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Determination of the DNA Cross-Linking Sequence Specificity of Reductively Activated Mitomycin C at Single-Nucleotide Resolution: Deoxyguanosine Residues at CpG Are Cross-Linked Preferentially

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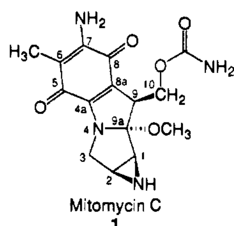
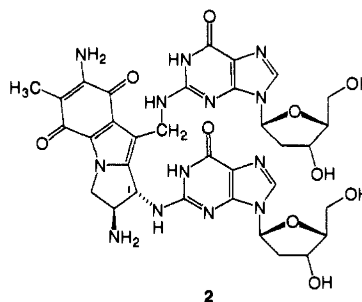
Abstract: Several synthetic oligodeoxyribonucleotide duplexes containing one or more 5′-CG and/or 5′-GC sites and singly radiolabeled at the 5′- or 3′-termini were subjected to interstrand cross-linking by mitomycin C activated with sodium dithionite in the absence of oxygen. Isolation of the interstrand cross-linked products by polyacrylamide gel electrophoresis (PAGE) was followed by fragmentation with Fe(II) EDTA/hydrogen peroxide/ascorbic acid and analysis of the resulting fragment sizes (by PAGE). It was demonstrated that reductively activated mitomycin C cross-links deoxyguanosine residues at duplex 5′-CG sites in strong preference to duplex 5′-GC sites and numerous A/T-containing sites. The identity of neighboring residues had a significant impact on the relative reactivity toward cross-linking of 5′-CG sites. Constrained molecular mechanics calculations on a model of the monoadduct of reductively activated mitomycin C with N2 of deoxyguanosine in a pentanucleotide duplex indicate a strong preference for the conformation required for cross-linking at 5′-CG over that for 5′-GC.

Mitomycin C (MC, 1) is a toxic antibiotic that is active against a wide variety of cancers.[1] Soon after the discovery of MC, it was shown to inhibit the synthesis of DNA in bacteria as well as cause extensive decomposition of DNA. The subsequent finding



that MC causes DNA interstrand cross-links in vivo,[2] as well as in vitro after reductive activation,[3] provided strong evidence that DNA is the relevant cellular target of MC. DNA cross-linking, a property common to many antitumor substances,[2,4] may play an important role in MC's antitumor activity. This paper presents studies which demonstrate that reductively activated MC forms interstrand deoxyguanosine-to-deoxyguanosine cross-links preferentially at the duplex DNA sequence 5′-CG in synthetic duplex DNA fragments.

The chemistry of reductively activated MC has been the subject of numerous studies.[1] Reductive activation of MC results in the expulsion of the elements of methanol from carbons 9 and 9a and alkylation reactions at carbons 1 and 10 resulting from ring opening of the aziridine and loss of the carbamate functions. In addition to monoadducts of reductively activated MC with deoxyguanosine,[5] Tomasz et al. have recently isolated and identified a conjugate of two deoxyguanosine residues and a single mitosene (2) from enzymatic digestion of the reaction product of in vitro chemical reduction of MC in the presence of poly[d(GC)].[6] This same diadduct was isolated by processing rat liver after injection of rats with MC.



The structure of diadduct 2 proves that the N2 amino groups of deoxyguanosine residues in DNA are sites of bifunctional alkylation by reduced MC; thus 2 is an excellent candidate for the structural nucleus of the interstrand cross-link. On the assumption that B-DNA is minimally reorganized by reaction with reduced MC, it has been proposed that the base sequences 5′-CG and 5′-GC in duplex DNA, in which the N2 amino groups of adjacent guanine residues on opposite strands are within 4 Å of one another, are potential sequences for MC interstrand G-to-G cross-linking.[6] In fact, a duplex DNA containing a single 5′-CG site afforded, after exposure to reductively activated MC and appropriate processing, the diadduct 2.[7] The analogous duplex bearing a 5′-GC site failed to provide 2. If the assumption that minimal DNA distortion occurs is relaxed, other base sequences need to be considered as interstrand cross-link sites.

We have recently reported a method for the determination at single-nucleotide resolution of the DNA sequence specificity of interstrand cross-linking agents.[8] The site of cross-linking is determined by fragmentation of a cross-linked DNA duplex using Fe(II) EDTA/hydrogen peroxide/ascorbic acid,[9] followed by

(1) Reviews: (a) Carter, S. *Cancer Chemother. Rep., Part 3* **1968**, *1*, 99. (b) Moore, G. E. *Cancer Chemother. Rep., Part 1* **1968**, *52*, 675. (c) Goldberg, I. H.; Friedman, P. A. *Annu. Rev. Biochem.* **1971**, *40*, 445. (d) Crooke, S. T.; Bradner, W. T. *Cancer Treat. Rev.* **1976**, *3*, 121. (e) Powis, G. *Pharmacol. Ther.* **1987**, *35*, 57.

