

Reaction of DNA with chemically or enzymatically activated mitomycin C: Isolation and structure of the major covalent adduct

(reductive activation/DNA adduct)

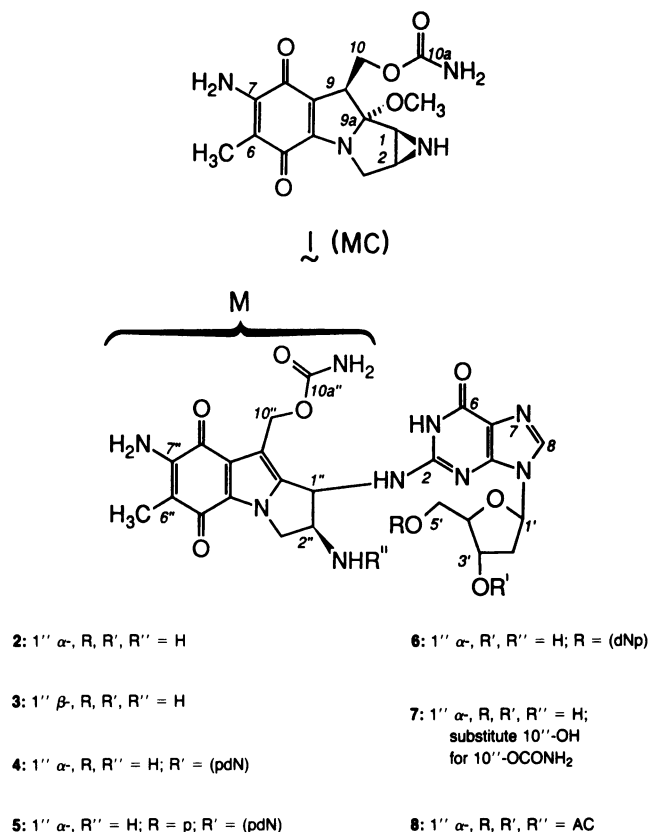
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ABSTRACT The antitumor antibiotic mitomycin C is shown to form a covalent complex with calf thymus DNA under anaerobic conditions in the presence of either NADPH cytochrome *c* reductase/NADPH, xanthine oxidase/NADH, or the chemical reducing system H₂/PtO₂. Digestion of the complex with DNase I/snake venom diesterase/alkaline phosphatase yields a single mitomycin deoxyguanosine adduct as the major DNA alkylation product, identified as N²-(2''β,7''-diaminomitosen-1''α-yl) 2'-deoxyguanosine (Structure 2). Two minor adducts, 2-5% each of the total adduct pool, are isolated and identified as the 1''β stereoisomer of 2 (Structure 3), and 10''-decarbamoyl-2 (Structure 7). The same results were obtained with M13 DNA and poly(dG-dC)·poly(dG-dC); however, in the latter case, a minor adduct apparently possessing two deoxyguanosine and one mitomycin unit is isolated. Digestion of the covalent mitomycin-calf thymus DNA complex with nuclease P1 yields four dinucleotide adducts, all of which consist of 2 linked at its 3' end to each of the four possible 5' nucleotides (A, T, G, and C). Upon treatment of each dinucleotide adduct with snake venom diesterase/alkaline phosphatase, 2 is released along with the corresponding free nucleoside. In apparent conflict with the present results, previous reports from another laboratory have indicated that modification of calf thymus DNA by mitomycin C under conditions identical to those described here result in the isolation of three mitomycin C mononucleotide adducts possessing linkages of the drug to N² and O⁶ of guanine and N⁶ of adenine. Evidence is shown suggesting that the latter adducts are actually three of the above four dinucleotide derivatives of 2 obtained independently by us and, thus, all of them in fact possess an identical N²-mitosenyguanone adduct moiety. Model-building studies indicate an excellent fit of the guanine N²-linked drug molecule inside the minor groove of B-DNA with no appreciable distortion of the DNA structure.

