD and alkaline phosphatase and HPLC of digests followed the protocol in **V** (linear gradient used was 6% to 18% over 30 minutes). Calculation of mole percent yields also followed the same protocol, single stranded oligonucleotide substituted duplex oligonucleotide where applicable.

RESULTS AND DISCUSSION

Synthesis of authentic ternary thiol-M-dG adducts. The ternary (mixed) adducts **4** (cys-M-dG) and **5** (GS-M-dG) were synthesized (Scheme I; p.102) to serve as authentic standards for the mixed adducts formed in the reactions of oligonucleotides and DNA with MC in presence of GSH (and cysteine). Both were formed essentially quantitatively in the reactions as seen by HPLC of the reaction mixtures (Figure 2, p.103). Their structures were assigned based on (a) their mode of formation in analogy to the simple mitosene-thiol adducts (Figure 24, p.74, part II); (b) their UV spectra, which were identical to UV spectrum of **2b** (Figure 3; p.104) and (c) the mass spectrum in the case of **5** (Figure 4 ; p.105).

Direct formation of ternary thiol-M-dG adducts in oligonucleotides and DNA. In control rections treatment of oligonucleotides (8-mer + 9-mer) with H_2/PtO_2 and cytochrome c reductase , in absence of GSH, gave the well characterized adduct **2b** after enzymatic digestion[55]. In presence of the bifunctional activator $Na_2S_2O_4$, however, **2b** and **3** were obtained (Figure 5; p.106). The percent yield of the adducts is indicated in Table 1 (p.116). A high yield of **2b** is obtained when H_2/PtO_2 was used as the activator. Although no **3** was formed in the H_2/PtO_2 reaction, only 3.7% was formed in enzyme activated and 18.1% in $Na_2S_2O_4$ activated reaction.

Total adduct yield in $Na_2S_2O_4$ reaction was only 28.6% as compared to 72% in H_2/PtO_2 and 42.3% in the enzymatic reactions.

When these reactions were conducted in presence of cysteine, formation of mixed adduct **4** (cys-M-dG) was observed in each case (Figure 6, p.107). The relative yield of **4** and monoadduct **2b** and cross-link adduct **3** is given in Table 1. **2b** is formed only in H_2/PtO_2 reaction but its yield is less than in the comparative control reaction. **3** is formed in both $Na_2S_2O_4$ and enzymatically activated reaction. The yield of **3** with $Na_2S_2O_4$ is more: 23% as compared to 18% in the control. Maximum yield of mixed adduct **4** (28%) was observed in the enzymatic reaction.

In presence of GSH instead of cysteine, a mixed adduct **5** (GS-M-dG) was formed in each reaction (Figure 7, p.108). The relative yields of **2b** and **3** decreased (Table 2). It is interesting to note that although the yield of **3** in cysteine reaction is similar to that in the glutathione reaction, (especially in the enzymatic reaction) that of mixed adduct **5** was less compared to the yield of **4**.

In control reactions with DNA, treatment of a mixture of MC and *M.luteus* DNA in neutral buffer with H_2/PtO_2, $Na_2S_2O_4$ or NADPH/cytochrome c reductase resulted in adduct formation of MC with DNA (Figure 8a,c,e, and 9a,d,g; p.109). In the

presence of high GSH concentration (216 mM) a good yield of mixed adduct **5** was obtained in all reactions (Figure 8). On the other hand no **5** was formed at lower GSH concentrations (1 or 5 mM) (Figure 9; p.110).

Formation of mixed adduct 5 between oligonucleotide-bound MC and GSH (Figure 10 ;p.111). Formation of mixed adduct **5** was observed when the single stranded oligonucleotide-monoadduct i.e. **6**, was treated with glutathione under both H_2/PtO_2 (Figure 11b, p.112), and $Na_2S_2O_4$ (Figure 12b, p.113) activating conditions. Percent yield of **5** in the H_2/PtO_2 reaction was much higher than in the $Na_2S_2O_4$ reaction (42% as compared to 7.7%). When reactions were conducted in presence of complementary strand mixed adduct **5** was not formed; only **2b** and **3** were detected (Figure 11c, 12c). These results are summarised in Table 4 (p.118). The results obtained with oligonucleotide-monoadduct **7** (not shown) were very similar to those with oligonucleotide-monoadduct **6**.

These results together indicate that ternary (mixed) adducts of thiol, MC and dG residues in DNA or oligonucleotides (**4** or **5**) are formed *in vitro* when the thiol is present at high concentration (216 mM) and MC is reductively activated. The mechanism probably involves, as *step (1)*, formation of DNA-monoadduct, e.g. **6** or **7** (p.93,114); *step (2)*, attack of thiol at C-10" of DNA-bound monoadduct which is still in the reduced (activated) state (p.114). This mechanism is based

on the facts that (i) preformed MC-GSH conjugates (**13,14,15**; part II) do not bind to oligonucleotides, under the same conditions (data not shown) and (ii) oligonucleotide-bound **2b** (e.g. **6** or **7**) does get converted to mixed adducts **4** or **5** upon treatment with thiols under reducing conditions (Figure 13; p.114).

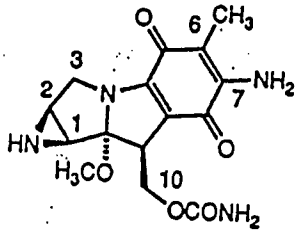
It is notable, that this conversion was best demonstrated in single-stranded oligonucleotides; in the double-stranded state, the conversion to interstrand cross-link (adduct **3**) by internal attack of the guanine-N^2 atom in the opposite strand competes effectively with the external attack by the SH of the thiol. This may be so however, only at the CpG sequence (required for interstrand cross-linking[57]). At TpG, for example, monoadduct **2b** cannot be converted to cross-link; consequently, conversion to mixed adduct should be more favorable. Such experiments were not conducted in the present work, however.

It remains to be seen whether the mixed adduct **5** occurs also in intact cells. The present results indicate that this is chemically feasible and provide the authentic standard to be used in search of adduct **5**. Such type of "mixed adducts" have been detected in intact cells upon treatment with cis-platin[58]. Guengerich and co workers have also observed it with 1,2-dibromoethane[50].

Modulation of the extent of overall binding of MC to DNA in the presence of GSH. This was measured by determining the binding ratio (BR) (mol MC bound/mol DNA mononucleotide) in the MC-DNA complexes formed under various conditions. The UV spectrophotometric method was used[54]. As seen in Table 3 (p.54), when lower concentrations of GSH are used, the data do not show a consistent trend: In some experiments GSH seems to inhibit the binding (BR is lowered); in others, it causes no change, or even an increase in binding. However, with higher concentration of GSH (216 mM), the data do show a consistent trend : BR is lowered in each case. Therefore, it can be concluded that higher concentrations of GSH inhibit the binding of MC to DNA. This is most likely due to the fact there is far more GSH around than DNA-guanine and GSH reacts with activated MC in a competition with DNA.

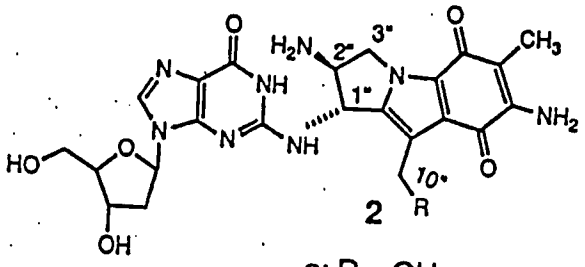
There is no indication that the mixed adduct GS-MC-DNA is mutagenic. However, these results suggest that increases in GSH levels do play a role in resistance, that cells develop towards the antitumor agent mitomycin C.

Figure 1.



1

MC

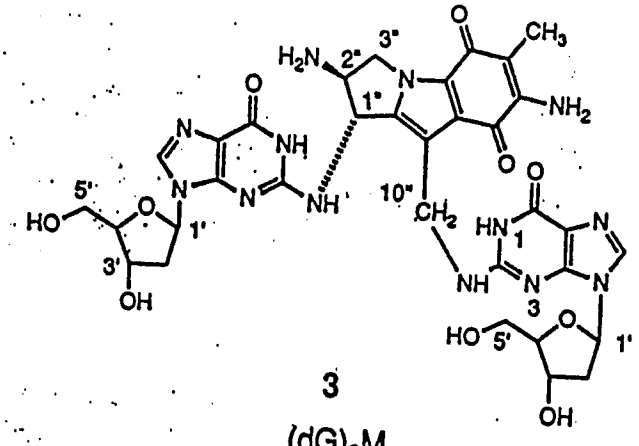


2

a: R = OH

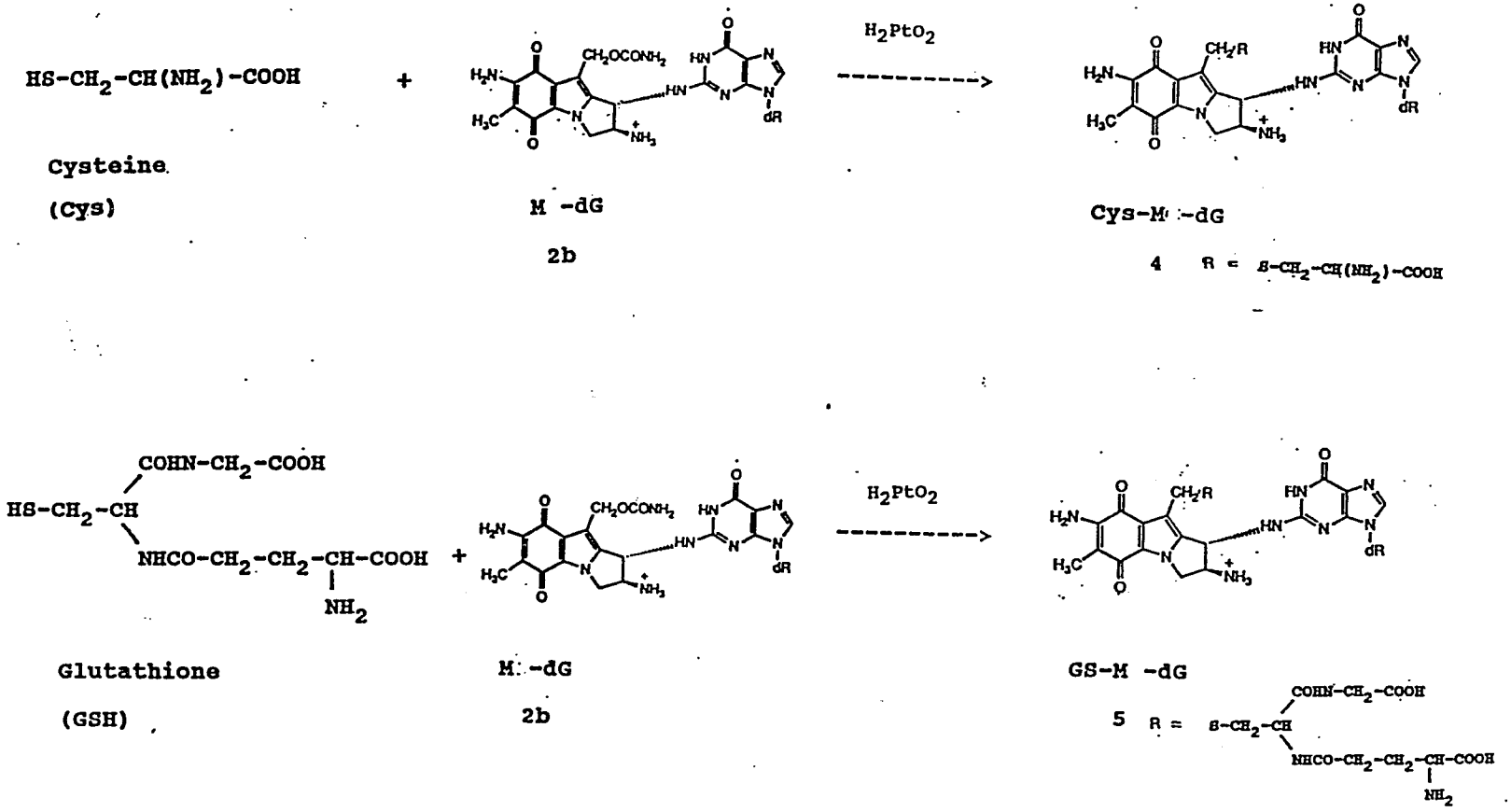
b: R = $OCONH_2$

c: R = HSO_3^-



3

$(dG)_2M$


HS-CH2-CH(NH2)-COOH
Cysteine
(Cys)
M -dG
2b
H2PtO2
Cys-M -dG
4 R = S-CH2-CH(NH2)-COOH
COHN-CH2-COOH
HS-CH2-CH
NHCO-CH2-CH2-CH-COOH
NH2
Glutathione
(GSH)
M -dG
2b
H2PtO2
GS-M -dG
5 R =

SCHEME I.

