VOLUME 118, NUMBER 39
OCTOBER 2, 1996

JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

2,7-Diaminomitosene, a Monofunctional Mitomycin C Derivative, Alkylates DNA in the Major Groove. Structure and Base-Sequence Specificity of the DNA Adduct and Mechanism of the Alkylation

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Received March 7, 1996[⊗]

Abstract: Reductive activation of the bifunctional alkylator and DNA cross-linking agent mitomycin C (MC) yields 2,7-diaminomitosene (2,7-DAM) as the major product of its activation both in cell-free systems and *in vivo*. 2,7-DAM lacks one of the alkylating functions of MC, the aziridine. We show that 2,7-DAM itself alkylates DNA monofunctionally upon reductive activation, to form a heat-labile adduct. A guanine-N7−2,7-DAM adduct was isolated from the drug−DNA complex upon heating. Nuclease digestion yielded this adduct both in the nucleoside (dG-2,7-DAM) and dinucleoside phosphate [d(GpG)-2,7-DAM] forms. The structures of all three forms were determined by LC−ESIMS and differential UV spectroscopy. The adduct is sequence-specific to guanines in $(G)_n$ tracts of DNA. A guanine-N7−2,7-DAM adduct in DNA was indirectly observed previously by Prakash et al. (Prakash, A. S.; Beall, H.; Ross, D.; Gibson, N. W. *Biochemistry* **1993**, *32*, 5518−5525). The results indicate that selective removal of the aziridine function of MC results in a switch from minor to major groove alkylation of DNA, and a switch of sequence specificity from guanines in CpG to guanines in $(G)_n$ tracts. They also show that the mitosene C-10 carbamate intrinsically reacts as an S_N2 alkylating agent, specific to guanine-N7.

Mitomycin C (MC;[2] **1**) is an antitumor antibiotic used clinically as a cancer chemotherapeutic agent. Its antitumor and cytotoxic activity is most likely based on its ability of alkylating and cross-linking DNA.[3] MC requires reductive activation for the DNA-alkylation process, as demonstrated both in cell-free systems[3] and intact cells.[4]

The structural and mechanistic aspects of the monofunctional and bifunctional alkylation of DNA by MC as well as the reductive activation of the drug itself have been extensively studied.[5] The active form of MC reacts at its C-1 and C-10 positions, exclusively with 2-amino groups of guanines in the

⊗ Abstract published in *Advance ACS Abstracts*, September 15, 1996.

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(2) Abbreviations: MC, mitomycin C; 2,7-DAM, 2,7-diaminomitosene; dG, 2′-deoxyguanosine; Gua, guanine; SVD, snake venom diesterase; AP, alkaline phosphatase; G-N7, guanine-N7 position; G-N^2, guanine-N^2 position; nt, nucleotide; ϵ_{312}, molar extinction coefficient at 312 nm.

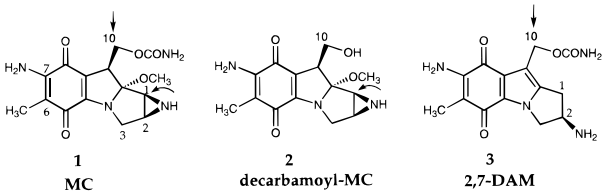
(3) Szybalski, W.; Iyer, V. N. In *Antibiotics 1: Mechanism of action*; Gottlieb, D., Shaw, P. D., Eds.; Springer-Verlag: New York, 1967; pp 230−245.

(4) Hoban, P. R.; Walton, M. I.; Robson, C. N.; Godden, J.; Stratford, I. J.; Workman, P.; Harris, A. L.; Hickson, I. D. *Cancer Res.* **1990**, *50*, 4692−4697. Marshall, R. S.; Paterson, M. C.; Rauth, A. M. *Biochem. Pharmacol.* **1991**, *41*, 1351−1360.

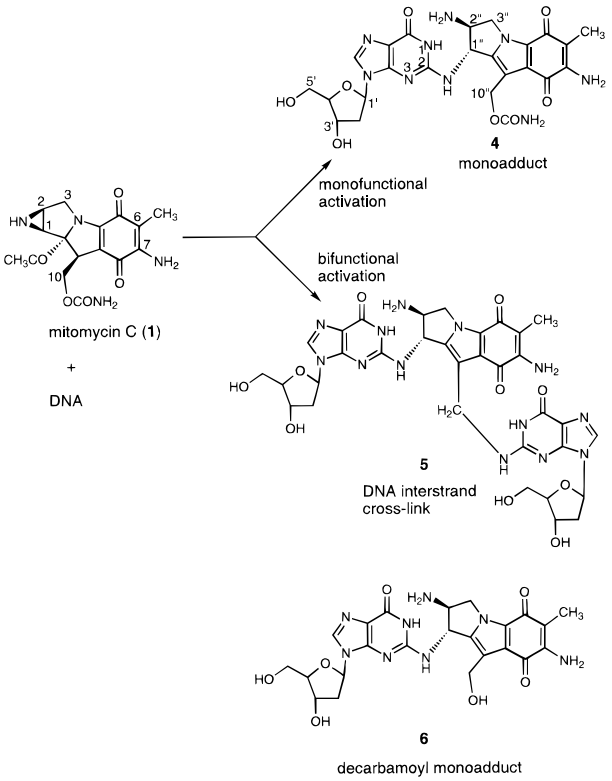
(5) For recent review, see: Tomasz, M. *Chem. Biol.* **1995**, *2*, 575−579.

S0002-7863(96)00740-8 CCC: $12.00

Chart 1



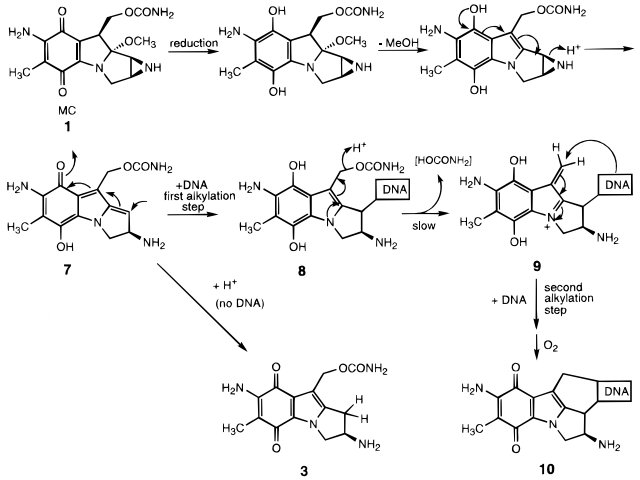
Scheme 1. Structures of Mitomycin C−DNA Adducts Obtained under Monofunctional and Bifunctional Activating Conditions



minor groove of DNA, to give monoadducts **4** and **6** and the bisadduct **5**; the latter constituting a DNA interstrand or intrastrand cross-link (Scheme 1).[6] By shortening the duration of the activated (reduced) state of the mitomycin, the intermediate monoadduct **4** is isolated as the single product, indicating that the first step of the alkylation takes place at the aziridine ring. The C-10 carbamate serves as an auxiliary alkylator function, only after the aziridine first monoalkylated the DNA (Scheme 2).[7]