Mitomycin C (MC; Structure 1), a potent antibiotic and clinically used antitumor agent, is known to interact covalently with DNA *in vivo* and *in vitro*. This is manifested by the reversible melting behavior of MC-exposed DNA, attributed to formation of covalent crosslinks between the complementary strands (1) and by covalent association of the ultraviolet chromophore of MC to DNA (2). These processes require reduction of MC into a transiently activated form, which is thought to occur in cells and can be mimicked easily *in vitro* chemically or enzymatically (1). The cytotoxicity of MC is most likely a direct result of DNA alkylation, as indicated by the parallels in biological activity of MC with a number of known "DNA damaging agents": selective inhibition of DNA replication (1), strong induction of the SOS response (3)



and sister chromatid exchange (4), and cross-resistance or cross-hypersensitivity of bacterial (1, 5) and mammalian (6) cells to UV light and MC.

The molecular nature of the covalent interactions between MC and DNA has remained elusive, mainly because of the difficulty of isolating low molecular weight adducts of MC with DNA. MC-modified DNA is rather resistant to degradation by nucleases, while chemical cleavage methods using acid or alkali irreversibly destroy the bound MC moiety (e.g., see ref. 2). Studies *in vivo* have especially been hindered by the unavailability of radioactive MC.

Recently, a deoxyguanosine-MC adduct arising from the model reaction of reductively activated MC (microsomes/NADPH or H₂/PtO₂) with dinucleoside phosphate d(GpC) was isolated in our laboratory. Its structure was rigorously established as N²-(2''β,7''-diaminomitosen-1''α-yl),2'-deoxyguanosine (the term "mitosene" refers to the bracketed structure in 2 without substituents at the 1'', 2'', and 7''

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Abbreviations: MC, mitomycin C; M, 2β,7-diaminomitosen-1-yl; SVD, snake venom diesterase; br, binding ratio (mol of MC per mol of mononucleotide); poly(dG-dC), poly(dG-dC)·poly(dG-dC).

positions) (Structure 2) (7, 8). [In our first report (7), the M moiety was stated to be linked to the O⁶ rather than N² position of guanine. A reinvestigation of this structural assignment, however, revealed that the correct structure is 2 (8).] With this reference adduct in hand, we turned to the isolation and characterization of adducts formed from treatment of DNA with MC under analogous activation conditions. We report here that reductively activated MC binds with extraordinary specificity to the N² position of guanine residues in DNA, yielding upon enzymatic digestion the previously elucidated monofunctional adduct 2; furthermore, 2 accounts singularly for 90% or more of the total adduct population.

These findings are in disagreement with reports from Shudo's laboratory, published while this study was in progress, stating that three MC DNA adducts are formed in roughly equal amounts under comparable conditions; i.e., bearing the M moiety linked to the N² and O⁶ positions of guanine, and the N⁶ position of adenine (9). Our thorough reinvestigation of the published experimental procedures (9) indicates that the three substances were apparently different dinucleotide derivatives of the same deoxyguanosine adduct 2 possessing the common general structure d[pG(M)pN] (Structure 5). Our findings presented here indicate that the N² position of guanine is virtually the exclusive site of attack on DNA by reductively activated MC.

MATERIALS AND METHODS

Materials and their sources are as follows: Calf thymus DNA (type I, sonicated before use), bacterial alkaline phosphatase (type III-R), and xanthine oxidase (grade III) were from Sigma; M13 DNA (strain BK8) was a gift of M. Z. Humayun (New Jersey College of Medicine, Newark, NJ); poly(dG-dC), d(GpA), d(GpT), d(GpC), d(CpG), and nuclease P1 were from Pharmacia P-L Biochemicals; DNase I and SVD (phosphodiesterase I) were from Cooper Biomedical (Malvern, PA). 10-Decarbamoyl-MC was synthesized as described (10). Authentic samples of adduct 2 were prepared as described (8).

Preparation of MC-DNA or MC-Poly(dG-dC)-Poly(dG-dC) [Poly(dG-dC)] Complexes under Various Reductive Activating Conditions. (i) H₂/PtO₂ activation: DNA (0.67 μmol/ml), MC (0.67 μmol/ml), and PtO₂ catalyst (100 μg per μmol of MC) were mixed in 0.015 M Tris·HCl (pH 7.4) and hydrogenated by a published procedure (7). Isolation and determination of the binding ratio (br; mol of MC per mol of mononucleotide) of the resulting MC-DNA complex was accomplished as described (2). (ii) NADH xanthine oxidase activation: DNA (1.0 μmol/ml), MC (1.0 μmol/ml), and NADH (2 μmol/ml) in 0.02 M sodium phosphate were incubated with xanthine oxidase (0.4 unit/ml) for 20 min at 37°C under a helium atmosphere. Isolation and determination of br of the complex was done as described in *i*. (iii) NADPH cytochrome *c* reductase activation was analogous to that in *ii*.

