A Mitomycin−N^6-Deoxyadenosine Adduct Isolated from DNA

Yolanda Palom,† Roselyn Lipman,† Steven M. Musser,‡ and Maria Tomasz*,†

Department of Chemistry, Hunter College, City University of New York, New York, New York 10021, and U.S. Food and Drug Administration, 200 C Street, S.W., Washington, D.C. 20204

Received November 13, 1997

A minor N^6-deoxyadenosine adduct of mitomycin C (MC) was isolated from synthetic oligonucleotides and calf thymus DNA, representing the first adduct of MC and a DNA base other than guanine. The structure of the adduct (**8**) was elucidated using submilligram quantities of total available material. UV difference spectroscopy, circular dichroism, and electrospray mass spectroscopy as well as chemical transformations were utilized in deriving the structure of **8**. A series of synthetic oligonucleotides was designed to probe the specificities of the alkylation of adenine by MC. The nature and frequency of the oligonucleotide−MC adducts formed under conditions of reductive activation of MC were determined by their enzymatic digestion to the nucleoside level followed by quantitative analysis of the products by HPLC. The analyses indicated the following: (i) $(A)_n$ sequence is favored over $(AT)_n$ for adduct formation; (ii) the alkylation favors the duplex structure; (iii) at adenine sites only monofunctional alkylation occurs; (iv) the adenine-to-alkylation frequency in the model oligonucleotides was 0.3−0.6 relative to guanine alkylation at the 5′-ApG sequence but only 0.02−0.1 relative to guanine alkylation at 5′-CpG. The 5′-phosphodiester linkage of the MC−adenine adduct is resistant to snake venom diesterase. The overall ratio of adenine to guanine alkylation in calf thymus DNA was 0.03, indicating that **8** is a minor MC−DNA adduct relative to MC−DNA adducts at guanine residues in the present experimental residues in the present experimental system. However, the HPLC elution time of **8** coincides with that of a major, unknown MC adduct detected previously in mouse mammary tumor cells treated with radiolabeled MC [Bizanek, R., Chowdary, D., Arai, H., Kasai, M., Hughes, C. S., Sartorelli, A. C., Rockwell, S., and Tomasz, M. (1993) *Cancer Res.* **53**, 5127−5134]. Thus, **8** may be identical or closely related to this major adduct formed in vivo. This possibility can now be tested by further comparison.

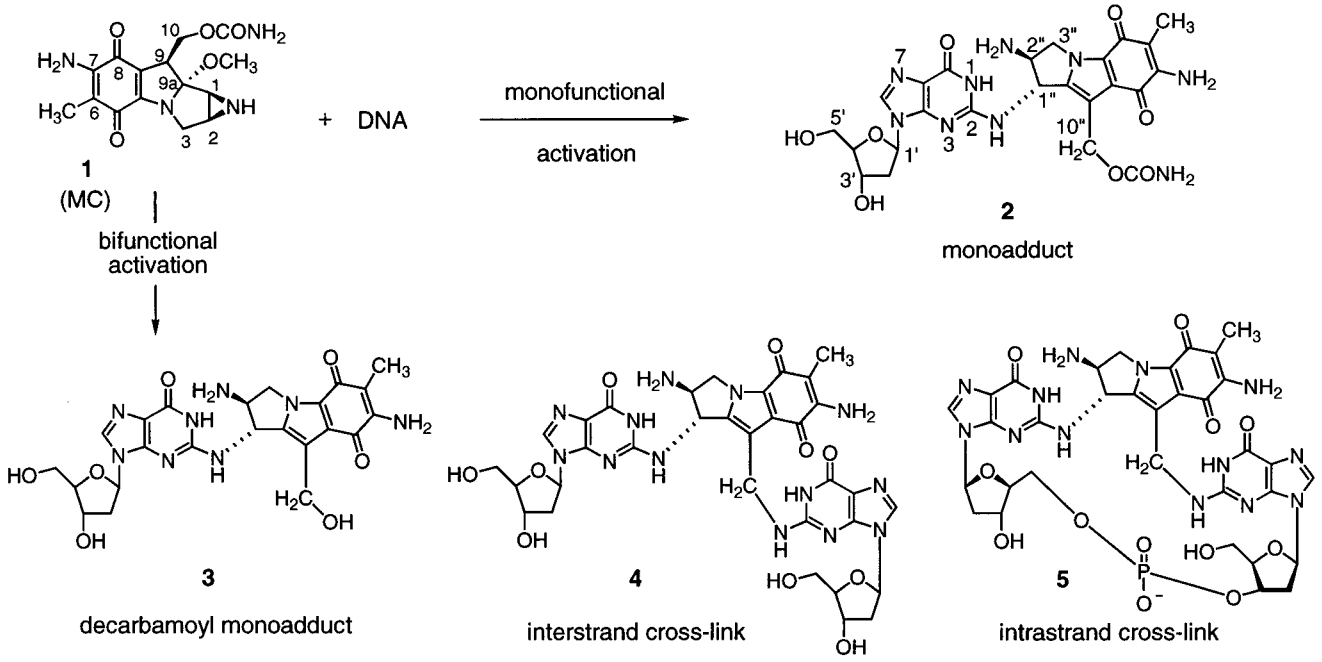
A large variety of complex natural products have been isolated from microorganisms which are capable of alkylating DNA. Such substances are highly cytotoxic and usually possess antitumor activity. A characteristic property of these natural products which contrasts them generally with synthetic DNA-reactive drugs and carcinogens is the high specificity of their covalent reaction with DNA. This is manifested by specificity to a single base and by base regioselectivity. Furthermore, selectivity is observed for a short DNA base sequence surrounding the target base. Typical examples of such selective natural DNA alkylators include the guanine N^2-specific drug anthramycin (*1*), the adenine N3-specific drugs CC-1065 (*1*) and duocarmycin (*2*), the guanine N7-specific family of the pluramycins (*3*), and the subject of the present report, mitomycin C. Mitomycin C (MC,[1] **1**), a clinically used anticancer agent, has been shown to alkylate DNA exclusively at the 2-amino group of guanines in in vitro systems. Its bifunctional alkylating activity results in the formation of several different guanine N^2 adducts: monoadducts **2** and **3** and the DNA interstrand (**4**) and DNA intrastrand (**5**) cross-link bisadducts (Scheme 1). The DNA interstrand cross-links are formed exclusively between two guanines in the CpG·CpG sequence. (For a recent review, see ref *4*.) High specificity, but of a different kind, was observed in the alkylation of DNA by the naturally occurring metabolite of MC, 2,7-diaminomitosene (**6**). This MC metabolite, which is formed in mammalian tumor cells and tissues from MC (*5*), lacks the primary alkylating function of MC, namely, the 1,2-aziridine. The remaining single alkylating function of the molecule, i.e., the C-10 carbamate, is activated by reduction to alkylate exclusively the guanine N7 position in the major groove, with a sequence specificity for guanines in $(G)_n$ runs (Scheme 2; *6*, *7*). All adducts of MC (Scheme 1; *8*−*10*) and of 2,7-DAM (Scheme 2; *11*) have been shown to be formed also in vivo.