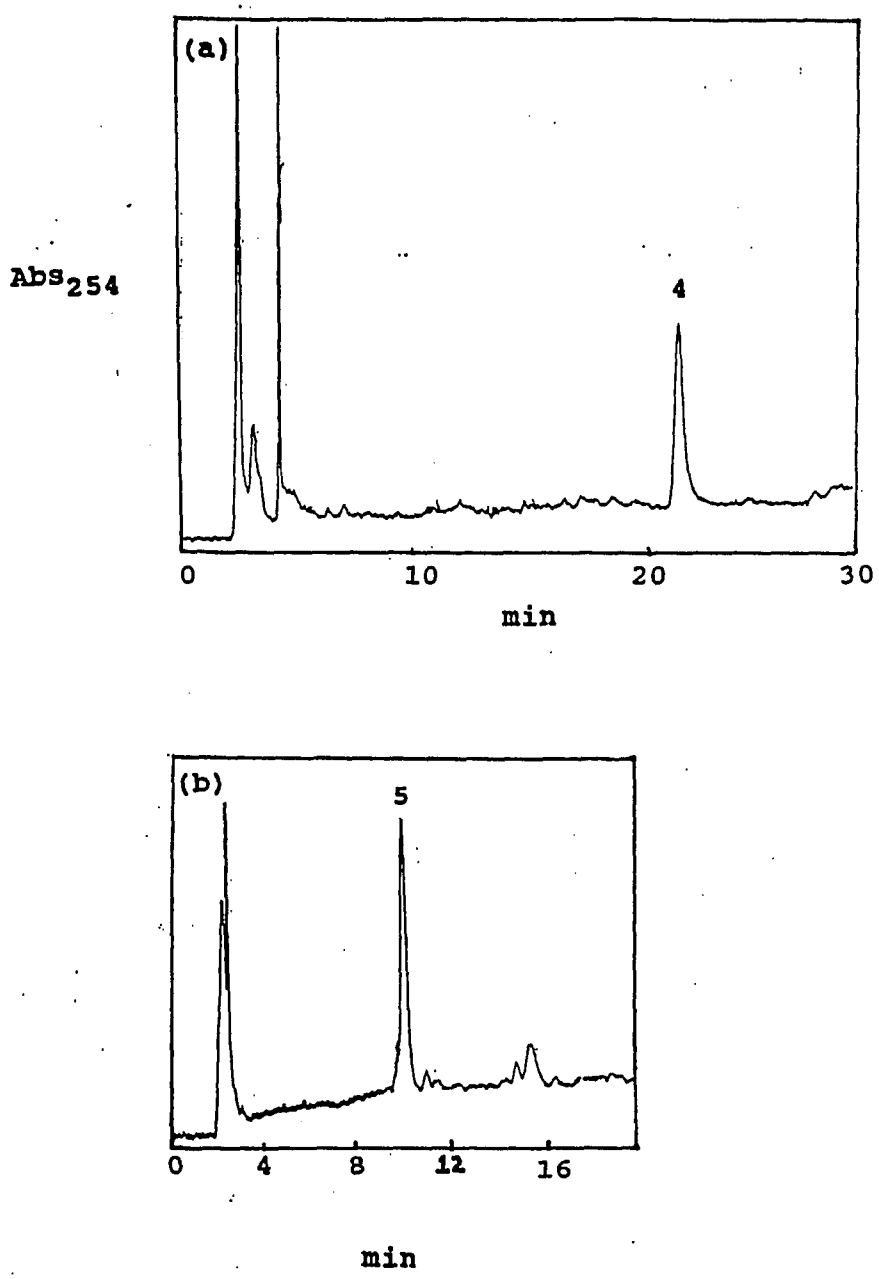


Figure 2. Analytical HPLC of; **(a)** Cys-M-dG **(4)**, linear gradient of CH_3CN/0.03 M KH_2PO_4, pH 5.0, 6% to 18% over 70 minutes. **(b)** GS-M-dG **(5)**, linear gradient of CH_3CN/0.03 M KH_2PO_4, pH 5.0, 6% to 18% over 30 minutes.

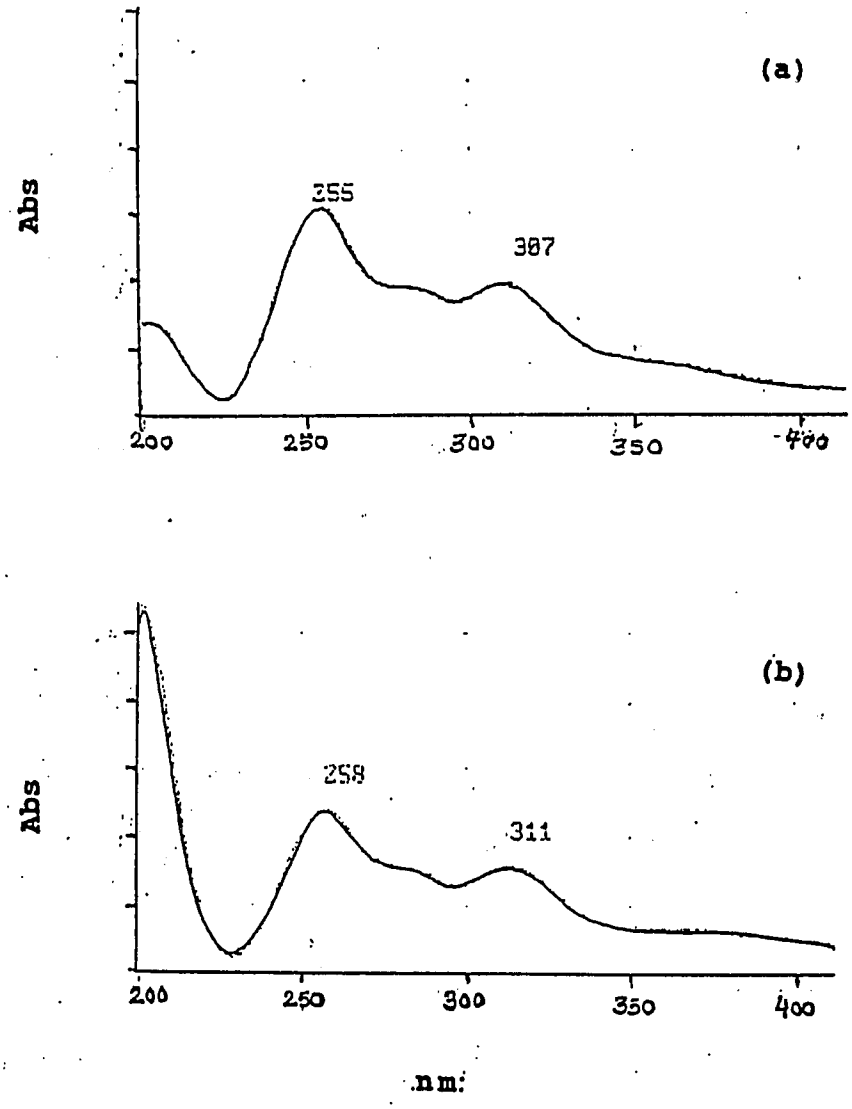


Figure 3. UV of (a) M-dG and (b) GS-M-dG in water.

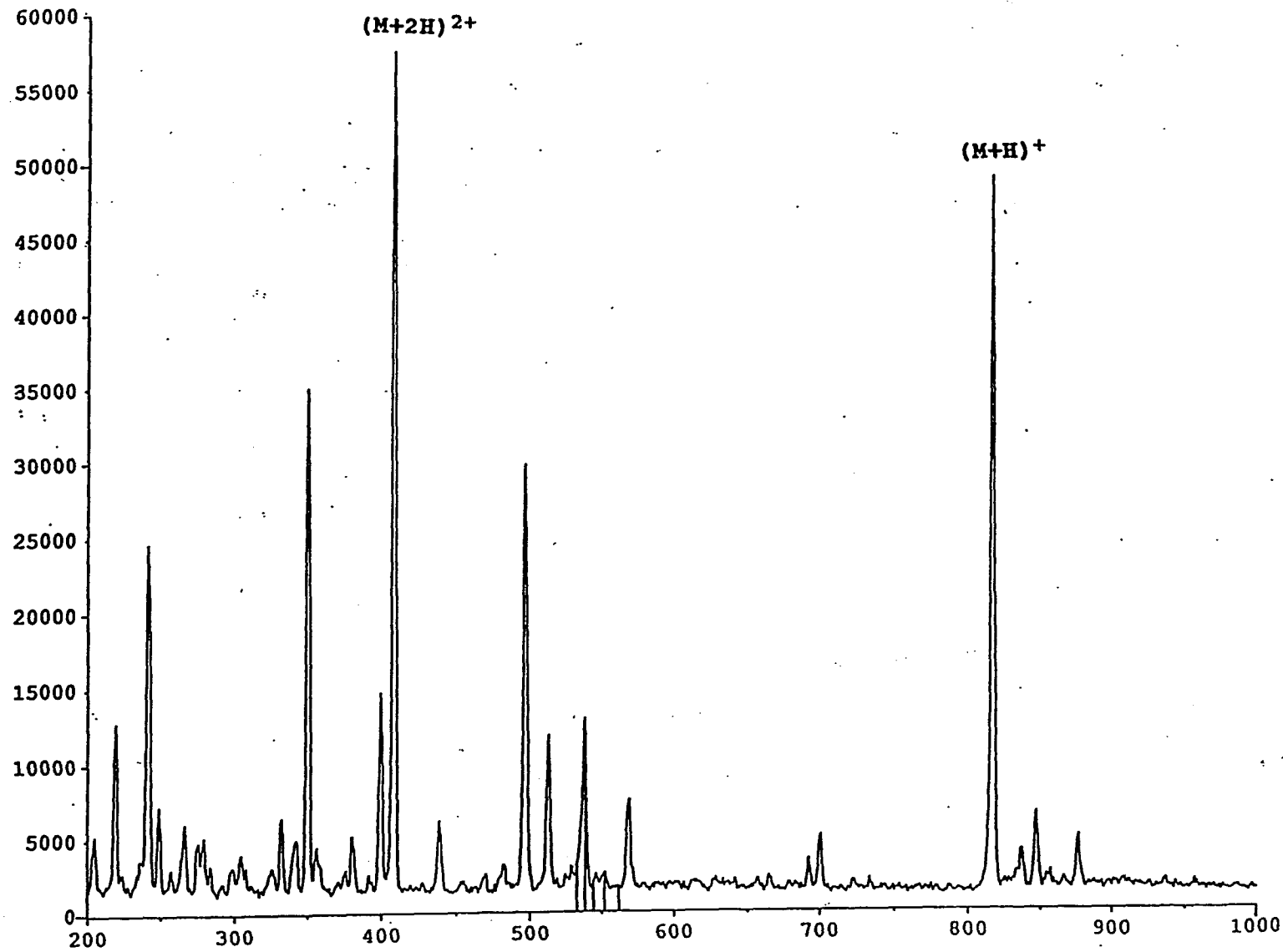


Figure 4. Electron spray mass spectrum of GS-M-dG (5)

