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Sequence Preferences of DNA Interstrand Cross-Linking Agents: dG-to-dG Cross-Linking at 5′-CG by Structurally Simplified Analogues of Mitomycin C†

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ABSTRACT: The nucleotide sequence preferences of the DNA interstrand cross-linking agents dehydroretronecine diacetate (DHRA), 2,3-bis(acetoxymethyl)-1-methylpyrrole (BAMP), dehydromonocrotaline, and dehydroretrorsine were studied by using synthetic DNA duplex fragments and polyacrylamide gel electrophoresis (PAGE). These agents have structural features in common with the reductively activated aziridinomitosene of mitomycin C (MC). Like MC, they preferentially cross-linked DNA duplexes containing the duplex sequence 5′-CG. For DHRA and BAMP interstrand cross-linked DNA duplexes, PAGE analysis of iron(II)–EDTA fragmentation reactions revealed the interstrand cross-links to be deoxyguanosine to deoxyguanosine (dG-to-dG), again analogous to DNA cross-links caused by MC. Unlike MC, DHRA could be shown to dG-to-dG cross-link a 5′-GC sequence. Furthermore, the impact of flanking sequence on the efficiency of interstrand cross-linking at 5′-CG was reduced for BAMP, with 5′-TCGA and 5′-GCGC being equally efficiently cross-linked. Possible origins of the 5′-CG sequence recognition common to all of the agents are discussed. A model is presented in which the transition state for the conversion of monoadducts to cross-links more closely resembles ground-state DNA at 5′-CG sequences.

The bifunctional alkylating agents are a class of drugs useful in the treatment of human cancer and include the chloroethylnitrosoureas, nitrogen mustards, and mitomycins. These drugs are widely believed to exert their cytotoxic action by reaction with DNA; numerous studies suggest that interstrand cross-linking of duplex DNA is important in this regard (Kohn, 1980). The chemical structures of the conjugates of these drugs and DNA have been extensively studied. Much less work has centered on the identification of sequences at which cross-links are preferentially formed (Mattes, et al., 1988). At the present time, the reactions with DNA of the psoralens are probably the best understood interstrand cross-linking reactions (Hearst, 1989). This paper concerns the mitomycins, cross-linking agents (Iyer & Szybalski, 1963, 1964; Matsumoto & Lark, 1963) about which much less is known. Three laboratories have reported that for reductively activated mitomycins, 5′-CG is the preferred cross-linked sequence (Chawla et al., 1987; Teng et al., 1989; Weidner et al., 1989; Millard et al., 1990b); the combined studies strongly suggest that the aziridinomitosene of mitomycin C (MC, Figure 1)[1] ultimately bridges the exocyclic amino groups (N2) of two deoxyguanosine residues by sequential formation and reaction with DNA of electrophilic sites at carbons 1 and 10 of the mitosene (Tomasz et al., 1987). Very recently, Norman et al. (1990) have reported NMR solution structure studies of an MC cross-linked hexanucleotide duplex and concluded that MC cross-linked DNA does possess this covalent connectivity, with the MC residue deeply nested in the minor groove of a right-handed double helix.

What is the mechanistic origin of 5′-CG recognition by reductively activated mitomycins? Particularly intriguing is the preference of 5′-CG over 5′-GC: Both of these sequences possess a pair of dG N2 groups centrally located in the minor groove of DNA and with similar interatomic spacing (3.6 and 4.0 Å, respectively) in canonical B DNA (Arnott et al., 1976). Furthermore, the sequence that flanks the core 5′-CG sequence

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[1] Abbreviations: BAMP, 2,3-bis(acetoxymethyl)-1-methylpyrrole; b, broad; d, doublet; DHRA, dehydroretronecine diacetate; DTE, dithioerythritol; DTT, dithiothreitol; m, multiplet; MC, mitomycin C; PAGE, polyacrylamide gel electrophoresis; q, quartet; s, singlet; t, triplet.

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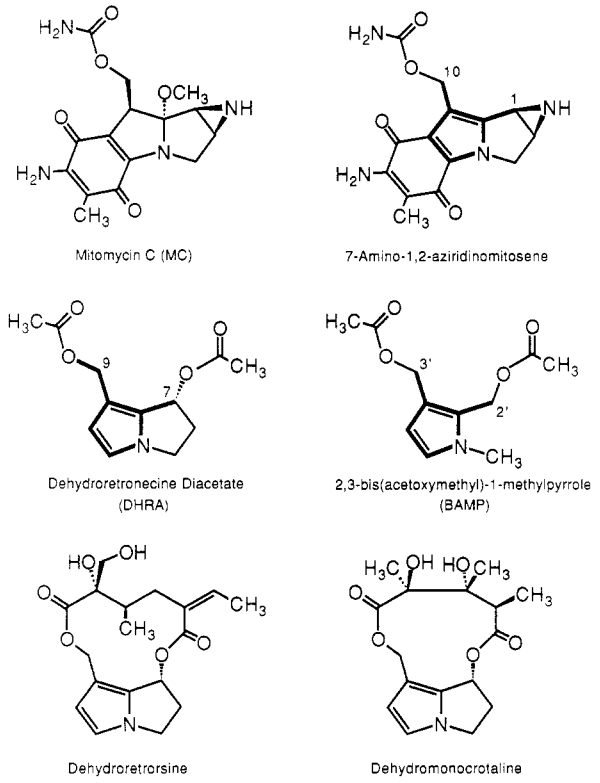


FIGURE 1: Structures of the DNA interstrand cross-linking agents used in this study.

can modulate the extent of cross-linking (Teng et al., 1989; Millard et al., 1990b; Borowy-Borowski et al., 1990). Whether these effects are due to differences inherent in the chemical reactivity of dG residues in different flanking sequences or features unique to the mitosene/DNA complex (hydrogen bonds, electrostatic or hydrophobic interactions, etc.) is unknown. To explore the structural requirements for 5′-CG versus 5′-GC recognition as well as flanking sequence effects, we have semiquantitatively evaluated the cross-linking sequence preferences of several substances that are structurally related to reductively activated mitomycins.

The pyrrolizidine alkaloids are a family of plant-derived toxins (Mattocks, 1986) that after metabolic activation bear a remarkable resemblance in both structure and reactivity to reductively activated mitomycins (Culvenor et al., 1969; Mattocks, 1969). Like the mitomycins, pyrrolizidine alkaloids cross-link DNA in vivo (Petry et al., 1984) and (after oxidative activation) in vitro (White & Mattocks, 1972; Reed et al., 1988). An analogue of the activated pyrrolizidines, dehydroretronecine diacetate (DHRA, Figure 1), bears reactive functionality at carbons 7 and 9 capable of sequential reactivity analogous to that at carbons 1 and 10 of an aziridinomitosene (Figure 1). As such, the dehydropyrrolizidines resemble reductively activated mitomycins but are completely devoid of the quinone ring. Consistent with a mechanistic similarity of DNA cross-linking reactions of mitomycins and pyrrolizidine alkaloids is the isolation of an N2 (deoxyguanosine)-to-C7 (dehydropyrrolizidine) conjugate (Robertson, 1982) analogous to the known N2 (deoxyguanosine)-to-C1 (mitosene) conjugate of mitomycin (Tomasz et al., 1986). An even simpler analogue of MC is 2,3-bis(acetoxymethyl)-1-methylpyrrole (BAMP, Figure 1), in which carbons 2′ and 3′ bear analogy to sites 1 and 10 in the aziridinomitosene. BAMP lacks both the quinone ring and the chirality associated with the secondary leaving groups of DHRA (at C7) and the aziridinomitosene (at C1). BAMP-treated DNA renatures anomalously quickly, suggesting that it, too, is an interstrand cross-linking agent (White & Mattocks, 1972). Experiments probing the DNA interstrand cross-linking preferences of DHRA and BAMP, as well as dehydroretrorsine and dehydromonocrotaline (Figure 1), are reported herein. Our results indicate that the full steric demands and complex functionality of mitomycin C are not necessary for preferential cross-linking of 5′-CG over 5′-GC. Even BAMP exhibits a remarkable preference for the sequence 5′-CG, albeit quantitatively reduced relative to mitomycins.

