Sequence-Specific DNA Interstrand Cross-Linking by an Aziridinomitosene in the *Absence* of Exogenous Reductant†

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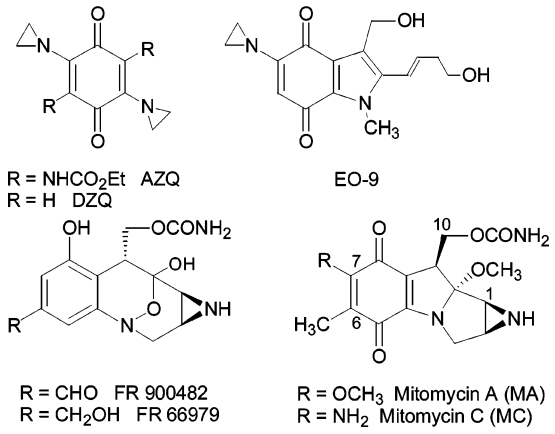
ABSTRACT: The aziridinomitosene derivative (1*S*,2*S*)-6-desmethyl(methylaziridino)mitosene (**4**) was shown to alkylate plasmid DNA at pH 7.4 *in the absence of a reducing agent* [Vedejs, E., Naidu, B. N., Klapars, A., Warner, D. L., Li, V. -s., Na, Y., and Kohn, H. (2003) *J. Am. Chem. Soc. 125*, 15796−15806], an activity not found in the parent mitomycins. We sought to evaluate aziridinomitosene **4** for the presence of DNA interstrand cross-linking activity using nonreductive reaction conditions. Radiolabeled DNA treated with **4** was analyzed by denaturing polyacrylamide gel electrophoresis (DPAGE), a technique that readily separates the less mobile cross-linked ds DNA from the more mobile ss DNA products. Nonreduced **4** produced an interstrand cross-link (ICL) in duplex DNA containing 5′-d(CG) sites, and the yield of ICL was comparable to that obtained from reduced MC under similar conditions. A ds DNA having the central tetranucleotide 5′-d(ACGT) provided the greatest ICL yield from both nonreduced **4** and reduced MC. Substitution of 5′-d(CG) with the inverted sequence 5′-d(GC) completely abolished interstrand cross-linking by **4**, revealing 5′-d(CG) as its specific site of ICL formation. Replacement of dG at 5′-d(CG) with 2′-deoxyinosine (dI), which lacks the exocyclic C_2 amino group present in dG, also prevented DNA ICL formation by **4**, revealing an essential role for the dG C_2 amino group in the interstrand cross-linking reaction between **4** and duplex DNA. This report directly demonstrates the presence of bifunctional alkylating activity in a nonreduced aziridinomitosene and clearly shows that unreduced **4** alkylates residues in the minor groove of ds DNA, cross-linking with the same 5′-d(CG) sequence specificity displayed by reduced MC.

Bioreductive antitumor agents are valuable tools used clinically in chemotherapy regimens to fight cancer (*1*). The bioreductive compounds shown in Chart 1 [aziridinylbenzoquinones AZQ[1] and DZQ, EO-9, FR 900482, FR 66979, mitomycin A (MA), and mitomycin C (MC)] are quite diverse in their molecular structures, yet all of these agents share the reductive mode of activation to elicit their antitumor and antibacterial properties (*2*−*4*). MC is regarded as *the* prototypical bioreductive alkylating agent requiring *in situ* one- or two-electron reduction of the quinone to generate mitosene intermediates that alkylate cellular macromolecules and produce DNA−DNA interstrand cross-links (*5*, *6*). The DNA interstrand cross-link (ICL) interferes with DNA replication and transcription within cells and is generally accepted as the primary cytotoxic DNA lesion (*7*, *8*).

Chart 1: Molecular Structures of Bioreductive Antitumor Agents: Aziridinylbenzoquinones AZQ and DZQ, EO-9, FR 900482, FR 66979, Mitomycin A (MA), and Mitomycin C (MC)



The proposed mechanism of DNA interstrand cross-linking by the two-electron reduced form of MC is illustrated in Scheme 1. Reduction of the MC quinone moiety is required

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[1] Abbreviations: aq, aqueous; AZQ, aziridinylbenzoquinone; BB, bromophenol blue; ddH_2O, deionized distilled water; dI, 2′-deoxyinosine; DMSO, dimethyl sulfoxide; DPAGE, denaturing polyacrylamide gel electrophoresis; ds, double stranded; DZQ, diazoaziridinylbenzoquinone; EDTA, ethylenediaminetetraacetic acid; ICL, interstrand cross-link; MA, mitomycin A; MC, mitomycin C; NH_4OAc, ammonium acetate; ntd, nucleotide; *Pu*, purine; *Py*, pyrimidine; RT, room temperature; ss, single stranded; TBE, Tris-Borate EDTA; XC, xylene cyanol; (*), 5′-^{32}P radiolabel.

