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# DNA Interstrand Cross-Linking Reactions of Pyrrole-Derived, Bifunctional Electrophiles: Evidence for a Common Target Site in DNA

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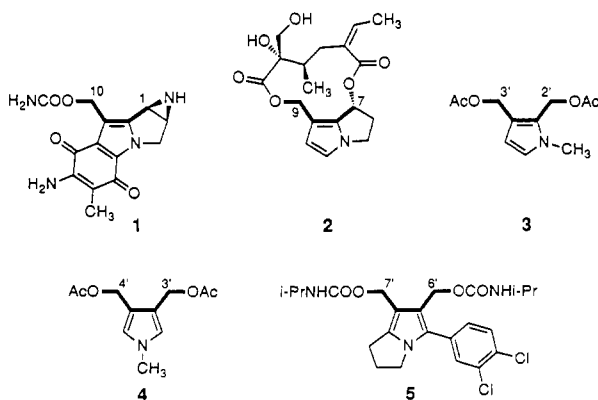
**Abstract:** The site of DNA interstrand cross-linking identified by a family of pyrrole-derived bifunctional electrophiles was studied in vitro in synthetic DNA duplexes. This family includes reductively activated mitomycin C (**1**), oxidatively activated pyrrolizidine alkaloids (e.g. **2**), the simple pyrroles 2,3- and 3,4-bis-(acetoxymethyl)-1-methylpyrrole (**3** and **4**), and the antitumor substance 2,3-dihydro-5-(3',4'-dichlorophenyl)-6,7-bis(hydroxymethyl)-1*H*-pyrrolizine bis-(isopropylcarbamate) (IPP, **5**). It is demonstrated herein that these agents preferentially cross-link a common target site in duplex DNA, the exocyclic amino groups of deoxyguanosine residues at the sequence 5'-d(CG). Specifically, **4** and **5** are shown to form interstrand cross-links in synthetic DNA, **4** and **5** (like **1** and **3**) are shown to preferentially cross-link deoxyguanosine residues at the duplex nucleotide sequence 5'-d(CG), and **3**, **4**, and **5** are shown to require both N<sup>2</sup> exocyclic amino groups of the potentially bridged deoxyguanosine residues for efficient interstrand cross-linking. Taken together, these results strongly implicate N<sup>2</sup> of deoxyguanosine residues at the sequence 5'-d(CG) as the common site of the covalent interstrand cross-linking reaction of these agents. This connectivity was unequivocally proven for the cross-link caused by **5** through isolation and characterization of the relevant lesion from an enzymatic digest of interstrand cross-linked DNA and comparison to an authentic sample prepared by rational chemical synthesis. New syntheses of **3** and **4** are reported.

## Introduction

Substances which are capable of covalently linking the strands of duplex DNA to one another have proven useful as both antitumor substances and in furthering our understanding of the structure and reactivity of nucleic acids. When the first members of this class were discovered some thirty years ago, the available technologies provided only a low-resolution view of DNA interstrand cross-linking reactions and their products.<sup>1</sup> During the intervening period, particularly the past decade, emerging technologies have markedly improved this situation. As our knowledge of these structures and the reactions which afford them increases, the search for unifying structure–activity relationships is facilitated. The subject of this paper is a common theme in the interstrand cross-linking reaction products of a structurally diverse family of pyrrole-derived, bifunctional alkylating agents. Some thirty years ago, a structural similarity between the reductively activated mitomycins (e.g. **1**) and

dine, e.g. **2**) was noted.<sup>2</sup> Several groups speculated that the shared interstrand cross-linking activity of these agents could be explained by the chemically reactive, pyrrole-containing substructure common to both.<sup>2,3</sup> This hypothesis is fully consistent with the subsequent findings that other agents possessing this substructure cross-link DNA (**3**,<sup>4,5</sup> **4**<sup>6</sup>) or have antitumor activity (e.g. **5**<sup>7</sup>), a property of many interstrand cross-linking agents. A consequence of this unifying hypothesis is that the DNA cross-links formed by all of these agents (**1**–**5**) will share a common covalent substructure and that the agents may cross-link a common target site (sequence of nucleotides and specific sites of alkylation), but this has never been directly demonstrated. We provide in this paper the first strong evidence that the pyrrole-derived bifunctional alkylating agents described herein do in fact preferentially cross-link synthetic DNA duplexes at a common target site, the exocyclic amino groups of deoxyguanosine residues at the sequence 5'-d(CG). The implications of these findings on the mechanistic origin of 5'-d(CG) selectivity are briefly discussed.

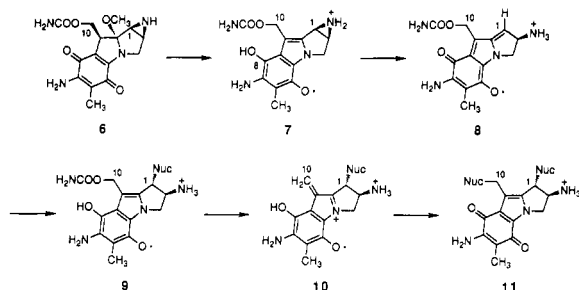
Of the cross-linking reactions discussed herein, the best characterized is that of mitomycin C (**6**, Scheme I), an antitumor antibiotic whose biological target is believed to be DNA.<sup>8</sup> From studies in several laboratories has emerged a mechanistic picture of the action of reductively activated mitomycin C (MC) as a bifunctional electrophile, summarized in Scheme I.<sup>9</sup> The precise redox state of the quinone function remains controversial,<sup>10</sup> and the one-electron-reduced form depicted is arbitrary. In overview,



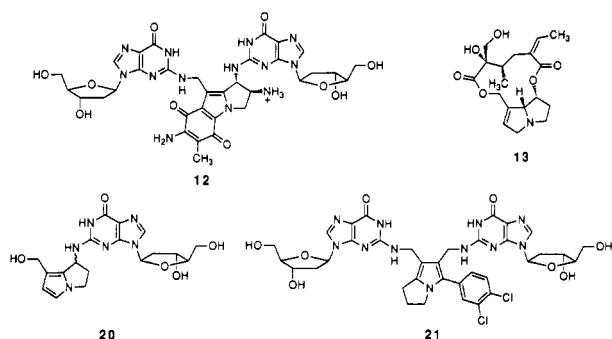
oxidatively activated pyrrolizidine alkaloids (dehydropyrrolizi-

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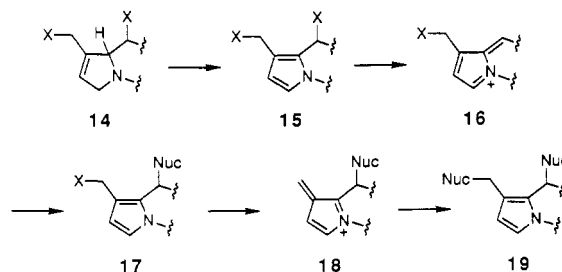
**Scheme I.** Presently Accepted Mechanistic Sequence for Interstrand Cross-Linking of DNA by Reductively Activated Mitomycin C

this process involves reductive activation of mitomycin C (**6**) which results in formation of the reduced pyrroloquinone **7**, followed by sequential departure of leaving groups coupled with entry of nucleophilic groups from the biopolymer (**7** → **8** → **9** → **10** → **11**). Particularly relevant to the present work is the isolation of **12** from nuclease/phosphodiesterase digestion of MC-



treated DNA in vitro or from the liver of MC-treated rats.<sup>11</sup> Conjugate **12** of two deoxyguanosine residues and mitomycin C clearly reveals the points of attachment of the biopolymer and drug to one another. Studies in three laboratories taken together indicate that lesion **12** is responsible for interstrand cross-linking and is formed preferentially at the duplex sequence 5'-d(CG).<sup>12-15</sup> There are no comparable reports of covalent characterization for any other pyrrole-derived interstrand cross-link.

It is well established that pyrrolizidine alkaloids (e.g. retrorsine, **13**) after oxidative activation are interstrand cross-linking agents.<sup>4,16,17</sup> Chemical oxidation in vitro<sup>4,16</sup> or hepatic oxidation in vivo<sup>17</sup> affords the relevant dehydro derivatives (dehydropyrrolizidines) analogous in structure to known interstrand cross-linking pyrrole **3**, both of which are generically depicted in Scheme II as **15**. The hypothesis of mechanistic and structural analogy between MC and the pyrrolizidines suggests the speculative cross-linking reaction mechanism outlined in Scheme II. Direct evidence for the reactions with DNA shown in this scheme in the pyrrolizidine alkaloid family is limited to the isolation or spectroscopic detection of monoadducts of all of the common

**Scheme II.** Probable Generic Mechanistic Sequence for Cross-Linking of DNA by Bifunctional Pyrroles Such as **2**, **3**, and **4**

deoxynucleosides with one or the other of C<sup>7</sup> and C<sup>9</sup> of the dehydropyrrolizidine nucleus,<sup>18</sup> for example, diastereoisomers **20**, in which N<sup>2</sup> of deoxyguanosine is conjugated at C<sup>7</sup> with dehydroretrorsine.<sup>19</sup> No conjugate of two deoxynucleosides with a single dehydropyrrolizidine has been reported. No attempts to isolate conjugates of **3** with DNA have been reported. Furthering the analogy with reductively activated MC, both the dehydropyrrolizidines and simple pyrrole **3** have been shown to preferentially cross-link synthetic duplex DNA fragments containing the sequence 5'-d(CG).<sup>5</sup> For pyrrole **3**, that linkage has been shown to bridge deoxyguanosine residues,<sup>5</sup> but the site of linkage on deoxyguanosine has not been unambiguously determined.

In the course of exploring the structure-biological activity relationships of pyrrole-derived bifunctional alkylating agents, a family of 6,7-disubstituted pyrrolizidine diesters has been synthesized and tested for antitumor activity.<sup>7</sup> Among these was the diisopropylcarbamate pyrrolizine **5** (IPP), which was especially impressive in its antitumor activity. This substance bears obvious structural analogy to pyrrole **4**. There are no prior reports concerning the DNA interstrand cross-linking reactions or resulting conjugates with DNA of pyrroles **4** and **5**, but it is natural to extend the hypothetical cross-linking reaction mechanism outlined in Scheme II to embrace these substances.

The goal of the present work was to establish unequivocally whether there exists a common structure for the interstrand cross-links formed by the pyrrole-based DNA interstrand cross-linking agents **1-5**. We report herein experiments which prove that 2,3- and 3,4-bis(acetoxymethyl)-1-methylpyrrole (**3** and **4**) both require both of the exocyclic amino groups (N<sup>2</sup>) of the deoxyguanosine residues at the sequence 5'-d(CG) for efficient interstrand cross-linking. The DNA interstrand cross-link afforded by IPP (**5**) is unequivocally shown herein to be due to the presence of residue **21**, as revealed by direct isolation of **21** from enzymatic digests of interstrand cross-linked DNA and comparison to an authentic sample obtained by rational chemical synthesis. Together, these data provide strong support for the argument that the DNA interstrand cross-links formed by this family possess a common substructure. We speculate that this structural parallel reflects an underlying mechanistic parallel in forming the lesions, the details of which remain to be elucidated.

