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Manipulative Interplay of Two Adozelesin Molecules with d(ATTAAT)$_2$ Achieving Ligand-Stacked Watson−Crick and Hoogsteen Base-Paired Duplex Adducts

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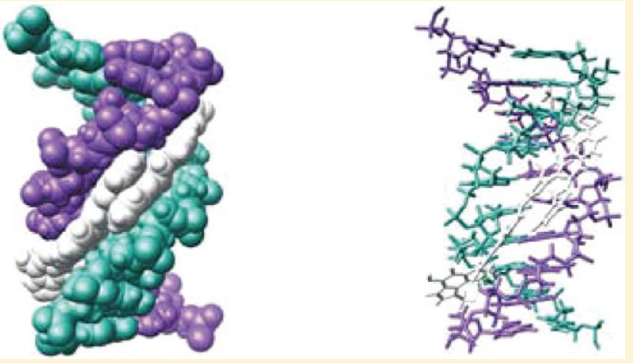
Supporting Information

ABSTRACT: Previous structural studies of the cyclopropapyrroloindole (CPI) antitumor antibiotics have shown that these ligands bind covalently edge-on into the minor groove of double-stranded DNA. Reversible covalent modification of the DNA via N3 of adenine occurs in a sequence-specific fashion. Early nuclear magnetic resonance and molecular modeling studies with both mono- and bis-alkylating ligands indicated that the ligands fit tightly within the minor groove, causing little distortion of the helix. In this study, we propose a new binding model for several of the CPI-based analogues, in which the aromatic secondary rings form π-stacked complexes within the minor groove. One of the adducts, formed with adozelesin and the d(ATTAAT)$_2$ sequence, also demonstrates the ability of these ligands to manipulate the DNA of the binding site, resulting in a Hoogsteen base-paired adduct. Although this type of base pairing has been previously observed with the bisfunctional CPI analogue bizelesin, this is the first time that such an observation has been made with a monoalkylating nondimeric analogue. Together, these results provide a new model for the design of CPI-based antitumor antibiotics, which also has a significant bearing on other structurally related and structurally unrelated minor groove-binding ligands. They indicate the dynamic nature of ligand−DNA interactions, demonstrating both DNA conformational flexibility and the ability of two DNA-bound ligands to interact to form stable covalent modified complexes.



Adozelesin (formerly U73975, Upjohn Co.) is a synthetic analogue of (+)-CC-1065,[1,2] a cyclopropapyrroloindole (CPI) drug containing the DNA-alkylating cyclopropa[*c*]pyrrolo[3,2]-indole-4(5*H*)-one subunit (Scheme 1). Adozelesin was originally synthesized to combat the delayed lethality problems associated with (+)-CC-1065.[3,4] It is an overall "right-handed" molecule, mimicking the pitch of B-form DNA and fitting snugly into the minor groove, with a sequence preference for 5′-Pu,Py/Pu,TTA*, where the asterisk denotes the site of covalent modification.[5,6]

Reaction with double-stranded DNA occurs via the N3 position of a reactive adenine, forming a covalent adduct that overlaps a 5 bp region in the minor groove.[7,8] The reaction is thought to be a multistep process using noncovalent interactions by hydrophobic and van der Waals forces between the three subunits and the DNA minor groove, followed by covalent reaction through the alkylating subunit. The reaction mechanism is shown in Scheme 1. Adduct formation results in positive charge that is delocalized within the adenine residue, and this gives rise to the limited stability of CPI duplex adducts. Heating a CPI duplex adduct to temperatures of >60 °C will result in depurination and strand breakage; even at NMR temperatures (5 °C), duplex adducts will be usable only for a couple of weeks before accumulation of breakdown products makes the data unusable.

Previous NMR studies with the 5′-d(CGTAAGCGCTTA*CG)$_2$−adozelesin adduct (the asterisk denotes the site of covalent modification) showed that adozelesin was located edge-on in the minor groove and bound to adenine bases, with Watson−Crick base pairing maintained throughout the adduct.[9] A high degree of hydrogen bonding was found to exist between phenolic protons and the phosphate backbone,[10] and a strong hydrogen bond was formed between the amide linker and a thymine (T10) on the modified strand. The amide interactions were thought to be the significant difference between adozelesin and (+)-CC-1065, and their absence in (+)-CC1065 was identified as the molecular cause of DNA overwinding and associated delayed lethality. Additionally, during molecular modeling experiments,[9] it was noted that a degree of "fishtailing" could be observed at the aromatic ring of the "C" subunit, which was pushed and/or positioned toward the modified strand to avoid the second adozelesin residue. As the sequence modeled in these studies was palindromic, only half of the adduct was modeled. However, on the basis of these studies, it was proposed that the overall length of the adozelesin

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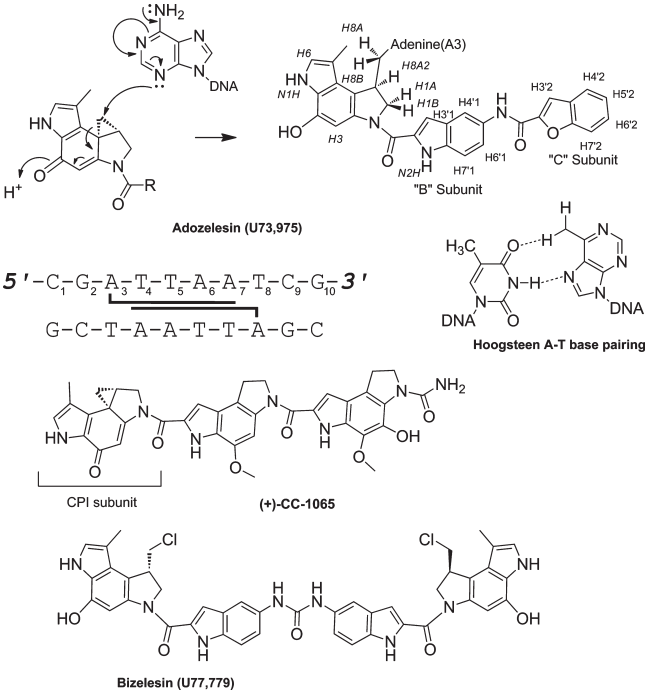
Scheme 1. Structures of the Monoalkylation Compounds Adozelesin, (+)-CC-1065, and Bisfunctional Cross-Linking Analogue Bizelesin[a]



[a] Shown is the site of the reaction between adenine A3 and adozelesin (including numbering for the duplex bases and adozelesin used in this NMR study; a Hoogseen A-T base pair is also shown).

ligand could result in a slight overlap of the aromatic benzofuran C rings within the adduct. Unfortunately, because the ends of the two drugs were identical, NOE evidence was inconclusive, and this was left as an observation of the modeling study.

