Sequence-Specificity for DNA Interstrand Cross-Linking by α,ω-Alkanediol Dimethylsulfonate Esters: Evidence for DNA Distortion by the Initial Monofunctional Lesion

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Abstract: The sequence specificity for DNA cross-linking by a series of α,ω-alkanediol dimethylsulfonate esters ($CH_3SO_2O-(CH_2)_n-OSO_2CH_3$) is described. The results show that bifunctional alkylating agents that produce 5- and 6-carbon interstrand linkages ($n = 5$ and 6) prefer to react at N7-guanine at 5′-GNC sites. When $n = 8$, a more random cross-linking pattern is observed at 5′-GNC and 5′-GC. As previously reported with the nitrogen mustard bis(2-chloroethyl)methylamine (mechlorethamine), the predominant site of cross-linking at 5′-GNC by the $n = 5$ compound is not consistent with the distance between the N7-G sites in B-DNA and the length of the covalent linkage.

Introduction

The equilibrium binding and covalent interactions of carcinogens and antineoplastic agents with double-stranded DNA involve complex and interactive steric and electronic factors. This is due in part to the ability of DNA to adopt different conformations in different macro- and micro-environments. An excellent example of this complexity is the efficient N7-**G**-to-N7-**G** interstrand cross-linking of DNA by the nitrogen mustard *N*,*N*-bis(2-chloroethyl)methylamine (mechlorethamine) (see Figure 1 for structures), at complementary 5′-**G**NC-3′/3′-CN**G**-5′ sequences (N = any nucleotide).[1−5] This cross-link product is highly deformed because the covalent linkage between the two strands is at least 1.4 Å too "short" to accommodate a classical B-DNA structure (Figure 2). Regardless, 5′-GNC is the predominant cross-link site rather than the 5′-GC site predicted from models of B-DNA.[6]

A priori there are two explanations for the unanticipated cross-link sequence specificity of nitrogen mustards: (i) DNA normally adopts nonclassical structures, and the 5′-GNC cross-link is the kinetically favored product; or (ii) the initial monofunctional mustard adduct induces a conformational perturbation in B-DNA that allows the 5′-GNC cross-link to efficiently form. The first explanation is not in accord with NMR and crystal structures of DNA which do not indicate the degree of conformational diversity required for the 5′-GNC cross-link.[7] More importantly, there is no data for a kinetic selection based on the monofunctional alkylation pattern by mechlorethamine.[3] On the other hand, there is circumstantial evidence that the dicationic monofunctional precursor of the interstrand cross-link (Figure 2) can alter DNA structure since 5-(ω-aminoalkyl)-2′-deoxypyrimidines residues, with cationic side chains located in the major groove, bend DNA as measured by gel mobility assays.[8,9] The reaction of nitrogen mustards with DNA results in monofunctional adducts that have both a cationic purine ring and a cationic side chain appendage (Figure 2). To address the role of the cationic purine on the sequence specificity of DNA interstrand cross-linking agents, we initiated a detailed study using α,ω-alkanediol dimethylsulfonate esters, $CH_3SO_2O-(CH_2)_n-OSO_2CH_3$ ($n = 4$, 5, 6, and 8). These compounds, some of which have been clinically used to treat acute myeloid leukemia,[10,11] cross-link DNA via monofunctional cationic N7-alkylguanine adducts, but do not have cationic side chains.