## Results and Discussion

**Stability of Duplex DNAs.** All of the DNAs studied herein were 18-mers in which only the central four residues were varied (see Table I). Accordingly, these central residues are used as descriptors herein. We verified at the outset that the duplex/single strand equilibria of these oligodeoxynucleotides did in fact reside predominantly on the side of duplex under the conditions of ionic strength and pH used for the cross-linking reactions. This was accomplished by determination of the thermal denaturation profiles monitored at 260 nm (Table I). The oligodeoxynucleotides AGCT and ACGT (see Table I), whose cross-linking efficiencies are compared below, exhibited very similar

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**Table I.** Interstrand Cross-Linking of DNAs with 3, 4, and 5

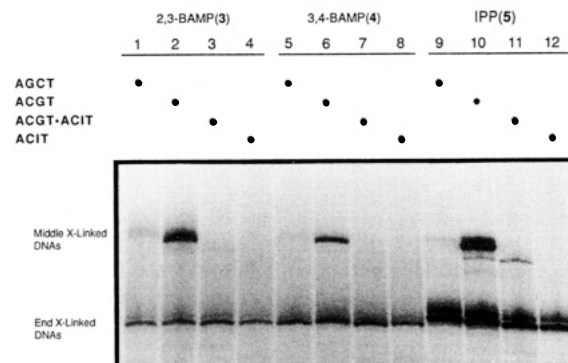
descriptor	DNA	cross-link yield (%) <sup>a</sup>			<i>T<sub>m</sub></i> (°C) <sup>a</sup>
		3	4	5	
AGCT	5'HO-TATAATAGCTATTATA-OH <sub>3'</sub> 3'HO-ATATTATCGATAATAT-OH <sub>5'</sub>	0.02 <sup>b</sup>	0.01 <sup>b</sup>	0.00	46
ACGT	5'HO-TATAATACGTATTATA-OH <sub>3'</sub> 3'HO-ATATTATGCATAATAT-OH <sub>5'</sub>	0.20	0.08	0.49	46
ACGT-ACIT	5'HO-TATAATACGTATTATA-OH <sub>3'</sub> 3'HO-ATATTATCATAATAT-OH <sub>5'</sub>	0.00	0.00	0.03 <sup>b</sup>	<i>c</i>
ACIT	5'HO-TATAATACITATTATA-OH <sub>3'</sub> 3'HO-ATATTATCATAATAT-OH <sub>5'</sub>	0.00	0.00	0.00	36

<sup>a</sup> See Experimental Section for conditions. <sup>b</sup> The indicated yield represents the most abundant electrophoretic band that is similar but not identical in mobility to cross-linked ACGT. The covalent structure of this substance is unknown. Not determined.

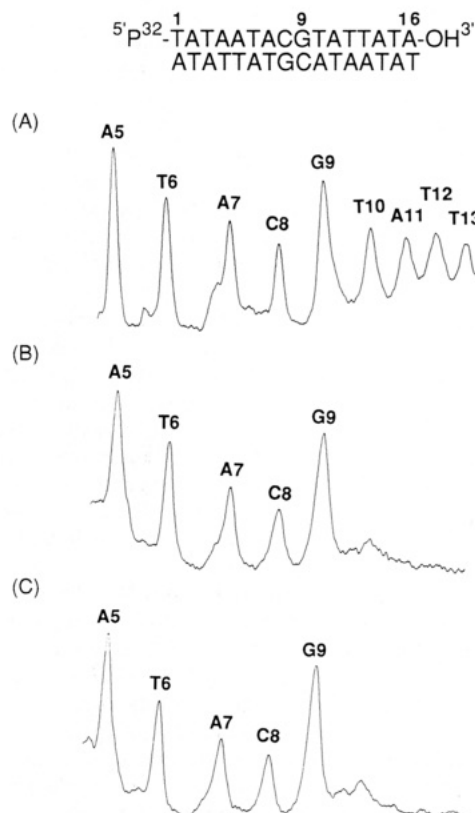
melting profiles, with, for our purposes, essentially identical *T<sub>m</sub>*'s (half maximal hyperchromicity using baseline correction at high- and low-temperature extremes<sup>20</sup>) of 46° at 300 mM sodium ion concentration, pH 7.1. The homoduplex of oligodeoxynucleotide ACIT, which failed to cross-link with the agents studied herein (see below), exhibited a *T<sub>m</sub>* of 36°, demonstrating that failure to cross-link was not a consequence of a lack of duplex structure. It is expected but was not experimentally verified that the melting temperature of the heteroduplex of ACIT with ACGT, which likewise failed to cross-link (see below), is intermediate between those of the homoduplexes of ACIT and ACGT, and that the failure to cross-link was again not a consequence of lack of duplex structure.

**Sequence Specificity and Functional Group Requirements of Cross-Linking.** The activity of 3,4-bis(acetoxymethyl)-1-methylpyrrole (4) and IPP (5) as DNA interstrand cross-linking agents had not previously been demonstrated. These substances were independently incubated with the 3'-<sup>32</sup>P-radiolabeled oligodeoxynucleotide duplexes ACGT and AGCT and analyzed by denaturing PAGE (Figure 1). Immediately noteworthy is that 3, 4, and 5 provide a "high-mobility" interstrand cross-link with all of the DNAs studied. This phenomenon is commonly observed with highly reactive electrophiles and has been clearly shown for 3 to be due to cross-linking near the strand termini of the duplex.<sup>5</sup> The biological relevance of these "end-cross-links", if any, is at present unknown. Because the focus of the present work was reactivity distant from DNA termini, these bands were not further studied. Instead, the less electrophoretically mobile bands previously proven for 3 to be dG-to-dG, "middle" cross-links were of greatest interest. As has been observed for 1,<sup>12-15</sup> 2,<sup>5</sup> and 3,<sup>5</sup> the 5'-d(CG)-containing DNA was cross-linked in at least a 10-fold preference to the 5'-d(GC)-containing DNA with both 4 and 5 (see Table I). Iron(II)-EDTA-promoted cleavage of ACGT cross-linked with 4 or 5 clearly revealed deoxyguanosine-to-deoxyguanosine connectivity (Figure 2).<sup>21</sup>

To probe the functional group requirements of dG-to-dG cross-linking by 3-5, we utilized substitution of the deoxyinosine residue for deoxyguanosine. These residues are identical to one another with the critical exception that deoxyinosine bears a hydrogen atom at C<sup>2</sup> where deoxyguanosine bears the exocyclic amino group which is necessary for MC cross-linking reactions. We attempted to cross-link the homoduplex of ACIT, which entirely lacks the minor-groove amino groups, as well as a heteroduplex of ACIT with ACGT, which possesses a single minor-groove amino group. The heteroduplex was obtained by admixture of a 100-fold molar excess of "cold" ACIT with 3'-<sup>32</sup>P-radiolabeled ACGT. Denaturing PAGE analysis (Figure 1) clearly revealed the importance of the minor-groove exocyclic amino groups on the reaction product distribution for 3, 4, and 5. Although the product distributions were subtly different in each case, the common result



**Figure 1.** DPAGE analysis of the interstrand cross-linking reactions of DNAs in Table I with bifunctional electrophiles 3, 4, and 5.

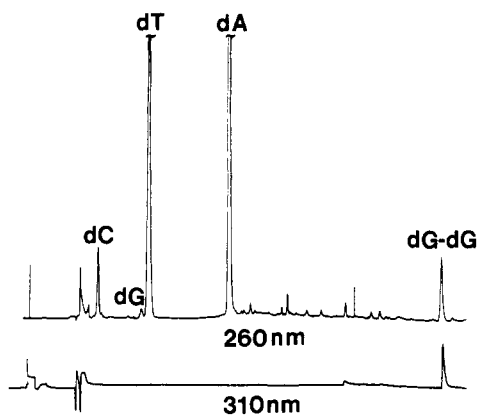


**Figure 2.** Densitometric scan of DPAGE-resolved 5'-<sup>32</sup>P-radiolabeled DNA fragments following iron(II)-EDTA-catalyzed cleavage: (A) before exposure to 4 or 5; (B) interstrand cross-linked by exposure to 4; (C) interstrand cross-linked by exposure to 5.

was a substantial reduction in the amount of cross-linked DNA with mobility previously shown to correspond to the dG-to-dG cross-link. These data clearly demonstrate that both exocyclic amino groups (N<sup>2</sup>) of deoxyguanosine are critical for the formation of the dG-to-dG interstrand cross-link. The simplest interpre-

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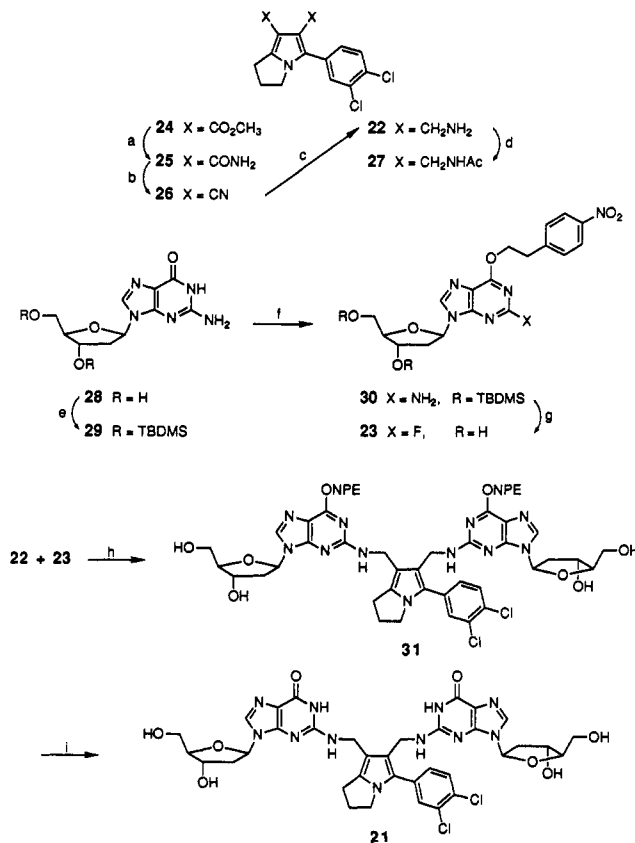
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**Figure 3.** HPLC analysis of enzymatic hydrolysate of interstrand cross-linked ACGT from treatment with **5**. Detection at 260 nm (upper) and 310 nm (lower). (Retention times increase to right).

tation of this observation is that the N<sup>2</sup> atoms of deoxyguanosine are the common sites of covalent attachment of **3**, **4**, and **5** to both strands of DNA.