(2) (a) Iyer, V. N.; Szybalski, W. *Proc. Natl. Acad. Sci. U.S.A.* **1963**, *50*, 355. (b) Matsumoto, I.; Lark, K. G. *Exp. Cell Res.* **1963**, *32*, 192.

(3) Iyer, V. N.; Szybalski, W. *Science* **1964**, *145*, 55.

(4) For reports emphasizing the biological significance of DNA monoalkylation by MC, see: (a) Small, G. D.; Setlow, J.; Kooistra, J.; Shapanka, R. *J. Bacteriol.* **1976**, *125*, 643. (b) Carrano, A. V.; Thompson, L. H.; Stetka, D. G.; Minkler, J. L.; Mazrimas, J. A.; Fong, S. *Mutat. Res.* **1979**, *63*, 175.

(5) (a) Hashimoto, Y.; Shudo, K.; Okamoto, T. *Tetrahedron Lett.* **1982**, *23*, 677. (b) Hashimoto, Y.; Shudo, K.; Okamoto, T. *Chem. Pharm. Bull.* **1983**, *31*, 861. (c) Tomasz, M.; Lipman, R.; Snyder, J. K.; Nakanishi, K. *J. Am. Chem. Soc.* **1983**, *105*, 2059. (d) Tomasz, M.; Lipman, R.; Verdine, G. L.; Nakanishi, K. *Biochemistry* **1986**, *25*, 4337. (e) Tomasz, M.; Chowdary, D.; Lipman, R.; Shimotakahara, S.; Veiro, D.; Walker, V.; Verdine, G. L. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 6702. (f) Pan, S.-S.; Tracki, T.; Bachur, N. R. *Mol. Pharmacol.* **1986**, *29*, 622.

(6) (a) Tomasz, M.; Lipman, R.; Chowdary, D.; Pawlak, J.; Verdine, G. L.; Nakanishi, K. *Science* **1987**, *235*, 1204. (b) Tomasz, M.; Chawla, A. K.; Lipman, R. *Biochemistry* **1988**, *27*, 3187.

(7) Chawla, A. K.; Lipman, R.; Tomasz, M. In *Structure and Expression, Volume 2: DNA and Its Drug Complexes*; Sarma, R. H., Sarma, M. H., Eds.; Adenine Press: Albany, NY, 1987.

(8) Weidner, M. F.; Millard, J. T.; Hopkins, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 9270.

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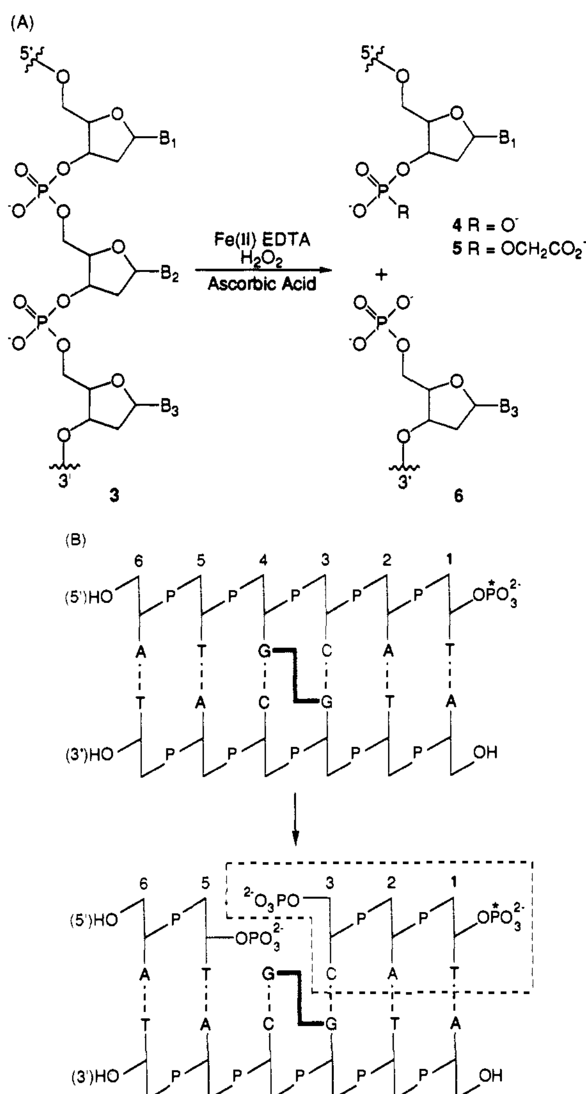


