DNA Interstrand Cross-Linking by 2,5-Bis(1-aziridinyl)-1,4-benzoquinone: Nucleotide Sequence Preferences and Covalent Structure of the dG-to-dG Cross-Links at 5′-d(GN$_n$C) in Synthetic Oligonucleotide Duplexes

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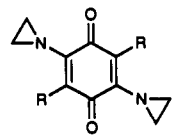
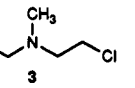
Abstract: The nucleotide sequence specificity of the DNA interstrand cross-linking reaction of 2,5-bis(1-aziridinyl)-1,4-benzoquinone (DZQ) and the covalent structure of the predominant cross-link were determined in synthetic DNA duplexes. A panel of synthetic DNAs was exposed to aqueous DZQ and analyzed for interstrand cross-links by denaturing polyacrylamide gel electrophoresis (DPAGE). Those containing the sequences 5′-d(GGCC), 5′-d(GGGCCC), and 5′-d(GGGGC) afforded relatively abundant interstrand cross-linked products. Exposure of some of these DZQ-cross-linked DNAs to hot aqueous piperidine followed by DPAGE analysis afforded DNA strand scission products with mobility identical to that of the products of the Maxam–Gilbert G reaction, consistent with a dG-to-dG nucleotide connectivity at the duplex sequences 5′-d(GN$_n$C), $n = 0$, 1, and 2, with some preference for $n = 1$. The covalent structure of the cross-link was assigned by a combination of spectroscopic measurements and chemical reactions as bridging N^7 atoms of dG residues on opposite strands of DNA through an intervening 2,5-bis(ethyleneamino)-1,4-benzoquinone unit. The origin of the nucleotide sequence preference of DZQ is discussed.

Introduction

The bifunctional alkylating agents occupy an important place among the drugs useful in cancer chemotherapy.[1] This class includes such structurally diverse substances as the nitrogen mustards, cisplatin, nitrosoureas, and mitomycin C. A substantial body of evidence suggests that the biologically important target of the antitumor alkylating agents is DNA. For example, agents in this class are commonly found to inhibit the template action of DNA, thus thwarting the synthesis of DNA, RNA, and protein. Chromosomal abnormalities have been observed in cells treated with these agents. Cell lines deficient in the ability to repair DNA damage are often unusually sensitive to these agents. DNA–DNA and DNA–protein cross-links have been documented in vivo and in vitro for this class. An attractive hypothesis holds that DNA–DNA interstrand cross-links might pose a special threat to cellular processes, serving not only to inhibit the template action of DNA[2] but also to fustrate the DNA repair apparatus. Interstrand cross-links may thus be especially important in the mechanism of action of some or all of these agents. We describe herein studies on the nucleotide sequence preferences of and structures which result from the DNA–DNA interstrand cross-linking reactions of the bifunctional alkylating agent 2,5-bis(1-aziridinyl)-1,4-benzoquinone (**1a**, commonly called diaziridinyl-benzoquinone or DZQ) in synthetic deoxyribonucleotide duplexes.



1a DZQ R = H
1b AZQ R = NHCOOCH$_2$CH$_3$



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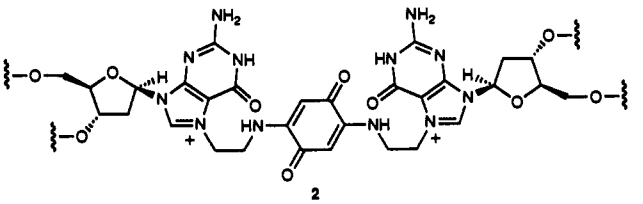

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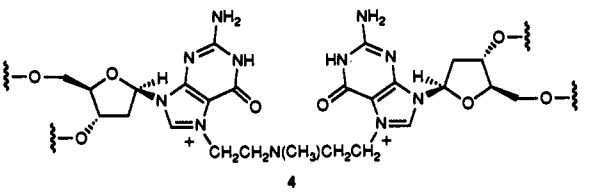

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The anticancer activity of diaziridinyl quinones has been known for many years. The parent compound DZQ (**1a**) is active,[3] but its high toxicity has precluded its use as an antitumor agent.[4] The variant AZQ (diaziquone, **1b**) has attracted considerably more attention. The report in 1976 that AZQ possesses activity against a mouse brain tumor model[5] has led in the intervening years to its testing against a variety of human tumors in numerous phase I and II clinical trials.[6] Most recently, a phase III trial comparing AZQ and BCNU (carmustine) found these two not to differ in efficacy against human brain tumors.[7]

As with the other alkylating agents which possess antitumor activity, it is likely that the molecular target of the aziridinyl quinones is DNA. DZQ causes chromosomal aberrations both

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in vitro[8] and in vivo.[9] DNA–DNA interstrand cross-linking by these agents has been observed in several laboratories.[10-13] The efficiency of alkylation and cross-linking is enhanced by reduction of the quinone moiety or lowering of the pH.[13,14] While it has also been suggested that the production of active oxygen species such as hydrogen peroxide and hydroxyl radical during redox cycling may play a role in the antineoplastic action of the aziridinyl quinones,[15] this activity has more commonly been associated with the undesirable toxic side effects.[16]

It appears to be generally accepted that the alkylation of DNA by aziridinyl quinones is the result of scission of one or both of the aziridine rings through attack at carbon of the aziridine rings by a nucleophilic site on DNA, a mechanism which is analogous to that of the reaction of nitrogen mustard and DNA.[17] The evidence for this is, however, quite indirect. The kinetics and products of solvolysis of DZQ in water[18] or alcohols[19] at neutral to acidic pH are fully consistent with this hypothesis, as is the UV spectrum of DZQ-treated calf thymus DNA.[13,20] Attempts to further characterize the molecular structures of the latter lesions following enzymatic digestion failed.[20] DNA adducts of AZQ have been detected by the ^{32}P postlabeling technique, but the structures were not identified.[21] It was noted that the aziridine function was critical for alkylation of DNA, as the corresponding bis(aminoalcohol) resulting from the hydrolysis of AZQ did not form these adducts. In no prior study has the lesion responsible for the DNA–DNA interstrand cross-link caused by a diaziridinylbenzoquinone been unequivocally identified, a deficiency addressed herein.

Our knowledge of the chemistries of formation and the structures of DNA–DNA interstrand cross-links has advanced considerably in recent years.[22] One example is the case of mechlorethamine (**3**), one of the simplest members of the nitrogn mustard family. This substance has been shown to cross-link the N^7 atoms of deoxyguanosyl residues on opposite strands of the duplex sequence 5′-d(GNC).[23] Available evidence suggests that this lesion distorts duplex DNA by imparting a bend in the helix axis, likely the result of the tether which bridges the strands being inadequate in length to span the sites of alkylation on DNA without a reduction in the interatomic spacing.[24] It has long been believed that the alkylation reactions of nitrogen mustards proceed through aziridinium ion intermediates.[25] As such, there is the possibility for a mechanistic analogy between the reactions of the nitrogen mustards and the diaziridinylbenzoquinones. For this reason, we have studied the DNA interstrand cross-linking reactions of DZQ (**1a**) in synthetic oligonucleotide duplexes.

We present evidence that the major interstrand cross-link caused by DZQ (**1a**) bridges the N^7 atoms of two deoxyguanosyl residues at the duplex sequences 5′-d(GN_nC), n = 0, 1, and 2, through an intervening 2,5-[*N*,*N*′-bis(ethyleneamino)]-1,4-benzoquione, as in **2**. This structure is the analog of the lesion **4** which has recently been shown to be responsible for the predominant interstrand cross-link in DNA formed by mechlorethamine (**3**).[23] The present study thus establishes the structural homology of the lesions formed in DNA by these two classes of antitumor agents.