Due to the unique bifunctional alkylation mechanism of mitomycin C, coupled with its clinical usefulness, intensive efforts have been made to design more efficacious mitomycin analogues based on the mode of action of the original drug.[8] For the rational design of structural MC analogues in which this mode of action is preserved, it is essential to understand the individual reactivities of the two alkylating functions of MC with DNA. The monofunctional MC derivative, in which *the carbamate function is absent* (**2**; decarbamoyl MC) has been found to monoalkylate DNA by its aziridine by a mechanism strictly analogous to that by MC.[6c,9] An alternative monofunctional mitomycin, **3** (2,7-diaminomitosene; 2,7-DAM),[10ai] in which *the aziridine function is absent* while the weaker, carbamate alkylator group is intact, presents an excellent opportunity to study the intrinsic reactivity of the MC carbamate alone toward DNA, independent of the aziridine. A direct comparison of the mitosene derivative **3** with mitosanes[10i] MC and **2** is appropriate since all three converge to the mitosene structure in the course of the reductive activation; Scheme 2. We report that the carbamate function of 2,7-DAM alkylates DNA in the major groove upon reductive activation, in sharp contrast to the exclusive minor groove reactivity of the carbamate of MC. We isolated the resulting single covalent adduct and determined its structure, mechanism of formation, and DNA sequence specificity. Formation of this adduct is also important in the biological context since 2,7-DAM is a major byproduct of the reductive activation process of MC (Scheme 2) in cell-free systems[10a,b,g,h] and in tumor tissues and cells.[11] Furthermore it is the major product upon complete reduction of aqueous solutions of MC in the absence of DNA.[16b] Therefore the adduct described herein is likely to occur in tissues and cells which had been exposed to MC treatment. Suggestive evidence for 2,7-DAM-mediated alkylation of DNA treated with MC in cell-free activation systems has been described.[12b]

Scheme 2. Mechanism of the Reductive Activation of MC and Reactions of the Activated Form **7** with DNA and H^+



Alkylation of DNA by 2,7-DAM has been reported previously from two laboratories.[12a,b,10d] The Remers group[12a] observed,

(6) (a) Tomasz, M.; Chowdary, C.; Lipman, R.; Shimotakahara, S.; Veiro, D.; Walker, V.; Verdine, G. L. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 6702−6706. (b) Tomasz, M.; Lipman, R.; Chowdary, D.; Pawlak, J.; Verdine, C. L.; Nakanishi, K. *Science* **1987**, *235*, 1204−1208. (c) Tomasz, M.; Lipman, R.; McGuiness, B. F.; Nakanishi, K. *J. Am. Chem. Soc.* **1988**, *110*, 5892−5896. (d) Bizanek, R.; McGuiness, B. F.; Nakanishi, K.; Tomasz, M. *Biochemistry* **1992**, *31*, 3084−3091. (e) Tomasz, M.; Lipman, R.; Verdine, G. L.; Nakanishi, K. *Biochemistry* **1986**, *25*, 4337−4344.

(7) Tomasz, M.; Chawla, A. K.; Lipman, R. *Biochemistry* **1988**, *27*, 3182−3187.

(8) Kasai, M.; Arai, H. *Expert Opin. Ther. Pat.* **1995**, *5*, 757−770.

(9) (a) Li, V.; Kohn, H. *J. Am. Chem. Soc.* **1991**, *34*, 7120−7126. (b) Li, V.; Choi, D.; Tang, M.-s.; Kohn, H. *Biochemistry* **1995**, *34*, 7120−7126.

(10) (a) Tomasz, M.; Lipman, R. *Biochemistry* **1981**, *20*, 5056−5061. (b) Siegel, D.; Gibson, N. W.; Preusch, P. C.; Ross, D. *Cancer Res.* **1990**, *50*, 7483−7489. (c) Beall, H. D.; Mulcahy, T.; Siegel, D.; Traver, R. D.; Gibson, N. W.; Ross, D.; *Cancer Res.* **1994**, *54*, 3196−3201. (d) Siegel, D.; Beall, H.; Senekowitsch, C.; Kasai, M.; Arai, H.; Gibson, N. W.; Ross, D. *Biochemistry* **1991**, *31*, 7879−7885. (e) Sharma, M.; Tomasz, M. *Chem. Res. Toxicol.* **1994**, *7*, 390−400. (f) Hoey, B. M.; Butler, J.; Swallow, A. J. *Biochemistry* **1988**, *27*, 2608−2614. (g) Pan, S.-S.; Andrews, P. A.; Glover, C. J.; Bachur, N. R. *J. Biol. Chem.* **1984**, *259*, 959−966. (h) Peterson, D. W.; Fisher, J. *Biochemistry* **1986**, *25*, 4077−4084. (i) The term "mitosene" defines mitomycin derivatives with indoloquinone chromophore (cf. **3**), while "mitosanes" are derivatives of 9,9a-dihydroindoloquinones, exemplified by MC (**1**).

(11) (a) Chirrey, L.; Cummings, J.; Halbert, G. W.; Smyth, J. F. *Cancer Chemother. Pharmacol.* **1995**, *35*, 318−322. (b) Cummings, J.; Chirrey, L.; Willmott, N.; Halbert, G. W.; Smyth, J. F. *J. Chromatogr.* **1993**, *612*, 105−113.

(12) (a) Iyengar, B. S.; Dorr, R. T.; Shipp, N. G.; Remers, W. A. *J. Med. Chem.* **1990**, *33*, 253−257. (b) Prakash, A. S.; Beall, H.; Ross, D.; Gibson, N. W. *Biochemistry* **1993**, *32*, 5518−5525. (c) Ueda, K.; Morita, J.; Komano, T. *Biochemistry* **1984**, *23*, 1634−1640.

in addition, that 2,7-DAM was cytotoxic and caused extensive DNA single-strand breakage in L-1210 leukemia cells. No adducts were isolated from alkylated DNA, however, in either investigation, and therefore, the chemical details of the alkylation remained uncertain. Our results unequivocally characterize the reaction by means of isolation and structure determination of the resulting DNA adduct and by observation of its DNA sequence specificity. The contrasting structures of the DNA adducts of the two closely related drugs MC and 2,7-DAM testify to a great complexity of factors governing covalent interactions of mitomycins with DNA.

Results

Reductive Alkylation of *M. luteus* DNA by 2,7-DAM and Detection of Several Adducts after Digestion of the Alkylated DNA. (i) DT-diaphorase/NADH as reductive activating agent: 2,7-DAM, DNA, NADH, and DT-diaphorase were incubated at 37 °C in neutral aqueous buffer for 1 h under argon. The resulting 2,7-DAM−DNA complex was enzymatically hydrolyzed using DNAse I, SVD, and alkaline phosphatase, and the hydrolysate was chromatographed by reverse-phase HPLC. The tracing (Figure 1a) indicated the formation of two major adducts marked **11** and **13** and several minor ones, one of them marked **12**. Conducting the reactions in the presence of air gave very similar results (data not shown). *pH dependence of the yield of the adducts:* When the reaction mixture was incubated at pH 5.8 instead of pH 7.4, the yield of each adduct was higher, as determined from the intensities of the adduct peaks relative to the peak corresponding to the deoxythymidine component of the digest (marked dT) on the tracings (Figure 1b). (ii) NADH−cytochrome *c* reductase/NADH or (iii) chemical reduction by H_2/PtO_2 as activating agents gave the same results as above, as shown in Figure 1c,d for the H_2/PtO_2 experiments. (iv) $Na_2S_2O_4$ activation at pH 7.4 resulted in barely detectable adducts while that at pH 5.8 yielded detectable adducts, but in considerably lower yields than the other activation procedures (data not shown).

Release of a Single Adduct by Heat from Alkylated DNA (Scheme 3). The above 2,7-DAM−DNA complexes were heated in neutral aqueous buffer at 90 °C for 30 min followed by HPLC of the mixture. Using 254 nm absorbance detection a single distinct component **13** was observable above a broad shallow absorbance spread due to degraded DNA. Monitoring the absorbance at 320 nm, near an absorbance maximum of 7-aminomitosenes,[10a] resulted in the detection only of adduct **13**, as expected since DNA itself has no absorbance at this wavelength (Figure 1e). When the heated 2,7-DAM−DNA complex was subsequently digested by DNAse I, SVD, and APase, HPLC indicated again the presence of only the single adduct **13** in the hydrolysate in addition to the four regular nucleosides (Figure 1f). These experiments led to the tentative conclusion that the alkylation by 2,7-DAM produced a single adduct **13** which was hydrolyzed from DNA non-enzymatically upon heat treatment while the other adduct components **11** and **12** (Figure 1a−d), which were observable only upon enzymatic digestion, represented higher order DNA fragments containing bound **13.** If so, these fragments should yield free **13** when heated (Scheme 3). This was demonstrated to be the case as follows.