**Isolation and Characterization of the dG-to-dG Lesion Formed by IPP (**5**) at 5'-d(CG).** In order to unequivocally determine the sites of covalent attachment of one of these pyrroles to interstrand cross-linked DNA, we isolated and characterized the covalent structure of one such lesion. The duplex DNA ACGT (90 OD units) was cross-linked with IPP (**5**) at pH 5.2, where the yield of the least electrophoretically mobile interstrand cross-linked product was found to be 4%. This band was separated from the resulting crude mixture using preparative, denaturing polyacrylamide gel electrophoresis to yield 1.0 OD unit of interstrand cross-linked DNA. This material was subjected to enzymatic degradation using a mixture of DNase I, snake venom phosphodiesterase, and calf intestinal alkaline phosphatase. The hydrolysate was analyzed by RP HPLC with dual detection at 260 nm (primarily nucleic acid) and 310 nm (pyrrole nucleus only) (Figure 3). Quantitation of the returned nucleosides at 260 nm revealed the molar ratio of 14.0 (dA):1.8 (dC):0.18 (dG):13.5 (dT), two deoxyguanosine residues short of the original calculated (and experimentally verified) ratio of 14:2:2:14. The near absence of deoxyguanosine in the digested cross-linked sample clearly indicated that the dG residues were the site of lesion formation. There was in addition a new peak having the long-wavelength chromophore of the cross-linking agent. Quantitation of this peak based on the reasonable assumption that the eluted substance has  $\epsilon_{260}$  approximately twice that of deoxyguanosine (the cross-linking agent **5** has a low absorbance at this wavelength) accounted for 80% of the "missing" deoxyguanosine residues. The retention time of this substance was considerably longer than that of the parent nucleosides, as is commonly seen in conjugates of this type.<sup>11,22,23</sup> The positive ion electrospray ionization mass spectrum of this substance gave parent ions  $M + H^+$  and  $M + Na^+$  for  $M = 809$ , the mass expected of two deoxyguanosine residues plus one of IPP (**5**) less 2 mol of *N*-isopropylcarbamic acid. Because deoxyguanosine is the most massive of the four common deoxynucleosides, no other pairing could provide a comparable mass. Fragment ions corresponding to loss of one and two sugar residues indicated that it was the heteroaromatic bases which were connected to one another, not the deoxyribose functions. The mass spectroscopic data was thus consistent with formulation of the lesion as **21** but did not distinguish this material from other covalent structures in which the bifunctional alkylating portion of IPP bridges the heteroaromatic bases of two deoxyguanosine residues. The UV spectrum of this substance was strongly pH dependent between pH 7 ( $\lambda_{max}$  254 and 280 nm) and



**Figure 4.** Synthesis of authentic **21**: (a) HCONH<sub>2</sub>, NaOCH<sub>3</sub>, 20% CH<sub>3</sub>OH/DMF; (b) (i) oxalyl chloride, DMF, CH<sub>3</sub>CN, (ii) pyridine; (c) LiAlH<sub>4</sub>, THF; (d) Ac<sub>2</sub>O, Et<sub>3</sub>N, DMAP, CH<sub>2</sub>Cl<sub>2</sub>; (e) TBDMSCl, imidazole, DMF; (f) *p*-nitrophenethyl alcohol, PPh<sub>3</sub>, DEAD, *p*-dioxane; (g) *t*BuONO, HF, pyridine; (h) Et<sub>3</sub>N, DMF; (i) DBU, DMF, CH<sub>3</sub>CN.

pH 12.5 ( $\lambda_{max}$  260 nm), indicating the presence of an ionizable functional group. The changes in maximum and extinction coefficient were qualitatively consistent with N<sup>2</sup> alkylation and inconsistent with O<sup>6</sup> alkylation.<sup>24</sup> The thermal stability of the original cross-link argues against N<sup>7</sup> as the alkylation site.

To unequivocally establish the covalent structure of the lesion responsible for DNA cross-linking by **5**, we synthesized an authentic sample of **21** for comparison with the material isolated from interstrand cross-linked DNA. This synthesis relied on the coupling of diamine **22** with 2 equiv of the protected 2-fluoro-2'-deoxyinosine derivative **23** (Figure 4). This approach assured the connection of the substituted pyrrole to what was at the outset N<sup>2</sup> of the deoxyguanosine derivatives. The known diester **24**<sup>25</sup> (Figure 4) was converted to diamine **22** by sequential processing to the diamide **25** and dinitrile **26**, followed by reduction to diamine **22**. The highly polar diamine **22** was characterized as its diacetate derivative **27**. O<sup>6</sup>-Protected 2-fluoro-2'-deoxyinosine **23** was prepared by a modification of the literature method from 2'-deoxyguanosine (**28**).<sup>26</sup> The 3'- and 5'-hydroxyl functions of 2'-deoxyguanosine were protected as the TBDMS derivatives; then O<sup>6</sup> was protected as the NPE derivative. Finally, the primary amine was converted to the requisite 2-fluoro derivative **23**. Coupling of the diamine **22** with an excess of the protected fluorodeoxyinosine **23** was achieved at 25 °C in Et<sub>3</sub>N/DMF to afford **31**, the NPE-protected form of **21**. The 500-MHz <sup>1</sup>H NMR and FAB ionization mass spectra of this substance were consistent with the indicated formulation. Removal of the NPE groups from **31** was achieved smoothly with DBU in CH<sub>3</sub>CN/

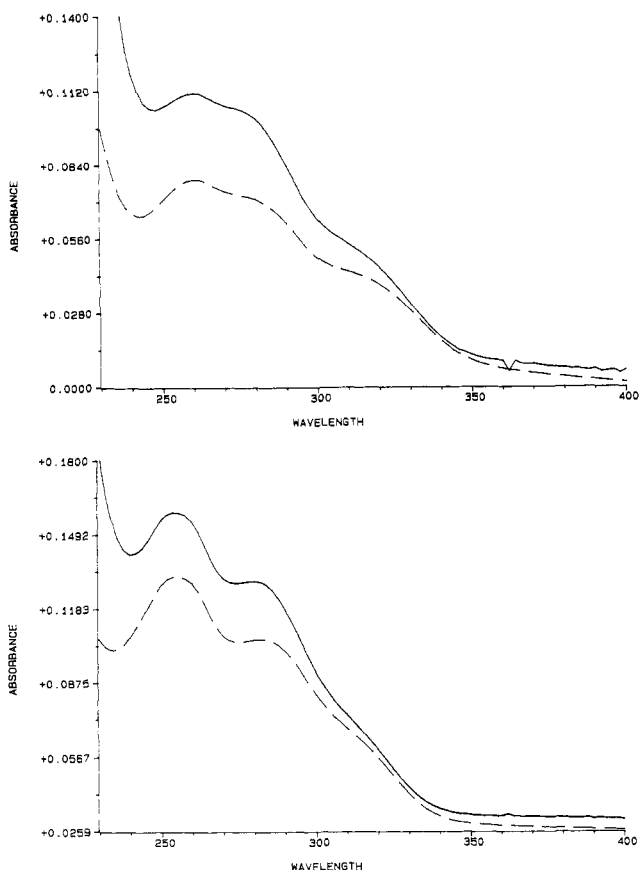
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**Figure 5.** Ultraviolet spectra of synthetic **21** (---) and **21** isolated from 5-cross-linked ACGT (—):(upper) pH 12.5; (lower) pH 7.0.

DMF to afford the authentic sample referred to hereafter as synthetic **21**.<sup>27</sup>

That synthetic **21** had the structure depicted and was additionally identical in all respects to the putative **21** from interstrand cross-linked DNA was demonstrated as follows: Synthetic **21** was a single substance on RP-HPLC, which in two gradient solvent systems coeluted with admixed **21** from interstrand cross-linked DNA. The electrospray ionization mass spectrum of synthetic **21** was fully consistent with the assigned structure, having in common with **21** from interstrand cross-linked DNA all of the key fragment ions. The UV spectra of synthetic **21** and **21** from interstrand cross-linked DNA at pHs 7.0 and 12.5 (Figure 5) further support the identity of the two samples.

**Molecular Modeling of Pyrrole-Cross-Linked DNA.** The AMBER<sup>28</sup> force-field was used to generate an energy-minimized, computer model of IPP (**5**)-cross-linked DNA (Figure 6). This model at once reveals the approximate alignment of the long axis of the agent with the minor groove of B-DNA, but with the pyrrolizine moiety substantially displaced from the center of the

groove. This situation is reminiscent of that suggested for the solution structure of the mitomycin C cross-link derived from a combination of solution <sup>1</sup>H NMR measurements and computerized energy minimization.<sup>29</sup> Two conformations were judged deserving of initial consideration, one in which the phenyl substituent projects toward the 3'-end of the strand with which the cross-linking agent makes more intimate contact and the alternative with the phenyl group projecting toward the 5'-end. Inspection of space-filling models and preliminary molecular mechanics calculations revealed that, in the former structure, there were substantial unfavorable van der Waals interactions of the phenyl substituent and sugar-phosphate backbone of B-DNA. The DNA portion of the resulting energy-minimized structure was very substantially distorted relative to B-DNA. This structure was not further studied. The alternative conformation in which the phenyl group projects toward the 5'-end of the more intimately contacted strand readily accommodated the phenyl substituent with some structural distortion of B-DNA (Figure 6). Widening of the minor groove was required to accommodate the three contiguous methylene groups of the pyrrolizine nucleus. The computed structure featured some bending of the helix axis away from the cross-linking agent. The significance of the latter is unknown, given the modest length of the modeled oligodeoxynucleotide (hexamer). The computer model predicts at most minor torsional reorganization of B-DNA in accommodating this cross-link.

