Synthesis and Characterization of Disulfide Cross-Linked Oligonucleotides

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Abstract: Disulfide cross-linked derivatives of the "Dickerson/Drew dodecamer" 5′-d(CGCGAATTCGCG)-3′ and the related decamer 5′-d(GCGAATTCGC)-3′ have been synthesized, each bearing a dithiobis(ethane) (C_2) or dithiobis(propane) (C_3) cross-link at the central adenine (A). NMR and CD spectroscopy indicate that these duplexes are essentially undistorted relative to native B-DNA. However, cross-linking does confer a large degree of stabilization upon the duplexes, as reflected in increased melting temperatures of 15–21 °C for the cross-linked oligonucleotides relative to those for the native sequences. In contrast, un-cross-linked tethers at the same adenines cause substantial decreases in the melting temperature. The cross-linked duplexes were also found to be stable to millimolar concentrations of β-mercaptoethanol, an important feature for future applications of these cross-links to the study of protein–DNA interactions.

Introduction

Site-specific cross-linking of DNA is a potentially valuable tool for the study of protein–DNA interactions. Many DNA-binding proteins, especially those that catalyze reactions such as transcription, replication, recombination, repair, and methylation, require strand separation or local helical distortion to perform their functions.[1-4] Mechanistic studies of these proteins would be facilitated by site-specific interstrand cross-links that could be incorporated into native DNA sequences without causing artifical structural distortions. The principle of using cross-linked DNA to study protein–DNA interactions has been demonstrated in several studies: an elongation complex of T7 RNA polymerase has been trapped using a psoralen cross-link[5] and the T4 and T7 DNA polymerases, as well as the polymerase and exonuclease activities of the Klenow fragment, have been studied using ethano-bridged cytosine–cytosine cross-links.[6] More recently, ethano-bridged guanine–cytosine cross-links have been incorporated into DNA and shown to interfere less with polymerase activity than cytosine–cytosine cross-links.[7]

Although cross-linking methods such as these have produced significant results, their use is complicated by two factors: difficulties in targeting the cross-link to a specific site within DNA and generation of structural distortions in DNA by the cross-link.[8] Targeting is particularly problematic for bis-electrophile cross-linking reagents, since they are often only specific for very short sequences and tend to produce mixtures of monoadducts, interstrand cross-links, and intrastrand cross-links.[9-14] Even reagents that show sequence-specificity, such as psoralen,[15] can only be targeted to a single site when their preferred site of cross-linking occurs once within the oligonucleotide being cross-linked; this places a severe limitation on the sequences that can be used in such experiments.[16-18]

The targeting problem can be circumvented by the introduction of a modified base at one specific site within an oligonucleotide, using solid-phase DNA synthesis technology. This modified base possesses functionality that, upon duplex formation, is capable of cross-linking to the opposite strand. An example of this strategy is the introduction of electrophilic aziridine groups at the exocyclic amines of 5-methylcytosine in synthetic oligonucleotides using phosphoramidite chemistry.[19,20] This ethano cytosine can form a site-specific cross-link to the complementary base by attack of a primary amino group from the opposite strand on the aziridine, but this requires a C–C or C–A mismatch and therefore results in non-native DNA. Such a cross-linked mismatch would be expected to distort the duplex DNA structure substantially, thereby complicating the interpretation of its interaction with proteins.[21]

As an alternative to these approaches, we have developed a method for site-specific cross-linking of DNA via formation of

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(21) An aziridine derivative of guanine (N^6,N^6-ethano-2,6-diaminopurine) has been used to form cross-links within G–C base pairs, but in this case, the modified guanine is incorporated into DNA as a triphosphate rather than as a phosphoramidite.[7] This makes targeting a particular site for cross-linking more difficult than using phosphoramidite chemistry. Another drawback is that the modified guanine may be incorporated opposite adenine and cross-linked to it as well as to cytosine, resulting in a mixed population of cross-links.

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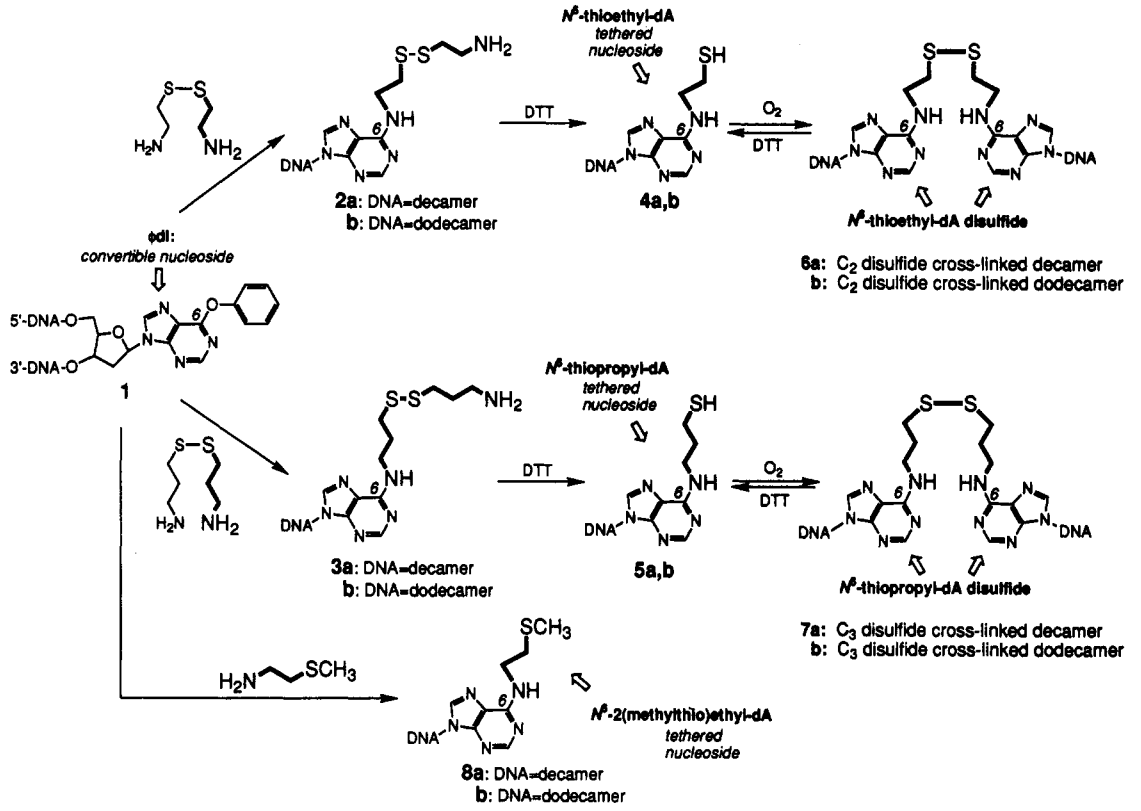
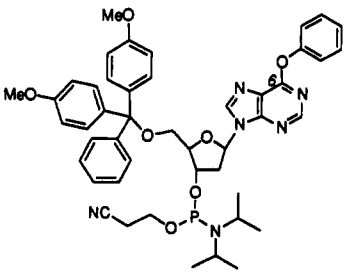
Scheme I



an interstrand disulfide bond.[22] In this method, alkane thiol tethers are attached to the exocyclic amino group of adenines located on opposite strands of the DNA duplex; these tethers are positioned such that they form a disulfide bond under oxidizing conditions. Since the tethers are attached to DNA site-specifically during solid-phase oligonucleotide synthesis and the redox chemistry is efficient and selective for the thiol function only, this synthetic scheme provides a means of achieving absolute sequence-specificity and high yields of homogeneous cross-linked DNA. Because the tethers are attached in such a way that they do not interfere with normal Watson–Crick base pairing, these disulfide cross-links are expected to confer minimal structural distortion upon DNA. Here we report the synthesis of four cross-linked oligonucleotides: the "Dickerson/Drew dodecamer" 5′-d(CGCGAATTCGCG)-3′ and the related decamer 5′-d(GCGAATTCGC)-3′, each bearing a dithiobis(ethane) (C_2) or dithiobis(propane) (C_3) cross-link at the indicated position (A).[23,24] We present general structural data on these duplexes as well as information about the duplex stability of the disulfide cross-linked oligonucleotides and the resistance of their disulfide bonds toward reduction.