Direct Proof that 11 and 12 are Heat-Labile Precursors of 13. Adduct **11** was heated at 90 °C for 30 min in water or at 85 °C for 5 min in 90% acetic acid. Under both conditions, it was completely converted to **13** and deoxyguanosine 3′-phosphate as seen by HPLC (Figure 2). Adduct **12** gave **13** but no dGp under similar heating conditions (not shown).

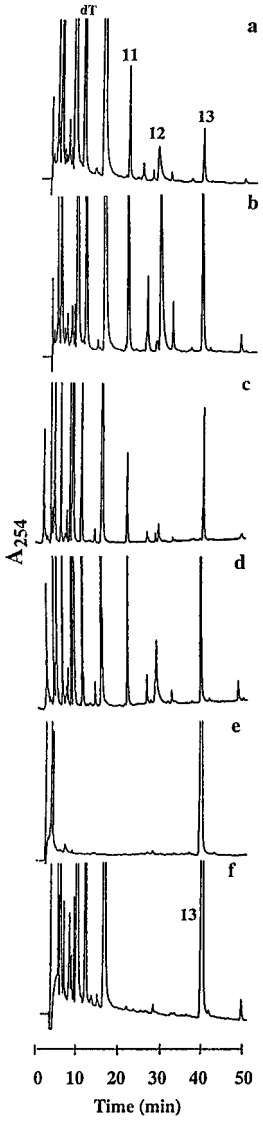
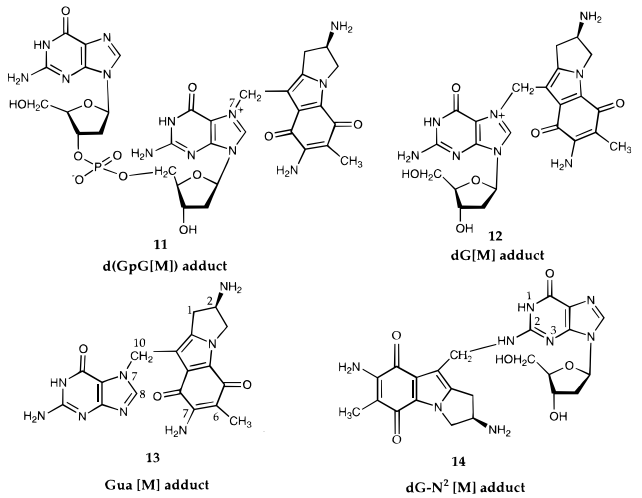


Figure 1. Isolation of adducts of 2,7-DAM and DNA by HPLC from enzymatic digests of 2,7-DAM−DNA complexes. Reductive activation conditions for adduct formation: (a) DT-diaphorase/NADH, pH 7.0; (b) same, pH 5.8; (c) H_2/PtO_2, pH 7.4; (d) same, pH 5.8. (a−d) Enzymatic digests of 2,7-DAM−DNA complexes. (e) Undigested 2,7-DAM−DNA complex of b was heated at 90 °C for 30 min, followed by HPLC of the reaction mixture. (f) Undigested 2,7-DAM−DNA complex of b was heated at 90 °C for 30 min then enzymatically digested, followed by HPLC of the resulting mixture.

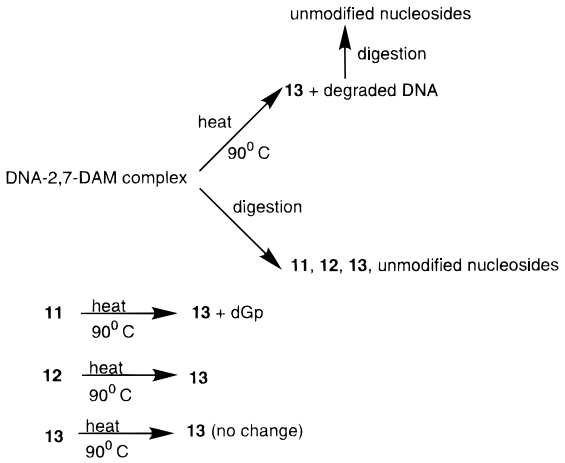
Structure of the Adducts. Adducts **11**, **12**, and **13** were isolated from enzymatic hydrolysates of 2,7-DAM−*M. luteus* DNA complexes, by collecting the appropriate HPLC peaks indicated in Figure 1. Adduct **13** was isolated in a better yield from undigested DNA by heating at 90 °C. Yields were typically 2 A_{260} units (∼100 μg) from 2.5 mg *M. luteus* DNA, less from calf thymus DNA. Both **11** and **12** were unstable, slowly decomposing in solution to give **13**. This fact, together with results of differential spectroscopy and LC−ESIMS experiments gave unequivocal proof of all three structures as follows.

Product 11: The UV spectrum qualitatively indicated a combination of deoxyguanosine and 2,7-DAM chromophores (Figure 3a,d,f). Subtraction of the UV spectrum of **12** yielded a spectrum corresponding to that of standard deoxyguanosine (**15**; Figure 3e), indicating that **11** has a deoxyguanosine chromphore added to the chromophore of **12**. ESIMS (Figure

Chart 2



Scheme 3. Degradations of the 2,7-DAM−DNA Complex



4) in the negative ion mode gave *m/z* 838 (100%), corresponding to $[M - 2H]^-$; in the positive ion mode, *m/z* = 840 (36%), corresponding to $[M]^+$ was observed. Additional peaks in the latter were *m/z* 689 (19%, $[M -$ guanine $+ 2H]^+$), 597 (20%, $[M -$ mitosene residue $+ H]^+$), 421 (100%, $[M]^{2+}$). Heating of **11** gave dGp and adduct **13** (Figure 2).

Product 12: The UV spectrum of **12** (Figure 3b) qualitatively indicated again a combination of dG and 2,7-DAM. Subtraction of the UV spectrum of 2,7-DAM yielded a spectrum corresponding to standard 7-ethyl-dG (**16**) (Figure 3f). Heating of **12** gave adduct **13** (data not shown). ESIMS (Figure 4c) in the positive ion mode gave *m/z* 511 (47%, $[M]^+$), 395 (100%, $[M -$ deoxyribose $+ 2H]^+$), 244 (88%, $[M -$ deoxyguanosine$]^+$).

Product 13: This substance was the stable end product of heating of its various precursors (Scheme 3). Its UV spectrum (Figure 3c) was a 1:1 composite of that of 2,7-DAM and standard 7-methylguanine (**18**) as shown by difference UV spectra (Figure 3d,g). ESIMS (Figure 4) in the positive ion mode gave *m/z* 395 (100%) corresponding to $[M + H]^+$.

The mass spectrometry results confirmed the molecular weight and provided evidence of the structural composition of each of the adducts. The heat lability of the glycosidic linkage of the adducted guanine is characteristic of either Gua-N7 or Gua-N3 substitution.[13b] The UV data, however, showed decisively that the guanine was substituted at its 7-position by 2,7-DAM as follows: Since each positional alkyl-substitution isomer of deoxyguanosine and guanine has a unique characteristic UV spectrum,[13a] differential UV spectroscopy is well-suited for defining the site of substitution in alkylated guanine derivatives as previously demonstrated.[14] This method was eminently applicable in the present investigation. Since the spectrum of 3-alkylguanine (Figure 3h) is vastly different from that of 7-alkylguanine, the results are only compatible with 7-substitution of Gua by 2,7-DAM. An additional test further confirmed guanine-N7 as the alkylation site: Alkylation of the synthetic oligodeoxynucleotide duplex **18** proceeded in 11% yield while the 7-deazaguanine-substituted analogous duplex **19** yielded no alkylation product (Figure 5).