In contrast to natural products, synthetic antitumor agents and carcinogens usually alkylate DNA with less specific reactivity, at several different nucleophilic sites of DNA. For example, *N*-alkyl-*N*-nitrosoureas alkylate A N3, G O^6, G N7, T O^2, C O^2, and phosphate groups (*12*). Aziridinylbenzoquinones DZQ and MeDZQ alkylate G N7 and an unknown position of adenines equally (*13*). The carcinogen styrene oxide reacts with the exocyclic amino groups of both guanine and adenine (*14*); a benzo[*a*]pyrenediol epoxide has been observed to react with three different positions of guanine as well as with

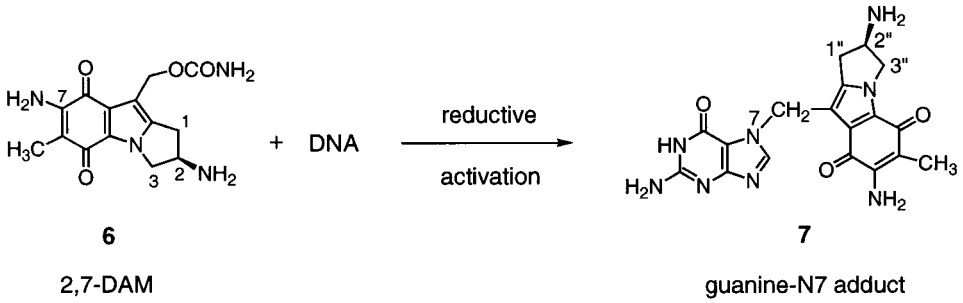
† Hunter College, City University of New York.
‡ U.S. Food and Drug Administration.
[1] Abbreviations: MC, mitomycin C; 2,7-DAM, 2,7-diaminomitosene; SVD, snake venom diesterase; AP, alkaline phosphatase; P_1, nuclease P_1; M, mitosene as substituent.

S0893-228x(97)00205-1 CCC: $15.00
Published on Web 02/06/1998

Scheme 1. MC−Guanine Adducts



Scheme 2. 2,7-DAM−Guanine Adduct



adenine (*15*). The greater specificity of natural antibiotics probably reflects evolutionary processes leading to the generation of effective cytotoxins for selective advantage to the producing microorganisms.

While these distinctions between natural and synthetic DNA-alkylating agents hold qualitatively, exceptions have been noted. For example, duocarmycin A [but not duocarmycin SA (*2*)] was reported to yield minor G N3 adducts besides major A N3 adducts (*16*). We report here that MC forms a minor adenine adduct, by alkylating the exocyclic 6-amino group of deoxyadenosine residues in DNA. Discovery of this minor adduct of MC is potentially important. Although the DNA cross-linking action of MC, resulting in the major bisguanine adduct **4**, is likely to be the critical cause of cell death, the relative roles of the various guanine monoadducts and cross-links in MC's cytotoxicity and mutagenicity have not been systematically assessed. It is possible that a minor adduct such as this one plays a disproportionately large role in one or another effect, as has been observed with other mutagens and cytotoxins. Its present characterization will aid in detecting its formation in vivo as well as in assessing its specific biological effects.

Experimental Section

Materials. Mitomycin C was obtained from Dr. D. M. Vyas, Bristol-Myers Squibb Co., Wallingford, CT.

Oligodeoxyribonucleotides were synthesized on an automated DNA synthesizer (model 380B, Applied Biosystems, Inc.), using the β-cyanoethyl phosphoramidite method. The crude products (1-μmol scale, "trityl-off"), after deprotection by concentrated NH_4OH overnight at 55 °C, were purified using a Sephadex G-25 (fine) column (2.5 × 56 cm; 0.02 M NH_4HCO_3 eluant). The void volume fraction containing the oligonucleotide was lyophilized. HPLC on a C-4 reverse-phase column indicated ≥95% purity of the oligonucleotides. Nuclease P_1, NADH-cytochrome *c* reductase, NADH, calf thymus DNA (type 1), 2′-deoxyadenosine, N^6-methyl-2′-deoxyadenosine, and N^6-(2-isopentenyl)adenosine were obtained from Sigma; snake venom diesterase (phosphodiesterase I) and *Escherichia coli* alkaline phosphatase (type III-R) were from PL-Worthington, Freehold, NJ. Calf thymus DNA was sonicated before use.

Methods. Quantitative Analysis. Quantities of nucleosides, oligonucleotides, DNA, and MC were measured by UV spectrophotometry using the following molar extinction coefficients: dA, 13 300; dG, 13 000; dT, 6600; dC, 6300 (all at 254 nm); calf thymus DNA, 6500 (260 nm); oligonucleotides, 10 000 (260 nm; average value for one mononucleotide unit; thus, 10 A_{260} units of an oligonucleotide correspond to 1 μmol in mononucleotide); 2,7-DAM (**6**), 10 300 (314 nm, H_2O); adducts **8** and **2**, 24 000 (254 nm).

Spectroscopic Techniques. UV spectra were determined by the HPLC diode array method: A Beckman System Gold 165 UV−visible diode array scanning detector was used for recording the spectra of substances eluted from a Beckman Ultrasphere ODS, C-18 HPLC column (4.5 × 250 mm) with 0.03 M potassium phosphate, pH 5.5, containing 6−18% acetonitrile in a linear

gradient. For calculating UV difference spectra, UV spectra were normalized to equal intensity at 320 nm before subtraction of one from the other, using the Beckman System Gold Chromatography software for these computations.

LC/ESIMS Analysis. A Hewlett-Packard (Palo Alto, CA) model 1050 LC pump was used to provide linear gradients and a constant flow rate of 200 μL/min. All chromatography was performed on a YMC Inc. (Wilmington, NC) J-sphere ODS-M80 column (2 × 250 mm) packed with 4-mm particles. A 40-min gradient starting with 8% MeCN−92% H_2O, 10 mM ammonium formate (pH 6.5) and ending with 30% McCN−70% H_2O, 10 mM ammonium formate (pH 6.5) was used for the elution of metabolites. Mass spectrometry was performed on a Finnigan (San Jose, CA) model TSQ-7000 triple-quadrupole mass spectrometer equipped with a standard Finnigan electrospray ion source. UV (254 nm)-absorbing compounds were detected in-line prior to entry into the mass spectrometer. Mass spectra were acquired in the positive ion mode at a rate of 2 scan/s over a mass range of 150−1200 Da.

Circular Dichroism Spectra. A Jasco model J710 spectropolarimeter connected to a Digital Equipment Corp. personal computer was employed.

HPLC. A Beckman instrument equipped with variable wavelength detector and peak integrator was used.