Results

Alkane thiol tethers were introduced into DNA via the convertible nucleoside approach.[25] A phosphoramidite of the convertible nucleoside O^6-phenyl-2′-deoxyinosine[26] (φdI phosphoramidite) was synthesized in six steps from 2′-deoxyinosine by standard methods. This was employed, along with commercial



φdI phosphoramidite

reagents, in the synthesis of two oligonucleotides, 5′-d(CGCGAφITTCGCG)-3′ and 5′-d(CGCGAφITTCGCG)-3′, which could then be treated with alkyl amines to convert the φdI moieties to adenines bearing tethers at the exocyclic amino groups (Scheme I). Cleavage of a synthetic oligonucleotide from the resin, deprotection of the phosphate groups, and removal of the base protecting groups all occur under basic conditions (usually NH_4OH); hence, it would seem possible to perform deprotection and φdI conversion in one step using a primary amine. However, it has been shown that undesired side reactions of N^4-protected dC residues can occur when a fully protected oligonucleotide is treated with a primary amine.[25,28] Therefore, we settled on the two-step method reported here: deprotection of all the bases (except the convertible nucleoside) with NH_4OH and then reaction of the convertible nucleoside with the desired amine.

In the oligonucleotide syntheses, phenoxyacetyl (PAC) phosphoramidites were used for C, G, and the unmodified A residue because these acyl protecting groups are sufficiently base-labile to allow for their complete removal prior to amine substitution of φdI: they are displaced within a few hours at room temperature

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in concentrated aqueous NH_4OH,[29] while the half-life of φdI under similar conditions is about 36 h.[26,30,31] The success of the ammonia-deprotection scheme in cleaving the DNA from the resin, deprotecting the phosphodiester backbone, and removing the PAC protecting groups—while leaving the φdI intact—was confirmed by nucleoside compostion analysis of the φdI-containing oligonucleotides after 4-h treatment with NH_4OH.[32] Digestion and HPLC analysis revealed the presence of the four native nucleosides, the φdI, and a second nucleoside having a UV spectrum like that of dG. This peak was identified as N^2-acetyl-dG, which arose from deprotection of PAC-dG by lutidine and subsequent capping during the oligonucleotide synthesis.[33] This side product does not present a problem because it is later converted to dG during the displacement of φdI with amine.

Reverse-phase HPLC purification of the tritylated DNA was performed prior to conversion of the φdI moiety to N^6-alkyl-dA because the trityl group was found to be cleaved partially under the strongly basic conditions of the conversion reaction. Acidic detritylation was performed under standard conditions, and the resulting oligonucleotide was carried on to the next step.

The φdI moiety was then converted to dA bearing a tether at N^6 by an addition–elimination reaction using a primary amine (Scheme I). Reactions with cystamine, 3,3′-dithiobis(propylamine), and 2-(methylthio)ethylamine all resulted in complete conversion to the desired tethered adenine without significant quantities of side products, as assayed by HPLC analysis of the nucleoside products from enzymatic digestion (Figure 1A). Particularly noteworthy is the absence of 2′-deoxyinosine, the product of hydrolysis of φdI. In nucleoside model studies examining substitution vs hydrolysis of φdI, amine concentrations such as those used here (1 M) were sometimes found to yield small amounts (<10%) of dI.[26] The substitution pathway seems to be favored even further in DNA, perhaps reflecting a general decrease in reactivity of φdI within a polynucleotide.[31] The excess amine was removed, and the DNA mixed disulfides **2** and **3** were cross-linked without further purification. DNA of sufficient purity for NMR studies was obtained by HPLC purification only (see above), with no purification after this conversion step or the subsequent cross-linking step. In cases where DNA of higher purity was required, the cross-linked DNA was purified by denaturing polyacrylamide gel electrophoresis (PAGE) at the end of the synthesis.

The sequences investigated in this study are self-complementary and thus spontaneously self-pair to form duplex DNA; in these duplexes, the tethered adenines are located on adjacent base pairs of opposite strands and are thus suitably disposed to form an interstrand disulfide cross-link (Figure 2). The ability to form cross-linked duplexes was examined by denaturing PAGE using oligonucleotides **2** and **3**, the tethers of which contained a mixed disulfide function. Brief treatment of the decamers **2a** and **3a** with 10 mM dithiothreitol (DTT), a disulfide reducing agent, led to a marked increase in mobility (band lower in gel; Figure 3, compare lanes 1 with 3 and 4 with 6) to a position similar to that of a control decamer (lane 7). Small amounts of material having the mobility of the bands in lanes 2 and 5 could also be seen in some instances (not shown). Removal of the reducing agent resulted in virtually quantitative conversion of the DTT-treated decamers to the retarded species (lanes 2 and 5), which had the mobility of a linear 15-mer (lane 8). Vigorous treatment of the

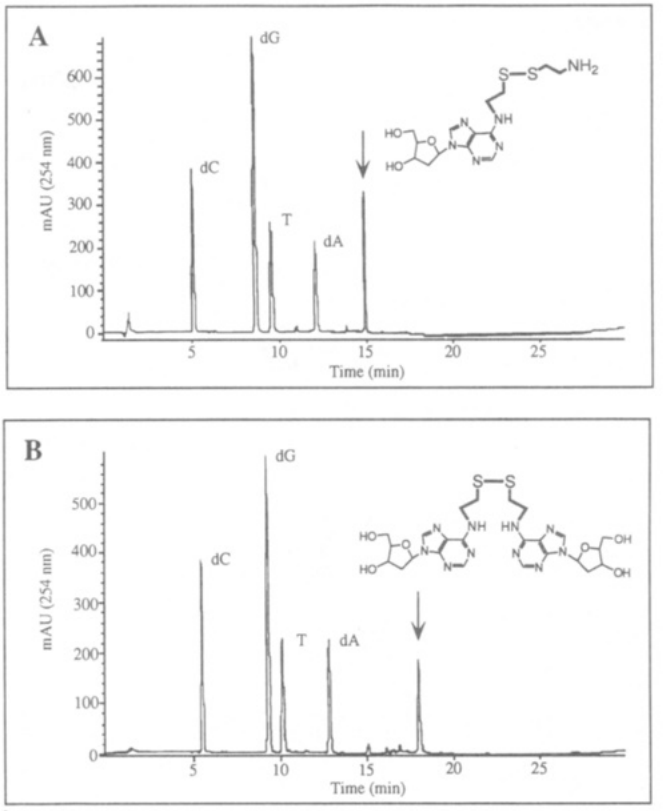

Figure 1. HPLC analysis of the products from enzymatic digestion with phosphodiesterase and alkaline phosphatase of (A) the decamer containing the C_2 mixed disulfide tether **2a** and (B) the C_2 cross-linked decamer **6a** (see Experimental Section for details). Arrows indicate peaks corresponding to the modified adenosines whose structures are shown.