#### Implications on the Mechanistic Origin of Sequence Specificity.

DNA interstrand cross-linking reactions are multistep processes which involve distinct monoadduct formation and monoadduct-to-cross-link formation steps. Sequence specificity could in principle arise in either or both of these events. For the best studied of the pyrrole-derived cross-linking agents, reductively activated mitomycin C, the net specificity has been experimentally demonstrated to have its origin in both selective monoadduct formation (ca. 10:1, flanking sequence-dependent preference for 5'-d(CG) over 5'-d(GC))<sup>30</sup> and a relatively more efficient progression of monoadduct to cross-link at 5'-d(CG) than at 5'-d(GC).<sup>31</sup> The underlying physical interactions which promote these specificities have been the subject of both experiment and speculation but remain incompletely understood. The more modest net interstrand cross-linking sequence specificity of the much simpler pyrroles **3**, **4**, and **5** for the 5'-d(CG) sequence has not yet been dissected into monoadduct- and cross-link-forming steps. The finding of a *reduced* rather than *eliminated* specificity is fully consistent with the notion that more than one factor contributes to the net high selectivity of mitomycin C. In other words, structural attributes of mitomycin C not present in **3**, **4**, and **5** which have been speculated to contribute to sequence specificity remain as candidates for a contribution but are clearly revealed not to be *solely responsible* for sequence specificity. Among these is the chirality at C<sup>1</sup>, which we have speculated enforces the minor-groove orientation of the monoadduct, which is required for cross-linking at 5'-d(CG) but which is inconsistent with cross-linking at 5'-d(GC).<sup>15</sup> It can likewise be concluded that one or more of the elements of mitomycin C missing in **3**, **4**, and **5** must in fact contribute to the high interstrand cross-linking sequence specificity of the former, because, in its (their) absence, the net specificity is reduced. More modest changes in the mitomycin skeleton will be necessary to delineate specifically which functionalities make critical contributions.

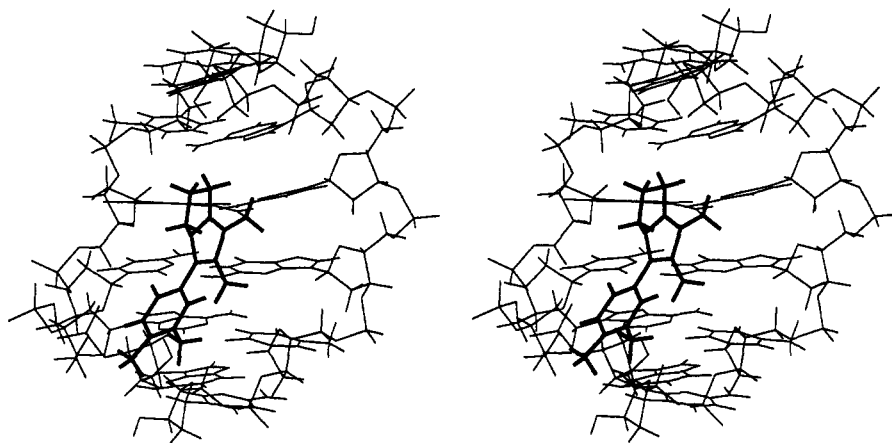
(27) We attempted to isolate and characterize the lesion responsible for interstrand cross-linking by **3**. This effort was plagued by the low yield of interstrand cross-linked DNA (less than 1%), which could not be improved by variation of exposure time, temperature, concentration of **3**, pH, or ionic strength. The HPLC profile of digests of 3-cross-linked DNA had in common with Figure 3 (for 5-cross-linked DNA) the substantial reduction in quantity of recovered dG, along with a peak which coeluted with a synthetic sample of the substance analogous to **21** synthesized by substituting 2,3-bis-(aminomethyl)-1-methylpyrrole for **22**. However, the size of the latter peak varied from one experiment to the next and never exceeded ca. 30% of the amount required for the expected stoichiometry. Additionally, there were ca. 5 other peaks of similar mobility and intensity. From the other evidence presented in this paper, it is highly probable that **3** cross-links DNA at 5'-d(CG), from dG-to-dG, N<sup>2</sup>-to-N<sup>2</sup>. We suspect but have not proven that the initially formed lesion scavenges further 3-derived electrophiles at a rate comparable with cross-linking. While this has precluded the covalent characterization of this lesion, it is in no way in conflict with the central conclusions of this paper.

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**Figure 6.** Stereoscopic representation of computer simulated, energy-minimized structure of the duplex 5'-d(GACGTC) interstrand cross-linked with **5** (bold). In this simulation, two methyl groups were substituted for the two chlorine atoms of **5**.

## Conclusion

We present herein evidence that the pyrrole-derived DNA interstrand cross-linking agents mitomycin C (**1**), the pyrrolizidine alkaloids (e.g. **2**), simple bifunctional pyrroles **3** and **4**, and the synthetic antitumor substance **5**, all of which in their active forms share a common bifunctional electrophilic substructure, preferentially cross-link a common target site in DNA, the exocyclic amino groups of deoxyguanosine residues at the duplex sequence 5'-d(CG). Specifically, substances **4** and **5** are shown herein to create interstrand cross-links which bridge deoxyguanosine residues in synthetic DNA fragments which contain that sequence, a fact shown in earlier work also to be true for pyrrole **3**. For all three of pyrroles **3**, **4**, and **5**, it is shown by substitution of deoxyinosine residues at the putative cross-link site that both N<sup>2</sup> amino groups of the potentially linked deoxyguanosine residues must be present or cross-linking efficiency is greatly reduced, consistent with the covalent linkage occurring at these sites. To unequivocally demonstrate this connectivity, the covalent nucleus of the interstrand cross-link formed by **5** was identified as **21** by direct isolation and comparison with an authentic synthetic sample. A computer model of this lesion in a short DNA fragment revealed that some structural distortion of B-DNA is required to accommodate this cross-link, a prediction which has not been experimentally pursued. The finding that the specificity for **3**, **4**, and **5** for dG-to-dG interstrand cross-linking at 5'-d(CG) over 5'-d(GC) (ca. 10:1) is considerably reduced but not eliminated relative to that of reductively activated mitomycin C (>100:1) is consistent with the model that more than one structural component of mitomycin C contributes to its net sequence specificity.

## Experimental Section

**Materials and Methods.** Materials and their sources were as described by Kirchner et al.<sup>22</sup> except as follows: [ $\alpha$ -<sup>32</sup>P] dATP, Amersham; DNase I, Pharmacia; IPP, a gift from Professor W. Anderson. For autoradiography, the dried gels were autoradiographed on Amersham Hyperfilm-MP. Densitometric (Hoeffer GS-300, interfaced to an IBM PC) data were smoothed and plotted using Spectra Calc (Galactic Industries Corp., Salem, NH). HPLC detection utilized sequential SSI 500 UV/vis (output to both an HP 3390A electronic integrator and a GOW-MAC Model 70-150 recorder) and RAININ Dynamax model UV-1 (output to a Linear Model 255/mm recorder) detectors. Solvent gradients for analytical separations and co-injections were run at 1 mL/min as follows. Gradient A: solvent A, 100 mM ammonium acetate (pH 7.0); solvent B, acetonitrile; isocratic 95% A for 4 min, 11-min linear gradient to 70% A, 15-min linear gradient to 60% A, 10-min linear gradient to 20% A, then a 10-min linear gradient to initial conditions. Gradient B: the same as gradient A except solvent A, 0.1 mM ammonium formate. Gradient C: solvent A, 0.1 mM ammonium formate; solvent B, methyl alcohol; 90% A for initial, 5-min linear gradient to 70% A, 15-min linear gradient to 10% A, then a 10-min linear gradient to initial conditions. Solvent gradients for preparative separations were run at 2 mL/min as follows. Gradient

D: solvent A, 0.1 mM ammonium formate; solvent B, acetonitrile; 90% A for initial, 5-min linear gradient to 70% A, 15-min linear gradient to 30% A, then a 10-min linear gradient to initial conditions.

General Synthetic Procedures were as follows: Air- or water-sensitive reactions were conducted under a positive-pressure argon atmosphere. Commercial reagents were used as received, except for the following: acetonitrile, CH<sub>2</sub>Cl<sub>2</sub>, and pyridine were distilled under argon from calcium hydride; deuterated chloroform, DMF, DMSO, and dioxane were dried over 4-Å molecular sieves; diethyl ether and tetrahydrofuran were distilled under argon from sodium benzophenone ketyl; methanol was dried over 3-Å molecular sieves. Column chromatography was performed under slight positive pressure on Merck silica gel 60 (230–400 mesh); thin-layer chromatography was performed on precoated silica gel 60 plates (0.25 mm). Infrared (IR) spectra were recorded as a thin film on a NaCl plate on a Perkin-Elmer Series 1600 FTIR spectrophotometer. Proton nuclear magnetic resonance spectra (<sup>1</sup>H NMR) were determined on a Bruker AC200 (200 MHz) or a Bruker AMX500 (500 MHz) spectrometer and, unless otherwise noted, are reported in parts per million downfield from internal tetramethylsilane (0.00 ppm). <sup>19</sup>F NMR spectra were determined on a Bruker AC200 (188.15 MHz) spectrometer and are reported in parts per million downfield from external trifluoroacetic acid (76.53 ppm). Coupling constants (*J*) are given in Hertz. Low-resolution mass spectra (LRMS) were measured on a KRATOS Profile HV3 mass spectrometer using direct sample insertion and electron impact (EI) ionization; high-resolution FAB mass spectra (FAB-HRMS) and low-resolution mass spectra (FAB-LRMS) were measured on a VG70SEQ double-focusing mass spectrometer. Electrospray mass spectra (ESMS) were recorded on a Sciex Atmospheric Pressure Ionization (API) triple-quadrupole mass spectrometer. Ultraviolet (UV) spectra were measured on a Hewlett-Packard Model 8452A UV/vis spectrophotometer and are reported as wavelength in nanometers.

**Preparation and Radiolabeling of DNA.** Oligodeoxynucleotides were synthesized using both Applied Biosystems Model 380A and 382 synthesizers and were purified as described by Kirchner et al.<sup>22</sup> For most experiments, the 16-mer DNAs in Table I were prepared in <sup>32</sup>P-labeled form by Klenow filling of the appropriate 15-mer duplex one residue shorter at the 3'-terminus using [ $\alpha$ -<sup>32</sup>P]dATP. This was conducted using 0.1 OD<sub>260</sub> of DNA in 20  $\mu$ L of Klenow buffer [10 mM Tris (pH 7.5), 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM DTE], 30  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]dATP, and 4 units of Klenow fragment at 25 °C for 0.33 h. Radiolabeling was stopped by addition of 10  $\mu$ L of 3 M aqueous sodium acetate (pH 5.2) and 70  $\mu$ L of water, followed by heating to 75 °C for 5 min. The resulting sample was treated with 0.7 mL of cold ethanol (–20 °C). After 1 h at –20 °C, the resulting precipitate was collected by centrifugation, redissolved in 0.1 mL of 0.3 M sodium acetate (pH 5.2), and precipitated by addition of 0.7 mL of cold ethanol. After 1 h at –20 °C, the precipitate was collected by centrifugation and used in the following experiments.

Cross-linked DNA used in the Fe(II)–EDTA fragmentation experiments was from 5'-radiolabeled duplex DNA prepared using [ $\gamma$ -<sup>32</sup>P]-ATP and T4 polynucleotide kinase.

