[Chem. Pharm. Bull. 31(3) 861—869 (1983)]

Modification of Deoxyribonucleic Acid with Reductively Activated Mitomycin C. Structures of Modified Nucleotides

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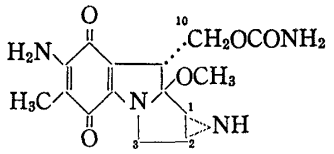
(Received August 19, 1982)

Mitomycin C (MMC) binds to deoxyribonucleic acid (DNA) after its reductive activation by catalytic hydrogenation with Pd on charcoal. Three modified nucleotides, named MG-1, MG-2 and MA, were isolated and purified by high performance liquid chromatography (HPLC) from the modified DNA after enzymatic hydrolysis to 5′-nucleotides with nuclease P1. Analysis of the proton nuclear magnetic resonance (^{1}H-NMR) and ultraviolet (UV) spectra, and studies of the acid and enzymatic hydrolysates of these modified nucleotides suggested that MG-1 and MG-2 are deoxyguanylic acid–MMC adducts bound at position 1 of mitosene and a heteroatom of the guanine moiety. Analogous binding to an adenine moiety is proposed for the adduct, MA. Chemical transformations (methylation, diazotization and thioketonization) were used to unambiguously determine the binding sites of the purine bases. The binding sites were identified as the N^2 atom of guanine for MG-1, the O^6 atom of guanine for MG-2, and the N^6 atom of adenine for MA. Thus, these three modified nucleotides were concluded to be 1,2-*trans*-2,7-diamino-1-(N^2-deoxyguanylyl)mitosene (MG-1), 2,7-diamino-1-(O^6-deoxyguanylyl)mitosene (MG-2), and 2,7-diamino-1-(N^6-deoxyadenylyl)mitosene (MA). These same modified nucleotides were identified in DNA extracted from the livers of rats treated with MMC.

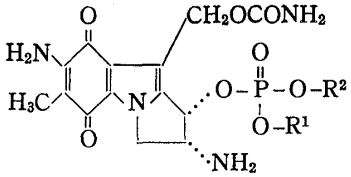
Keywords——mitomycin C; DNA; modification of DNA; bioreductive alkylation; antitumor agent

Introduction

Mitomycin C (MMC) is a potent antibiotic and is also used clinically as an antitumor agent in spite of its rather high toxicity. Many derivatives of MMC have been synthesized in attempts to develop more effective antitumor agents with higher antitumor activity and lower toxicity.[1] It is well established that MMC requires reductive activation to become an alkylating agent of biological macromolecules, especially deoxyribonucleic acid (DNA).[2] The action of MMC as a bioreductive alkylating agent of DNA is believed to be, at least in part, responsible for its effectiveness as an antitumor agent. Bifunctional alkylation of DNA leading to cross-linking of the two strands was considered to be the direct cause of the cytotoxicity of the drug,[3] although the monofunctional attachment, which occurs 10 to 20 times more frequently than the cross-linking, has also been implicated as biologically significant damage to DNA.[2] Elucidation of molecular aspects of this alkylation reaction of DNA by MMC should provide basic information for the development of more effective antitumor agents. Recently, Moore[4] proposed MMC acts as a reductively activated bifunctional alkylating agent. He suggested that positions 1 and 10 are binding sites of MMC. Though there have been several studies on the sites of binding of MMC and DNA, no unambiguous evidence for the chemical structure of the modified DNA has yet been obtained. In addition, the redox chemistry of MMC itself is very complex and not well understood. Only a few products have been characterized from any reaction of reduced MMC.[5–7] Very recently, we reported the alkylation of 5′-guanylic acid by reductively activated MMC (catalytic reduction) and showed the structure of MMC-bound 5′-guanylic acid to be 1,2-*cis*-2,7-diamino-1-(5′-guanylyl)mitosene (**2**).[8] The other nucleic acid derivatives modified with MMC are uridyl derivatives (**3**—**5**), reported by Tomasz and Lipmann,[9] but the products were obtained by reaction of MMC and uridylic acid



1: MMC



2: R^1=guanyl-5′-, R^2=H
3: R^1=uridyl-5′-, R^2=H
4: R^1=uridyl-5′-
R^2=uridyl-3′-
5: R^1=O(OH)-P-O-P(OH)-O-(uridyl-5′)-
R^2=H

Chart 1

derivatives under acidic conditions and without reductive activation of MMC.