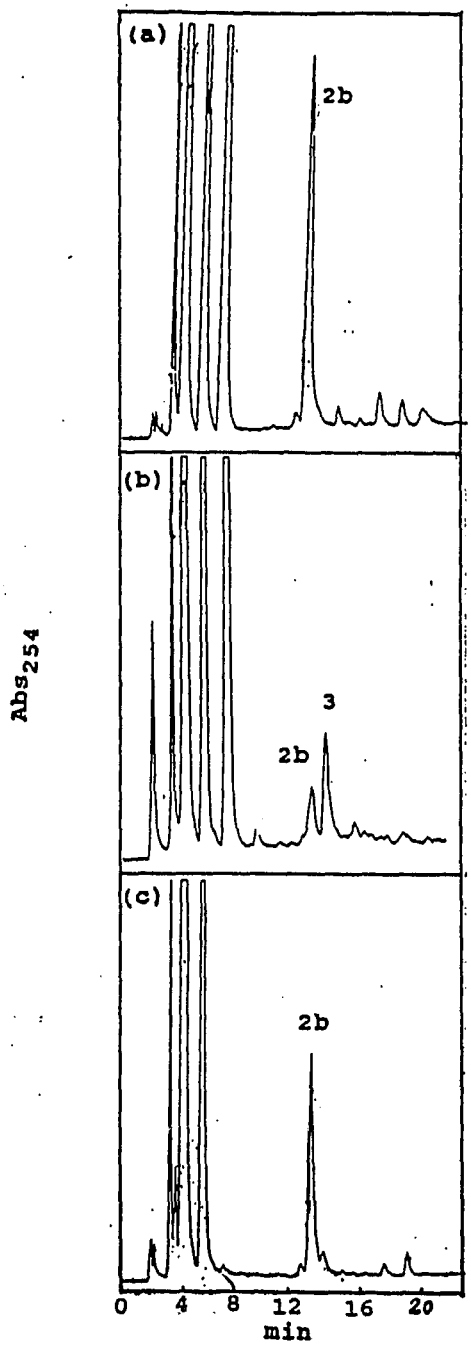


Figure 5. Analytical HPLC analysis of the digestion of the MC-Oligo complexes formed under;

(a) H_2/PtO_2 reduction conditions

(b) $Na_2S_2O_4$ reduction conditions

(c) Enzymatic (cytochrome c reductase / NADH) reduction conditions.

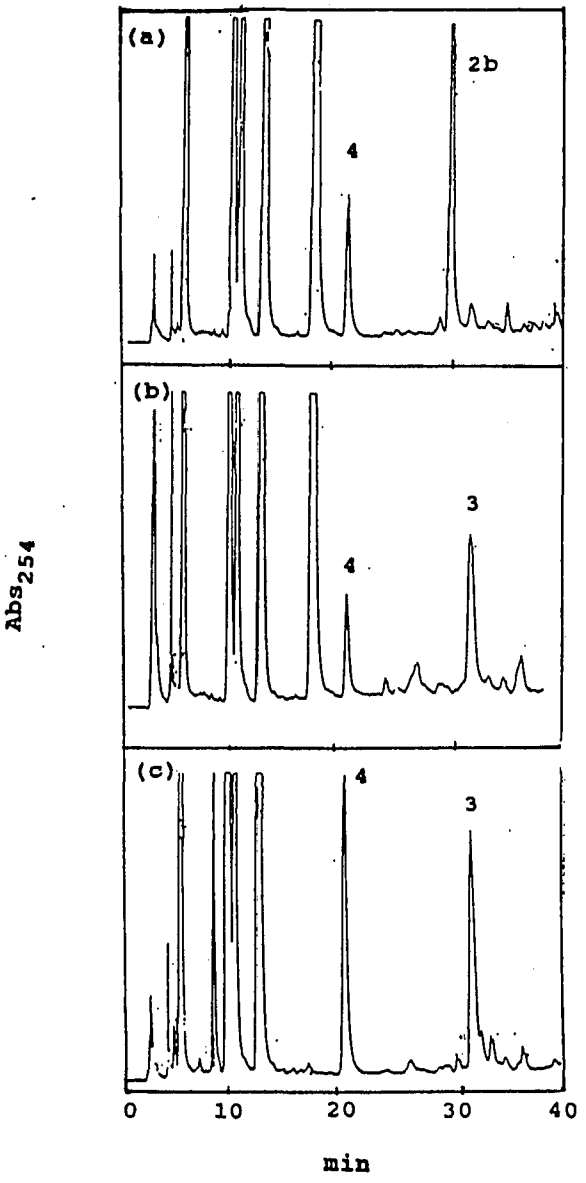


Figure 6. Analytical HPLC analysis of the digestion of Cys-MC-Oligo complexes formed in the presence of 216 mM cysteine under;

(a) H_2/PtO_2 reduction conditions

(b) $Na_2S_2O_4$ reduction conditions

(c) Enzymatic (cytochrome c reductase / NADH) reduction conditions (dA deaminated to dI during reduction).

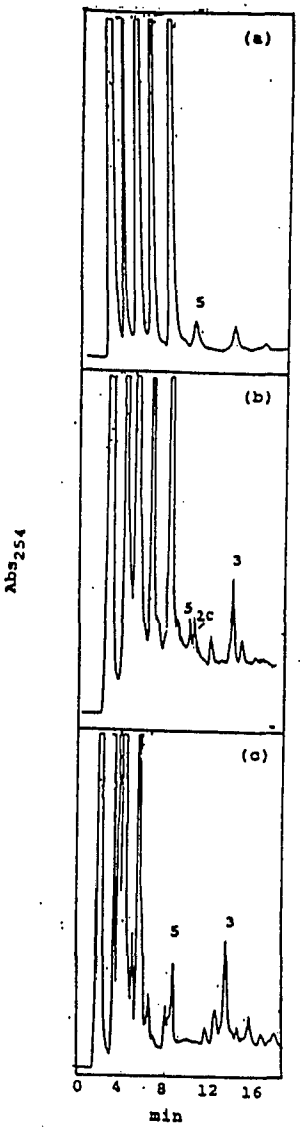


Figure 7. Analytical HPLC analysis of the digestion of GS-MC-Oligo complexes formed in the presence of 216 mM GSH under;

(a) H_2/PtO_2 reduction conditions

(b) $Na_2S_2O_4$ reduction conditions

(c) Enzymatic (cytochrome c reductase / NADH) reduction conditions (dA deaminated to dI during reduction).

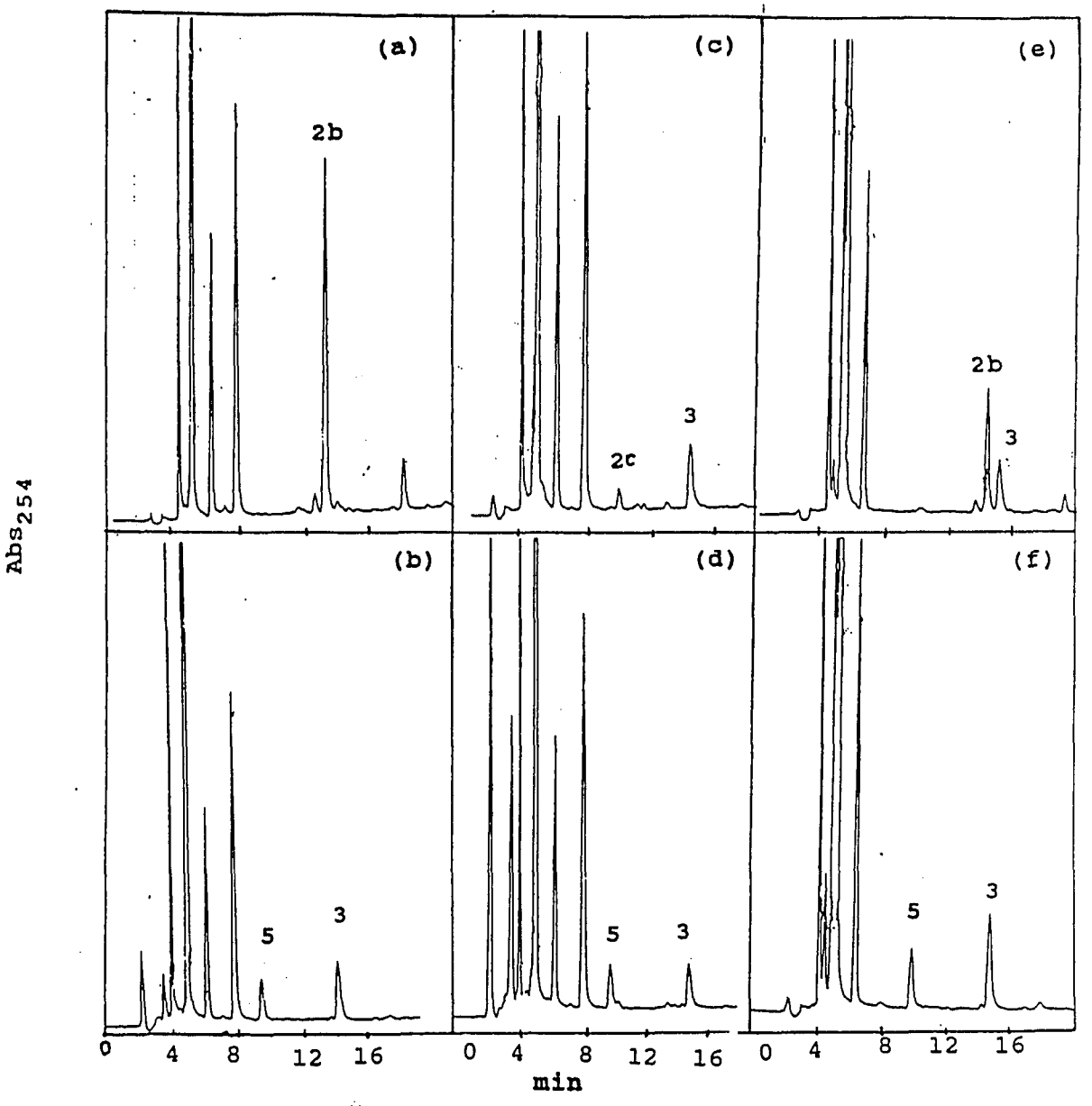


Figure 8. Analytical HPLC analysis of the digestion of the MC-DNA and GS-MC-DNA complexes from the reactions conducted at 8 mM MC, 2.86 mM DNA and 216 mM GSH formed under **(a)** H_2/PtO_2 reduction conditions in absence of GSH; **(b)** same, in presence of GSH; **(c)** $Na_2S_2O_4$ reduction conditions in absence of GSH; **(d)** same, in presence of GSH; **(e)** Enzymatic (cytochrome c reductase / NADH) reduction conditions in absence of GSH; **(f)** same, in presence of GSH.