Experimental Section

Mechlorethamine and 1,4-butanediol dimethylsulfonate ester were purchased (Aldrich Chemicals, St. Louis, MO). The remaining α,ω-alkanediol dimethylsulfonate esters were synthesized using previously described methods.[12] The oligodeoxynucleotides (ODNs) were synthesized using standard phosphoramidite chemistry and purified by reverse phase cartridge and demonstrated to be homogeneous by polyacrylamide electrophoresis (PAGE). ODN-1 (50 nmol) was dissolved in water and incubated with 50 units T4 kinase (New England Biolabs, Beverly, MA) in the presence of [^{32}P]-γ-ATP (10 pmol, specific activity 6000 Ci/mmol, Amersham, Arlington Heights, IL) at 37 °C for 30 min. Labeled ODN-1 was then purified using 20% PAGE (19:1, acrylamide: bisacrylamide). The purified ODN-1 was eluted off the gel and then relabeled with T4 kinase in the presence of γ-ATP (90 pmol, specific activity 6000 Ci/mmol) as described above and purified using a MicroSpin G-25 column (Pharmacia Biotech, Piscataway, NJ).

Molecular modeling was done using SYBYL software (Tripos Associates, St. Louis, MO) and structures imported from the Protein Database (http://www.rcsb.org/). Using the distance monitoring function, the interstand distances between N7-G sites was measured.

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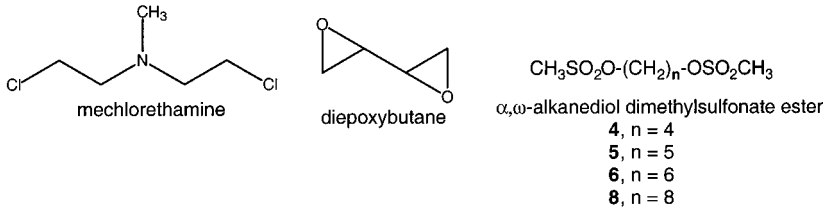


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                                        111213   151617
ODN-1 = 5′-d(A-A-C-C-T-A-T-A-T-T-G-G-G-C-G-G-G-A-T-T-A-A)-3′
ODN-2 =       3′-d(G-A-T-A-T-A-A-C-C-C-G-C-C-C-T-A-A-T-T-A-A)-5′
ODN-3 =       3′-d(G-A-T-A-T-A-A-C-C-C-D-C-C-C-T-A-A-T-T-A-A)-5′
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Figure 1. Structure of compounds and ODNs (**D** in ODN-*3* is 7-deazaguanine).

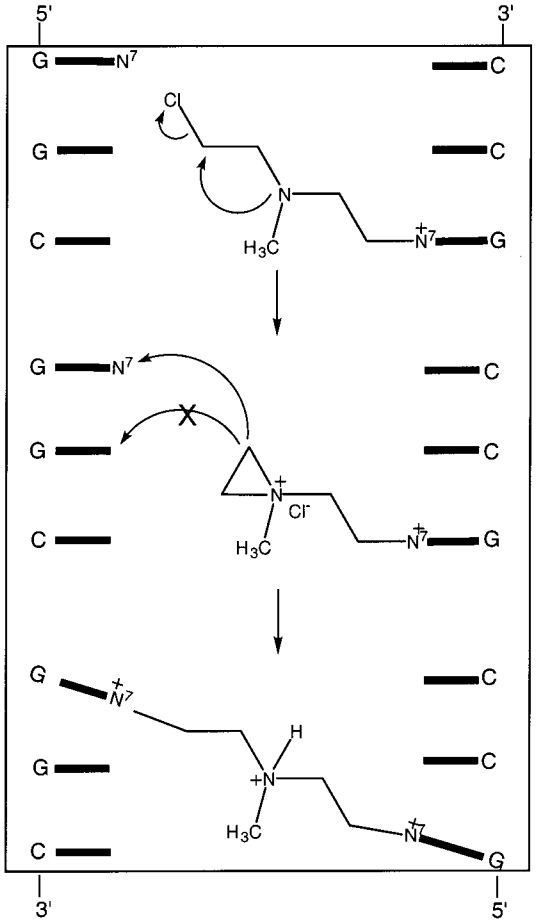

Figure 2. Scheme for cross-linking and perturbation of DNA by mechlorethamine.