This study is concerned with the isolation and structural identification of monofunctional alkylation products of DNA obtained with reductively activated MMC.[10] DNA was modified chemically by catalytic reduction, as well as *in vivo* in male Wistar rats.[11]

Experimental

Materials——MMC was supplied by Kyowa Hakko Kogyo Co. Calf thymus DNA (Type I, sodium salt) and alkaline phosphatase (Type I, from bovine intestine) were purchased from Sigma Co. 3-Methyladenine, 7-methyladenine, 1-methylguanine and 7-methylguanine were supplied by Dr. K. Kohda, Nagoya City University. 3-Methylguanine and 6-thioguanine were prepared essentially by the methods of Townsend and Robins,[12] and Elion and Hitchings,[13] respectively. Nuclease P1[14] and male Wistar rats were purchased from Yamasa Shoyu Co. and Nippon Seibutsu Zairyo Center, respectively. 1,2-*cis*-2,7-Diamino-1-hydroxymitosene (7) was prepared by the method of Taylor and Remers.[15]

High Performance Liquid Chromatography (HPLC)——A Shimadzu LC-2 apparatus equipped with an SPD-1 UV spectrophotometer as a detector and a Polygosil $_5C_{18}$ column (4.6 $\phi \times$ 250 mm for analysis, 8.0 $\phi \times$ 250 mm for preparative use, eluted with 3—10% CH_3CN in 0.3% NH_4Cl aq.) was used. UV spectra were obtained by the stop-flow method in an SPD-1 spectrophotometer.

Reaction of MMC with Calf Thymus DNA——An aqueous solution of MMC (1 mg/ml, 50 ml) was mixed with an aqueous solution of DNA (2 mg/ml, 50 ml). The resulting blue solution was vigorously stirred with 25 mg of 5% Pd on charcoal under a hydrogen atmosphere at room temperature. After about 10 min, just when the reaction mixture changed from blue to reddish-purple, the mixture was filtered through a filter paper to remove the catalyst. The reaction should be stopped at this stage, since longer reduction (30—60 min) gave a colorless modified DNA that did not yield soluble modified nucleotides on enzymatic hydrolysis. The reaction was repeated 20 times (total DNA used, 2.0 g). Cold EtOH (4 l) was added to the combined filtrate, and the resulting insoluble red modified DNA was collected by centrifugation (1.65 g). Modified DNA was purified by reprecipitation from aqueous EtOH or by gel filtration chromatography on Sephadex G-50 (H_2O). The yield of modified DNA was 1.6 g. About one molecule of MMC was bound per 200—300 nucleotides of DNA, as estimated from the UV spectrum of the modified DNA (ratio of absorbance at 260 nm to that at 310 nm).

Enzymatic Hydrolysis of Modified DNA——Modified DNA (1.75 g) was dissolved in 200 ml of dilute CH_3COOH (pH 5.5). To this viscous solution, 10 mg of nuclease P 1 was added, and the mixture was warmed at 55°C for 2 h. During the hydrolysis, a small amount of dark red insoluble material (probably MMC-bound nucleic acid derivatives) was formed, and this was removed by centrifugation; the amount of precipitate increased when the reduction time was longer (30—60 min). The mixture was lyophilized and the residue was washed with MeOH and *N*,*N*-dimethylformamide (DMF) successively. The resulting mixture was dissolved in H_2O–MeOH (9: 1 v/v) at 55°C. On cooling of the resulting red solution, white precipitates (a part of the normal nucleotides) were formed, and these were removed by centrifugation. Analysis of the red supernatant by HPLC showed the presence of three modified nucleotides named MG-1, MG-2 and MA. These modified nucleotides eluted from the column after normal nucleotides. The hydrolysis mixture thus

obtained contained MG-1, MG-2, MA and normal nucleotides (0.6 g after lyophilization).

Isolation of Modified Nucleotides——The nucleotides mixture (0.6 g) was dissolved in 40 ml of H_2O, and small portions (150—200 μl each) were subjected to preparative HPLC (7.5% CH_3CN–0.1% NH_4Cl–H_2O). Fractions containing MG-1, MG-2 and MA were collected and lyophilized. The residues were each dissolved in 0.1 ml of dimethyl sulfoxide (DMSO), and then 0.5 ml of H_2O was added to precipitate chromatographically pure red MG-1 (1.2 mg, dry weight), MG-2 (1.1 mg) and MA (0.8 mg).

