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Sequence Preferences of DNA Interstrand Cross-Linking Agents: Importance of Minimal DNA Structural Reorganization in the Cross-Linking Reactions of Mechlorethamine, Cisplatin, and Mitomycin C

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Summary: Interstrand cross-linking of DNA is believed to account for the cytotoxicity of many bifunctional alkylating agents, some of which are useful in the treatment of human cancer. The nucleotide sequences at which these cross-links are formed have been defined at single nucleotide resolution in DNA fragments for several agents, including mechlorethamine, cisplatin, mitomycin C, and some structurally related agents. Taken together, the structure of duplex DNA, the sequences which are cross-linked, and the atomic sites on DNA which are linked, indicate that cross-linking occurs preferentially at locations which will result in minimal distortion of B-DNA. The proposal that this preference is primarily expressed by minimizing the energy of the transition state for conversion of monoadducts to cross-links is supported by experiments with mechlorethamine. It is suggested that extension of the modest sequence-recognizing capacity of these cross-linking agents by conjugation to highly sequence-selective "delivery vehicles" may yield second generation, targeted antitumor drugs.

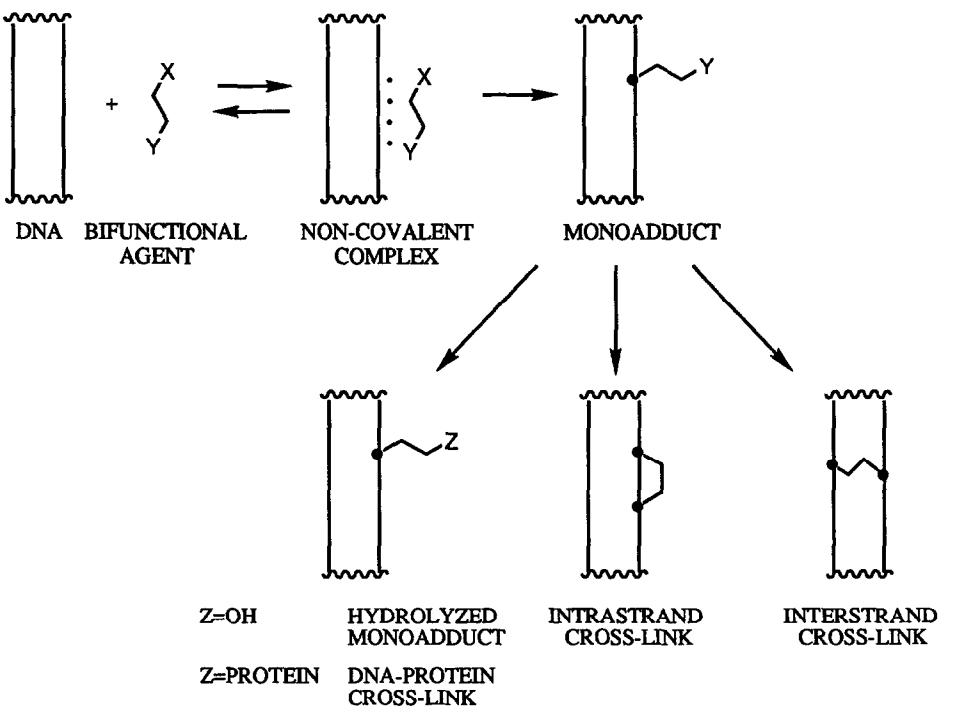
Introduction

The double helical structure of deoxyribonucleic acid offers many advantages as a repository for the genetic code. This structure protects the nitrogenous bases from undesirable reactions. Furthermore, when a lesion is produced on one strand, repair with high fidelity is conceptually straightforward: the damaged nucleotide can be enzymatically removed and discarded. Installation of a fresh nucleotide is then guided by the template information on the undamaged partner strand. Perhaps most importantly, the double helix provides a simple mechanism for its own accurate molecular reproduction: separation of the strands, followed by template-directed synthesis to yield two double helical progeny.

But Nature's system is not foolproof. In the event that *both* strands of DNA sustain lesions in close proximity to one another, neither strand can serve as a faithful template for reproduction of the other. If the strands become unable to separate from one another, as might happen if they were covalently connected, both repair and reproduction are frustrated. Both of these seemingly unlikely scenarios are in fact believed to account for the high cytotoxicity of many bifunctional alkylating agents. These agents have been shown to inflict DNA interstrand cross-links *in vitro* and *in vivo*, which in turn correlate with cytotoxicity.[1] The selective toxicity of some bifunctional alkylating agents has provided valuable drugs for the treatment of human cancer, including the nitrogen mustards, cisplatin, mitomycin C, and the nitrosoureas.[2] The bifunctional alkylating agents also, however, include such deadly toxins as the pyrrolizidine alkaloids, which are a leading cause of livestock poisoning deaths in the Northwest United States.[3]

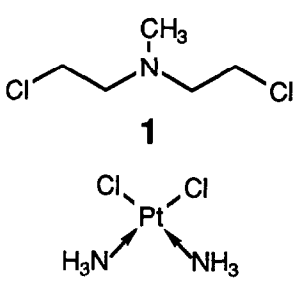
The reactions of bifunctional alkylating agents with DNA present questions of structure and mechanism on many levels. Which atomic sites in DNA are chemically modified? What sequences of nucleotides are the preferred sites of attack and of interstrand cross-linking? Is this sequence preference important in toxic or therapeutic action? This line of inquiry is doubly rewarding, contributing fundamentally to our understanding of the structure and chemical reactivity of DNA and potentially exposing the mechanisms by which antitumor agents operate.

