Diepoxybutane Cross-Links DNA at 5′-GNC Sequences†

Julie T. Millard* and Maude M. White

Department of Chemistry, Colby College, Waterville, Maine 04901

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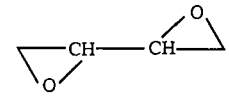
ABSTRACT: Epoxides are cancer-causing agents chemically analogous to the nitrogen mustards, a family of powerful antitumor drugs. We found that the DNA interstrand cross-linking sequence preference of diepoxybutane is the same as that of the mustard mechlorethamine: 5′-GNC. Therefore, the genomic site of cross-linking alone cannot explain why some interstrand cross-linkers act as antitumor agents whereas others are deadly toxins.

Biological effects of epoxides include the production of DNA point mutations and deletions, chromosomal aberrations, and induction of cancer (Ehrenberg & Hussain, 1981). Mutagenic epoxides are used as pesticides and sterilizing agents for food and medical equipment and are found naturally as mold contaminants and as metabolic breakdown products during detoxification in mammalian tissue. The widespread exposure of the public to these lethal compounds warrants a detailed study of their biochemical mode of action.

Diepoxybutane (**1**, DEB) was the first epoxide reported to be carcinogenic (Hendry et al., 1951). In 1967 Lawley and Brookes proposed that DEB acts as a chemical cross-linker in a manner similar to nitrogen mustards such as mechlorethamine (**2**, *N*-methylbis(2-chloroethyl)amine). Nitrogen mustards, the first clinically useful antitumor drugs (Haskell, 1985), difunctionally alkylate the N7 position of deoxyguanosine residues (Brookes & Lawley, 1960; 1961a; 1961b). Isolation of the conjugate **3** from DEB-treated DNA hydrosylates suggested that diepoxides also alkylate the N7 position of guanine residues on opposite strands of DNA (Lawley & Brookes, 1967). A major structural difference between DEB and mechlorethamine is the length of their main alkyl chains: DEB's four-carbon atom chain is shorter than that of mechlorethamine by one nitrogen atom. This difference could be crucial in determining the DNA sequence cross-linked: a single nitrogen mustard can span only 7.5 Å, yet the minimal N7-to-N7 distance is 7.7 Å, found in the sequence 5′-GpC (Brookes & Lawley, 1961a). It has been questioned whether DNA's macromolecular structure is flexible enough to accommodate a DEB cross-link with its even shorter alkyl chain (Lawley & Brookes, 1967). However, a recent study has shown that mechlorethamine does not in fact preferentially cross-link adjacent deoxyguanosine residues at all, but rather cross-links duplex DNA fragments through distal deoxyguanosine residues at the sequence 5′-GNC (N = G or C) in preference to 5′-GC or 5′-CG sequences (Millard et al., 1990a). Mustard's ability to span this distance of about 8.9 Å provides evidence for considerable conformational flexibility of DNA, which may well permit DEB to cross-link an unexpectedly long distance as well.

We have examined the sequence preferences of diepoxybutane's interstrand cross-linking on duplex DNA fragments of defined sequence in order to compare its mode of action to that of the nitrogen mustards. Despite their similar structural chemistry, epoxides and mustards have vastly different biological effects. Comparison of the DNA sequence preferences of these compounds may reveal whether the genomic site of cross-linking accounts for such agents acting as antitumor drugs or as deadly toxins.

Chart I



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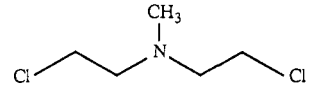

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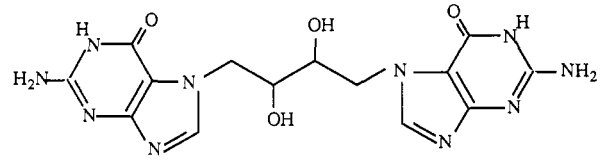