^{1}H-NMR of Modified Nucleotides——The dried modified nucleotides were each placed in an NMR tube connected to a vessel containing DMSO-d_6 with CaH, or CF_3COOD. Dry DMSO-d_6 (0.5 ml) and CF_3COOD (0.025 ml) were transferred to the NMR tube by cooling the tube with liquid nitrogen under a vacuum. The 400 MHz ^{1}H-NMR spectra were obtained with a JNM-FX-400 apparatus.

Selective Hydrolysis of Modified Nucleotides——Method A: Each modified nucleotide (3μg) was dissolved in 100 μl of 1 N HCl. The red solution was heated at 80°C for 40 min, by which time the red color and absorption at 310 nm had disappeared. The mixture was cooled and analyzed by HPLC (3% CH_3CN–0.3% NH_4Cl–H_2O). Nucleic acid bases were identified.[16]

Method B: Each modified nucleotide (10 μg) in a minimum amount of H_2O was subjected to a silica gel thin-layer chromatography (TLC) (iso-PrOH/conc.NH_4OH=1: 1 v/v). The red band was collected and extracted with H_2O–EtOH–NH_4OH mixture and the extract was concentrated under reduced pressure. The residue was dissolved in a minimum amount of DMSO and analyzed by HPLC. The modified nucleotides all gave 10-decarbamoyl-1,2-*cis*-2,7-diamino-1-hydroxymitosene (**6**) in yields of 30—40%, and this compound was identified[16] by comparison with an authentic sample prepared by hydrolysis of MMC. ^{1}H-NMR (DMSO-d_6): 1.82 (s, 3H, 6-CH_3), 3.59 (d, J=6 Hz, 3-Ha), 3.83 (dd, J=6 Hz and 12 Hz, 3-Hb), 4.40 (dd, J=12 Hz and 5 Hz, 2-H), 4.72 (s, 10-CH_2–), 4.80 (d, J=5 Hz, 1-H), 6.60 (br s).

Method C: Samples of modified nucleotides (3 μg) were each dissolved in 100 μl of aqueous NH_4OH (pH 10). Alkaline phosphatase was added and the mixture was warmed at 40°C for 15 min. The solution was analyzed by HPLC. The modified nucleotides were stable under these conditions without the enzyme.

Methylation of Modified Nucleotides——Method A: MG-1 or MG-2 (100 μg) was dissolved in 200 μl of DMSO and 200 μg of trimethylsulfoxonium iodide[17] was added. The solution was heated at 100°C for 1 h. HPLC showed that the mixture contained 5—10% of the starting material but no guanine or methylguanines. Next, 50 μl of CH_3COOH and then 300 μl of 1 N HCl were added, and after heat treatment at 100°C for 20 min, the mixture was analyzed by HPLC (1.5% CH_3CN–0.3% NH_4Cl–H_2O). Guanine, xanthine and methylguanines were identified.[16]

Method B: MA (100 μg) was dissolved in 200 μl of DMSO and 650 μg of trimethylsulfoxonium iodide was added. Work-up and identification of methylated bases were performed as described in method A.

Reactions of MG-1 and MG-2 with P_2S_5——MG-1 or MG-2 (10 μg) was suspended in dry pyridine (100 μl) and 200 μg of P_2S_5 was added. The suspension was heated at 100°C for 5 h, and then evaporated under reduced pressure. After analysis by HPLC to confirm the absence of 6-thioguanine, the residue was dissolved in 100 μl of 1 N HCl. The solution was heated at 100°C for 1 h, and then the hydrolysate was analyzed by HPLC (3% CH_3CN–0.3% NH_4Cl–H_2O) to identify 6-thioguanine.[16]

Diazotization of MG-1 and MG-2——MG-1 or MG-2 (0.8 μg) was dissolved in 0.2 ml of dilute HCl (pH 1—2) and 100 μg of $NaNO_2$ was added. After 2 h at room temperature, urea was added in small portions to decompose excess nitrite (monitored with KI-starch). After analysis by HPLC to confirm the absence of guanine or xanthine, 0.5 ml of 1 N HCl was added and the mixture was heated at 100°C for 1 h. The resulting mixture was analyzed by HPLC (3% CH_3CN–0.3% NH_4Cl–H_2O) to identify guanine or xanthine.[16]

Identification of Modified Nucleotides in Rat Liver DNA treated with MMC——A sample of 10 mg of MMC in 3 ml of 0.15 M KCl was injected intraperitoneally into a male Wistar rat (150 g). The liver (8 g) was removed after 3 h and homogenized. DNA was extracted from the homogenate by Kirby's PAS–phenol method.[18] The DNA fraction thus obtained (8 mg) was treated with RNase and dissolved in 4 ml of dilute CH_3COOH (pH 5.5). Nuclease P1 (0.1 mg) was added and the mixture was warmed at 55°C for 2 h, then lyophilized. The residue was dissolved in 0.5 ml of H_2O and analyzed by HPLC to identify MG-1, MG-2 and MA.[16]