Our laboratory is studying the nucleotide sequence preferences of DNA interstrand cross-linking reactions. A generic mechanism for this multistep process is shown in Scheme I. Association of a bifunctional reagent and DNA may lead to a non-covalent complex, which then reacts to form a covalently bound monoadduct. Three fates for the monoadduct are commonly observed: reaction with an external nucleophile such as solvent or protein, reaction with the same DNA strand a second time to afford an intrastrand cross-link, or reaction with the opposite strand to yield an interstrand cross-link. This last pathway, though of great toxicological interest, is the exception with most agents. The interstrand cross-link is thus the minor component of a complex mixture.



Scheme I. Generic Mechanistic Pathway for the Reaction of Interstrand Cross-Linking Agents with Duplex DNA.

In principle, any combination of one, several, or all of the steps in Scheme I could be important in defining the interstrand cross-linking sequence preference of a bifunctional alkylating agent. We present experimental evidence in this paper supporting the generalization that the conversion of monoadducts to cross-linked molecules is a critical reaction in determining sequence preference. We propose that transition states for this reaction which minimally reorganize the structure of double helical DNA are preferred. The generality of this proposal is suggested by the structural diversity of the agents to which we find it to apply, including mechlorethamine (1), cisplatin (2), mitomycin C (3), and the pyrrolizidine alkaloid retrorsine (4).



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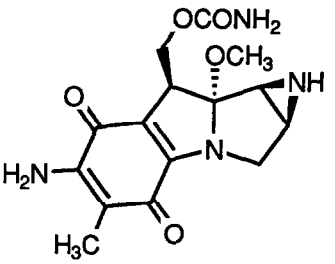

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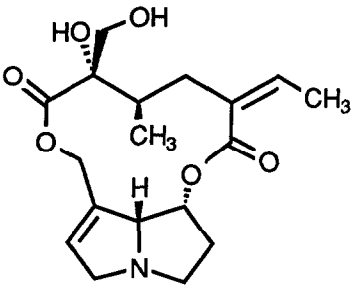

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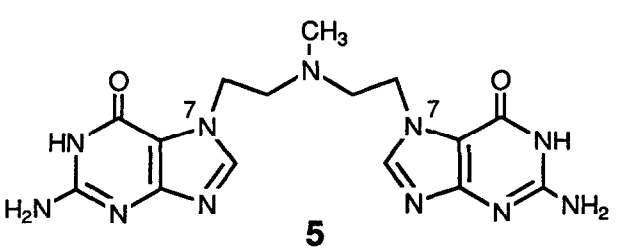
Mechlorethamine

Mechlorethamine (1) was the first clinically useful antitumor drug; members of the mustard family remain among the most useful drugs for the treatment of cancer.[4] The ability of mechlorethamine to produce interstrand cross-links in DNA was first inferred from the anomalously fast renaturation kinetics of mechlorethamine-treated DNA.[5] The isolation from a hydrolysate of mechlorethamine-treated yeast RNA of the conjugate **5** containing two guaninyl residues linked to the termini of the mustard skeleton suggested the possibility that mechlorethamine creates deoxyguanosine to deoxyguanosine (dG-to-dG) interstrand cross-links.[6,7] Consideration of the limited molecular reach of the two alkylating functions of mechlorethamine (7.5 Å, N7 to N7 in **5**) and the spacing of N7 atoms of dG in duplex DNA led to the further speculation that the base pairs involved would necessarily reside in close proximity[6] and that the minimal N7 to N7 distance in the duplex sequence 5'-GC would best match the short mustard chain. We have tested this proposal using synthetic oligonucleotide duplexes.[8]



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Three synthetic duplexes containing, respectively, the nucleotide sequences 5'-CCGGCCGG, 5'-GGCCGGCC, and 5'-GGGCGGG were treated with mechlorethamine hydrochloride and the product mixtures were studied by denaturing polyacrylamide gel electrophoresis (PAGE). Under these conditions, duplex DNA denatures to single strands (and presumably monoadduct-bearing single strands) which migrate relatively rapidly through the gel. The covalent bridge which links the strands of interstrand cross-linked DNA prevents strand separation, markedly diminishing the mobility of these molecules. This method serves as both an analytical and a preparative method for interstrand cross-linked DNA. Several interstrand cross-linked products were evident in each duplex, but one or two predominated in each case. The major interstrand cross-linked products were isolated, and processed as described below to define the sites of cross-links at nucleotide resolution.

We used the base lability of N7-alkylated dG residues to establish the sites of mechlorethamine cross-linking. Aqueous piperidine cleaves the DNA strand at these lesions.[9] Denaturing PAGE readily distinguishes DNA molecules differing in length by a single residue, allowing this technique to define the resulting fragment lengths, and thus pinpoint the originally alkylated deoxyguanosine residues. This analysis revealed a remarkable consistency in the three mechlorethamine cross-linked duplexes: distal deoxyguanosine residues at the sequence 5'-GNC were preferentially cross-linked. In the example shown in Figure 1A, G2 in the upper strand and the central, isolated G in the lower strand were linked to one another. These cross-links were virtually completely cleaved by piperidine at the indicated dG residues, implying that the linkages are N7 to N7 as in **5**. Loechler *et al.* have independently reported that the reaction of a synthetic oligonucleotide duplex with mechlorethamine affords an N7 to N7 cross-link at 5'-GNC, in this case, 5'-GAC,[10] suggesting that the 5'-GNC preference may be general.