Materials and Methods

Materials and their sources were as follows: DNA synthesis reagents, Applied Biosystems; [α-^{32}P]dATP and [γ-^{32}P]ATP, New England Nuclear; Klenow fragment and T4 polynucleotide kinase, Boehringer Mannheim; mitomycin C, Sigma or Bristol Myers; 2,3-bis(hydroxymethyl)-1-methylpyrrole, a gift from Professor A. R. Mattocks. "Purified water" was from a Millipore Milli-Q deionizer. Oligonucleotides were synthesized on an Applied Biosystems Model 380A synthesizer. All other chemicals were of commercial origin and were used as received. Samples were concentrated in vacuo with a Savant Speed Vac Concentrator. Except for purification of synthetic oligonucleotides, all PAGE was conducted on a Hoeffer thermojacketed Poker Face gel stand. Formamide dye solution was a 9:1 mixture (v/v) of deionized formamide and 100 mM Tris (pH 7.5), 1% xylene cyanole, 1 mM EDTA. Gels were dried by using a Bio-Rad model 583 gel drier onto Whatman 3MM paper; autoradiograms were with Kodak XAR-5 film. Densitometry was performed on a Hoeffer GS 300 densitometer interfaced to an IBM PC. Data were smoothed and plotted by using the program Spectra Calc. Scintillation counting utilized a Packard TRI-CARB 2000CA scintillation counter. ^{1}H NMR spectra were recorded on a Bruker AC200 spectrometer and unless otherwise stated were referenced to internal tetramethylsilane, δ 0.

Preparation of DHRA and BAMP. Both DHRA and BAMP were prepared by in situ acetylation of diols: An accurately weighed 2–3-mg aliquot of 2,3-bis(hydroxymethyl)-1-methylpyrrole or dehydroretronecine [prepared by saponification of retrorcine followed by bromanil-promoted dehydrogenation (Mattocks et al., 1989) of the resulting retronecine] in 300 μL of $CDCl_3$ at 25 °C was treated sequentially with 5.0, 1.0, and 2.5 molar equiv of triethylamine, 4-(dimethylamino)pyridine, and acetic anhydride, respectively. After 1.0 h, the ^{1}H NMR spectrum of this solution revealed replacement of the starting material resonances [2,3-bis(hydroxymethyl)-1-methylpyrrole ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.57 (1 H, d, H5), 6.08 (1 H, d, H4), 4.58 (2 H, s, CH_2OH), 4.53 (2 H, s, CH_2OH), 3.67 (3 H, s, CH_3); dehydroretronecine ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.56 (1 H, d, H3), 6.17 (1 H, d, H2), 5.28 (1 H, dd, H7), 4.74 (1 H, d, H9), 4.62 (1 H, d, H9), 4.16 (1 H, m, H5), 3.92 (1 H, m, H5), 2.83 (1 H, m, H6), 2.47 (1 H, m, H6)] by resonances for the corresponding diacetate [BAMP ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.63 (1 H, d, H5), 6.17 (1 H, d, H4), 5.15 (2 H, s, CH_2OAc), 5.06 (2 H, s, CH_2OAc), 3.64 (3 H, s, CH_3), 2.07 (3 H, s, OAc), 2.04 (3 H, s, OAc); DHRA ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.67 (1 H, d, H3), 6.30 (1 H, d, H2), 6.13 (1 H, dd, H7), 5.08 (1 H, d, H9), 4.98 (1 H, d, H9), 4.15 (1 H, m, H5), 3.97 (1 H, m, H5), 2.92 (1 H, m, H6), 2.52 (1 H, m, H6), 2.05 (6 H, s, 2 OAc)]. The concentration of BAMP or DHRA in these solutions was calculated by comparison of the integrated values of the diacetate resonances of DHRA and aromatic ring hydrogen resonances of 4-(dimethylamino)pyridine.

Preparation of Dehydromonocrotaline and Dehydroretrorsine. Dehydromonocrotaline and dehydroretrorsine were

prepared by bromanil-promoted dehydrogenation of the parent alkaloids (Mattocks et al., 1989). The resulting products were of greater than 90% purity (200-MHz ^{1}H NMR analysis) and were used without further purification. Dehydromonocrotaline: ^{1}H NMR (200 MHz, $CDCl_3$, reference to internal $CHCl_3$, δ 7.24) δ 6.57 (1 H, d, H3), 6.26 (1 H, d, H2), 6.05 (1 H, dd, H7), 5.67 (1 H, d, H9), 4.54 (1 H, d, H9), 4.13 (1 H, m, H5), 3.98 (1 H, m, H5), 3.08 (s, impurity), ca. 3.0 (1 H, m, H6), 2.92 (1 H, q, H14), 2.53 (1 H, m, H6), 2.29 (s, impurity), 1.54 (s, 3 H, H15 or H16), 1.47 (s, 3 H, H15 or H16), 1.30 (3 H, d, H17), 1.22 (m, impurity). Dehydroretrorsine: ^{1}H NMR (200 MHz, $CDCl_3$, reference to internal $CHCl_3$, δ 7.24) δ 6.63 (1 H, d, H3), 6.28 (1 H, d, H2), 5.87 (1 H, d, H7), 5.82 (1 H, bq, H20), 5.38 (1 H, d, H9), 5.04 (1 H, d, H9), 3.98 (2 H, m, H5), 3.75 (1 H, d, H18), 3.67 (1 H, d, H18), 2.96 (1 H, m, H6), 2.59 (1 H, m, H6), 2.33 (1 H, d, H14), 1.93 (3 H, d, H21), 1.82 (1 H, m, H13), 1.65 (1 H, dd, H14), 0.88 (3 H, d, H19). The concentration of $CDCl_3$ solutions of dehydromonocrotaline and dehydroretrorsine used for cross-linking was determined by addition of a known volume of a 1% solution of acetone in $CDCl_3$ followed by ^{1}H NMR integration.