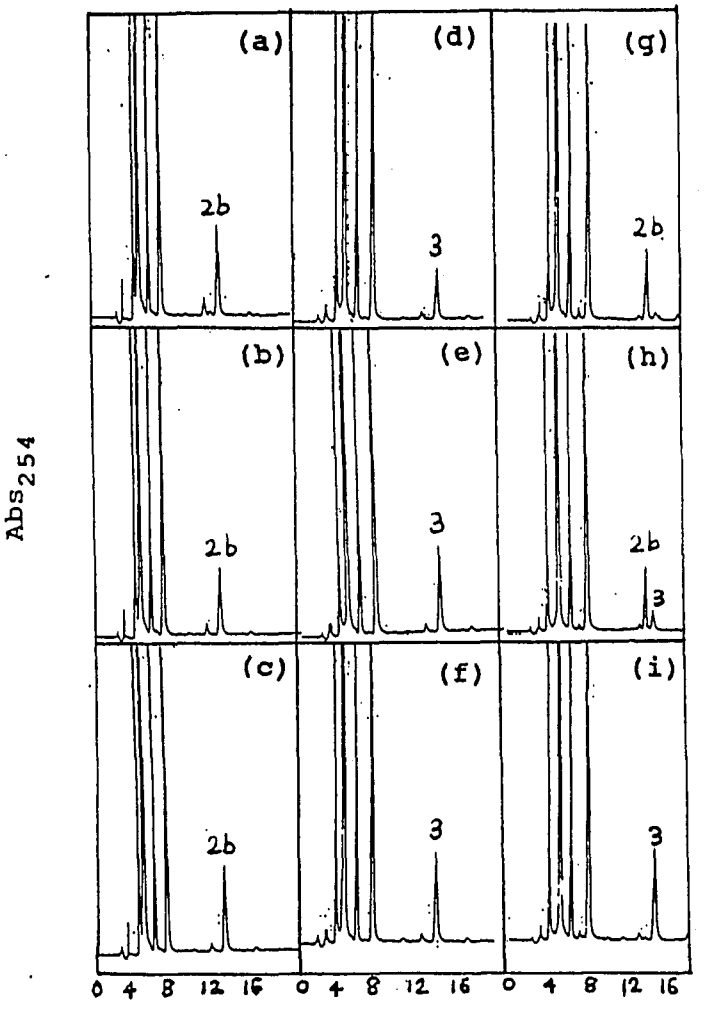
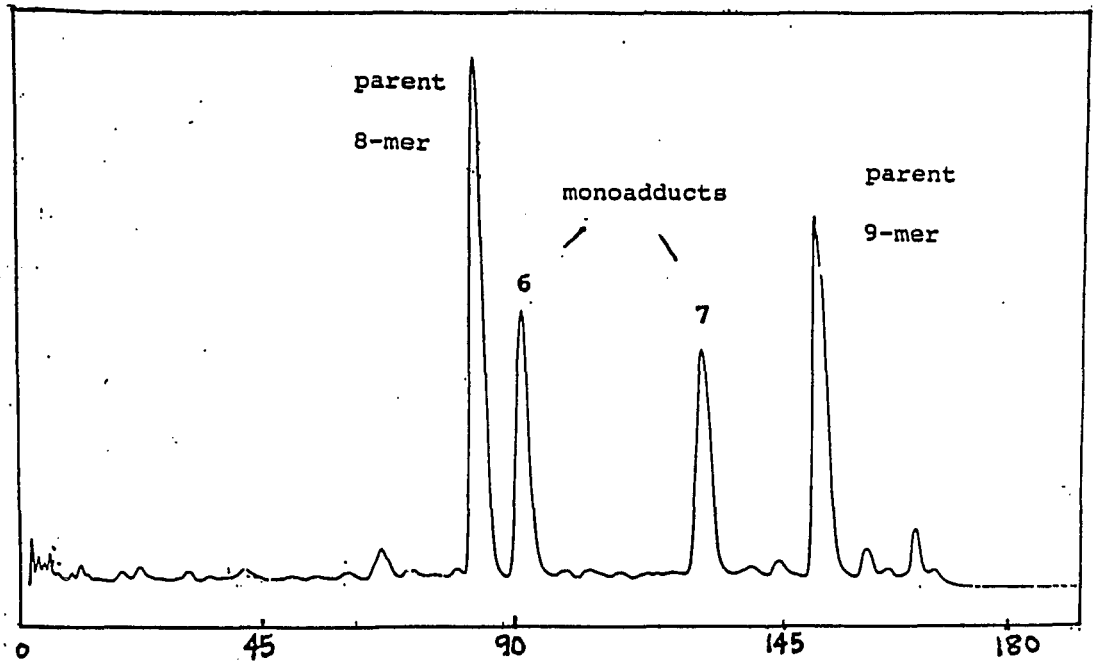


Figure 9. Analytical HPLC analysis of the digestion of the MC-DNA and complexes from the reactions conducted at 0.33 mM MC, 0.66 mM DNA and 1 or 5 mM GSH. **(a)** H_2/PtO_2 reduction conditions in absence of GSH; **(b)** same, in presence of 1 mM GSH; **(c)** in presence of 5 mM GSH. **(d)** $Na_2S_2O_4$ reduction conditions in absence of GSH; **(e)** in presence of 1 mM GSH; **(f)** in presence of 5 mM GSH. **(g)** Enzymatic (NADH-cytochrome c reductase/NADH) reduction conditions in absence of GSH; **(f)** in presence of 1 mM GSH and **(i)** in presence of 5 mM GSH.



Oligonucleotides

5′- TIACGTIT -3′ 8-mer

3′- TACTGCACA -5′ 9-mer

Monoadducts

6 = 5′- TIACGTIT -3′
M

7 = 3′- TACTGCACA -5′
M

Figure 10. Semi-preparative HPLC of aerobic reaction of 8-mer and 9-mer oligonucleotides with MC (reducing agent $Na_2S_2O_4$) resulting in the formation of monoadducts **6** and **7**.

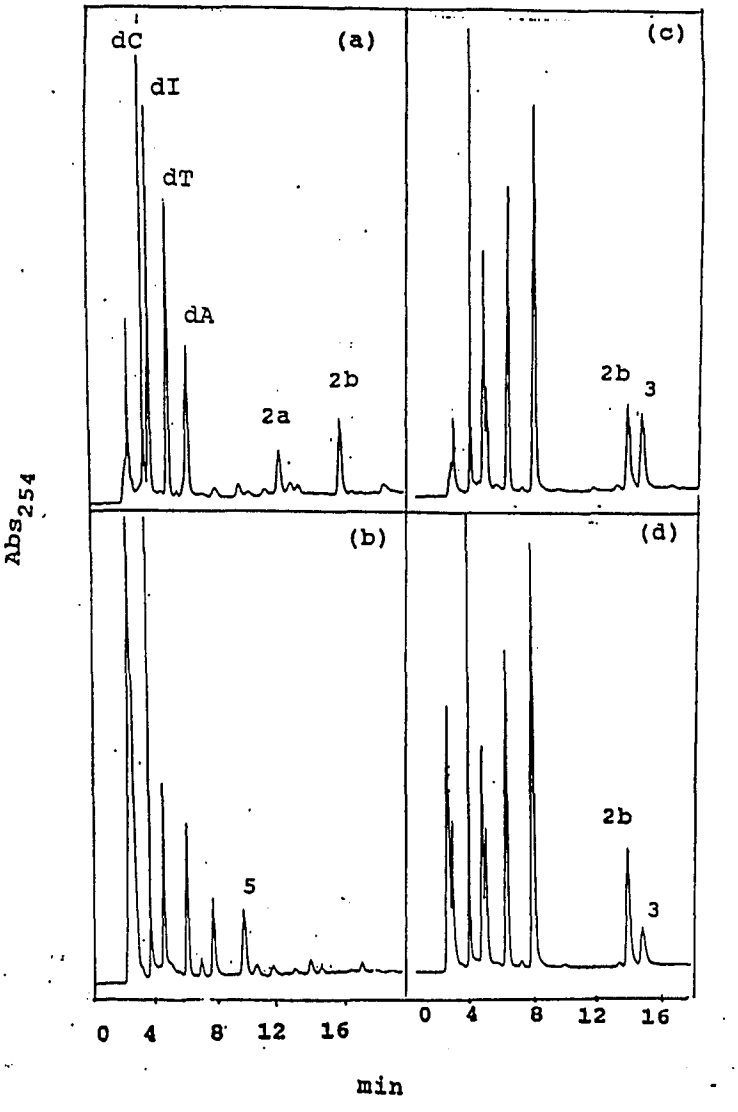


Figure 11. Analytical HPLC analysis of the digestion of the oligonucleotide-monoadduct **6** in absence and presence of GSH and complementary strand (9-mer) ;

(a) monoadduct + H_2/PtO_2

(b) monoadduct + H_2/PtO_2 + GSH

(c) monoadduct + complementary strand + H_2/PtO_2

(d) monoadduct + complementary strand + H_2/PtO_2 + GSH

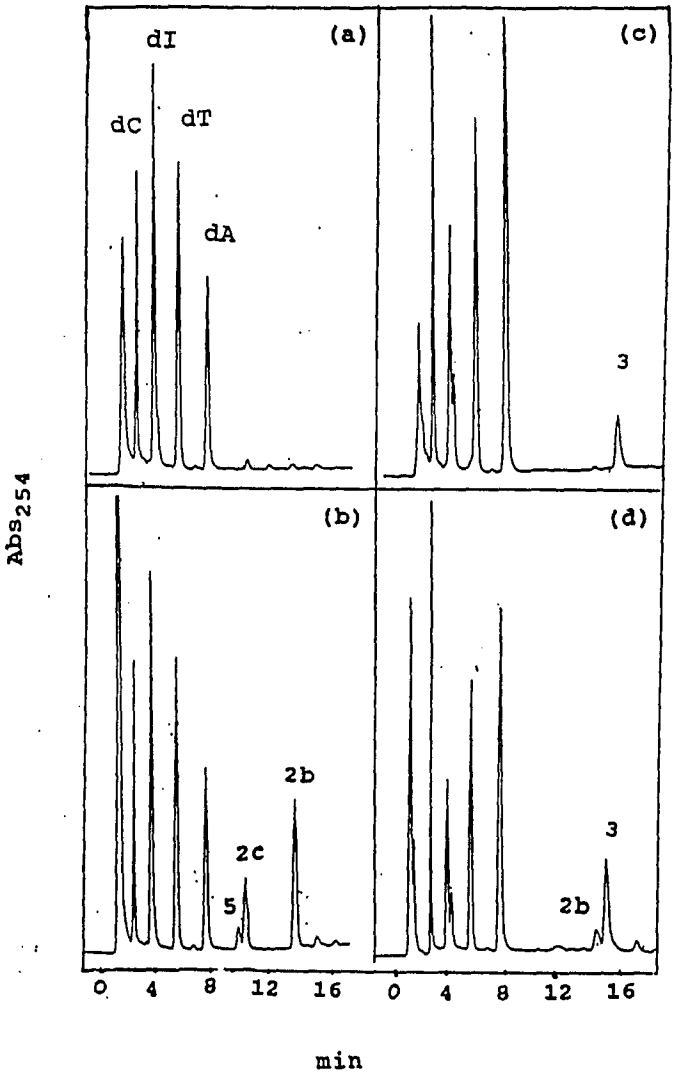


Figure 12. Analytical HPLC analysis of the digestion of the oligonucleotide-monoadduct **6** in absence and presence of GSH and complementary strand (9-mer) ;

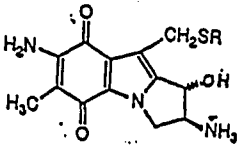
(a) monoadduct + $Na_2S_2O_4$

(b) monoadduct + $Na_2S_2O_4$ + GSH

(c) monoadduct + complementary strand + $Na_2S_2O_4$

(d) monoadduct + complementary strand + $Na_2S_2O_4$ + GSH

Figure 13.