Reductive Alkylation of Adenine Residues of Oligonucleotides by MC. Isolation of the Products by HPLC after Enzymatic Digestion of the Alkylated Oligonucleotides. (i) Monofunctional MC-Activating Conditions (substoichiometric $Na_2S_2O_4$) (*18*): Oligonucleotide (2 mM in mononucleotide units, usually 1 μmol; 10 A_{260} unit scale), MC (8 mM), and $Na_2S_2O_4$ (2 mM; taken from a more concentrated freshly prepared anaerobic solution) in 0.1 M sodium phosphate, pH 7.5, buffer were incubated for 1 h at 0 °C. Under these conditions complementary oligonucleotides were in the duplex form. After 1 h the reaction mixture was passed through a Sephadex G-25 (fine) column (2.5 × 56 cm) and eluted with 0.02 M NH_4HCO_3. The void volume fraction containing both modified and unmodified oligonucleotides was lyophilized and digested by two different protocols. (1) SVD/AP protocol: 1 A_{260} unit of oligonucleotide, 1 unit of SVD, and 0.5 unit of AP in 0.1 M Tris−2 mM $MgCl_2$, pH 8.2 (200 μL) were incubated for 4 h at 45 °C. (2) Nuclease P_1/SVD/AP protocol: 1 A_{260} unit of oligonucleotide and 1 unit of nuclease P_1 were incubated at 37 °C for 2 h in 0.8 mL of 0.02 M ammonium acetate, pH 5.5; 0.1 M $MgCl_2$ (20 μL) was added, and the pH was adjusted to 8.2 by addition of 20 μL of 0.2 M NaOH. SVD (2 units) and AP (2 units) were added, and incubation was continued at 37 °C for 2.5 h. Both digestion mixtures were directly analyzed by HPLC using a Rainin Microsorb C-18 reverse-phase column (0.46 × 25 cm). The elution system was 6−18% acetonitrile in 0.03 M potassium phosphate, pH 5.4, in 60 min, 1 mL/min flow rate.

The molar proportions of the separated nucleosides and nucleoside−MC adducts present in the digests were calculated from the relative HPLC peak areas divided by the corresponding extinction coefficients at 254 nm. Adenine adduct frequency, defined as mol of adduct/mol of adenine residue, was calculated from the above HPLC data simply by dividing the molar proportion of **8** by the molar proportion of dA. Guanine adduct frequency was obtained analogously.

For preparative purposes, larger scale alkylation reactions were carried out (e.g., 100 A_{260} units of oligonucleotide) and a 10- × 250-mm (semipreparative) C-18 HPLC column was used. The elution system was 3−18% NH_4OAc in 90 min at 4.0 mL/min flow rate. Fractions were collected manually and were desalted by passing through a 1.5- × 56-cm Sephadex G-25 column (0.02 NH_4HCO_3 as eluant). Adducts **8** and **9a**,**b** each eluted *after* the salt-containing fraction. Lyophilization yielded typically 100−200 μg of adduct material.

(ii) Bifunctional MC Activation: Activation using excess anaerobic $Na_2S_2O_4$ conditions for reduction (*19*) was employed for alkylation of oligonucleotides by the previously described protocol.

Formation and Isolation of Adenine Adducts of MC from Calf Thymus DNA. H_2/PtO_2 Activation (*20*): Calf thymus DNA (0.67 mM mononucleotide), MC (1.34 mM) and PtO_2 (100 μg/μmol of MC) in 0.015 M Tris, pH 7.4, buffer were mixed, and after deaeration H_2 gas was bubbled through the mixture for 5 min at room temperature. After a further 5-min incubation under argon, the mixture was filtered and passed through Sephadex G-25. The lyophilized void volume fraction was digested as follows: 1 A_{260} unit of DNA and 1 unit of nuclease P_1 in 400 μL of 0.02 M NH_4Ac, pH 5.5, were incubated at 37 °C for 2 h. $MgCl_2$ was added to 2 mM, and the pH was adjusted to 8.2 by addition of 8 μL of 0.2 M NaOH; then SVD (2.2 units) and AP (2.5 units) were added. Incubation was carried out for 2.5 h. The digest was separated into its components by HPLC, as described for digests of alkylated oligonucleotides above.

Enzymatic Activation (*20*): An identical MC−calf thymus DNA reaction mixture was activated by 2 mM NADH and 0.44 unit of NADH-cytochrome *c* reductase per μmol of MC under argon, and the reaction mixture was incubated for 20 min at 37 °C. Further processing of the alkylation reaction mixture was the same as above.

Hydrolysis of the MC−Dinucleoside Phosphate Adducts 9a,b to the MC−Deoxyadenosine Adduct 8 by Nuclease P_1/AP. 9a or **9b** (0.03 A_{260} unit) were digested by 0.5 unit of nuclease P_1 in 100 μL of 0.02 M NH_4Ac, pH 5.5, at 37 °C for 2 min; then 100 μL of 0.5 M Tris, pH 8.2, and 1 unit of AP were added. After digestion for 2 h at 37 °C, the digest was analyzed by HPLC. Digestion with nuclease P_1 alone (no AP added) to yield the 5′-phosphate of adduct **8** was performed and evaluated by HPLC in the same way.

Preparation of Adduct 8 from 2′-deoxyadenosine and MC. 2′-Deoxyadenosine (10 mg) and MC (2.8 mg) were dissolved in 1 mL of H_2O. PtO_2 (1 mg) was added, and after deaeration the mixture was hydrogenated by bubbling H_2 through the solution for 5 min. After a further 5-min incubation under argon, the mixture was filtered and fractionated by semipreparative scale HPLC (see above). Adduct **8** was isolated in 1.2% yield (55 μg). The two major products were 2β,7-diamino-1α- and -1β-hydroxymitosene (*21*).

Results

Detection of Two Unknown MC Adducts in HPLC of Digests of Oligonucleotides Treated with MC and $Na_2S_2O_4$. The two new adducts were first noted in the reaction of oligonucleotide 1 and MC which was run to prepare a homogeneous, G-substituted oligonucleotide, containing adduct **2**. Since the G in the 5′-AGA sequence has very low reactivity with MC (*18*), a relatively large quantity of crude digested reaction was injected for detection of adduct **2** by HPLC analysis (Figure 1a). In addition to **2** later-eluting unknown components marked **9a** and **8** appeared in the HPLC tracing, all at comparable peak intensities. The calculated yield of **2** was 1−2% (i.e., 1−2% total G residue was converted to **2**), and it was apparent that the new products were formed at similarly low yields. Oligonucleotide 2 which had the same nucleotide composition and same central AGA sequence gave similar results (Figure 1f).

Relationship of 9a,b to 8. When the alkylated oligonucleotide mixtures were digested by adding P_1 nuclease to the usual SVD/AP digestion protocol, the first-eluted unknown adduct (**9a** or **9b**) was converted quantitatively to the second one (**8**) (Figure 1b,h). This suggested that **9a**,**b** contained a SVD-resistant phosphodiester linkage which was, however, further hydrolyzed by nuclease P_1 and AP to a single MC−nucleoside adduct, **8** (Scheme 3).