Preparation of Radiolabeled DNA Duplexes. Oligonucleotides (1-μmol scale synthesis) were purified by denaturing PAGE (ca. 80 OD of crude DNA, 20% polyacrylamide, 19:1 acrylamide:bis(acrylamide), 8 M urea, 1.5 mm thick, 14 × 16 cm, using a five-toothed comb) run until the xylene cyanol dye had traveled 8–9 cm from the origin. DNA was visualized by UV shadowing (Maxam & Gilbert, 1980). DNA was isolated from the gel by a crush and soak procedure: gel slices were crushed with a glass rod into fine particles and incubated at 37 °C for at least 1 h in 0.5 M aqueous NH_4OAc and 1 mM aqueous EDTA followed by an ethanol precipitation (Maniatis et al., 1981). Radiolabeling was at either a 3′-terminus [10 mM Tris (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTE, with [α-^{32}P]dATP and Klenow fragment] or at a 5′-terminus [50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.1 mM spermidine, 5 mM DTT, 0.1 mM EDTA, with [γ-^{32}P]ATP and T4 polynucleotide kinase]. In all cases, unincorporated radionuclides were removed by at least three ethanol precipitations, followed by an 85% aqueous ethanol rinse of the DNA pellet. Because the interpretation of data derived from these DNA duplexes is independent of whether they are radiolabeled in one, both, or a mixture of one and both strands, this issue was not directly addressed. For convenience only, the duplexes are illustrated in the work that follows as having a single radiolabeled strand.

Preparation of Cross-Linked DNA Duplexes. Synthetic, radiolabeled DNA (70 nmol of base pairs) in 25 μL of 50 mM aqueous sodium citrate buffer (pH 5.0), 5 mM aqueous NaCl, and 5 mM aqueous $MgCl_2$ was agitated (vortex mixer) at 25 °C with 0.4–0.5 mL of a $CDCl_3$ solution containing 3.5 μmol of DHRA or BAMP for 0.75 h. Cross-linking reactions with dehydromonocrotaline and dehydroretrorsine were identical except that the DNA was in 50 mM Tris (pH 7.5) and 100 mM aqueous NaCl. (Cross-linking reactions with mitomycin C are described below.) The DNA solution from the cross-linking mixture was ethanol precipitated, the supernatant removed, and the pellet dried. The pellet was dissolved in 100 μL of purified water. A 5-μL aliquot was dried and Cerenkov counted and the remaining sample was ethanol precipitated and the pellet dried. The pellet was dissolved in 18 μL of 5 M aqueous urea, heated to 90 °C for 2 min, and iced 2–3 min. The resulting solution was analyzed by denaturing PAGE. Included in neighboring lanes were (a) 18 μL of formamide dye solution and (b) a mitomycin C cross-linked oligonucleotide (N_4 = TCGA, see Figure 2) prepared according to Millard et al. (1990b). Denaturing PAGE was conducted on a 25% gel (19:1 acrylamide:bis(acryamide), 8 M urea, 0.35 mm thick, 33 × 41 cm, using a 20-toothed comb) at 65–70 W and ca. 65 °C until the xylene cyanole had run 14 cm. Autoradiography was used to visualize the single-stranded and cross-linked DNA. Cross-linked DNA bands were identified by comparison to the mitomycin C cross-linked DNA standard.

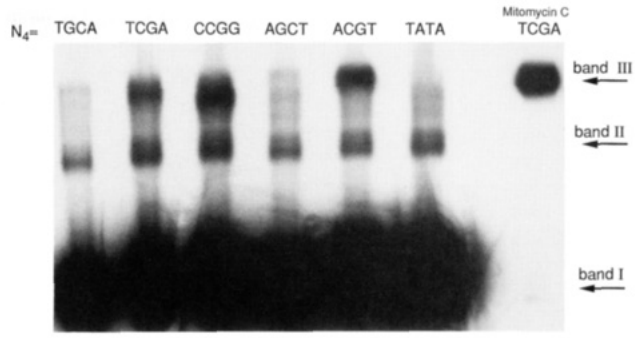


FIGURE 2: Overexposed autoradiograph of denaturing PAGE analysis of DHRA cross-linking reactions of six 3′-end radiolabeled DNAs, 5′-d[AATATAAT(N_4)ATTAT], N_4 as indicated above gel lanes. The right lane is a purified, mitomycin C cross-linked DNA included as a reference.

Mitomycin C Cross-Linking Reactions. A siliconized test tube, capped with a rubber septum, containing radiolabeled DNA (70 nmol of base pairs) in 50 μL of 50 mM aqueous sodium citrate buffer (pH 5.0), 5 mM aqueous NaCl, 5 mM aqueous $MgCl_2$, and 0.5 mL of a $CDCl_3$ solution containing 3.5 μmol of mitomycin C (prepared by dilution of 20 mM mitomycin C in CH_3OH with $CDCl_3$) was flushed with argon for ca. 5 min. Three 50-μL aliquots of freshly prepared 70 mM aqueous $Na_2S_2O_4$ were added at 15-min intervals while the mixture was agitated (vortex mixer) at 25 °C for 0.75 h. The aqueous layer was separated and ethanol precipitated and the pellet rinsed with 85% ethanol, dried, suspended in 18 μL of 5 M aqueous urea, heated to 90 °C for 2 min, iced 2–3 min, and loaded onto a 25% denaturing gel (19:1 acrylamide:bis(acrylamide), 8 M urea, 0.35 mm thick, 33 × 41 cm, using a 20-toothed comb) run at 65–70 W and ca. 65 °C until the xylene cyanole had migrated 14 cm. A 15-h autoradiograph was used to visualize cross-linked DNA for excision from the gel. DNA was extracted from the gel slice by a crush and soak procedure. The samples were reexposed to radiolabeling (see below) and again subjected to 25% PAGE. A 3-h autoradiograph showed cross-linked DNA bands for both oligonucleotides N_4 = TCGA and TGCA (see Figure 2 legend for DNA sequence). Iron(II)–EDTA and guanine-specific cleavage reactions (see below) were performed on cross-linked and native DNAs.

Excision of Cross-Linked DNA Bands and Relabeling. Gel slices of approximate dimensions 2.5 × 1 cm containing cross-linked DNA were excised from the above-described 25% denaturing polyacrylamide gels. DNA was eluted from the gel slice by using a crush and soak procedure, ethanol precipitated, dried, and reexposed to radioactive labeling by using the appropriate radionuclide, buffer, and enzyme (described above).

DNA Cleavage Reactions. Iron(II)–EDTA cleavage of DNA was generally as described by Tullius and Dombroski (1985) with the following modifications: To a solution of DNA in 14 μL of 10 mM Tris (pH 7.5) and 10 mM NaCl was added 2 μL each of 10 mM aqueous ascorbic acid, 0.3% aqueous H_2O_2, and 1 mM aqueous $(NH_4)_2Fe(SO_4)_2$. After 1 min at

25 °C, the reaction was stopped by the addition of 2 μL of 0.1 M aqueous thiourea. These samples, along with comparison lanes containing uncut, cross-linked, and native (un-cross-linked) DNA and a Maxam–Gilbert guanine-specific sequencing reaction (Maxam & Gilbert, 1980) on native DNA, were dried, suspended in 4 μL of formamide dye solution, heated to 90 °C for 2 min, iced for 2–3 min, loaded onto a 25% denaturing gel (19:1 acrylaide:bis(acrylamide), 8 M urea, 0.35 mm thick, 33 × 41 cm, using a 32-toothed comb), and electrophoresed at 65–70 W, at ca. 65 °C, until the xylene cyanole dye had run 14–16 cm from the origin. The gel was dried and autoradiographed. Band identification was made by reference to the Maxam–Gilbert guanine-specific sequencing reaction. Gel lanes derived from iron(II)–EDTA fragmentation of cross-linked DNAs revealed that the major product was the starting, cross-linked DNA. Bands were clearly evident not only for fragments smaller than single strand but also for fragments of mobility bracketed by cross-linked duplex and full-length single strand. The latter presumably represent fragments that retain one full-length strand covalently cross-linked to a portion of the opposing strand. Although in principle analysis of these bands could provide further information on cross-link location, in practice the resulting pattern is not easily interpreted and as such is not presented herein.