Some mechanistic insight into the 5'-GNC preference of mechlorethamine was gained by an experiment which probed the relative importance of the initial alkylation and the subsequent conversion to interstrand cross-link. At one extreme, it is possible that sequence selective monoadduct formation strongly biases the cross-link site. At the opposite extreme, monoalkylation may be sequence random,[11] with the subsequent conversion to cross-link favoring 5'-GNC. We tested this by independently evaluating the sequence dependence of monoadduct formation (by piperidine fragmentation of the total DNA following treatment with mechlorethamine) and cross-link formation (by fragmentation of all interstrand cross-links). Because the number of monoadducts (as detected by piperidine cleavage) greatly exceeds that of crosslinks (quantified by denaturing PAGE), the distribution of total alkylation products, without separation of cross-linked molecules, is a reasonable approximation of monoalkylation frequency. For the duplex shown in Figure 1A, piperidine cleavage of total DNA, and thus monoalkylation, was rather uniformly distributed among dG residues (Figure 1B). A three-fold bias against alkylation at G3 relative to G2 was evident, however, and accounts in part for the preference for 5'-GNC vs. 5'-GC cross-linking. For the cross-link population, piperidine fragmentation led to a decidedly non-random distribution of products: consistent with 5'-GNC being the globally preferred sequence for interstrand cross-linking by mechlorethamine, almost half of the cleavage was found at G2. Especially interesting is the difference in ratios of G2 to G3 in the

monoadduct (3:1) and cross-link populations (22:1). This outcome suggests that the conversion of monoadducts to crosslinks is seven-fold more efficient at G2 (5'-GNC) than at G3 (5'-GC), and that this step is the primary determinant of interstrand cross-linking sequence preference by mechlorethamine. Adjustment of these figures to account for what is anticipated to be a minor reaction pathway,[11] cross-linking by monoalkylation of the bottom strand (Figure 1) followed by reaction with the top strand, is unlikely to alter this conclusion. Appreciable fragmentation of the cross-link population at G1 and G6 suggests that this sample contains as yet structurally uncharacterized interstrand cross-links.

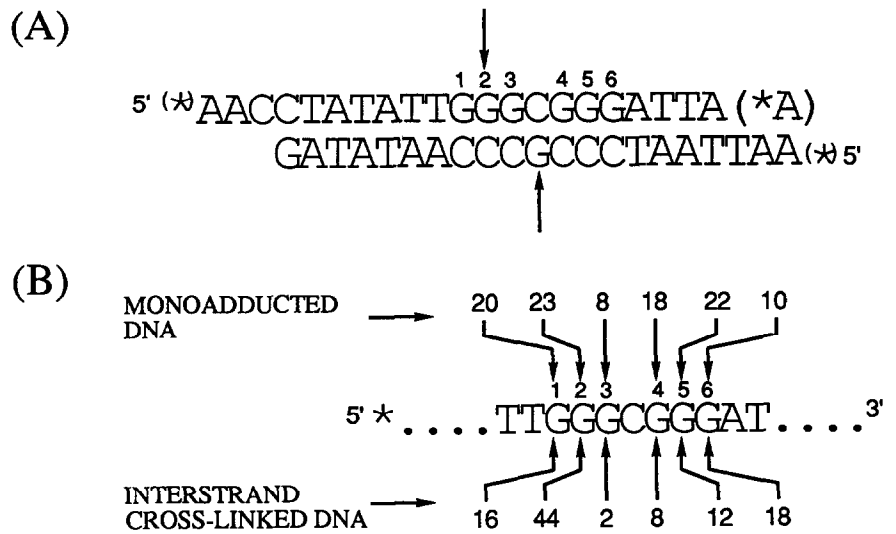


Figure 1. (A) Arrows mark location of deoxyguanosine residues covalently bridged in the major mechlorethamine cross-linked product of the indicated DNA duplex. This conclusion was consistent with three experiments in which the indicated (*) strand termini were independently radiolabeled. (B) Relative extent of alkylation (monoadduct formation, upper, %) and cross-linking (lower) by mechlorethamine as a function of nucleotide position as measured by piperidine fragmentation.

Figure 2 illustrates the close proximity of distal deoxyguanosine N7 atoms in the nucleotide sequence 5'-GNC in duplex DNA. The apparent inconsistency with the now thirty year old prediction that 5'-GC sequences[6] would be cross-linked did not concern us: This model arose from consideration of static B-DNA.[12] We now know that duplex DNA in solution[13] or single crystals[14] possesses subtle sequence-dependent conformational variation. Modeling based on the average B-DNA conformation derived from fiber diffraction studies (Arnott B-DNA)[12] will thus be imperfect. Even allowing for subtle conformational variation, however, it is clear that *any* duplex DNA sequence must substantially distort if a single mustard molecule is to bridge N7 atoms on opposite strands, distortion which will appear in any chemically reasonable transition state (tethered aziridinium ion or direct displacement of the primary chloride) for the

conversion of monoadduct to cross-link. Taken together with our current poor understanding of the conformational deformations available to duplex DNA, distinguishing the subtly different spacing of N7 atoms at 5'-GC and 5'-GNC is doubtless premature. We speculate that some combination of propeller twisting in excess of that seen in single crystals (which diminishes the spacing of N7 atoms at 5'-GNC and causes the N7 lone pairs to approach one another as required in the cross-link) and kinking DNA in a direction to close the major groove may be critical for cross-linking. The possibility that the electrophile may be forced by steric demands of duplex DNA to approach N7 out of the plane of the heterocycle containing N7 (e.g. perpendicular to the floor of the major groove), making use of some π- rather than exclusively σ-electron density at N7, is also worthy of consideration. In that circumstance, it could be argued that *less* deformation would be required at 5'-GNC than at 5'-GC for closure of monoadduct to interstrand cross-link.