Digestion of MC-DNA Complexes by Various Nuclease Combinations. (i) DNase/SVD/alkaline phosphatase: MC-DNA complex in 0.005 M Tris·HCl/0.001 M MgCl₂, pH 7.0 (3 A₂₆₀ units/ml), was digested at 37°C with enzymes according to the following protocol: DNase I (16 units/A₂₆₀ unit) at 0 hr and 1 hr; SVD (1.25 units/A₂₆₀ unit; pH increased to 8.2) at 2 hr and 5 hr; alkaline phosphatase (0.5 unit/A₂₆₀ unit) at 7 hr, incubation continuing until 24 hr. (ii) Nuclease P1: MC-DNA complex (3 A₂₆₀ units/ml) in dilute acetic acid (pH 5.5) was incubated with nuclease P1 (0.5 unit/A₂₆₀ unit) at 55°C for 2 hr. In another experiment designed to duplicate the previously reported conditions (9), 8 mg of MC-DNA complex per ml and 6 mg of nuclease P1 per mg of DNA was used, corresponding to a 50-fold higher concentration of

DNA and 16-fold higher concentration of nuclease P1 than above. (iii) Nuclease P1 alkaline phosphatase: The nuclease P1 digest solutions were brought to pH 8.2 by addition of 0.5 M Tris·HCl (pH 8.2). Alkaline phosphatase (1.6 units/A₂₆₀ unit) was added and the digestion was allowed to proceed for 2 hr at 37°C.

HPLC Separations. A reversed-phase column (Beckman Ultrasphere ODS; 1.0 × 25 cm) was used; flow rate was 2.0 ml/min; eluant was 8:92 CH₃CN/0.02 M potassium phosphate, pH 5.0, unless otherwise noted.

Synthesis of the MC-Dinucleoside Phosphate Adducts 4 and 6. MC was allowed to react with either d(GpC), d(GpA), d(GpG), d(GpT), or d(CpG) under activation by H₂/PtO₂ as described for the reaction of MC with d(GpC) (7). The resulting adducts were purified to homogeneity by HPLC.

Digestion of MC-Dinucleoside Phosphate Adducts 4 and 6 by Various Nuclease Systems. (i) The adduct (0.5–3.0 A₂₆₀ units/ml), SVD (2.5 units/A₂₆₀ unit), and alkaline phosphatase (1.3 units/A₂₆₀ unit) were incubated in 0.005 M Tris·HCl/0.001 M MgCl₂, pH 8.5, at 37°C for 2 hr. (ii) Nuclease P1/alkaline phosphatase digestion was carried out as described above for MC-DNA complexes.

RESULTS

Formation of MC-DNA Complexes under Chemical or Enzymatic Reductive Activation Conditions. Brief treatment of a mixture of MC and calf thymus DNA in neutral buffer with either H₂/PtO₂, NADPH cytochrome *c* reductase, or xanthine oxidase resulted in covalent complex formation of MC with DNA. Such variation of the reductive activating agent did not significantly affect the br, which was typically in the range 0.04–0.07. M13 DNA and denatured calf thymus DNA gave similar results. Poly(dG-dC), however, formed complexes that exhibited a higher br (0.10–0.12).

Adduct HPLC Patterns from Various DNA-MC Complexes Digested with DNase I/SVD/Alkaline Phosphatase. The digest of the calf thymus DNA-MC complex (br, 0.07) was analyzed by HPLC (Fig. 1*a*). With the exception of the early-eluting unmodified nucleosides, only one major peak is evident in the pattern, at 45 min. The two minor peaks, at 39 and ≈120 min, each represent <5% of the peak area of the 45-min major peak.

MC-calf thymus DNA complexes formed under enzymatic activation (NADPH cytochrome *c* reductase or xanthine oxidase) yielded patterns (Fig. 1*b* and *c*) essentially identical to those obtained using H₂/PtO₂ (Fig. 1*a*). M13 DNA and denatured calf thymus DNA also gave similar patterns (not shown). MC-poly(dG-dC) complexes (H₂/PtO₂ or NADPH cytochrome *c* reductase) also yielded the same pattern of adducts (Fig. 1*d*), except for an additional minor adduct peak marked X.