While this study was in its final stages, Berardini, Hartley, et al. reported independent studies of the interstrand cross-linking of synthetic DNA duplexes by DZQ.[26] Unlike the present study, theirs included investigation of both the quinone and hydroquinone forms of this agent. A smaller range of DNA sequences was investigated by Berardini, Hartley, et al., but they mapped a larger number of sites in the DNAs studied. Berardini, Hartley, et al. selected DNA sequences containing 5′-d(GN_nC) sequences embedded in an AT-rich flanking context. We studied similar sequences but in addition included 5′-d(GN_nC) sequences embedded in a GC-rich context, because it has long been known that runs of deoxyguanosine are unusually susceptible to alkylation.[27] Nevertheless, the central conclusions of this work concerning the sequence preference for DNA interstrand cross-linking by DZQ are in full agreement with those reported by Berardini, Hartley, et al. That study did not concern itself with proving the covalent structure of the resulting lesion, a central point of the present work.

Table 1. Panel of DNA Duplexes

5′TACAAN$_6$TTGT
TGTTN$_6$AACAT5′

entry	N_6	entry	N_6
1	CGATCG	8	CACGTG
2	CCATGG	9	CGGCCG
3	CTATAG	10	CCGCGG
4	CAATTG	11	CAGCTG
5	CGCGCG	12	GAATTC
6	CCCGGG	13	GAGCTC
7	CTCGAG	14	GGGCCC

Results

Nucleotide Sequence Specificity. We chose to survey the nucleotide sequence preference of DZQ as an interstrand cross-linking agent using a panel of synthetic oligodeoxyribonucleotide duplexes. This approach often directly provides the consensus sequence required for cross-linking[28] and allows subsequent experiments to define the precise nucleotide connectivity of the cross-link and the covalent structure of the lesion. However, relative to the use of much larger DNAs such as plasmids (ca. 4000 bp), this approach screens a relatively limited range of DNA sequences in each duplex studied. In order not to bias the search in favor of any one result, we chose for initial evaluation a panel composed primarily of DNA duplexes (entries 1–11, Table 1) which were designed and synthesized in the course of studies on formaldehyde–DNA interactions.[29] This set was supplemented with three new members, entries 12–14. The total set of fourteen

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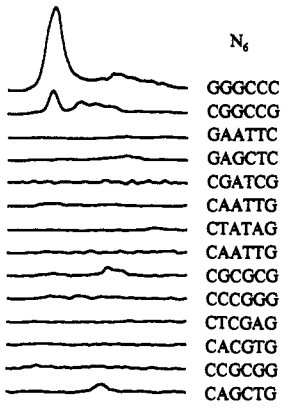


Figure 1. Densitometric traces (from phosphorimage) of interstrand cross-link-containing region of DPAGE of 14 self-complementary DNAs 5′-d(TACAAN$_6$TTGT), N$_6$ as shown, following exposure to DZQ (**1a**).

Table 2. Some DNA Duplexes Used in This Study

DNA sequence	descriptor
1 2 3 5′TATGGGCCCATA ATACCCGGGTAT5′ 3 2 1	GGGCCC
1 2 3 4 5′ATATTGGGGCAATAT TATAACCCCGTTATA5′	GGGGC

self complementary DNAs varied only with respect to the identity of the central six base pairs. While these did not represent a complete "sequence search", they did contain representatives of the majority of all possible short sequences: Represented were (a) all 10 of the dinucleotide duplexes, (b) 9 of the 10 trinucleotides consisting of all possible N^1 and N^3 in the trinucleotide duplex sequence N^{1}XN^3, (c) 8 of the 10 tetranucleotides consisting of all possible N^1 and N^4 in the tetranucleotide sequence N^{1}XXN^4, and (d) 25 of the 32 trinucleotide duplexes. These DNAs in 5′-radiolabeled form were exposed to 1 mM DZQ in 100 mM sodium acetate buffer, pH 5.0, for 1 h at 25 °C. Following isolation of the DNA by ethanol precipitation samples were analyzed by 20% denaturing polyacrylamide gel electrophoresis (DPAGE), which revealed interstrand cross-linked products as bands of roughly half the mobility of the starting single strands. Densitometric traces of the region of the gel containing interstrand cross-links are shown in Figure 1. Twelve of the fourteen duplexes were interstrand cross-linked in a total yield of less than ca. 0.5% with no single band in excess of 0.2%. Those products were not further studied. The remaining two duplexes, N$_6$ = CGGCCG and GGGCCC, were interstrand cross-linked in a significantly higher yield of ca. 3 and 5%, respectively, with the major band in each case being 1 and 4%, respectively. This broad and relatively unbiased search established an analogy to the case of mechlorethamine, in which a 5′-offset of two reactive deoxyguanosyl residues was preferred for relatively efficient interstrand cross-linking.[23] In the case of mechlorethamine, the specific preference was for connection of two deoxyguanosyl residues at the sequence 5′-d(GNC). To define the precise nucleotide connectivity in the case of DZQ, we proceeded as follows.

Nucleotide Connectivity of Cross-Links. Two DNA duplexes were employed in nucleotide connectivity studies, a self-complementary 12-mer duplex hereafter referred to as "GGGCCC" and a non-self-complementary 15-mer duplex hereafter called "GGGGC" (see Table 2). As in the case with mechlorethamine, advantage was taken of the lability toward depurination and subsequent strand cleavage of DNAs containing N^7-alkylated deoxyguanosyl residues. It seemed probable that DZQ-derived cross-links would, in fact, be the result of N^7 alkylation, given that a variey of related electrophiles react at this site and that DNA treated with aziridinyl quinones had previously been found to undergo thermally induced strand scission.[14] A 5′-radiolabeled sample of GGGCCC (Table 2) was exposed to 0.5 mM DZQ at pH 4.8. Under these conditions, DPAGE analysis (phosphorimagery) revealed the recovery of some 75% of single-strand DNA identical in mobility to the DNA starting material and inert to treatment with hot aqueous piperidine, as expected for the starting material. About 20% of the returned product was slightly less electrophoretically mobile than starting single strands (two bands). Although the identity of these materials is not critical in the present context, we tentatively suggest on the basis of their gel mobility and behavior on exposure to hot aqueous piperidine that these substances are monoadducts or intrastrand cross-links. The remaining 5% was present as interstrand cross-linked material, which appeared as two bands in an ca. 4:1 (more mobile to less mobile) ratio. The analysis which follows concerns primarily the major of these two bands. The low conversion (75% recovered starting material) was purposely employed here to maximize chances that the interstrand cross-linked products were predominantly "singly hit". Despite this, preliminary analyses of the type described in the following paragraph were consistent with the minor band of the 4:1 mixture being composed of some three or more products, some or all of which were multiply alkylated. This minor band was not further studied.