This, together with the demonstrated conversions of the adducts (Scheme 3), defines the three structures as the dideoxyguanosine phosphate−2,7-DAM adduct **11**, the deoxyguanosine−2,7-DAM adduct **12**, and the guanine−2,7-DAM adduct **13**.

DNA Sequence Specificity of the Alkylation by 2,7-DAM. Poly(dG)·poly(dC) and poly(dG-dC)·poly(dG-dC) were alkylated under identical conditions, and the resulting complexes were digested and analyzed by HPLC. The analysis indicated that poly(dG)·poly(dC) was extensively alkylated (9% of the guanine residues) while the alternating sequence poly(dG-dC)·poly(dG-dC) was alkylated to a much lesser extent (Figure 6). Also, while adduct **11** was prominent among the three adducts in the digest of poly(dG)·poly(dC), it was not present in that of poly(dG-dC)·poly(dG-dC), consistent with its assigned structure. Poly(dA-dT)·poly(dA-dT) yielded no adducts (Figure 5). It was concluded that guanine in a GpG sequence of DNA is a more favorable substrate for alkylation than in GpC or CpG. To further refine the sequence specificity, a series of self-complementary duplex oligonucleotides were alkylated, digested, and analyzed by HPLC. The results were as follows (Figure 5): Guanines spaced singularly (**15a**,**b**) had very low reactivity, if any. Increasing the number of adjacent guanines in the sequences resulted in a dramatic increase of the average yield of alkylation per guanine residue. These results, taken together with those of the synthetic polynucleotide series above, show that alkylation by 2,7-DAM is favored at guanines located in longer $(G)_n$ tracts of DNA.

Lack of Alkylation of Deoxyguanosine and d(GpC) by Reduced 2,7-DAM. All attempts to detect reaction products formed between dG and 2,7-DAM failed, as described in detail in the Experimental Section. Several attempts using d(GpC) as the substrate gave similar negative results. *Lack of solvolysis of the carbamate* of reductively activated 2,7-DAM was revealed by the same experiments based on the following: HPLC analysis of the reaction mixtures in which H_2/PtO_2 or DT-diaphorase were used as reducing agents indicated quantitative recovery of 2,7-DAM after the reduction/reoxidation cycle.

pH Dependence of the Alkylation of DNA by 2,7-DAM. Alkylation of *M. luteus* DNA was carried out at pHs ranging from 5.0 to 7.4, using either DT-diaphorase, NADH−cytochrome *c* reductase, or H_2/PtO_2 as reductive activator. The yield of adducts decreased with increasing pH 10−20-fold within this range regardless of the method of activation (Figure 7).

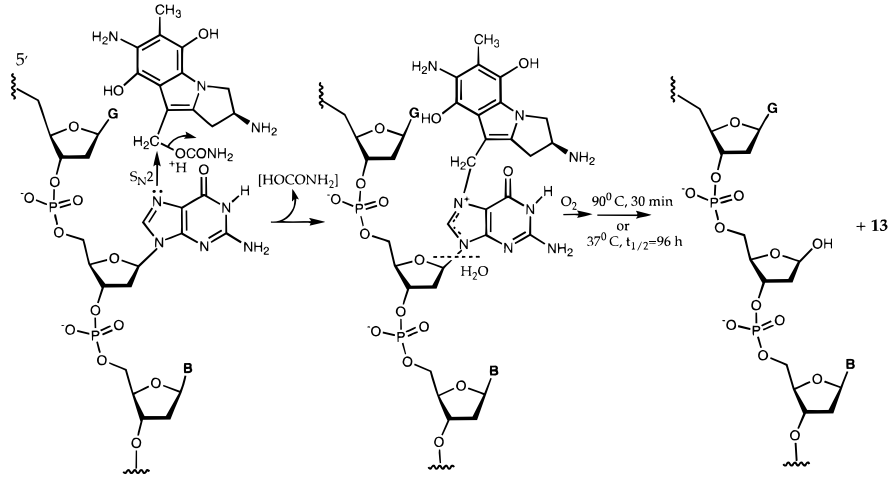
Hydrolysis Rate of the Glycosidic Linkage of the Alkylated Guanines in *M. luteus* DNA at 25 and 37 °C. This was determined by incubation of 2,7-DAM−*M. luteus* DNA complexes in pH 7.4 buffer and analysis of the amount of hydrolytically released adduct **13** by HPLC. The results

(13) (a) Singer, B.; Grunberger, D. *Molecular Biology of Mutagens and Carcinogens*; Plenum Press: New York, 1983; pp 295−334. (b) *Ibid.* pp 15−44. (c) *Ibid.* pp 354.

(14) Tomasz, M.; Lipman, R.; Verdine, G. L.; Nakanishi, K. *J. Am. Chem. Soc.* **1985**, *107*, 6120−6121.

(15) Suresh Kumar, G.; He, Q.-Y.; Behr-Ventura, D.; Tomasz, M. *Biochemistry* **1995**, *34*, 2662−2671.

Scheme 4. DNA Alkylation by Reductively Activated 2,7-DAM and Subsequent Hydrolysis of the Adduct



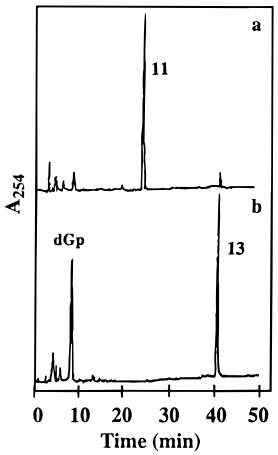


Figure 2. HPLC assay of the conversion of adduct **11** to adduct **13** upon heating. Reaction conditions: H_2O, 90 °C, 30 min.

indicated half-lifes of 400 and 96 h of the glycosidic linkage at 25 and 37 °C, respectively.

Discussion

The bifunctional DNA alkylator and cross-linker MC is converted by simple chemical or enzymatic reduction to 2,7-DAM.[10a,b,d,f−h] This reaction is interesting mechanistically since it proves the intermediacy of quinone methide **7** in the reductive activation of MC (Scheme 2).[10a,h,16] It also represents the major path of the reductive metabolism of MC in tumor cells and tissues.[11] Evidence that 2,7-DAM may also act as a reductive DNA alkylator was first provided by Remers and his colleagues,[12a] reporting an irreversible association of the mitosene chromophore with DNA after incubation with 2,7-DAM and $Na_2S_2O_4$. Similarly, Ross, Gibson, and co-workers, investigating the reductive metabolism of MC as mediated by the quinone oxidoreductase DT-diaphorase, discovered that 2,7-DAM formed from MC could itself be activated reductively to alkylate DNA.[10d,12b] Evidence for this was (i) the observed association of radioactivity with calf thymus DNA after incubation with [^{3}H]-2,7-DAM[10d] and (ii) hot piperidine-induced cleavage of 2,7-DAM-treated DNA as detected by gel electrophoresis of the resulting ^{32}P-labeled DNA fragments.[12b] On the basis of the fragment pattern, the site of alkylation was proposed to be N7 of guanine in the major groove. Guanine-specific cleavage