Identification of the Major MC-DNA Adduct as 2. The major component of each of the adduct patterns described above (45-minute peak; Fig. 1*a–d*) was isolated by either HPLC or Sephadex G-25 chromatography (7). Direct comparisons of this material with authentic 2 (7, 8) were made with respect to the following criteria: (i) HPLC elution time: identical (Fig. 1*e*); single homogeneous peak when mixed with authentic 2. (ii) Sephadex G-25 elution volume (7): identical. (iii) UV spectra (Fig. 2) obtained at three pH values: identical. (iv) Conversion to adduct triacetate 8 shows a very good comparison between the ¹H NMR spectra of 8 obtained in this work and an authentic sample (8) (Fig. 3). Furthermore, the HPLC retention times, fast atom bombardment mass spectra, and Fourier transform IR spectra (not shown; see ref. 8) of authentic and DNA-derived 8 were virtually identical.

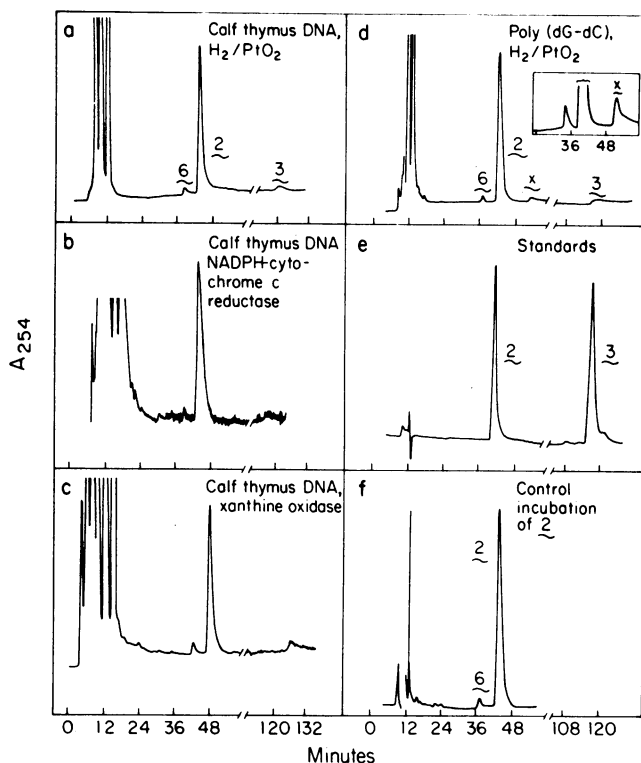


FIG. 1. HPLC patterns from DNase I/SVD/alkaline phosphatase digests of various MC-DNA and MC-poly(dG-dC) complexes. Digests of MC-calf thymus DNA complexes formed under reductive activation by H_2/PtO_2 (a), NADPH cytochrome *c* reductase (b), xanthine oxidase (c). (d) Digest of MC-poly(dG-dC) complex (H_2/PtO_2 activation). (e) Authentic standards 2 and 3. (f) Adduct 2 incubated with DNase I/SVD/alkaline phosphatase.

Elucidation of Minor Adducts. The minor adduct eluting at 120 min (Fig. 1 *a-d*) was identified as 3, the 1'- β stereoisomer of 2 by direct comparison of its HPLC retention time (Fig. 1*e*) and characteristic UV with authentic 3 (8). The 39-min minor adduct (Fig. 1 *a-d*) was identified as 7, the 10'-decarbamoyl derivative of 2 by the following: (i) When authentic pure 2 was incubated under the same conditions as those used to digest the MC-DNA complex, a minor peak appeared in the HPLC at 39 min, in addition to major unchanged 2 at 45 min (Fig. 1*f*). (ii) This same 39-min peak was the single major adduct obtained when 10'-decarbamoyl-MC was substituted for MC in the complexation reaction with DNA or poly(dG-dC) (data not shown). These results strongly suggest that 7 is formed from 2 via hydrolysis of the 10'-carbamoyl substituent during the enzymatic digestion of the MC-polynucleotide complex.

Nuclease P1 Digestion Products of the MC-DNA Complex.