The major interstrand cross-linked product was isolated from the gel and subjected to strand scission conditions, 1 M aqueous piperidine, 90 °C, 45 min. Quantitative DPAGE analysis (phosphorimagery) of the resulting fragment distribution revealed virtually complete strand scission, with less than 1% recovery of cross-links or single strands. Instead, fragments corresponding to cleavage at G^2 and G^3 were observed in an ca. 1.5:1 ratio. There was essentially no cleavage at G^1. This result is inconsistent with cross-linking at any *single* site among 5′-d(GN$_n$C), n = 0, 1, or 2, and also is inconsistent with appreciable cross-linking at 5′-d(GN$_n$C), n = 3 or 4, but is consistent with a *mixture* of G^2 to G^2 cross-links at 5′-d(GNNC) admixed with either or both G^2 to G^3, 5′-d(GNC) and G^3 to G^3, 5′-d(GC) cross-links. Strong evidence supportive of this structural heterogeneity was obtained by partitioning of the major gel band representing interstrand cross-linked material into three approximately equal portions differing only slightly in electrophoretic mobility. DPAGE analysis of the piperidine hydrolysate of these portions gave G^2/G^3 ratios varying from ca. 3:1 (necessarily richer in 5′-d(GNNC) cross-link) to 1:2 (necessarily richer in 5′-d(GC) cross-link). The results of experiments commencing with 3′-radiolabeled DNA were in quantitative agreement, showing a 1.5:1 ratio of G^2/G^3 in the total interstrand cross-linked sample, with this ratio varying on partitioning of the product. These data assured the presence of 5′-d(GNNC) and 5′-d(GC), dG-to-dG cross-links but, because of the possible presence of 5′-d(GNC) cross-links, did not allow the product ratio to be defined.

The ambiguity with regard to ratio of products of varying nucleotide connectivity was resolved by employing the non-self-complementary DNA GGGGC (Table 2). This DNA was interstrand cross-linked by DZQ in ca. 4% total yield, with three quarters of this being a single band. The analysis which follows concerns this major band. A sample originating from duplex which was originally 5′-radiolabeled in the lower strand revealed quantitative fragmentation at the lone dG residue in this strand, assuring this as the connection point on that strand. Duplexes originally radiolabeled in either the 5′- or 3′-ends afforded on piperidine fragmentation of the isolated interstrand cross-linked product a 3:1 ratio of cleavage at G^3 and G^4. There was essentially no cleavage at G^1 or G^2. (That the same fragment ratio was obtained with the radiolabel originally at alternate ends assures that these samples were singly alkylated.) Thus, in this case dG-to-dG linkages at 5′-d(GNC) predominated somewhat over 5′-d(GC) linkages, with 5′-d(GNNC) being not appreciably represented.

Taken together, these data prove the existence of dG-to-dG, DZQ-derived interstrand cross-links at the sequences 5′-d(GN$_n$C), n = 0, 1, and 2. The distribution of these isomers is a function

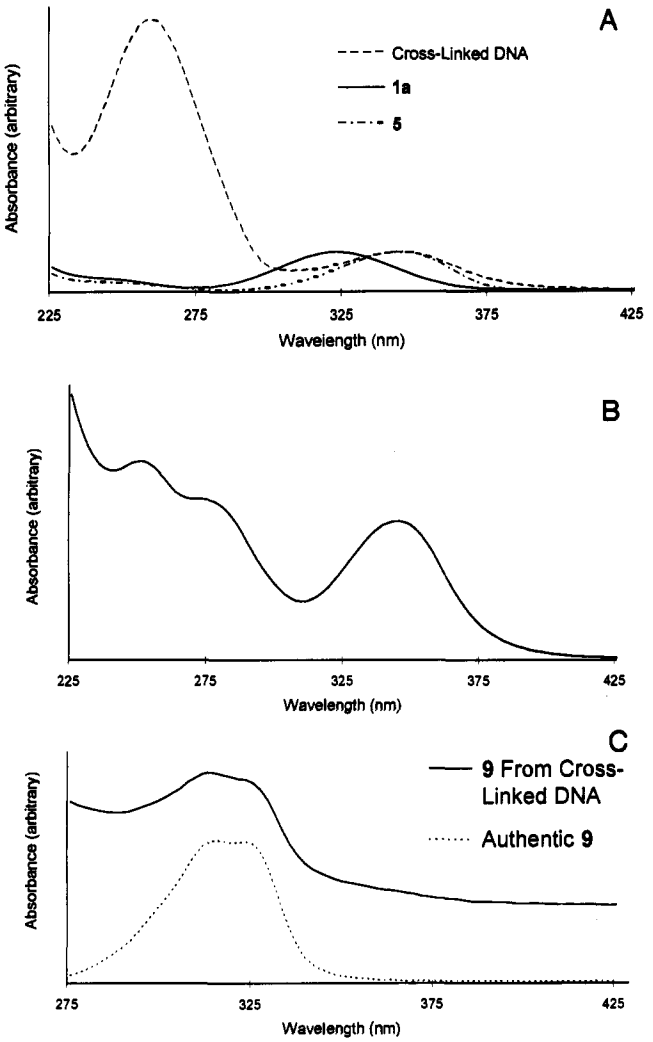
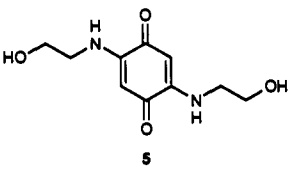


Figure 2. Ultraviolet spectra. (A, top) DZQ-interstrand cross-linked DNA GGGCCC (- - -) overlaid with DZQ (**1a**, —) and 2,5-bis((2-hydroxyethyl)amino)-1,4-benzoquinone (**5**, ···). (B, middle) **8** isolated by HPLC from an enzymatic digest of interstrand cross-linked DNA GGGCCC. (C, bottom) Basic solution of 2,5-dihydroxy-1,4-benzoquinone (**9**) derived from DZQ-interstrand cross-linked DNA GGGCCC (—) overlaid with authentic sample (···).

of sequence, which we can at present neigher confidently rationalize nor even predict empirically. In the one case which could be well defined, the order of cross-linking efficiency was $n = 1 > 0 \gg 2$ and 3.

Covalent Structure of the Cross-Link. Mechanistic analogy to the case of nitrogen mustard would suggest **2** as the covalent structure of DZQ-derived DNA–DNA interstrand cross-links. Preliminary evidence supportive of this came from the UV spectrum (Figure 2A) of the interstrand cross-linked DNA GGGCCC (Table 2) isolated from DPAGE. In addition to absorbances due to DNA, which does not absorb appreciably beyond 300 nm, this spectrum contained an absorbance with λ_{max} 344 nm (see Figure 2a). Lusthoff et al. have observed this same chromophore in DZQ-treated calf thymus DNA as well as in adducts released by enzymatic hydrolysis of the sugar phosphate backbone of that sample.[13,20] This band was strongly suggestive of the presence of the quinone ring derived from DZQ, as depicted in structure **2**. The position of this band was red shifted by 20 nm relative to the corresponding band in DZQ itself (λ_{max} 324 nm) but was coincident with that of 2,5-bis((2-hydroxyethyl)-amino)-1,4-benzoquinone (**5**). This chromophore was reversibly bleached by addition of excess sodium dithionite followed by exposure to air, as expected for redox interchange of a quinone and hydroquinone. The simplest explanation of this observation is the presence in the cross-linked DNA of the 2,5-bis(alkylamino)-



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benzoquinone function common to **2** and **5**, indicating ring opening of the two aziridine rings of the parent diaziridinyl quinone. Using a calculated extinction coefficient (ϵ) of 191 000 M^{-1} cm^{-1} for this DNA at 260 nm and the measured ϵ value of 28 000 M^{-1} cm^{-1} for quinone **5**, a quinone-to-duplex DNA molar ratio of 1.07 ± 0.07:1.0 could be calculated, in good agreement with the 1:1 ratio expected for structure **2**.