Densitometry. One-dimensional (linear) densitometry of autoradiograms was performed by scanning down the center of the bands in a lane. Two-dimensional densitometry was used to evaluate the data on the effect of flanking sequence preference on the DNA cross-linking of BAMP by using the following procedure: A series of sequential linear scans, from one side of a lane on the autoradiograph, moving 2 mm per scan to the opposite side of a lane, generated a series of stacked plots for iron(II)–EDTA cleaved, cross-linked, and native DNA. The plots were smoothed and the integrated peak areas (Spectra Calc) for each nucleotide of the cross-linked or native DNA were summed. Linear film response was verified by duplicate densitometry on 1.0- and 2.0-h autoradiographic exposures.

Results

Core Sequence Preference of Dehydroretronecine Diacetate (DHRA). Six self-complementary (5′-overhang), 3′-^{32}P end radiolabeled oligonucleotides (Figure 2) in 50 mM aqueous sodium citrate buffer (pH 5.0) were vigorously agitated with a chloroform solution of a large excess (50:1 DHRA:base pairs) of dehydroretronecine diacetate (DHRA) at 25 °C. The DNA in these solutions was isolated by precipitation and then analyzed by 25% denaturing PAGE. An overexposed autoradiogram is shown in Figure 2. In addition to recovered single strands (band I, presumably including monoadducted single strands), less electrophoretically mobile, interstrand cross-linked products were formed. All six oligonucleotides showed similar amounts of a higher mobility, interstrand cross-linked DNA (band II). Varying amounts of a major, lower mobility, interstrand cross-linked product (band III) were also formed.

The nucleotide connectivity of band II (Figure 2) DNA, formed in significant amounts from all DNAs, was established for N_4 = TGCA. DNA eluted from the excised gel band was fragmented with iron(II)–EDTA (Weidner et al., 1989; Millard et al., 1990b). High-resolution denaturing PAGE analysis indicated the formation of products identical in mobility with native (un-cross-linked) DNA for cleavage from the radiolabeled end through A4 (Figure 3). The lower abundance of fragments derived from cleavage at T3 and A2 in the cross-linked sample relative to native sample strongly

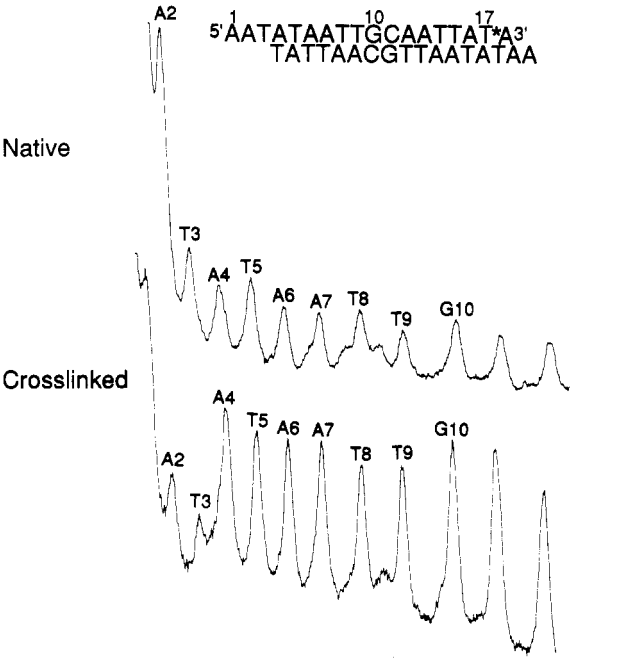


FIGURE 3: Partial fragmentation patterns of 3′-end radiolabeled (* = ^{32}P), native, and DHRA cross-linked DNA (band II), N_4 = TGCA.

suggests that band II arises from cross-linking at the common end sequence of all DNAs studied. At least partial cross-linking at A4 is suggested by the reduced intensity relative to native for fragmentation at T3. Partial overlap in the gel of fragments derived from cleavage at A1 with a full-length single strand precludes meaningful further analysis of the connectivity of these terminally cross-linked DNAs. The presence of this full-length *single strand* in an iron(II)–EDTA fragmentation reaction that started exclusively with cross-linked DNA suggests that band II cross-links may at least partially revert to intact single strands under these conditions. Because several lines of evidence suggested that band II DNA (Figure 2) was peculiar to the reactivity of the termini of duplex DNA fragments (see discussion), and thus of low relevance to cross-linking in duplex regions, band II DNA was not studied further.

The nucleotide connectivity of the band III DNA (Figure 2) for N_4 = TCGA and TGCA was established by iron-(II)–EDTA fragmentation analysis. Excision of these gel bands and elution of the DNA afforded insufficient material for iron(II)–EDTA fragmentation. Accordingly, in both cases the DNA eluted from band III was reexposed to Klenow polymerase/[α-^{32}P]dATP, and this product was subjected to iron(II)–EDTA fragmentation. High-resolution denaturing PAGE (Figure 4) afforded in both cases fragments identical in mobility with those derived from native (un-cross-linked) DNA for scissions from the radiolabeled end through G11 (TCGA) or G10 (TGCA). Thus, band III represents in these two DNAs dG-to-dG interstrand cross-links, at the sequences 5′-CG and 5′-GC, respectively. Importantly, band III (Figure 2) is most intense in the three sequences containing 5′-CG (N_4 = TCGA, ACGT, and CCGG) and considerably less intense in those containing 5′-GC or no dG residues (N_4 = TGCA, AGCT, TATA). Thus, cross-linking occurs with a preference for 5′-CG over 5′-GC.

The yield of dG-to-dG cross-linking was approximated for the examples in Figure 2 by Cerenkov counting, comparing an accurately measured aliquot of the crude cross-linking reaction mixture (prior to resoluion of cross-linked and un-cross-linked DNA by electrophoresis) and the excised gel band