Interstrand Cross-Linking. 5′-[^{32}P]ODN-1 (30 μM) was annealed with excess of ODN-*2* (90 μM) or ODN-*3* (90 μM) in 40 mM sodium cacodylate buffer (pH 8.0). The duplex was incubated with mechlorethamine (40 μM in DMSO) at 37 °C for 3 h. In the case of α,ω-alkanediol dimethylsulfonate esters **4**−**6** and **8**, ODNs were annealed in a solution of 100 mM triethanolamine (pH 8.0) containing 1 mM EDTA. The duplex was incubated with 20 mM sulfonate ester in DMSO at 37 °C for various times. At the end of the incubation period, the DNA was precipitated with NaOAc (0.3 M) in 75% EtOH and washed with cold EtOH.

Isolation of Cross-Linked and Monoalkylated Products. The precipitated DNA was resuspended in 5 M urea and run on a 20% denaturing PAGE (19:1 acrylamide/bisacrylamide, 7.8 M urea, 55 W, 1.5 h). The bands corresponding to cross-linked and monofunctionally adducted DNA were then eluted off the gel.[13] After precipitation, each DNA sample was subjected to either: (i) neutral thermal hydrolysis (90 °C for 15 min) followed by heating in 10% piperidine (90 °C for 30 min) or (ii) 10% hot piperidine (90 °C for 30 min). Each sample was resuspended in formamide and heat denatured at 90 °C for 3 min before electrophoresis on 20% denaturing PAGE. Maxam−Gilbert G and G + A lanes were included as reference markers.[14] The location and quantitation of the bands on the gels was visualized using a Molecular Dynamics Phosphorimager (Sunnyvale, CA).

Results

The procedures and DNA targets used in this study of the α,ω-alkanediol dimethylsulfonate esters are based on those previously described to determine the sequence specificity for mechlorethamine cross-linking.[3] Mechlorethamine was included as a "positive" control, and compared to the cross-linking sulfonate esters with 4-, 5-, 6-, and 8-carbon linkers (Figure 1). As reported,[3] the mustard mechlorethamine efficiently cross-linked DNA at a 40 μM dose with a 3 h incubation (Figure 3). The second band in Figure 3 is attributed to undenatured duplex since the DNA samples were not heat denatured prior to loading on the gel in order to minimize destruction of the cross-linked lesions. The preferred site for cross-linking is at N7-G in a 5′-GNC sequence (Figure 4, Table 1). The preference for 5′-GNC is approximately 6-fold relative to 5′-GC (Table 1). To obtain significant (≥2%) cross-linking with the α,ω-alkanediol dimethylsulfonate esters, concentrations of 20 mM were used along with incubation times of up to 30 h. There was a time course and dose response for cross-linking, but the 30 h incubation period allowed us to use sufficiently low doses of the sulfonate esters to maintain an aqueous buffered reaction. In all cases denaturing PAGE was used to separate the cross-linked DNA from unmodified DNA and DNA with monofunctional and/or intrastrand cross-links. To enhance the specific activity of the cross-linked DNA, we adopted a two-step labeling procedure. ODN-1 was end-labeled as usual and then purified on a gel. The DNA was eluted off the gel and then relabeled with γ-[^{32}P]-ATP using T4 kinase and purified on a size exclusion column. The result is DNA that has >3-fold increase in specific activity. This enhanced specific activity significantly facilitates the number of experiments that can be performed since the cross-linked oligomers are generated in low yield (Figure 3).