13 + oligonucleotide —red. agent—✕→ 5

R = $-CH_2CH(CONHCH_2COOH)NHCOCH_2CH_2CH(NH_2)COOH$

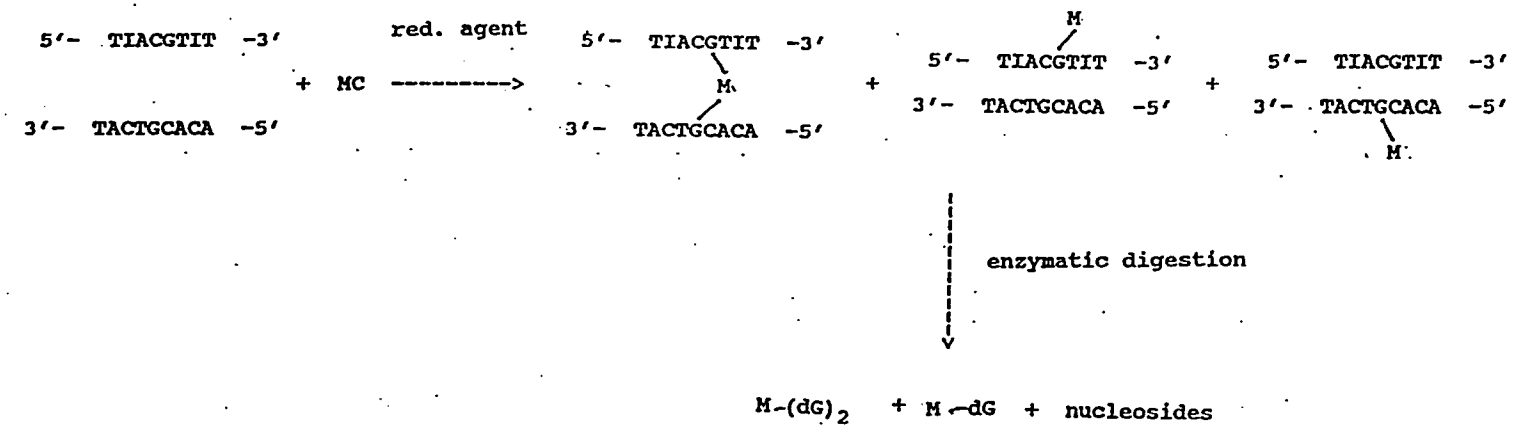
GSH
red. agent

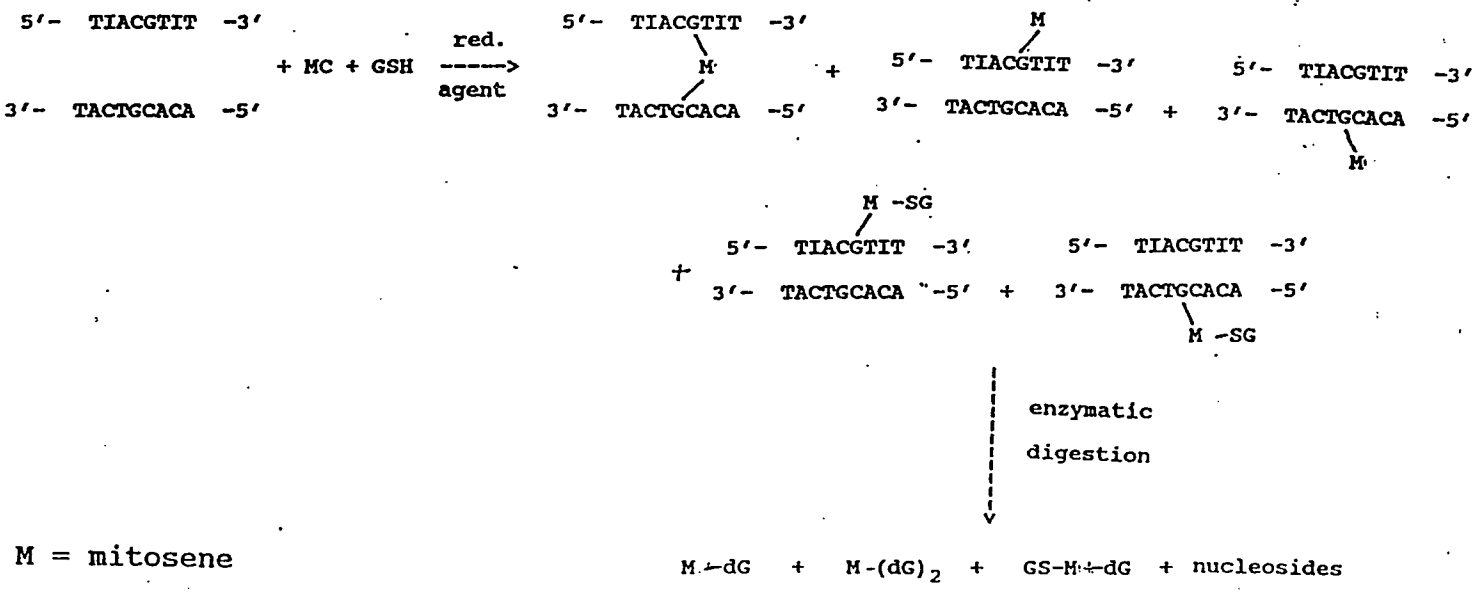
5′- TIACGTIT -3′ ----------> 5

M

6

SCHEME II.





M = mitosene

TABLE 1.

REACTION	% YIELD (-) CYS			% YIELD (+) CYS			
	2a	2b	3	4	2a	2b	3
Oligo/MC/H_2/PtO_2		72%		13.3%		37%	
Oligo/MC/$Na_2S_2O_4$		7.7%	18%	10.4%			23%
Oligo/MC/Enzyme		42%	3.7%	28.4%			24%

TABLE 2.

REACTION	% YIELD (-) GSH			% YIELD (+) GSH			
	2a	2b	3	5	2a	2b	3
Oligo/MC/H_2/PtO_2		72%		10.0%	7.1%		
Oligo/MC/$Na_2S_2O_4$		7.7%	18%	2.0%	2.0%		3.5%
Oligo/MC/Enzyme		42%	3.7%	18.0%			28%

TABLE 3. Binding ratios (BR) determined in the reactions of MC with DNA in absence and presence of GSH.

	0.33 mM MC (MC:DNA = 1:2) (Fig.6)			8 mM MC (MC:DNA = 1:2) (Fig.5)	
RED.AGENT	-GSH	+GSH		-GSH	+GSH
		1 mM	5 mM		216 mM
H_2/PtO_2	0.106	0.078	0.095	0.364	0.129
$Na_2S_2O_4$	0.050	0.090	0.088	0.102	0.079
Enzyme	0.087	0.080	0.109	0.154	0.090

TABLE 4.

REACTION	% YIELD - GSH					% YIELD + GSH				
	-compl.			+compl.		-compl.			+compl.	
	2a	2b	3	2b	3	5	2a	2c	2b	3
Mono/H_2/PtO_2	19.5	-	-	53.2	45.5	42.3	-	-	78	22
Mono/$Na_2S_2O_4$	-	-	-	-	25	7.7	24.3	52	21	66

Mono = monoadduct 6 , compl. = complementary strand

PART IV. ALKYLATION OF PROTEINS BY MITOMYCIN C (PRELIMINARY REPORT).

Introduction

(a) MC AND METALLOTHIONEIN

Resistance to antineoplastic agents is the major obstacle to curative therapy of cancer. Increased levels of cellular constituents containing sulfhydryl groups may interact with the drug and prevent its binding to target DNA. Protection against the cytotoxicity induced by alkylating agents has been demonstrated in animals by pretreatment with thiol compounds with high affinity for alkylating agents, e.g. cysteine[59].

Among the cellular components which recently have been associated with such detoxification mechanisms is a group of small molecular weight (MW 6000 to 7000) soluble cytoplasmic proteins known as metallothioneins (MT's). They are extremely rich in sulfhydryl groups (cysteine accounting for approximately 25 to 30% of the amino acid residues) and show no disulfide linkages[59].

One biological function of MT's is apparently their involvement in the cellular metabolism and detoxification of metals. Cultured cells can increase their resistance to cadmium with simultaneous synthesis of cytoplasmic MT[60]. A high degree of resistance against the anticancer drug cis-

dichlorodiamine platinum, an alkylator-like agent has been demonstrated in MT-containing cells[61]. In the resistant cells, a significant fraction of the cytosolic platinum was bound to MT; however, it could not be determined whether the platinum sequestered by MT only represented metal derived from the compound or the intact platinum complex[61].

Further, investigation was conducted to examine whether enhanced level MT-containing cells also show resistance against an alkylating agent containing no metal and if MT in the cytoplasm of these cells has the capacity to bind the drug or its metabolites. For this purpose, chlorambucil, a nitrogen mustard derivative widely used in the treatment of various malignant diseases, was incubated with cells containing elevated levels of cytoplasmic metallothionein. Following 1- and 24-hr incubations of cells with [^{14}C] chlorambucil and subsequent ultracentrifugation and gel filtration of the cytosols, about 20 to 40% of the ^{14}C-derived radioactivity coeluted with the metallothionein from the resistant strains. The results indicated that intracellular MT sequesters chlorambucil or its toxic metabolites and that the protein thus contributes to the resistance against this drug[59].

The aim of the present investigation was to determine whether mitomycin C (Figure 1a, p.133), an alkylating agent like chlorambucil, would also bind metallothionein (Figure

2,p.134). Mitomycin C alkylates glutathione. Metallothionein maybe similar to glutathione in providing a nucleophilic sink, and binding of mitomycin C to MT may represent an additional detoxification mechanism for mitomycin C. In addition to MC , interaction of mitomycin A (MA; the most potent analog belonging to the family of mitomycins, Figure **1b**) was also studied with MT.

(b). MC AND PROTEIN KINASE C

The enzyme family protein kinase C (PKC) occupies a central role in the transduction of signals from a variety of mediators across the cell membrane. Receptor occupation by a number of hormones, cytokines, neurotransmitters, and growth factors results in activation of PKC via activation of phospholipase C through either a G protein mechanism or a tyrosine kinase mechanism. PKC then propagates the signal by phosphorylation of proteins on serine or threonine, with ATP as cosubstrate, resulting in modification of the properties of these proteins. Thus PKC appears to regulate mechanisms of cell proliferation, secretion and gene expression. Inhibition of PKC may provide therapy for diseases such as rheumatoid arthritis (through inhibition of T-cell proliferation), cancer, and AIDS (through inhibition of viral entry and viral gene expression)[62].