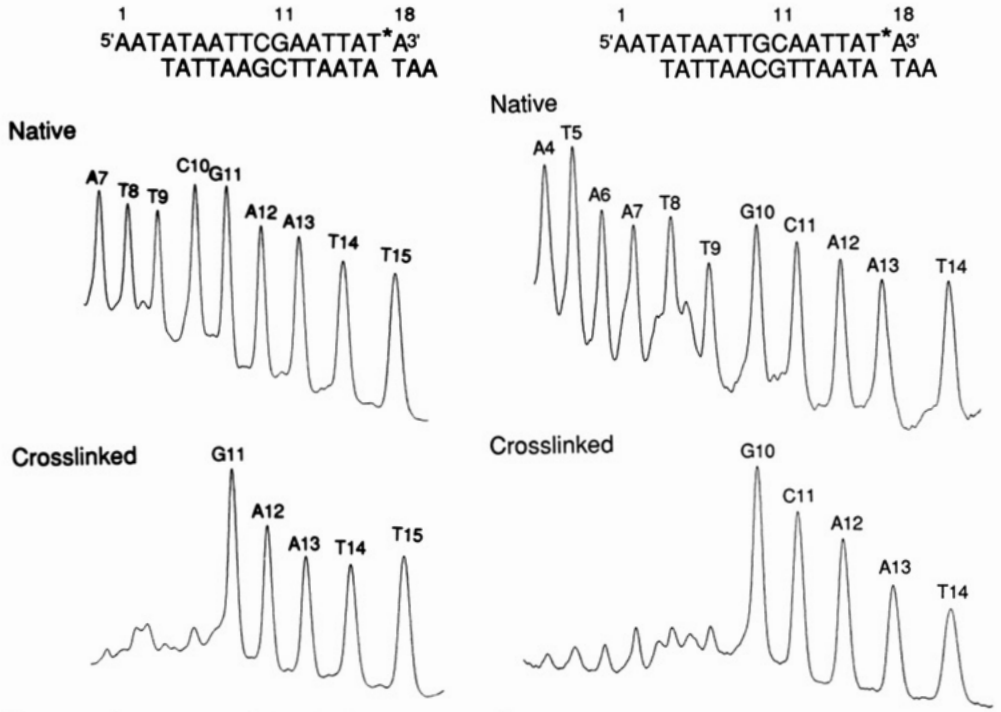


FIGURE 4: Partial fragmentation patterns of 3′-end radiolabeled (* = ^{32}P), native, and DHRA cross-linked DNA (band III), N_4 = TCGA and N_4 = TGCA.

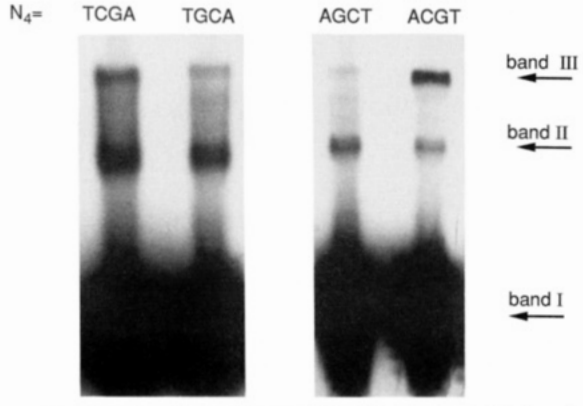


FIGURE 5: Overexposed autoradiographs of denaturing PAGE analysis of BAMP cross-linking reactions of four radiolabeled DNAs, 5′-d-[AATATAAT(N_4)ATTAT], N_4 as indicated above gel lanes.

III (Figure 1). In all cases, the yield of band III cross-linked product was less than 0.2%.

Core Sequence Preference of 2,3-Bis(acetoxymethyl)-1-methylpyrrole (BAMP). The ability of DHRA to distinguish 5′-CG and 5′-GC sequences in its cross-linking reactions prompted us to evaluate the cross-linking sequence preferences of 2,3-bis(acetoxymethyl)-1-methylpyrrole (BAMP). Four end-radiolabeled DNA duplexes (Figure 5) were incubated with BAMP under the conditions described above for DHRA. Denaturing PAGE analysis afforded a distribution of products (Figure 5) similar to those formed by DHRA. It was assumed that band II (Figure 5), by analogy (see above), represented terminally interstrand cross-linked fragments. For the BAMP-cross-linked sequence N4 = TCGA, the nucleotide connectivity of the least mobile band (III, Figure 5) was determined by iron(II)–EDTA fragmentation of samples originally radiolabeled independently at the 3′- and 5′-termini. As was the case with DHRA cross-linked samples, reexposure of the purified band III (Figure 5) DNA to radiolabeling conditions was necessary to obtain sufficient material for iron(II)–EDTA fragmentation analysis. The largest fragment smaller than full-length single strand derived in both cases from cleavage at G11 (Figure 6), indicating that, as in the case of DHRA, the band III DNA was dG-to-dG cross-linked. Again by Cerenkov counting, the yield of band III DNA was found to be less than 0.2%. The preferential formation of band III DNA in 5′-CG-containing sequences (Figure 5) indicated a significant preference of BAMP for cross-linking at that sequence.

Relative Selectivity for 5′-CG versus 5′-GC of Mitomycin C (MC), Dehydroretronecine Diacetate (DHRA), and 2,3-Bis(acetoxymethyl)-1-methylpyrrole (BAMP). Teng et al. (1989) have excised and Cerenkov counted PAGE bands representing cross-linked products from 5′-CG- and 5′-GC-containing DNA fragments treated with reductively activated mitomycin C. From this, a conservative estimate of 10:1 in favor of 5′-CG over 5′-GC can be made. From the present study, excision and Cerenkov counting of gel bands III for N_4 = TCGA versus TGCA indicate a selectivity for the former of only 2:1 to 3:1 for both DHRA and BAMP (data not shown). Similar results were obtained with N_4 = ACGT versus AGCT. A somewhat higher selectivity of ca. 8:1 for 5′-CG over 5′-GC was obtained by one-dimensional densitometry (Figure 7). The difference in these two estimates no doubt reflects the impact of baseline selection, which relegates to background those counts not associated with a distinct peak.

At least for DHRA, the data in Figure 4 suggest that dG-to-dG cross-links can be formed at 5′-GC sequences. To evaluate whether the same is true of reductively activated mitomycin C, we exposed the DNAs shown in Figure 4 (N_4 = TCGA, 3′-end radiolabeled, and TGCA, independently 3′- and 5′-end radiolabeled) to a large excess of reductively activated mitomycin C. As expected, denaturing PAGE analysis revealed a substantial quantity (0.5% yield as measured by Cerenkov counting) of a cross-linked DNA for the former and only traces of radioactivity at the same gel position for the latter. Nevertheless, the gel position anticipated for the N_4 = TGCA dG-to-dG cross-link was excised, eluted, and relabeled with Klenow polymerase/[α-^{32}P]dATP. Denaturing PAGE analysis revealed a diffuse band similar in mobility to the corresponding N_4 = TCGA cross-link. In contrast to the