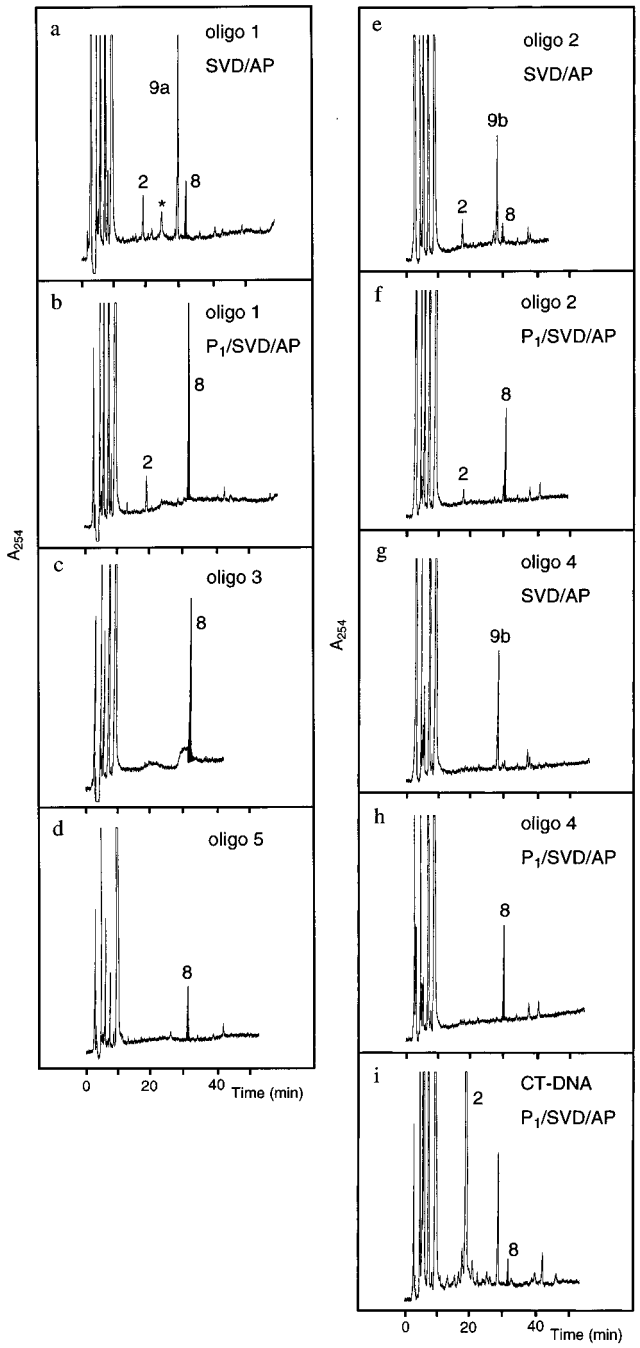


Figure 1. Isolation of MC adducts by HPLC from enzymatic digests of MC−oligonucleotide and MC−calf thymus DNA complexes (see Table 1 for oligonucleotide sequences): (a) SVD/AP digest of oligonucleotide 1 (Table 1), peak marked with asterisk is an artifact; (b) P_1/SVD/AP digest of oligonucleotide 1; (c) P_1/SVD/AP digest of oligonucleotide 3; (d) P_1/SVD/AP digest of oligonucleotide 5; (e) SVD/AP digest of oligonucleotide 2; (f) P_1/SVD/AP digest of oligonucleotide 2; (g) SVD/AP digest of oligonucleotide 4; (h) P_1/SVD/AP digest of oligonucleotide 4; (i) P_1/SVD/AP digest of calf thymus DNA.

Proof of Structure of 8. (1) 8 Is an Adenine Adduct. This was indicated by observing that oligonucleotides 3−5 all yielded adduct **8** under the same reaction and digestion conditions (Figure 1c,d,h). Furthermore, **8** was also obtained from 2′-deoxyadenosine (see below).

(2) Structural Characterization of 8. Approximately 50 μg (1.5 A_{260} units) of this substance was prepared from MC and 2′-deoxyadenosine (Methods). The UV spectrum qualitatively indicated a combination of

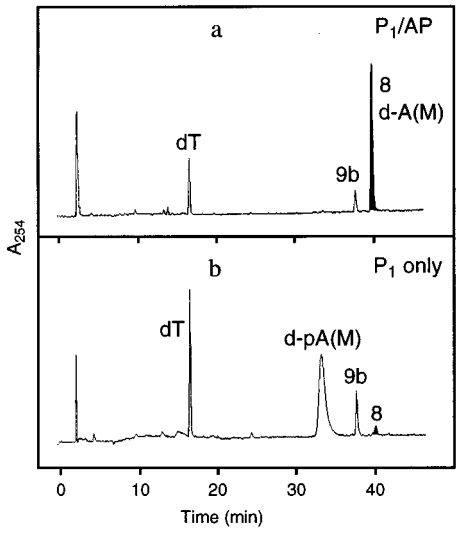
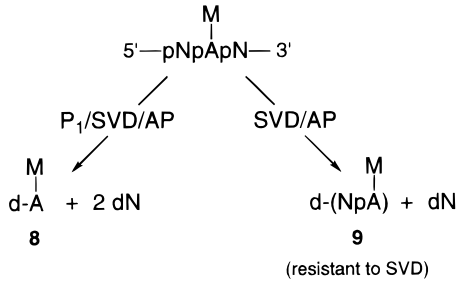
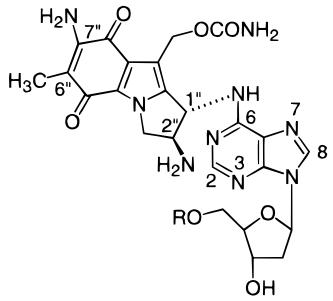


Figure 2. Enzymatic conversion of d-TpA(M) (**9b**) to dT + d-pA(M), demonstrated by HPLC: (a) P_1/AP digestion of **9b**, (b) P_1 digestion of **9b**. HPLC conditions: 0−6% acetonitrile in 0.03 M potassium phosphate, pH 5.5, 0−15 min; 6−18% acetonitrile in the same, 15−40 min.

Scheme 3. Differential Susceptibility of the DNA Adenine N^6 Adduct of MC to SVD and P_1 Cleavage





8 (adenine-N^6 adduct): R= H

9 a: R= dAp-

b: R= dTp-

deoxyadenosine-type (λ_{max} 267 nm) and 7-aminomitosene-type (λ_{max} 310 nm) chromophores (Figure 3a). Subtraction of the UV spectrum of 2,7-DAM (**6**) normalized to that of **8** at 320 nm (Figure 3a) gave the UV difference spectrum in Figure 3b. Its λ_{max} (267 mm) and shape matched closely the spectra of the N^6-alkylated adenosines N^6-methyldeoxyadenosine (λ_{max} 266 nm) and N^6-(2-isopentenyl)adenosine (λ_{max} 269 nm). Adenosines which are unsubstituted or alkylated at other positions (N1, N3, or N7), as well as various disubstitued adenosines, have different UV spectra which are readily distinguishable from that of the N^6-alkyladenosines. For example, the absorption maximum of $N1$-alkyladenosines is approxi-