After the DNA was isolated, it was treated directly with hot piperidine or subjected to neutral thermal hydrolysis followed by hot piperidine to expose the sites of cross-linking. The former procedure specifically generates strand breaks at N7-G,[14] while the latter gives cleavage at all thermally labile *N*-alkylpurine lesions.[15] Under the same conditions, the relative cross-linking efficiency of sulfonate esters **6**, **5**, **8,** and **4** is 22:6:1:not detected, respectively (Figure 3). The failure to observe cross-linking with

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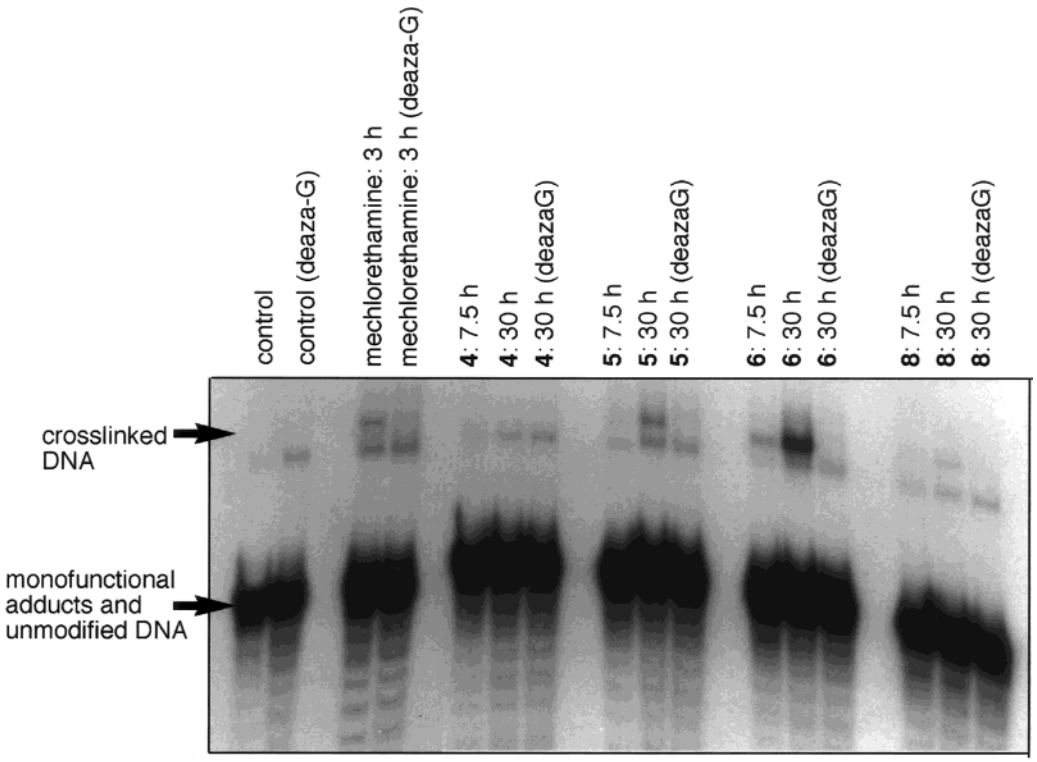

Figure 3. Autoradiogram of denaturing PAGE analysis of the reaction of 40 μM mechlorethamine (3 h incubation) and 20 mM α,ω-alkanediol dimethylsulfonate esters with 5′-[^{32}P]-ODN-1+2 (7.5 and 30 h incubation) and 5′-[^{32}P]-ODN-1+3 (30 h incubation).

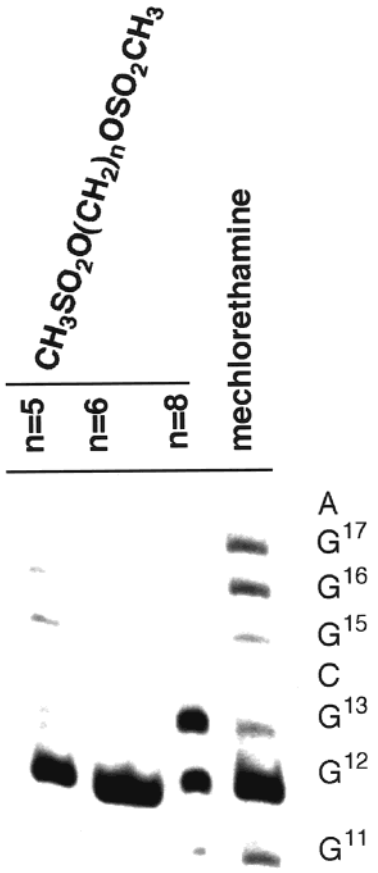

Figure 4. Sequence specificity for 40 μM mechlorethamine and 20 mM α,ω-alkanediol dimethylsulfonate esters induced interstrand DNA cross-links.