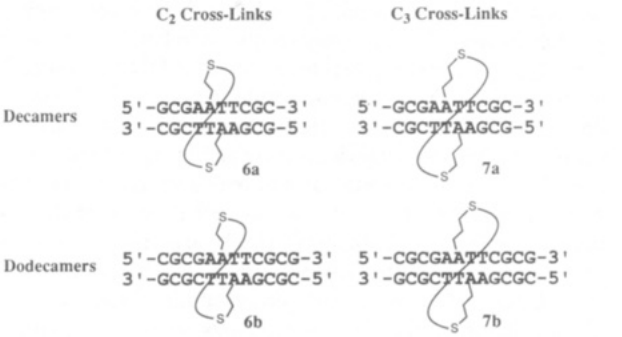

Figure 2. Disulfide cross-linked oligonucleotides synthesized in this study.

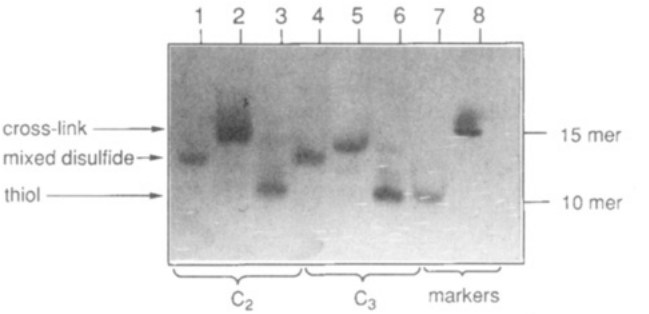

Figure 3. Denaturing polyacrylamide gel of cross-linking reactions. Lanes 1 and 4 show the decamers containing C_2 and C_3 mixed disulfide tethers, respectively (**2a** and **3a**, Scheme I); in lanes 2 and 5 are the disulfide cross-linked products **6a** and **7a** of reduction/oxidation of **2a** and **3a**. Lanes 3 and 6 contain the products of reduction of cross-links **6a** and **7a** with 10 mM DTT. Single-stranded 10-mer and 15-mer size markers are shown in lanes 7 and 8, respectively.

retarded species with DTT resulted in a regeneration of the fastest migrating species. These behaviors are consistent with the expected thiol–disulfide chemistry: (i) reduction of the mixed

(29) Schulhof, J. C.; Molko, D.; Teoule, P. *Nucl. Acids Res.* **1987**, *15*, 397–416.

(30) The widely used benzoyl protecting groups require overnight incubation in NH_4OH at 55–65 °C for their removal, during which time φdI would be converted to adenine.

(31) Addition–elimination reactions of convertible nucleosides seem to proceed more slowly and hence more selectively in DNA than on the nucleoside; see: MacMillan, A. M.; Verdine, G. L. *Tetrahedron* **1991**, *47*, 2603–2616.

(32) Additional data are available in the supplementary material.

(33) C. Brush, personal communication. This side reaction can be avoided by using phenoxyacetic anhydride rather than acetic anhydride in the capping reagent.

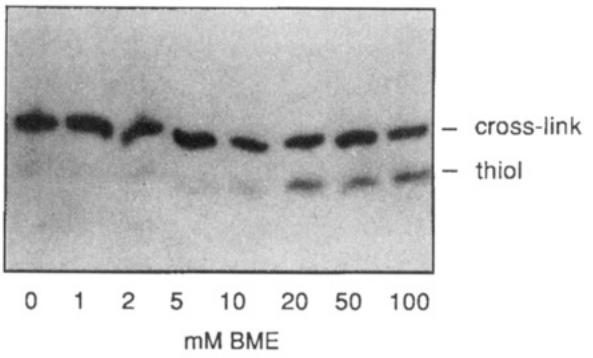

Figure 4. Reduction of C_2 disulfide cross-linked dodecamer with the indicated concentrations of β-mercaptoethanol overnight at room temperature under argon (see Experimental Section for details).

disulfide tethers in **2a** and **3a** releases the positively charged amine function, which is accompanied by an increase in mobility (increase in the charge/mass ratio) of the oligonucleotides; (ii) removal of the reducing agent results in spontaneous oxidation of the thiol tethers to a disulfide; and (iii) reduction of the disulfide cross-linked DNA regenerates the thiol-tethered decamers. Even though the disulfide cross-linked molecules possess 20 nucleotide units, their mobility is more like that of a linear 15-mer. This behavior, which probably arises from their inherently higher compactness relative to that of linear molecules of the same size or their propensity to form transient duplex species even under the denaturing conditions of gel electrophoresis, contrasts with the results seen for mitomycin C cross-linked duplexes, in which the cross-linked products showed lower mobilities than a single-stranded sequence having the same number of nucleotide units.[34]

The cross-linked dodecamers **2b** and **3b** exhibited markedly different behavior. Namely, brief treatment of **2b** and **3b** with DTT resulted in formation of roughly equal amounts of the reduced and cross-linked species (**4b**/**5b** and **6b**/**7b**, respectively; not shown). As with the decamers, air oxidation of the dodecamers resulted in virtually quantitative conversion to the corresponding disulfide cross-linked duplexes, which was reversible by treatment with DTT. The direct cross-linking of **4b** and **5b** upon treatments with dilute DTT probably results from DTT-catalyzed disulfide exchange; the increased propensity of the dodecamers to undergo this reaction as compared to that of the decamers can be ascribed to the increased stability of the dodecamer duplexes and the resulting higher intramolecularity of disulfide formation. For the same reason, the disulfide bond of the cross-linked dodecamers **6b**/**7b** is more resistant to reduction than that of the cross-linked decamers **6a**/**7a**, as discussed below.

Direct evidence for the oligonucleotides being cross-linked was gained by nucleoside composition analysis, which revealed the presence of a non-native nucleoside in roughly the same amount as dA (Figure 1B).[32] This additional species was found to be identical to a disulfide-linked thioalkyl-dA dimer by HPLC retention time and high resolution mass spectral analysis.[32] The oligomerization state of the cross-linked duplexes was established by nondenaturing gel electrophoresis. In this analysis, the cross-linked oligonucleotides exhibited similar mobilities to control duplexes, indicating that they are simple dimers and do not form multimers by disulfide cross-linking between duplexes.

In agreement with observations during the cross-linking reactions, addition of the two extra C–G base pairs substantially stabilizes the cross-linked dodecamers to reducing agents as compared to the decamers (Figure 4). Significant reduction of the C_2 cross-linked decamer was observed at 5 mM BME,[32] but four times this concentration of reducing agent was required to reduce the C_2 cross-linked dodecamer significantly.

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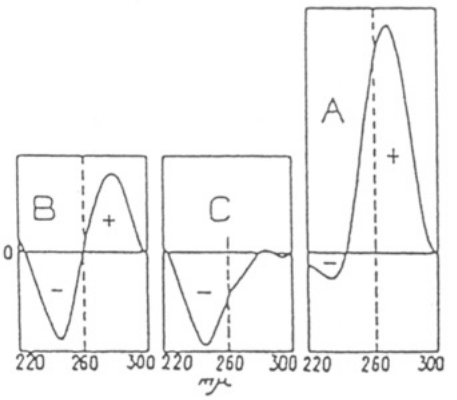
Table I. Melting Temperatures of Decamers and Dodecamers (°C)[a]

	native	C_2 cross-link (**6**)	C_3 cross-link (**7**)	MTE[b] tether (**8**)
[5′-d(GCGAATTCGCG)-3′]$_2$	61	82	80	43
[5′-d(CGCGAATTCGCG)-3′]$_2$	70	89	86	45

[a] Melting temperatures were obtained at a 0.040 ± 0.007 mM concentration of the duplex. For buffer conditions, see the Experimental Section. [b] 2-(Methylthio)ethyl.