Figure 1. (A) Likely principal products of cleavage of a DNA strand by Fe(II) EDTA/H_2O_2/ascorbic acid. (B) Cleavage at residue 4 of a duplex DNA covalently cross-linked (bold) at residues 4 and 10 followed by denaturation provides a radiolabeled trinucleotide (box) (P = phosphate, * = radiolabel).

analysis of the resulting fragment size distribution by denaturing high-resolution PAGE. The cleavage reaction is generally believed to be mediated by hydroxyl radical and to result in degradation of the sugar (Figure 1A), breaking the original DNA chain (3) to yield two new DNA fragments, one bearing a 5'-terminal phosphate (6) and the other a mixture of 3'-terminal phosphate (4) and phosphoglycolate (5).[10] Denaturing electrophoresis of singly end-radiolabeled DNA strands subjected to this cleavage followed by detection by autoradiography affords an equimolar mixture of all fragment sizes up to and including the full-length single strand. In contrast, interstrand cross-linked DNA duplexes yield short radiolabeled fragments only for cleavage at or to the radiolabeled side of the cross-linked residue. Cleavage at any other residue generates a radiolabeled fragment larger by virtue of the cross-link and less electrophoretically mobile than the intact single strand. This analysis predicts, for example (Figure 1B), that a singly end-radiolabeled hexamer duplex cross-linked through nucleotides 4 and 10 will yield radiolabeled fragment sizes of 0 (phosphate)-3 residues for cleavage at nucleotides 1-4, respectively. Cleavage at any other nucleotide will afford a radiolabeled fragment 6 residues or larger. The absence of 4- and 5-residue radiolabeled fragments pinpoints nucleotide 4 of the radiolabeled strand as the cross-link site. We report here application of fragment distribution analysis to the MC/DNA cross-linking reaction.

(9) (a) Tullius, T. D.; Dombroski, B. A. *Science* **1985**, *230*, 679. (b) Tullius, T. D.; Dombroski, B. A.; Churchill, M. E. A.; Kam, L. *Methods Enzymol.* **1987**, *155*, 537. (c) Tullius, T. D. *Annu. Rev. Biophys. Chem.* **1989**, *18*, 213.

(10) (a) Hertzberg, R. P.; Dervan, P. B. *Biochemistry* **1984**, *23*, 3934. (b) Giloni, L.; Takeshita, M.; Johnson, F.; Iden, C.; Grollman, A. P. *J. Biol. Chem.* **1983**, *256*, 8608.

Experimental Section

Materials and their sources were as follows: DNA synthesis reagents, Applied Biosystems; [γ-^{32}P]ATP and [α-^{32}P]dATP, New England Nuclear; Klenow fragment and T4 polynucleotide kinase, Boehringer Mannheim; mitomycin C, Sigma or Bristol-Myers.

Preparation of Radiolabeled DNA Duplexes. Oligonucleotides were synthesized[11] (Applied Biosystems Model 380A) on a 1-μmol scale. Purification was by denaturing polyacrylamide gel electrophoresis (20% PAGE, 25:1 acrylamide/bisacrylamide, 40% urea) followed by passage through a Sep-Pak C_{18} cartridge [Waters; elution with (1) 10 mM aqueous NH_4OAc, (2) 25% acetonitrile/water]. Radioisotopic labeling of 3'-ends was with [α-^{32}P]dATP/Klenow fragment; 5'-ends were labeled with [γ-^{32}P]ATP/polynucleotide kinase.[12] In all cases, labeling was followed by ethanol precipitation.

Preparation of Mitomycin C Cross-Linked DNA Duplexes. Radiolabeled DNA was added to 0.7 mM (base pairs) duplex DNA and 1.4 mM MC (from a 20 mM stock in 33% aqueous MeOH) in 15 mM Tris (pH 7.5); the total volume was 110 μL. Samples were incubated at 37 °C for 1 h prior to bubbling argon through the solution for 20 min. Three equal aliquots of freshly prepared aqueous sodium dithionite (Baker, 0.05 M stock solution in deoxygenated water) were added at 15-min intervals to the DNA/MC mixture, which was maintained at 37 °C (data in Figures 2 and 3) or 0 °C (data in Figures 4 and 5). Fifteen minutes after the final addition (total 0.14 μmol, 1:1 molar ratio of dithionite to MC), the sample was ethanol-precipitated and then subjected to denaturing 20% PAGE. Cross-linked material, which was visualized by autoradiography and had roughly half the mobility of the corresponding single strands, was isolated from the gel.