4 is consistent with a previous study using linearized pBR322 DNA as a target.[16] The cross-linking by **5**, **6**, and **8** occurred at N7-G as shown by Maxam−Gilbert G-lane chemistry, i.e., direct treatment with piperidine (Figure 4). The same sites were observed using neutral thermal hydrolysis followed by piperidine. Additional evidence for the involvement of N7-G sites in the cross-linking reaction was obtained by exchanging G[33] in ODN-2 with a 7-deazaG residue (ODN-3) (Figure 1). This substitution inhibited the formation of the low mobility bands in the PAGE (Figure 3). Compounds **5** and **6** both selectively cross-linked DNA at the 5′-GNC sequence (Figure 4). Interestingly, **8** with the longer 8-carbon linker reproducibily afforded almost equal amounts of cross-linking at 5′-GNC and 5′-GC, with only a trace of cleavage at 5′-GNNC (Figure 4, Table 1). Thus, the compound with the longer linker gives more of the "shorter" cross-link product.

Table 1. Relative Sequence Specificity for DNA Cross-Linking and Monofunctional Adduction by Mechlorethamine and Sulfonate Esters **4**, **5**, **6**, and **8**

	relative band intensity					
	cross-link			monofunctional		
compd	G-11	G-12	G-13	G-11	G-12	G-13
mechlorethamine	1.5 ± 0.1	6.4 ± 1.5	1.0 ± 0.0	1.0 ± 0.0	1.5 ± 0.2	1.4 ± 0.2
4	n.d.	n.d.	n.d.	1.0 ± 0.0	1.3 ± 0.1	1.5 ± 0.3
5	1.1 ± 0.1	10.7 ± 3.4	1.1 ± 0.1	1.6 ± 0.6	1.5 ± 0.0	1.6 ± 0.6
6	1.0 ± 0.0	28.1 ± 9.4	2.5 ± 0.3	1.4 ± 0.4	1.5 ± 0.1	1.4 ± 0.4
8	1.0 ± 0.0	5.9 ± 4.3	5.6 ± 3.5	1.0 ± 0.0	1.6 ± 0.4	2.4 ± 1.3

It was reported that exposure of linearized pBR322 plasmid DNA to compounds **6** and **8** did not afford detectable monofunctional adducts using G-lane chemistry.[17] Since these compounds did generate cross-links, it was suggested that sites of covalent modification were not at N7-G.[17] Our results with ODN-1 + 2 clearly show that monofunctional adducts are generated and are responsible for most of the cross-linking (Figure 5).

To determine if the preferred cross-link site reflects sequence selective formation of monofunctional adduct, the monofunc-