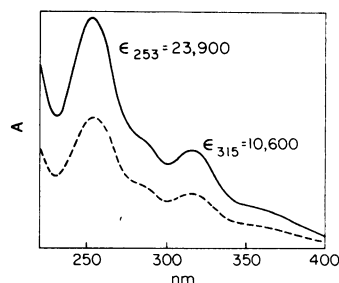


FIG. 2. Comparison of the UV spectra of synthetic 2 with that obtained from enzymatic digestion of MC-calf thymus DNA. Buffer: 0.01 M potassium phosphate (pH 7.0).

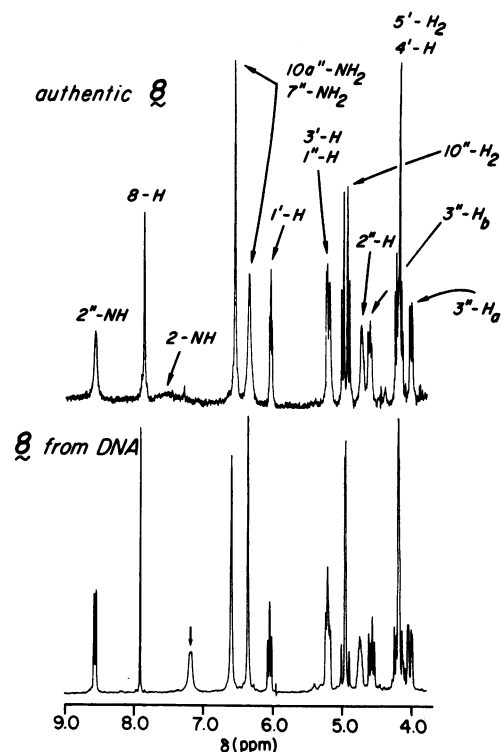


FIG. 3. 1H NMR spectra (9.0–3.7 ppm region) of adduct triacetate 8. Upper spectrum: authentic 8 (8). Lower spectrum: 8 obtained by acetylation (8) of DNA-derived adduct 2. Solvent: Me_2SO-d_6 ; chemical shifts: ppm downfield from external tetramethylsilane. The upper spectrum was recorded on a JEOL FX-400 spectrometer (400 MHz); the lower one was recorded on a Bruker WM-250 instrument (250 MHz); because of the difference in field strength, signals in the upper spectrum appear narrower. The 2-NH signal is broadened in the spectrum of authentic 8 relative to DNA-derived 8 (arrow); differences in the width and position of exchangeable protons are routinely observed depending on sample preparation (e.g., differences in H_2O and traces of acid).

A MC-calf thymus DNA complex was prepared by careful reiteration of the experimental procedure of Hashimoto and co-workers including the use of 5% Pd on charcoal, instead of PtO_2 as hydrogenation catalyst (9). The MC-modified calf thymus DNA thus obtained (br, 0.02) was digested with nuclease P1 strictly according to Hashimoto *et al.* (ref. 9; see also *Materials and Methods*). An aliquot of this digest was analyzed by HPLC (10:90 $CH_3CN/0.3\%$ aqueous NH_4Cl ; ref. 9), giving rise to the pattern in Fig. 4*a*. With a more polar eluant (6:94 $CH_3CN/0.3\%$ aqueous NH_4Cl) to distribute the products over a larger retention time, the apparent triplet at >11 min (Fig. 4*a*) resolved into a complex series of products with various peak intensities and widths; thus, it is clear that the "triplet" in Fig. 4*a* is actually composed of a larger number of overlapping components.

Dephosphorylation of Nuclease P1 Digestion Products with Alkaline Phosphatase. Another aliquot of the nuclease P1 digest was treated with alkaline phosphatase. HPLC of this digest resulted in the trace shown in Fig. 4*b*. Peaks 1–4 exhibited elution times that were identical to members of the authentic synthetic dinucleoside phosphate adduct series 4 (i.e., dN = dA, dG, dC, and dT; Fig. 4*b*, *Inset*). No MC adduct was eluted before peak 1.

Nuclease P1/alkaline phosphatase digestion of a MC-poly(dG-dC) complex (br, 0.12) gave rise to a single detectable MC-linked dinucleoside phosphate 4 (dN = dC) (Fig. 4*d*), which was identical to peak 1 in Fig. 4*b*.