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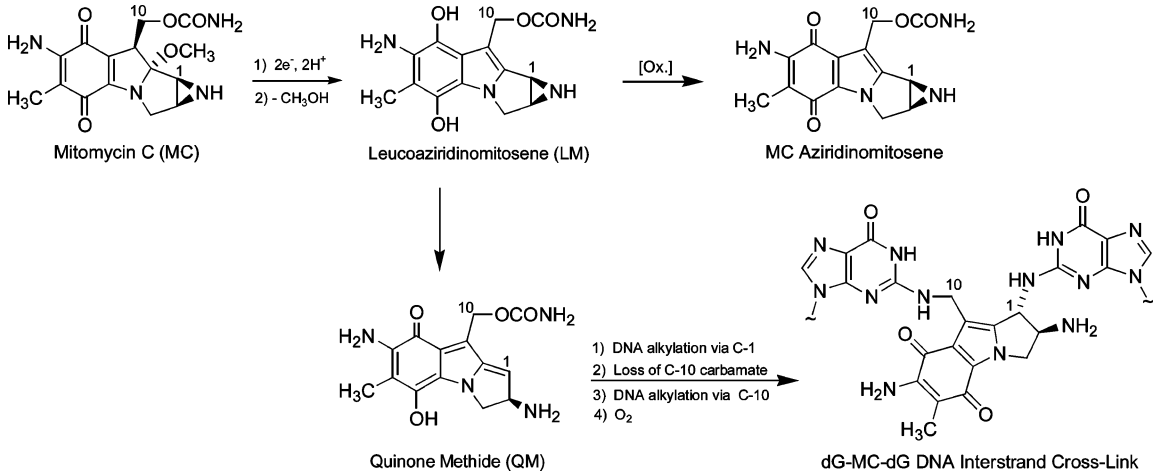
Scheme 1: General Mechanism of DNA Interstrand Cross-Linking by Reductively Activated MC[a]



[a] Sequential alkylation of duplex DNA by C-1 and C-10 in the reduced intermediates followed by quinone oxidation produces the covalent dG-MC-dG DNA interstrand cross-link. Oxidation of LM generates MC aziridinomitosene.

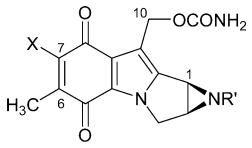
to facilitate the loss of the angular methoxy group to form mitosene intermediates [leucoaziridinomitosene (LM) (*9*) and quinone methide (QM)]. Sequential alkylation of opposing DNA strands through the electrophilic C_1 and C_{10} atoms produces the lethal DNA−DNA ICL lesion. Activated MC specifically targets the minor groove of duplex DNA, alkylating the C_2 amino groups of two dG residues at 5′-d(CG)·5′-d(CG) duplex sites to form the covalent dG-MC-dG DNA interstrand cross-link shown in Scheme 1 (*10*, *11*). Back oxidation via redox cycling of the reduced intermediates to the parent quinone forms an MC aziridinomitosene (upper pathway in Scheme 1) that remains structurally equipped with aziridine and carbamate leaving groups essential for bifunctional alkylating potential.

As a prodrug, MC must be activated to alkylate and cross-link DNA. Under acidic conditions (pH ≤ 5) and without the addition of a reducing agent, MC reacts primarily with nucleophilic sites in the major groove of duplex DNA (*12*, *13*), while at neutral pH, unreduced MC is inactive as an alkylating agent. Reductive activation of MC is required under neutral conditions to generate electrophilic intermediates that alkylate the C_2 amino position of dG and cross-link within the minor groove of duplex DNA (*5*).