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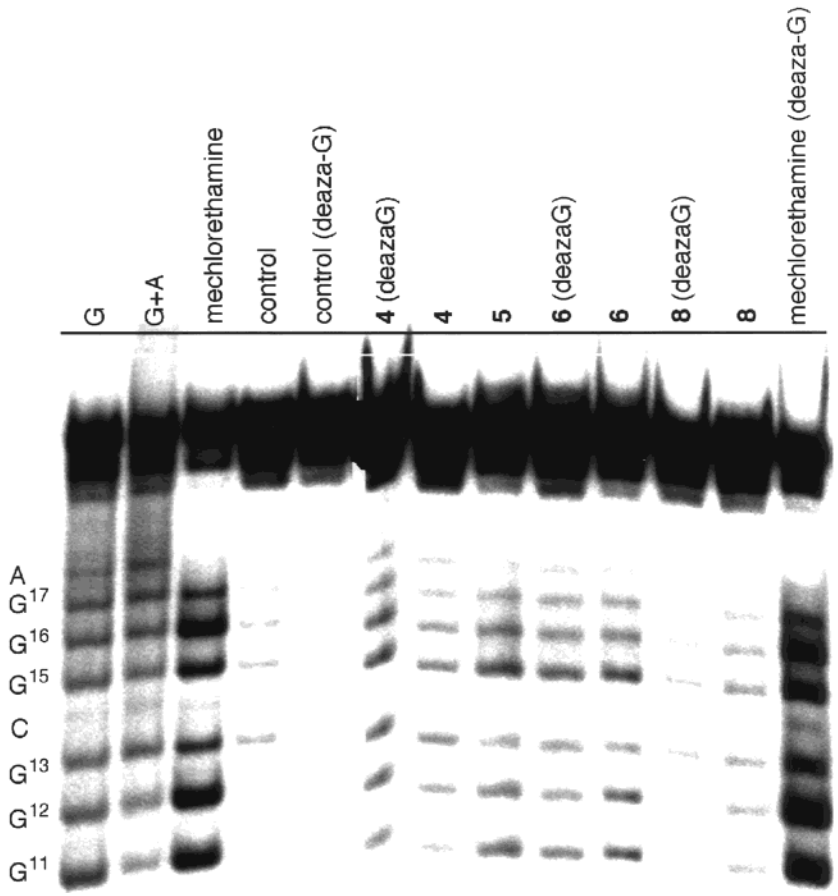

Figure 5. Sequence specificity for 40 μM mechlorethamine (3 h incubation) and 20 mM α,ω-alkanediol dimethylsulfonate esters (30 h incubation) induced monofunctional lesions in 5′-[^{32}P]1+2 and 5′-[^{32}P]1+3 containing an N7-deazaguanine residue (Figure 1).

tional lesions were also quantitated (Figure 5, Table 1). The results indicate that the preference for monofunctional and cross-link modifications do not overlap. The difference between the most and least reactive site for monofunctional alkylation by **5** and **6** in the G$^{11-13}$ run in ODN-1 is approximately 2-fold (Table 1). In contrast, the cross-linking specificity is 10-fold higher for the 5′-G^{12}NC relative to the 5′-G^{13}C site (Table 1). The deazaguanine substitution in ODN-3 does not effect monofunctional alkylation in ODN-1 (Figure 5).

Discussion

It has been known for some time that the cytotoxicity of nitrogen mustards results from their ability to form interstrand N7-G-$(CH_2)_2-(NR)-(CH_2)_2$-N7-G cross-links.[18] The toxicity of α,ω-alkanediol dimethylsulfonate esters has also been correlated to the formation of cross-links, with the 6−8 carbon linkers being the best in vitro cross-linking agents and the most cytotoxic.[19]