The 5′-d(CGATTAATCG)$_2$ duplex used within this study was designed to investigate the possibility of an overlap of the aromatic rings of the B and C subunits within a DNA−adozelesin adduct. Adozelesin is known to span 5 bp, so the new DNA sequence was designed to be 4 bp shorter than that previously studied by Cameron.[9] The predicted reaction site for adozelesin within the proposed duplex would be the N3 position of adenine residue A3 (Scheme 1). For a palindromic adduct to form, significant overlap would be required between the B and C subunits of the two adozelesin molecules.

EXPERIMENTAL PROCEDURES

Chemicals. Adozelesin was a gift from the former Upjohn Co. and was used without additional purification. Reagents used to prepare the NMR buffers, sodium hydrogen phosphate (99.99%), sodium chloride (99.99%), and EDTA (99.99%), were purchased from Aldrich. HPLC grade acetonitrile, acetic acid, and methanol were purchased from Aldrich. Reagents and solvents for automated DNA synthesis were purchased from Applied Biosystems/Cruachem. DNA grade Bio-Gel hydroxyapatite was purchased from Bio-Rad.

Oligonucleotide Preparation and Purification for NMR Studies. The single strand for the self-complementary intrastrand adduct duplex 5′-d(CGATTAATCG)$_2$ was synthesized on a 4 × 10 μmol scale using automated solid-phase phosphotriester and phosphoramidite chemistry[11] on an Applied Biosystems automated DNA synthesizer (model 391A), leaving the trityl protecting group on each of the strands. The DNA was deprotected overnight with saturated aqueous ammonia, followed by purification by reversed-phase HPLC, as outlined in the previous study;[12] the DNA was then detritylated using 50% aqueous acetic acid and evaporated to dryness, followed by further purification by HPLC. The self-complementary 5′-d(CGATTAATCG)$_2$ sample was then desalted (dialysis) and annealed in deuterated NMR buffer [10 mM NaH_2PO_4, 100 mM NaCl, and 0.1 mM EDTA (pH 6.85)]; only duplex signals were observed, and a full complement of NMR data was recorded. This procedure was repeated in a 10% D_2O/90% H_2O mixture to provide data on exchangeable protons.

Preparation and Purification of Adducts for NMR Studies. The adozelesin adduct was prepared by stirring adozelesin (4.0 mg) in deuterated DMF (0.2 mL) with 30 μmol of the purified duplexes in twice-concentrated deuterated NMR buffer (pH 6.85, 500 μL). The reactions were followed by ^{1}H NMR; after 10 days at 5 °C, none of the duplex signals could be observed and two discrete sets of adduct signals were present. The rate of this reaction was much slower than that observed by Cameron and Thompson in the previous adozelesin study, which went to completion in 48 h.[9] The samples were lyophilized to remove the solvent (DMF) and resuspended in D_2O (500 μL). The sample was again lyophilized to dryness and desalted. Excess drug was then removed on C18 Sep-Pak cartridges, and the sample was lyophilized to dryness and then redissolved in deuterated NMR buffer (500 μL). The final stage of this procedure was repeated using a 10% D_2O/90% H_2O mixture to provide data on exchangeable protons.

Proton NMR Experiments. One- and two-dimensional 600 MHz NMR data sets were recorded in H_2O- and D_2O-buffered solutions on a Varian Inova 600 (600 MHz) spectrometer. Proton signals were recorded in parts per million and referenced relative to the residual water signal (δ 4.71). Phase sensitive two-dimensional NOESY spectra (Varian) were recorded with mixing times of 50, 100, 250, and 300 ms (Varian) with 32 scans at each of 1024 T_1 values at a spectral width of 10.002 ppm at a relaxation delay of 2.0 s between scans. Two-dimensional NOE spectra in 90% H_2O with mixing times of 150 and 300 ms were recorded; HDO suppression was achieved by using Varian presaturation suppression or a 1−1 echo read pulse sequence[13,14] with a 1.5 s pulse repetition delay and a sweep width of 25 ppm. Spectra were processed with the Inova spectrometer (VNMR 6.1c) and also in the TRIAD module of the SYBYL software suite. During data processing, a 90°-shifted sine-bell function was used in both F_1 and F_2 dimensions. The FID in F_1 was zero-filled to 2K, prior to FT to give a 2K × 2K spectrum. DQF-COSY, TOCSY, and ROESY spectra were also recorded on the Varian 600 MHz spectrometer and were used to confirm assignments made in the NOESY spectra. In addition, selected ^{13}C resonances were identified using a heteronuclear multiple-quantum coherence experiment in D_2O.[15]

Molecular Modeling. Additional base pairs (G-C) were added to the top and bottom of the duplexes to give 14-mers; this approach significantly reduced the possibility of terminal base pairs fraying within the calculations and kept the central 10-mer free from artificial restraints. The DNA duplexes were built using the BioPolymer module of SYBYL, and the adozelesin ligand was docked and bound into the minor groove. Charges were then calculated for the complete complexes using the

Table 1. Chemical Shifts (parts per million) for the Adozelesin in the 5′-d(CGATTAATCG)$_2$−Adozelesin Hoogsteen and Watson−Crick Adducts

	CPI subunit									benzoindole B subunit					benzofuran C subunit				
	CH_3	H1A	H1B	H3	H6	H8A	H8B	H8A2	N1H	H3′1	H4′1	H6′1	H7′1	N2H	H3′2	H4′2	H5′2	H6′2	H7′2
Hoogsteen adduct	2.83	3.89	3.89	8.10	8.28	5.35	5.35	3.90	11.75	7.28	7.80	8.37	8.34	11.19	7.72	8.11	8.16	7.38	7.45
Watson−Crick adduct	2.70	3.51	3.51	8.14	8.28	5.50	5.50	4.20	12.55	7.92	6.96	8.10	8.14	13.79	7.28	7.80	7.46	7.53	8.10

Gasteiger−Hückel set of charges.[16,17] Counterions were placed on the O−P−O bisector 6.0 Å from the phosphorus atom prior to solvation.[18] The systems were solvated as a droplet, using six layers of solvent via the Molecular Silverware algorithm.[19]