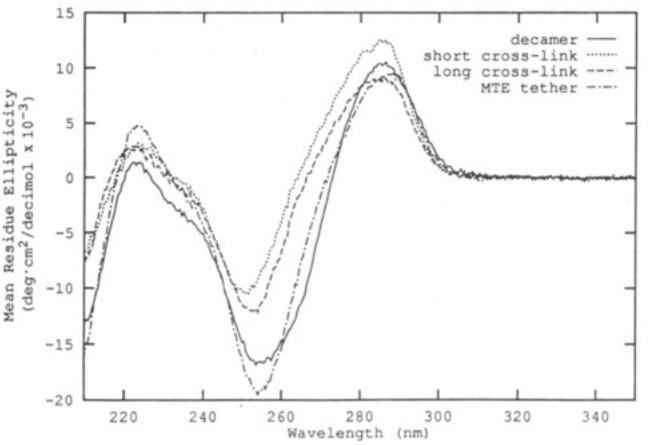


Figure 5. Circular dichroism spectra of native (solid line), MTE-tethered (**8**, dot–dash line), C_2 cross-linked (**6a**, dotted line), and C_3 cross-linked decamers (**7a**, dashed line) at room temperature. Idealized spectra of A-, B-, and C-form DNA are shown for comparison; these are reproduced from ref 35 with permission.

The stability of the cross-linked duplexes to thermal denaturation was also investigated. Cross-linking increased the UV melting temperatures (T_m) of the oligonucleotides by 16–21 °C, a dramatic stabilization of the duplex form (Table I). In both the decamers and dodecamers, the melting temperature of the C_2 cross-linked oligonucleotide exceeds that of the C_3 cross-linked molecule by 2–3 °C, a small but reproducible margin. In contrast, oligonucleotides containing 2-(methylthio)ethyl (MTE) tethers (**8**, Scheme I), which are sterically similar to the C_2 cross-link but are incapable of cross-linking, cause a decrease of 18–25 °C in the T_m relative to the native duplexes (Table I).

The observed cooperative melting behavior provided preliminary evidence that the cross-linked oligonucleotides assume a duplex structure. Evidence for their possessing a B-form-DNA structure was gained from their CD spectra (Figure 5).[32] There are, however, a few differences between the cross-linked and uncross-linked duplexes: the mean residue ellipticity is smaller for the cross-linked duplexes than for the native sequences and the spectrum is shifted to shorter wavelengths. These effects are not observed in the MTE-tethered oligonucleotides and may be due to the contribution of the chiral disulfide to the CD spectrum.[36,37]

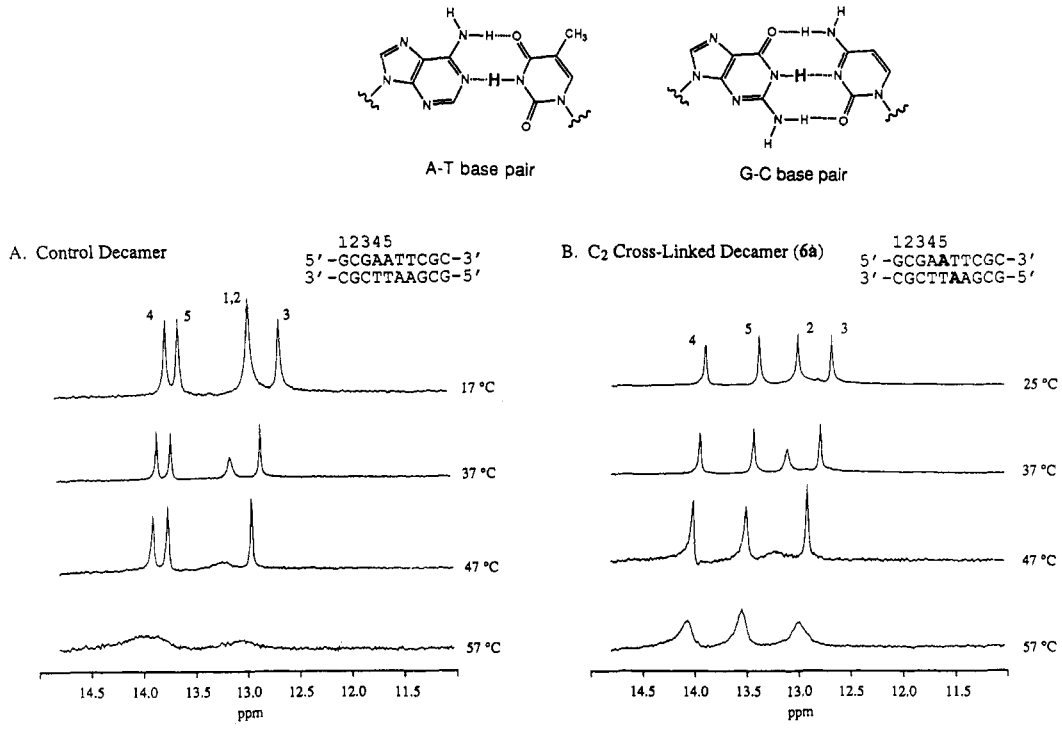


Figure 6. Imino proton spectra of (A) the control decamer and (B) the C_2 cross-linked decamer **6a** as a function of temperature. The imino protons in the A–T and G–C base pairs are shown above the spectra. A lower temperature spectrum (at 17 °C) is shown for the control decamer, in which the imino protons in the terminal base pairs are beginning to appear.

Decreased ellipticity would also be in agreement with an unwinding of the cross-linked duplex as compared to that of the native structure.

The ^{1}H NMR spectra of the imino protons (Figure 6) gave further insight into several structural features. First, the very presence of the imino protons in the low-temperature spectra confirmed that the cross-linked oligonucleotides were stable Watson–Crick base-paired duplexes (Figure 6; data shown for **6a** only).[38] In fact, only the imino proton in the base pair containing the cross-linked adenine (base pair 5) showed a chemical shift that differed from that of the corresponding proton in the control decamer. This change in chemical shift was likely due to some small perturbation in the structure caused by cross-linking. Variable-temperature NMR studies of the imino protons confirmed that the cross-links stabilize the duplexes toward thermal denaturation. Dissociation of a base pair is observed as the disappearance of the peak for the imino proton in that base pair. A comparison of the behavior of the control decamer with that of the C_2 cross-linked decamer **6a** shows that the terminal two base pairs fray at approximately the same temperature (37–47 °C) in these two decamers. However, as the temperature is increased further, the central six base pairs in **6a** persist at temperatures above the temperature at which the control decamer is completely dissociated (57 °C). This means that the change in T_m observed by UV is largely attributable to the increased stability of the center of the duplex, not to changes in the behavior of the terminal two base pairs. In other words, these disulfide cross-links do not stabilize the duplex over long distances of DNA but only at the base pairs immediately adjacent to the cross-link.

Discussion

In this study, we have used the convertible nucleoside O^6-phenyl-2′-deoxyinosine (ϕdI) to insert disulfide-containing tethers into DNA at the N^6 position of adenines. We have shown that these tethers, when introduced as pairs into duplex DNA, can undergo efficient redox chemistry resulting in the formation of an interstrand disulfide cross-link. This approach has been used to synthesize the four modified versions of the "Dickerson/Drew dodecamer"[23,24] sequence 5′-d(CGCGAATTCGCG)$_2$-3′ (**6a**, **6b**, **7a**, and **7b**) shown in Figure 2.