The activation of PKC by its cofactors, calcium and diacylglycerol, most likely occurs as the enzyme transiently

and weakly associates with membrane phospholipids. This membrane bound form is susceptible to proteolysis, by calpain or other enzymes, to give a cytosolic form (PKM) which is fully active in the absence of diacylglycerol or phospholipid. The physiological role of PKM is unknown; however, if both active forms of the enzyme (PKC and PKM) are to be inhibited, either the protein sustrate site or the ATP binding site should be blocked[62].

Dequalinium[63], staurosporine[64] and a series of maleimides are the known inhibitors of PKC[62]. Maleimides are DNA alkylating agents like MC. This provided the lead to explore the possibility that MC (**1a**) or MA (**1b**) are inhibitors of protein kinase C (Figure 3, p.135).

EXPERIMENTAL SECTION

MATERIALS

Metallothionein (from rabbit liver, mixture of MT I and MT II, containing 7% cadmium and zinc), and xanthine oxidase, were from Sigma Chemicals. NADH was from Boehringer. Dialysis membrane (MW cut-off 3000 and 1000) was purchased from Spectrum. MC was a generous gift from Dr. Vyas of Bristol-Myers-Squibb Co., Wallingford, CT. Mitomycin A (MA) was synthesized from MC by a published procedure[38] and was provided by Dr. Shiv Kumar of this lab.

Dialysis of MT to remove metals[65] : Metallothionein (15 mg) was dissolved in 15 ml of 0.05 N HCl and dialyzed against 200-fold volume of 0.05 N HCl with one change of HCl, transferred into 0.015 M Tris buffer pH 7.4 and allowed to equilibrate overnight. UV of MT after dialysis indicated loss of metals (indicated by loss of absorbance at 280 nm). Protein concentration of dialyzed MT was determined by the Bradford method[40] and was found to be 0.337 mg/ml. Molar concentration of -SH in dialyzed MT was found to be 0.68 umol/ml by the DTNB assay[66].

Protein kinase C was isolated from C3H10T1/2-PKC-4 cells by the method of Rotenberg et al.[63] and was provided by Dr. S Rotenberg. Protein content was determined by the Bio-Rad protein assay[40] with lysozyme as the standard. Crude preparation of PKC-Bi (0.3 ug/ml) had specific activity 6-17 units/mg, where 1 unit is 1 nmol $^{32}P_i$ transferred to substrate per minute.
[γ^{32}P]ATP (0.5-3 Ci/mmol) was bought from Amersham, histone III-s and phosphatidylserine were purchased from Sigma.
The MC derivative BMY-25282, was provided by Dr. Vyas, Bristol-Myers-Squibb Co., Wallingford, CT.

Protein kinase C assay. PKC activity was taken as the difference in the amount of $^{32}P_i$ transferred from [γ^{32}P]ATP to histone III-S (15, 21 or 42 uM) (average MW 16,000), in the presence and absence of phosphatidylserine (PS). The

reaction medium (0.12 ml), placed in 100-mm disposable glass test tubes, consisted of 20 mM Tris, pH 7.5, 10 mm $MgCl_2$, 1 mM $CaCl_2$, and the following components were added in order : 0.1 mg/ml histone III-S, 10 ug PS or 10 ul H_2O, 10 ul enzyme (3.8 ug), and 10 ul of test compound. For each inhibitor concentration, duplicate sets of tubes were prepared with and without phospholipid. PS working solutions were prepared by drying, the stock PS (dissolved in chloroform), using N_2, adding 1 ml sterilized, deionized H_2O, and sonicating with three 10 s bursts with a sonifier cell disrupter. Following the addition of test compound. The kinase reaction was initiated by the addition of 73 uM [γ^{32}P]ATP (approximately 200 cpm/pmol) to each tube, which was immediately transferred to a 30^{o}C waterbath for 10 min. The reaction was quenched by transferring 40 ul of the reaction medium to a 3 x 3 cm square of phosphocellulose paper and immersing it in a 1.0 liter beaker of tap water. After terminating all reactions,the squares were washed 5 times with 1 L of water. Finally, each square was placed in a scintillation vial containing 7 ml Hydrofluor and was measured for ^{32}P content. The results for each duplicate set of tubes were averaged. The difference between the (+) PS and (-) PS tubes was judged to be Ca^{2+}/PS dependent activity. The presence of PS typically stimulated the kinase reaction 6- to 10-fold with the histone III S substrate.

METHODS

Metallothionein experiments.

SET I : Reactions conducted in this set utilized a molar equivalent ratio MT-SH:MC = 0.01:1 ; reaction volume = 1 ml; incubation for 30 min at 37°C.

(i) *Control : Incubation of MT at 37°C.*

MT (0.01 mM in -SH) in 0.015 M Tris buffer, pH 7.4 was incubated at 37°C for 30 min, under anaerobic conditions (deaeration with helium).

(ii) *Control : Incubation of xanthine oxidase/NADH.*

NADH (10 mM) was dissolved in 0.015 M Tris, pH 7.4, placed at 37°C and deaerated for 10 minutes, when xanthine oxidase (5 units/ml; dissolved in a small amount of the same buffer) was added to the solution at 37°C. Incubation under anaerobic conditions was continued until 30 min.

(iii) *Incubation of MT with xanthine oxidase/NADH*

MT (0.01 mM -SH) was mixed with 10 mM NADH in Tris buffer as above and deaerated at 37°C. Xanthine oxidase (5 units/ml) was added at 10 min and incubation continued for 20 min.

(iv) *Incubation of MC with MT*

MT (0.01 mM -SH) was mixed with 10 mM MC in Tris buffer and incubated as in **(i)**.

(v) *Incubation of MC with xanthine oxidase/NADH*

Same as in (ii) except MC (10 mM) was also included.

(vi) *Incubation of MT with MC and xanthine oxidase/NADH*

Reaction (v) was repeated with addition of 0.01 mM MT-SH.

(vii) *Incubation of MT with MA (mitomycin A)*

MT (0.01 mM -SH) was mixed with 10 mM MA in Tris buffer. Incubation proceded as in (i).

(viii) *Incubation of MA with xanthine oxidase/NADH*

Reaction (v) was repeated using MA in place of MC.

SET II. Same as in set I except that reactions in set II involved molar ratio MT-SH:MC = 0.01:100 using 0.002 mM MT-SH and 20 mM MC. Reaction volume was 5 ml; incubation for 1 hr at 37°C.

All reactions were conducted in duplicate in <u>both</u> sets and the results are reported as the average of the two measurements.

Sephadex chromatography

All reaction mixtures were chromatographed on G-25 (fine) column. Eluant was 0.02 M NH_4HCO_3. MT and xanthine oxidase elutes in the void volume. Void volume fractions were pooled and concentrated.

Dialysis

Metallothionein and xanthine oxidase fractions were dialyzed overnight in 200-fold their volume 0.02 M NH_4HCO_3.

Determination of binding of drug to protein by spectrophotometery.

UV of metallothionein and xanthine oxidase fractions were determined before and after dialysis on a Varian cary-3

spectrophotometer. Absorbance at 310 nm (lambda max. for reduced mitomycin C, i.e. 7-aminomitosenes) and 285 nm (lambda max. for reduced MA i.e. apomitomycin A or 7-methoxy mitosenes) was determined and the total absorbance (A x ml volume) at these wavelengths was calculated in each case. MT has no absorbance max. at 310 or 285 nm. Binding of MC or MA to MT and xanthine oxidase is indicated by increase in absorbance at 310 nm and 285 nm, respectively, calculated by subtracting absorbance of appropriate control "reaction mixtures". (Table 1 shows the actual data; Table 2 shows the calculated results.)

PKC experiments

(i) *Control : Incubation of protein kinase C in Tris buffer in the absence of mitomycins.*

A 50 ul solution of PKC (6-17 units/mg) in buffer A (20 mM Tris pH 7.5, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, 10 ug/ml leupeptin, 10 ug/ml soybean trypsin inhibitor) was dissolved in 20 mM Tris pH 7.5 to a total volume of 0.5 ml; placed in ice and deaerated (under helium) for 1 hr.

(ii) *Incubation of PKC with mitomycins.* The above incubation was repeated with 4.5 mM MC.

(iii) *Incubation* (i) was repeated in presence of 4.5 mM BMY 25282.

(iv) *Incubation* (i) was repeated in presence of 4.5 mM MA.

RESULTS AND DISCUSSION

Elution profiles of gel chromatography of reactions of some reaction mixtures [MT and xanthine oxidase, MT and MC (enzymatic activation by xanthine oxidase/NADH), and MA and xanthine oxidase/NADH] are given in Figure 4 (p.136). The first UV peak (void volume) in each case represents mixtures of MT, MT associated with the drug and xanthine oxidase, i.e. the protein fraction. Fractions in this void volume were pooled concentrated and dialyzed. UV absorbance was recorded before and after dialysis (Figure 5-7, p.137-139). Dialysis was performed to eliminate any non-covalent binding of MC or mitosenes with MT or xanthine oxidase. Total absorbance (at 310 nm and 285 nm) are given in Table 1 (p.140).

Total absorbance (Table 1) before and after dialysis clearly indicate that there was no or very little non-covalent binding of drug to protein in any of the reactions. An exception was the reaction of MT (0.002 mM) with MC (20 mM) in absence of reducing agent xanthine oxidase (Set II) in which loss of absorbance occurred in the protein fraction at 367 nm after dialysis (this is not seen from the data in Table 1, but is illustrated in Figure 6, p.138). Unreduced mitomycin C has an absorbance maxima at 367 nm. Before dialysis, this was seen only in the UV of the reaction MT/MC (Set II). The loss of 367 nm absorbance after dialysis

clearly indicates that when in excess MC binds non-covalently to MT.

Covalent binding to MT (Table 1, Table 2). Total A_{310} of MC/xanthine oxidase was subtracted from total A_{310} of the MT/MC/xanthine oxidase reaction (Table 1) to obtain total A_{310} of MC bound to MT (Table 2; Set II). This was found to be 2.2 total A_{310} per 0.005 mg MT, corresponding to 0.2 umol mitosene per 0.005 mg MT. It may be pointed out however that the values (Table 2; Set II) obtained in the reaction MC/xanthine oxidase/NADH and MC/MT/xanthine oxidase/NADH employing excess MC (Set II) were lower than those in set I. Part of it is accounted by the loss of protein during centrifugation (centrifugation was necessary before application to column, because of a lot of precipitation in the reaction mixture). However, the fact that excess MC was inhibitory to MT is not ruled out.

An interesting observation was made when this study was conducted. MC was found to bind not only to MT but also to the enzyme xanthine oxidase (Table 1 and Table 2, Set Ia). Therefore when xanthine oxidase reduces (activates) MC, some of the reduced MC binds to the enzyme. This was concluded from the following results: Incubation of enzyme (xanthine oxidase/NADH) was conducted to serve as control for this experiment. As seen in Table 2, Set Ia, total A_{310} of enzyme was subtracted from total A_{310} of MC/enzyme to obtain total

A_{310} of mitosene (bound to the enzyme). This was found to be 2.9 total A_{310} per 4 mg xanthine oxidase, corresponding to approximately 0.27 umol MC (mitosene) bound per 4 mg xanthine oxidase.