To simplify unambiguous identification of this lesion, we separated the conjugate **6** of two guanyl residues bound to a single DZQ from DNA. An aqueous solution of the interstrand cross-linked DNA GGGCCC (Table 2) was thermolyzed at 90 °C for 1 h to cause depurination of the alkylated and thus labilized purines. As has been found with related compounds, low water solubility complicated manipulation of this lesion.[17,23] Specifically, application of the hydrolysate to DEAE Sephacel (to separate the anionic DNA from the neutral **6**) followed by elution with a gradient of aqueous salt solution afforded no fractions with significant UV absorbance beyond 300 nm, despite the persistence of this chromophore after the hydrolysis. If instead the hydrolysate was treated with 100 mM glyoxal for 48 h at 25 °C to form the putative bis(dihydroxyethylene) derivative[30] **7** form **6** (Scheme 1), chromatography on DEAE Sephacel afforded essentially quantitative recovery of the putative quinone chromophore. The absorption maxima for this sample of 284 and 344 nm, respectively, were diagnostic for the N^7-alkylated guanyl residues[31] and bis(alkylamino)benzoquinone function of **2**, **6**, and **7**. Attempts to obtain an electrospray ionization mass spectrum (ESIMS) of substance **7** were unsuccessful, presumably due to both the small quantity in which it was isolated and its chemical instability. This point was not pursued because success was achieved by the alternative approach described below.

As an alternative to the extrusion of the cross-link from DNA by direct scission of the glycosyl bond, we employed enzymatic digestion of the sugar phosphate backbone. The interstrand cross-linked DNA GGGCCC was treated with a cocktail of DNase I, DNase II, snake venom phosphodiesterase, and calf intestinal alkaline phosphatase.[32] HPLC analysis of the resulting mixture revealed the presence of the four common deoxynucleosides, with dG in deficiency, as expected on the basis of its consumption in forming a cross-link. In addition, several strongly retained substances were formed, which we hypothesized to include **8** along with other substances less completely degraded. The most abundant of these substances was isolated and analyzed by UV and ESIMS. The UV spectrum was consistent with an N^7-alkylated deoxyguanosine, with λ_{max} 252, 274 (shoulder),[31] and a 2,5-diamino-1,4-benzoquinone chromophore, λ_{max} 344 (Figure 2b). This substance afforded on ESIMS a base peak m/e 493, consistent with the protonated substance **6** derived from solvolysis of the glycosyl bonds. Signals for the sodium and potasssium ion adducts of **6** were also prominent, as were fragments corresponding to further loss of one (m/e 342) and two (m/e 191) guainine units from protonated **6**. Protonated guanine (m/e 152) was also present. These results prove that the cross-link is the result of reaction of DZQ at deoxyguanosine residues and that the full mass of DZQ is present in the lesion, as in **2**.

Final conclusive evidence that the covalent structure of the interstrand cross-link is as depicted in **2** followed from quantitation

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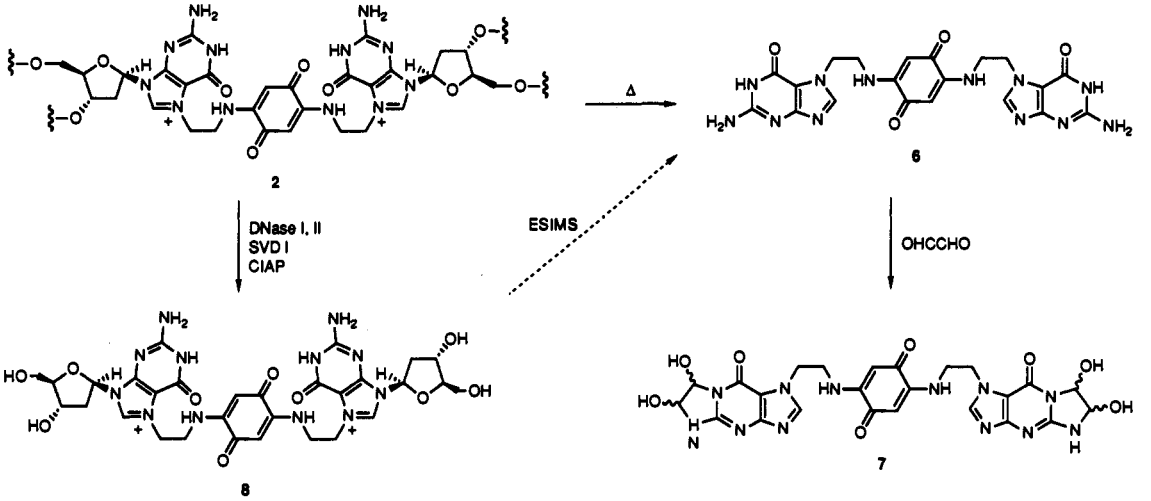
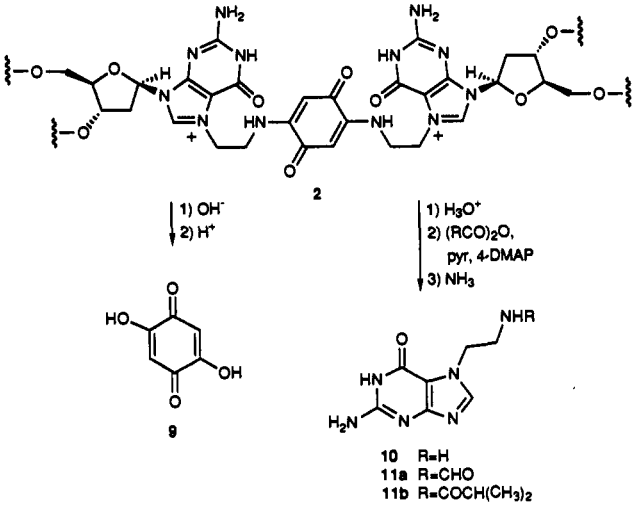
Scheme 1



of fragments derived from **2** following hydrolysis of the vinylogous amide functions therein. Evidence for the presence of a 2,5-disubstituted quinone in the cross-linked sample was obtained following treatment with aqueous base (see Figure 2c). It is known that treatment of 2,5-bis((2-hydroxyethyl)amino)-1,4-benzoquinone (**5**) with base affords 2,5-dihydroxy-1,4-benzoquinone (**9**).[18] Thus, the cross-linked DNA GGGCCC was treated



with 5% aqueous KOH for 12 h. The acidified hydrolysate was extracted with organic solvent. The UV spectrum of a basic aqueous solution of the residue from concentration of this extract gave a UV spectrum with features diagnostic for 2,5-dihydroxy-1,4-benzoquinone. This chromophore has been previously observed upon base treatment of HPLC fractions from the enzymatic hydrolysate of DZQ-treated calf thymus DNA.[20] These chemical reactivity and spectroscopic data are fully consistent with **2** as the structure of the DZQ cross-link, providing especially strong support for the presence of the 2,5-diamino-1,4-benzoquinone substructure.