Results

Binding of MMC to DNA

MMC binds to DNA only after reductive activation. We used catalytic hydrogenation with 5% Pd on charcoal and H_2 gas for reductive activation of MMC. Other reducing systems, such as $Na_2S_2O_4$, KBH_4 and $NaBH_4$, did not give similar results. Reaction of reductively activated MMC with DNA occurred under neutral conditions. The modified DNA has no absorption maximum at 350 nm (characteristic of the mitosane skeleton), but it possesses an absorption maximum at 310 nm which is characteristic of the mitosene skeleton. About one

molecule of MMC was bound per 200—300 nucleotides of DNA, as estimated from the UV spectrum of the modified DNA (ratio of absorbance at 260 nm to that at 310 nm).[19] Increase in the acidity of the reaction mixture did not enhance the binding. Analysis of an enzymatic hydrolysate of the modified DNA showed the presence of three modified nucleotides, MG-1, MG-2 and MA. The amounts of these three modified nucleotides could be estimated from their peak heights on HPLC and their molar extinction coefficients (Table I). More than 80% of the binding of MMC to DNA could be accounted for by the formation of MG-1, MG-2 and MA.

UV Spectroscopy

The UV spectra of MG-1, MG-2 and MA (Table I) have absorption maxima at *ca.* 310 nm, suggesting that these modified nucleotides contain a mitosene moiety, because the mitosene chromophore has an absorption maximum at 310 nm.[20] The UV spectra of MG-1 and MG-2 were similar, both having an absorption shoulder at *ca.* 275 nm (except at pH 1), the position of the absorption maximum of the deoxyguanylic acid chromophore. The UV spectrum of MA suggested that the nucleic acid base moiety of MA is adenine, because it had an absorption maximum only at 258 nm, and the absorption at *ca.* 310 nm had a molecular extinction coefficient similar to that of MG-1 and MG-2.

Table I. UV Data for MG-1, MG-2 and MA (nm)

	pH 1	pH 7	ε	pH 10
MG-1	260	255	3.8×10^4	255
	275 (sh)	277 (sh)		270 (sh)
	312	310	0.6×10^4	312
MG-2	260	255	3.8×10^4	255
	310	275 (sh)		275 (sh)
		308	0.6×10^4	310
MA	258	258	3.6×10^4	258
	310	310	0.6×10^4	312

Identification of Nucleic Acid Base Moieties

MG-1, MG-2 and MA were hydrolyzed completely by treatment with 1 N HCl. MG-1 gave guanine (45%) and xanthine (15%), and MG-2 gave guanine quantitatively, suggesting that MG-1 and MG-2 are deoxyguanylic acid–MMC adducts. MA gave adenine quantitatively, suggesting that MA is a deoxyadenylic acid–MMC adduct. The modified nucleotides MG-1 and MA were stable under weakly acidic conditions (pH 2, 50°C, 30 min), *i.e.*, conditions which are reported to hydrolyze the glycosidyl bond of 7-alkylated purine nucleotides and nucleosides.[21] Even the most unstable nucleotide, MG-2, decomposed under these conditions only to the extent of about 15%.

Phosphomonoester Group

When these modified nucleotides were treated with alkaline phosphatase, less polar products (probably the corresponding nucleosides) were obtained quantitatively. This finding and the observation that these nucleotides were all resistant to phosphodiesterase and the established actions of nuclease P1 suggest that these compounds have a mono-phosphate group at the 5′-position of 2′-deoxyribose.

^{1}H-NMR Spectroscopy

^{1}H-NMR spectra were obtained in DMSO-d_6–CF_3COOD. The use of completely anhydrous solvent and exchange of hydrogen of NH and OH made it possible to assign all the signals. The results of ^{1}H-NMR experiments are shown in Fig. 1 and Table II. Assignment of proton signals was performed by homonuclear decoupling experiments and by comparing

the spectra obtained with those of model compounds such as mitosene and nucleotides. The ^{1}H-NMR spectra of MG-1 and MG-2 showed a singlet at δ 9.10 and 9.20, respectively, which is characteristic of the proton at position 8 of the guanine moiety. This observation in addition to the results described above suggests that the binding sites of the deoxyguanylic acid moiety of MG-1 and MG-2 are at one of the heteroatoms of guanine, but not the C^8 position. The ^{1}H-NMR spectrum of MA showed two characteristic singlet signals of positions 2 and 8 of adenine (δ 8.58 and 8.65). Thus, the binding site of the deoxyadenylic acid moiety of MA is also a heteroatom of adenine. The chemical shifts of protons at positions 1, 2, and 3 of the mitosene rings of these modified nucleotides were different from each other, but those of position 10 were nearly the same (δ 5.02—5.04), which is close to that of **7**, but not **6**. This suggests that the binding sites of the mitosene moiety of these modified nucleotides are at not position 10.