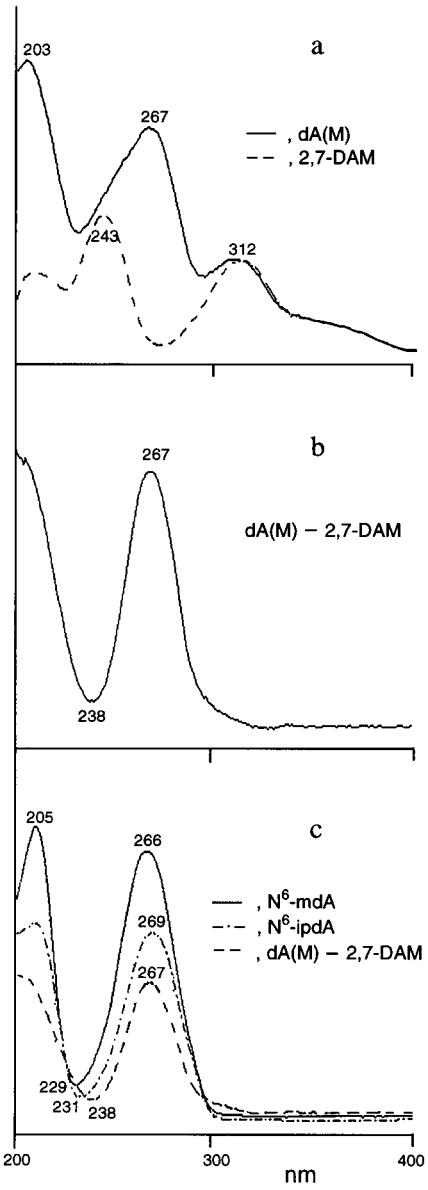


Figure 3. UV spectra and computed UV difference spectra of the MC−deoxyadenosine adduct **8**, 2,7-DAM, and deoxyadenosine. Comparison with standard N^6-alkyldeoxyadenosines: (a) UV spectra of dA(M) (adduct **8**) and 2,7-DAM (**6**), (b) difference spectrum of dA(M) − 2,7-DAM (**8** − **6**), (c) spectra of N^6-mdA, N^6-ipdA, and their comparison with the difference spectrum of dA(M) − 2,7-DAM in panel b.

mately 10 nm lower (257 nm) than that of N^6-alkyladenosines (*22*). Thus adduct **8** appeared to be an N^6-substituted deoxyadenosine by a mitosene.

(3) Heat Stability. No adduct **8** was released hydrolytically when the alkylated oligonucleotide 1 was heated at 90 °C at neutral pH in the absence of enzymatic digestion, as indicated by HPLC of an aliquot of the heated solution (data not shown). The remainder of the heated solution was digested. HPLC of the heated and digested sample showed the same adducts as the unheated digested sample control displayed in Figure 1a. This result independently excluded substitution at the adenine N3 or N7 position since the glycoside bonds of such derivatives are readily cleaved upon heating (*12*). Had such hydrolysis occurred, the HPLC pattern of the digest would have lacked the peak corresponding to **8**, which is a nucleoside; rather, another new peak (deglycosylated adduct) would have appeared.

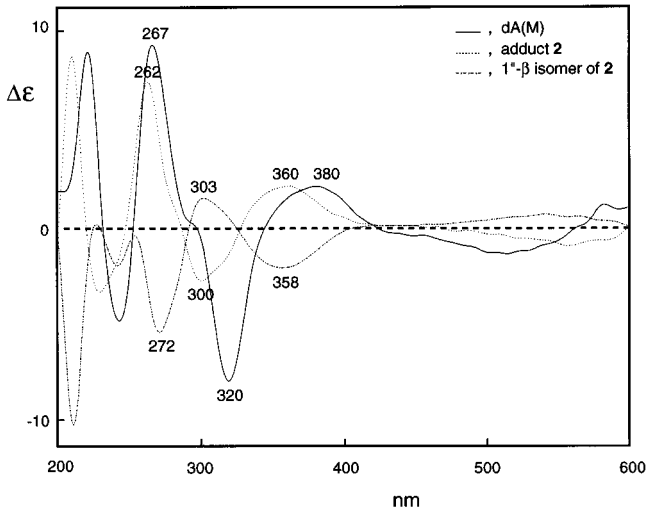
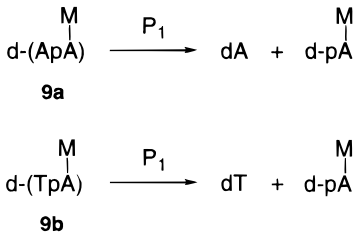


Figure 4. Establishment of the C-1″ stereochemistry of the MC−deoxyadenosine adduct **8**: comparison with the CD spectra of the stereoisomeric MC−deoxyguanosine adduct **2** and its C-1″ stereomer.

Scheme 4. Proof of the Sequence of SVD-Resistant Adducts 9a,b



Prolonged heating (100 °C, 2 h) of isolated **8** alone converted approximately 40% to a faster eluting substance. ESIMS indicated that this is the 10″-decarbamoylated derivative (**10b**) of adduct **8**.

(4) Mass Spectra. The compositions of **9a**,**b** corresponding to the dinucleoside phosphate *plus* 2,7-DAM and the composition of **8** corresponding to deoxyadenosine *plus* 2,7-DAM were confirmed by the results of the electrospray MS as follows: **9a**, calcd MH^+ 866, found 866; **9b**, calcd MH^+ 857, found 857; **8**, calcd MH^+ 553, found 553; **10b**, calcd MH^+ 511, found 511.

(5) Circular Dichroism Spectra (Figure 4). The sign of the 500−600-nm shallow, broad Cotton effect of 1-substituted 7-aminomitosenes is known to be diagnostic of the absolute stereochemistry of the 1-substituent (*23*). The 1″α and 1″β configurations give negative and positive Cotton effects, respectively, in this region as exemplified by the CD of the diastereomeric adduct **2** and its 1″β stereomer (Figure 4). The negative Cotton effect at 510 nm of adduct **8** indicates that the mitosene 1″-carbon has the α configuration. This result excludes the possibility of a regioisomeric structure, i.e., the reversal of the 1″- and 2″-substituents: were the amino group also located in the 1″-position, this 1″-substituent would have the β configuration, giving a Cotton effect with opposite sign.