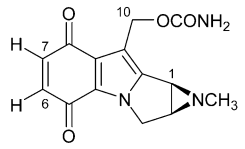
The search for new therapeutic agents has included studies of synthetic aziridinomitosenes derived from MC (**1**) (*14*, *15*), *N*-methyl MC (**2**) (*14*, *16*), and *N*-methyl MA (**3**) (*15*−*17*) (Chart 2). Biological testing of reductively activated **1**−**3** revealed varying levels of antibacterial and tumorogenic properties (*15*, *16*), which suggested the presence of bifunctional alkylating activity in these aziridinomitosenes. Teng et al. used DPAGE to conclusively demonstrate that reduced **3** cross-linked DNA specifically at 5′-d(CG) sites (*18*), the same dinucleotide sequence targeted by reductively activated MC.

Reports of aziridine ring opening in *unreduced* aziridinomitosenes **1** and **2** at neutral pH (*14*) suggested the presence of novel electrophilic characteristics that could afford DNA alkylation and cross-linking potential in the native aziridinomitosenes. The Kohn and Vedejs research groups specifically evaluated DNA alkylation by unreduced aziridinomitosenes **1** (*19*), **3** (*19*), and (1*S*,2*S*)-6-desmethyl-(methylaziridino)mitosene (**4**) (*20*, *21*), an unsubstituted

Chart 2: Structures of Synthetic Aziridinomitosenes



1 X = NH_2, R' = H MC aziridinomitosene
2 X = NH_2, R' = CH_3 MC (methylaziridino)mitosene
3 X = OCH_3, R' = CH_3 MA (methylaziridino)mitosene



4 (1*S*,2*S*)-6-desmethyl-(methylaziridino)mitosene

quinone form of *N*-methyl MA. Compounds **1**, **3**, and **4** were found to covalently modify dG residues within 5′-d(CG) sequences in ds plasmid DNA, results that clearly established these nonreduced aziridinomitosenes as DNA alkylating species. However, the question remained as to whether an aziridinomitosene could cross-link duplex DNA under neutral conditions in the absence of a reductant.

The goal of this work is to conclusively demonstrate whether nonreduced aziridinomitosene **4** can interstrand cross-link synthetic ds DNA under aerobic, nonreductive reaction conditions. DPAGE is used to analyze *in vitro* reactions between radiolabeled self-complementary DNA and nonreduced **4** for the presence of DNA interstrand cross-linking properties. In this paper, we provide the first direct evidence of DNA interstrand cross-linking by aziridinomitosene **4** in the absence of a reducing agent. We report that exogenous reductants are *not required* for the C_6−C_7 unsubstituted aziridinomitosene **4** to form interstrand cross-linked DNA specifically at 5′-d(CG) sites. Unreduced **4** displays the same *Pu*CG*Pyr*·*Pu*CG*Pyr* sequence preference for ICL formation that was found for reductively activated MC (*22*). These data are consistent with the presence of DNA sequence recognition and interstrand cross-linking motifs in both reduced MC and unreduced **4** despite their different redox environments.

MATERIALS AND METHODS

Chemicals and Biochemicals. Materials and their sources were as follows: synthetic DNA, Keystone Labs and Midland Certified Reagent Company, Inc.; [γ-^{32}P]ATP (3000 Ci/mmol), Perkin−Elmer; MC, Fisher Scientific; **4** was generously provided by the Vedejs laboratory; recombinant T4 polynucleotide kinase, New England Biolabs; 40% (19:1) acrylamide/bisacrylamide solution, BioRad; RNase/DNase-free formamide, Fisher Scientific; and MicroSpin G-25 columns, Amersham Biosciences. All other reagents were commercial and used as received. House deionized water was glass distilled (ddH_2O) and autoclaved prior to laboratory use.

Biochemical Equipment and Methods. Samples were concentrated in vacuo using a Savant Speed-Vac Plus SC110A vacuum concentrator linked to a Savant Universal Vacuum Source UVS400. UV spectra were measured on a Hewlett−Packard 8453 UV−vis spectrophotometer running HP UV−vis Chem Station Software (rev. A.02.05) and interfaced with an Intel Pentium processor.

DPAGE Electrophoretic Materials and Methods. Denaturing preparative gels (17 cm × 15 cm × 1.5 mm thick; 5 tooth comb) consisted of 20% (19:1) acrylamide/bisacrylamide, 48% urea, and 0.09 M Tris-borate/2.0 mM EDTA at pH 8.3 (1× TBE) buffer. All sequencing gel electrophoresis was conducted on a BioRad Sequi-Gen GT sequencing gel system (38 cm × 50 cm × 0.35 mm thick) using a 32-tooth comb. Samples in 50% (aq) formamide were heat-denatured and loaded onto a 20% DPAGE gel with 1× TBE running buffer (*23*). The 20% DPAGE sequencing gels were dried using a BioRad Model 583 dryer onto BioRad sequencing gel filter paper; autoradiograms were obtained with KODAK X-OMat AR film. Digitization of dried gels was achieved using a Storm 840 PhosphorImage system, and computations were performed using ImageQuant and Excel software.