The initial step in the cross-linking process for mechlorethamine is the formation of a monofunctional N7-G adduct (Figure 2). This monofunctional adduct can then react with another N7-G site, if one is available, to afford a cross-link, or undergo solvolysis to yield a monofunctional lesion. The first and second covalent steps in the cross-linking process are assumed to involve aziridinium ion intermediates.[20] Based on the length of the $-CH_2-CH_2-NR-CH_2-CH_2-$ linkage, which is ∼7.5 Å, it was assumed for many years that the cross-link formed between N7-G's at a 5′-**G**C dinucleotide (Figure 2).[6] Molecular modeling of B-DNA shows that this would afford the most logical product since the cross-link would not perturb the B-DNA structure. However, due to the independent work of the Loechler[1,2] and Hopkins[3−5] groups, it was determined that the predominant site of interstrand cross-linking is between N7-G's at 5′-**G**NC. This result was quite unanticipated because the 5′-GNC cross-link requires a significant distortion of B-DNA: the distance between the N7-G sites at 5′-GNC is ∼8.9 Å.[7] Thus, the cross-link is ∼1.4 Å too short to bridge the atoms in a classical B-DNA structure.[3−5] When one considers that the second step in cross-link formation involves an aziridinium ion, the disparity in the distance is actually even greater. The sulfonate esters, which react with DNA via an S_N2 pathway,[21] show the same type of distance discrepancy. The 5-carbon linkage in **5** is also ∼1.4 Å shorter than what would be required in a B-DNA structure. In fact, gel mobility studies show that the mechlorethamine cross-linked DNA product is bent.[5] The magnitude of the bend may be as low as 4° or as large as 28° depending on the direction of the bend, which was not determined.[5] Since it is not possible to stretch the bonds in the linker, *the DNA must be distorted prior to the closure of the second linkage* so that the two eventual sites of alkylation are close enough in the transition state to efficiently form the cross-link. In the case of mechlorethamine it is likely that the cationic monofunctional adduct is involved in generating the required distortion, since DNA substituted with cationic side chains, i.e., 5-(3-aminopropyl)-2-deoxyuridine, is bent by approximately 8°.[8,9] On the basis of the cross-linking preference of **5**, it appears

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that the formation of a charged purine can also contribute to DNA distortion that is necessary to allow the 5′-GNC cross-link to form. The span of the linkage in **6** (8.8 Å) is close to the N7-G-to-N7-G interstrand distance of the 5′-GNC cross-link, and this compound is more efficient than the other α,ω-alkanediol dimethylsulfonate esters tested in cross-linking at 5′-GNC based on this and a previous study.[19]

The formation of the 5′-GNC cross-link with **5** is not because G^{12} in the G_3 run in ODN-1 is more reactive to the sulfonate ester since compounds **5** and **6** show little sequence selectivity in terms of monofunctional alkylation at the G^{11-13} stretch. A similar outcome has been reported for their reaction with linearized pBR322.[17] In addition, **8** actually gives almost equal amounts of cross-linking at $G^{13}$and G^{12}, indicating that G's other than G^{12} can form cross-links with G^{33} in the complementary strand. In any event, the difference in the yields of cross-linked products from **5** and **6**, which is approximately 4-fold lower for **5** than for **6**, must originate from the relative rates for cross-link closure and/or hydrolysis of the monofunctional lesion to the alcohol.

The cross-linking preference for 5′-GNC originally observed for mechlorethamine and now for **5** has also been reported for cross-linking by 1,2,3,4-diepoxybutane.[22] The results with diepoxybutane are quite striking in that a 4-atom linker with a 4 Å length is able to bridge the ~8.9 Å distance required for a 5′-GNC cross-link. It should be noted that the efficiency for cross-linking by the diepoxide is relatively low: 250 mM concentration and pH 5.0 buffer are required. This concentration is 3 orders of magnitude higher than that required for mechlorethamine, and acidic pH is needed to accelerate the normally sluggish reaction of the epoxides. Despite the short 4-carbon length of the diepoxybutane linker, the anticipated 5′-GC site is only a minor cross-linking product. Moreover, cross-linking by 1,2,5,6-diepoxyhexane and 1,2,7,8-diepoxyoctane has also been reported.[23] As with diepoxybutane, both the hexyl and octyl compounds showed a preference for cross-linking at 5′-GNC, although 5′-GNNC was also a significant target. The efficiency for cross-linking followed the order: butyl > octyl > hexyl, and none of the compounds showed any sequence specificity in terms of monofunctional lesions. The relationship between interstrand cross-linking yields and linker length for the series of diepoxide alkanes is reversed for the sulfonate esters. Compound **6** is most efficient at cross-linking DNA, and no cross-linking is observed with **4**. Still, it is puzzling that neither **4** nor 1,2,3,4-diepoxybutane forms any N7-G cross-links across the 5′-GC sequence. Contrary to what would be expected, **8** with the longer linker is approximately 6-fold less efficient than **5** at cross-linking DNA, and the 5′-GC and 5′-GNC sites are involved in equal amounts. Little cross-linking at the more distal G^{11} (Figure 1) at the 5′-GNNC site is observed. We assume that the lipophilicity of **8** accounts for the ineffective monofunctional (Figure 5) and cross-link formation (Figure 3). Why the longer linker affords more of the shorter cross-link product is unclear. In this regard the diepoxides and disulfonate esters are also quite different as diepoxyoctane shows a relative preference for 5′-GNC:5′-GNNC:5′-GC of 3:2:1.[22,23] The diepoxides and disulfonate esters share a common characteristic in that the monofunctional adducts generated from both compounds must cause a significant distortion of DNA. The difference in the relationship between cross-linking specificity for the two classes of compounds may be attributed to the nature of the tethers (alkane vs hydroxyalkane), and the electrophilic reacting groups (sulfonate ester vs epoxide), which could affect the local conformation of the DNA and/or the nonbonded association of the linkage with the DNA.