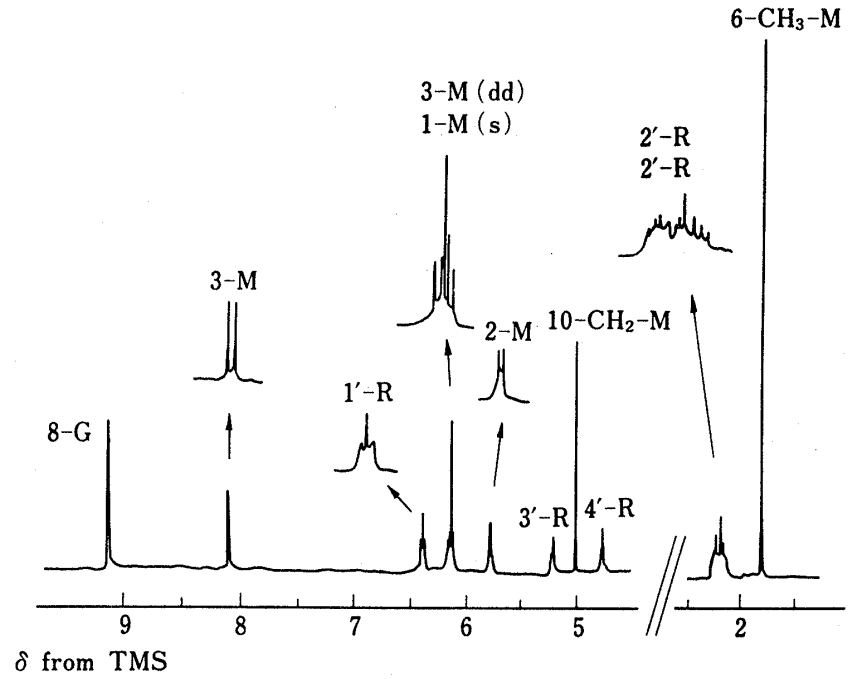


Fig. 1. ^{1}H-NMR (400 MHz) Spectrum of MG-1 in 5% CF_3COOD-d_6-DMSO
M; mitosene: G; guanine: R; deoxyribose.

TABLE II. ^{1}H-NMR (400 MHz) Spectra of MG-1, MG-2 and MA in 5% CF_3COOD–DMSO-d_6
Chemical Shifts from TMS and Coupling Constants

	Mitosene moiety						Deoxyribose moiety					Purine base moiety
	1-H	2-H	3-Ha	3-Hb	6-CH_3	10-CH_2–	1'-H	2'-H	3'-H	4'-H	5'-H	
MG-1	6.18 s	5.81 d 5 Hz	6.13 dd 5 Hz 8 Hz	8.11 d 8 Hz	1.82 s	5.04 s	6.38	2.25 2.30	5.16	4.79	4.50	9.10(s) 8-H of guanine
MG-2	6.38 s	5.78	6.16 dd 5 Hz 7 Hz	8.04 d 7 Hz	1.82 s	5.02 s	6.28	2.30	5.20	4.80	4.40	9.20(s) 8-H of guanine
MA	6.28 s	5.74	6.30 dd 5 Hz 7 Hz	8.90 d 7 Hz	1.81 s	5.02 s	6.40	2.12	5.12	4.78	4.48	8.58(s) 8.65(s) 2-H and 8-H of adenine

s, singlet; d, doublet; dd, double doublet.