Purification of Crude DNA Syntheses. Each of the ss DNA sequences used in these studies was commercially synthesized on a 1 μmol scale and received in crude form (Table 1). Crude DNA (1 μmol scale) in 100 μL of sterile ddH_2O was admixed with an equal volume of formamide, and the sample was heat-denatured (90 °C, 1−2 min) and then quick-cooled on ice before loading onto a preparative 20% DPAGE gel. Xylene cyanol (XC) and bromophenol blue (BB) in formamide were loaded into the empty center lane, and the gel was run at 450 V for 1.5 h or until the BB dye migrated 13−14 cm. DNA bands were visualized by UV shadowing, and the ss DNA product was eluted from the acrylamide gel slices by rocking samples at 22 °C in 0.5 M NH_4OAc/10 mM MgOAc/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 (aq) NH_4OAc, (2) water, and (3) 25% (aq) CH_3CN. DNA concentrations were calculated from UV $A_{260\ nm}$ readings using the ss DNA molar absorptivities provided by the manufacturer.

Preparation of 5′-[^{32}P]-Radiolabeled (*) ss DNA. Synthetic DNA was purified twice by preparative 20% DPAGE prior to use in the radiolabeling reactions. Double-pure DNA (20 pmol ss, dry) in T4 kinase buffer [70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 5 mM dithiothreitol] was 5′-end-labeled with [γ-^{32}P]ATP (60 μCi [γ-^{32}P]ATP) using 10 units of recombinant T4 polynucleotide kinase (10 μL total reaction volume). Incubation (30 min at 37 °C) was followed by heat inactivation of the enzyme (70 °C, 20 min). The reaction volume was adjusted to 50 μL with water and the sample loaded onto a prespun (1 min at 735*g*) MicroSpin G-25 column. The loaded column was spun (2 min at 735*g*) in a Heraeus Biofuge 15R centrifuge (4 °C) to remove unincorporated nucleotides. The recovered *DNA was dried in a Savant Speed-Vac under medium heat, and the dried pellet was resuspended in 20 μL of ddH_2O for use in DNA−aziridinomitosene cross-linking experiments.

*Treatment of *DNA with **4** in the Absence of Exogenous Reductants.* Solid **4** was dissolved in DMSO and stored at −80 °C prior to use in the DNA experiments. A preliminary investigation revealed pH 6.0 as the optimum pH for interstrand cross-linking by compound **4**. All reactions were performed in duplicate. Synthetic ss *DNA 5′-d(TTATAN$_4$-TATAA)-3′ (1−2 pmol) was annealed to unlabeled DNA (3.0 nmol ss DNA) in 50 mM (aq) sodium cacodylate/1.0 mM EDTA buffer (pH 6.0) by heating the sample to 90 °C for 2 min and then cooling the reaction slowly to RT. Water was added to the ds DNA/buffer mixture followed by **4** (9 nmol in DMSO) for a final reaction volume of 30 μL [final reaction conditions: 25 mM sodium cacodylate (pH 6.0)/0.5 mM EDTA, 0.05 mM ds DNA, 0.3 mM **4**, and 1.75% DMSO]. The samples were incubated at 20 °C for 48 h. Cold carrier DNA (3 nmol of ds synthetic DNA in 10 mM NH_4OAc) was added to each reaction, and the samples were placed on ice for 15 min, followed by overnight storage at −20 °C. Each product mixture was combined with an equal volume of neat formamide, heat-denatured (1 min, 90 °C; onto ice), and directly analyzed on a 20% DPAGE sequencing gel (0.35 mm × 35 cm × 50 cm gel; 32 tooth comb). Sequencing gels were run at 85 W (constant)/2800−3000 V/45−55 °C, until the BB dye migrated ∼23 cm (∼2.5−3 h). The gels were transferred to Saran Wrap and BioRad sequencing gel filter paper and then dried under vacuum (cycle 1, 80 °C) for 2 h. Dried gels were visualized by autoradiography on Kodak X-OMat AR film and analyzed on a Storm 840 PhosphorImage System. Data were acquired using Molecular Dynamics ImageQuant software (version 1.2), and computations were performed using ImageQuant and Excel software. ICL yields were determined as the ratio of *ICL products to total *DNA.