Adducts from Nuclease P1 Digestion Are Further Degradable to 2 by SVD/Alkaline Phosphatase. An aliquot of the

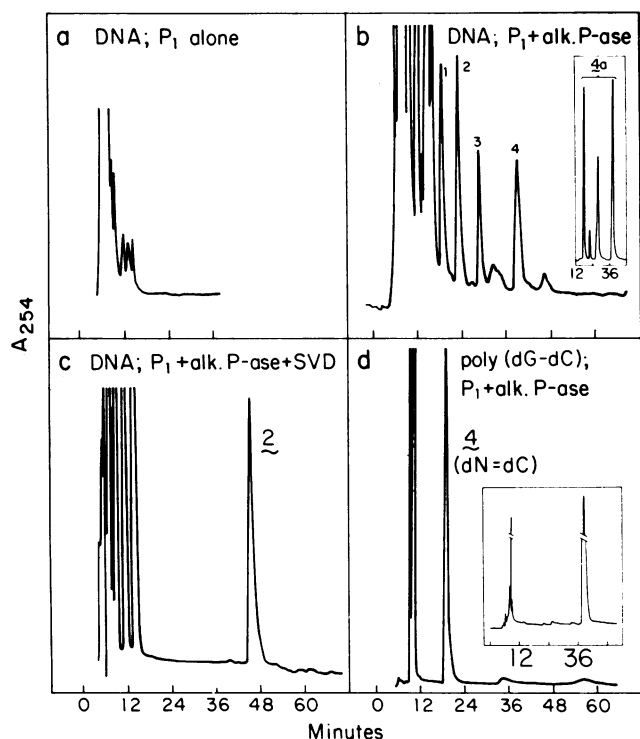


FIG. 4. HPLC of digests of MC-calf thymus DNA and MC-poly(dG-dC) using several nuclease treatment combinations. (a) MC-calf thymus DNA complex (br, 0.02; $H_2/Pd-C$ activation) digested with only nuclease P1. (b) Complex in a digested further with alkaline phosphatase. (Inset) The four synthetic adducts, 4 (dN = dC, dG, dT, and dA, in respective order of increasing elution time). (c) Same complex as in a and b digested further with SVD/alkaline phosphatase. (d) MC-poly(dG-dC) complex (br, 0.12; H_2/PtO_2 activation) digested with nuclease P1/alkaline phosphatase. (Inset) Further digestion of the purified 4 peak (dN = dC) with SVD/alkaline phosphatase; the early eluting peak is deoxycytidine and the late eluting peak is 2.

above nuclease P1 digest was adjusted to 0.001 M $MgCl_2$ and pH was adjusted to 8.5, and was incubated with alkaline phosphatase (0.5 unit/ A_{260} unit) and SVD (2.5 units/ A_{260} unit) for 2 hr at 37°C. Direct HPLC analysis (Fig. 4c) indicated essentially a single adduct, 2, as judged by its spectral and chromatographic properties. Application of the same digestion conditions to any of the four isolated nuclease P1/alkaline phosphatase products (Fig. 4b; peaks 1-4) results

in the quantitative release of 2 and the corresponding nucleoside in a 1:1 ratio; for example, treatment of pure peak 1 (4; dN = dC) (Fig. 4d) gave rise to the early eluting deoxycytidine peak and a later eluting peak, 2 (Inset).

Summary: The Adducts from Nuclease P1/Alkaline Phosphatase Digestion Are MC-Dinucleoside Phosphates, 4. This is concluded from their identity with the authentic synthetic substances with respect to the following properties: (i) HPLC retention times; (ii) degradation by alkaline phosphatase/SVD to 2 and deoxynucleoside (molar ratio, 1:1) (e.g., see Fig. 4d); (iii) resistance to nuclease P1.

Differential Susceptibility of Dinucleoside Phosphate Adducts d[G(M)pN] 4 and d[NpG(M)] 6 to Nuclease P1 but Not to SVD. Both 4 (dN = dC) and 6 (dN = dC) were incubated with nuclease P1, followed by alkaline phosphatase, and analyzed directly by HPLC. Although 4 remained unchanged even in the presence of a 100-fold increase of nuclease P1 concentration, 6 was degraded readily to 2 and deoxycytidine. On the other hand, SVD/alkaline phosphatase degraded both 4 (dN = dC) and 6 (dN = dC) to 2 and deoxycytidine.

Characterization of the Minor Adduct X (Fig. 1d). The UV spectrum of X was very similar to that of 2 (Fig. 2) except that the A_{252}/A_{310} ratio was higher in the former (2.8 vs. 2.3, respectively). Base analysis (not shown) indicates guanine as the only base in X.