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MATERIALS AND METHODS

Preparation of Radiolabeled DNA Duplexes. Oligonucleotides were purchased from Operon Technologies, Inc. and purified through denaturing polyacrylamide gel electrophoresis (PAGE; 20%, 25:1 acrylamide/bisacrylamide, 40% urea, 1.5 mm thick, 17 × 15 cm). DNA-containing gel slices (as visible through UV shadowing) were incubated in TE buffer (10 mM Tris Cl, 1 mM EDTA, pH 7.6) overnight, followed by passage of the eluant through a Sep-Pak C_{18} cartridge [Waters; elution with (1) 10 mM aqueous NH_4OAc, (2) 25% (v/v) acetonitrile/water] and lyophilization. Radioisotopic labeling of 3′-ends was with [α^{32}P]dATP/Klenow fragment, and 5′-ends were labeled with [γ-^{32}P]ATP/T4 polynucleotide kinase under standard conditions (Sambrook et al., 1989). In all cases, labeling was followed by ethanol precipitation with 0.3 M NaOAc. Self-complementary duplexes are illustrated below as having a single radiolabeled strand for convenience only. Radiolabeling of one, both, or a mixture of one and both strands would not affect the interpretation of the data.

Diepoxybutane Cross-Linking. 0.5 OD_{260} radiolabeled duplex DNA (0.4 mM) was incubated in 0.3 mM sodium acetate (pH 5) and 250 mM DEB (100 μL total volume) for 2 h at 37 °C. We found these conditions to maximize the

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* Author to whom correspondence should be addressed.

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efficiency of the cross-linking reaction, possibly due to acid-catalyzed ring opening of the epoxide. Incubation was followed by ethanol precipitation and lyophilization. To calculate cross-linking efficiencies, the resulting pellet was dissolved in 100 μL of purified water, and a 5-μL aliquot was removed for Cerenkov counting to determine total cpm in the reaction. The remaining sample was lyophilized for denaturing polyacrylamide gel electrophoresis (PAGE).

Mitomycin C Cross-Linking. Mitomycin C-cross-linked DNAs were prepared as previously described (Millard et al., 1990b; 1991). Mitomycin C (MC) was added to 1 OD_{260} radiolabeled duplex DNA (0.7 mM) in 15 mM Tris (pH 7.5) to achieve a 1.8 mM drug concentration (110 μL total volume). Samples were incubated at 37 °C for 1 h, deaerated by purging with argon for 15 min, and put on ice. The MC was activated through three subsequent additions at 15-min intervals of 1 equiv of fresh sodium dithionite (33 mM stock solution in deoxygenated water; final concentration 5.4 mM). Samples were then ethanol precipitated and subjected to denaturing 20% PAGE.

Denaturing Polyacrylamide Gel Electrophoresis. Cross-linked samples were resuspended in 5 M aqueous urea and loaded onto a 20% polyacrylamide gel (19:1 acrylamide/bisacrylamide, 50% urea, 0.35 mm thick, 41 × 37 cm) run on a Hoefer thermojacketed Poker Face gel stand at ca. 65 W and 65 °C. Autoradiography was used to visualize the single-stranded and cross-linked DNA of roughly half the mobility of the corresponding single strands. Centrally cross-linked DNA was located by comparison to an MC-cross-linked DNA standard. Gel slices of approximately equal dimensions from the appropriate region of the gel (with respect to the MC standard) were excised and Cerenkov counted. The percentage of cross-linked DNA was determined from the ratio of counts in the gel slices to the total counts in the reaction. Experiments were repeated at least five times and standard deviations calculated as reported.

DNA Cleavage Reactions. Cross-linked DNA was excised and eluted from denaturing gels run as described above. DNA was heated at 90 °C in aqueous piperidine to cleave at sites of N7 alkylation (Maxam & Gilbert, 1980). The resulting fragments were purified to single-base resolution through 25% denaturing PAGE run as above. Following autoradiography, bands were assigned by reference to a Maxam–Gilbert guanine-specific sequencing reaction (Maxam & Gilbert, 1980) on radiolabeled native duplex DNA. The gel was dried (Hoefer Drygel Sr.) onto Whatman 3MM paper. Densitometry (Hoefer GS-300, interfaced to a Macintosh II computer) data were smoothed, plotted, and integrated (Hoefer Scientific GS370 Densitometry Program, Version 2.0) to obtain plots of cleavage intensity for each nucleotide.