ICL Yield as a Function of pH. Buffer solutions of 25 mM sodium cacodylate/0.5 mM EDTA with pH values ranging from pH 5.0 to 7.4 were used to evaluate interstrand cross-linking activity of compound **4** with the self-complementary DNA duplex ACGT [5′-d(TTATA**ACGT**TATAA)]$_2$. All reactions were performed in duplicate and analyzed on 20% DPAGE sequencing gels, and gel data were quantified as previously described.

2′-Deoxyinosine (dI) Substitution Experiments. The radiolabeled dI/dG DNA heteroduplex (N$_4$ = *ACIT·ACGT) was treated with **4** and analyzed on a DPAGE sequencing gel as described above.

Reductively Activated MC-DNA Cross-Linking Reactions. In separate reactions, individual ss *DNA sequences (3 pmol ss) were annealed to the corresponding unlabeled ss DNA (3.0 nmol ss DNA) in 50 mM (aq) sodium cacodylate/1.0 mM EDTA (pH 6.0) buffer (90 °C, 2 min; cool slowly to RT). Water was added to the ds DNA/buffer mixture followed by MC [42 nmol in 33% (aq) CH_3OH] for a final

Table 1: Synthetic Self-Complementary DNA Sequences Used in the Assessment of DNA Interstrand Cross-Linking by Nonreduced **4**

ss DNA sequences (5′ → 3′) 5′-d(TTATA**N**$_4$TATAA)-3′	decriptor N_4 (5′-NNNN)
TTATA**TCGA**TATAA	TCGA
TTATA**ACGT**TATAA	ACGT
TTATA**CCGG**TATAA	CCGG
TTATA**GCGC**TATAA	GCGC
TTATA**AGCT**TATAA	AGCT

reaction volume of 30 μL [final reaction concentrations: 25 mM sodium cacodylate (pH 6.0), 0.5 mM EDTA, 0.05 mM ds DNA, 1.4 mM MC, and 2.3% CH_3OH]. Reactions were sparged with argon (20 min) at RT prior to adding 14 nmol of sodium dithionite (1 μL of 14 mM $Na_2S_2O_4$ stock prepared in argon-sparged water) at 15 min intervals for a total of 3 aliquots (42 nmol $Na_2S_2O_4$). Reduced MC reactions were incubated for an additional 15 min at RT and subsequently stored at −20 °C until analyzed on a 20% DPAGE sequencing gel as described above.

RESULTS AND DISCUSSION

*Nonreduced **4** Generates an ICL in ds DNA Containing 5′-d(CG) Dinucleotide Sites.* We tested aziridinomitosene **4** for its ability to cross-link self-complementary duplex DNA having the general form [5′-d(TTATA**N**$_4$TATAA)-3′]$_2$ (Table 1). These self-complementary DNA sequences contain 14 of the 16 possible dinucleotide combinations of C, G, T, and A. Given that nonreduced **4** has a reported half-life of ∼6.67 h at pH 6.0 (*20*), we performed the experiments with **4** at pH 6.0 and allowed reactions to incubate at RT for 2 days. In contrast, MC was combined with ds DNA in anaerobic buffer (pH 6.0), activated by sodium dithionite, and incubated at RT for 1 h.

Direct analysis of the DNA products by DPAGE reveals that **4** produces DNA−DNA interstrand cross-links only in duplex DNA having the sequence 5′-d(CG) as the minimum common element (lanes 1−4, Figure 1A), the same dinucleotide sequence targeted by reduced MC (lanes 1−4, Figure 1B). The duplex DNA derived from AGCT (Table 1) has the inverted dinucleotide sequence 5′-d(GC) and affords no detectable interstrand cross-linked DNA after treatment with either nonreduced **4** (lane 5, Figure 1A) or dithionite-reduced MC (lane 5, Figure 1B). The two dinucleotide sites [5′-d(TG) and 5′-d(CA)] not represented in Table 1 were evaluated using nonself-complementary duplexes, and when treated under the same conditions with **4** or reduced MC, these DNA duplexes did not produce ICL (data not shown). These data confirm the presence of DNA bis-alkylating activity in nonreduced aziridinomitosene **4** and provide the first direct evidence of sequence-specific DNA interstrand cross-linking by an aziridinomitosene *without* prior activation by a reducing agent.