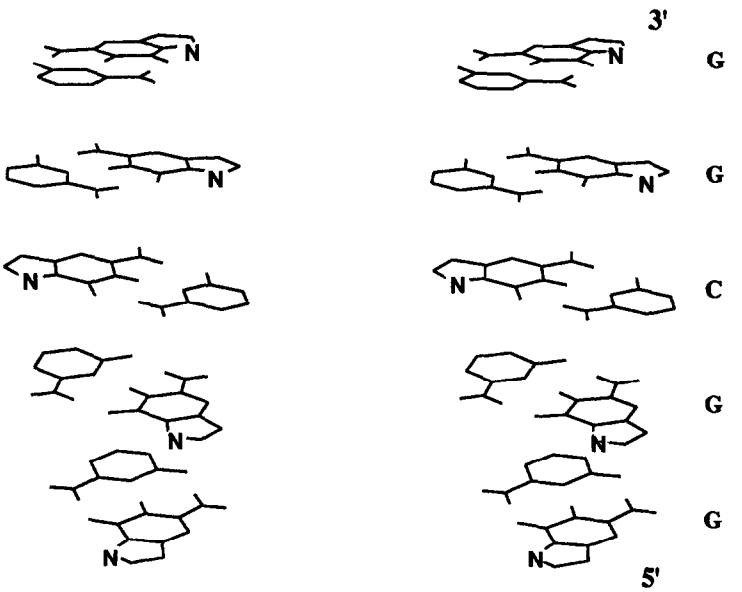
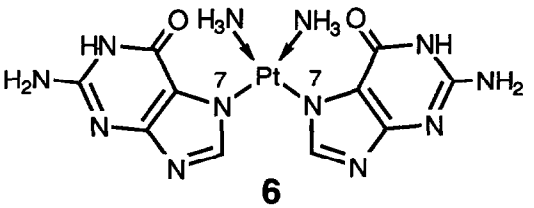


Figure 2. Stereoscopic representation of the positions of the heterocyclic bases in Arnott B-DNA at the nucleotide sequence 5'-GGCGG. N7 atoms of deoxyguanosine residues are indicated.

The important conclusions of the experiments with mechlorethamine are thus that (a) mechlorethamine preferentially cross-links the duplex DNA sequence 5'-GNC from dG-to-dG and likely N7 to N7, (b) this preference derives substantially from the efficiency with which monoadducts at this sequence are converted to cross-links, and (c) within the limits of our current knowledge of DNA's sequence dependent structure, the sequence preference for interstrand cross-linking matches what would be expected based on the concept of minimal reorganization of DNA during the latter reaction.

Cisplatin

cis-Diamminodichloroplatinum(II) (cisplatin, **2**) is a clinically useful antitumor drug.[15] DNA exposed to cisplatin suffers both intrastrand and interstrand cross-linking, with the intrastrand cross-link vastly predominating.[16,17] The relative importance of these two cross-links in cytotoxicity remains a matter of debate. From a hydrolysate of a sample of cisplatin-treated DNA "enriched" in interstrand cross-link, the diadduct **6** has been isolated;[18] this substance is thus an excellent candidate for the nucleus of the interstrand cross-link. Like mechlorethamine, then, cisplatin may produce interstrand cross-links at opposing dG residues by linking N7 atoms. Consistent with this, short DNA duplexes such as 5'-$(CG)_5$ have been shown to yield interstrand cross-links on treatment with cisplatin,[19] suggesting that either or both of the sequences 5'-GC and 5'-CG might be cross-linked. We have tested this hypothesis.



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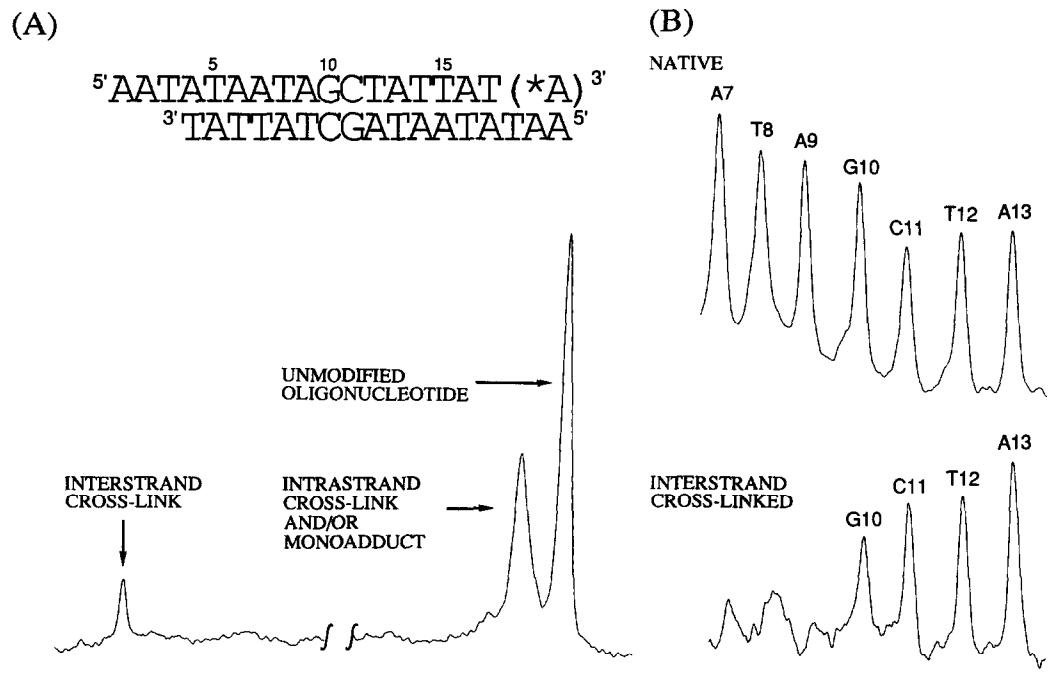


Figure 3. (A) Densitometric scan of denaturing PAGE analysis of indicated oligonucleotide duplex after 16 h exposure to excess cisplatin (see Experimental Section). (B) Partial fragmentation patterns of 3'-end radiolabeled (* = ^{32}P) native and cisplatin interstrand cross-linked DNA shown in (A).