DISCUSSION

These results demonstrate unambiguously that the major covalent adduct formed between MC and DNA *in vitro* in a variety of systems is the guanine N^2 -linked mitosene 2. The other two minor adducts also bear a guanine N^2 linkage (3, 7); 7 is a secondary degradation product of 2, whereas 3 is the 1'- β stereoisomer of 2, arising from attack of the guanine NH_2 group from the β rather than the α side of activated MC. Model-building studies (not shown) indicate severe steric hindrance to this mode of binding to native B-DNA structure. Therefore, 3 may actually be formed preferentially in transiently denatured segments of DNA. Accordingly, twice as much 3 was obtained from denatured as from native DNA. The reaction between reductively activated MC and deoxyguanosine itself yields 2 and 3 in almost equal amounts (8).

Our finding that the N^2 position of guanine is the only detectable site of binding of reductively activated MC in DNA is in conflict with a previous report by Shudo and coworkers (9). Reinvestigation of the reported work revealed a likely source of error as follows: according to the authors (9), nuclease P1 digestion of MC-modified calf thymus DNA

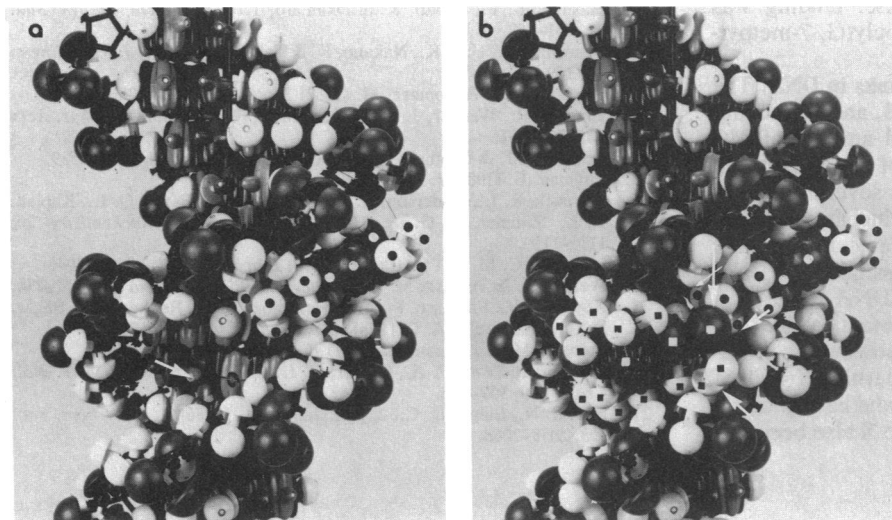


FIG. 5. Proposed model of the MC-DNA complex using space-filling models (Molecular Design, Inc.; Academic Press). (a) View of adduct 2 incorporated into DNA; the bound M moiety is indicated by dots; the 2'- NH_3^+ group is located on the left, the 10'- $CONH_2$ group is on the right. (b) Two molecules of adduct 2 incorporated into DNA at a $-C_1G_1C_2G_2-$ sequence. Dots and squares indicate the atoms of M_1 and M_2 , respectively. Arrows (clockwise from top): H bond between 10a'-carbonyl oxygen of M_2 and 2'- NH_3^+ of M_1 ; two H bonds between 2'- NH_3^+ of M_1 and O^2 atoms of C_2 and the C opposite G_1 ; H bond between 10a'- NH_2 of M_2 and 3'-O of G_2 . Also apparent in the model but occluded in the photo is an H bond between 10'-O of M_2 and 2- NH_2 of the G opposite C_1 . The M molecules are shown in the probable "head-to-tail" orientation; each M occupies exactly the minor groove space associated with 2 base pairs.

gave a three-peak pattern on HPLC, the components of which were used directly for structural studies and were concluded to be mononucleotides substituted by MC at N² and O⁶ of guanine and N⁶ of adenine, respectively. In our hands, this nuclease P1 digest gave a similar HPLC pattern (Fig. 4a). Upon further analysis, however, we have shown that these three peaks actually consist of four MC dinucleotide adducts, rigorously identified by several lines of evidence as 4 (dN = dG, dA, dC, and dT; all four are formed in native DNA; Fig. 4b). No conditions could be found for hydrolysis of the 3'-phosphodiester bond in 4 (isolated or synthetic) or in 5 by nuclease P1. Apparently, the presence of a N²-M-Gua residue totally inhibits the cleavage of its 3' phosphodiester linkage by nuclease P1. Thus, the previous structure determination (9) may have inadvertently used MC dinucleotides 5 rather than what were believed to be MC mononucleotides, leading to conflicting interpretations of the resulting data. Direct comparison of samples from the two laboratories is planned for definitive conclusion on this issue.