The two iminoethyl groups joining the deoxyguanosyl residues to the benzoquinone ring as proposed in structure **2** were quantitated following acid hydrolysis of DZQ-cross-linked DNA. The interstrand cross-linked DNA GGGCCC was treated with 6 N aqueous hydrochloric acid to hydrolyze the glycosyl linkages as well as the vinylogous amide functions believed to be present in **2**. Reversed-phase (RP-HPLC) analysis with UV detection clearly revealed the presence of the released bases and a peak which coeluted with an authentic sample of 7-(2-aminoethyl)-guanine (**10**) prepared by a literature procedure.[33] Because of overlap of the peaks representing **10** and Thy, this mixture could not be conveniently quantified. This was instead achieved on the formamide and isobutyramide derivatives **11a** and **11b**, respectively. A formic acid hydrolysate of cross-linked DNA GGGCCC was treated with acetic formic anhydride and then partially deformylated to reveal the guanyl amino group by exposure to concentrated aqueous ammonia at 55 °C for 2 h. RP-HPLC analysis afforded in addition to the four standard bases a peak which coeluted with an authentic sample of the formyl derivative **11a** of 7-(2-aminoethyl)guanine in a ratio of 7.0:4.2:7.2:6.0:1.6 (Cyt/Gua/Thy/Ade/**11a**), in reasonable agreement with the calculated ratio of 6:4:6:6:2 for **2** in the DNA GGGCCC (Figure 3). The outcome of derivatization of the hydrolysate to form the isobutyryl derivative **11b** gave analogous results. These data unambiguously define N^7 as the site of alkylation on both strands of DNA and carbons of the azidine rings of DZQ as the sites of alkylation of the cross-linking agent.

Discussion

We report herein a study of the DNA interstrand cross-linking reaction of 2,5-bis(1-aziridinyl)-1,4-benzoquinone (**1a**, DZQ) in the well-defined setting of synthetic oligodeoxynucleotide duplexes. Using a panel of 14 self-complementary DNA duplexes containing a variety of central "targets" for DZQ cross-linking which were flanked by duplex termini effectively inert to interstrand cross-linking, it was shown that DNAs containing the sequences 5′-d(CGGCCG) and 5′-d(GGGCCC) were interstrand cross-linked in the greatest yield. The nucleotide connectivity of the most abundant of these cross-links was studied by strand cleavage with aqueous piperidine. In one DNA, strong evidence for dG-to-dG linkages at the sequences 5′-d(GN_nC) $n = 0$ and 2 was found, with the possible presence of $n = 1$. In that DNA, connection with $n = 3$ or 4 was precluded. In a second DNA, dG-to-dG linkage at 5′-d(GN_nC), $n = 0$ and 1, predominated, with a preference for the latter. In that case no appreciable connections with $n = 2$ or 3 were observed.

The presence in duplex DNAs of 5′-d(GN_nC), $n = 0$, 1, or 2, sequences was not sufficient for optimally efficient cross-linking. These sequences were only efficiently cross-linked when embedded in a dG-rich context. This is probably a consequence of the enhanced nucleophilicity of N^7 of dG in runs of dG.[27] The dG-rich DNA commonly present in upstream control regions of genomic DNA may thus be an optimal target for DZQ interstrand cross-linking.[27b] The structural heterogeneity of interstrand cross-linked samples with respect to nucleotide connectivity which arises in these dG-rich regions may complicate eventual high-resolution structural studies, as structurally homogeneous samples are normally employed.

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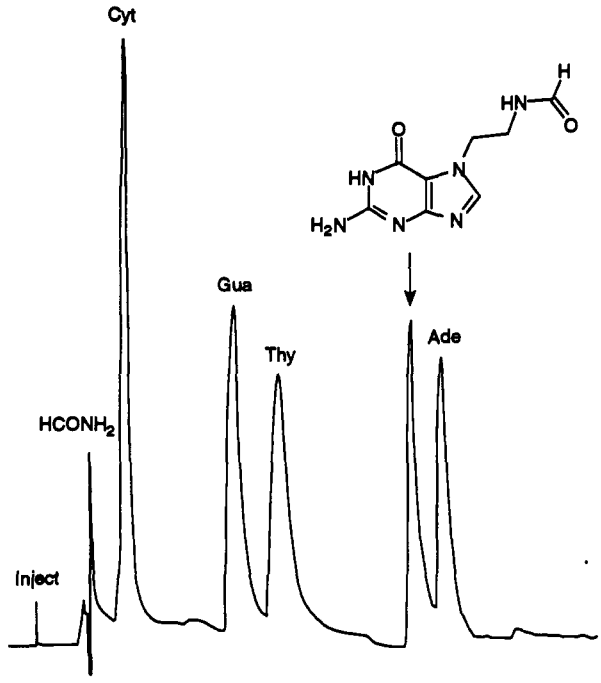

Figure 3. HPLC profile of formylated hydrolysate of DZQ-interstrand cross-linked DNA GGGCCC (see text). Retention time increases to right.

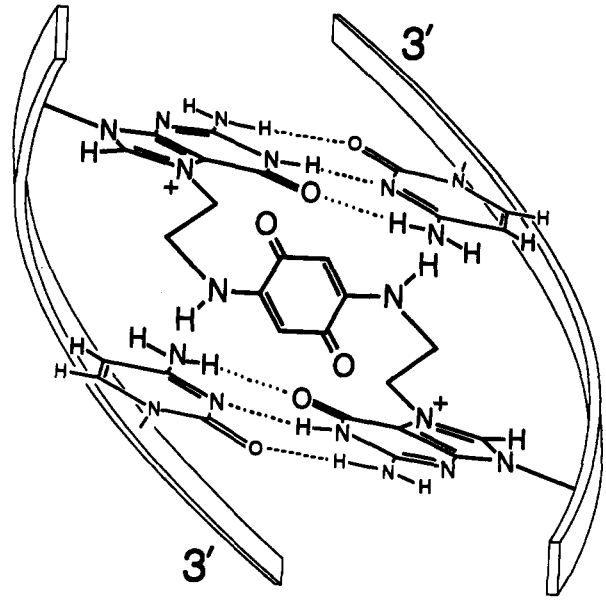

Figure 4. Schematic depiction of DZQ-cross-linked duplex DNA with the covalent connectivity as proven herein. The helical twist of ca. 72° and the interbase distance along the helix axis of ca. 6.8 Å illustrated in the cross-linked base pairs are as found at the sequence 5'-d(GNC) in canonical B DNA. The intervening base pair is omitted for clarity.

Many lines of evidence pointed to **2** as the nucleus of the most abundant interstrand cross-link. The position and intensity of a 344-nm absorbance which could be bleached by addition of sodium dithionite in interstrand cross-linked DNA was diagnostic for the presence of 1 mol of a 2,5-bis(alkylamino)-1,4-benzoquinone. As expected for **2**, this chromophore was released on thermolysis, revealing after its separation from excess DNA, a 284-nm absorbance diagnostic for an N^7 alkylated guanine. The electrospray ionization mass spectrum of a sample of this lesion isolated by HPLC from an enzymatic digest of the sugar phosphate backbone clearly revealed the lesion to be composed of one DZQ and two guanine moieties. The vinylogous amide function of **2** was evidenced by basic hydrolysis releasing 2,5-dihydroxy-1,4-benzoquinone (**9**) and acidic hydrolysis affording 2 mol of 7-(2-aminoethyl)guanine (**10**) per mole of interstrand cross-linked duplex.

DZQ-interstrand cross-linked DNA provides yet a further example of the major groove interstrand cross-linked DNAs, substances which without exception have a 5'-offset of the tethered nucleotides. This family includes the interstrand cross-links derived from nitrogen mustard,[23] cisplatin,[34] azinomycin,[35] and formaldehyde.[29] Possible origins of this uniformly observed 5'-offset are suggested by inspection of scale models of B DNA. A consequence of the helical twist of B DNA is that the minimal spacing of functional groups on opposite strands in the major groove occurs when these functional groups are offset from one another in the 5'- rather than 3'-direction (see Figure 4), an effect which is reversed in the minor groove. We have previously noted that the negative propeller twist commonly present in DNA causes any two reactive residues on opposite strands in the major groove and offset toward the 5'-ends to approach one another, an effect which may be important in the present case.