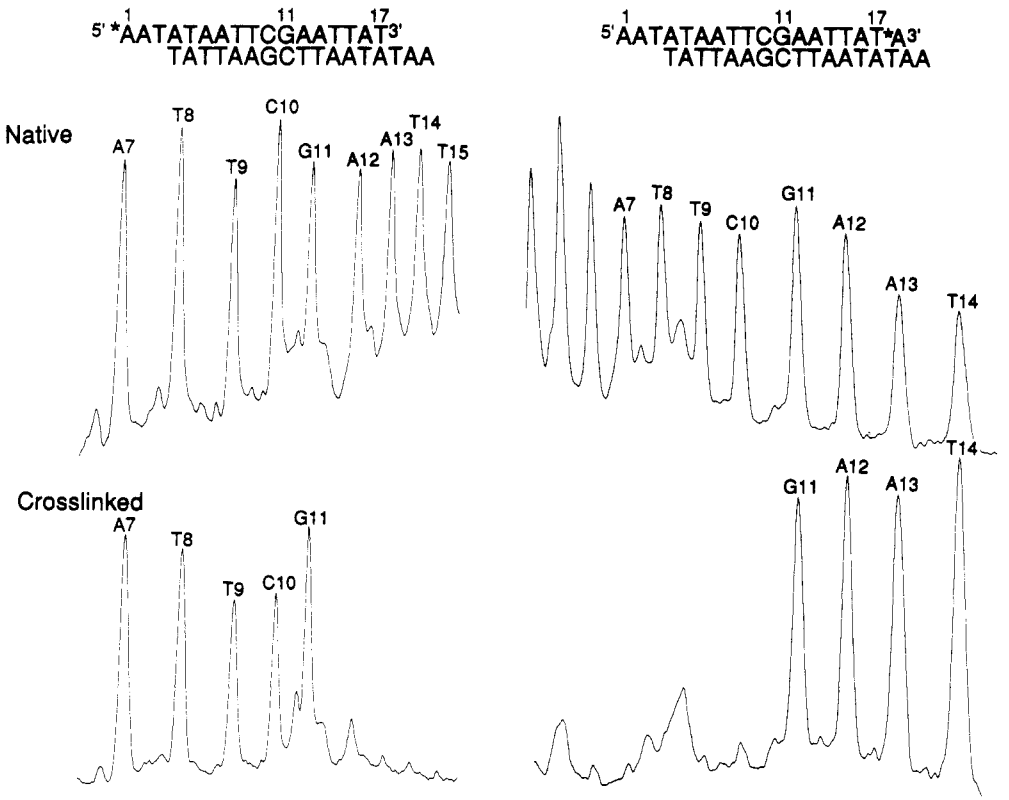


FIGURE 6: Partial fragmentation patterns of 5′-end radiolabeled (* = ^{32}P) and 3′-end radiolabeled (* = ^{32}P) native and BAMP cross-linked DNA (band III), N_4 = TCGA.

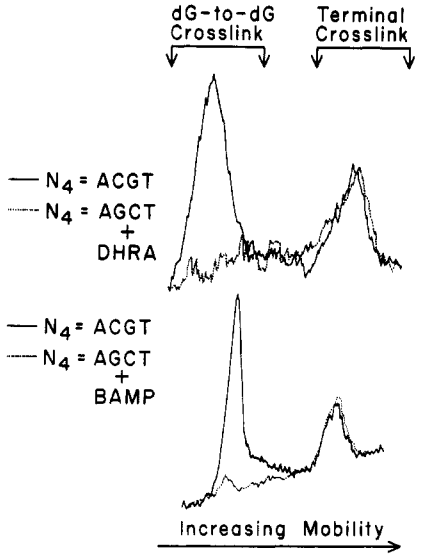


FIGURE 7: Densitometer traces of the cross-linked region of autoradiographs for DHRA and BAMP cross-linking reactions with the DNA oligonucleotides, 5′-d[AATATAAT(N_4)ATTAT], N_4 = ACGT and N_4 = AGCT.

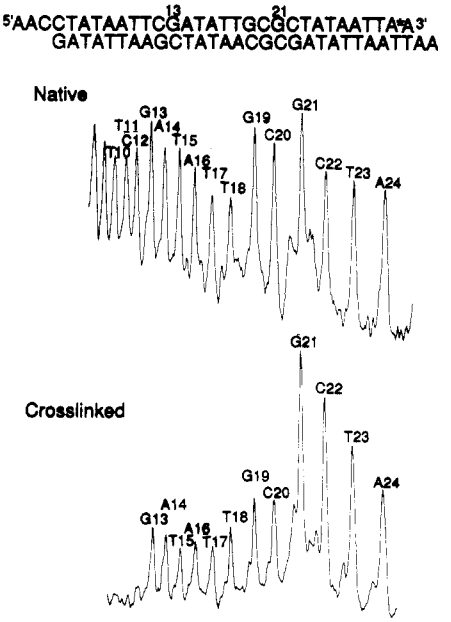


FIGURE 8: Partial fragmentation patterns of 3′-end radiolabeled (* = ^{32}P), native, and BAMP cross-linked DNA.

iron(II)–EDTA fragmentation data in Figure 4, the N_4 = TGCA sample did not reveal a homogeneous linkage pattern and was indicative instead of a heterogeneity of cross-link position possibly involving several residues from A7 to A12 (data not shown). It was concluded that reductively activated mitomycin C can in low yield cross-link sequences other than 5′-CG but that dG-to-dG linkage at 5′-GC is not favorable.

Flanking Sequence Preference in 2,3-Bis(acetoxymethyl)-1-methylpyrrole. With reductively activated mitomycin C, the yield of cross-linked product at 5′-CG sequences is dependent upon flanking sequence (Teng et al., 1989; Borowy-Borowski et al., 1990). We have demonstrated that the sequence 5′-GCGC is cross-linked in 2–3-fold preference over 5′-TCGA when both sequences are in the same DNA fragment (Millard et al., 1990b; J. T. Millard, M. F. Weidner, J. J. Kirchner, S. Ribeiro, and P. B. Hopkins, manuscript in preparation). The impact of flanking sequence on BAMP cross-linking at 5′-CG was studied in this same fragment. The 3′-end radiolabeled DNA duplex shown in Figure 8 was exposed to a large excess of BAMP. As in previous BAMP cross-linking reactions, denaturing PAGE revealed a high mobility, presumably terminally cross-linked product and a low-mobility, cross-linked product. The low-mobility product was excised, eluted from the gel, and subjected to iron(II)–EDTA fragmentation. As shown in Figure 8, relative to native

Table I: Quantitation of Iron(II)-EDTA Cleavage Data from Figure 8

		25					20					15		
cleavage site	3′	T	A	T	C	G	C	G	T	T	A	T	A	G
R^a		100	87	100	113	93	59	54	61	50	48	43	56	43
av				100						52				

[a] R is 100 times the ratio of the yield of a given fragment in cross-linked and native DNA. R values were normalized such that the average of C22–T25 is 100. G13 and G21 were excluded in the normalization due to the presence at this site of the cross-linking agent in an appreciable fraction of the DNA sample.

(un-cross-linked) DNA, the abundance of fragments fell roughly in half following G21 (5′-GCGC sequence) and dropped to baseline following G13 (5′-TCGA sequence). A more rigorous integration of the autoradiogram was achieved by creating a stacked plot of sequential one-dimensional scans, the integrated peak areas of which were summed. Relative to native DNA, the bands A14–C20 were thus shown to fall in intensity by on average 52% (Table I). The sequences 5′-TCGA and 5′-GCGC were thus shown, in this instance, to be of indistinguishable reactivity toward cross-linking by BAMP.

Cross-Linking of DNA Fragments by Dehydromonocrotaline and Dehydroretrorsine. Two-phase cross-linking reactions using the 3′-end radiolabeled oligonucleotides N_4 = TCGA and TGCA and dehydromonocrotaline and dehydroretrorsine were conducted in 50 μM Tris (pH 7.5) and 100 mM NaCl/$CDCl_3$. Isolation of the DNA by precipitation followed by denaturing PAGE revealed a banding pattern similar to that found with DHRA and BAMP (Figure 9). If it is assumed that the low-mobility bands represent dG-to-dG cross-linking, the qualitative preference for 5′-CG over 5′-GC is seen to be retained in the reactions of these substances. Cerenkov counting of cross-linking reactions before and after isolation of the low-mobility cross-link revealed yields of under 0.6%.