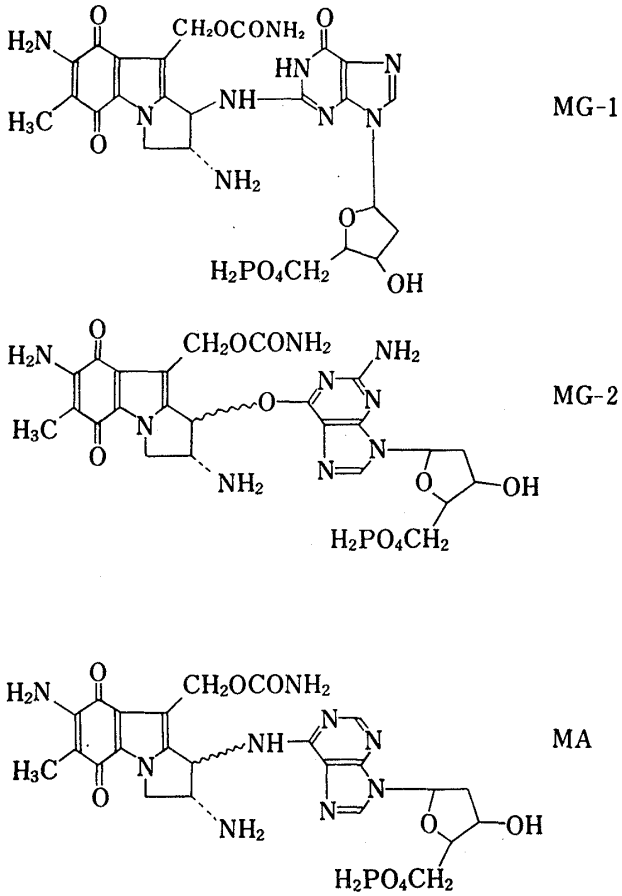
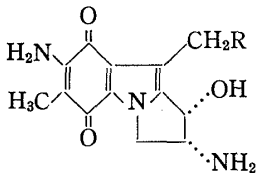


Chart 2. Structures of Modified Nucleotides

The configuration at positions 1 and 2 of mitosene of MG-1 was expected to be *trans* from the small coupling constant (less that 2 Hz) between the protons at positions 1 and 2. When the configuration of positions 1 and 2 is *trans*, the dihedral angle of protons at positions 1 and 2 should be about 95° on the basis of Dreiding-molecular model studies, if the molecule possesses a sterically stable conformation. If the configuration of positions 1 and 2 is assumed to be *cis*, the dihedral angle of protons at positions 1 and 2 in the most sterically stable conformation should be 25—27° (about 6 cps). Most aziridine-opening reactions of MMC derivatives are known to give *cis* isomers as the major products, along with minor amounts of the *trans* isomers.[15] However the aziridine-opening reaction to give a *trans* isomer is not incompatible with other cases. The signals of the protons at positions 1 and 2 of mitosene of MG-2 and MA were broad and the coupling constants could not be measured.

Binding Site of the Mitosene Moiety

The binding sites of the mitosene moiety in these modified nucleotides seem to be at position 1, judging from the ^{1}H-NMR spectra. For confirmation of this deduction, these modified nucleotides were hydrolyzed under mild conditions. The hydrolysates of MG-1, MG-2 and MA by treatment with silica gel–iso-PrOH–NH_4OH gave **6** in yields of 30—40%. Thus, the binding site of the mitosene moiety of these modified nucleotides is at position 1 or 10. This experiment alone did not exclude position 10 as a possible binding site. MG-2, which is the most acid-labile modified nucleotide, gave **7** as well as **6** on treatment with 0.5 N HCl at 0°C for 20 min, though the production of **7** was transient.[22] This shows that the binding site of the mitosene moiety of MG-2 is at position 1. The similar chemical shifts of the protons at position 10 in all these modified nucleotides strongly suggests that these compounds all possess a carbamoyl group at position 10 of the mitosene moiety.



6: R=OH
7: R=$OCONH_2$

Chart 3

Binding Site of the Purine Base Moiety of Modified Nucleotides

The ^{1}H-NMR spectra and the results on hydrolysis of MG-1, MG-2 and MA suggest that the binding sites of nucleic acid base moieties are at heteroatoms of the purine rings. These modified nucleotides were hydrolyzed with 1 N HCl after methylation with trimethylsulfoxonium iodide in DMSO. Both MG-1 and MG-2 gave guanine (4—10%), 3-methylguanine (1%), 1-methylguanine (2—3%), and 7-methylguanine (4—5%). MG-1 also gave xanthine (4%).

MA gave adenine (trace), 1-methyladenine (14%), 3-methyladenine (11%), and 7-methyladenine (0.5%). In all cases, some other products were observed, but not O^6-methylguanine from MG-2 and not N^6-methyl- or N^6,N^6-dimethyladenine from MA. These results suggest that the methylated positions (the 1-, 3-, and 7-positions) and the glycosylated position (the 9-position) of the purine rings are not the binding sites. Therefore, the binding site of MA is at the N^6 atom of adenine, and those of MG-1 and MG-2 are at the O^6 or N^2 atom of guanine.