RESULTS

Self-complementary, 5′-^{32}P-end-labeled oligonucleotides, differing only in their four central bases, were cross-linked with diepoxybutane at pH 5.0. One apparent difference between DEB and mechlorethamine is the relative concentrations of the two required for significant cross-link production. Mechlorethamine at 0.25–2.5 mM results in ca. 1% interstrand cross-linked DNA (Millard et al., 1990a), whereas 250 mM DEB was needed to achieve significant cross-linking in these studies. Using 2.5–25 mM DEB resulted in the same pattern of cross-linked products but a lower amount of all products (data not shown). It has previously been noted that on a concentration–time basis DEB is a much less efficient cross-linker than nitrogen mustard (Verly & Brakier, 1969).

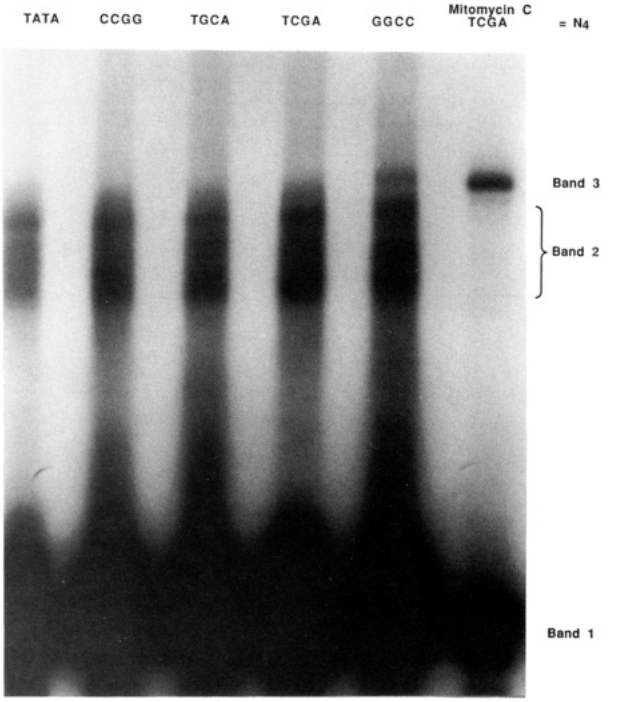

FIGURE 1: Overexposed autoradiogram of denaturing PAGE analysis of DEB-cross-linked reactions of five 5′-end-radiolabeled DNAs: 5′-d[AATATAT(N_4)ATAT], N_4 as indicated above gel lanes. The right lane is a mitomycin reaction with the duplex of core TCGA for comparison.

DEB-treated DNA was isolated by precipitation and analyzed by 20% denaturing PAGE. The resulting autoradiogram, which also includes a mitomycin C-cross-linking reaction for comparison, is shown in Figure 1. In addition to recovering single strands and presumably monoadducts (band 1), a variety of less mobile interstrand cross-linked products were also formed in the DEB reaction (band 2). Diffuse bands such as these are also found for mechlorethamine reactions and are indicative of less sequence-specific agents such as bis(acetoxymethyl)pyrroles (Weidner et al., 1990) and nitrous acid (Kirchner & Hopkins, 1991). Highly sequence-specific cross-linking agents such as mitomycin C (as shown in Figure 1) and psoralens invariably produce a cross-linked product that migrates as a single, narrow band when provided with a single potential site for cross-linking (Millard et al., 1991). Band 3, the least mobile band, comigrated with a mitomycin C-cross-linked duplex containing a single 5′-CG central site, the drug's preferred sequence (Chawla et al., 1987; Teng et al., 1989; Weidner et al., 1989). Centrally cross-linked DNAs have been shown to have a lower mobility on a denaturing gel than identical duplexes cross-linked toward the ends (Millard et al., 1991). Thus, only band 3 was quantified in order to distinguish between the relative cross-linking efficiencies of the variable core sequences in the duplexes studied.