*5′-d(CG) Flanking Nucleotides Modulate DNA−DNA ICL Production by Nonreduced **4**.* The yield of ICL from each 5′-d(CG)-containing ds DNA treated with **4** or activated MC varied from 2- to 3-fold, depending on the identity of the nucleotides [purine (*Pu*) and pyrimidine (*Py*)] flanking the 5′-d(CG) site (Figure 1). Inspecting the tetranucleotide

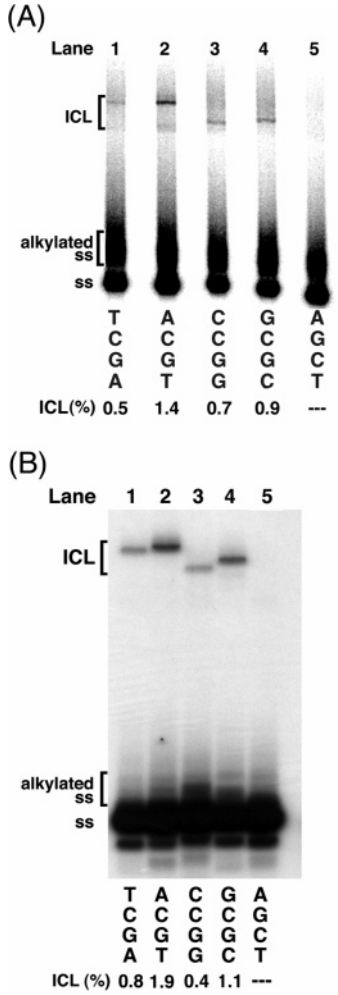


FIGURE 1: Autoradiographs of DNA alkylation products formed at pH 6.0 by unreduced **4** (A) and reductively activated MC (B) with ds *DNA (from Table 1). The specific self-complementary DNA duplex studied is indicated by the descriptor (N_4) under each lane. (A) *DNA was incubated with unreduced **4** for 48 h at 21 °C under aerobic conditions. (B) *DNA was incubated with MC and 1 equiv of $Na_2S_2O_4$ for 1 h at 21 °C under anaerobic conditions. The percent yield of ICL was determined by densitometric analysis of PhosphorImage gel data using ImageQuant software.

context of each 5′-d(CG) site revealed that the efficiency of the interstrand cross-linking was greater in ds DNA having 5′-d(*Pu*CG*Py)* tetranucleotides, with the maximum ICL yield obtained from DNA duplex ACGT treated with either **4** (lane 2, Figure 1A) or with reductively activated MC (lane 2, Figure 1B). These data support a shared mode of target recognition that directs bisalkylation of DNA by reduced MC (*11*, *18*, *22*) and aziridinomitosene **4** toward the ACGT tetranucleotide to afford maximum cross-linking efficiency. Interactions between the MC carbamate and the C_2 amino group of the dG residue present in the strand opposite the first alkylation are known to direct the second dG−dG interstrand cross-linking step at 5′-d(CG) by MC (*24*). One could speculate that a carbamate-dG C_2 amino interaction takes place between DNA dG residues and **4** to direct the bisalkylation reaction, but the factors that control the observed sequence-specific interstrand cross-linking by the unreduced aziridinomitosene have yet to be fully revealed.

*dG C_2 Amino Group Is Essential for the Formation of an ICL in ds DNA by Unreduced **4**.* To test the role of the dG

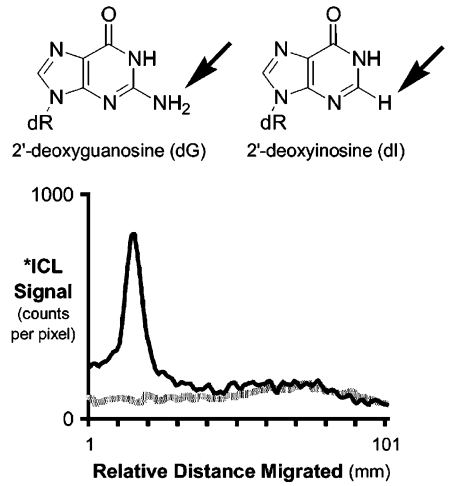


FIGURE 2: Relative abundance of DNA ICL produced in the reaction of **4** with *DNA duplex ACGT (Table 1), which contains a *d(CG)·d(CG) interstrand cross-link site (black line), versus the reaction of **4** with the DNA heteroduplex [*5′-d(TTATA**ACIT**TATAA)·5′-d(TTATA**ACGT**TATAA), gray line], which contains a *d(CI)·d(CG) site. The ICL abundance was determined for each reaction through densitometric analysis of PhosphorImage gel data using ImageQuant software.