For unambiguous determination of the binding sites of the guanine moieties of MG-1 and MG-2, the guanine moieties were chemically transformed. Acid hydrolysis of MG-1 and MG-2 after treatment with P_2S_5 gave 6-thioguanine from MG-1 (30%), but this was not obtained from MG-2. This suggests that the O^6 atom of the guanine moiety is blocked in MG-2 but not in MG-1. In addition, MG-1 and MG-2 were hydrolyzed with 1 N HCl after treatment with $NaNO_2$. The free 2-NH_2 group of guanine should be diazotized easily. Guanine (10%) together with xanthine (20%) was obtained from MG-1, but only xanthine (30%), not guanine, was obtained from MG-2. From these results, the binding sites of MG-1 and MG-2 were determined to be at the N^2 and O^6 atoms, respectively. Therefore, the structures of MG-1, MG-2 and MA are as shown in Chart 2.

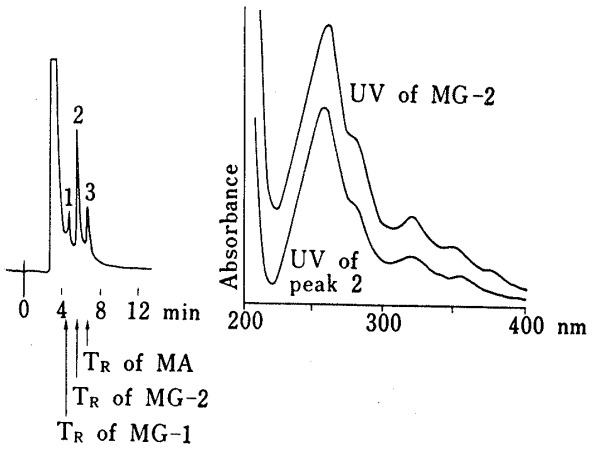


Fig. 2. HPLC of Hydrolysate of DNA extracted from the Liver of a Rat treated with MMC and the UV Spectra of Peak 2 and MG-2

Column; Polygosil $_5C_{18}$ 4.6ϕ × 250 mm.
Solvent; 10% CH_3CN in 0.3% NH_4Cl aq.
Flow rate; 0.8 ml/min.
Detection; absorption at 310 nm.

Modification of DNA with MMC *in Vivo*

DNA extracted from the liver of a rat treated with MMC was modified with MMC. About one molecule of MMC was bound per $1—2\times10^4$ nucleotides, as estimated from the UV spectrum of the modified DNA (ratio of absorbance at 260 nm to that at 310 nm). Enzymatic hydrolysis gave MG-1, MG-2 and MA in a ratio of 1 : 5 : 2 (Fig. 2). The yields of these three modified nucleotides account for most of the MMC bound to DNA; the amounts of other nucleotides modified with MMC, if any, were very small. MMC bound preferentially to guanine in DNA, especially at the O^6 atom.

Discussion

The structures of the three modified nucleotides isolated from DNA alkylated with reductively activated MMC were determined to be as shown in Chart 2. When MMC was reductively activated with $Na_2S_2O_4$, KBH_4 or $NaBH_4$, the soluble modified nucleotides (MG-1, MG-2 and MA) or other similar modified nucleotides were not isolated. Use of a buffer or addition of an inorganic salt, such as NaCl, did not change the amount of binding or the product ratio.

The mechanism of formation of MG-1, MG-2 and MA was not established unequivocally, but in view of previous results,[2,4–11] we propose that the mechanism of the reaction is as shown in Chart 4. MMC was reduced by catalytic reduction to the corresponding hydroquinone (**8**). The reduction enhances the electron-donating ability to eliminate the methoxy group. The second product (**9**) facilitates the cleavage of the aziridine ring: position 1 is now benzylic and is activated by many electron-donating groups. The carbonium ion (**10**) formed by opening of the aziridine ring binds covalently at position 1 of mitosene with a heteroatom at