Discussion

We have studied the sequence preferences with which several pyrrole-based bifunctional alkylating agents introduce interstrand cross-links into synthetic DNA duplex fragments. These agents were selected on the basis of similarities in structure and reactivity to the reductively activated aziridinomitosene of mitomycin C (MC). Like reductively activated MC, it was found that dehydroretronecine diacetate (DHRA) and 2,3-bis(acetoxymethyl)-1-methylpyrrole (BAMP) introduce dG-to-dG cross-links at the base sequence 5′-CG in duplex DNA. The most striking result of this study was that like MC, both DHRA and BAMP cross-linked 5′-CG in preference to 5′-GC. As discussed in greater detail below, this suggests that 5′-CG recognition does not require the structural and functional complexity of mitomycins and that a fundamentally simple explanation may at least in part account for this selectivity.

Although we have emphasized the similarities of cross-linking reactions of MC, DHRA, and BAMP, there are clear differences among these reactions of these substances. Firstly, in all oligonucleotide duplexes studied, DHRA and BAMP yielded interstrand cross-links not only between dG residues at target 5′-CG or 5′-GC sequences but also within a few residues of the exclusively dA/dT-containing duplex termini. These terminally cross-linked duplexes possessed a higher mobility in denaturing PAGE than the same duplexes cross-linked more centrally. In control experients with MC, which is highly selective for 5′-CG sequences, we demonstrated independently that the mobility of MC/cross-linked DNAs is a function of the position of the cross-link, with lower mobility

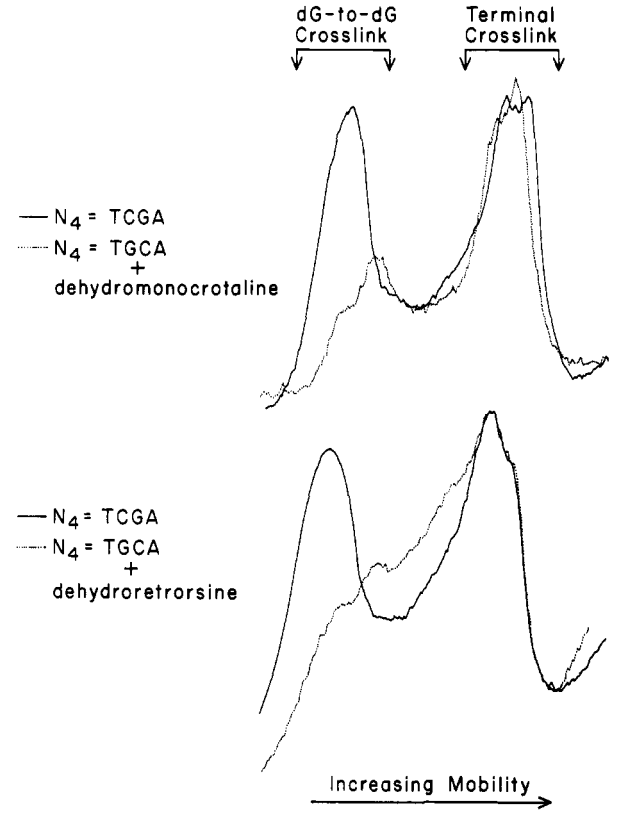


FIGURE 9: Densitometer traces of the cross-linked region of autoradiographs for dehydromonocrotaline and dehydroretrorsine cross-linking reactions with the 3′-end radiolabeled DNA oligonucleotides, 5′-d[AATATAAT(N_4)ATTAT], N_4 = TCGA and N_4 = TGCA.

in denaturing PAGE being correlated with more centrally located cross-links (J. T. Millard, M. F. Weidner, J. J. Kirchner, S. Ribeiro, and P. B. Hopkins, manuscript in preparation). In only one instance did an MC cross-linking reaction afford appreciable quantities of a terminally cross-linked DNA, in that case a DNA duplex containing a single dG-dC base pair at the duplex terminus (not at the sequence 5′-CG). A similar hyperreactivity toward cross-linking of the ends of duplex fragments has also been observed with the mechanistically distinct agent bis(2-chloroethyl)methylamine (nitrogen mustard) (Millard et al., 1990a). From this, we conclude that the reactivity of residues in the termini of duplex DNA fragments is higher than, and thus not representative of, that in the duplex regions. DHRA and BAMP were more reactive toward duplex termini than was reductively activated MC.

A second difference between reductively activated MC and the analogues studied here involves quantitative aspects of cross-linking selectivity. The selectivity of MC for dG-to-dG cross-linking at 5′-CG over 5′-GC was found to be higher for MC than for the pyrrole-based analogues. For MC, we and others have been unable to obtain appreciable quantities, much less a homogeneous sample, of a DNA cross-linked dG-to-dG at 5′-GC. Chawla et al. (1987) have incubated hexanucleotide DNA duplexes, one containing a central 5′-CG and the other

5′-GC, with reductively activated MC. After hydrolytic workup, only the former yielded a diadduct of two deoxyguanosine residues with the aziridinomitosene of MC. We attempted to isolate from denaturing PAGE a DNA of mobility expected for a dG-to-dG cross-link at 5′-GC in a duplex fragment. Analysis of this material by iron(II)–EDTA fragmentation indicated cross-linking in the vicinity of the 5′-GC sequence but heterogeneous with respect to cross-link location. This is in contrast to an analogous experiment in which DHRA yielded a cross-linked DNA duplex with clear dG-to-dG connectivity. Incubation of a 5′-GC containing DNA duplex with BAMP likwise afforded on denaturing PAGE a discernible band of mobility appropriate for a dG-to-dG cross-link. Thus, the 5′-CG versus 5′-GC selectivities of DHRA and BAMP are lower than those of MC. It should be noted that in no case have we established the cross-linking connectivity at the atomic level; the resolution of the iron(II)–EDTA method is the nucleotide level. As such, the dG-to-dG cross-links at 5′-GC sequences could represent some connectivity other than N2 to N2, thus complicating the mechanistic interpretation of the lower 5′-CG versus 5′-GC selectivity relative to MC of DHRA and BAMP.

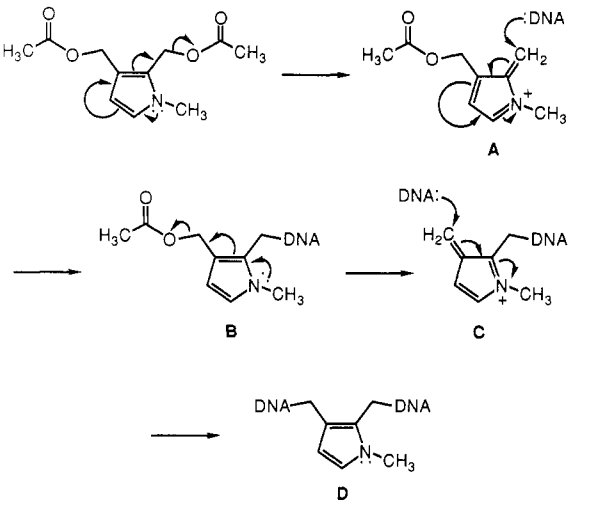
A third difference between reductively activated MC, DHRA, and BAMP is in the yield of cross-linked product obtained at 5′-CG. Under otherwise identical experimental conditions, mitomycin/dithionite gave a 5-fold greater yield of cross-linked dG-to-dG connected product (Cerenkov counting of excised gel bands) in N_4 = TCGA than did DHRA. In all of our experiments, the extent of cross-linking achieved by DHRA and BAMP as measured by Cerenkov counting was comparable. Variables such as the partition coefficient (chloroform versus water) of the relevant cross-linking agents and the instability toward hydrolysis of these substances complicates interpretation of differences in extent of cross-linking.