Two duplex DNAs (Figure 3A), one containing a single central 5'-GC sequence and the other the isomer bearing 5'-CG, were independently incubated with 6 molar equivalents of cisplatin. Analysis by denaturing PAGE revealed that both duplexes were converted as a function of time predominantly to a species of only slightly retarded electrophoretic mobility (relative to the starting single strand), likely a monoadduct or intrastrand cross-link. The 5'-GC-containing sequence also produced a product of mobility appropriate for an interstrand cross-linked molecule (Figure 3). At long reaction times, both sequences afforded numerous bands of low electrophoretic mobility, likely representing interstrand cross-link positional isomers.

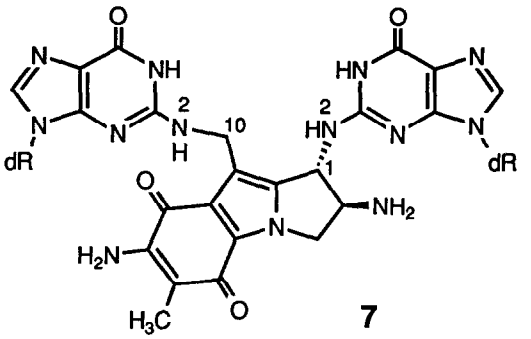
This most rapidly formed interstrand cross-linked product from the 5'-GC sequence was isolated by denaturing PAGE and subjected to limited, random fragmentation using the iron(II)/EDTA protocol.[20] This technique is diagnostic for the position of interstrand cross-links.[21] In this case, radiolabeled fragments shorter in length than single strand were obtained for cleavage from the radiolabeled end through G10 (Figure 3B), with the relative intensity of the cleavage band at G10 roughly half of that obtained with the native sample. The most reasonable interpretation of these data is that the cross-linked sample is linked dG-to-dG at the sequence 5'-GC. The reduced intensity of the G10 cleavage band in the cross-linked DNA would then follow from reactivity differences of cross-linked and native DNA due to structural or dynamical differences. An alternative, less likely explanation, which would account for the diminished intensity of the G10 band, is that G10 on one strand is linked to its base pairing partner C11 on the opposite strand. This would then suggest that even isolated GC base pairs should be interstrand cross-linked with similar efficiency. This interpretation is inconsistent with the failure of the duplex containing the 5'-CG sequence as well as others (data not shown) containing isolated GC base pairs to afford appreciable interstrand cross-linked products.

Taken together, these data and the diadduct **6** in which two guanines are linked to a single platinum atom through their N7 atoms strongly suggest that although cisplatin produces several isomeric interstrand cross-links on exposure to synthetic DNA duplex fragments, the sequence 5'-GC is especially efficiently cross-linked dG-to-dG. In B-DNA, N7 atoms of dG residues on opposing strands are minimally separated in this sequence relative to any other (Figure 2). The mandatory structural rearrangement which would accompany such a reaction may then be minimized at this sequence. This experimental result is opposite that predicted on the basis of somewhat complex theoretical considerations.[18]

Pyrrole-Based Cross-Linking Agents: Mitomycin C, Pyrrolizidine Alkaloids, and Structurally Simplified Analogs

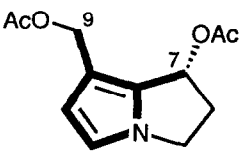
Mitomycin C (**3**) has been used in the treatment of a wide variety of cancers.[22] After reductive activation, it is a DNA interstrand cross-linking agent both *in vivo* and *in vitro*.[23] Tomasz and Nakanishi have isolated the conjugate **7**, in which the N2 atoms of two dG residues are covalently linked to carbons 1 and 10 of the mitomycin nucleus.[24] Consideration of the molecular dimensions of DNA and mitomycin C suggests that the sequences 5'-CG and 5'-GC could accommodate such an interstrand cross-link, because

both possess a pair of N2 atoms with an appropriate spacing of about 4 Å.[25] We have tested this idea with synthetic oligonucleotides.[21,26]



Several synthetic duplex DNA fragments have been treated with reductively activated mitomycin C.[26] Interstrand cross-linked DNAs were isolated by denaturing PAGE, and the sites of cross-linking were determined at nucleotide resolution by the iron(II)/EDTA protocol.[21] In every case tested, 5'-CG was the predominant site of cross-linking in duplex DNA. In one DNA offering only a 5'-GC site, cross-linking at least an order of magnitude less efficient than that at 5'-CG was seen, and a structurally heterogeneous interstrand cross-linked product was obtained, suggesting that 5'-GC is very poorly cross-linked relative to 5'-CG.[27] The groups of Tomasz[28] and Crothers[29] have independently reached the same conclusions.