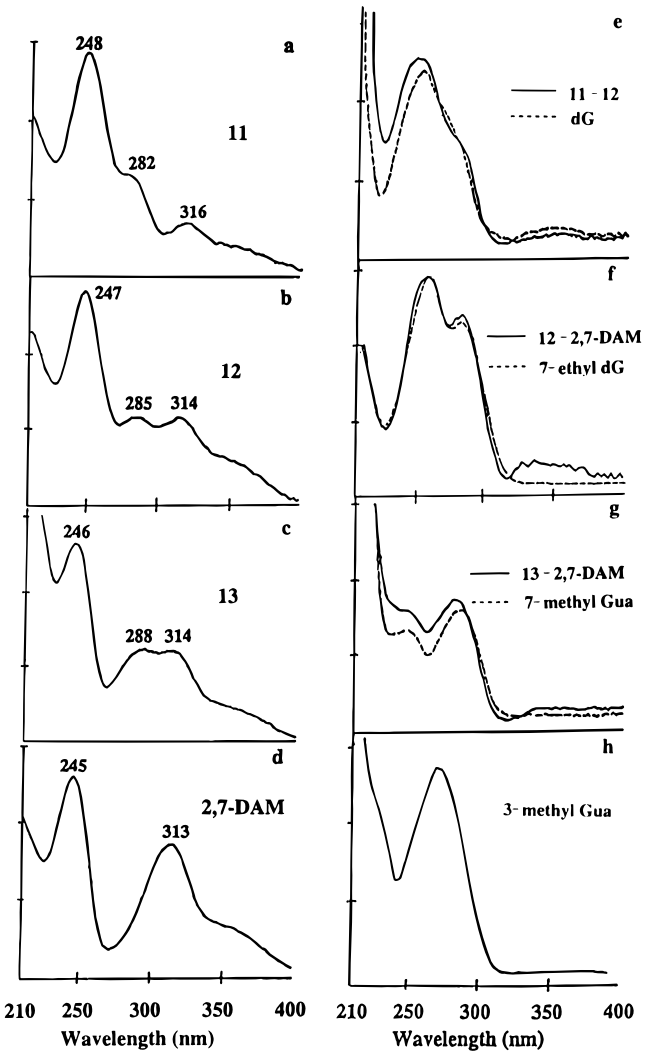


Figure 3. UV spectra and computed UV difference spectra of 2,7-DAM, its DNA adducts, and alkylated guanine derivatives. Spectra were determined by the LC-diode array method. The 315 nm absorption maximum of the mitosene chromophore was used for the weighted subtractions of spectra: (a) **11**; (b) **12**; (c) **13**; (d) 2,7-DAM; (e) —, difference spectrum **11**−**12**; - - -, dG; (f) —, difference spectrum **12** − 2,7-DAM; - - -, 7-ethyl-dG; (g) —, **13** − 2,7-DAM; - - -, 7-methylguanine; (h) 3-methylguanine.

of DNA treated with MC and $NaBH_4$, reported earlier,[12c] may also have originated from alkylation by 2,7-DAM, formed from MC in the reaction mixture. However, these experiments did

(16) (a) Kohn, H.; Zein, N. *J. Am. Chem. Soc.* **1983**, *105*, 4105−4106. (b) Schiltz, P.; Kohn, H. *J. Am. Chem. Soc.* **1993**, *115*, 10510−10518. (c) *Ibid.* **1992**, *114*, 7958−7959.

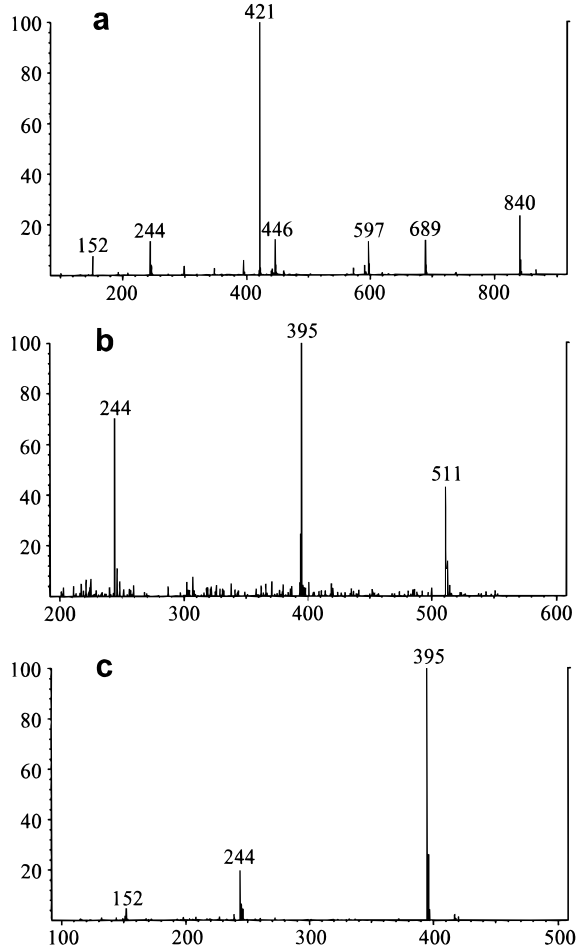


Figure 4. ESIMS of 2,7-DAM−DNA adducts: (a) adduct **11**, positive ion mode; (b) adduct **12**, positive mode; (c) adduct **13**, positive mode.

not reveal the structure of the adduct. Furthermore, any piperidine-resistant alkylation site (such as guanine-N^2, in the minor groove, for example) would have been undetected. Adducts from alkylated DNA were not isolated in either of these studies. The Remers group[12a] isolated and characterized a guanine-N^2 adduct (**14**) from the reaction between reduced 2,7-DAM and deoxyguanosine as the major product, clearly different from the adducts proposed by the Ross and Gibson group. Thus, taking these findings together, the nature of adducts formed between 2,7-DAM and DNA remained open and somewhat controversial. The present approach, accomplishing the isolation and structure elucidation of all 2,7-DAM−DNA fragments both from enzymatically hydrolyzed DNA and from DNA upon heating shows decisively that upon reductive activation a single adduct **13** is formed between 2,7-DAM and DNA. Use of this type of double protocol to search for DNA alkylation products is necessary when one aims at isolating all potential adducts rather than just heat-labile ones.

Evidence that the Carbamate of 2,7-DAM Alkylates DNA by the S_N2 Mechanism. The regioselective alkylation of the N7-position of guanine in the DNA major groove by the C-10 carbamate function of reduced 2,7-DAM is in sharp contrast to the regioselectivity by the C-10 carbamate of reduced MC to the N^2-position of guanine, in the minor groove. In the case of MC the first alkylation step is executed by the C-1 (aziridine) function of the activated MC **7** at guanine-N^2. This traps the C-10 carbamate in the minor groove, and thus, the second alkylating step, performed by this function, is facilitated as an intramolecular reaction at the N^2-position of a second guanine (**9** → **10**), favorably positioned for the bonding. Such favorable