**UV-Monitored Thermal Denaturation.** Samples were prepared by dissolving 0.7 OD<sub>260</sub> of DNA (ca. 2.66 nmol of duplex; 5'- and 3'-termini as free hydroxyls) in 1500  $\mu$ L of pH 7.1, 50 mM MOPS buffer containing 5 mM MgCl<sub>2</sub> and 300 mM NaCl. *T*<sub>m</sub>'s were determined as described by Kirchner et al.<sup>22</sup> The resulting data are shown in Figure 7.



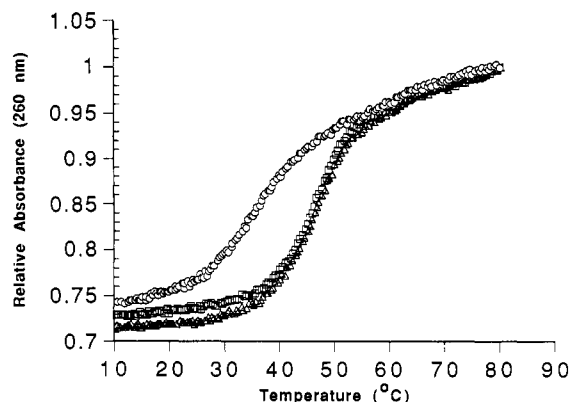


Figure 7. UV-monitored thermal denaturation profiles of ACGT (□), AGCT (Δ), and ACIT (O) (see Table I).

**Reaction of 3, 4, and 5 with DNA.** One-tenth of the previously  $3'$ -radiolabeled DNA ( $\sim 0.01$  OD<sub>260</sub>) was placed in a microfuge tube. To the tube was added 180  $\mu$ L of reaction buffer [50 mM MOPS (pH 7.1), 5 mM MgCl<sub>2</sub>, 300 mM NaCl] containing 1 OD<sub>260</sub> of the unirradiated, complementary 16-mer DNA. The samples were heated to 90 °C and cooled to 25 °C over 3 h, followed by treatment with 20  $\mu$ L of 10 mM 3 (or 4) in CD<sub>3</sub>CN at 25 °C. After being vortexed, the mixture was allowed to stand for 10 min at 25 °C. The reaction was stopped by addition of 1.2 mL of cold ethanol ( $-20$  °C). After 1 h at  $-20$  °C, the resulting precipitate was collected by centrifugation and rinsed with 80% cold ethanol ( $-20$  °C). The rinsed and dried sample was analyzed using 25% DPAGE. For IPP (5), the reaction time was extended to 16 h.

**DPAGE Analysis of Cross-Link Yield.** Pelleted DNA was dissolved in 10  $\mu$ L of loading buffer [90% deionized formamide, 10 mM Tris (pH 7.5), 0.1% xylene cyanol, 0.1 mM EDTA]. The samples were denatured at 90 °C for 1 min and chilled on ice prior to 25% PAGE (19:1 acrylamide/bisacrylamide, 50% urea, 0.35 mm thick, 41  $\times$  37 cm). Gels were prepared as follows: 23.75 g of acrylamide, 1.25 g of bisacrylamide, and 50 g of urea were dissolved in 10 mL of 10  $\times$  TBE and 20 mL of water. The volume was brought to 100 mL with water, and 350  $\mu$ L of 20% aqueous ammonium persulfate was added prior to filtering through Whatman #5 filter paper. To the solution was added 25  $\mu$ L of TEMED to induce polymerization, and the gel was poured. Pre-run of electrophoresis on a Hoeffer thermostateded Poker Face gel stand was performed for 1 h. Samples were loaded using flat sequencing tips (Marsh Biomedical) on a Rainin P-200 Pipetman, and the gel was run at 80 W with a Bio-Rad Model 3000 XI power supply until the xylene cyanol dye traveled 14–16 cm ( $\sim 4$  h). The gel was transferred onto filter paper covered with Saran Wrap and dried for 1 h followed by autoradiography or phosphorimager. Autoradiography was used to visualize the single-strand and cross-linked DNA.

**Iron(II)–EDTA–Promoted Fragmentation.** Gel slices containing  $5'$ -radiolabeled cross-linked DNA were excised from the above described 25% DPAGE. DNA was eluted from the gel slice using the crush and soak procedure described by Weidner et al.<sup>5</sup> In order to obtain sufficiently radiolabeled cross-linked DNA for cleavage reactions, the purified cross-linked DNA was reradiolabeled at the  $5'$ -end using [ $\gamma$ -<sup>32</sup>P]ATP and T4 kinase. Cleavage reactions of the cross-linked DNA duplexes were conducted on  $>20$  000 cpm (Geiger counter) of DNA in 14  $\mu$ L of 10 mM Tris (pH 7.5) and 10 mM NaCl. To this DNA solution was added 2  $\mu$ L each of 10 mM ascorbic acid, 0.3% hydrogen peroxide, and 0.5 mM (NH<sub>4</sub>)<sub>2</sub>Fe(SO<sub>4</sub>)<sub>2</sub> containing 1 mM EDTA. After 1 min at 25 °C, the reaction was stopped by the addition of 2  $\mu$ L of 0.1 M thiourea. These samples, along with comparison lanes containing uncleaved, cross-linked, and native (uncross-linked) DNA, a Maxam–Gilbert guanine-specific sequencing reaction on native DNA, and an iron(II)–EDTA-promoted fragmentation on native DNA, were dried, suspended in 4  $\mu$ L of loading buffer, heated to 90 °C for 1 min, iced, and loaded onto a 25% DPAGE. The electrophoresis was carried out at 65 W, at ca. 65 °C until the xylene cyanol dye had run 10–12 cm from the origin. The gel was dried and autoradiographed.

**Enzymatic Hydrolysis and Quantitation of Nucleosides by HPLC.** Cross-linked (0.1 OD<sub>260</sub>) or synthetic ACGT (0.1 OD<sub>260</sub>) in 30  $\mu$ L of aqueous 10 mM MgCl<sub>2</sub>, 50 mM Tris (pH 8.9) was treated with 1  $\mu$ L (10 units) of DNase I, 1  $\mu$ L (12.7 units) of alkaline phosphatase, and 4  $\mu$ L (2 units) of phosphodiesterase I at 37 °C for 4 h. HPLC analysis was carried out on 10  $\mu$ L of the above reaction mixture using gradient A. Peaks were identified by comparison of retention times to those of

authentic, commercial samples. Quantitation was based on the peak area ratios obtained from a standard, equimolar mixture prepared by weight of dC, dG, dT, and dA at 260 nm, which were as follows: dC, 1.0; dG, 1.5; dT, 1.1; dA, 1.8. Using these response factors, the uncross-linked DNA analyzed as 1.0 (dC): 1.0 (dG):6.6 (dT):7.0 (dA) (calcd: 1.0:1.0:7.0:7.0); cross-linked DNA analyzed as 1.8 (dC):0.18 (dG):13.5 (dT):14.0 (dA) (calcd: 2.0:0.0:14.0:14.0).

**Preparative DNA Cross-Linking.**  $3'$ -Radiolabeled ACGT (5 OD<sub>260</sub> per tube; 19 nmol of duplex per tube) was placed in each of 18 microfuge tubes. To each tube was added 180  $\mu$ L of 0.3 M aqueous sodium acetate (pH 5.2) and 300 mM NaCl, followed by treatment with 20  $\mu$ L of 25 mM IPP (5) in CD<sub>3</sub>CN at 25 °C. After being vortexed, the mixtures were allowed to stand for 16 h at 25 °C. Each resulting sample was treated with 1.2 mL of cold ethanol ( $-20$  °C), followed by cooling to  $-20$  °C for 1 h and then centrifugation for 15 min (4 °C). The supernatant was removed and discarded. The precipitated DNA samples were each dried, dissolved in 8  $\mu$ L of water, 8  $\mu$ L of 10 M urea, and 1  $\mu$ L of loading buffer, and analyzed by 25% DPAGE as described above with the exception that, instead of transferring the gel to filter paper, one glass plate was removed from the gel and the gel was covered with Saran Wrap followed by autoradiography to visualize the cross-linked DNA. The film was then aligned with the gel, and gel slices containing cross-linked DNA were excised. Gel slices were crushed in a 1.5-mL-size micropipette tip packed with glass wool and fit with a test tube, suspended in ca. 1 mL of 0.5 M NH<sub>4</sub>OAc/1 mM EDTA, and allowed to stand for 16 h at 37 °C. The eluent was collected in a test tube after centrifugation. The DNA was loaded onto a Sep-Pak and treated as described by Kirchner et al.<sup>22</sup> under Preparation and Radiolabeling of DNA. After concentration of the acetonitrile/water eluant to dryness, the residue was dissolved in 800  $\mu$ L of water.

**Isolation and Characterization of 21 from ACGT.** Cross-linked ACGT (0.6 OD) in 30  $\mu$ L of aqueous 10 mM MgCl<sub>2</sub>, 50 mM Tris (pH 8.9), was treated with 1  $\mu$ L (10 units) of DNase I, 1  $\mu$ L (12.7 units) of alkaline phosphatase, and 4  $\mu$ L (2 units) of phosphodiesterase I at 37 °C for 4 h. The substance **21** was isolated by HPLC using gradient A. The most strongly retained peak, with a retention time of 33.5 min, was collected and concentrated in a speed-vac. The concentrated sample was redissolved in 0.5 mL of water and lyophilized to remove HPLC buffer. Characterization of this substance and its synthetic version is described below.

**2,3-Dihydro-5-(3',4'-dichlorophenyl)-1H-pyrrolizine-6,7-diamide (25).** A solution of diester **24'** (3.47 g, 9.43 mmol) and formamide (2.5 mL, 62 mmol) in DMF (9.0 mL) at 100 °C was treated with a 25% solution of NaOMe in MeOH (2.8 mL) portionwise over 0.5 h.<sup>32</sup> The temperature was maintained for an additional 2.5 h at 100 °C, after which the mixture was cooled to 25 °C. The slurry was diluted with CH<sub>2</sub>Cl<sub>2</sub> (50 mL), and the solid was collected by filtration, washed with CH<sub>2</sub>Cl<sub>2</sub> (10 mL) and methanol (2  $\times$  10 mL), and dried in vacuo. The white solid (3.04 g) possessed limited solubility in common solvents and was used in the next step without further purification or characterization.