To explore the origins of mitomycin C recognition of 5'-CG sequences, we have tested structurally simplified analogs.[27] The choice of analogs was a consequence of the now well understood mechanism by which mitomycins yield interstrand cross-links.[22] Partial reduction of the quinone function of mitomycin C (**3**, Scheme II) results in expulsion of the elements of methanol to form an aziridinomitosene (**8**). The heteroatoms bound to carbons 1 and 10 thus become activated as leaving groups owing to the resonance stabilization of the resulting carbocations by conjugation to the pyrrole moiety. Sequential departure of leaving groups and capture by nucleophiles on DNA affords in turn intermediates **9**, **10**, **11**, and **12**. Ejection of an electron from **12** (or some earlier intermediate) returns the quinone oxidation level and accounts for the isolation of **7** on hydrolysis.[24] This mechanism suggests a simple question: Does the geometry and spacing of carbons 1 and 10 of mitomycins confer 5'-CG selectivity? We tested the interstrand cross-linking sequence preferences of pyrroles **13** (dehydroretronecine diacetate) and **14** (bis-2,3-acetoxymethyl-1-methylpyrrole) to address this question. These substances possess a spatial relationship of the two potentially electrophilic centers identical to that found in aziridinomitosenes; a mechanism for interstrand cross-linking by **14** analogous to that for mitomycin (Scheme II) is suggested in Scheme III.



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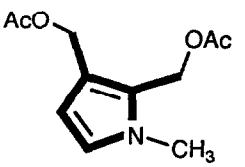

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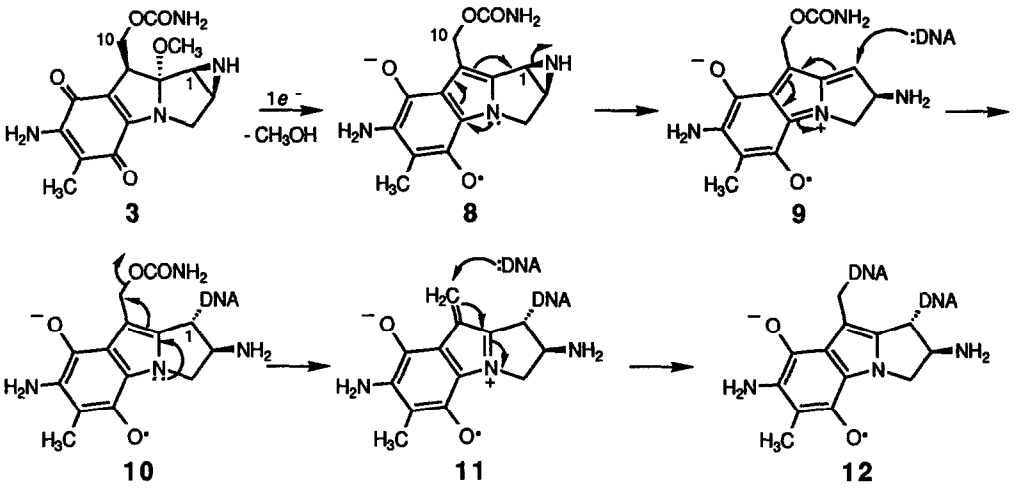
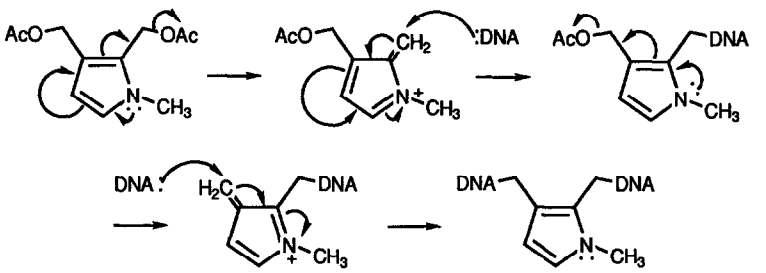


Scheme II. A Possible Mechanistic Sequence for Interstrand Cross-Linking of DNA by Reductively Activated Mitomycin C.



Scheme III. Likely Mechanism for Interstrand Cross-Linking of Duplex DNA by 2,3-Bis(acetoxymethyl)-1-methylpyrrole.

Pyrrole **13** was exposed to DNA duplex fragments containing 5'-CG and 5'-GC sequences.[27] These included 5'-d(AATATAATN$_4$ATTAT), where N$_4$ was ACGT, AGCT, TCGA, TGCA, CCGG or TATA. Denaturing PAGE analysis clearly revealed that **13** was less selective in its reactions than reductively activated mitomycin C, giving multiple interstrand cross-linked products. Nevertheless, dG-to-dG interstrand cross-linking at the sequence 5'-CG, as in mitomycin C, was clearly preferred. Pyrrole **14** was tested with fewer oligonucleotides, but the results and conclusions were essentially the same as for **13**.[27] The 5'-CG vs. 5'-GC selectivity of both **13** and **14** was not as high as that of mitomycin C. With mitomcyin C, the difficulty of quantifying the minor amount (if any) of cross-linking at 5'-GC has precluded assignment of an upper limit to its selectivity. In contrast, the 5'-CG vs. 5'-GC selectivities of **13** and **14** were roughly 8:1.