Proof of the Sequence of the SVD-Resistant Dinucleoside Phosphate Adducts 9a,b. Nuclease P_1 digestion of **9b** yielded dT and d-pA(M) rather than d-pT and dA(M) as concluded from HPLC of the products, utilizing the authentic standards dT, d-pT, and dA(M) to establish product identities (Scheme 4). Similarly, **9a** yielded dA and d-pA(M). This proved that **9a**,**b** have the

Table 1. Frequencies of Adenine and Guanine Alkylation Adducts by Activated MC in Oligonucleotide Substrates and Calf Thymus DNA

		frequency, %[a]			
		SVD/AP digest		P_1/SVD/AP digest	
oligonucleotide	sequence of oligonucleotide substrate	dA(M) (**8**)	d[NpA(M)] (**9a**,**b**)	dA(M) (**8**)	dG(M) (**2**)
1	5′-AAAAAAGAAAAAA[b] 3′-TTTTTTCTTTTTT	0.25	0.7	1.1	1.7, 2.1
2	5′-TATATAGATATAT[b] 3′-ATATATCTATATA	0	0.45, 0.5	0.6	1.8
3	5′-AAAAAAAAAAAAA[b] 3′-TTTTTTTTTTTTT	0.35	0.5	0.9, 1.0	
4	5′-TATATATATATA[b] 3′-ATATATATATAT	0	0.45, 0.5	0.5	
5	5′-AAAAAAAAAAAA[b] (single-stranded)	0.06	0.15	0.3	
6	5′-AAAAACGAAAAAA[b] 3′-TTTTTGCTTTTTT	0.6	1.4	2.3	22, 22
7	5′-ATACGTAT 3′-TATGCATA			0.7	30
	calf thymus DNA			0.20,[c] 0.24[d]	9.0,[c] 9.3[d]

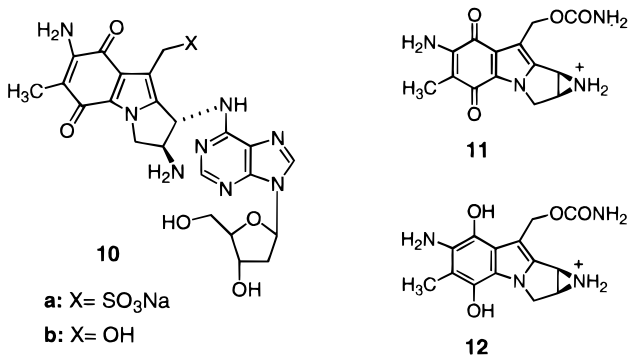
[a] Mol of adduct/mol of adenine (or guanine) residue × 100. [b] Activation of MC: monofunctional ($Na_2S_2O_4$). [c] Activation of MC: monofunctional (H_2/PtO_2). [d] Activation of MC: monofunctional (NADH-cytochrome *c* reductase).

sequence d[NpA(M)]. Therefore it is concluded that the 5′-phosphodiester of MC-modified adenine residues is resistant to SVD (but not to nuclease P_1).

Base Sequence Selectivity of Adenine Alkylation by MC in Oligonucleotides (Table 1). Adenines in $(A)_n$ runs were alkylated approximately 2-fold more extensively than those in $(AT)_n$ runs. This was concluded from comparing the observed adenine adduct frequencies of oligo 1 (1.1% of A residues) and oligo 3 (0.9% of A residues) with those of oligo 2 (0.6% of A residues) and oligo 4 (0.5% of A residues). The single-stranded $(A)_n$ run $(dA)_{13}$ appears to be less reactive (0.3% of A residues) than the corresponding duplex $(dA)_{13}\cdot(dT)_{13}$ (0.9% of A residues).

Adenine Alkylation Frequency Relative to Guanine Alkylation Frequency (Table 1). It is known that alkylation of guanine by MC is highly selective at the 5′-CpG sequence. 5′-GpG, 5′-TpG, and 5′-ApG are 10−50-fold less reactive (*18*). Accordingly, the ratio of adenine to guanine alkylation frequency varied, depending on which 5′-NpG sequence was present in the oligonucleotide substrate. Oligonucleotides 6 and 7 both contained a 5′-CpG site which was alkylated at a high frequency (22% and 30% of G residues, respectively); therefore, their ratios of adenine/guanine alkylation frequency were relatively low (0.1 and 0.02, respectively). However, in oligonucleotides 1 and 2 which contained only the low-reactivity guanine sites 5′-ApG, this ratio was considerably higher: 0.6 and 0.3, respectively. In calf thymus DNA the overall ratio of adenine-to-guanine alkylation frequency was lower [0.03 in both experiments (see Table 1, Figure 1i]. One possible explanation may be that the higher frequencies of the adenine adduct in the oligonucleotides reflect an "end effect": chain-terminal adenines may be more reactive than internal ones. This is suggested by the formation of a substantial amount of **8** in the SVD digests of oligonucleotide 1 which has a 5′-terminal A residue (Figure 1). When nuclease P_1 is not included in the digestion mixture, adduct **8** can only originate from 5′-terminal A adducts since the 5′-phosphodiester of the adduct is resistant to SVD, as discussed above.

Chart 1



Lack of Cross-Linking Activity of MC−Adenine Adducts. Under bifunctional MC-activating conditions (excess anaerobic $Na_2S_2O_4$) which result in guanine-to-guanine cross-links, both substrates 3 and 4 yielded a new addduct, which, however, was ruled out as a bisadenine adduct. Its HPLC elution time was 10 min *earlier* than that of adenine monoadduct **8**. This is opposite to the shift to later elution time by the bisguanine adduct **4** relative to guanine monoadduct **2**. The large shift of the new adduct to an *earlier* time suggests that the 10″-carbamate of **8** was displaced by the polar, negatively charged bisulfite group to give **10a** (Chart 1), in analogy to the 10″-bisulfite adduct obtained from the guanine monoadduct **2** under similar conditions (*24*). To further rule out cross-link formation by MC−adenine adducts, a DPAGE test (*25*) indicated no cross-linked oligonucleotide after a treatment of d(TA) with activated MC by a high-efficiency two-step cross-linking protocol which is known to result in quantitative cross-linking of $d(CG)_n$-containing oligonucleotides (*19*).

Discussion

Mitomycin C (**1**) is a prodrug since it contains two masked alkylating functions: the C-1,C-2 aziridine ring and the C-10 carbamate which become activated upon reduction of its quinone system (*4*). Enzymatic or chemical reduction triggers several consecutive chemical trans-

formation steps leading to a highly reactive bifunctional alkylating species (**12**) and a reactive monofunctional alkylating form (**11**)[2] (Chart 1). The balance of the two forms depends on the conditions of the reduction (*26*). Two different sets of DNA adducts result from the two different activated species (Scheme 1). Detection of the same adducts formed in vivo gave evidence that both monofunctional and bifunctional reductive activation of MC occurs in mouse mammary tumor cells (*9*) and various other mammalian systems (*10*, *27*). The reductively activated species of MC are extremely reactive. The bifunctional alkylating form **12** has never been isolated or spectroscopically characterized; its lifetime has been estimated as a few seconds (*28*). The $t_{1/2}$ of the monofunctional form **11** was reported as 3 min at 37 °C at pH 7.0 (*29*). The great specificity of their reaction in vitro with DNA at guanine N^2 is therefore quite remarkable. Noncovalent affinity of the MC active form to the minor groove of duplex DNA, including specific H-bonding to the 2-amino group of guanines, accounts partially for this specificity (*30*, *31*). The present adenine N^6 adduct is the only DNA major groove product of reductively activated MC observed so far. [A recently reported major groove guanine-*N*7−mitosene adduct (*7*) is not a product of activated MC but rather a product of the MC derivative 2,7-diaminomitosene.] The fact that it is formed only to the extent of a few percent among the major guanine N^2 adducts in calf thymus DNA underlines the intrinsic DNA guanine specificity of MC. The formation of the adenine adduct nevertheless shows a preference for duplex DNA and a sequence preference of -AAA- over -TAT-, suggesting a precovalent affinity of reduced MC to the major groove of DNA as well. The lack of detection of any bisadenine adduct in calf thymus DNA under bifunctional MC activation conditions testifies to the G specificity of the MC cross-links in DNA.