The cross-linking methodology is simple and efficient, and affords high yields of cross-linked products. Incorporation of the mixed disulfide tethers into the DNA after automated syntheis ensures complete site-specificity of the cross-links, while relying on redox chemistry for the cross-linking reaction avoids side reactions with the rest of the DNA. Although all the cross-links discussed here are across 5′-AT-3′ steps, the methodology can be readily extended to other sequences: the ϕdI convertible nucleoside could be used to cross-link any two adenines, such as those in 5′-TA-3′ steps or within longer stretches of DNA. Our laboratory has synthesized analogous convertible nucleosides for the modification of cytosines and guanines at their exocyclic amino groups.[25,31,39] Thus, 5′-CG-3′ and 5′-GC-3′ steps can also be cross-linked between cytosines in the major groove or between guanines in the minor groove. How readily the cross-links are formed seems to depend upon the distance between the tethered amines and the length of the linker. For example, the cross-links described here form readily upon oxidation of the thiol tethers (**4** and **5**, Scheme I), and the distance between the adenine groups in 5′-AT-3′ steps is about 3.5 Å. Guanine cross-links in 5′-CG-3′ steps are also easily formed between amino groups separated by 4.0 Å;[39] however, cytosine C_2 cross-links in 5′-CG-3′ steps, in which the amines are 5.9 Å apart, are considerably more difficult to form,[28] as are cross-links in which the tethered amines are separated by one or more base pairs.[40] One could also envision forming cross-links between adenines and cytosines in 5′-AG-3′ and 5′-GA-3′ steps. The ability to form strained cross-links is important for the design of non-ground-state DNA structures in oligonucleotides, while cross-links in the minor groove are a potentially valuable tool for the study of proteins that bind DNA

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(37) Ludescher, U.; Schwyzer, R. *Helv. Chem. Acta* **1971**, *54*, 1637–1644.
(38) At room temperature (25 °C), the terminal imino protons are not observed because of their rapid exchange with solvent, but as the temperature is lowered further, the resonance for the terminal GC base pair can be observed (e.g., the 17 °C spectrum of the control decamer in Figure 6).[32]
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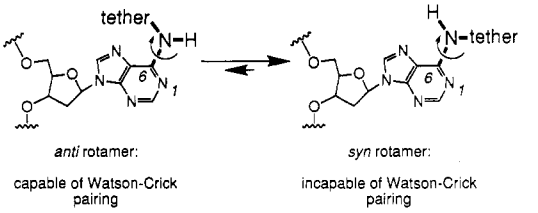
in the major groove. In addition, analogous RNA convertible nucleosides have been synthesized in our laboratory, which will enable us to engineer RNA structure by disulfide cross-linking.[41]

In order for a cross-link to be useful in studying a DNA-binding protein, it must be stable under the same conditions as is the protein. A specific concern in using disulfide cross-links is that the reducing agents often required to maintain the protein in an active form may cause reductive cleavage of the cross-link to a dithiol. To address this issue, we examined the stability of the cross-linked decamers and dodecamers to β-mercaptoethanol (BME), a reducing agent commonly used in protein work. By subjecting the C_2 cross-linked decamer and dodecamer **6a** and **6b** to various concentrations of BME for a prolonged period of time and analyzing the products by denaturing PAGE, we found that the cross-links were remarkably stable to reducing conditions (Figure 4). Moreover, cross-links in the more stable duplex, the dodecamer, showed a lower susceptibility to reduction than those in the decamer. This is likely because the gel analysis reflects an equilibrium between the reduced (**4**) and oxidized (**6**) forms of the cross-link and thus depends upon the rate of oxidation of the reduced species as well as the rate of reduction of the cross-link. Oxidation of the thiol tethers in turn depends upon the stability of the duplex: if the duplex is unstable and is denatured when the cross-link is cleaved, then oxidation of the thiol tethers would have to proceed via an intermolecular reaction; however, if the reduced DNA is a stable duplex, then oxidation could occur through an intramolecular reaction. Thus, a more stable duplex confers an entropic advantage upon the oxidation reaction that converts the thiol tethers into cross-links. One could then expect that disulfide cross-links within GC-rich regions of DNA would be more stable to reduction than those in AT-rich sequences and that longer oligonucleotides should favor disulfide cross-link formation over shorter ones. On the basis of the stability of the cross-linked dodecamer to BME, we expect that tolerance of the disulfide cross-links to the millimolar quantities of reducing agent used to stabilize proteins should not be problematic. The cross-links' stability to reducing agents might be increased further if a negatively-charged reducing agent were chosen, which would be repelled by the negatively-charged DNA backbone. It should be noted that these experiments do not allow for measurement of the rate of thiol–disulfide interchange but only reveal the equilibrium distribution of the products. These cross-links may be able to interconvert with free thiol tethers when catalytic amounts of reducing agent are present.

Another factor important for utilizing these cross-links in studying protein–DNA interactions is the overall stability of the duplexes to thermal denaturation. The T_ms of the cross-linked oligonucleotides were compared to those of the native sequences and control oligonucleotides containing 2-(methylthio)ethyl tethers at the position of the cross-link (**8a**, **8b**; see Table I). The latter serve as thermodynamic controls to measure the effect of un-cross-linked tethers upon duplex stability and allow for separation of the effect of tether attachment from that of cross-linking. The dramatic destabilization of the duplexes containing MTE tethers may be attributed to enthalpic factors: in order to form Watson–Crick hydrogen bonds, the tether must be *anti* to N1 of adenine, and this is the higher energy rotamer around the C6–N^6 bond (Scheme II).[42] Once the duplex has melted, the tether may adopt the lower energy *syn* conformation. These two factors lead to a smaller change in enthalpy during melting of the tethered DNA as compared to that of the native sequence and hence a lower T_m. The stabilization conferred by cross-linking can then be measured as the increase in T_m of the cross-linked duplexes over that of the tethered oligonucleotide.

The large increase in T_m of the cross-linked oligonucleotides can logically be attributed to the entropic destabilization of the

Scheme II



denatured duplex when it is cross-linked. Dissociation of the cross-linked DNA duplex involves a monomeric transition to a more disordered state and is therefore accompanied by a much smaller increase in entropy than is the bimolecular dissociation of an un-cross-linked duplex into two single strands. This change in the entropy factor for the free energy of melting would disfavor the denaturation of a cross-linked duplex and raise its T_m. The entropic disadvantage of melting the cross-links far outweighs the enthalpic advantage of melting the base pairs containing tethered adenines. A more subtle difference in entropy terms may account for the C_3 cross-linked sequences having slightly lower T_ms than the corresponding C_2 cross-linked sequences: because there are more degrees of freedom in the C_3 cross-links than in the C_2 cross-links, there could be a slightly greater gain in entropy upon melting the C_3 cross-links than the C_2 cross-links, which would result in a more favorable melting transition (lower T_m). These rationalizations have yet to be tested experimentally, since direct determination of the free energy stabilization of the cross-linked oligonucleotides into their enthalpic and entropic components is complicated by the fact that the melting transition of cross-linked duplex to single-stranded DNA is monomeric. Since the T_m is independent of the DNA concentration, the thermodynamics cannot be determined by standard UV melting analyses of the concentration dependence of T_m. Cross-links containing tethers that have no enthalpic contribution to the melting behavior would be expected to confer even greater stabilization upon duplex DNA than the ones described here; for example, the attachment of cross-links at C5 of cytosine or uridine would be uncomplicated by the thermodynamics of rotamerization.