Restrained Molecular Dynamics Simulations. Interproton distance constraints were generated from the NOESY data in TRIAD and were incorporated into the adduct model using the weak, medium, and strong methodology.[20,21] Distance restraints were primarily taken from 50 and 100 ms NOESY data sets and were distributed according to cross-peak intensities as strong (1.75−2.90 Å), medium (2.00−3.50 Å), and weak (2.25−4.50 Å), and additional restraints were added from a 300 ms NOESY data set (NOEs not observed in 50 or 100 ms data sets) and set at 3.00−4.50 Å. Distance restraints from exchangeable protons were incorporated with distance constraints of 1.75−4.5 Å to avoid errors resulting from water suppression. The decision to use the weak, medium, and strong methodology was based on the complexity of the spectra and the difficulty in obtaining clean NOE volume data. Although this approach will lead to the development of spin diffusion artifacts in NOE NMR data at long mixing times, the vast majority of the NOE distance restraints came for the 50 and 100 ms NOESY spectra where spin diffusion artifacts were less likely to be an issue. In many cases, the NOE signals were partially overlapped and the weak, medium, and strong approach used within this study allowed manual estimation of the contribution to the NOE peaks. The direct spin−spin systems (H5−C1, H6−C1 and H5−C9, H6−C9) along with direct spin−spin couplings from the ortho aromatic protons on the bound adozelesin residues (when clear from overlap) were used to provide an internal reference. The rMD calculations were performed using the Tripos Associates force fields within the SYBYL software suite on Intel quad core workstations (SUSE LINUX). The molecular dynamics calculations (in aquo) were performed at a constant volume, using 2.0 fs time steps. The equilibrium protocol consisted of 100 steps of steepest descent minimization, followed by conjugate gradient minimization, applied to the solvent molecules to relax possible steric clashes at the adduct−solvent interface.[22] The water was then thermalized at 300 K for 5.0 ps using a Boltzmann initial velocity distribution, constant dielectric function, and fixed DNA with counterions. Finally, the molecular dynamics was performed with only the DNA fixed, with the counterions and water mobilized at 300 K for 5.0 ps. The rMD calculations on the complete solvated complex were started at 0 K and ramped over 50 ps in 10 steps to 300 K. Alternative starting structures were Watson−Crick and Hoogsteen base-paired versions of (1) adozelesin 14-mer nonrestrained molecular dynamics products and (2) minimized B-DNA adducts. For the Watson−Crick base-paired self-complementary adduct, during the first 50 ps, the 194 NMR-derived distance restraints, 38 of which were related to the bound adozelesin (Table 1), were applied to the system along with additional distance restraints, which were applied between the base pairs and the counterions. During the next 5.0 ps, the non-NMR-derived distance restraints were removed from all but the two 5′ and 3′ terminal bases (G-C), which were left in place to prevent the duplex from fraying at elevated temperatures. The Hoogsteen adduct was treated the same way; 184 NMR-derived distance restraints, 44 of which were related to the bound adozelesin, were used (Table 2). The in vacuo and solvated systems were then held at 300 K for 60 ps, and the data from the last 50 ps (501 structures) were averaged and minimized to generate the averaged in vacuo and solvated structures.

RESULTS

Upon addition of the adozelesin to the purified duplex, the proton NMR signals disappeared from the reaction mixture within 48 h, giving a mixture of several products (this initial mixture was deemed to be unassignable). The mixture/suspension was left in the cold room for several days and then re-examined; some simplification had occurred, and we decided to allow the mixture to continue to react. After 8 days, the mixture had considerably simplified (although clearly contained more than one duplex adduct) and was prepared for NMR analysis.

On the basis of these observations, we have speculated that the initial species formed are monoadducts (at the preferred and possibly nonpreferred sites) and this reaction rate (loss of duplex) is similar to that observed in previous CPI studies.[12,24,25] At this point, there was probably a mix of monoadducts, and the two bisadducts reported, hence the number of signals observed within the mixture. The simplification of the mixture occurred as the adozelesin within monoadducts relocated (if necessary to the preferred binding site) and reacted to give the two bisadducts reported. This second phase of the reaction was much slower, perhaps reflecting the change in conformation of the DNA and first bound ligand allowing initial noncovalent binding of the second adozelesin molecule and subsequent covalent reaction.

Identification of the Two 10-mer Duplex Adducts. *Symmetry of the 5′-d(CGATTAATCG)$_2$−Adozelesin Adduct: Identification of Two Symmetrical Adducts.* The one-dimensional ^{1}H NMR nonexchangeable spectra and a peak count in the aromatic region in the duplex spectra gave approximately 15 peaks. This information, along with the three clear thymine methyl peaks that can be observed between δ 1.00 and 1.60, provides evidence of the purity of this duplex. The duplex adduct(s) spectrum had greatly increased in complexity, and because of overlaid signals, it is not possible to complete an accurate peak count in the aromatic region. However, when the number of peaks was estimated, it was apparent that the number of aromatic signals was approximately twice the expected number; on the basis of this observation, we concluded that more than one species was present in the reaction mixture.

The 5′-d(CGATTAATCG)$_2$−adozelesin adduct(s) was further investigated using COSY and NOESY NMR techniques. An expansion of the two-dimensional (2D) NOESY adduct and duplex spectra, H6/H8−H1′ region, are shown in Figure 1. Superimposition of the duplex 2D NOESY spectrum onto the adduct

Table 2. NOE Connectivities between the Drug Protons and the DNA Duplex in the 5′-d(CGATTAATCG)$_2$−Adozelesin Hoogsteen Adduct[a]

	H1a/b	H8a/b	H8A2	CH_3	H6	H3	H3′1	H4′1	H6′1	H7′1	H3′2	H4′2	H5′2	H6′2	H7′2
A3 H8				W											
A3 H1′		O	W												
A3 H4′		M		W	W										
A3 H2	M	W	M												
T4 H1′	O					W	M								
T4 H3								W							
T4 H4′			O												
T5 H1′												W	W		
T5 H3′								W					W		
T5 H4′								W					M		
T5 H6							M					O			
A6 H1′								O			W	O			
A6 H8								O			W				
A6 H4′								W							
A7 H2	O						O	O				O	M		
A7 H1′								M			M	W	M	M	W
A7 H3′								W							M
A7 H4′												W		W	W
T8 H1′	W						M			W					
T8 H6													O		

[a] Abbreviations: S, strong; M, moderate; W, weak; O, overlaid, but significant contribution to peak volume. This table is available in cartoon form as Supporting Information.