MA binding to MT and xanthine oxidase. Incubation of MA with MT did not reveal any significant non-covalent binding with MT (Table1; MT/MA reaction mixture). A remarkable finding was made, however, when MA was reduced (activated) with the enzyme xanthine oxidase/NADH (Table 1; MA/X.OX. reaction mixture). All of the MA was bound to xanthine oxidase. No reduced MA was recovered after the completion of reaction as seen from the sephadex chromatography pattern (Figure 4c, p.136) which shows just two peaks: protein fraction (xanthine oxidase) and NADH. The first peak (xanthine oxidase) area is increased considerably due to covalent binding of reduced mitomycin A. It was calculated (Table 2, p.141; Set Ib) that approximately 0.82 umol reduced MA is bound to 4 mg of xanthine oxidase.

Inactivation of protein kinase C by MA. Reaction mixtures MC/PKC, BMY-25282/PKC and MA/PKC were assayed for inhibition of protein kinase C with ATP and histone III S as substrates. MA showed good inhibitory activity: PKC was only 66% active after reaction with MA. 75% activity was observed after reaction with BMY 25282 but no significant loss in activity was seen with MC. When reactions were conducted in

presence of reducing agents H_2/PtO_2 and $Na_2S_2O_4$, no conclusive observations could be made because both of these agents were found to inhibit PKC.

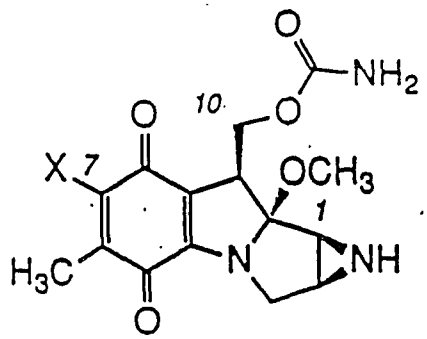
Significance

The major focus of this preliminary study was to address the question whether metallothionein could detoxify MC by reaction with MC via its sulfhydryl groups. The preliminary results indicate that this is a viable possibility, since we observed covalent binding of MC to MT upon reduction of the drug.

The data provide preliminary evidence that MC and MA bind the reductase xanthine oxidase, in a manner resembling suicide inactivation, since they are substrates of this reductase. In future work, inactivation of xanthine oxidase should be tested directly in order to substantiate this hypothesis. The observed covalent binding of these drugs to xanthine oxidase, the enzyme which reduces them to alkylating agents resembles the binding of MC to DT-diaphorase, another reductase, reported by Seigel[71].

MA is generally considered more toxic and therefore less effective against tumors than its clinically useful cousin, MC[67]. The reason(s) for this variation in activity between

the two antibiotics is currently unknown. *In vitro* both MA and MC cross-link DNA to an equal extent[68]. Other factors besides DNA alkylation probably influence the activity of these two mitomycins *in vivo*. Possibly, the difference in hydrophilicity between the two 7-position groups (amino versus methoxy) effects the drug's cellular uptake[69]. In this way, MC may be taken up more selectively by tumor cells while MA may be more indiscriminate, entering both normal cells and tumor cells. Another factor is thought to be the higher redox potential of MA (- 0.19 V for MA versus - 0.40 V for MC), making it more easily activated by reductase[70]. The results in this part of the thesis provide only preliminary evidence for interaction of MC and MA with proteins, but they appear to suggest that differential binding to proteins could also explain differential effectiveness of MC over MA.



1a (MC ; X = $-NH_2$)

1b (MA ; X = $-OCH_3$)

1c (BMY 25282 ; X = $-N=CHN(CH_3)_2$

FIGURE 1. Structures of MC (1a), MA (1b) and BMY-25282 (1c)

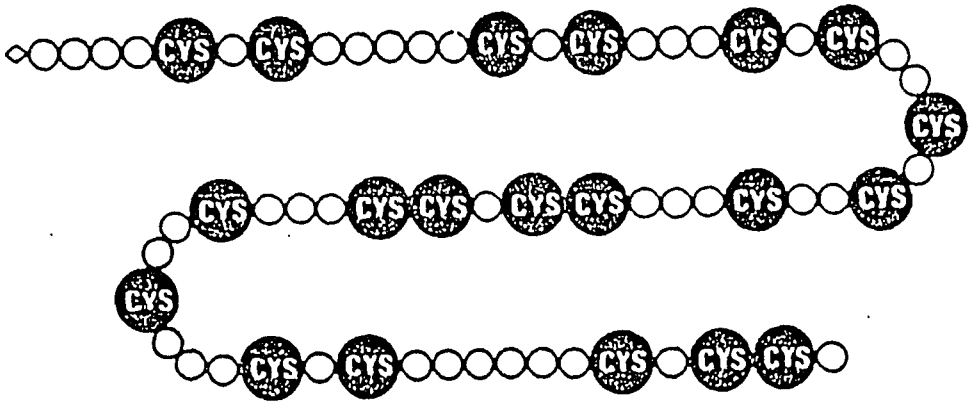


FIGURE 2. Mode of distribution of cysteine residues in mammalian metallothionein (Mol. Wt. 6000-7000).

(REFERENCE: CELL 52: 343-354, 1988)

NOTES:

Cysteine residues (C) are shown in bold print.
Tryptic peptides containing cysteine residues are underlined with a wavy line (~~~).
Anion-rich sequences are bounded by vertical dashed lines.
Sites of auto-phosphorylation are designated with an asterisk (*).
The pseudosubstrate sequence is boxed.

FIGURE 3. Deduced amino acid sequence of PKC-B1.

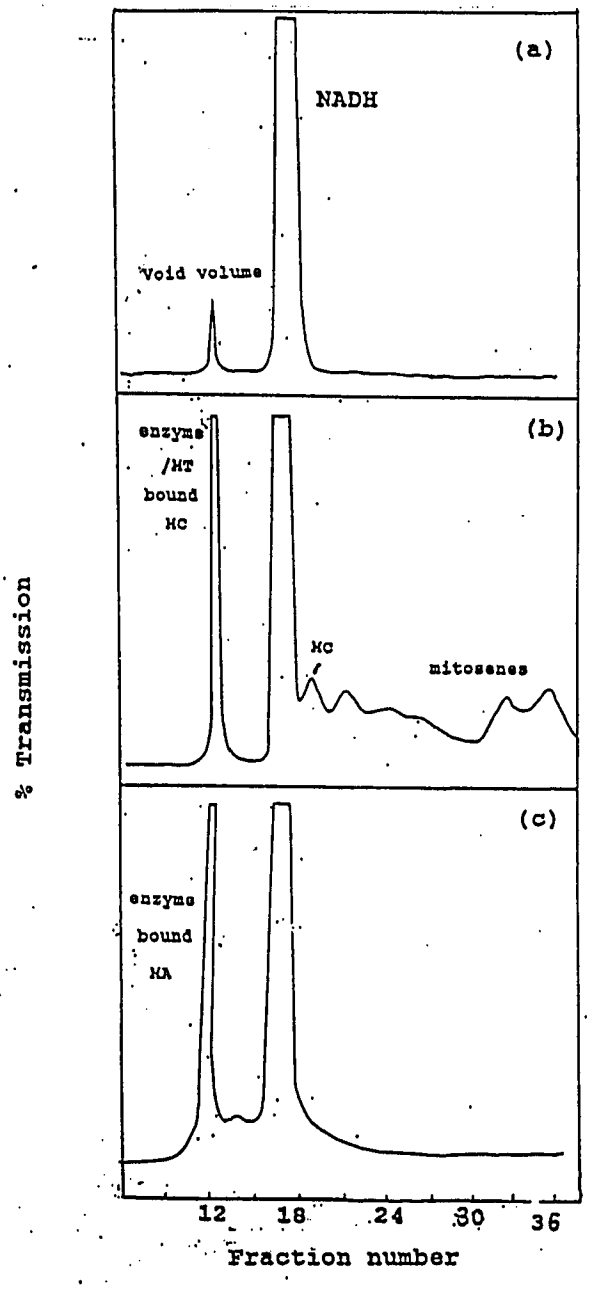
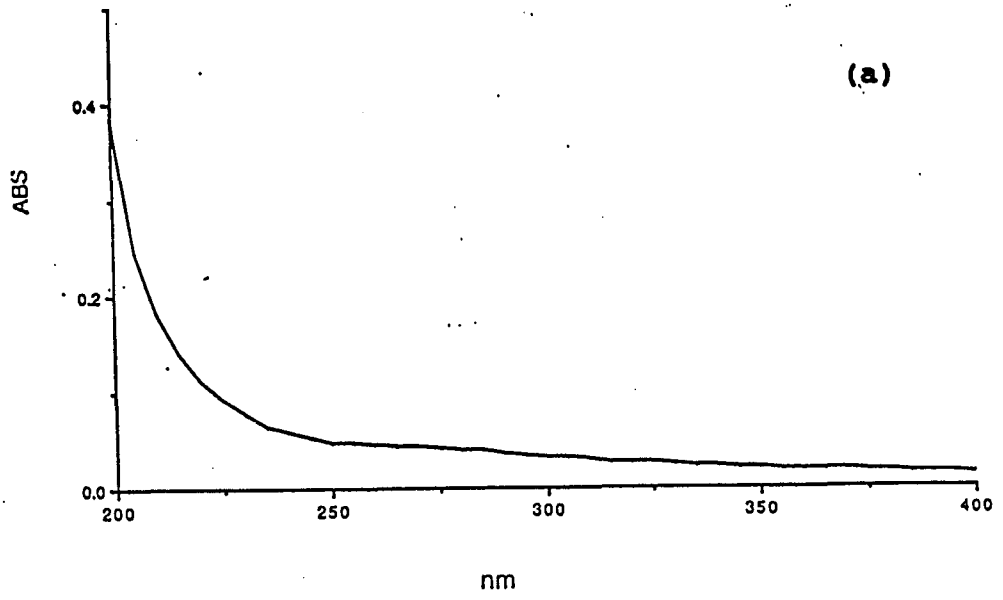


FIGURE 4. Sephadex G-25 chromatography patterns of the following reaction mixtures,