Hydroxyl Radical Fragmentation and Electrophoretic Analysis. Fe(II) EDTA cleavage reactions[9] were with 50 μM $(NH_4)_2Fe(SO_4)_2$, 100 μM EDTA, 1 mM sodium ascorbate, 10 mM H_2O_2, and 5 mM Tris (pH 7.5) for 1 min at 25 °C. Reactions were stopped with excess thiourea. Samples were lyophilized, resuspended in a mixture of 90% deionized formamide, 10 mM Tris (pH 7.5), 0.1% xylene cyanole, and 0.1 mM EDTA, heat-denatured at 90 °C for 3 min, and cooled on ice prior to loading on a 25% polyacrylamide gel (19:1 acrylamide/bisacrylamide, 50% urea, 0.35 mm thick, 41 × 37 cm), which was run on a Hoeffer thermojacketed Poker Face gel strand at ca. 65 W and 65-70 °C. The gel was dried (Bio-Rad Model 583) onto Whatman 3MM paper and autoradiographed on Kodak XAR-5 film. Bands were assigned by reference to a Maxam-Gilbert guanine-specific sequencing reaction[13] on un-cross-linked, radiolabeled duplex. Densitometry (Hoeffer GS-300, interfaced to an IBM PC) data were smoothed and plotted by using Spectra Calc (Galactic Industries Corp., Salem, NH).

Molecular Mechanics Calculations. All-atom minimizations were performed on a Microvax II using the program Macromodel 2.5 (Clark Still, Columbia University).[14] The AMBER[15] force field was used in the absence of solvent and counterions, with dielectric R_{ij} and with cutoffs of 7 Å (van der Waals) and 12 Å (electrostatic). Charges were from AMBER. Minimizations were conducted by using Polak-Ribiere conjugate gradient to an rms gradient of 0.01 kcal mol^{-1} Å^{-2}.

Results

Deoxyguanosine Residues at 5'-CG are Cross-Linked in Preference to Those at 5'-GC. To test the proposal that 5'-CG and/or 5'-GC sites in duplex DNA might be cross-linked by reductively activated MC, we prepared the 3'- and 5'-end-radiolabeled du-

(11) (a) Letzinger, R. L.; Lunsford, W. B. *J. Am. Chem. Soc.* **1976**, *98*, 3655. (b) Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859.

(12) Maniatis, T.; Fritsch, E. G.; Sambrook, J. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1981.

(13) Maxam, A. M.; Gilbert, W. *Methods Enzymol.* **1980**, *65*, 499.

(14) Still, W. C.; Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Lipton, M.; Liskamp, R.; Chang, G.; Hendrickson, T.; DeGunst, F.; Hasel, W. *MacroModel*; Version 2.5; Department of Chemistry, Columbia University: New York, 1988.

(15) (a) Weiner, S. J.; Kollman, P. *J. Comput. Chem.* **1981**, *2*, 287. (b) Weiner, S. J.; Nguyen, D.; Case, D. A.; Kollman, P. A. *J. Comput. Chem.* **1986**, *7*, 230.

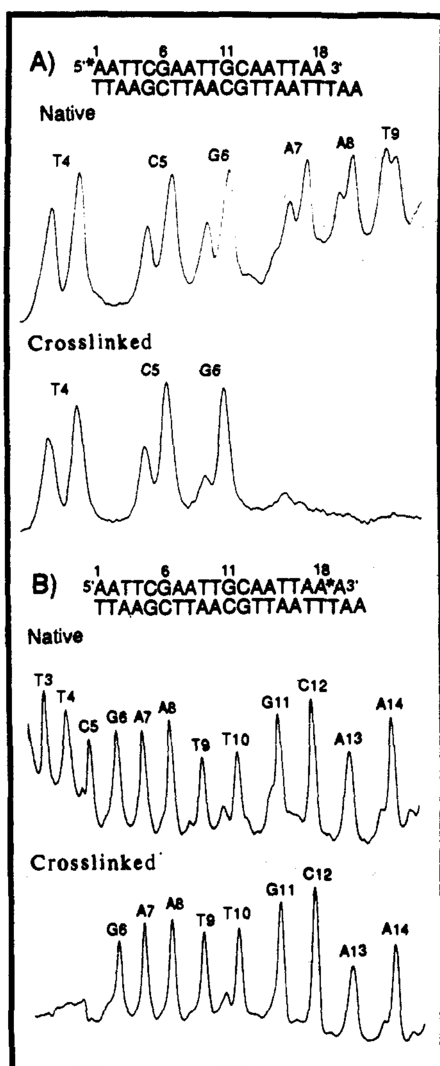


Figure 2. Partial fragmentation patterns for a radiolabeled (* = ^{32}P) native and mitomycin C cross-linked DNA duplex containing a single 5′-CG and a single 5′-GC site: (A) 5′-end-labeled and (B) 3′-end-labeled.