The results described here indicate that the cross-linked duplexes are essentially B-form DNA. The CD spectra of the native, tethered, and cross-linked duplexes all have the general profile of B-DNA (Figure 5). In addition, the two-dimensional ^1H–^1H COSY and ^1H–^1H NOESY spectra of the cross-links are very similar to those of the unmodified sequence, indicating that the structures are very similar to one another.[43] Some differences in chemical shift are seen between the imino protons of the cross-linked base pairs and the corresponding protons in the control sequence (Figure 6). The C_3 cross-linked AT base pairs show an imino proton chemical shift between that of the C_2 cross-linked base pairs and that of the native decamer.[32] Yet, all are in the chemical shift range characteristic of Watson–Crick hydrogen-bonded protons. The upfield shift in the cross-linked base pairs relative to the native base pairs could be due to a slight lengthening of the hydrogen bond or a change in the position of the thymine relative to the adjacent bases in the helix. Upfield shifts of imino protons are associated with the more interior base pairs in a duplex,[44] so the upfield shift may simply be a consequence of better base stacking in the duplexes that are stabilized by cross-linking. Thus, for applications in which the effect of cross-linking on DNA structure is a concern, the chemistry reported here has advantages over a more recently described method in which the cross-link necessarily disrupts Watson–Crick base pairing.[45] Ours is not, however, the only method for appending thiol tethers to DNA without disrupting base pairing: thiol tethers

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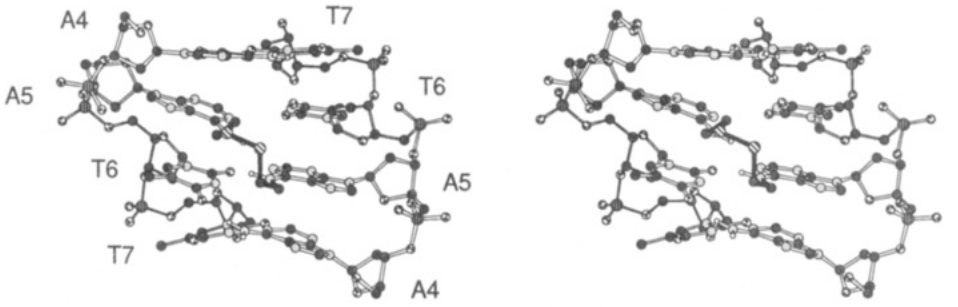

Figure 7. Stereoview of the central four base pairs of the C_2 cross-linked decamer **6a** at 300 K from molecular dynamics using CHARMM.[47] In this view, the cross-link is in the center of the duplex (blacked-out bonds). The duplex is Watson–Crick hydrogen-bonded, and there is little overall distortion of the DNA from the B-form.

have been introduced at the phosphodiester backbone by reaction of an H-phosphonate internucleotide linkage with cystamine.[46]

The lack of duplex perturbation indicated by the NMR spectra is in agreement with the results of molecular modeling of these cross-linked duplexes using the CHARMM program (Figure 7).[47] The models generated in that study indicate that small structural deviations from native B-DNA are localized around the cross-linked base pairs and involve slight changes in the roll angle and propeller twisting. This agrees with the crystal structure of the methylated *Eco*R I site in the Dickerson dodecamer, in which there is little perturbation aside from a slight increase in roll to lengthen the N^6–N^6 distance between the adenines.[48] Thus, the structural changes induced by cross-linking may simply be a consequence of having tethers at N^6 of the adenines and are not correlated with cross-linking per se. Another observation from the modeling was a constraint in the twisting motion around the central base pairs, which indicates that cross-linking may have an impact upon the dynamic structure of the DNA even though it does not greatly affect the average structure of the duplex. The ability to constrain the motions within DNA without significantly altering its structure could be a valuable tool for studying the requirements of proteins for DNA flexibility in protein–DNA complexation. Another indication that the disulfide cross-links do not grossly perturb the DNA structure is found in examining the melting profiles of the imino protons shown in Figure 6. As the base pairs dissociate during heating, the imino protons of the terminal bases are observed to broaden first due to fraying of the terminal base pairs in the helix followed by dissociation of the central six base pairs. This trend holds whether the DNA is cross-linked or not. In contrast, the cross-linking reagent psoralen is known to disrupt the melting of the duplex by conferring asymmetry upon the DNA near the site of cross-linking.[8] It is worth noting that only the melting of the central base pairs is shifted to a higher temperature by cross-linking, while the melting of the terminal base pairs is unaffected (compare peak 2 in Figures 6A and 6B at 37 and 47 °C). This means that the cross-links cause only local stabilization within the central core of the decamer without exerting a stabilizing influence over an extended region of DNA. Thus, disulfide cross-links such as the ones reported here provide a means for covalent attachment of complementary strands of DNA with only highly localized changes in DNA stability.

Conclusion

C_2 and C_3 disulfide cross-linked decamers, 5′-d(GCGAAT-TCGC)-3′, and dodecamers, 5′-d(CGCGAATTCGCG)-3′ (Table I), have been synthesized, and their stabilities to reduction and thermal denaturation have been investigated. The synthesis utilized the convertible nucleoside ϕdI to attach thiol tethers to the exocyclic amino groups of the adenines to be cross-linked. These thiol tethers were readily oxidized to a cross-link under oxidizing conditions. The cross-links are B-form DNA and are much more stable to thermal denaturation than the native oligonucleotides or MTE-tethered control duplexes, most likely because denaturation of the cross-linked duplexes is entropically disfavored. Moreover, the stabilization appears to occur only within a central core of six base pairs surrounding the cross-link and does not act upon more distant base pairs. We believe that such cross-links as these will be useful in studying protein–DNA interactions for several reasons: (1) they allow for covalent connection of complementary strands of DNA without significant perturbation of the duplex structure; (2) the method described here may be readily extended to other sequences, including C–G base pairs, and may be used to construct cross-links in the minor groove of DNA; and (3) the cross-links are stable to the concentrations of reducing agents required for the study of proteins. In addition, the possiblity that disulfide cross-links may constrain the dynamic motions of DNA may open up new possibilities for the study of the dynamic requirements for protein–DNA interactions.

More detailed structural investigations of these cross-linked species are under way using NMR spectroscopy and X-ray crystallography. In addition, detailed thermodynamic characterization of the melting transition by differential scanning calorimetry is being undertaken.

Experimental Section

General Methods. Spectroscopy. ^{1}H NMR spectra were recorded on a Bruker AM-300, AM-400, or AM-500 spectrometer and are reported in parts per million (ppm) with reference to $CHCl_3$ (δ 7.24). ^{13}C NMR spectra were recorded on a Bruker AM-400 (100 MHz) spectrometer and are reported in ppm with reference to $CDCl_3$ (δ 77.0). ^{31}P NMR spectra were recorded on a Bruker WM-300 (121 MHz) spectrometer and are reported with reference to an external standard of 85% H_3PO_4. High-resolution mass spectra (FAB, glycerol matrix) were obtained by Dr. Andrew Tyler of the Harvard University Department Mass Spectrometry Facility on a Kratos MS50 mass spectrometer. IR spectra were recorded on a Nicolet 5PC FT–IR spectrometer. CD spectra were recorded on an AVIV Model 60DS or 60HDS spectropolarimeter, and each spectrum shown is an average of three scans.

Oligonucleotide Quantification. The extinction coefficient of each oligonucleotide was determined by phosphate analysis.[49] The resulting values for the duplexes at 260 nm and 25 °C are as follows: 1.467×10^5 M^{-1} cm^{-1} for the decamer, 1.176×10^5 M^{-1} cm^{-1} for the C_2 cross-linked decamer, 1.103×10^5 M^{-1} cm^{-1} for the C_3 cross-linked decamer, 1.737×10^5 M^{-1} cm^{-1} for the dodecamer, 1.697×10^5 M^{-1} cm^{-1} for the C_2 cross-linked dodecamer, and 1.592×10^5 M^{-1} cm^{-1} for the C_3 cross-linked dodecamer. Concentrations of oligonucleotides were then determined from the UV absorbance at 260 nm and 25 °C as measured on a Hewlett-Packard 8452A diode array spectrophotometer.