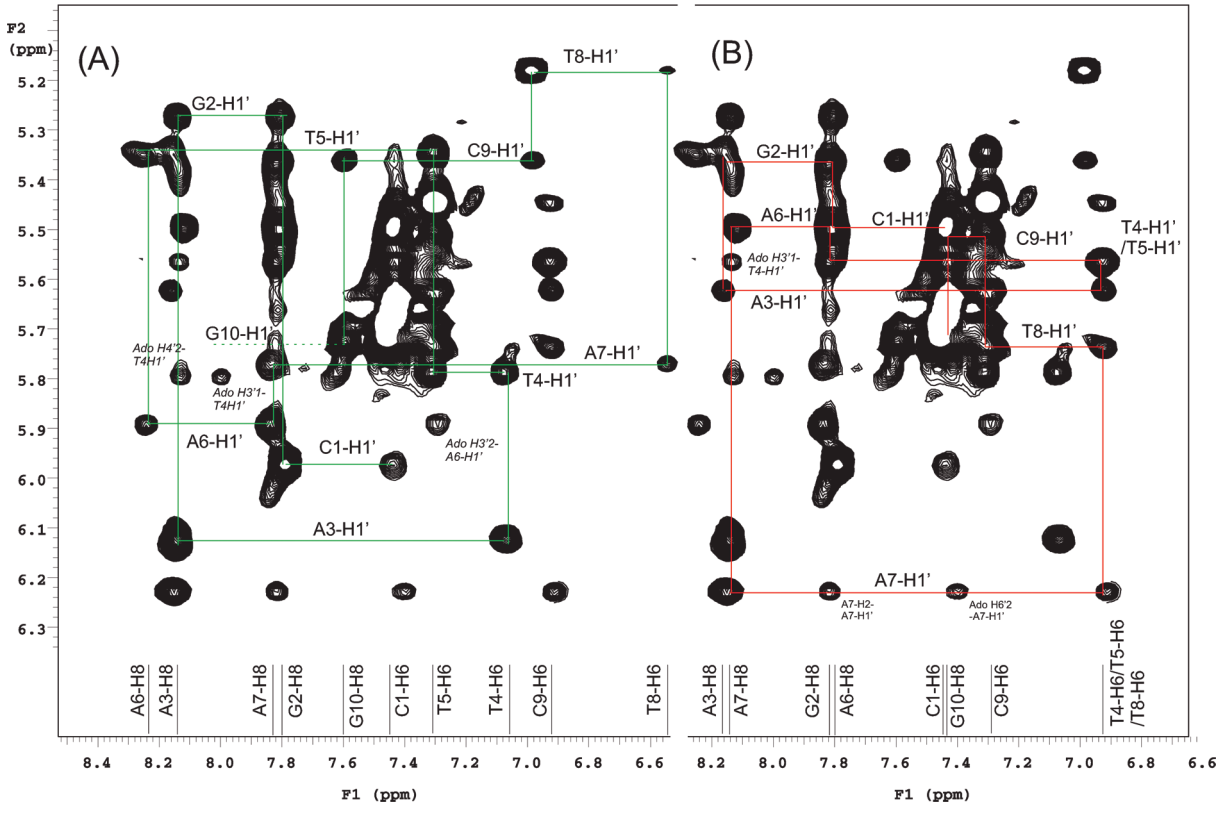


Figure 1. Two-dimensional phase sensitive NOESY spectrum (250 ms mixing time) expanded contour plot of the adozelesin duplex adducts in buffered D_2O solution (pH 6.75) at 300 K, displaying connectivities from aromatic Pu H8/Py H6 to deoxyribose H1′ and deoxyribose H1′ of the 5′-neighbor for the Watson−Crick adduct (A) and for the Hoogsteen adduct (B).

spectrum confirmed that the reaction had proceeded to completion, as there was no evidence of remaining unreacted duplex cross-peaks. Analysis of the 2D NOESY and COSY spectra confirmed the presence of two self-complementary and palindromic DNA−adozelesin adducts. There are four clear COSY cross-peaks visible at F_1 and F_2 coordinates of δ 7.44 and 5.70, δ 7.44 and 5.65, δ 7.31 and 5.45, and δ 6.98 and 5.18, respectively, confirming the presence of four distinct cytosine resonances

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(two from each adduct). In addition, two complete "walks" are possible in the aromatic H6/H8−H1′ region of the NOESY spectrum (Figure 1). Resonances from adozelesin protons in both adducts confirm the presence of two adozelesin residues (one in each adduct) that are in close association with the DNA duplex.

Identification of the Covalent Sites in Both DNA−Adozelesin Adducts. As discussed previously, adozelesin is known to react with the N3 position of an adenine base in the base of the minor groove, with the adduct spanning a total of 5 bp. It has been shown in previous gel electrophoresis[10] and NMR[9] studies to have a preference for 5′-ATT-3′ sequences. As such, upon reaction with the 5′-d(CGA*TTAATCG)$_2$ duplex, the adozelesin molecule should have just one preferred reaction site at base A3.

The alkylation of DNA has been observed to proceed through opening of the cyclopropyl ring and formation of a methylene bridge from C8 to N3 of the modified adenine. This is confirmed in this case by the observation of downfield shifts of the adozelesin cyclopropyl ring protons Ado H8A and Ado H8B. The shifts of Ado H8A and Ado H8B in unreacted adozelesin would be expected at δ 1.47 and 2.02, respectively.[23] In the 5′-d(CGATTAATCG)$_2$−adozelesin adduct, these proton resonances are shifted downfield to around δ 5.5, in the case of the first adduct, and δ 5.3 for the second. This is also analogous to the shifts for the 5′-d(CGTAAGCGCTTACG)$_2$−adozelesin adduct, in which the Ado H8A and Ado H8B proton resonances were found at δ 5.06 and 5.15, respectively,[9,24,25] and confirms that the covalent bond with adenine has formed via the C8−N3 methylene bridge.