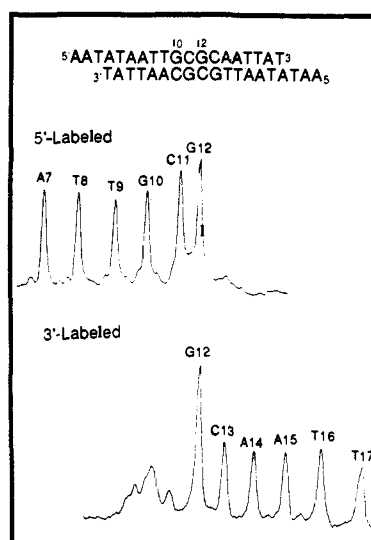


Figure 3. Partial fragmentation patterns for a radiolabeled, self-complementary DNA duplex cross-linked with mitomycin C.

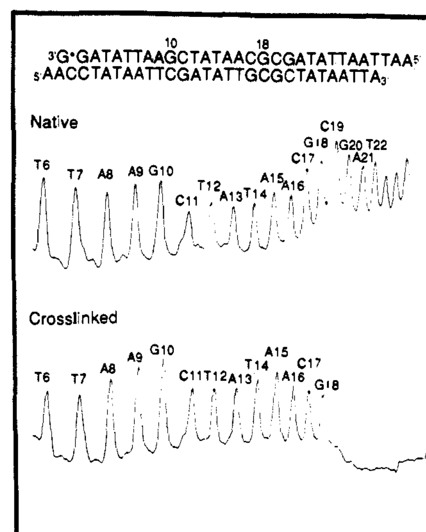


Figure 4. Partial fragmentation pattern for a 3′-end-radiolabeled (* = ^{32}P) DNA duplex cross-linked with mitomycin C.

plexes shown in Figure 2.[16] The aqueous duplexes admixed with a large excess of MC were treated with sodium dithionite. The resulting DNAs were analyzed by denaturing 20% PAGE, which revealed in addition to recovered single-stranded DNA (the major radioactive product) a low yield of a material of roughly half the mobility of the starting single strand. This less mobile, interstrand cross-linked product was isolated from the gel and subjected to Fe(II) EDTA cleavage[9] followed by sequential denaturing high-resolution PAGE, autoradiography, and scanning densitometry. The results (Figure 2) offer very strong support for the presence of a cross-link to residue G6 of the radiolabeled strand (the 5′-CG site). No abnormality was seen at G11 of the 5′-GC site, suggesting limited if any cross-linking at this site.

To explore the generality of the preference for cross-linking at 5′-CG over 5′-GC, a duplex containing the base sequence 5′-GCGC was prepared in both 5′- and 3′-end-radiolabeled forms (Figure 3). The MC cross-linked duplex was prepared by the standard protocol. Fe(II) EDTA cleavage/electrophoretic analysis from both ends pinpointed G12 as the cross-linked residue of this self-complementary sequence. (Significant cross-linking at one of the two symmetry-related 5′-GC sequences would require in the 5′-labeled cross-linked sample diminished intensity of fragments representing cleavage at C11 and G12 and in the 3′-labeled sample significant fragments for cleavage at G10 and C11.) The 5′-CG site is thus the preferred cross-link sequence when surrounded by AT-rich (Figure 2) or GC-rich (Figure 3) flanking bases.

Effect of Surrounding Base Sequence on CG Cross-Linking. The role of flanking bases in modulating the overall efficiency of cross-linking at 5′-CG sites was studied in duplexes bearing multiple 5′-CG sites. A MC cross-linked duplex bearing both 5′-TCGA and 5′-GCGC sites was prepared by the standard protocol (Figure 4). Because the efficiency of cross-linking is quite low (ca. 1%), these duplexes are expected to contain almost exclusively a single cross-link per molecule. Extension of the analysis accompanying Figure 1 for a duplex containing a cross-link at a single location within a duplex to the case of duplexes that contain a single cross-link but at different sites from one molecule to the next predicts that the yield of fragments to the nonradiolabeled side of a given cross-link site will diminish in proportion to the efficiency of cross-linking at that site. The cleavage fragment distribution showed almost no drop in intensity (relative to the native duplex) following G10 of the 5′-TCGA site but a complete lack of fragments following G18 of the 5′-GCGC site. Thus, although the sequence 5′-TCGA is capable of being cross-linked (Figure 2), the sequence 5′-GCGC is cross-linked preferentially when both are present (Figure 4). A duplex containing four 5′-CG sites (5′-TCGG, 5′-ACGT, 5′-GCGG, 5′-ACGA) was cross-linked (Figure 5). From the fragmentation patterns of both independently 5′-end-radiolabeled strands, it is obvious that cross-linking at no single site dominates but rather

(16) The peak label for each fragment denotes the residue cleaved by Fe(II) EDTA/H_2O_2/ascorbic acid cleavage.