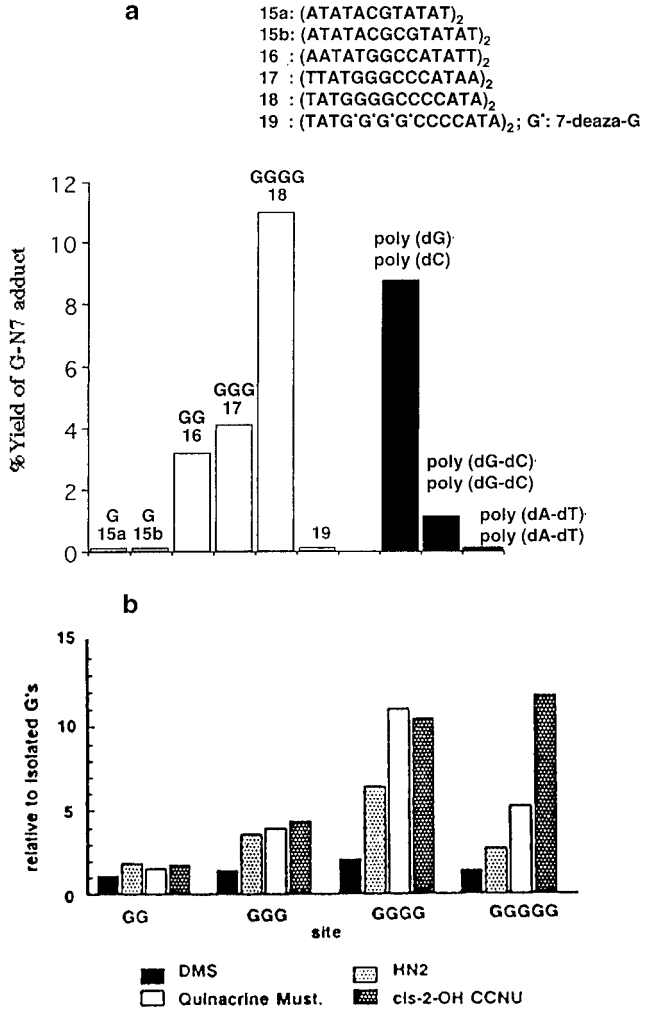


Figure 5. Sequence specificity of the yield of alkylation of DNA at guanine-N7 (a) by 2,7-DAM and (b) by several common synthetic alkylating agents as reproduced from ref 20: DMS, dimethyl sulfate; Quinacrine Must., quinacrine mustard; HN2, mechlorethamine; cis-2-OH CCNU, [N^1-*cis*-(2-hydroxycyclohexyl)-N^2-nitroso-N^2-(2-chloroethyl)]urea.

condition exists exclusively at the CpG·CpG sequence.[17] In the case of 2,7-DAM, however, which lacks the aziridine, the alkylation of guanine-N^2 by the carbamate is not facilitated by any such intramolecular constraint and favorable alignment. Our results show, furthermore, that the switch from G-N^2 to G-N7 alkylation is not due simply to the lack of favorable positioning but rather to the intrinsic S_N2-type reactivity of 2,7-DAM which renders it to selectively alkylate the much more nucleophilic G-N7.[21] This is apparent from the following:

As shown in Figure 5, increasing the length of the $(G)_n$ tract in the oligonucleotide series resulted in an increase of the average intensity (and average rate[18]) of the alkylation of the guanine residues. It appears that two adjacent G's is a minimum requirement for alkylation in the series under our conditions. This is in excellent agreement with the earlier study of Prakash et al.,[12b] who observed -GG-specificity of 2,7-DAM by their DNA cleavage assay (*vide supra*). However, another sequence, -GTC-, also seen to be cleaved in their study, was entirely unreactive in our system, when incorporated in the oligonucle-

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(18) The observed extent of alkylation under standard, first-order kinetic conditions (large excess of activated MC over guanine sites) may be regarded as proportional to the rate of the alkylation (Borowy-Borowski, H.; Lipman, R.; Tomasz, M. *Biochemistry* **1980**, *29*, 2999−3004).

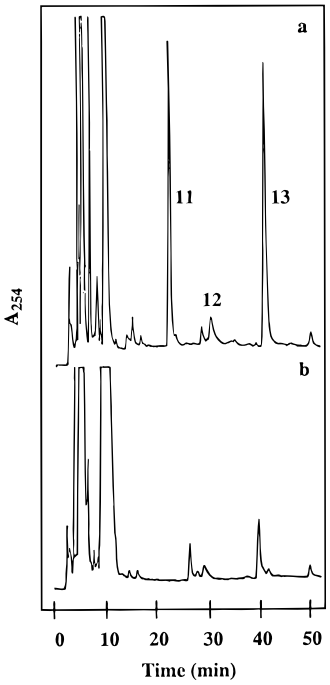

Figure 6. Comparison of adduct formation of 2,7-DAM with (a) poly(dG)·poly(dC) and (b) poly(dG-dC)·poly(dG-dC) by HPLC of the enzymatic digests of the 2,7-DAM−polynucleotide complexes.

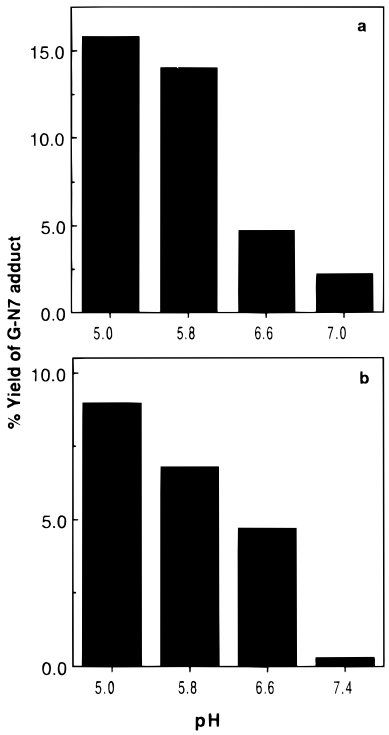

Figure 7. pH dependence of the yield of adduct **13** in the alkylation of *M. luteus* DNA by 2,7-DAM: (a) activator DT-diaphorase/NADH. (b) activator NADH−cytochrome *c* reductase. The yields were determined from HPLC of 2,7-DAM−DNA complexes which were first heated then enzymatically digested prior to HPLC, yielding the general pattern seen in Figure 1f.

otide 5′-d(TAATAT*GTC*ATATT)·5′-d(AATATGACATATTA) (data not shown). Specificity to $(G)_n$ sequences is further demonstrated by the observed 10-fold increase of alkylation of poly(dG)·poly(dC) compared to poly(dG-dC)·poly(dG-dC) (Figures 5 and 6). Enhancement of guanine-N7 reactivity in $(G)_n$ tracts was first discovered by Kohn and his colleagues,[19] using as alkylators a wide variety of important carcinogens, mutagens, and antitumor agents. This is exemplified by their data in Figure 5b.[20] The alkylating specificity of 2,7-DAM (Figure 5a) shows a striking similarity qualitatively and quantitatively to these and other G-N7 alkylating agents. A basis for the enhancement of G-N7 reactivity in $(G)_n$ tracts was proposed by Pullmann and Pullman,[21] who showed by theoretical calculations that the guanine-N7 sites have the highest negative molecular electrostatic potential in DNA and that the magnitude of this property is *sequence-dependent*, i.e. guanines surrounded by other guanines exhibit more negative molecular potential minima at N7 than single guanines. In experimental corroboration, Kohn et al.[22] demonstrated a quantitative correlation between the observed alkylating intensities of various nitrogen mustards and the calculated electrostatic potentials of guanine-N7 at increasing length of $(G)_n$ sequences. It is now general knowledge that guanine-N7 atoms in $(G)_n$ tracts are the most nucleophilic sites in B-DNA and that selectivity for guanine-N7 in $(G)_n$ tracts is characteristic of S_N2-type alkylation.[23] From the observed behavior of 2,7-DAM, it is apparent that it selectively reacts only with the strongest nucleophile in DNA, i.e. G-N7 in $(G)_n$ tracts, indicative of the S_N2-type mechanism. This conclusion is supported by additional, independent evidence as follows: (i) Reduced 2,7-DAM alkylates strong model nucleophiles, e.g. thiolates, by the S_N2 mechanism.[24,10e] (ii) When 2,7-DAM is reduced in the absence of DNA or strong nucleophiles, no solvolytic displacement (hydrolysis) of the carbamate is observed. (iii) 2′-Deoxyguanosine, which is a weaker nucleophile than guanine residues in duplex DNA, did not react with 2,7-DAM in our reduction systems. (We failed to obtain a product (**14**) as reported by Iyengar et al.[12a]) These facts together provide substantial support for the S_N2-type reactivity of 2,7-DAM in the physiological milieu. The observed inverse dependence of the rate[18] on pH (Figure 7) may be due to more facile reduction at lower pH,[16b] requirement of protonation of the 2-amino group of 2,7-DAM (pK_a 7.5),[15] or protonation of the carbamate leaving group.[12b]