Nucleoside Composition Analysis. An oligonucleotide (20 nmol) in distilled water (33 μL) was incubated with 1 M Tris·Cl, pH 9.0 (5 μL),

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1 M NaCl (5 μL), 140 mM $MgCl_2$ (5 μL), and snake venom phosphodiesterase (2 units) at room temperature for 2 h. To this were added 1 M Tris·Cl, pH 9.0 (15 μL), 10 mM $ZnCl_2$ (1 μL), 1 M $MgCl_2$ (1 μL), water (33 μL), and calf intestine alkaline phosphatase (1 unit), and incubation was continued at 37 °C for another 2 h. Enzymes were removed by filtration through a Millipore Ultrafree-MC 10-kDa molecular weight cutoff filter. HPLC analysis of the resulting nucleosides was performed on a Hewlett-Packard HP 1090 liquid chromatograph equipped with a diode array detector and a 4.6- × 250-mm Beckmann C_{18} Ultrasphere column (2 mL/min). Gradient: 100% 0.02 M KH_2PO_4, pH 5.4, at 0 min; 25% 60:40 methanol:water in 0.02 M KH_2PO_4 at 10 min; 100% 60:40 methanol:water at 15 min; 100% 60:40 methanol:water at 25 min; and 100% 0.02 M KH_2PO_4 at 30 min. Retention times: dC, 5.1 min; dG, 8.7 min; T, 9.6 min; dA, 12.1 min; N^6-(6-amino-3,4-dithiahexyl)-dA (C_2 mixed disulfide dA), 14.9 min; N^6-(8-amino-4,5-dithiaoctyl)-dA (C_3 mixed disulfide dA), 16.1 min; N^6-thioethyl-dA disulfide (C_2 cross-linked dA), 18.0 min; and N^6-thiopropyl-dA disulfide (C_3 cross-linked dA), 19.9 min. Assignments were based upon comparison of the UV spectra and the retention times of the DNA-derived nucleosides with those of authentic synthetic standards. The assignments of peaks corresponding to modified adenosines were confirmed by mass spectral analysis of the eluted material.

Gel Electrophoresis. Oligonucleotides were electrophoresed in 20% (19:1) denaturing (7 M urea) or nondenaturing polyacrylamide gels. Samples were prepared by adding an equal volume of loading dye to the reaction mixture (95% formamide with 20 mM EDTA and 0.05% bromophenol blue and xylene cyanole for denaturing gels; glycerol for nondenaturing gels). Gels were prerun for 1 h prior to loading the DNA. Analytical samples (200 pmol/lane) were visualized by staining with 0.2% (w/v) methylene blue. Preparative (3 mm) gels were run at 4 °C, and the samples were viewed under UV light, cut out, crushed, and soaked overnight in 1 M triethylammonium bicarbonate (TEAB), pH 7.5, at room temperature. After this time, the supernatant was removed and the DNA was further extracted once with 1 M TEAB and three times with 25 mM TEAB. This solution of DNA was loaded onto a C_{18} Sep-Pak cartridge (Waters), which was washed with 20 mL of 25 mM TEAB. The DNA was then eluted in 6 mL of 30% CH_3CN in 100 mM TEAB. The resulting fractions were lyophilized.

Phosphoramidite Synthesis. O^6-Phenyl-2′-deoxyinosine (φdI) was synthesized from 2′-deoxyinosine as reported previously.[26]

5′-O-(4,4′-Dimethoxytrityl)-O^6-phenyl-2′-deoxyinosine. O^6-Phenyl-2′-deoxyinosine (6.2 g, 19 mmol) was dissolved in 50 mL of freshly distilled pyridine. 4,4′-Dimethoxytrityl chloride (6.75 g, 20 mmol) and 4-(dimethylamino)pyridine (100 mg, 0.8 mmol) were added. The reaction mixture was stirred under nitrogen at room temperature in the dark for 16 h and was monitored by TLC (R_f of product = 0.55 in ethyl acetate). The pyridine was removed *in vacuo* as an azeotrope with heptane. Flash chromatography of the product with 90:5:5 ethyl acetate:methanol:triethylamine yielded 5′-O-(4,4′-dimethoxytrityl)-O^6-phenyl-2′-deoxyinosine (9.4 g, 15 mmol, 79%) as a white foam. ^{1}H NMR ($CDCl_3$): δ 2.55–2.61 (m, 1H, H2′), 2.82–2.89 (m, 1H, H2′), 3.36–3.45 (m, 2H, H5′), 3.76 (s, 6H, $-OCH_3$), 4.17–4.21 (m, 1H, H4′), 4.68–4.72 (m, 1H, H3′), 6.50 (dd, J = 6.5, 6.5 Hz, 1H, H1′], 6.78–7.45 (m, 18H, aryl Hs), 8.16 (s, 1H, H8), 8.42 (s, 1H, H2). ^{13}C NMR ($CDCl_3$): δ 40.2, 55.2, 63.7, 72.4, 84.6, 86.2, 86.6, 113.1, 121.8, 125.8, 126.9, 127.9, 128.0, 129.1, 129.6, 129.9, 135.5, 141.6, 144.4, 152.0, 152.2, 158.5, 160.1. HRMS calcd for $C_{37}H_{34}N_4O_6$ (M + H) 631.2556, found 631.2564.

5′-O-(4,4′-Dimethoxytrityl)-O^6-phenyl-2′-deoxyinosine 3′-(2-cyanoethyl-N,N-diisopropyl) Phosphoramidite (φdI Phosphoramidite). 5′-O-(4,4′-Dimethoxytrityl)-O^6-phenyl-2′-deoxyinosine (6.3 g, 10 mmol) was dissolved in 60 mL of methylene chloride under nitrogen. N,N′-Diisopropylethylamine (9.5 mL, 55 mmol) was added followed by 2-cyanoethyl phosphorochloridate (2.4 mL, 11 mmol). After being stirred for 2 h at room temperature in the dark, the reaction mixture was concentrated and the product purified by flash chromatography with 90:5:5 ethyl acetate:methanol:triethylamine. The phosphoramidite was recrystallized from ethyl acetate–hexanes to yield 8.0 g (9.6 mmol, 96%, R_f = 0.74 in ethyl acetate) of material of sufficient purity for use in DNA synthesis. It can be stored stably at −20 °C for years without significant decomposition. ^{1}H NMR ($CDCl_3$): δ 1.10–1.30 (m, 12H, $(CH_3)_2CHs$), 2.47 and 2.61 (2t, J = 6.3 Hz), 2H, CH_2CN), 2.64–2.75 (m, 1H, H2′), 2.91–2.99 (m, 1H, H2′), 3.35–3.90 (m, 6H, H5′′s, $POCH_2$–, $(CH_3)_2CHs$), 3.77 (m, 6H, $-OCH_3$s), 4.32–4.36 (m, 1H, H4′), 4.79–4.83 (m, 1H, H3′), 6.50–6.54 (m, 1H, H1′), 6.77–7.45 (m, 18H, aryl Hs), 8.21 (d, J = 8.8 Hz, 1H, H8), 8.43 (d, J = 1.7 Hz, 1H, H2). ^{13}C NMR ($CDCl_3$): δ 20.2, 20.3, 22.9, 24.5, 39.5, 43.1, 43.3, 55.2, 58.1, 58.2, 58.3, 58.4, 63.3, 63.5, 73.4, 73.6, 74.1, 74.2, 84.8, 85.8, 86.0, 86.4, 113.1, 117.4, 117.6, 121.8, 122.2, 125.7, 126.9, 127.8, 128.1, 129.1, 129.6, 130.0, 135.6, 141.7, 144.4, 152.0, 152.3, 152.5, 158.5, 160.2. ^{31}P NMR ($CDCl_3$): δ 149.3, 149.4. HRMS calcd for $C_{46}H_{51}N_6O_7P$ (M + H) 831.3635, found 831.3646.