Further evidence exists for adenine A3 as the site of covalent modification in the observation of NOE connectivities from the adenine H2 proton of the modified adenine into the CPI head unit. NOEs can be found from A3 H2 to Ado H8A2 and into Ado H1A/B in both adducts. A lack of any cross-peak between A3 H2 and the adozelesin H3 proton is consistent with previous studies on the 5′-d(CGTAAGCGCTTACG)$_2$−adozelesin adduct and indicates that the drug is bound in an edge-on orientation in the minor groove.[9]

Several models were examined to explain the presence of two species in the 5′-d(CGATTAATCG)$_2$−adozelesin adduct. The first is that there is simply unreacted DNA duplex remaining in the reaction mixture. As discussed above, this can be ruled out on the basis of a comparison between the 2D NOESY spectrum of the duplex and that of the mixture after reaction with adozelesin. The second possibility is that of bisalkylation (bisalkylation is required to maintain a self-complementary duplex) at different adenine residues, namely, A6 or A7. Stereochemistry within the adozelesin molecule directs the reaction with DNA, and in both cases, adducts would be formed with the B and C subunits pointing out and away from the center of the duplex. Simple models for each of these adducts were produced in SYBYL. Within the A6 bisalkylated adduct, there was overlap of the nonplanar A rings while the C subunit in the bis-A7-alkylated adduct protruded from the end of the duplex. Both models could also easily be eliminated on the basis of the NMR NOE data. The site of covalent modification in the case of both adducts was finally confirmed as A3 by NOE contacts back into the modified base and DNA duplex.

The possibility of monoalkylation also had to be eliminated because it was conceivable that either two monoalkylated adducts or a mixture of one monoalkylated and the expected bisadduct could have formed. Examination of the 2D NOESY spectrum of the reaction mixture again discounts this option. Observation of the two complete self-complementary symmetrical adducts confirms that both adducts contain two adozelesin residues each reacting with the equivalent adenine. In the case of a monoadduct, the symmetry of the species would be lost and the two DNA strands would no longer be equivalent.

It is also possible to envisage two adducts in which the benzofuran C rings of adozelesin were overlapped differently. In one model, the whole of the adozelesin residue would be closely associated with the modified strand. In the second, the residue would associate with the modified strand near the site of covalent attachment (CPI-ring) but would then cross over, and the benzofuran C ring would associate with the opposite strand. These options were investigated extensively; however, it was clear from NOE connectivity between the bound adozelesin residues and the duplex that the drug was most closely associated with the modified strand in both adducts (Figures 6 and 7).

Mixed adducts were formed in previous experiments by Seaman and Hurley[26,27] involving the reaction of a 5′-d(CGTAATTACG)$_2$ duplex with the dimeric CPI analogue bizelesin. In this study, approximately 60% consisted of an adduct in which both adenine residues of the central TA step were Hoogsteen base-paired to thymines, while the remaining 40% existed in an open conformation. It was suggested that the insertion of bizelesin into the minor groove provides the impetus for reorientation of the central adenine residues and stabilizes the resulting Hoogsteen base pairs upon completion of the cross-link.[26]

This paper suggested three further possibilities for the identification of the two adducts present within this study: (1) the formation of a Watson−Crick bisadduct with overlap of the C subunits as predicted, (2) the formation of a base-paired overlapping adduct in which the central TA step displays Hoogsteen base pairing, and (3) formation of an overlapped bisadduct with an open base pair conformation.

In this 5′-d(CGATTAATCG)$_2$−adozelesin adduct, the overlap of the benzofuran C subunits should give an adozelesin complex that is similar in overall length to that of bizelesin, and it is feasible that the Hoogsteen and open base pair adducts could be "frozen out" in the same way that was observed in the previous study of bizelesin.[26] The Hoogsteen rotation is a stable duplex response to the steric stress associated with cross-linkage,[26,27] and as the overlapped region of the adozelesin drug moieties unquestionably results in a large increase in the degree of steric crowding in this region, it is unsurprising that the duplex DNA may adopt this conformation as a solution. Quinoxaline intercalators have also been shown to stabilize partial Hoogsteen base pairs to the 3′-side of intercalation;[28,29] however, with these compounds, the insertion of the intercalator disrupts base stacking and base−backbone interactions in the Hoogsteen region. The overlapped adozelesin molecules will not intrude into the base stacking of the Watson−Crick to Hoogsteen base pair transition region, and therefore, this type of Hoogsteen stabilization is unlikely for the adozelesin. It was considered more likely that any Hoogsteen type base pairs would be stabilized in a similar manner to that previously observed with bizelesin; however, unlike the bizelesin adduct, the urea linker, believed to be partly responsible for stabilizing the bizelesin adducts, is not present.

The possibility of open base pairs was investigated using 2D ROESY spectroscopy; this would also indicate if exchange (on the NMR time scale) was occurring between the observed

Watson−Crick and Hoogsteen adducts. Studies on the bizelesin adducts[26] showed multiple possible conformations for the open base pairs, with movement (on an NMR time scale) between these conformations. ROESY experiments were used to study this conformational exchange. However, ROESY data for the 5′-d(CGATTAATCG)$_2$−adozelesin mixed adduct provided no evidence of conformational exchange in either adduct, and the possibility of open base pairs was ruled out on the basis of this result. It was, therefore, concluded that the mixed adozelesin adduct consisted of two adducts, in almost equal quantities, one maintaining Watson−Crick base pairing throughout and the other exhibiting the formation of Hoogsteen base pairs at the two AT base pairs forming the central TA step in analogy with previous studies on bizelesin.[26,27] Final confirmation of the species present was established by full analysis of the respective NOESY and COSY data sets.

Assignments of the Proton Resonances for the Adozelesin Watson−Crick Duplex Adduct. Proton chemical shifts in the 5′-d(CGATTAATCG)$_2$−adozelesin adducts relative to those of duplex DNA can be found in Table 3. Downfield shifts of the protons from the opened adozelesin cyclopropyl ring Ado H8A and Ado H8B (δ 5.50) relative to unbound adozelesin (δ 1.47 and 2.02, respectively)[23] confirm ring opening, while NOE interactions of the modified base and surrounding bases confirm the site of covalent attachment as adenine A3 N3 and C8 of the adozelesin molecule. Large upfield shifts are observed in the signals from the T8 H6 nucleotide aromatic and T8 H1′ protons, which are situated directly opposite the modified A3 base. The NOE intensities mentioned above (T8 H6−T8 H1′ and T8 H6−A7 H1′) can be seen in Figure 1, and it is noted that they are also relatively weak compared to other DNA aromatic−H1′ NOE intensities. This suggests a degree of backbone distortion as a result of adduct formation. One possible explanation is that the drug molecules are required to "push back" to achieve π-stacking between the aromatic benzofuran C rings. This movement of drug molecules in the narrow minor groove causes distortion along the backbone of the DNA duplex, which centers on the T8 nucleotide. This is supported by further downfield shifts of 0.68 ppm of the signal for the aromatic proton of the C9 nucleotide (C9 H6), indicating T8 and C9 as the probable centers for any distortion. Molecular modeling, with the inclusion of π-stacking restraints at the overlapped drug region, was used to support this hypothesis.