**2,3-Dihydro-5-(3',4'-dichlorophenyl)-1H-pyrrolizine-6,7-dinitrile (26).** A solution of DMF (0.80 mL, 10 mmol) in acetonitrile (15 mL) at 0 °C was treated dropwise with oxalyl chloride (0.86 mL, 9.8 mmol) over 10 min, accompanied by gas evolution and formation of a precipitate. This slurry was treated with diamide **25** (1.00 g, 2.96 mmol) in portions over 5 min and stirred for 0.65 h, after which pyridine (1.6 mL, 20 mmol) was added dropwise over 5 min.<sup>33</sup> This slurry was warmed to 10 °C over 0.5 h and partitioned between 1 N aqueous HCl (10 mL) and CH<sub>2</sub>Cl<sub>2</sub> (30 mL), the aqueous layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (2  $\times$  10 mL), and the combined organic phases were washed with brine (10 mL) and dried (MgSO<sub>4</sub>). The solvent was removed in vacuo, the residue was triturated with methanol (20 mL) and filtered, and the solid was washed with methanol (2  $\times$  5 mL) and dried to give 0.762 g (81% yield, two steps) of **26** as a white solid, a portion of which was recrystallized from methanol to obtain a sample for spectroscopic characterization: <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>)  $\delta$  2.67 (2 H, quin,  $J$  = 7, H<sup>2</sup>), 3.11 (2 H, t,  $J$  = 7, H<sup>1</sup>), 4.14 (2 H, t,  $J$  = 7, H<sup>3</sup>), 7.40 (1 H, dd,  $J$  = 2, 8, H<sup>6</sup>), 7.58 (1 H, d,  $J$  = 8, H<sup>5</sup>), 7.59 (1 H, d,  $J$  = 2, H<sup>2</sup>); IR 2222, 1426, 1028, 901, 828 cm<sup>-1</sup>; LRMS  $m/e$  303 (M<sup>+</sup> + 2), 301 (M<sup>+</sup>, B), 266, 230, 75, 36, 28.

**6,7-Bis(aminomethyl)-2,3-dihydro-5-(3',4'-dichlorophenyl)-1H-pyrrolizine (22) and 6,7-Bis(*N*-acetamidomethyl)-2,3-dihydro-5-(3',4'-dichlorophenyl)-1H-pyrrolizine (27).** A suspension of LiAlH<sub>4</sub> (0.058 g, 6.56 mmol) in THF (5 mL) at 25 °C was treated with a suspension of dinitrile **26** (0.725 g, ca. 2.4 mmol) in THF (20 mL) over 5 min. This mixture was heated under reflux for 2 h and cooled to 0 °C, and the

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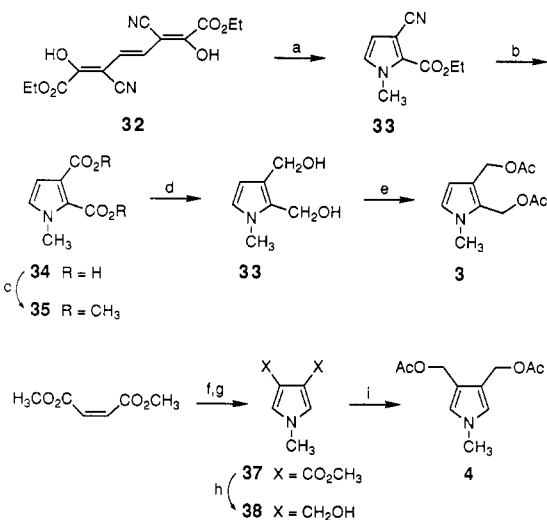
excess  $\text{LiAlH}_4$  was quenched by the careful addition of 20% aqueous  $\text{NaOH}$  solution followed by  $\text{CH}_2\text{Cl}_2$  (60 mL). This mixture was stored at 4 °C for 10 h, and the insoluble material was collected by filtration, washed with  $\text{CH}_2\text{Cl}_2$  ( $2 \times 20$  mL), and discarded. The solvent was removed in vacuo to give 0.67 g of **22** as an orange oil which solidified upon standing at 4 °C.

This sample was analyzed as the diamide **27**. A solution of **22** (0.055 g, 0.177 mmol), triethylamine (0.30 mL, 2.1 mmol), and a trace (ca. 1 mg) of 4-(dimethylamino)pyridine in  $\text{CH}_2\text{Cl}_2$  (3.0 mL) was treated with acetic anhydride (0.17 mL, 1.8 mmol) at 25 °C. After 2 h, the volatiles were removed in vacuo and the residue was partitioned between 0.1 N aqueous  $\text{HCl}$  and  $\text{CH}_2\text{Cl}_2$ . The organic phase was washed successively with saturated aqueous  $\text{NaHCO}_3$  and brine and then dried ( $\text{Na}_2\text{SO}_4$ ). The solvent was removed in vacuo. Chromatography of the residue on silica gel (5% methanol/ $\text{CH}_2\text{Cl}_2$ ) afforded 0.022 g (31%) of **27** as a white solid whose resonances in the  $^1\text{H}$  NMR corresponded to the major component in the sample before chromatography. Compound **27** was chromatographically (TLC) homogenous but had ca. 10% unidentified impurities as judged by NMR:  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  1.92 (s, impurity), 1.95 (s, impurity), 1.96 (3 H, s,  $\text{CH}_3$ ), 1.99 (3 H, s,  $\text{CH}_3$ ), 2.47 (2 H, quin,  $J = 7$ ,  $\text{H}^2$ ), 2.90 (2 H, t,  $J = 7$ ,  $\text{H}^1$ ), 3.87 (2 H, t,  $J = 7$ ,  $\text{H}^3$ ), 4.26 (2 H, d,  $J = 6$ ,  $\text{CH}_2$ ), 4.30 (2 H, d,  $J = 6$ ,  $\text{CH}_2$ ), 4.31–4.36 (m, impurity), 6.56 (1 H, b t, NH), 6.63 (1 H, b t, NH), 7.23 (1 H, dd,  $J = 2$ , 8,  $\text{H}^6$ ), 7.25–7.43 (m, impurity), 7.45 (1 H, d,  $J = 2$ ,  $\text{H}^2$ ), 7.48 (1 H, d,  $J = 8$ ,  $\text{H}^5$ ); IR 3285, 1646, 1560, 1459  $\text{cm}^{-1}$ ; LRMS  $m/e$  396 ( $\text{M}^+ + 3$ ), 394 ( $\text{M}^+ + 1$ ), 360, 337, 335.

**3'-O, 5'-O-Bis(tert-butyldimethylsilyl)-2'-deoxyguanosine (29).**<sup>34</sup> Deoxyguanosine monohydrate (Fluka) (**28**, 2.00 g, 7.01 mmol), *tert*-butyldimethylsilyl chloride (3.50 g, 23.19 mmol), and imidazole (3.15 g, 46.3 mmol) were suspended in DMF (7.0 mL) at 25 °C. Most of the solid dissolved within 10 min, but after that time a new precipitate was observed. After 25 h, the mixture was diluted with ethanol (15 mL) and stored at –23 °C for 10 h. The solid was collected by filtration, washed with cold ethanol (7 mL), and dried in vacuo to give 2.52 g (73%) of **29** as a white solid:  $^1\text{H}$  NMR (200 MHz,  $\text{CDCl}_3$  and 3 drops of  $\text{DMSO}-d_6$ ,  $\text{DMSO}-d_5$  at  $\delta$  2.49 ppm)  $\delta$  –0.02 (6 H, s,  $\text{Si}(\text{CH}_3)_2$ ), 0.01 (6 H, s,  $\text{Si}(\text{CH}_3)_2$ ), 0.81 (18 H, s,  $\text{Si}(\text{CH}_3)_3$ ), 2.21 (1 H, ddd,  $J = 3$ , 6, 13,  $\text{H}^2$ ), 2.39 (1 H, m,  $\text{H}^2$ ), 3.65 (2 H, m,  $\text{H}^3$ ), 3.85 (1 H, m,  $\text{H}^4$ ), 4.46 (1 H, m,  $\text{H}^3$ ), 5.64 (2 H, b s,  $\text{NH}_2$ ), 6.13 (1 H, t,  $J = 6$ ,  $\text{H}^1$ ), 7.64 (1 H, s,  $\text{H}^8$ ), 10.64 (1 H, b s, NH); FAB-LRMS  $m/e$  396 ( $\text{M}^+ + 1$ ), 152 (B).

**3'-O, 5'-O-Bis(tert-butyldimethylsilyl)-O<sup>6</sup>-(2''-(4'''-nitrophenyl)ethyl)-2'-deoxyguanosine (30).** A suspension of **29** (2.50 g, 5.04 mmol), *p*-nitrophenethyl alcohol (1.01 g, 6.05 mmol), and triphenylphosphine (1.59 g, 6.05 mmol) in *p*-dioxane (80 mL) was stirred for 1 h at 25 °C, followed by the addition of diethyl azodicarboxylate (0.953 mL, 6.05 mmol) in one portion.<sup>35</sup> After 24 h, the solvent was removed in vacuo and the residue dissolved in ether (30 mL) and stored at –23 °C for 12 h. The solid was removed by filtration and washed with cold ether (ca. 4 mL). The filtrate was concentrated in vacuo, and the residue was chromatographed on silica gel (2% methanol/ $\text{CH}_2\text{Cl}_2$ ) to give 1.94 g of **30** as a pale-yellow oil which crystallized upon standing. Rechromatography of other fractions containing **30** yielded an additional 0.69 g (total yield 81%):  $^1\text{H}$  NMR (200 MHz,  $\text{CDCl}_3$ )  $\delta$  0.08 (6 H, s,  $\text{Si}(\text{CH}_3)_2$ ), 0.10 (6 H, s,  $\text{Si}(\text{CH}_3)_2$ ), 0.91 (18 H, s,  $\text{Si}(\text{CH}_3)_3$ ), 2.34 (1 H, ddd,  $J = 4$ , 6, 13,  $\text{H}^2$ ), 2.57 (1 H, ddd,  $J = 6$ , 7, 13,  $\text{H}^2$ ), 3.28 (2 H, t,  $J = 7$ ,  $\text{OCH}_2\text{CH}_2$ ), 3.78 (2 H, m,  $\text{H}^5$ ), 3.98 (1 H, m,  $\text{H}^4$ ), 4.58 (1 H, m,  $\text{H}^3$ ), 4.73 (2 H, t,  $J = 7$ ,  $\text{OCH}_2\text{CH}_2$ ), 4.80 (2 H, b s,  $\text{NH}_2$ ), 6.32 (1 H, t,  $J = 6$ ,  $\text{H}^1$ ), 7.48 (2 H, d,  $J = 9$ , ArH), 7.91 (1 H, s,  $\text{H}^8$ ), 8.16 (2 H, d,  $J = 9$ , ArH); FAB-LRMS  $m/e$  645 ( $\text{M}^+ + 1$ ), 301 (B).