The common unanticipated cross-linking sites for a variety of agents, i.e., nitrogen mustards, epoxides, and α,ω-alkanediol dimethylsulfonate esters, suggests that a common mechanism is at play. One important factor that determines the sites involved in the cross-link attachment is the orientation of the monofunctional N7-G lesion. This adduct is predicted to point in the 3′-direction (Figure 2) due to unfavorable steric interactions between the side chain and the 5′-residue,[24] and ensures that cross-linking in duplex-1+2, which must involve G^{33} in ODN-2, is at G^{11-13} and not G^{15-17} in ODN-1. To explain the unanticipated preference for reaction at G^{12}, we propose that the formation of the cationic N7-G monofunctional adduct, which is common to the different classes of compounds, causes local distortion in the DNA, i.e., a static kink or bend, or anisotropic flexibility.

Little is known about the effect of N7-alkylguanine adducts on DNA structure or stability. Ezaz-Nikpay and Verdine introduced an N7-methylguanine lesion into a self-complementary Dickerson dodecamer using a gap-filling approach.[25] Thermodynamic studies show that the N7-methylguanine causes a 4.5 °C decrease in T_M, although at room temperature the stability of the N7-methylguanine modified and unmodified DNA are the same: $\Delta G°$ for the two oligomers are identical. While $\Delta G°$ is unchanged, the enthalpic and entropic contributions to duplex stability are very different. The introduction of the methyl group and the associated positive charge on the guanine causes a 7.7 kcal/mol decrease in $\Delta H°$ and a 7.5 kcal/mol ($T = 298$ °K) decrease in $T\Delta S°$. The decrease in entropy is attributed to a disruption of base stacking interactions and/or a decrease in solvent stabilization.[25] The decrease in $\Delta H°$ is interpreted to mean that the N7-methylguanine:C base pair is more stable than a normal Watson−Crick G:C pair.

It has been proposed, based on molecular modeling calculations, that the cross-linking specificity of nitrogen mustards at 5′-GNC could involve a noncovalent association between the drug and DNA.[26] These studies do not address why the 5′-GC cross-link does not form to any appreciable extent. Moreover, it is unlikely that DNA equilibrium binding complexes involving sulfonate esters and cationic nitrogen mustards will have much in common.

In summary, we have demonstrated that the cross-linking of DNA by α,ω-alkanediol dimethylsulfonate ester **5** follows the same general pattern seen with other cross-linking agents, i.e., mechlorethamine and diepoxybutane, that react via N7-G alkylation. The final interstrand cross-linked structures are not consistent with canonical B-DNA, and the transition state requires a significant distortion of the DNA. We propose that the sequence specificity involves the local disruption of DNA structure by the initial monofunctional cationic N7-alkylguanine lesion prior to closure of the interstrand linkage in the second covalent reaction. Additional structural characterization of an N7-alkylguanine lesion is planned.

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