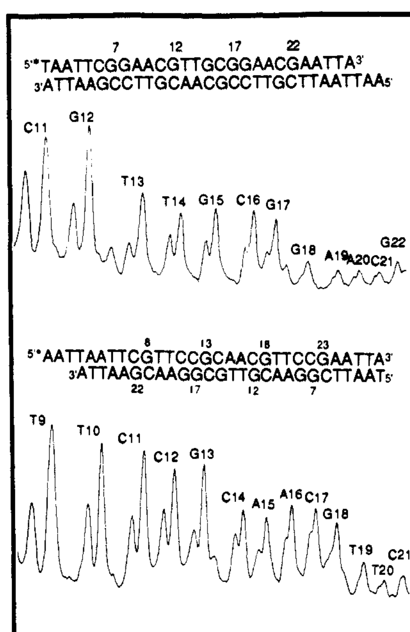


Figure 5. Partial fragmentation pattern for both strands (5'-labeling, * = ^{32}P) for a DNA duplex cross-linked with mitomycin C.

Table I. Energies (kcal/mol) of Minimized DNA Monoadducts

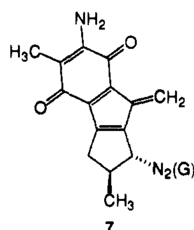
```
        M
 1  2  |  4  5
5' G  C  G  C  G

   C  G  C  G  C  5'
  10  9  8  7  6
```

atoms allowed to move[a]	E_{tot}, 5'MG[b]	E_{tot}, 3'MG[c]	Δ
M	−209	−193	−16
M, 3, 8	−248	−241	−7
M, 2–4, 7–9	−325	−320	−5

[a] M = mitosene; numbers refer to DNA residues. [b] Exocyclic methylene of 7 oriented toward 5'-end of attached strand. [c] Exocyclic methylene of 7 oriented toward 3'-end of attached strand.

that all sites show some degree of cross-linking. Notably, the 5'-GCGG and 5'-ACGT sites were cross-linked approximately equally and in preference to the 5'-ACGA and 5'-TCGG sites.

Molecular Modeling. The impact on cross-linking sequence specificity of conformational preferences in the monoadduct of reductively activated MC with DNA duplexes was evaluated by using molecular mechanics calculations. Coordinate sets were generated in which a pentanucleotide duplex (5'-GCGCG·5'-CGCGC, Arnott B-DNA[17]) was attached to 7, a model for the intermediate immediately preceding formation of the second covalent bond to DNA, by a single covalent bond which replaced that normally to the non-hydrogen-bonded hydrogen on N2 of the central deoxyguanosine residue. To diminish the impact of



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electrostatic effects in these calculations, we replaced the nitrogen of the pyrrole and the exocyclic ammonium group of MC with carbon. Conformations in which the exocyclic methylene group

(17) Arnott, S.; Campbell-Smith, P.; Chandresekharan, P. *CRC Handbook of Biochemistry*; CRC: Boca Raton, FL, 1976; Vol. 2, pp 411–422.

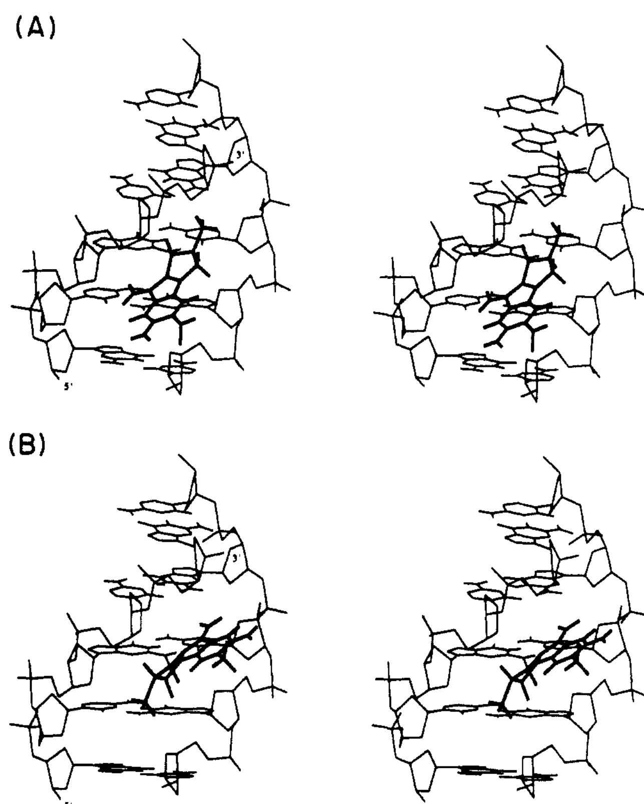

Figure 6. Stereoviews of energy-minimized minor-groove-aligned conformations of 7 covalently attached to a B-DNA pentanucleotide duplex (atoms of DNA constrained during minimization). (A) 5'MG, conformer with reactive methylene oriented toward the 5'-end of the attached strand, and (B) 3'MG, with reactive methylene toward the 3'-end. Hydrogens except on the drug and on NH or NH_2 of bases are omitted for clarity. The drug is shown in bold. Termini of the drug-bound strand are labeled.