A partial structural overlap of representatives of the pyrrolizidine alkaloid and mitomycin families prompted us to explore the sequence preferences of activated forms of retrorsine (**4**) and monocrotaline. These substances undergo *in vivo* oxidative activation, analogous to the reductive activation of mitomycins, revealing a pyrrole function which labilizes the leaving groups. The resulting dehydropyrrolizidine is then functionally analogous to pyrrole **13**. Both dehydromonocrotaline and dehydroretrorsine preferentially produced interstrand cross-links in DNA duplexes containing the nucleotide sequence 5'-CG.[27]

How do such structurally simple substances as pyrroles **13** and **14** distinguish 5'-CG and 5'-GC sequences in duplex DNA? Mechanistic arguments concerning the origin of the cross-linking sequence selectivity for these agents are necessarily speculative. We currently lack any information concerning the sequence preferences of monoadduct formation for **13** and **14**. Furthermore, conjugates of **13** and **14** with DNA, analogous to **7**, which have the potential to conclusively demonstrate N2 to N2 cross-linking, have not yet been isolated. The structural similarities of **13**, **14**, and the aziridinomitosene of mitomycin C nevertheless suggest a common site of attack and mechanism of sequence recognition.

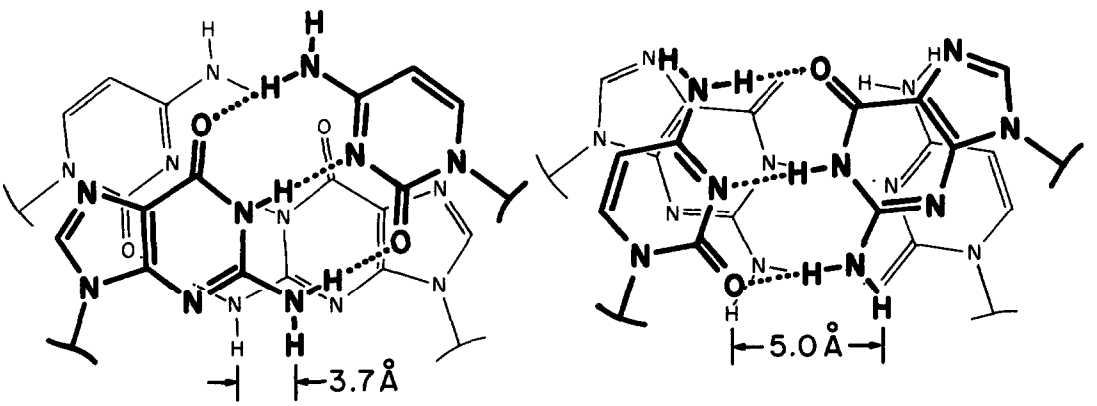


Figure 4. Relative atomic positions in Arnott B-DNA at 5'-CG (left) and 5'-GC (right) steps.

Two factors may contribute to the mitomycin's high preference for interstrand cross-linking at 5'-CG. The first bond linking mitomycin C and DNA forms with the stereochemistry at C1 of the mitosene shown in Scheme II (**10**),[30] possibly the result of a steric effect, with the bulky nucleophile, DNA, approaching mitomycin C opposite the solvated ammonium cation. To avoid distortion of DNA, this monoadduct must align itself with the minor groove. Of the two possible orientations, that which directs the remaining reactive carbamate moiety toward the 3'-end of the opposite strand, as required for 5'-CG cross-linking, appears from inspection of models to be preferred as a consequence of this stereochemistry at C1 of the mitosene.[26,28,30]

While this argument *could* in principle explain the sequence preference of pyrrole **13**, it necessitates that the achiral cation derived from ionization of the secondary acetate of **13** be attacked predominantly from the α-face by DNA. Models suggest no basis for this. The monoadduct of **14** has no such stereochemistry, and computer modeling offers no evidence for a preferred groove orientation. How does pyrrole **14** recognize 5'-CG? Models of DNAs interstrand cross-linked N2 to N2 at 5'-CG and 5'-GC by **14** suggest one explanation. Less distortion of DNA is required at 5'-CG, a result of the orientation of the bonds which hold the cross-linking agent to the nucleic acid: At 5'-CG, these bonds are parallel, a situation easily accommodated by **14**. The divergence of these bonds at 5'-GC (Figure 4) is incompatible with the structural constraints of **14**. It is possible that the transition state which determines sequence specificity may resemble the cross-link itself. One particularly simple possibility is that this transition state involves attack of a dG (N2) σ-lone pair, available through ionization or tautomerization, at the trigonal carbon of an ionized monoadduct (Figure 5). Ionized, minor groove-aligned monoadducts at 5'-CG possess an orbital spacing and orientation favorable for cross-link formation without DNA distortion (Figure 5, lower monoadduct). In contrast, the geometry of 5'-GC steps would mandate more extensive, and presumably energetically costly, reorganization to achieve a cross-link (Figure 5, upper monoadduct). Thus, the conversion of monoadducts to cross-links at 5'-CG steps may be favored.

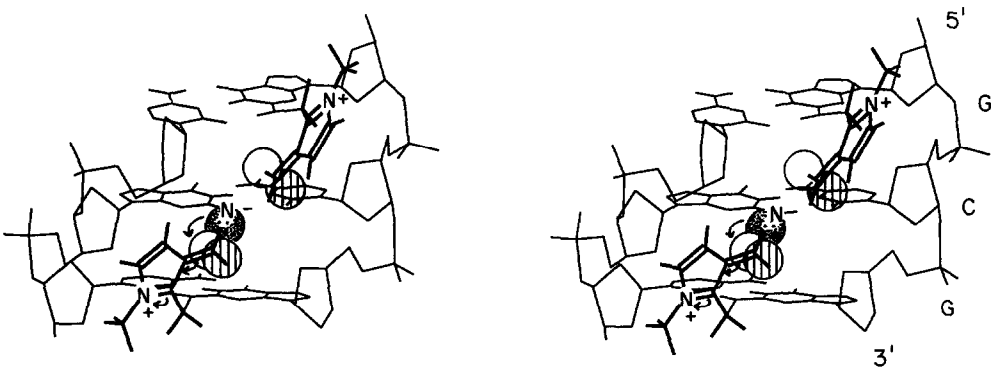


Figure 5. Stereoscopic representation of a hypothetical transition state for dG-to-dG cross-linking of DNA by **14**. Minor groove-aligned, ionized monoadducts of **14** (bold) are shown attached to the uppermost dG (5'-GC step) and lowermost dG (5'-CG step) residues in an Arnott B-DNA trinucleotide duplex. The stipled, σ-lone pair (sp^2) on N2 of the central dG residue is properly oriented for bonding to the π-orbital (open and hatched circles) of the lower monoadduct, as required for formation of a cross-link at 5'-CG without distortion of the DNA.