C_2 amino in the DNA cross-linking reaction of **4**, we replaced the dG residue within the 5′-d(A**CG**T) target sequence with 2′-deoxyinosine (dI), a nucleotide that lacks a C_2 amino group (Figure 2). DPAGE analysis of the reaction products clearly revealed ICL production from the reaction between **4** and duplex *ACGT·ACGT. In contrast, no ICL signal was detected from DNA heteroduplex *ACIT·ACGT treated with nonreduced **4**. These data support an essential role for the dG C_2 amino group in the covalent cross-linking of ds DNA at 5′-d(CG) by nonreduced **4** and are consistent with the **4**-derived ICL spanning the minor groove of duplex DNA. The thermal stability observed for interstrand cross-linked DNA produced by **4** provides additional support for the minor groove dG C_2 amino alkylation by the native aziridinomitosene.

From the data obtained thus far, it could be concluded that **4** reacts with duplex DNA at both C_1 and C_{10} positions of the aziridinomitosene. However, the specific atoms covalently modified in **4** when it forms a DNA interstrand cross-linkage remain to be determined.[2]

*ICL Reaction between ds DNA and Nonreduced **4** Is pH-Dependent.* Under nonreducing conditions, DNA interstrand cross-linking activity between **4** and ds DNA ACGT was found to increase as pH decreased with a maximum ICL yield obtained at pH ~6.1 (Figure 3). These results are consistent with acid catalysis of DNA cross-linking by nonreduced **4**. The less than 10-fold increase in the DNA cross-link yield observed from pH 7.0 to 6.0 suggests the presence of competing reactions that reduce the catalytic effect of increasing hydrogen ion concentration on the DNA cross-linking reaction by unreduced **4**.

[2] As mentioned by a reviewer, it is possible that the cross-link does not involve the carbamate but rather Michael addition to the quinone ring of **4** by DNA. Because the exact structure of the covalent adduct is unknown, the Michael addition mechanism is plausible and is currently being explored. However, preliminary evidence with the decarbamoyl version of **4** suggests that the carbamate is vital for cross-link formation.

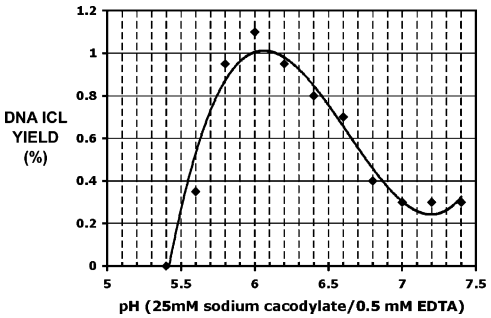


FIGURE 3: Yield of ICL formed by nonreduced **4** with duplex *DNA **ACGT·ACGT** (see Table 1) as a function of pH.

CONCLUSIONS

This work is the first demonstration of sequence-specific DNA interstrand cross-linking by an aziridinomitosene in the absence of a reducing environment. It is important to recognize the sensitivity of the aziridinomitosene activity to the specific conditions used in the cross-linking experiments. The mechanism of ICL formation for the reactions of **4** with duplex DNA containing 5′-d(CG) is acid-catalyzed over the pH range studied and involves noncovalent atomic interactions that direct bisalkylation toward 5′-d(CG) sequences. The discovery of DNA interstrand cross-linking properties in unreduced aziridinomitosene **4** under mildly acidic conditions suggests that structurally related aziridinomitosene intermediates generated from reductive activation of MC may function as bifunctional DNA alkylating species.

Additional research is required to reveal the nature of the covalent connectivity between **4** and the nucleotide residues in interstrand cross-linked DNA, to determine which molecular features dictate the sequence-specific interaction between **4** and its duplex DNA target, and to ultimately reveal the mechanism that is responsible for bifunctional activity of **4** in a nonreducing environment. These studies and evaluation of DNA cross-linking activity for reduced **4** are underway, and the results will be reported in due course.

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