of 7 was oriented toward the 3'-end (3'MG) and 5'-end (5'MG) of the attached DNA chain were generated. Energy minimizations using AMBER were conducted without positional constraint of (a) drug-derived atoms, (b) drug-derived atoms and DNA residues 3 and 8, or (c) drug-derived atoms and DNA residues 2–4 and 7–9. Remaining atoms were constrained with a fixed-atom penalty of 100 kcal/Å^2. As shown in Table I, the lower energy conformation of each paired calculation was that with the reactive methylene oriented toward the 5'-end of the attached strand, as would be necessary for cross-linking at the sequence 5'-CG. This preference diminished, but was not eliminated, as fewer residues were constrained. Calculated structures in which only drug-derived atoms were unconstrained are shown as stereoviews in Figure 6.

Discussion

Deoxyguanosine Residues at 5'-CG Are Cross-Linked in Preference to Those at 5'-GC. Chawla et al. have concluded that MC cross-links deoxyguanosine residues on opposite strands of DNA through N2 at the sequence 5'-CG but not at 5'-GC on the basis of analysis for diadduct 2 in digests of two synthetic DNA duplexes exposed to reductively activated MC. Using the fragment abundance assay described here, we have directly confirmed that interstrand cross-linking is greatly preferred at 5'-CG over 5'-GC sites in two oligonucleotide duplexes, each of which contained at least one 5'-CG and one 5'-GC sequence. In each case studied, the covalent connection was to deoxyguanosine residues. It is important that for a given strand both 5'- and 3'-end-labeled samples afforded a largest fragment representing cleavage at the same deoxyguanosine residue. This eliminates the possibility that a "footprinting" of covalently bound MC has compromised location of the covalently bound residue. In fact, we have observed *enhancements* of cleavage frequency (relative to native duplex) in proximity of the MC cross-link (for example, Figure 3, bottom trace). The source of this enhancement is being pursued.

During these studies, Teng et al.[18] reported results relevant to our studies. In 17 DNA duplexes subjected to reductively activated mitomycins, only those containing 5′-CG afforded a product of electrophoretic mobility consistent with cross-linking; those that lacked 5′-CG, including those that did contain 5′-GC, showed at most traces of cross-linked product. In their study, the locations of these cross-links were inferred to be at deoxyguanosine of 5′-CG sequences. Essentially identical conclusions are thus reached in three distinct studies.

Effect of Surrounding Base Sequence on 5′-CG Cross-Linking. We have semiquantitatively evaluated the relative reactivity toward MC cross-linking of several 5′-CG sequences in distinct flanking contexts. The central 5′-CG unit of 5′-GCGC was found to be cross-linked in preference to 5′-TCGA. This is consistent with the report of Teng et al. that as n in the sequence 5′-$(CG)_n$ (embedded in an otherwise AT-bearing oligonucleotide duplex) is increased, the relative reactivity toward cross-linking per CG unit increases.[18] Any generalization that 5′-CG in a GC-rich context is more reactive is apparently an oversimplification, however, because 5′-ACGT and 5′-GCGG had roughly equivalent reactivity that was greater than that of 5′-TCGG and 5′-ACGA, which were themselves roughly equivalent. Further study of the flanking sequence is clearly warranted.

Molecular Modeling. Defining the molecular basis for the 5′-CG selectivity of the overall MC cross-linking reaction is complicated by the multiplicity of steps that lead to a cross-link. In pinpointing the relevant steps, the finding of Teng et al.[18] that the aziridinomitosene of MC possesses 5′-CG selectivity eliminates at least the early steps in activation of MC. They have further concluded that differential noncovalent binding of MC or some activated intermediate cannot account for the selectivity. Neither of the two alkylation events required for cross-linking at 5′-CG appears to be reversible, and as such it seems reasonable to inspect the relative energies of transition states leading to monoadduct and cross-link.