The 5′-d(CGATTAATCG)$_2$−adozelesin Watson−Crick adduct displays a surprising lack of changes in chemical shift compared to the duplex values in the central region of the duplex in the area of drug overlap. These central protons remain at a chemical shift very similar to that found in the duplex. The ability of the minor groove to open by up to 17 Å has been noted in previous studies on stacked lexitropsin adducts.[30]

The assignment of the complete 2D NOESY and COSY spectra for the two 5′-d(CGATTAATCG)$_2$−adozelesin adducts provided many drug−DNA interproton connectivities, which confirm the location of the drug in the minor groove of the DNA duplex. All drug−DNA connectivities for the adozelesin Watson−Crick adduct can be found in Table 4. NOE cross-peaks between the DNA duplex and the A subunit of adozelesin confirm the association of the drug molecule with the base protons at the site of covalent modification. In particular, an intense NOE between A3 H2 and Ado H1a/b provides conclusive evidence of the covalent modification site at A3.

Association of the Watson−Crick Adduct with the Modified Strand of DNA and Confirmation of Benzofuran C Ring Stacking. NOE connectivities between the protons of the benzofuran C subunit (Ado H4′2, Ado H5′2, Ado H6′2, and Ado H7′2) and bases A6, A7, and T8, as shown in Table 4 and Figure 6, confirm a close association of the drug with the modified strand. NOE interactions between the Ado H6′1 and Ado H3′1 protons of the indole B subunit and the aromatic protons of the benzofuran C subunit, such as from Ado H6′1 to Ado H4′2 and from Ado H6′1 to Ado H6′2, confirm the overlap of the B and C subunits. These connectivities can arise from the overlap of the B and C subunits in the respective molecules of adozelesin only in the bis adduct, as the protons are too spatially separated from each other to exhibit connectivities within the same drug molecule. These observations provide evidence that the subunits are substantially overlapped, and the relative intensities confirm the orientation and probability of π-stacking of the C units (Figure 6).

Refined Molecular Model of the 5′-d(CGATTAATCG)$_2$−Adozelesin Watson−Crick Adduct. The 5′-d(CGATTAATCG)$_2$−adozelesin Watson−Crick adduct was modeled using the SYBYL software suite, as outlined in Experimental Procedures. Figure 5 shows the in aquo computer model of the adozelesin Watson−Crick adduct. Adozelesin is colored white, with the two DNA strands colored green and purple. As with previous adozelesin adducts, it is clear that Watson−Crick base pairing has been maintained, with retention of the β-helical structure that allows sequential assignment of the DNA duplex.

Figure 6 shows the central region of the duplex, with A7 H1′, Ado H3′2, and Ado H7′2 highlighted. NOE connectivities between the adozelesin aromatic protons and A7 H1′ are a clear indication of the association of the drug molecule with the strand with which it has reacted covalently; any other arrangement would render these protons too spatially separated to exhibit NOE connectivity in this manner.

Assignments of the Proton Resonances for the Adozelesin Hoogsteen Duplex Adduct. The chemical shifts of the proton signals in the 5′-d(CGATTAATCG)$_2$−adozelesin Hoogsteen adduct, relative to those of duplex DNA, can be found in Table 3. As expected, some large shift changes can be observed, because of the accommodation of the adozelesin molecule in the minor groove. A large upfield shift of 0.50 ppm can be observed in the signal from H1′ of the A3 residue; this residue is the site of covalent modification, and as such, large shifts here are not unexpected and were previously observed in the 5′-d(CGTAAGCGCTTACG)$_2$−adozelesin adduct.[9]

Large changes in the chemical shift of the signal for T5 H8, as well as in those for A6 H1′ and A7 H1′, were also observed in the 5′-d(CGATTAATCG)$_2$−adozelesin Hoogsteen adduct. The T5 and A6 nucleotides are the sites of the Hoogsteen base pair, and the large changes in chemical shifts provide evidence of a change in environment at this point. In addition, these central protons are all situated in the vicinity of the overlapping benzofuran C subunits of the adozelesin molecules, so large changes in chemical shift could be indicative of some steric crowding and shielding and/or deshielding from the aromatic rings thereof.

Assignment of the 2D NOESY and COSY spectra for the 5′-d(CGATTAATCG)$_2$−adozelesin Hoogsteen adduct gave many interproton connectivities from drug to DNA, confirming the location of the drug in the minor groove. All the drug−DNA connectivities are listed in Table 2. Numerous connectivities between the duplex and the CPI subunit of adozelesin once again

Table 3. Chemical Shifts (parts per million) for the 5′-d(CGATTAATCG)$_2$ Duplex, the 5′-d(CGATTAATCG)$_2$−Adozelesin Hoogsteen Adduct, and the 5′-d(CGATTAATCG)$_2$−Adozelesin Watson−Crick Adduct (bold)[a]