Of the interstrand cross-linking agents which have been studied in detail, DZQ is among the *least* sequence selective. Unlike agents such as cisplatin,[34] formaldehyde,[29] nitrous acid,[36] and the pyrrole-derived agents[37] such as mitomycin and FR900482,[38] which possess an appreciable preference for cross-linking one nucleotide sequence with a specific connectivity (e.g. dG to dG at 5'-d(CG)), the duplex DNAs studied herein were cross-linked in three connectivities, dG to dG at 5'-d(GN_nC), n = 0, 1, and 2. The situation is no different for the reduced form of DZQ: while this manuscript was in review, Haworth et al. reported that the DNA interstrand cross-linking reaction of the hydroquinone form of DZQ occurs with two connectivities, n = 0 and 2, for the cases studied.[39] It seems likely that this is the result of another difference between DZQ and the other agents mentioned, a substantially greater distance which can be spanned by the two electrophilic centers. For example, the "linking arms" in cisplatin and formaldehyde cross-links are composed of a single atom, while that of the pyrrole cross-linking agents is four atoms and includes constraint to an eclipsed conformation of one of the internal dihedral angles. In contrast, DZQ has a 10-atom linking chain, with 3 dihedral constraints associated with the quinone ring. Inspection of scale molecular models suggests that, in fully extended form, the DZQ-derived tether, as in **2**, can bridge a pair of nucleophiles separated by some 12 Å. We suggest that this flexible chain affords opportunity for cross-linking reactions which proceed through a variety of conformations and thus bridge nucleophilic centers on DNA with various spacings. The interatomic spacings of N^7 atoms of pairs of dG residues at the sequences 5'-d(GN_nC) with n = 0, 1, and 2, the sequences observed herein to be cross-linked, are ca. 8, 9, and 11 Å, respectively, in B DNA[40] and are thus a good match to the DZQ structure. Inspection of models indicates that these distances are in reasonable agreement with what might be spanned by a DZQ monoadduct in the transition state for progression to a cross-link, although it may be that for the longest distance (n = 2) some reorganization of DNA to diminish the spacing of the bridged N^7 atoms is required.

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Experimental Section

Materials and Methods. Materials and their sources were as follows: DNA synthesis reagents, Applied Biosystems; [γ-^{32}P]ATP, [α-^{32}P]dATP, and [α-^{32}P]dTTP, New England Nuclear; T4 polynucleotide kinase, Amersham; Klenow fragment and alkaline phosphatase (calf intestinal), Boehringer Mannheim; DNAse I and DNase II, Sigma; phosphodiesterase I (Crotalus adamanteus venom), Pharmacia. Water was purified on a Millipore Milli-Q deionizer. All other reagents were commercial and used as received except for pyridine, which was distilled under nitrogen from calcium hydride. Unless otherwise specified, solutions were aqueous. Samples were concentrated on a Savant Speed Vac concentrator. Except for purification of synthetic oligonucleotides, all DPAGE was performed on a Hoeffer thermojacketed Poker Face gel stand. Loading buffer was 90% aqueous deionized formamide containing 10 mM Tris/Tris·HCl (pH 7.5), 0.1% xylene cyanol, and 0.1 mM Na_2EDTA. TE buffer was 10 mM Tris/Tris·HCl (pH 7.5) and 1.0 mM Na_2EDTA. Kinase and Klenow buffers were both 50 mM NaCl, 10 mM Tris/Tris·HCl (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT. 1 × TBE buffer was 90 mM Tris/90 mM boric acid (pH 7.5) and 1.8 mM Na_2EDTA. Hydrolysis buffer was 50 mM Tris/Tris·HCl (pH 8.5) and 10 mM $MgCl_2$. Autoradiography was performed on Amersham Hyperfilm-MP. For phosphorimagery, gels were dried (Bio-Rad Model 583) onto Whatman 3MM paper and scanned by a Molecular Dynamics 400A Phosphorimager. All scanning operations, data display, and analysis were performed using Molecular Dynamics' Image Quant software (v. 3.1 or 3.2) operating on Intel 80386 or 80486 microprocessors using MS-DOS 5.0 and MS-Windows 3.0 or 3.1 operating systems. UV spectra were measured on a Hewlett-Packard 8452A or a Perkin-Elmer Lambda 3A spectrophotometer. Proton nuclear magnetic resonance spectra (^{1}H NMR) were measured on a Bruker AC200 (200 MHz) or AM500 (500 MHz) spectrometer and, unless otherwise noted, are reported in parts per million downfield from internal tetramethylsilane (0.00 ppm). Low-resolution mass spectra (LRMS) were measured on a KRATOS Profile HV3 mass spectrometer using direct sample insertion and electron impact (EI) ionization. Operations, data display, and analysis were performed by KRATOS' MACH 3 software operating on a Sun Workstation. Electrospray ionization mass spectra (ESIMS) were measured on a Sciex Atmospheric Pressure Ionization triple-quadrupole mass spectrometer. Operations, data display, and analysis were performed using Sciex Tune (v. 2.3) and MacSpec (v. 3.21) software operating on a Macintosh IIfx. Except where otherwise noted, selected ions, diagnostic for the substance of interest, are reported. HPLC was performed on an Alltech, 5-μm, C18, 250 mm × 4.6 mm Econosphere column using either a Beckman 110B dual-pump system with a Beckman 421A controller and a Waters Lambda-Max 481LC UV/vis detector (output to a Linear 156 recorder and an HP 3390A electronic integrator) or an SSI 200B/220B dual-pump system with an SSI controller and sequential SSI 500 UV/vis (output to a Linear 255/M recorder and an HP 3390A electronic integrator) and Rainen UV-D II (output to a Linear 156 recorder and an HP 3390A electronic integrator) detectors. Solvent gradients were run at 1 mL/min. Gradient A: solvent A, 100 mM ammonium formate (pH 5.1); solvent B, 80% methanol; isocratic 100% A for 15 min, isocratic 100% B for 10 min, and immediate return to initial conditions. Gradient B: solvent A, 10 mM ammonium acetate; solvent B, 100% acetonitrile; isocratic 92% A for 7 min, a 13-min linear gradient to 70% A, a 10-min linear gradient to 60% A, and a 10-min linear gradient to initial conditions. Gradient C: solvent A, 100 mM ammonium acetate (pH 7.0); solvent B, 50% acetonitrile; isocratic 99% A for 12 min, an 8-min linear gradient to 70% A, and a 5-min linear gradient to initial conditions.

DZQ (**1a**) was synthesized according to literature methods from 2,5-dichloro-1,4-benzoquinone and azirdine.[41] Purity was checked by ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.25 (8H, s, CH_2), 5.95 (2H, s, CH); LRMS (EI) m/e 190 (M^+); UV λ_{max} 324 nm (H_2O).

7-(2-Aminoethyl)guanine (**10**) was synthesized according to literature methods from calf thymus DNA and aziridine and purified by HPLC using gradient A[33] and hereafter is referred to as "authentic **10**". Purity was checked by ^{1}H NMR (500 MHz, D_2O, external DSS = 0.00 ppm) δ 2.95 (2H, t, CH_2), 4.15 (2H, t, CH_2), 8.0 (1H, s, CH); LRMS (EI) m/e 194 (M^+); ESIMS (methanol) m/e 195 ($M + H^+$); UV λ_{max} 284 nm, λ_{min} 260 (H_2O); λ_{max} 268 (formic acid); λ_{max} 281, λ_{min} 259 (30% NH_4OH).