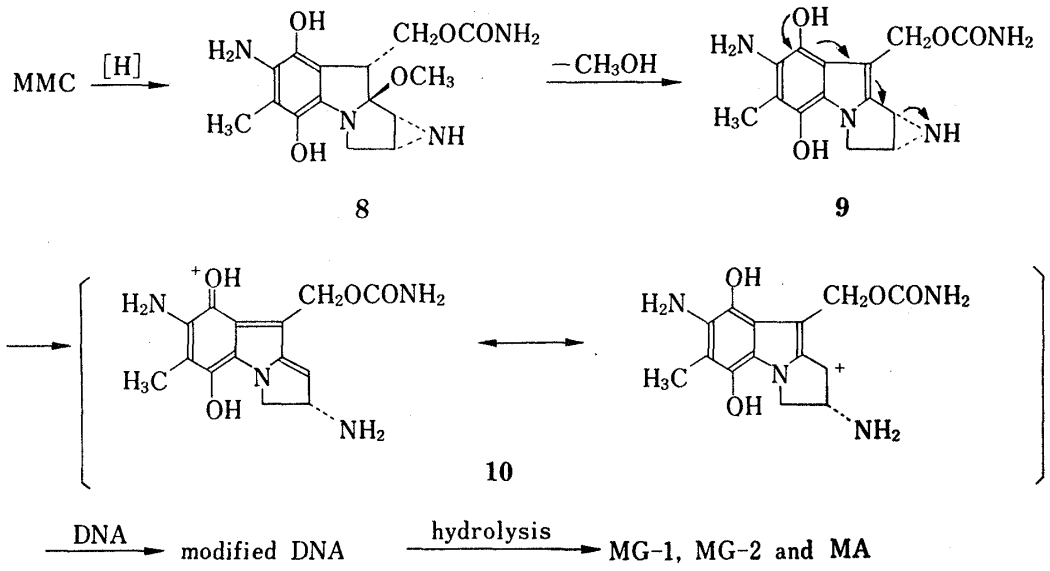


Chart 4

position 2 or 6 of the purine bases. The products might be oxidized by excess MMC or MMC derivatives, or during the work-up procedures to give MG-1, MG-2 and MA.

It is significant that this reaction, established chemically, was also found to occur *in vivo*. The same modified nucleotides, MG-1, MG-2 and MA, were the major modified nucleotides *in vivo* in rats. Incubation of MMC with a rat liver homogenate also gave MG-1, MG-2 and MA in nearly the same ratio as that of the modified nucleotide mixture isolated from liver DNA of a rat treated with MMC. Details of the metabolic pathway *in vivo* are unknown, but the catalytic reduction seems to be a very good model of *in vivo* activation. The report by Tomasz and Lipman[7] that an enzymatic metabolite mixture of MMC resembles the mixture obtained from MMC by catalytic reduction, but not that obtained by reduction with $Na_2S_2O_4$ or $NaBH_4$, suggests that the reaction we have established is an appropriate model of the alkylation reaction of MMC *in vivo*.

We did not obtain any evidence for the participation of position 9a or 10 of mitosene as a binding site in the reaction with DNA, as proposed by Moore[4] and Hornemann *et al.*[5] Tomasz and Lipman reported that all the products of the catalytic reduction have a carbamoyl group at position 10, and the participation of position 10 is secondary.[7] The insoluble precipitate formed by hydrolysis of modified DNA could be cross-linked nucleotides, though a similar precipitate was not formed from the DNA modified *in vivo*.

In the reaction of reductively activated MMC with monomeric 5′-guanylic acid and poly G, modified nucleotides such as MG-1 and MG-2 were not observed; the binding only occurs in natural DNA. Covalent binding of MMC to DNA seems to occur after intercalation of the activated form of MMC into double-stranded DNA.[23] The situation is similar to the cases of some muta-carcinogens.[24] The conformation of the intercalated complex of activated MMC and DNA might determine the configuration and binding site of the products. Elucidation of the conformation of the intercalated complex is a challenging problem. The presence of a masked leaving group, activation of the leaving ability by reduction, and subsequent formation of a strong electrophile are essential molecular requirements. The molecular size and planarity of the activated form of MMC, which regulate the intercalation into DNA, should be important for the action of MMC as a strong alkylating agent.[23]

The present finding represent a first step in understanding the molecular basis of the action of MMC as a bioreductive alkylating agent. The reductive activation and the mode of modification of nucleotides in DNA must, at least in part, play a role in the carcinostatic or

carcinogenic mechanisms of MMC. These findings may contribute to the development of more effective antitumor-active MMC derivatives.

Acknowledgement We are grateful to Kyowa Hakko Kogyo Co. for a generous supply of MMC, and to Dr. Kohfuku Kohda, Faculty of Pharmaceutical Sciences, Nagoya City University, for gifts of methyladenines and methylguanines, and also to the NMR Laboratory of the Institute of Applied Microbiology at the University of Tokyo for measuring the 400 MHz ^{1}H-NMR.

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