Conclusion

We have shown using three structurally distinct bifunctional alkylating agents that the sequence preferences of DNA interstrand cross-linking agents can be accounted for by assuming that DNA will be minimally reorganized during the conversion of monoadducts to cross-links. For those agents in which a short tether must span the major groove, this amounts to little more than finding the shortest route from one strand to the other. The minor groove cross-linking agents likewise seek on DNA transition state structures which best complement their own ground state structure.

At one level, it is remarkable that such small molecules as the cross-linking agents discussed herein can "recognize" DNA sequence information. At another level, however, the size of the message read is disappointing, being grossly inadequate for the highly specific genomic targeting which is a goal of future therapeutic approaches. Fortunately, it is not difficult to imagine extending the limited sequence recognition of these interstrand cross-linking agents by linkage to other sequence recognizing "delivery vehicles". The resulting conjugates have the potential to produce difficultly repaired interstrand cross-links at precisely defined genomic locations. As our understanding of interstrand cross-linking reactions of DNA grows, so grows the prospect of therapeutically useful second generation, interstrand cross-linking antitumor drugs.

Experimental Section

Materials and Methods. Materials and their sources were as follows: DNA synthesis reagents, Applied Biosystems; [α-^{32}P] dATP and Klenow fragment, Amersham; *cis*-diamminodichloroplatinum(II) (cisplatin), Aldrich. All other reagents were from commercial sources and used as received. Loading buffer was 90% aqueous deionized formamide containing 10 mM Tris (pH 7.5), 0.1% xylene cyanole, and 0.1 mM sodium EDTA. For autoradiography, gels were 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[9] on uncross-linked, radiolabeled DNA. Densitometric (Hoeffer GS-300, interfaced to an IBM PC) data were smoothed and plotted using Spectra Calc (Galactic Industries Corp., Salem, NH).

Preparation of Radiolabeled DNA Duplexes. Oligonucleotides were synthesized (Applied Biosystems Model 380A) using the phosphoramidite method on a 1 μmol scale and were purified by denaturing PAGE (20% gel cross-linked with 5% bisacrylamide, 48% urea). DNA was visualized by UV shadowing and eluted from crushed gel slices by incubation at 37° in 0.5 M NH_4OAc/1 mM sodium EDTA for 16 h. The eluate was passed through a Waters Sep-Pak C_{18} cartridge, and eluted sequentially with (1) 10 mM aqueous NH_4OAc, (2) water, (3) 25% CH_3CN/water. DNA was recovered by concentration of the CH_3CN/water eluent. DNA (1.0 O.D., 7.2 nmol) in 20 μL of Klenow buffer was 3'-end radiolabeled by exposure to 30 μCi [α-^{32}P] dATP and 10 units of Klenow fragment for 0.25 h at 37°. Labeling was stopped by addition of 10 μL of 3 M sodium acetate buffer (pH 5.2), 10 μL of 0.2 M sodium EDTA (pH 8.0), and 60 μL of water followed by 700 μL of ethanol at -20°. After 0.5 h at -20°, the resulting precipitate was collected, redissolved in 100 μL of 0.3 M sodium acetate buffer (pH 5.2) and precipitated by addition of 700 μL of ethanol at -20°. After

0.5 h at -20°, the collected precipitate was suspended in 1 mL of 75% aqueous ethanol at -20°. The precipitate was collected and used in the following experiments.

Reaction of Cisplatin with DNA. Radiolabeled DNA (0.36 nmol of duplex) was incubated with cisplatin (2.16 nmol) in 50 μL of an aqueous buffer containing 20 mM $NaClO_4$, 1 mM potassium phosphate, pH 7.6. At time intervals ranging from 1 min to 4 d, aliquots were taken and DNA was precipitated by addition of cold ethanol. The dried, precipitated samples were stored at -20° until all aliquots had been taken, then dissolved in 5 μL of loading buffer and analyzed by denaturing PAGE (25% gel cross-linked with 5% bisacrylamide containing 48% urea on a Hoeffer thermojacketed gel stand). Products were visualized by autoradiography. The product with mobility roughly half that of single strands, which was presumably interstrand cross-linked, was isolated from the gel by the crush and soak procedure described above.

Iron(II) EDTA Fragmentation Analysis of Cislatin Interstrand Cross-linked DNA. Cleavage reactions of the cross-linked DNA duplexes were conducted on 100,000 cpm (geiger counter) of DNA in 20 μL of an aqueous solution containing 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°. Reactions were stopped by addition of 2 μL of 50 mM aqueous thiourea, lyophilized, dissolved in 5 μL of loading buffer, heated to 90° for 3 min, cooled to 0°, and analyzed by denaturing PAGE (25% gel cross-linked with 5% bisacrylamide containing 48% urea, 0.35 mm thick, 41 X 37 cm, run at 2000 V on a Hoeffer thermojacketed Poker Face gel stand at 64-68°).

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