Oligonucleotide Synthesis. Synthesis and Purification. The oligonucleotides 5′-DMT-d(GCGAφITTCGC)-3′ and 5′-DMT-d(CGCGAφITTCGCG)-3′ (φI = O^6-phenylinosine) were synthesized on an Applied Biosystems Model 381A DNA synthesizer using phenoxyacetyl-protected phosphoramidites (PAC amidites, Pharmacia), φdI phosphoramidite (above), and benzoyl-dC or -dG CPG resin (10-μmol columns, Applied Biosystems). φdI phosphoramidite was used as a 0.1 M solution in acetonitrile, and the 10-μmol synthesis cycle was modified to add an extra 2 min to the coupling time for this base. With this modification, there is no notable difference between the efficiency of coupling for this amidite and for commercially available ones (the phosphoramidite of unmodified 2′-deoxyinosine also requires a longer time for complete coupling; Applied Biosystems, manual for 381A DNA synthesizer). The resin from each synthesis was incubated in concentrated ammonium hydroxide (6 mL) at room temperature for 4 h, after which time the supernatant was removed and the resin washed with NH_4OH (3 mL). Triethylamine (200 μL) was added to the ammonium hydroxide solution, which was then lyophilized to dryness. The residue was taken up in 0.1 M triethylammonium acetate (TEAA), pH 8.0 (1.5 mL), and triethylamine (50 μL) for purification by reverse-phase HPLC using a 7.0- × 305-mm Hamilton PRP-1 column (45 °C, 2.5 mL/min) on a Hewlett-Packard HP 1090 liquid chromatograph. Gradient: 20% acetonitrile in 0.1 M TEAA, pH 8.0, at 0 min; 30% acetonitrile at 10 min; 30% acetonitrile at 25 min; and 20% acetonitrile at 30 min. Fractions containing trityl-on oligonucleotide, which eluted at 12–15 min, were lyophilized to yield purified DNA. To detritylate the oligonucleotide, it was taken up in water (0.97 mL) and the pH was lowered to 3.0 with glacial acetic acid (120 μL). The DNA was incubated at room temperature under these conditions for 10 min, the reaction was quenched by freezing on dry ice, and the mixture was lyophilized. The residue was taken up in water (1 mL) and extracted three times with ethyl acetate (1 mL). This yielded 3.5–4.5 μmol (90–92% stepwise yield) of detritylated oligonucleotide per 10-μmol synthesis.

Preparation of Oligonucleotides Containing N^6-(2-(Methylthio)ethyl)-2′-deoxyadenosine. Oligonucleotides containing φdI (375–400 nmol) were dissolved in 500 μL of 1 M 2-(methylthio)ethylamine and incubated at 65 °C for 16 h. The reactions were neutralized with acetic acid, diluted to 20 mL with 25 mM TEAB, and loaded onto a C_{18} Sep-Pak (Waters). The Sep-Pak was washed with 20 mL more of 25 mM TEAB to remove excess amine, and the DNA was eluted in 6 mL of 30% CH_3CN in 100 mM TEAB, which was then lyophilized to dryness. The resulting tethered DNA was purified by gel electrophoresis (see the General Methods above). This yielded 200–300 nmol (53–75%) of product.

Preparation of Mixed Disulfides 2 and 3. Oligonucleotides containg φdI were dissolved in 1 M amine (cystamine or 3,3′-dithiobis(propylamine), 1 mL per μmol of DNA. Mixtures were incubated at 65 °C for 18 h. The reaction was then neutralized with acetic acid, and the excess amine was removed by repeated centrifugal dialysis (Centricon-3 microconcentrator, Amicon) or using a C_{18} Sep-Pak cartridge, as described above. Nucleoside analysis confirmed that the φdI residue has been cleanly converted to N^6-(6-amino-3,4-dithiahexyl)-dA or N^6-(8-amino-5,6-dithiaoctyl)-dA (see Figure 1). This resulted in a 95–100% yield of oligonucleotides containing mixed disulfides that were used without further purification.

Preparation of Disulfide Cross-Linked Oligomers. Oligonucleotides containing mixed disulfides (1 μmol per mL of solution) were incubated in 10 mM dithiothreitol (DTT) or β-mercaptoethanol (BME) in 10×TE (100 mM Tris·Cl and 10 mM EDTA, pH 8.0, degassed) under nitrogen at 37 °C for 10–12 h. The reducing agent was removed by dialysis (Centricon-3) or C_{18} Sep-Pak cartridge under aerobic conditions so as to allow air oxidation to the disulfide cross-link. This yielded double-stranded cross-linked oligonucleotides (80–100% yield) with no detectable amounts of starting material as assayed by analytical gel electrophoresis. The resulting cross-linked decamers were typically of sufficient purity to be used directly in NMR studies without purification.

Oligonucleotide Characterization. T_m Measurements. Thermal denaturation curves were acquired on a Perkin-Elmer Model 575 spectrophotometer equipped with a thermoelectrically controlled cell holder and interfaced to a computer. Samples were 30–60 μM in duplex concentration, 1 M in NaCl, 1 mM in EDTA, and 10 mM in sodium phosphate buffer at pH 7. Data were obtained by heating the samples

from 5 to 110 °C at 0.5 °C/min. Melting temperatures were obtained from the data as described previously.[50]

CD Spectroscopy. Samples for CD spectra were 1 M in NaCl, 0.1 mM in EDTA, and 10 mM in sodium phosphate buffer (pH 7). Duplex concentrations were 35–50 μM. Spectra were obtained from 210 to 350 nm at temperatures low enough to insure that the duplex was completely intact: 25 °C for the cross-links and native duplexes, 20 °C for the MTE-dodecamer, and 10 °C for the MTE-decamer. Data were normalized to account for concentration differences between samples.

Reduction with β-Mercaptoethanol (BME). The C_2 cross-linked decamer and dodecamer (40 pmol/μL) were incubated at room temperature for 12 h in 1, 2, 5, 10, 20, 50, and 100 mM BME in 1×TE, pH 8.0 (20 μL total volume). Samples were analyzed on a 20% denaturing polyacrylamide gel as described under the General Methods above.

Imino Proton Spectra. Samples (1.0–1.6 μmol of duplex) were dissolved in 0.4 mL of 10 mM sodium phosphate buffer, pH 7.5, lyophilized, and resuspended in 360 μL of H_2O and 40 μL of D_2O. All spectra were recorded on a Bruker AM-500 spectrometer. One-dimensional ^1H spectra and ^1H–^1H NOESY spectra of the imino protons were obtained using a 1331 pulse sequence for water suppression.[27] NOESY spectra were acquired at 12 °C with a mixing time of 200 ms. Data were collected into 2048 complex points in t_2 and 450 points in t_1. For each value of t_1, 32 scans were acquired with a relaxation delay of 2.0 s.

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Supplementary Material Available: Experimental procedures for the synthesis of 3,3′-dithiobis(propylamine), NMR spectrum of φdI phosphoramidite, data for the cross-linked dodecamers **6b** and **7b**, results from nondenaturing gel electrophoresis of all the cross-linked duplexes and from reduction of the C_2 cross-linked decamer **6a** with BME, mass spectra of cross-linked nucleoside dimers isolated from **6a** and **7a**, two-dimensional NOESY spectra of the control decamer and the C_2 and C_3 cross-linked decamers **6a** and **7a**, and imino proton spectra of **7a** (14 pages). Ordering information is given on any current masthead page. The material may also be obtained from the authors by FAX at (617)495-8755.