The duplex with the core GGCC (which contains mechlorethamine's preferred site, GNC) showed the greatest amount of band 3 DEB-cross-linked product by more than 4-fold over the TCGA- and TGCA-containing DNAs. Even this preferred sequence, however, demonstrated less than 0.5% cross-linking through Cerenkov counting. Table I shows the average relative cross-linking efficiencies for the core sequences tested, which were as follows: GGCC ≫ TGCA, TCGA > CCGG > TATA.

We used the base lability of N7-alkylated dG residues to pinpoint the sites of DEB cross-linking. These lesions are cleaved by aqueous piperidine (Maxam & Gilbert, 1980). Single-base-resolving PAGE on the resulting fragments allows

Table I: Average DEB Cross-Linking Efficiencies of DNAs with Sequence 5′-d[AATATAT(N_4)ATAT], N_4 As Indicated

N_4	cross-linking efficiency[a] (%)
TATA	0.027 ± 0.003
CCGG	0.035 ± 0.003
TGCA	0.045 ± 0.006
TCGA	0.058 ● 0.014
GGCC	0.26 ± 0.08

[a] From Cerenkov counting of gel region where centrally cross-linked material is expected.

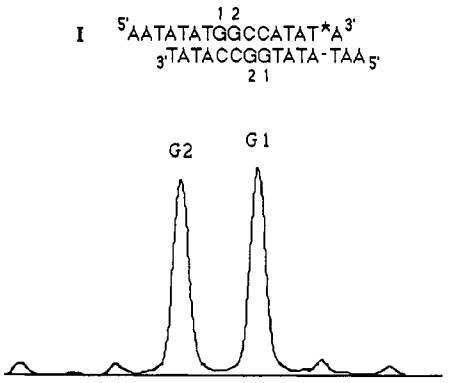

FIGURE 2: Densitometer scan of the denaturing PAGE analysis of cleavage products of DNA I (3′-end radiolabel: * = ^{32}P). Lettering denotes the residue cleaved. Cleavage bands of equal intensities indicate linkage between G^1 and G^2 on opposite strands, implying cross-linking at the sequence 5′-GNC.

assignment of the sites of cleavage and thus the originally alkylated dG residues. This analysis was performed on DNAs containing the cores GGCC, CCGG, TGCA, and TCGA.

Denaturing PAGE on the piperidine products from DEB-cross-linked duplex I (core of GGCC) showed approximately equal cleavage at G^1 and G^2 (Figure 2). Cross-linking of 5′-GC would not produce the G^1 fragment. However, linkage of G^1 to G^2 on opposite strands, as for cross-linking at 5′-GNC (N = G or C), is consistent with these results. Therefore, despite the short chain length of DEB, like nitrogen mustard (Millard et al., 1990a; Ojwang et al., 1989) it can cross-link the sequence 5′-GNC.

For less efficiently cross-linked G-containing oligomers, the entire smear of cross-linked products was isolated from the gel and subjected to piperidine cleavage. Bands corresponding to cleavage at guanine were produced in each case (data not shown). However, intact cross-linked DNA remained after piperidine cleavage, indicating that not all cross-linking was at guanine residues. This verifies that DEB is somewhat flexible in its cross-linking and is a less sequence-specific agent than mitomycin C, for example.

The 5′-GNC preference of DEB was confirmed in duplex II (Figure 3). Cross-linking, isolation of the least mobile band, and piperidine cleavage of this duplex in three radiolabeled states (5′-radiolabeled top strand, 5′-labeled bottom strand, and 3′-labeled top strand) pinpointed the single central dG on the bottom strand and G^2 on the top strand as the predominant linked residues. Thus, 5′-GGC was cross-linked in preference to 5′-GGGC, 5′-GC, 5′-CG, 5′-CGG, and 5′-CGGG. Similar results have been shown for mechlorethamine (Millard et al., 1990a).