As might be expected on the basis of the findings with DHRA and BAMP, dehydroretrorsine and dehydromonocrotaline were likewise found to afford more low-mobility, presumably dG-to-dG cross-linked DNA with a 5′-CG than with a 5′-GC containing duplex fragment. Dehydropyrrolizidine alkaloids such as these provide an intriguing potential mechanism for significant flanking sequence recognition: Ionization of one of the ester linkages (presumably at C7) affords a chirality-rich, functionalized side chain, which might recognize minor groove sequence information. As such, the sequence preferences of these agents deserve further study.

We have in one pair of 5′-CG sequences with different flanking sequences, 5′-GCGC and 5′-TCGA, compared cross-linking reactions of MC and BAMP. While reductively activated MC preferred cross-linking of 5′-GCGC over that of 5′-TCGA by a 2:1 margin (Millard et al., 1990b; J. T. Millard, M. F. Weidner, J. J. Kirchner, S. Ribeiro, and P. B. Hopkins, manuscript in preparation), BAMP cross-linked these sequences almost equally well (1:1). This difference could simply reflect a greater chemical reactivity and hence lower selectivity of BAMP or may indicate that some functionality of MC not present in BAMP, such as the quinone or chiral aziridine, is mediating MC recognition. The finding that BAMP, a much smaller molecule than MC, is less discriminating of flanking sequence is also consistent with the suggestion that the wider minor groove of GC-rich DNA is responsible for MC's preference for 5′-CG in a GC-rich flanking sequence (Teng et al., 1989).

We demonstrate here that, like reductively activated MC, several pyrrole-based DNA interstrand cross-linking agents

Scheme I: Possible Mechanism for DNA Interstrand Cross-Linking by BAMP[a]

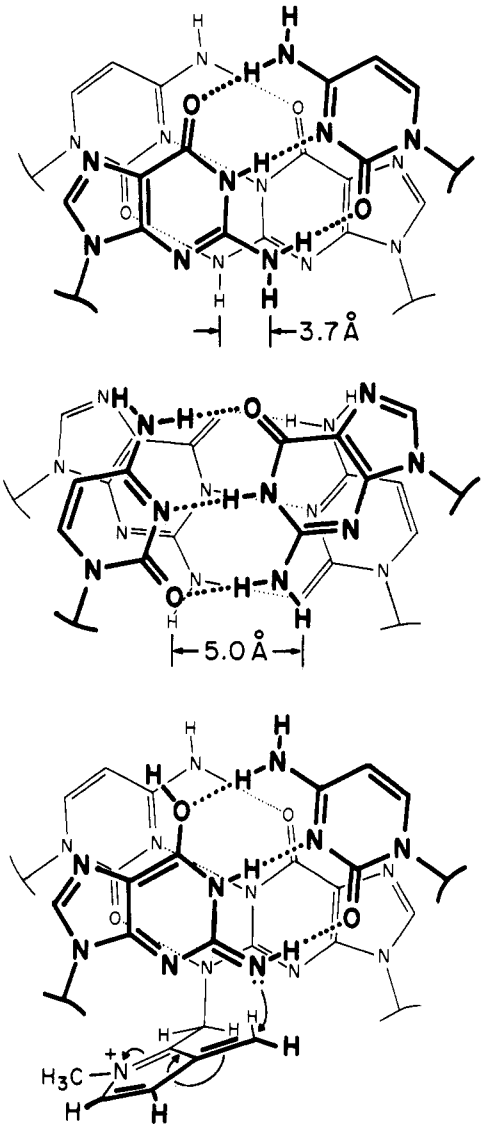


[a] Ordering of ionization steps can be reversed without altering the arguments discussed herein.

afford dG-to-dG cross-links at the DNA duplex sequence 5′-CG. This suggests that, as has been shown for MC, the cross-links of the pyrrole-based agents bridge N2 atoms of dG residues on opposing strands (Tomasz et al., 1987; Norman et al., 1990). The preference for 5′-CG over 5′-GC is then impressive, as both of these sequences possess dG N2 atoms centrally located in the minor groove of B DNA and with nearly equal intertomic spacing (3.6 and 4.0 Å). Analogy to mitomycins suggests a minimal mechanism as shown in Scheme I. Detailed molecular modeling of this reaction sequence is complicated by a lack of information concerning the conformation(s) of DNA involved and the trajectory with which N2 attacks the electrophiles (through the π-electron density or through a σ-lone pair exposed by deprotonation or tautomerization of N2).

Although none of the reaction steps in Scheme I may at this time be eliminated from consideration as sequence preference determining, inspection of space-filling or computer-generated models of putative intermediate C and cross-link D at 5′-CG and 5′-GC steps in B DNA yields a particularly simple explanation for the observed selectivity. Computer or CPK model building studies of N2-to-N2, dG-to-dG BAMP cross-links at these two sequences indicate that BAMP, like the aziridinomitosene of MC (Teng et al., 1989; Millard et al., 1990b), requires much less distortion of B DNA for crosslinking at the sequence 5′-CG than at 5′-GC. This is primarily a consequence of the relative orientation of the bonds that hold the presumably replaced dG N2 hydrogens in these two sequences (Scheme II): In 5′-CG these bonds are roughly parallel, resulting in a hydrogen-to-hydrogen interatomic distance of 3.7 Å (Scheme II, top), a reasonably good match for the 3.2-Å spacing predicted for the spacing of the acetoxy-bearing carbons of BAMP. This alignment is also present in the recently reported, NMR-based structure of an MC cross-linked duplex (Norman et al., 1990). In contrast, the divergent geometry of these same bonds in 5′-GC results in a 5.0-Å hydrogen-to-hydrogen spacing (Scheme II, middle) (an even larger spacing of carbons bound at this position is expected due to the longer N–C bond) that cannot be bridged without appreciable DNA reorganization. This suggests that the transition state that determines the cross-linking sequence specificity may resemble the cross-link itself. This is most easily accommodated in a transition state model involving an ionized monoadduct (C) and an ionized or tautomerized,

Scheme II: Relative Atomic Positions in B DNA at 5′-CG (Upper) and 5′-GC (Middle) Steps and (Lower) a Possible Geometry for the Conversion of an Ionized Monoadduct (C, See Scheme I) to a Cross-Link at 5′-CG



diagonally opposite dG residue reacting through a σ-lone pair on N2. This transition state (Scheme II, bottom) is only subtly different from the cross-linked structure itself and is expected to be favored at 5′-CG over 5′-GC because of the structural reorganization necessary for the latter. This kinetic model for 5′-CG selectivity suggests that monoadducts at 5′-CG are converted to cross-links faster than monoadducts at 5′-GC, an experimentally testable question, which is under investigation.

Acknowledgments

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