	H6/H8 aromatic			H1′			CH3/H5/H2			H2′			H2″			H3′			H4′			imino		
	duplex	adduct		duplex	adduct		duplex	adduct		duplex	adduct		duplex	adduct		duplex	adduct		duplex	adduct		duplex	adduct	
C1	7.40	7.41	*0.01*	5.47	5.51	*0.04*	5.67	5.68	0.01	1.65	1.63	*0.02*	2.16	2.22	*0.06*	4.51	4.69	*0.20*	3.52	3.89	*0.37*	–	–	
		7.40	***0.00***		**5.99**	***0.52***		**5.67**	**0.00**		**2.19**	***0.54***		**2.45**	***0.29***		**4.51**	***0.00***		**3.80**	**0.28**		–	
G2	7.80	7.79	*0.01*	5.36	5.34	*0.02*	–	–	–	2.55	2.60	*0.05*	2.66	2.70	*0.04*	4.83	4.23	*0.60*	4.17	*	–	12.60	+	
		7.76	***0.04***		**5.29**	***0.07***	–	–	–		**2.55**	***0.00***		**2.64**	***0.02***		**4.14**	***0.69***		**3.04**	0.13		+	
A3	8.10	8.15	*0.05*	6.13	5.63	*0.50*	7.40	7.28	0.12	2.54	2.24	*0.30*	2.78	2.62	*0.16*	4.89	4.97	*0.08*	4.00	3.91	0.09	–	–	
		8.11	***0.01***		**6.13**	***0.00***		**7.40**	**0.00**		**2.57**	***0.03***		**2.78**	***0.00***		**4.89**	***0.00***		**3.90**	0.10		–	
T4	7.00	6.90	*0.10*	5.71	5.58	*0.13*	1.17	1.16	0.01	1.79	1.25	*0.49*	2.32	1.40	*0.08*	4.71*	4.91	*0.20*	4.00	3.50	0.50	13.30	10.13	*3.37*
		7.05	***0.05***		**5.79**	***0.08***		**1.11**	**0.06**		**1.77**	***0.02***		**2.34**	***0.02***		**4.03**	–		**3.49**	**0.51**		**12.80**	***0.50***
T5	7.16	6.92	*0.24*	5.52	5.58	*0.06*	1.43	1.32	0.11	1.93	1.62	*0.31*	2.28	1.83	*0.45*	4.71*	4.28	*0.43*	3.96	3.32	0.64	13.40	12.90	*0.50*
		7.29	***0.13***		**5.34**	***0.18***		**1.49**	**0.06**		**2.09**	***0.16***		**2.41**	***0.13***		**4.21**	–		**3.31**	**0.65**		**9.76**	***3.64***
A6	8.10	7.80	*0.30*	5.76	5.39	*0.37*	+	7.43	–	2.58	2.57	*0.01*	2.74	2.70	*0.04*	4.33	4.88	*0.55*	4.28	3.80	0.52	–	–	
		8.22	***0.12***		**5.90**	***0.14***		**7.56**	–		**2.53**	***0.05***		**2.66**	***0.08***		**4.85**	***0.52***		**3.76**	**0.52**		–	
A7	7.97	8.14	*0.17*	5.96	6.23	*0.27*	7.65	7.81	0.16	2.36	2.51	*0.15*	2.71	2.59	*0.12*	4.82	4.40	*0.42*	4.00	3.32	0.68	–	–	
		7.81	***0.16***		**5.78**	***0.18***		**7.83**	**0.18**		**2.09**	***0.27***		**2.41**	***0.30***		**4.40**	***0.42***		**3.00**	**1.00**		–	
T8	6.94	6.90	*0.04*	5.70	5.74	*0.04*	1.09	1.13	0.04	1.78	2.13	*0.35*	2.22	2.20	*0.02*	4.90	4.09	*0.81*	3.96	4.01	0.05	13.50	11.17	*2.33*
		6.51	***0.43***		**5.18**	***0.52***		**0.97**	**0.12**		**1.34**	***0.44***		**1.84**	***0.38***		**4.21**	***0.69***		**4.00**	**0.04**		**11.02**	***2.48***
C9	7.28	7.29	*0.01*	5.53	5.51	*0.02*	5.43	5.45	0.02	1.83	1.84	*0.01*	2.18	2.22	*0.04*	4.71*	3.96	*0.75*	3.90	3.89	0.01	–		
		7.96	***0.68***		**5.38**	***0.15***		**5.18**	**0.25**		**1.49**	***0.34***		**1.89**	***0.29***		**4.29**	–		**3.80**	**0.10**			
G10	7.75	7.41	*0.34*	5.94	5.68	*0.26*	–	–	–	2.18	1.68	*0.50*	2.44	2.09	*0.35*	4.51	4.40	*0.11*	3.90	3.55	0.35	+	+	
		7.56	***0.19***		**5.73**	***0.21***		–	–		**2.07**	***0.11***		**2.31**	***0.13***		**4.34**	***0.17***		**3.51**	**0.39**		+	

[a] Chemical shift differences between the duplex and duplex adducts are shown in italics and are underlined if the difference was greater than 0.25 ppm. A plus means no peak was found. An asterisk means a peak at 4.71 ppm (not observed because of water suppression) was found.

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Table 4. NOE Connectivities between the Drug Protons and the DNA Duplex in the 5′-d(CGATTAATCG)$_2$−Adozelesin Watson−Crick Adduct[a]

	H1a/b	H8a/b	H8A2	CH_3	H6	H3	H3′1	H4′1	H6′1	H7′1	H3′2	H4′2	H5′2	H6′2	H7′2
A3 H8				O											
A3 H1′				O											
A3 H4′				O											
A3 H2	S	M					W								
T4 H1′		O				W	M								
T4 H3′							W								
T5 H1′												M			
T5 H3′													W		
T5 H4′								W							
A6 H1′			M								W	O			
A6 H8											W				
A6 H4′												W			
A7 H2	M		M				O	W				O	O	M	
A7 H1′								W			M	W	M	W	
A7 H3′								W							M
T8 H1′										W					
T8 H4′									W					W	

[a] Abbreviations: S, strong; M, moderate; W, weak; O, overlaid, but significant contribution to peak volume. This table is available in cartoon as Supporting Information.

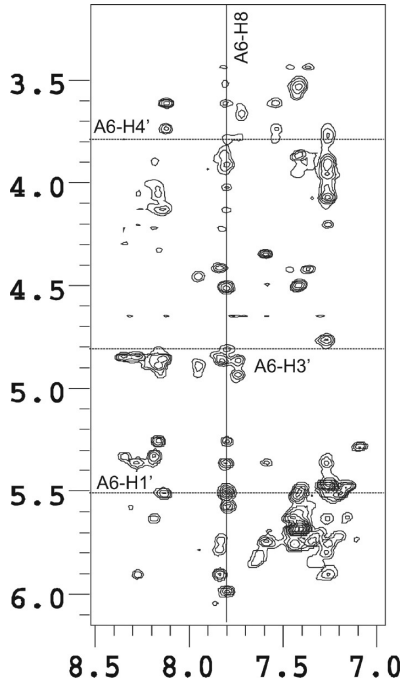

Figure 2. Two-dimensional phase-sensitive NOESY spectrum (200 ms mixing time) expanded contour plot of the adozelesin duplex adducts in a buffered D_2O solution (pH 6.75) at 300 K. Expansion showing NOE connectivities arising from the A6 H8 (Hg) resonance. Cross-peaks to protons in the minor groove are weakened, while the cross-peak from A6 H8 (Hg) to A6 H1′ (Hg) is intensified.

confirm the covalent modification of the A3 base. As expected, NOE connectivities from the CPI subunit are predominantly to bases A3 and T4 of the DNA duplex, because of the close association of the head unit with the modified strand. Evidence of the overlap of the benzofuran C subunits exists in the form of NOE connectivities between protons on the B and C subunits that as reported for the Watson−Crick adduct could not exist unless two adozelesin molecules were overlaid. NOE connectivities can be seen between Ado H4′1 of the indole subunit and the aromatic Ado H4′2, Ado H5′2, and Ado H6′2 protons of the benzofuran subunit. In addition, a cross-peak can be seen as a result of the spatial proximity of Ado H6′2 and Ado H6′1. This NOE interaction can arise from the overlap of these subunits only from the respective molecules of adozelesin (Figure 7).