The different relative product distributions for radiolabeling of the 5′- versus the 3′-end of the top strand of duplex II can be explained by monoalkylation accompanying cross-linking. Because only alkylations closest to the radiolabel are detected, monoalkylation at G1 would result in elevated cleavage at

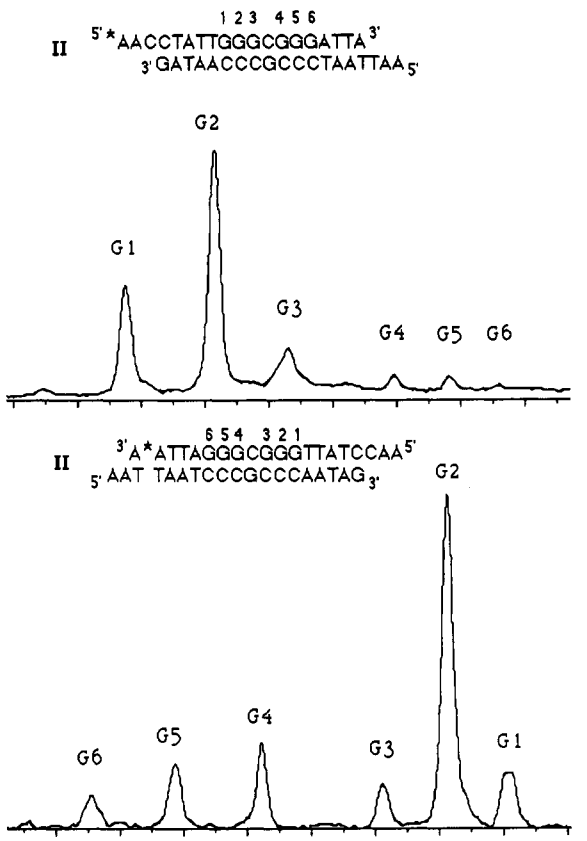

FIGURE 3: Densitometer scans of the denaturing PAGE analysis of radiolabeled (* = ^{32}P) cleavage products of DNA II. Lettering denotes the residue cleaved.

this site in the 5′-labeled sample relative to the 3′-labeled sample, as shown in Figure 3. Similarly, monoalkylation at G4, G5, or G6 would result in elevated cleavages at these residues only in the 3′-labeled sample. Such monoalkylation does not affect the interpretation of 5′-GNC being the favored site for cross-linking.

The cross-linking preference of DEB could arise from sequence-selective monoalkylation, or covalent binding to only one DNA strand, the first step of cross-link formation. This is true for mitomycin C (Li & Kohn, 1991; Kumar et al., 1992). Alternatively, monoalkylation could be sequence random with the subsequent formation of cross-link favoring 5′-GNC. Mechlorethamine is intermediate between these two extremes, with conversion of monoadducted DNA to cross-links being the predominant factor in interstrand cross-link sequence preference (Hopkins et al., 1991). We tested the relative importance of DEB's initial alkylation and the subsequent formation of interstrand cross-links by comparing piperidine fragmentation of the total DEB-treated DNA to the fragmentation of only the interstrand cross-links. Because the cross-linking efficiency is so low, the distribution of total alkylation is a reasonable approximation of monoalkylation frequency. The average monoalkylation frequency for duplex II, as measured by piperidine cleavage of both 5′- and 3′-end-labeled total DNA, was fairly uniform among dG residues (Figure 4). However, fragmentation of the cross-linked DNA led to a decidedly nonrandom product distribution, with almost half of the cleavage occurring at G^2, consistent with 5′-GNC as the preferred cross-linking site. Appreciable fragmentation at other sites suggests that other undefined sequences are also cross-linked. This experiment indicates that the primary determinant of DEB's interstrand cross-linking preference occurs at the step of monoadduct conversion to cross-links.

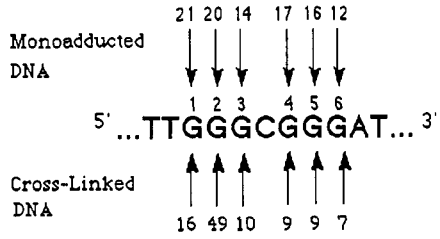

FIGURE 4: Average relative percentages of monoadduct formation and cross-linking by diepoxybutane as a function of nucleotide position. Piperidine cleavage products of both 5′- and 3′-end-labeled duplex II (multiple trials of each) were averaged.