Evidence that the Carbamate of MC Alkylates DNA by the S_N1 Mechanism. As a result of the first alkylation step by the aziridine a monofunctionally linked intermediate is formed in the minor groove (**8** in Schemes 2 and 5; cf. **4** in Scheme 1). Provided that **8** is linked to guanine at a CpG·CpG sequence the carbamate function alkylates the N^2 of the guanine in the opposite strand (**8** → **9** → **10**), leading to the bisadduct **5** (Scheme 5, arrow 2). At other sequences, where it is not juxtaposed directly to a G-N^2, *it undergoes solvolysis* (Scheme 5, arrow 1) leading to the monoadduct **6** (Scheme 1).[6b,7] Formation of this solvolytic product strongly suggests that the mechanism of the carbamate displacement involves the iminium ion intermediate **9**, constituting S_N1 displacement of the carbamate overall as first proposed by Zein and Kohn.[34] The origin of this different behavior of MC from that of 2,7-DAM in its DNA alkylation remains to be elucidated. High local

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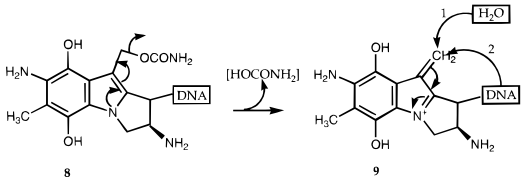
Scheme 5. S_N1 Displacement of the C-10 Carbamate of the MC−DNA Monoadduct



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acidity in the minor groove at G·C basepairs,[35] the site of MC monoadducts, may be responsible.

Role of Pre-covalent Intercalation of 2,7-DAM in its G-N7 Alkylation Regioselectivity. Steric accessibility or specific pre-covalent binding may modulate or even dominate site selection of an agent in its covalent reaction with DNA. 2,7-DAM binds to DNA and synthetic polynucleotides by nonspecific intercalation in a K_b range 10^4-10^5 M^{-1} nt^{-1}.[15] Peterson and co-workers[27] observed binding of 2,7-DAM to DNA in its protonated form (pK_a 7.5) with binding constant 2.5×10^{-4} M, in excellent agreement with our results.[28] However, since this non-covalent binding is not sequence-specific, it does not account for the sequence specificity of 2,7-DAM alkylation to poly(dG)·poly(dC) or $(G)_n$ tracts of oligonucleotides. Assuming that the reduced, hydroquinone form of 2,7-DAM also intercalates into DNA, this binding interaction may facilitate the alkylation kinetically. It is noteworthy that a similar mechanism is known to operate in the case of alkylation of DNA by aflatoxin B_1 8,9-epoxide.[36]

Hydrolytic Lability and Lack of Guanine-N^2 Alkylation of 2,7-DAM-Adducted DNA. The facile hydrolytic release of the 7-alkylated guanine adduct **13** from DNA ($t_{1/2}$ at 37 °C is 96 h) and from the DNA digestion products **11** and **12** is in accordance with the characteristic lability of the glycosidic linkage of 7-alkyldeoxyguanosine derivatives.[13b] 7-Alkylated guanine sites of DNA give rise to abasic sites upon hydrolysis (Scheme 4) which are themselves labile to heat or alkali, leading to cleavage of DNA.[13a] Hot piperidine treatment also cleaves DNA at 7-alkylated guanine sites; this method was used by Prakash et al.[12b] to detect and characterize the alkylation of DNA by 2,7-DAM, as discussed above. Their conclusion that 2,7-DAM leads to G-N7 adducts is fully verified by the present work. No evidence was found for guanine-N^2 alkylation by 2,7-DAM as seen from the total lack of heat-stable adducts in enzymatic digests of the alkylated DNA.

Biological Significance of the Alkylation of DNA by 2,7-DAM. The alkylation is likely to occur in cells and organisms treated with MC, since MC is metabolized to 2,7-DAM.[10,11] Side-by-side formation of minor and major groove DNA adducts in cell-free MC activation systems has been observed.[12b,30] Remers and co-workers reported that 2,7-DAM is cytotoxic and produces DNA single-strand breaks in tumor cells.[12a] The latter effect is particularly interesting since it may be related to the G-N7 alkylation described herein. An intriguing possibility that 2,7-DAM is primarily mutagenic, in analogy to aflatoxin and in contrast to the bifunctional MC which is primarily cytotoxic, remains to be tested.

Significance for Drug Design. Numerous mitosenes[10i] with various types of alkylating groups in the C-1 and/or C-10 positions have been designed and synthesized previously as potential antimicrobial or antitumor agents.[31] The present work highlights the S_N2 reactivity of the C-10 carbamate of a mitosene without a C-1 alkylating function and shows how it differs from the reactivity of the C-10 carbamate of reduced MC which possesses the C-1,2-aziridine ring in addition. The comparison of the DNA adducts of the two related drugs MC and 2,7-DAM demonstrates the mechanistic complexity involved in the alkylation of DNA by mitomycins. The conclusions should be taken into account for DNA receptor-based drug design of monofunctional or bifunctional alkylating agents based on the mechanism of MC.

Experimental Section

Materials. 2,7-DAM was synthesized from MC as previously described.[10a] DT-diaphorase (NAD(P)H-quinone oxidoreductase; EC I.6.99.2) from rat Walker 256 carcinoma cells was prepared as reported.[32] Its activity was assayed as described by Benson et al.[33] NADH−cytochrome *c* reductase and NADH were purchased from Sigma, St. Louis, MO. Poly(dG)·poly(dC), poly(dG-dC)·poly(dG-dC), and poly (dA-dT)·poly(dA-dT) were obtained from Pharmacia Biotech, Piscataway, NJ. All other materials were obtained as previously described.[26]

Quantitative Analysis. Quantities of nucleotides, oligonucleotides, polynucleotides, DNA, and 2,7-DAM were measured by UV spectrophotometry, using their known molar extinction coefficients, as described in detail previously.[15] The percent yields of 2,7-DAM adducts **11**, **12**, and **13** designate the mole percent adducted guanine of total guanine nucleotide residues present in the oligonucleotide or DNA substrates. These yields were determined by HPLC of enzymatic digests of the adducted nucleic acids or oligonucleotides, based on HPLC peak area measurements of the appropriate components of the digests, as described in detail elsewhere.[26]

Spectroscopic Techniques. The UV spectra were determined using a Cary 3 spectrophotometer (Varian) or by diode array scanning of the substance during its elution from HPLC.

LC−MS Analysis. A Hewlett-Packard (Palo Alto, CA) Model 1050 HPLC pump was used to provide linear gradients and a constant flow rate of 200 μL/min. All chromatography was performed on a YMC Inc. J-sphere ODS-M80 HPLC column (2 × 250 mm). Chromatography conditions for electrospray analysis started with 12% CH_3CN with a linear gradient to 36% CH_3CN in 39 min; a buffer concentration of 2 mM ammonium acetate was maintained throughout the run. Under these conditions adduct **11** elutes at 13.4 min, compound **12** elutes at 21.1 min, and adduct **13** elutes at 29.3 min. The column effluent was first passed through a Kratos Spectroflow 783 UV detector set at 254 nm then into the electrospray ion source. MS conditions used a Finnigan Model TSQ-7000 triple-quadrupole mass spectrometer with the standard Finnigan electrospray ion source. Nitrogen was used as a nebulizing gas, and the capillary temperature was 240 °C. The instrument was scanned over the range 110−900 amu at 1.5 s/scan.

HPLC. A Beckman System Gold 125 instrument, equipped with a diode array detector System Gold 165 and controlled by System Gold Chromatography Software, was used.