The three-dimensional features of adduct 2 in double-stranded DNA were studied by space-filling molecular models, leading to the remarkable result that in one unique conformation the N²-Gua-bound M residue fits snugly into the minor groove without appreciable perturbation of DNA structure (Fig. 5). In the MC model, the 2'' NH₃⁺ group lies inside the minor groove and four H bonds are seen between functional groups of the M residue and the DNA (see legend of Fig. 5). This model is consistent with a number of previous experimental observations about MC-modified DNA as follows: (i) Intercalation by covalently bound M residues was ruled out by linear flow dichroism studies (11). (ii) Binding of MC was uninhibited to T2 DNA (12), even though the major groove is severely blocked by glucosylated 5-hydroxymethyl cytosines. (iii) MC-modified DNA is more stable than native DNA, as indicated by increased melting temperature (2, 13); the favorable secondary interactions of covalently anchored M units with the surrounding minor groove readily account for this fact. The location of the 2'' NH₃⁺ group inside the groove—i.e., the site of minimum electrostatic potential—is especially favorable (14). The stabilizing effect of bound MC on DNA duplex structure is analogous to that of anthracycline, a molecule of similar size and binding site (15). (iv) The experimental saturation binding ratio of MC to poly(dG-dC) is 0.25 (i.e., one MC every 2 base pairs) (16). According to the proposed model, at a (CG)_n sequence the guanine of every second base pair may be substituted by MC; this way, the drug molecules fill the minor groove completely, with head-to-tail H bonds to one another (Fig. 5b), in agreement with the experimental saturation br.

Results with synthetic polyribonucleotide analogs are also consistent with guanine-N² as binding site: binding was uninhibited to poly(O⁶-methylguanine) or poly(G, 7-methylguanine) but inhibited to poly(I) (17).

On the Origin of the MC-Induced Crosslinks in DNA. The monofunctional adducts reported here, 2, 3, and 7, account essentially quantitatively for the observed amount of MC bound to DNA. What is then the origin of the interstrand crosslinks of MC, which are detectable by several physical methods (1) but the molecular structure of which has not been demonstrated to date? Presumably, the resolution of this conflict lies in the very low incidence of crosslinking of DNA by MC: typically, <1 crosslink per 10⁶-Da DNA (1); thus, a "crosslink"-type adduct could escape detection by UV methods. In the MC-poly(dG-dC) digests, however, a promising crosslink candidate, X, is clearly discernible (≈5% of monofunctional adducts) [based on the UV and base analysis data (see Results)]. A peak corresponding to X also becomes

evident in native DNA-MC complexes, which have higher br values (data not shown).

Activation of MC *in Vitro* vs. *in Vivo*. MC-reducing activity, which catalyzes the formation of MC metabolites as well as covalent binding and crosslinking of MC to DNA, has been demonstrated in bacterial and mammalian cell extracts (1). NADPH cytochrome *c* reductase was specifically implicated in intracellular activation of MC (18). Microsomal preparations (19) as well as purified flavoenzymes (xanthine oxidase, NADPH cytochrome *c* reductase; ref. 20) or H₂/PtO₂ (19) catalyze the formation of an identical series of MC metabolites and, as shown here, identical DNA modifications *in vitro*. It is most likely, therefore, that the DNA-modifying effects of MC *in vivo* are similar or identical to those described here. The elucidation of the nature of the adducts and their analytical properties now opens the way to MC adduct analysis *in vivo*. Such analyses will be essential to the search for correlations between the biological effects of MC and its covalent modifications of DNA. Furthermore, these methods of adduct analysis should be adaptable to MC analogs, currently of considerable interest in cancer chemotherapy.

Note. After this manuscript was completed, molecular mechanics simulation of M bound to N² of guanine in d(GC)_n was published (21). The orientation of M is very similar to that in our model, but only two of the four H bonds (Fig. 5) were observed by these authors.

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