**2-Fluoro-O<sup>6</sup>-(2''-(4'''-nitrophenyl)ethyl)-2'-deoxyinosine (23).**<sup>25,26</sup> A 70% HF/pyridine solution in a plastic tube at –78 °C was diluted dropwise with pyridine (1.33 mL). This solution was warmed to –25 °C, and **30** (1.29 g, 2.0 mmol) was added in one portion, followed by *tert*-butyl nitrite (0.36 mL, 3.0 mmol). This mixture was stirred for 0.25 h, during which time it warmed to –20 °C, then poured into 100 mL of crushed ice/water, and exhaustively extracted with  $\text{CH}_2\text{Cl}_2$ . The combined organic phases were washed successively with water (30 mL), saturated aqueous  $\text{NaHCO}_3$  ( $2 \times 10$  mL), brine (20 mL), and dried ( $\text{Na}_2\text{SO}_4$ ). The solvent was removed in vacuo, and the residue was chromatographed on silica gel (5% methanol/ $\text{CH}_2\text{Cl}_2$ ) to give 0.272 g of **23** (32%) as a colorless oil which crystallized upon standing:  $^1\text{H}$  NMR (200 MHz,  $\text{CDCl}_3$ )  $\delta$  2.87 (1 H, ddd,  $J = 4$ , 6, 13,  $\text{H}^2$ ), 2.93 (1 H, ddd,  $J = 5$ , 9, 13,  $\text{H}^2$ ), 3.32 (2 H, t,  $J = 6.7$ ,  $\text{OCH}_2\text{CH}_2$ ), 3.81 (1 H, m,  $\text{H}^5$ ), 3.98 (1 H, dd,  $J = 2$ , 13,  $\text{H}^5$ ), 4.19 (1 H, m,  $\text{H}^4$ ), 4.79 (1 H, m,  $\text{H}^3$ ), 4.85 (2 H, t,  $J = 6.7$ ,  $\text{OCH}_2\text{CH}_2$ ), 6.33 (1 H, dd,  $J = 5$ , 6,  $\text{H}^1$ ), 7.50 (2 H, d,  $J = 8.9$ , ArH), 8.02 (1 H,



**Figure 8.** Syntheses of 2,3- and 3,4-bis(acetoxymethyl)-1-methylpyrrole (**3** and **4**, respectively): (a)  $\text{CH}_3\text{NH}_2$ ,  $\text{EtOAc}$ ,  $\text{EtOH}$ ,  $\text{H}_2\text{O}$ ; (b)  $\text{NaOH}$ ,  $\text{H}_2\text{O}$ ; (c)  $\text{TMSCl}$ ,  $\text{MeOH}$ ; (d)  $\text{LiAlH}_4$ ,  $\text{Et}_2\text{O}$ ; (e)  $\text{Ac}_2\text{O}$ ,  $\text{Et}_3\text{N}$ , DMAP,  $\text{CDCl}_3$ ; (f) tosylmethyl isocyanide,  $\text{NaH}$ ,  $\text{Et}_2\text{O}$ , DMSO; (g)  $\text{CH}_3\text{I}$ ,  $\text{NaH}$ , DMF; (h)  $\text{LiAlH}_4$ ,  $\text{Et}_2\text{O}$ ; (i)  $\text{Ac}_2\text{O}$ ,  $\text{Et}_3\text{N}$ , DMAP,  $\text{CDCl}_3$ .

s,  $\text{H}^8$ ), 8.18 (2 H, d,  $J = 8.9$ , ArH);  $^{19}\text{F}$  NMR (188.15 MHz,  $\text{CDCl}_3$ )  $\delta$  103.3 (s); FAB-LRMS  $m/e$  420 ( $\text{M}^+ + 1$ ), 304 (B), 155; FAB-HRMS  $m/e$  420.1298 (calcd 420.1319).

**Compound 31.** A solution of the protected fluorinosine **23** (0.029 g, 0.068 mmol) and the crude diamine **22** (0.011 g) in DMF (0.07 mL) was treated with triethylamine (0.015 mL, 0.108 mmol) and stirred at 25 °C for 70 h.<sup>36</sup> The mixture was partitioned between  $\text{CH}_2\text{Cl}_2$  (5 mL) and 0.1 N aqueous  $\text{HCl}$  (2 mL), and the organic phase was washed successively with saturated aqueous  $\text{NaHCO}_3$  (2 mL) and brine (2 mL) and dried ( $\text{Na}_2\text{SO}_4$ ). The solvent was removed in vacuo, and the residue was chromatographed on silica gel (7% methanol/ $\text{CH}_2\text{Cl}_2$ ) to give 0.0176 g of **31** (47%) as a pale-yellow solid as well as 0.0093 g of recovered **23**. The resulting solid was dissolved in 10% methanol/ $\text{CH}_2\text{Cl}_2$ , treated with activated charcoal, and filtered through a pad of Celite to give **31** as a white solid after evaporation of the solvent:  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$  and 4 drops of methanol- $d_4$ )  $\delta$  2.04 (2 H, m,  $\text{H}^2$ ), 2.46 (2 H, m,  $\text{CH}_2\text{CH}_2\text{CH}_2$ ), 2.58 (1 H, m,  $\text{H}^2$ ), 2.65 (1 H, m,  $\text{H}^2$ ), 2.82 (2 H, t,  $J = 7$ ,  $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$ ), 3.12 (2 H, t,  $J = 7$ ,  $\text{OCH}_2\text{CH}_2$ ), 3.15 (2 H, t,  $J = 7$ ,  $\text{OCH}_2\text{CH}_2$ ), 3.59 (2 H, m,  $\text{H}^5$ ), 3.76 (2 H, m,  $\text{H}^5$ ), 3.90 (2 H, m,  $\text{CH}_2\text{CH}_2\text{N}$ ), 3.99 (2 H, m,  $\text{H}^4$ ), 4.40 (2 H, s,  $\text{NHCH}_2$ ), 4.43 (2 H, s,  $\text{NHCH}_2$ ), 4.50 (4 H, m,  $\text{OCH}_2\text{CH}_2$ ), 4.60 (2 H, m,  $\text{H}^3$ ), 6.08 (2 H, m,  $\text{H}^1$ ), 7.14 (1 H, dd,  $J = 2$ , 8, ArCHCl), 7.18–7.32 (impurity), 7.37 (6 H, m, ArCHCHCNO<sub>2</sub> and ArCHCHCl), 7.60 (2 H, s,  $\text{H}^8$ ), 8.07 (2 H, d,  $J = 8$ , ArCHCHCNO<sub>2</sub>), 8.08 (2 H, d,  $J = 8$ , ArCHCHCNO<sub>2</sub>); FAB-LRMS  $m/e$  1110 ( $\text{M}^+ + 3$ ), 1108 ( $\text{M}^+ + 1$ ), 1076, 1074, 694, 692 (B), 660, 658, 578, 576, 544, 542; FAB-HRMS  $m/e$  1108.3198 (calcd 1108.3235).

**Synthetic 21.** A solution of **31** (ca. 1 mg, 0.9 mmol) in 9% DMF/acetone nitrile (77  $\mu\text{L}$ ) at 25 °C was treated with DBU (5  $\mu\text{L}$ , 33 mmol). The reaction was monitored by HPLC using the 250 mm  $\times$  10 mm column and gradient D and was judged to be complete after 12 h. The mixture was diluted with an equal volume of 50% methanol/water and purified by HPLC, **21** eluting with retention time of ca. 19.8 min. Concentration in vacuo afforded **21** as a solid, characterization of which is described in the following section.

**Comparison of 21 from IPP-Cross-Linked ACGT and Chemical Synthesis.** ESMS (1:1 methanol/water, inlet voltage 250 V, needle voltage 5000 V, 3  $\mu\text{L}/\text{min}$ ).  $m/e$  synthetic: 812 and 810 ( $\text{M}^+ + \text{H}^+$ ); 696 and 694 ( $\text{M}^+ - \text{deoxyribose} + 2\text{H}^+$ ); 580 and 578 ( $\text{M}^+ - 2 \text{ deoxyribose} + 3\text{H}^+$ ); 505; 429 and 427 ( $\text{M}^+ - \text{deoxyguanosyl} - \text{deoxyribose} + 1\text{H}^+$ ).  $m/e$  from ACGT: 834 and 832 ( $\text{M}^+ + \text{Na}^+$ ); 812 and 810; 696 and 694; 580 and 578; 429 and 427. UV (50% methanol/20 mM triethylammonium acetate, pH 7.0 or 50% methanol/saturated aqueous  $\text{Ca}(\text{OH})_2$ , pH 12.45). Synthetic  $\lambda_{\text{max}}$  254, 282 (pH 7.0), 260 (pH 12.45). From ACGT:  $\lambda_{\text{max}}$  254, 280 (pH 7.0), 260 (pH 12.45). Synthetic **21** and **21** from ACGT coeluted on HPLC using gradients B (32.0 min) and C (24.2 min).

**Syntheses of 2,3- and 3,4-Bis(acetoxymethyl)-1-methylpyrrole (3 and 4).** Syntheses of **3** and **4** are summarized in Figure 8 and are described below.

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**2-Carboxy-3-cyano-1-methylpyrrole (33).** A suspension of **32**<sup>37</sup> (23.16 g, 75.6 mmol) in ethyl acetate (200 mL) at reflux was treated with a solution of 40% aqueous  $\text{CH}_3\text{NH}_2$  (12.4 mL, 144 mmol) in 50 mL of ethanol over 1 h. After 4 h the mixture was cooled to 25 °C and the insoluble material removed by filtration and discarded. The filtrate was concentrated in vacuo, and the residue was partitioned between diethyl ether (50 mL) and water (30 mL). The aqueous layer was extracted with diethyl ether (2 × 20 mL). The combined organic layers were washed successively with 1 N aqueous HCl (20 mL), saturated aqueous  $\text{K}_2\text{CO}_3$  (20 mL), and brine (10 mL). After drying ( $\text{MgSO}_4$ ), the mixture was concentrated to ca. 10 mL and the resulting suspension stored at -23 °C for 10 h, after which the solid was removed by filtration and washed with cold diethyl ether (ca. 5 mL), to give 4.88 g of **33** as an off-white solid. The second and third crop were obtained by repeating this procedure to give an additional 1.56 g (total yield 48%):  $^1\text{H}$  NMR (200 MHz,  $\text{CDCl}_3$ )  $\delta$  1.44 (3 H, t,  $J = 7.1$ ,  $\text{CH}_3\text{CH}_2$ ), 3.97 (3 H, s,  $\text{NCH}_3$ ), 4.40 (2 H, q,  $J = 7.1$ ,  $\text{CH}_3\text{CH}_2$ ), 6.49 (1 H, d,  $J = 2.8$ , ArH), 6.77 (1 H, d,  $J = 2.8$ , ArH); LRMS  $m/e$  178 ( $\text{M}^+$ ), 150, 133 (B), 106, 78, 64, 52, 28.