The 5′-phosphodiester linkage of the MC−adenine adduct is resistant to SVD (Scheme 3), in contrast to that of MC−N^2-guanine adducts which is cleaved by this enzyme. Conversely, however, the guanine N^2 adducts are resistant to cleavage by nuclease P_1 at their 3′-phosphodiester linkage (*20*), while the adenine N^6 adduct is cleaved by P_1. The explanation for these intriguing observations is not known.

Our main interest in the minor adenine adduct of MC stems from our detection of an unknown MC adduct in vivo. In a previous study (*9*) we analyzed the DNA adducts formed in EMT6 mouse mammary tumor cells upon treatment with ^{3}H-labeled MC. HPLC of nuclease digests of DNA isolated from the treated cells indicated a distinct fraction of radiolabeled substance, marked **X**, the elution time of which did not correspond to any known MC adduct. No UV signal was detectable in association with the radiolabel, since in cells DNA adducts are formed in low quantities in general. The proportion of substance **X** to the other adducts was dramatically enhanced under certain conditions of the MC treatment; such conditions also enhanced the cytotoxicity of MC (*9*). An adduct with similar properties was observed in porfiromycin-treated tumor cells (*32*, *9*). The presently described MC−N^6-deoxyadenosine adduct shows the same HPLC elution time as substance **X**, as confirmed by coelution of the radiolabel of **X** collected from the HPLC of the cellular digests with the UV absorbance of authentic adduct **8** (Palom and Tomasz, unpublished results). Further tests are necessary to establish the identity of adduct **8** with this unknown, inducible in vivo adduct of MC. However, the discovery of the adduct **8** provides a potential in vitro source for **X** which could be difficult to collect from in vivo sources in sufficient quantities for characterization.

The present work demonstrates a successful application of the enzymatic and microscale UV and CD methods developed previously by the Tomasz and Nakanishi laboratories for the isolation and elucidation of complex mitomycin−DNA adducts on low scale (*31*, *34*, *35*). For example, the enzymatic transformations in Schemes 3 and 4 were established using less than 10 μg of material. Similar amounts were used for the UV, CD, and mass spectra (less in the latter), resulting altogether in the derivation of the adduct structure **8**. This approach should be readily applicable to microscale elucidation of DNA adducts of a number of new, recently designed antitumor agents under investigations based on the mitomycin structural model of action (*36*−*39*).

Acknowledgment. This work was supported by a grant from the NIH (CA28681) and a Research Centers in Minority Institutions award from the NIH Division of Research Resources (RR03037), both to M.T.

References

(1) Warpehoski, M. A., and Hurley, L. H. (1988) Sequence selectivity of DNA covalent modification. *Chem. Res. Toxicol.* **1**, 315−333.
(2) Boger, D. L., Johnson, D. S., and Yun, W. (1994) (+)- and *ent*-(−)-Duocarmycin SA and (+)- and ent-(−)-N-BOC-DSA DNA alkylation properties. Alkylation site models that accommodate the offset AT-rich adenine N3 alkylation selectivity of the enantiomeric agents. *J. Am. Chem. Soc.* **116**, 1635−1656.
(3) Sun, D., Hansen, M., and Hurley, L. H. (1995) Molecular basis for the DNA sequence specificity of the pluramycins. A novel mechanism involving groove interactions transmitted through the helix via intercalation to achieve sequence selectivity at the covalent bonding step. *J. Am. Chem. Soc.* **117**, 2430−2440.
(4) Tomasz, M., and Palom, Y. (1997) The mitomycin bioreductive antitumor agents: Cross-linking and alkylation of DNA as the molecular basis of their activity. *Pharmacol. Ther.* **76**, 73−87.
(5) Chirrey, L., Cummings, J., Halbert, G. S., and Smyth, J. F. (1995) Conversion of mitomycin C to 2,7-diaminomitosene and 10-decarbamoyl 2,7-diaminomitosene in tumor tissue in vivo. *Cancer Chemother. Pharmacol.* **5**, 318−322.
(6) Prakash, A. S., Beall, H., Ross, D., and Gibson, N. W. (1993) Sequence-selective alkylation and cross-linking induced by mitomycin C upon activation by DT-diaphorase. *Biochemistry* **32**, 5518−5525.
(7) Suresh Kumar, G., Musser, S. M., Cummings, J., and Tomasz, M. (1996) 2,7-Diaminomitosene, a monofunctional mitomycin derivative alkylates DNA in the major groove. Structure and base-sequence specificity of the DNA adduct and mechanism of the alkylation. *J. Am. Chem. Soc.* **118**, 9209−9217.
(8) Tomasz, M., Lipman, R., Chowdary, D., Pawlak, J., Verdine, G. L., and Nakanishi, K. (1987) Isolation and structure of a covalent cross-link adduct between mitomycin C and DNA. *Science* **235**, 1204−1208.
(9) Bizanek, R., Chowdary, D., Arai, H., Kasai, M., Hughes, C. S., Sartorelli, A. C., Rockwell, S., and Tomasz, M. (1993) Adducts of mitomycin C and DNA in EMT6 mouse mammary tumor cells: Effects of hypoxia and dicumarol on adduct patterns. *Cancer Res.* **53**, 5127−5134.
(10) Warren, A. J., Maccubbin, A. E., and Hamilton, J. W. (1997) Detection of mitomycin C-DNA adducts in vivo by ^{32}P-postlabeling: Time course for formation and removal of adducts and biochemical modulation. *Cancer Res.*, in press.
(11) Palom, Y., Suresh Kumar, G., Belcourt, M. F., Arai, H., Kasai, M., Sartorelli, A. C., Rockwell, S., and Tomasz, M. (1997) A

[2] In our previous publications the quinone methide derivative of **12** has been designated as the bifunctional alkylating species. The modified reductive activation mechanism of MC features **12** itself as such (*26*, *39*).