2,5-Bis((2-hydroxyethyl)amino)-1,4-benzoquinone (**5**) was synthesized according to literature methods from ethanolamine and 1,4-benzoquinone.[18] Purity was checked by ^{1}H NMR (200 MHz, d_6-DMSO) δ 3.2 (4H, q, CH_2), 3.55 (4H, q, CH_2), 4.92 (2H, t, OH), 5.29 (2H, s, CH), 7.53 (2H, t, NH); LRMS (EI) m/e 226 (M^+); ESIMS (methanol) m/e 227 ($M + H^+$); UV λ_{max} 344 (ϵ_{344} 28 000 M^{-1} cm^{-1}), 212 nm, λ_{min} 286 (H_2O).

Preparation and Radiolabeling of DNA. Oligodeoxynucleotides were synthesized on an Applied Biosystems Model 392 synthesizer on a 1-μmol scale and purified by DPAGE (ca. 40 o.d. of crude DNA, 20% polyacrylamide (19:1 acrylamide/bis(acrylamide), and 8 M urea in 1 × TBE buffer, 1.5-mm thick, 14 × 16 cm^2, 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[42] and isolated from the gel by a crush and soak procedure: the DNA was cut from the gel, crushed with a glass rod into fine particles, and incubated at 37 °C for 12–18 h in TE buffer. The supernatant was removed and the gel incubated with fresh TE buffer for an additional 2 h. The supernatants were combined and passed through a Waters Sep-Pak C_{18} cartridge previously washed with 10 mL of acetonitrile and 10 mL of water, followed by 10 mL of 10 mM aqueous ammonium acetate and 10 mL of water, and the DNA was eluted with 4 mL of 25% aqueous acetonitrile. DNA was recovered by concentration of the acetonitrile/water eluent. DNA was labeled at the 5′-terminus using 0.01 o.d. of DNA in 10 μL of kinase buffer, 10 μCi of [γ-^{32}P]ATP, and 5 units of T4 polynucleotide kinase at 37 °C for 45 min. Radiolabeling was terminated by heating at 90 °C for 1.5 min followed by the addition of 1 μL of 3 M sodium acetate and precipitation with 1 mL of absolute ethanol at –20 °C. After 10 min at –20 °C, the precipitate was pelleted by centrifugation at 4 °C for 10 min and the supernatant discarded. The remaining solid was suspended in 1 mL of 85% aqueous ethanol at –20 °C. After 10 min at –20 °C, the precipitate was pelleted by centrifugation at 4 °C for 10 min and the supernatant discarded. The remaining precipitate of radiolabeled DNA was used in the following experiments. Labeling at the 3′-terminus was performed on 0.01 o.d. of duplex DNA (possessing a one-base 5′ overhang on the unlabeled complementary strand) in 10 μL of Klenow buffer, 10 μCi of [α-^{32}P]dATP or -dTTP, and 2 units of Klenow fragment at 37 °C for 45 min. Termination and precipitation were performed as described above for 5′-terminus labeling.

General Conditions for Preparation, Isolation, and Characterization of Interstrand Cross-Linked DNA Duplexes. For analytical scale reactions, 0.03 o.d. of radiolabeled DNA and 0.03 o.d. of unlabeled complementary DNA were dissolved in 25 μL of 100 mM sodium acetate (pH 4.8) and 0.5 μL of DZQ (25 mM stock solution in acetonitrile). After 2 h at 25 °C, the cross-linking mixture was ethanol precipitated, the supernatant removed, and the pellet dried. Pelleted DNA was dissolved in 7 μL of loading buffer and analyzed by DPAGE. DPAGE was conducted on a 25% gel (19:1 acrylamide/bis(acrylamide) and 8 M urea in 1 × TBE buffer, 0.35-mm thick, 33 × 41 cm, using a 20-toothed comb) at 45–55 W at 20 °C until the xylene cyanol dye had traveled 15–18 cm from the origin. Samples were not thermally denatured prior to loading, and electrophoresis was performed at 20 °C to minimize the thermally promoted decomposition of the cross-linked DNA. Autoradiography was used to visualize the single-strand and cross-linked DNA. Yields of interstrand cross-links were determined by phosphorimagery. The data displayed in Figure 1 were normalized to equate the total integral in each lane and then smoothed by the method of Savitzky–Golay.[43]

For preparative-scale reactions, 50 o.d. of unradiolabeled DNA was dissolved in 740 μL of water, 100 μL of 1 M sodium acetate (pH 4.8), and 160 μL of DZQ (25 mM stock solution in acetonitrile). After 2 h at 25 °C, the cross-linking mixture was split into four samples of 250 μL each and ethanol precipitated, the supernatant removed, and the pellet dried. Pelleted DNA was dissolved in 30 μL of 90% aqueous deionized formamide containing 10 mM Na_2EDTA and subjected to DPAGE as above. UV shadowing was used to visualize the cross-linked DNA.

Gel slices of approximate dimensions of 1.0 cm × 0.3 cm containing cross-linked DNA were excised from the above-described gels. DNA was eluted from the gel slice using the crush and soak procedure and the supernatant desalted by passage through a Sep-Pak, as described above under Preparation and Radiolabeling of DNA. Cross-linked DNA was recovered by concentration of the acetonitrile/water eluent.

The quinone and duplex DNA chromophores in cross-linked DNA were quantified by using extinction coefficients estimated as follows: the extinction coefficient of **5** (determined above to be 28 000 M^{-1} cm^{-1}) at 344 nm was used as the extinction coefficient of the quinone component of cross-linked DNA. The extinction coefficient of the duplex DNA component at 260 nm was estimated by determining the single-strand

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extinction coefficient,[44] multiplying by 2, subtracting 3300 M^{-1} cm^{-1} for every AT base pair and 2000 M^{-1} cm^{-1} for every GC base pair due to the hyperchromicity change upon formation of the duplex,[45] and decreasing the extinction coefficient of each of the modified guanines by half due to the N^7 modification.[31] Applying this formula to cross-linked duplex DNA GGGCCC results in a value of 191 000 M^{-1} cm^{-1}. The molar ratio of quinone and duplex DNA found in cross-linked DNA GGGCCC was as follows: duplex DNA, 1.0; quinone 1.07 ± 0.07 (calcd 1.0:1.0). The 344-nm chromophore, believed to be due to the quinone functionality present in cross-linked DNA, was bleached by sodium dithionite as follows: cross-linked DNA GGGCCC (0.2 o.d. at 344 nm in 150 μL of water) was placed in a 4-mL cuvette fitted with an airtight stopcock and degassed under vacuum for 15 min. Water was sparged with argon and degassed under vacuum while being sonicated. Sodium dithionite was added to the water, and the solution was mixed and cannula-transferred to the cuvette, bringing the final dithionite concentration to 1 mM. The UV spectrum was immediately measured and showed the peak at 344 nm to have been eliminated. Upon reexposure to air, the peak at 344 nm returned with its original intensity.

Piperidine Cleavage of 5′- or 3′-Labeled Cross-Linked DNA. Radiolabeled, cross-linked DNA isolated from the gel as described above was dissolved in 100 μL of 1 M aqueous piperidine and heated to 90 °C in a sealed microfuge tube for 45 min. After concentration to dryness, the mixture was sequentially concentrated to dryness twice from 20 μL of water, dissolved in 5 μL of loading buffer, and analyzed by 25% DPAGE at 55 °C as described above. The gel was dried and quantified by phosphorimagery.