**2,3-Dicarboxy-1-methylpyrrole (34).** A suspension of cyanoester<sup>38</sup> **33** (2.00 g, 11.23 mmol) in water (10 mL) at 135 °C was treated with a solution of NaOH (3.5 g, 87.5 mmol) in water (10 mL) over 0.35 h, after which the solution had become homogenous. After 6.5 h the solution was cooled to 0 °C, diluted with water (45 mL), and acidified with 10 N aqueous HCl. The resulting suspension was kept at 4 °C for 12 h, and the white solid was collected by filtration, washed with cold water (ca. 5 mL), and dried in vacuo to give 1.86 g (98%) of **34** as a white solid:  $^1\text{H}$  NMR (200 MHz,  $\text{DMSO}-d_6$ ,  $\text{DMSO}-d_5$  at  $\delta$  2.49 ppm)  $\delta$  3.90 (3 H, s,  $\text{NCH}_3$ ), 6.62 (1 H, d,  $J = 2.8$ , ArH), 7.16 (1 H, d,  $J = 2.8$ , ArH); LRMS  $m/e$  169 ( $\text{M}^+$ ), 151, 125, 108 (B), 80, 53, 44, 39, 28.

**2,3-Dicarbomethoxy-1-methylpyrrole (35).** A suspension of diacid **34** (1.5 g, 8.89 mmol) in methanol (90 mL) was treated dropwise with trimethylsilyl chloride (6.75 mL, 53.23 mmol), and this mixture was stirred at 25 °C for 24 h.<sup>39</sup> The volatiles were removed in vacuo, and silica gel (ca. 1 g) was added. Three portions of methanol (10 mL) were sequentially added and evaporated. The residue was loaded on top of a silica gel column and eluted with 1% methanol/ $\text{CH}_2\text{Cl}_2$  to yield 1.64 g (94% yield) of **35** as a colorless oil which crystallized upon concentration from diethyl ether:  $^1\text{H}$  NMR (200 MHz,  $\text{CDCl}_3$ )  $\delta$  3.82 (6 H, s,  $\text{OCH}_3$ ), 3.88 (3 H, s,  $\text{NCH}_3$ ), 6.47 (1 H, d,  $J = 2.7$ , ArH), 6.66 (1 H, d,  $J = 2.7$ , ArH); LRMS  $m/e$  197 ( $\text{M}^+$ ), 166 (B), 136, 107, 81, 67, 53, 42, 28.

**2,3-Bis(hydroxymethyl)-1-methylpyrrole (36).** A suspension of  $\text{LiAlH}_4$  (0.378 g, 9.97 mmol) in ether (10 mL) was treated dropwise with a solution of diester **35** (0.473 g, 2.40 mmol) in ether (5 mL). After being stirred at 25 °C for 0.25 h, the mixture was heated at reflux for 1.5 h, cooled to 25 °C, and quenched by the careful addition of  $\text{H}_2\text{O}$  (0.5 mL), followed by 20% aqueous NaOH until evolution of gas ceased. This mixture was stirred for 10 min, and then the solids were removed by filtration and washed with  $\text{CHCl}_3$  (2 × 30 mL). The filtrate was dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated in vacuo to give 0.331 g (100%) of **36** as a pale-yellow oil which crystallized upon standing at -23 °C:  $^1\text{H}$  NMR (200 MHz,  $\text{CDCl}_3$ )  $\delta$  3.67 (3 H, s,  $\text{NCH}_3$ ), 4.56 (2 H, s,  $\text{CH}_2$ ), 4.62 (2 H, s,  $\text{CH}_2$ ), 6.09 (1 H, d,  $J = 2.7$ , ArH), 6.56 (1 H, d,  $J = 2.7$ , ArH); LRMS  $m/e$  141 ( $\text{M}^+$ , B), 122, 110, 94, 82, 67, 53, 42.

**2,3-Bis(acetoxymethyl)-1-methylpyrrole (3).** A solution of diol **36** (0.052 g, 0.37 mmol), triethylamine (0.26 mL, 1.8 mmol), and ca. 1 mg of 4-(dimethylamino)pyridine in  $\text{CDCl}_3$  (1 mL) at 25 °C was treated with acetic anhydride (0.087 mL, 0.925 mmol). After 1 h the reaction was judged complete by  $^1\text{H}$  NMR and the organic phase was washed with water (3 × 0.5 mL) and the solvent removed in vacuo. The residue was dissolved in  $\text{CDCl}_3$  (0.7 mL) and washed with water (0.7 mL) and the solvent removed in vacuo. The residue was dissolved in  $\text{CD}_3\text{CN}$  (3.69 mL), and this solution was used directly in cross-linking reactions:  $^1\text{H}$  NMR (200 MHz,  $\text{CD}_3\text{CN}$ ,  $\text{CD}_2\text{HCN}$  at  $\delta$  1.94)  $\delta$  1.91 (3 H, s,  $\text{CH}_3$ ), 1.96 (3 H, s,  $\text{CH}_3$ ), 3.54 (3 H, s,  $\text{NCH}_3$ ), 4.92 (2 H, s,  $\text{CH}_2$ ), 5.03 (2 H, s,  $\text{CH}_2$ ), 6.03 (1 H, d,  $J = 2.7$ , ArH), 6.62 (1 H, d,  $J = 2.7$ , ArH); LRMS  $m/e$  225 ( $\text{M}^+$ ), 166, 123 (B), 107, 96, 94, 43.

**3,4-Dicarbomethoxy-1-methylpyrrole (37).** A suspension of NaH (0.300 g, 12.5 mmol) in ether (10 mL) at 0 °C was treated with a solution

of dimethyl maleate (0.63 mL, 5.0 mmol) and tosylmethyl isocyanide (0.976 g, 5.0 mmol) in ether (15 mL) and DMSO (6 mL) over 0.25 h.<sup>40</sup> The mixture was then warmed to 25 °C over 0.5 h, followed by addition of water (15 mL). The mixture was extracted with  $\text{CH}_2\text{Cl}_2$  (2 × 20 mL). The aqueous phase was filtered, and the resulting solid was washed with a few drops of ethanol and dried to yield 0.415 g of a solid that was used without purification in the next step. This solid, in DMF (2.5 mL) was treated sequentially with NaH (0.163 g, 6.8 mmol) and MeI (0.423 mL, 6.8 mmol) at 25 °C. After 2.5 h, the mixture was diluted with 0.5 N aqueous HCl (30 mL) and exhaustively extracted with  $\text{CH}_2\text{Cl}_2$ . The combined organic phases were dried ( $\text{Na}_2\text{SO}_4$ ), the volatiles were removed in vacuo, and the residue was chromatographed on silica gel (50% EtOAc/hexanes) to give 0.212 g of **37** as a white solid (25%, two steps):  $^1\text{H}$  NMR (200 MHz,  $\text{CDCl}_3$ )  $\delta$  3.67 (3 H, s,  $\text{NCH}_3$ ), 3.82 (6 H, s,  $\text{OCH}_3$ ), 7.18 (2 H, s, ArH); LRMS  $m/e$  197 ( $\text{M}^+$ ), 166 (B), 136, 108, 80, 53, 42, 28.

**3,4-Bis(hydroxymethyl)-1-methylpyrrole (38).** A suspension of  $\text{LiAlH}_4$  (0.025 g, 0.659 mmol) in ether (1 mL) was treated dropwise with a suspension of diester **37** (0.022 g, 0.112 mmol) in ether (1.5 mL) at 25 °C, followed by heating at reflux for 0.5 h. After being cooled to 25 °C, the mixture was quenched by dropwise addition of 20% aqueous NaOH (ca. 0.2 mL). The solvent was decanted from the insoluble material, which was in turn washed with  $\text{CH}_2\text{Cl}_2$  several times. Concentration of the combined organic phases in vacuo yielded 0.016 g (100%) of **38** as a white solid:  $^1\text{H}$  NMR (200 MHz,  $\text{CDCl}_3$ )  $\delta$  3.21 (2 H, b s, OH), 3.57 (3 H, s,  $\text{NCH}_3$ ), 4.51 (4 H, s,  $\text{CH}_2$ ), 6.54 (2 H, s, ArH).

**3,4-Bis(acetoxymethyl)-1-methylpyrrole (4).** A solution of diol **38** (0.007 g, 0.05 mmol), triethylamine (0.070 mL, 0.50 mmol), and ca. 1 mg of 4-(dimethylamino)pyridine in  $\text{CDCl}_3$  (1 mL) at 25 °C was treated with acetic anhydride (0.024 mL, 0.250 mmol). After 1 h, the reaction was judged complete by  $^1\text{H}$  NMR. The organic phase was washed with water (3 × 0.5 mL) and the solvent removed in vacuo. The residue was dissolved in  $\text{CDCl}_3$  (0.7 mL) and washed with water (0.7 mL), and the volatiles were removed in vacuo. The residue was dissolved in  $\text{CD}_3\text{CN}$  (0.5 mL), and this solution was used directly in cross-linking reactions:  $^1\text{H}$  NMR (200 MHz,  $\text{CD}_3\text{CN}$ ,  $\text{CD}_2\text{HCN}$  at  $\delta$  1.94)  $\delta$  1.96 (6 H, s,  $\text{CH}_3$ ), 3.57 (3 H, s,  $\text{NCH}_3$ ), 4.93 (4 H, s,  $\text{CH}_2$ ), 6.67 (2 H, s, ArH); LRMS  $m/e$  225 ( $\text{M}^+$ ), 166, 123 (B), 107, 94, 43.

**Computer Modeling.** Computation was performed on a Silicon Graphics 4D-25 IRIS workstation, using the Biopolymer and Discover modules of Insight II (Biosym Technologies, Versions 1.1.0 and 2.6.0, respectively) for model building and energy minimization. A double-strand, B-form oligodeoxynucleotide structure consisting of the sequence 5'-d(GAGTC) and 3,4-dimethylpyrrole were separately constructed. The amino group nitrogens of the dG residues on opposing strands at the sequence 5'-d(CG) were linked to the carbon atoms of the methyl groups of the pyrrole to afford a covalently cross-linked structure. This structure was energy minimized with the AMBER force field using a distance-dependent dielectric in the absence of solvent and counterions. The minimization used initially a steepest descents algorithm using diagonal terms only and a simple harmonic potential for 1000 iterations or until an RMS derivative of 0.01 kcal/(mol Å) was reached followed by ca. 7000 iterations of conjugate gradient minimization again until the same RMS derivative was reached. To the resulting structure was appended the three-carbon bridging unit and the 3,4-dimethylphenyl ring. This structure was further minimized using the above conditions, the result of which is shown in Figure 6.

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