(a) MT / xanthine oxidase / NADH

(b) MT / MC / xanthine oxidase / NADH

(c) MA / xanthine oxidase / NADH



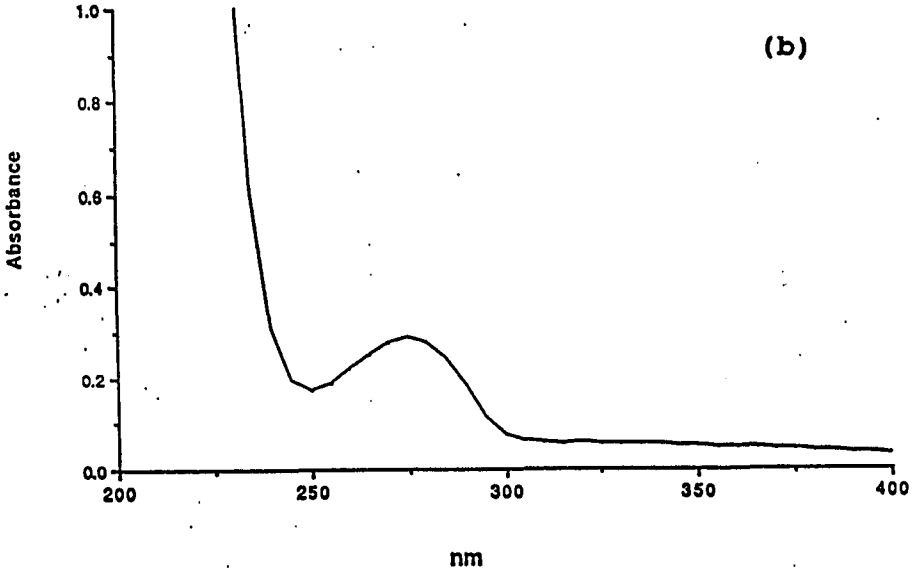
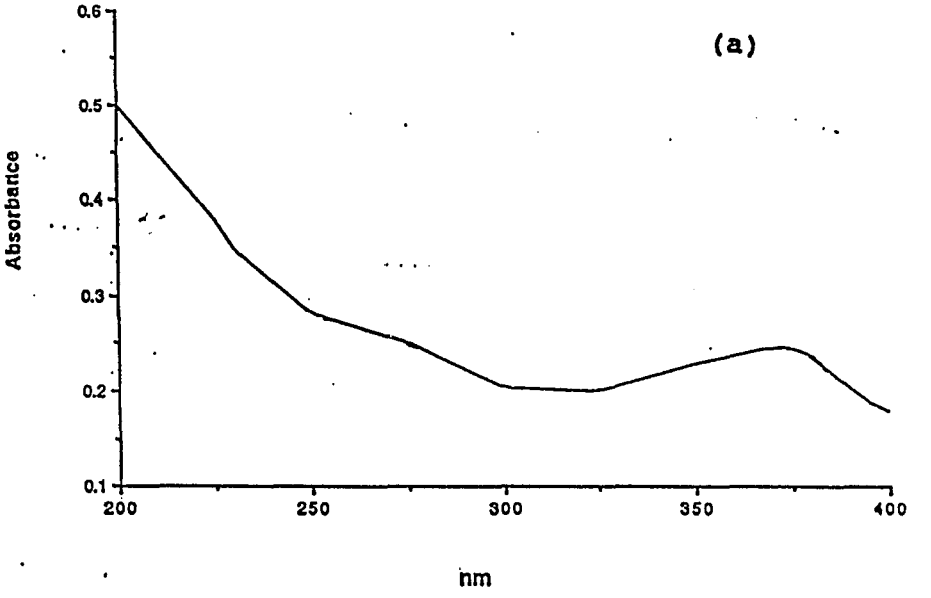


FIGURE 5. (a) UV of metallothionein (after dialysis in 0.05 N HCl to remove metals). (b) UV of enzyme xanthine oxidase, in 0.02 M NH_4HCO_3 (1 mg/ml).



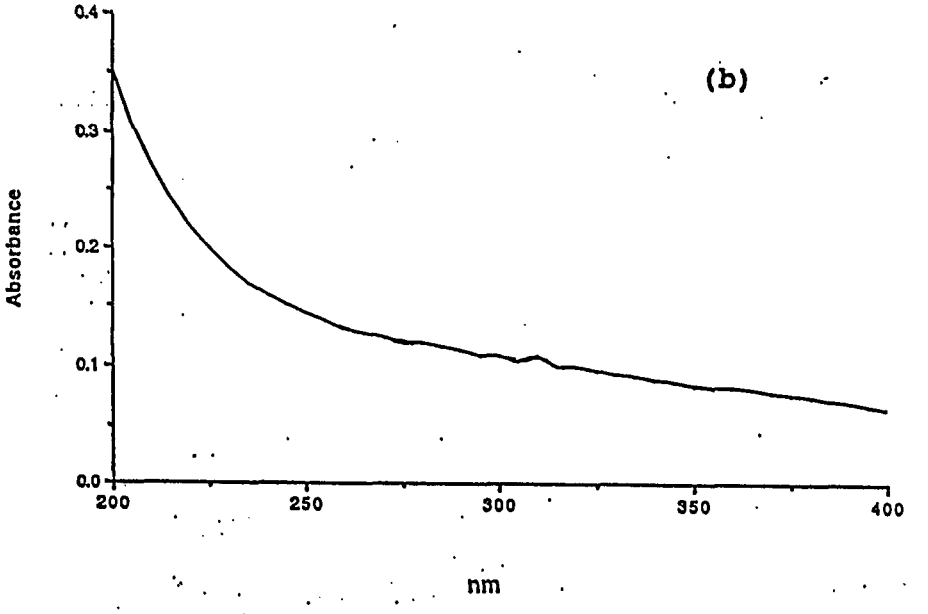


FIGURE 6. UV (in 0.02 M NH_4HCO_3) of MT fractions from MC/MT reaction (set II);

(a) Before dialysis , showing absorbance of MC at 367 nm.

(b) After dialysis , Showing loss of absorbance at 367 nm.

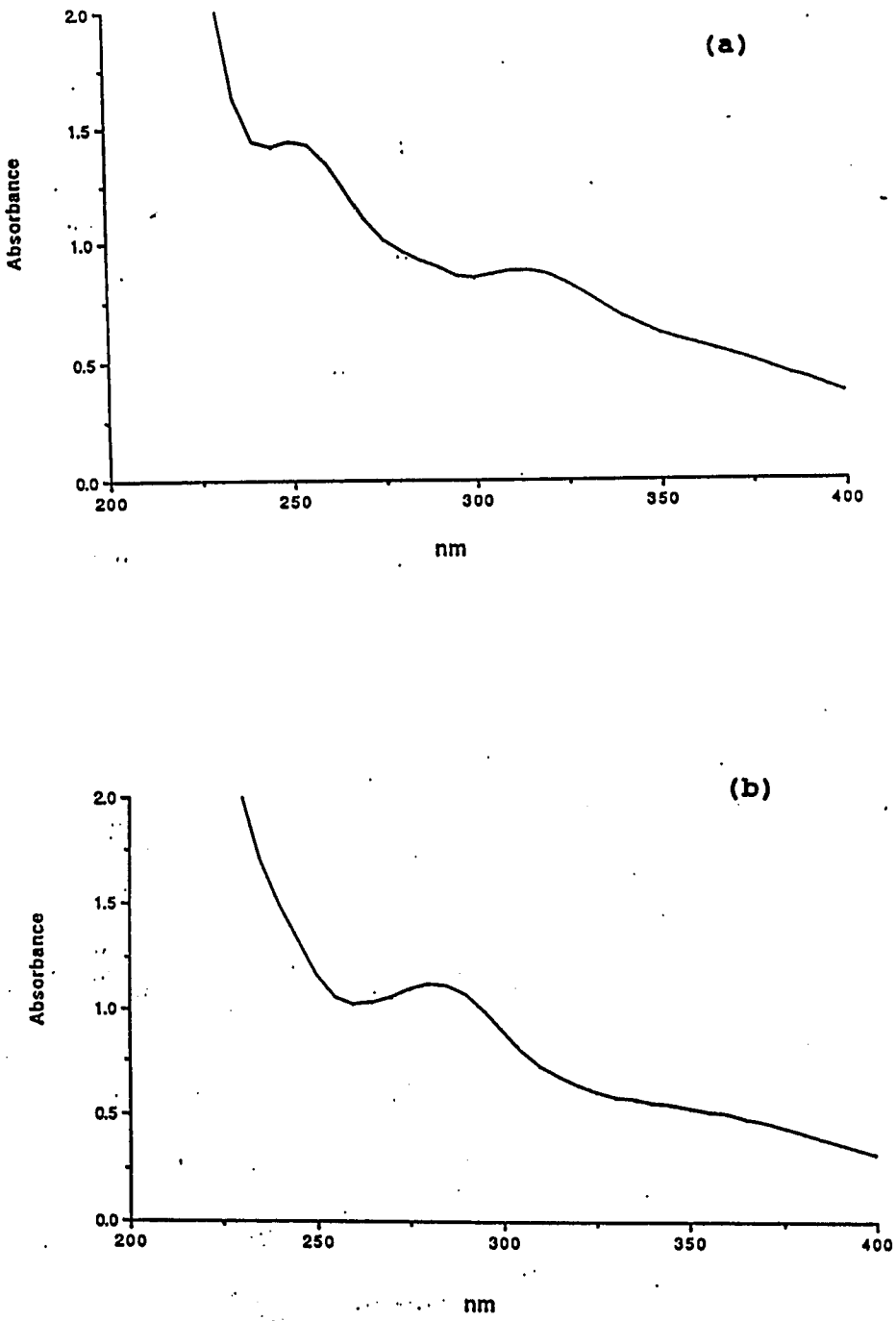


FIGURE 7. UV (in 0.02 M NH_4HCO_3) of Sephadex protein (void volume) fractions from

(a) MC/MT/xanthine oxidase/NADH reaction (set I).

(b) MA/xanthine oxidase/NADH reaction (set I).

TABLE 1. Total absorbance (A_{310} and A_{285}) of MT and xanthine oxidase reaction mixtures with MC or MA before and after dialysis.

	SET I				SET II			
	BEFORE DIALYSIS		AFTER DIALYSIS		BEFORE DIALYSIS		AFTER DIALYSIS	
RX	Total A_{310}	A_{285}	Total A_{310}	A_{285}	Total A_{310}	A_{285}	Total A_{310}	A_{285}
MT	0.168	0.249	0.100	0.128	0.222	0.357	0.148	0.236
X.OX	0.544	2.303	0.425	1.768	0.632	2.380	0.456	1.800
MT/ X.OX	1.275		0.567		1.256		0.448	
MT/ MC	0.228		0.175		0.546		0.288	
MC/ X.OX	4.182		3.360		1.485		0.711	
MT/ X.OX/ MC	6.542		5.125		4.002		2.637	
MT/ MA		0.468		0.168		0.393		0.189
MA/ X.OX		12.91		10.81				

* RX = reaction mixture

X.OX = enzyme xanthine oxidase/NADH

TABLE 2. Calculated binding of MC and MA to MT or xanthine oxidase, based on A_{310} and A_{285} measurements in Table 1.

	BEFORE DIALYSIS	AFTER DIALYSIS	NET TOTAL A/mg protein	umol mitosene/ mg protein
SET I*				
Net A_{310}:				
(a) MC/X.OX - X.OX				
= MC/X.OX (net)	3.638	2.935	2.93/4	0.27/4
(b) MT/MC/X.OX -(a)				
= MC/MT (net)	2.904	2.190	2.19/0.005	0.20/0.005
Net A_{285}:				
(c) MA/X.OX - X.OX				
= MA/X.OX (net)	10.607	9.042	9.042/4	0.82/4
SET II**				
Net A_{310}:				
(a) MC/X.OX - X.OX				
= MC/X.OX (net)	0.853	0.255	0.255/4	0.02/4
(b) MT/MC/X.OX - (a)				
= MC/MT (net)	3.149	2.382	2.38/0.005	0.21/0.005

* Set I : MT-SH:MC molar ratio = 0.01:10; reaction vol. = 1 ml, incubation for 30 min at 37°C.
** Set I : MT-SH:MC molar ratio = 0.01:100; reaction vol. = 5 ml, incubation for 1 hr at 37°C.

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73. The term mitosene signifies indoloquinone derivatives of MC. Mitosene itself is the indolquinone as in structure **2b** (p.101), but without substituents in the 1"-, 2"-, and 7"-positions[56]. In a less rigorous sense, we use this term as the DNA-bound mitomycin unit in general. Absorbance maximum of mitosene is at 310 nm (E_{310} = 11000) which may be used to determine umol mitosene present in a monoadduct.