guanine-N7 adduct of 2,7-diaminomitosene is a dominant DNA lesion in mitomycin C-treated EMT6 mouse mammary tumor cells. *Proc. Am. Assoc. Cancer Res.* **38**, 228.
(12) Singer, B., and Grunberger, D. (1983) *Molecular Biology of Mutagens and Carcinogens*, p 68, Plenum Press, New York.
(13) Lee, C.-S., Hartley, J. A., Berardini, M. D., Butler, J., Siegel, D., Ross, D., and Gibson, N. W. (1992) Alteration in DNA cross-linking and sequence-selectivity of a series of aziridinylbenzoquinones after enzymatic reduction by DT-diaphorase. *Biochemistry* **31**, 3019−3025.
(14) Stone, M. P., and Feng, B. (1996) Sequence and stereospecific consequences of major groove α-(N^6-adenyl)-styrene oxide adducts in an oligodeoxynucleotide containing the human *N-ras* codon 61 sequence. *Magn. Reson. Chem.* **34**, 5105−5114.
(15) MacLeod, M. C., Evans, F. E., Lay, J., Chiarelli, P., Geacintov, N. E., Powell, K. L., Daylong, A., Luna, E., and Harvey, R. G. (1994) Identification of a novel, N7-deoxyguanosine adduct as the major DNA adduct formed by a nonbay-region diolepoxide of benzo[*a*]pyrene with low mutagenic potential. *Biochemistry* **33**, 2977−2987.
(16) Asai, A., Nagamura, S., and Saito, H. (1994) A novel property of duocarmycin and its analogues for covalent reaction with DNA. *J. Am. Chem. Soc.* **116**, 4171−4177.
(17) Ramos, L. H., Lipman, R., Tomasz, M., and Basu, A. K. (1998) The major mitomycin C-DNA monoadduct is cytotoxic but not mutagenic in *Escherichia coli*. *Chem. Res. Toxicol.* **11**, 64−69.
(18) Kumar, S., Lipman, R., and Tomasz, M. (1992) Recognition of specific DNA sequences by mitomycin C for alkylation. *Biochemistry* **31**, 1399−1407.
(19) Borowy-Borowski, H., Lipman, R., and Tomasz, M. (1990) Recognition between mitomycin C and specific DNA sequences for cross-link formation. *Biochemistry* **29**, 2999−3004.
(20) Tomasz, M., Chowdary, D., Lipman, R., Shimotakahara, S., Veiro, D., Walker, V., and Verdine, G. L. (1986) Reaction of DNA with chemically or enzymatically activated mitomycin C: Isolation and structure of the major covalent adduct. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6702−6706.
(21) Tomasz, M., and Lipman, R. (1981) Reductive metabolism and alkylating activity of mitomycin C induced by rat liver microsomes. *Biochemistry* **20**, 3901−3907.
(22) Singer, B., and Grunberger, D. (1983) *Molecular Biology of Mutagens and Carcinogens*, p 313, Plenum Press, New York.
(23) Tomasz, M., Jung, M., Verdine, G. L., and Nakanishi, K. (1984) Circular dichroism spectroscopy as a probe for the stereochemistry of aziridine cleavage reactions of mitomycin C. Applications to adducts of mitomycin with DNA constituents. *J. Am. Chem. Soc.* **106**, 7367−7370.
(24) McGuinness, B. F., Lipman, R., Nakanishi, K., and Tomasz, M. (1991) Reaction of sodium dithionite activated mitomycin C with guanine at non-cross-linkable sequences of oligonucleotides. *J. Org. Chem.* **56**, 4826−4829.
(25) Teng, S. P., Woodson, S. A., and Crothers, D. M. (1989) DNA sequence specificity of mitomycin cross-linking. *Biochemistry* **28**, 3901−3907.
(26) Suresh Kumar, G., Lipman, R., Cummings, J., and Tomasz, M. (1997) Mitomycin C-DNA adducts generated by DT-diaphorase. Revised mechanism of the enzymatic reductive activation of mitomycin C. *Biochemistry* **36**, 14128−14136.
(27) Pan, S., Yu, F., and Hiphser, C. (1993) Enzymatic and pH modulation of mitomycin C-induced DNA damage in mitomycin C-resistant HCT 116 human colon cells. *Mol. Pharmacol.* **43**, 870−877.
(28) Cera, C., Egbertson, M., Teng, S. P., Crothers, D. M., and Danishefsky, S. J. (1989) DNA cross-linking by intermediates of the mitomycin activation cascade. *Biochemistry* **28**, 5665−5669.
(29) Han, I., and Kohn, H. (1991) 7-Aminoaziridines: Synthesis, structure and chemistry. *J. Org. Chem.* **56**, 4648−4653.
(30) Sastry, M., Fiala, R., Lipman, R., Tomasz, M., and Patel, D. J. (1995) Solution structure of the monoalkylated mitomycin C-DNA complex. *J. Mol. Biol.* **247**, 338−359.
(31) Gargiulo, D., Musser, S. S., Yang, L., Fukuyama, T., and Tomasz, M. (1995) Alkylation and cross-linking of DNA by the unnatural enantiomer of mitomycin C: Mechanism of the DNA sequence specificity of mitomycins. *J. Am. Chem. Soc.* **117**, 9388−9398.
(32) Pan, S. (1990) Porfiromycin disposition in oxygen modulated P388 cells. *Cancer Chemother. Pharmacol.* **27**, 187−193.
(33) Tomasz, M., Lipman, R., Snyder, J. K., and Nakanishi, K. (1983) Full structure of a mitomycin C dinucleoside phosphate adduct. Use of differential FT-IR in microscale structural studies. *J. Am. Chem. Soc.* **105**, 2059−2063.
(34) Tomasz, M., Lipman, R., Lee, M. S., Verdine, G. L., and Nakanishi, K. (1987) Reaction of acid-activated mitomycin C with calf thymus DNA and model guanines. Elucidation of the base-catalyzed degration of N7-alkylguanine nucleosides. *Biochemistry* **26**, 2010−2027.
(35) Hendriks, H. R., Pizao, P. E., Berger, D. P., Kooistra, K. L, Bibby, M. C., Boven, E., Meulen, H. C. D.-V. D., Henrar, R. E. C., Fiebig, H. H., Double, J. A., Hornstra, H. W., Pinedo, H. M., Workman, P., and Schwarts-Mann, G. (1993) EO9: A novel bioreductive alkylating indoloquinone with preferential solid tumor activity and lack of bone marrow toxicity in preclinical models. *Eur. J. Cancer* **29A**, 897−906.
(36) Schulz, W. G., Nicman, R. A., and Skibo, E. B. (1995) Evidence for DNA phosphate backbone alkylation and cleavage by pyrrolo[1,2-*a*]benzimidazoles: Small molecules capable of causing base-pair-specific phosphodiester bond hydrolysis. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 11854−11858.
(37) He, Q.-Y., Maruenda, H., and Tomasz, M. (1994) Novel bioreductive activation mechanism of mitomycin C derivatives bearing a disulfide substituent in their quinone. *J. Am. Chem. Soc.* **116**, 9349−9350.
(38) Lee, J.-H., Naito, M., and Tsuruo, T. (1994) Nonenzymatic reductive activation of 7-N-{2-{[2-(γ-L-glutamylamino)ethyl]-dithio}ethyl}mitomycin C by thiol molecules: A novel mitomcyin C derivative effective on mitomycin C-resistant tumor cells. *Cancer Res.* **54**, 2398−2403.
(39) Schiltz, P., and Kohn, H. (1993) Studies on the reactivity of reductively activated mitomycin C. *J. Am. Chem. Soc.* **115**, 10510−10518.

TX970205U