Both monolinked and cross-linked adducts relevant to cross-link nucleus **2** have been studied by space-filling models and molecular mechanics energy minimization. The two groove-aligned conformers of the monoadduct through guanine N2 and mitosene C1 (stereochemistry as in **2**) have been compared by using space-filling models, with the result that only one of these, that in which the carbamate function is oriented toward the 3′-end of the attached strand, fits snugly in the minor groove with minimal reorganization of B-DNA.[5e] This conformer was energy-minimized in another report but not compared to the alternate groove-aligned conformer.[19] The need for relatively little net reorganization of B-DNA to accommodate cross-linking at 5′-CG with nucleus **2** has been noted on the basis of molecular modeling involving a cross-link at 5′-CG.[6a] Finally, in the only study in which the nucleus of **2** was incorporated at both 5′-CG and 5′-GC sites in several DNA duplexes followed by energy minimization, no consistent preferred orientation was found.[18] It was, however, again concluded that less reorganization of B-DNA is required for incorporation of the cross-link at the 5′-CG site.

We compare here the calculated structures and energies of monoadducts of **7** at guanine N2 in a pentanucleotide duplex in the two minor-groove-aligned conformations that would lead to 5′-CG and 5′-GC cross-links (Figure 6). We find that 5′MG, the conformer aligned for 5′-CG cross-linking, is preferred to 3′MG, that conformer aligned for 5′-GC cross-linking. At least in the calculation in which all DNA-derived atoms were constrained not to move, the physical basis of the energy difference is clear. The N2 (guanine) to C1 (mitosene) bond vector is displaced in the plane of the base pair toward the alkylated strand relative to the dyad that interchanges the two sugar–phosphate backbones. Given the chirality at C1 of the mitosene (R), one rotamer places the bulk of the mitosene centrally in the minor groove and orients the small H1 of the mitosene toward the spatially proximal alkylated strand. The alternate rotamer is a poorer structural match, placing the bulk of the mitosene in the limited space near the sugar–phosphate backbone of the alkylated strand. The resulting steric repulsion distorts the bond angles of the mitosene, particularly at C1. Although the energetic impact is predicted to be diluted by allowing reorganization of the DNA, in none of the minima was it eliminated. This relationship of chirality at mitosene C1 to groove orientation is entirely analogous to that proposed[20] and experimentally verified[21] in the pyrrolo[2,1-*c*][1,4]benzodiazepine (anthramycin family) antitumor antibiotics. The results of these calculations are not directly comparable to those of Teng et al. on cross-linked duplexes, where no consistent difference between 5′-CG and 5′-GC cross-links was found, because in the latter study no positional constraints were employed.

On the basis of these calculations, we suggest that if the transition state leading to the monoadduct of MC with DNA resembles B-DNA and if the chirality at C1 of the mitosene is as in **2**, then the monoadduct will be formed with the conformation appropriate for cross-linking at 5′-CG, with the carbamate moiety directed to the 3′-terminus of the attached strand. Overall specificity for cross-linking at 5′-CG would then follow either from a fast second alkylation relative to conformational interchange or from destabilization of the transition state leading to the 5′-GC cross-link by factors present in the related monoadduct conformer.

Conclusions

We demonstrate here that reductively activated mitomycin C cross-links opposite strands of duplex DNA fragments through deoxyguanosine residues at the sequence 5′-CG in strong preference to both 5′-GC and numerous A/T-bearing sequences. This conclusion is in full agreement with those independently drawn by two other groups,[7,18] each using an experimentally distinct approach. The surrounding base sequence affects the extent of cross-linking at a 5′-CG site; for example, 5′-GCGC is more extensively cross-linked than 5′-TCGA. Constrained molecular mechanics calculations on the monoadduct at N2 of deoxyguanosine in a B-DNA duplex with a model for reductively activated mitomycin C suggest an energetic preference for the groove-aligned conformer expected to lead to a 5′-CG rather than a 5′-GC cross-link. A steric effect involving the chirality at mitosene C1 of the monoadduct and the orientation of the deoxyguanosine-to-mitosene bond in the minor groove is responsible for the theoretical result and may play a significant role in guiding the mitosene C to preferentially cross-link 5′-CG rather than 5′-GC sequences.

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(18) Teng, S. P.; Woodson, S. A.; Crothers, D. M. *Biochemistry* **1989**, *28*, 3901.
(19) Rao, S. N.; Singh, U. C.; Kollman, P. A. *J. Am. Chem. Soc.* **1986**, *108*, 2058.
(20) Petrusek, R. L.; Anderson, G. L.; Garner, T. F.; Fannin, Q. L.; Kaplan, D. J.; Zimmer, S. G.; Hurley, L. H. *Biochemistry* **1981**, *20*, 1111.
(21) Graves, D. E.; Stone, M. P.; Krugh, T. R. *Biochemistry* **1985**, *24*, 7573.