Glyoxal-Derivatized 1,4-Bis[(2-(N^7-guaninyl)ethyl)amino]benzoquinone from Cross-Linked DNA. Isolated cross-linked DNA GGGCCC (ca. 2.0 o.d.) was dissolved in 1 mL of 300 mM triethylammonium bicarbonate (pH 8.6) and heated at 90 °C for 1 h to release the cross-link conjugate. Upon cooling, 100 μL of 1 M glyoxal was added and left at 25 °C for 2 days. The sample was then applied to a DEAE Sephacel column (prepared in a Pasteur pipet: previously washed with 3 mL of 0.6 M NaCl/TE (pH 7.6), 3 mL of TE (pH 7.6), and 3 mL of 0.1 M NaCl/TE (pH 7.6)) and eluted with 4 mL of 0.1 M NaCl/TE (pH 7.6) to recover the gloxal-derivatized cross-link conjugate, 3 mL of 0.3 M NaCl/TE (pH 7.6), and 4 mL of 0.6 M NaCl/TE (pH 7.6) to recover the depurinated DNA.[46] The eluent containing the conjugate was concentrated to 200 μL, and an equal volume of methanol was added. This solution was applied to a sephadex LH–20 column (0.5 cm × 40 cm; previously washed with at least 3 column volumes of 1 mM methanolic ammonium acetate) and eluted with 1 mM methanolic ammonium acetate. Fractions containing conjugate (UV) were concentrated to dryness and redissolved in water, and the UV spectrum of each was measured (λ_{max} 284, 344 nm).

Enzymatic Hydrolysis of Cross-Linked DNA. Isolated cross-linked DNA GGGCCC (1.0 o.d.) was hydrolyzed using 100 μL of hydrolysis buffer, 50 units of DNAse I, 21 units of DNase II, 5 units of snake venom phosphodiesterase I, and 15 units of calf intestinal alkaline phosphatase at 25 °C for 18 h. The largest peak retained beyond the four common nucleosides, retention time 19.5 min, was isolated by HPLC using gradient B. The peak was collected and concentrated to dryness, the residue redissolved in water, and the UV spectrum measured. On a subsequent run, the peak was collected and directly analyzed by electrospray ionization mass spectrometry. These measurements were both consistent with its assignment as structure **8**: UV 252 (274 sh), 344 nm; ESIMS (180-V inlet voltage, 4400-V needle voltage, 3 μL/min, sum of 50 scans) *m/e* 493 (M^{2+} + H^+ − 2deoxyribosyl$^+$), 342 (M^{2+} − deoxyribosyl$^+$ − deoxyguanosine), 191 (M^{2+} − H^+ − 2deoxyguanosine), 152 (Gua + H^+).

2,5-Dihydroxy-1,4-benzoquinone from 2,5-Bis(2-hydroxyethyl)amino)-1,4-benzoquinone and Cross-Linked DNA. Isolated cross-linked DNA GGGCCC (0.7 o.d.) or **5** (1 mg) was dissolved in 5% KOH and allowed to stand at 25 °C for 12–14 h. The reaction mixture was acidified with 1 M aqueous HCl and extracted sequentially with two equal volumes of ethyl acetate. The ethyl acetate layers were combined, the solvent removed under a stream of air, the residue dissolved in 1 mL of water, a drop of saturated aqueous sodium bicarbonate added, and the UV spectrum measured. Both the sample derived from **5** and the sample derived from cross-linked DNA GGGCCC had maxima at 316 and 324 nm (a shoulder at 324 nm for the material derived from cross-linked DNA), identical to the UV spectrum of 2,5-dihydroxy-1,4-benzoquinone (**10**) in the identical solvent system.

Preparation of Derivatized 7-(2-Aminoethyl)guanine from Cross-Linked DNA and Quantification by HPLC. The formyl derivative **11a** was prepared as follows: cross-linked DNA GGGCCC (1.0 o.d.) was dissolved in 400 μL of 88% aqueous formic acid and heated to 140 °C in a sealed, Teflon-lined vial for 2 h. After concentration to dryness, the mixture was dissolved in 100 μL of pyridine and cooled to 0 °C in an ice–water bath, and 100 μL of acetic formic anhydride[47] was added dropwise over 2–4 min while stirring. The bath was allowed to warm to 25 °C over the course of 1 h, 100 μL of water was added, and the mixture was stirred an additional 10 min. After concentration to dryness, the mixture was sequentially concentrated to dryness three times from 50 μL of methanol and dissolved in 200 μL of 30% NH_4OH. After 2.5 h at 55 °C, the mixture was concentrated to dryness, sequentially concentrated to dryness three times from 50 μL of water, and dissolved in 20 μL of formamide.

Alternatively, the isobutyryl derivative **11b** was prepared as follows: cross-linked DNA GGGCCC (1 o.d.) was dissolved in 100 μL of 6 N aqueous HCl and heated to 95 °C in a sealed microfuge tube for 2 h. After concentration to dryness, the sample was sequentially concentrated to dryness twice from 50 μL of water, once from 100 μL of 30% aqueous NH_4OH, and three times from 100 μL of pyridine. The sample was then dissolved in 100 μL of pyridine, 100 μL of isobutyric anhydride, and 4 μL of 5 mM 4-(dimethylamino)pyridine in pyridine. After stirring for 1 h at 25 °C, 20 μL of water was added and the mixture was stirred an additional 10 min. After concentration to dryness, the mixture was sequentially concentrated to dryness three times from 50 μL methanol and dissolved in 200 μL of 30% aqueous NH_4OH. After 12–18 h at 55 °C, the mixture was concentrated to dryness, sequentially concentrated to dryness three times from 50 μL of water, and dissolved in 20 μL of formamide.

HPLC analysis was performed on 5-μL aliquots of the formyl- (**11a**) and isobutyryl- (**11b**) derivatized acid hydrolysates of cross-linked DNA GGGCCC using gradient C. Quantitation, performed at 284 nm for all samples, was based on the peak area ratios obtained from two standard, equimolar mixtures prepared by weight of Ade, Cyt, Gua, Thy, and 7-methylguanine (me^7Gua; Ade and me^7Gua are coincident in gradient C), which were as follows: Ade, 1.0; Cyt, 1.3; Gua, 2.4; Thy, 1.5; me^7-Gua, 3.4. The hydrolysate of cross-linked DNA GGGCCC which had been formylated analyzed as 6.0 (Ade), 7.0 (Cyt), 4.2 (Gua), 7.2 (Thy), and 1.6 (**11a**). The isobutyrylated sample analyzed as 6.0 (Ade), 2.4 (Cyt), 4.2 (Gua), 5.4 (Thy), and 2.4 (**11b**). In both cases the calculated ratio was 6:6:4:6:2.

Authentic **10** was treated as described above to yield either **11a** or **11b**. Both derivatives of authentic **10** had the same UV spectral characteristics (λ_{max} 284 nm, λ_{min} 260 (H_2O)) as authentic **10**. **11a** from authentic **10** was shown to coelute with **11a** from formic acid hydrolyzed, formyl-derivatized, cross-linked DNA GGGCCC in gradient C. **11b** from authentic **10** was shown to coelute with **11b** from HCl-hydrolyzed, isobutyryl-derivatized, cross-linked DNA GGGCCC in gradient C. Unmodified authentic **10** was shown to coelute with **10** and Thy from an underivatized HCl hydrolysate of cross-linked DNA GGGCCC in gradient C. The four bases recovered from either the formyl- or isobutyryl-derivatized acid hydrolysates were shown to coelute with the four bases used in the standard quantitation mixture in gradient C.

Computer Modeling. Molecular modeling was performed on a Silicon Graphics 4D-25 IRIS workstation using the Biopolymer module of Insight II (v. 2.0.0).

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