DISCUSSION

We have examined the DNA cross-linking preferences of diepoxybutane, the simplest diepoxide. This compound is analogous to the nitrogen mustards in that its epoxide rings should behave chemically similar to the aziridinium ion formed in an activated mustard (Rutman et al., 1969). We have found that DEB, despite its shorter chain length, does form cross-links at the preferred 5′-GNC sequence of nitrogen mustard.

We compared the cross-linking of a series of duplexes different only in their central four bases. In all of the DNA oligonucleotides examined, diepoxybutane produced not only cross-links involving central dG residues but also cross-links involving terminal dA/dT residues. These latter products possessed a higher mobility in denaturing PAGE than those more centrally cross-linked. Gel mobility varying with cross-link position has previously been described for mitomycin C-cross-linked duplexes (Millard et al., 1991). The observed hyperreactivity of duplex termini, which may not be representative of duplex regions, has also been noted for mechlorethamine (Millard et al., 1990a), nitrous acid (Kirchner & Hopkins, 1992), and several pyrrole-based mitomycin C analogues (Weidner et al., 1990). These less sequence-specific agents appear to be more reactive toward duplex termini than highly specific agents such as mitomycin C.

Quantitation of the least mobile cross-linked product led to the following DEB preferences: GGCC ≫ TGCA, TCGA > CCGG > TATA. This is consistent with a core preference of 5′-GNC, similar to that of mechlorethamine (Millard et al., 1990a), although DEB also cross-links other sequences less efficiently. Virtually complete piperidine fragmentation verified that the linkage for the GGCC-containing oligomer was in fact N7-to-N7 at the sequence GNC.

The GNC core preference of DEB was confirmed in another duplex, which allowed the comparison of a variety of G-containing sequences within the same molecule. Again, the preferred site of cross-linking was 5′-GNC, whereas monoadduct formation occurred approximately equally at all dG sites. This indicates that the conversion of monoadducts to cross-links is more efficient at 5′-GNC sequences than at 5′-GC (by almost 5-fold in the duplex examined), despite the longer distance that the relatively short DEB must span.

These results are inconsistent with the previous prediction that 5′-GC sequences are cross-linked exclusively (Brookes & Lawley, 1961), which is still widely cited. However, this model arose from consideration of static B-DNA (Arnott et al., 1976) and did not account for subtle sequence-dependent conformational variations demonstrated by duplex DNA (Wing et al., 1980; Wemmer et al., 1985). Modeling clearly shows that any DNA must distort substantially to permit a single mustard molecule to link distal guanines through N7 atoms in the duplex sequence 5′-GNC in the B conformation (Millard et al., 1990a). Cross-linking by diepoxybutane's shorter chain would result in even more distortion. Hopkins and co-workers (1991) have speculated that DNA propeller twisting and kinking may combine to close the major groove and drive the relevant guanines' N7 lone electron pairs together to facilitate mustard cross-linking. They further propose that the transition state for cross-link formation may involve significant π-electron density at N7, which would result in the necessity of less DNA deformation at the sequence 5′-GNC than at 5′-GC. Such reasoning may also explain diepoxybutane's preference for cross-linking a distance that it could not span in static B-DNA.

Diepoxybutane is a much less efficient cross-linker than nitrogen mustard (Verly & Brakier, 1969), as verified by the high concentrations needed for these studies. We have shown that monoadducts form relatively randomly, so that it is the closure to cross-link that determines DEB's sequence preference. It is likely that the steric difficulty of accommodating a four-carbon chain across the preferred 5′-GNC site in part dictates the weak interstrand cross-linking capacity of diepoxybutane relative to nitrogen mustard.

We have shown that, despite their vastly different biological effects, diepoxybutane and mechlorethamine seem to target the same genomic sites of DNA. The interstrand cross-linking sequence preferences of these compounds alone therefore cannot explain their differing selective toxicities.

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