Reductive Alkylation of *M. luteus* DNA by 2,7-DAM and Detection of Products by HPLC after Digestion of the Alkylated DNA. (i) DT-diaphorase/NADH as activating agent: 2,7-DAM (1.26

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mM), *M. luteus* DNA (1.25 mM nucleotide), NADH (2.5 mM), and DT-diaphorase (0.35 unit/μmol of 2,7-DAM) in 0.01 M KH_2PO_4 + 0.001 M Na_4 EDTA, either pH 5.8 or pH 7.0, buffer were incubated at 37 °C for 1 h under argon. Analytical-scale reactions typically utilized 0.5 μmol of DNA-nucleotide in a 0.4 mL reaction volume, and preparative-scale reactions contained 25 μmol of DNA-nucleotide in a 20 mL volume. The analytical-scale reaction mixture was chromatographed over a 2.5 × 25 cm column of Sephadex G-100, using 0.02 M NH_4HCO_3 as the eluant. The void volume fraction was lyophilized and digested to nucleotides and adducts using DNAse I (50 units/OD_{260}; 345 units/μmol of DNA), SVD (3 units/OD_{260}; 21 units/μmol of DNA), and APase (0.75 unit/OD_{260}; 5.2 units/μmol of DNA) by a previously described protocol.[6a] The digest was fractionated by HPLC. For analytical purposes, a C-18 reverse-phase column (Beckman Ultrasphere ODS; 0.45 × 15 cm) was used; the elution system was 3−12% CH_3CN in 0.03 M KH_2PO_4, pH 5.5 in 55 min, with a flow rate of 1 mL/min. The absorbance of the eluate was monitored at 254 and 320 nm. For preparative-scale reactions, a semipreparative C-18 column (Beckman Ultrasphere, 10 mm × 25 cm) was employed; the elution system was 3−18% CH_3CN in 0.02 M NH_4Ac in 90 min, flow rate of 4.0 mL/min. Fractions were collected manually and lyophilized to yield homogenous material for further study. In a typical experiment, 3.06 A_{312} units of **13** was obtained, estimated as 120 μg by employing ϵ_{312} of mitosene = 10 000. Approximately 10% each of **11** and **12** were also isolated. Poly(dG)·poly(dC) gave a 10-fold greater yield of adduct **11** (cf. Figure 6). (ii) NADH−cytochrome *c* reductase as activating agent: All procedures were identical to those above, except NADH−cytochrome *c* reductase was used instead of DT-diaphorase. (iii) H_2/PtO_2 as activating agent: 2,7-DAM (1.26 mM), *M. luteus* DNA (1.27 mM nucleotide), and PtO_2 (100 μg/μmol of 2,7-DAM) were dissolved in the same buffer as in i, and the mixture was deaerated by purging it with helium for 10 min. H_2 gas was passed through the solution for 4 min, followed by purging by helium for 10 min. PtO_2 was removed by filtration. The mixture was further processed by Sephadex G-100 chromatography, etc., as described in i above. (iv) $Na_2S_2O_4$ reduction: Anaerobic conditions. A mixture of 2,7-DAM (5.0 mM) and *M. luteus* DNA (1.27 mM nucleotide) in the same buffers as in iii were deaerated as above at 37 °C, and then a fresh anaerobic solution of $Na_2S_2O_4$ was added (to 9 mM) in one portion. The mixture was chromatographed after 1 h as in i. Aerobic reduction: The reaction was completed following the same protocol as above except that the reaction was carried out in an open flask with stirring.

Analysis of recovery of excess 2,7-DAM from a reaction mixture of alkylation of *M. luteus* DNA was carried out after a reaction in which NADH−cytochrome *c* and NADH was used as reducing agent, in pH 5.1 buffer. The preparative-scale reaction mixture was chromatographed on a 5 × 56 cm Sephadex G-25 column using 0.02 M NH_4HCO_3 as the eluant. This method results in separation of 2,7-DAM, 10-decarbamoyl-2,7-DAM,[10a] and the other reaction components in one step. 2,7-DAM was recovered in 82% yield. A small amount of 10-decarbamoyl-2,7-DAM was recovered which corresponded to that present in the original 2,7-DAM as an approximately 10% contaminant.

Specific Generation of Adduct 13 from DNA by Heating. In an alternative procedure, the above 2,7-DAM−DNA complexes were not submitted to enzymatic digestion but rather to heating in 0.02 M NH_4HCO_3 or water for 30 min at 90 °C. The resulting mixture was directly chromatographed by HPLC. In two other variations, the 2,7-DAM−DNA complex was (i) *heated first*, then digested, followed by analysis by HPLC; (ii) *digested first*, then heated, followed by HPLC.

Reductive Alkylation of Poly(dG)·poly(dC) and other synthetic polynucleotides and detection of the adducts by HPLC after enzymatic digestion was carried out by the same protocol as for DNA. NADH−cytochrome *c* reductase/NADH was used for activation.

Reductive Alkylation of Self-complementary Duplex Oligonucleotides By 2,7-DAM. 2,7-DAM (3.33 mM), oligonucleotide (8.33 mM nucleotide), NADH (6.67 mM), and DT-diaphorase (0.35 units/μmol of 2,7-DAM) in 0.1 M KH_2PO_4−0.001 M Na_4EDTA, pH 5.8, buffer were incubated for 2 h at 5−10 °C under purging with argon. The reaction mixture was chromatographed over a Sephadex G-25 (fine) column (2.5 × 56 cm) using 0.02 M NH_4HCO_3 (pH 8.1) as the eluant; the void volume fraction was lyophilized, and the residue was heated in H_2O solution at 90 °C for 30 min. The heated mixture was digested enzymatically with SVD and APase by a protocol described before,[26] and the digest was analyzed by HPLC.

Attempted Alkylation of 2′-Deoxyguanosine by 2,7-DAM under Reductive Conditions. (i) $Na_2S_2O_4$ activation:[12a] 2,7-DAM (2 mg) and 2′-deoxyguanosine monohydrate (12.5 mg) were dissolved in H_2O (1.25 mL), and the solution was purged with N_2 for 60 min at 40 °C. A fresh, purged solution of $Na_2S_2O_4$ (12.5 mg, in 0.125 mL of H_2O) was added, and the decolorized mixture was kept for 45 min under N_2, followed by stirring in air for 15 min. The purple mixture was analyzed by HPLC. Several components were apparent in the tracing in increasing order of elution time: deoxyguanosine, one major and a minor unknown, 10-decarbamoyl 2,7-DAM, and 2,7-DAM. A control reaction from which deoxyguanosine was omitted gave the same products, except deoxyguanosine. From this it was concluded that the two unknowns were not dG-2,7-DAM adducts. The 10-decarbamoyl-2,7-DAM component was originally present in 2,7-DAM as a contaminant. (ii) H_2/PtO_2 reduction: The same procedure as that used for alkylation of *M. luteus* DNA was employed, except that the DNA component was substituted by 2′-deoxyguanosine. The filtered reaction mixture was directly analyzed by HPLC. Only starting materials were seen in the HPLC tracing. (iii) Enzymatic activation: DT-diaphorase/NADH, NADH−cytochrome *c* reductase/NADH, and NADPH−cytochrome *c* reductase/NADPH were used in separate experiments, employing the same protocols as those for alkylation of *M. luteus* DNA, except that the latter was substituted by 2′-deoxyguanosine. In some runs, 10-fold molar excess of dG over 2,7-DAM was used. HPLC analysis indicated starting materials only in all experiments.

Attempted Alkylation of d(GpC) by 2,7-DAM under various conditions as above similarly failed.

Acknowledgment. We thank Dr. D. M. Vyas of Bristol-Myers Squibb Co., Wallingford, CT, for a generous gift of mitomycin C. This research was supported by a grant (CA28681) from the National Cancer Institute and a Research Centers in Minority Institutions award (RR003037) from the Division of Research Resources, NIH (both to M.T.).

JA9607401