When the NOE connectivities between the drug and the DNA backbone are examined, cross-peaks are observed between A7 H1′ and the aromatic Ado H5′2 and Ado H6′2 protons of the adozelesin benzofuran subunit. Such dipolar coupling of adozelesin aromatic protons with the base protons of the covalently modified strand provides unequivocal evidence of the close association of the drug molecule with the covalently modified strand (Figure 7).

The most diagnostic cross-peaks for a Hoogsteen base pair come from A6 H2 and A8 H8 protons, as seen in previous studies involving assignment of Hoogsteen duplexes.[26,27] In a Watson−Crick duplex, the A H2 proton is found in the minor groove, while the major groove accommodates the A H8 proton. When a Hoogsteen base pair (Scheme 1) is formed, *anti* to *syn* rotation around the glycosidic bond (C1′−N9) moves the A H2 proton to a major groove position, similar to that previously occupied by the A H8 proton, and relocates the A H8 proton into the minor groove. The new minor groove position of *syn* A H8 results in the classical connectivities to major groove protons, such as DNA H3′, H4′, H2′, and H2″, becoming greatly weakened, while the NOE cross-peak from A6 H8 to A6 H1′ is intensified because of the relatively shorter distance from A6 H1′ to *syn* A6 H8 (Figure 2). In addition to weakened peaks associated with A6 H8, NOE resonances can be observed between A6 H2 and H2′ and H2″ of the 3′ neighboring base (T5) (Figure 3). In its new location within the major groove, *syn* A6 H2 is placed in the vicinity of these protons and so exhibits NOE connectivities with them. This is in analogy to previous studies on a Hoogsteen adduct performed by Seaman and Hurley.[26] Unlike the previous study with bizelesin, we were able to identify and assign the exchangeable imino signal for the Hoogsteen base pair within this adduct via an NOE contact to adenine H8. This NOE contact is the equivalent of the H2−imino NOE normally observed in Watson−Crick paired AT base pairs and is characteristic of Hoogsteen base pairing (Figure 4).

Refined Molecular Model of the 5′-d(CGATTAATCG)$_2$–Adozelesin Hoogsteen Adduct. The 5′-d(CGATTAATCG)$_2$–adozelesin Hoogsteen adduct was modeled using the SYBYL software suite, as outlined in Experimental Procedures. The Hoogsteen model was created by modifying the Watson–Crick adduct models created above and then adjustment to incorporate the central Hoogsteen base pairs. Figure 5 shows the in aquo computer model of the adozelesin Hoogsteen adduct. Adozelesin is colored white, with the two DNA strands colored green and purple. With the exception of the central A6 Hoogsteen base pairs, the duplex has retained β-helical structure. The central Hoogsteen pairs are slightly distorted but show minimal disruption to surrounding base pairs and accommodate the drug overlap by providing a slight widening of the minor groove. Figure 7a shows the central region of the duplex, with A7 H1′, Ado H5′2, and Ado H6′2 highlighted. NOE connectivities between the adozelesin aromatic protons and A7 H1′ are a clear indication of the association of the drug with its own covalently modified strand. Figure 7b also shows the central region of the duplex. Ado H4′2, Ado H5′2, Ado H6′2, and Ado H6′1 are colored red. These protons were found to be dipolar coupled, exhibiting NOESY cross-peaks that provide confirmation of the overlap of the adozelesin benzofuran C rings.

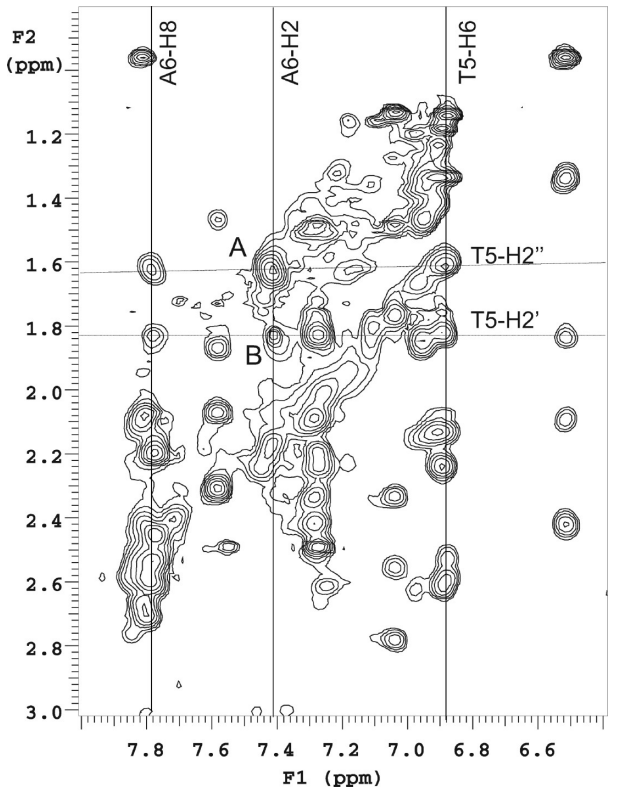

Figure 3. Two-dimensional phase-sensitive NOESY spectrum (200 ms mixing time) expanded contour plot of the adozelesin duplex adducts in a buffered D_2O solution (pH 6.75) at 300 K, Expansion of the H6/H8–H2′/H2″ region showing NOE connectivity of A6 H2 (Hg) with T5 H2′ (Hg) and T5 H2″ (Hg) (peaks A and B, respectively).

DISCUSSION

NMR studies of adducts formed in the reaction of adozelesin with 5′-d(CGATTAATCG)$_2$ confirmed that stacking of the C rings is possible. However, rather than the predicted single self-complementary adduct, two structurally related self-complementary adozelesin-stacked adducts are formed in almost equal quantities. The first adduct was identified as the predicted Watson–Crick base-paired adduct in which only minor distortion of the duplex is observed. Both adozelesin residues are sitting in the minor groove with the two adozelesin benzofuran C rings stacked edge-on to the groove and stabilized by π-stacking between the rings. The second adduct was considerably more difficult to identify. It was established quickly that the overall structure of this adduct was similar to that of the first adduct with alkylation occurring at the predicted adenine residues. However, it was also evident that an unusual base-pair conformation was present within the two central A-T base pairs. It was also

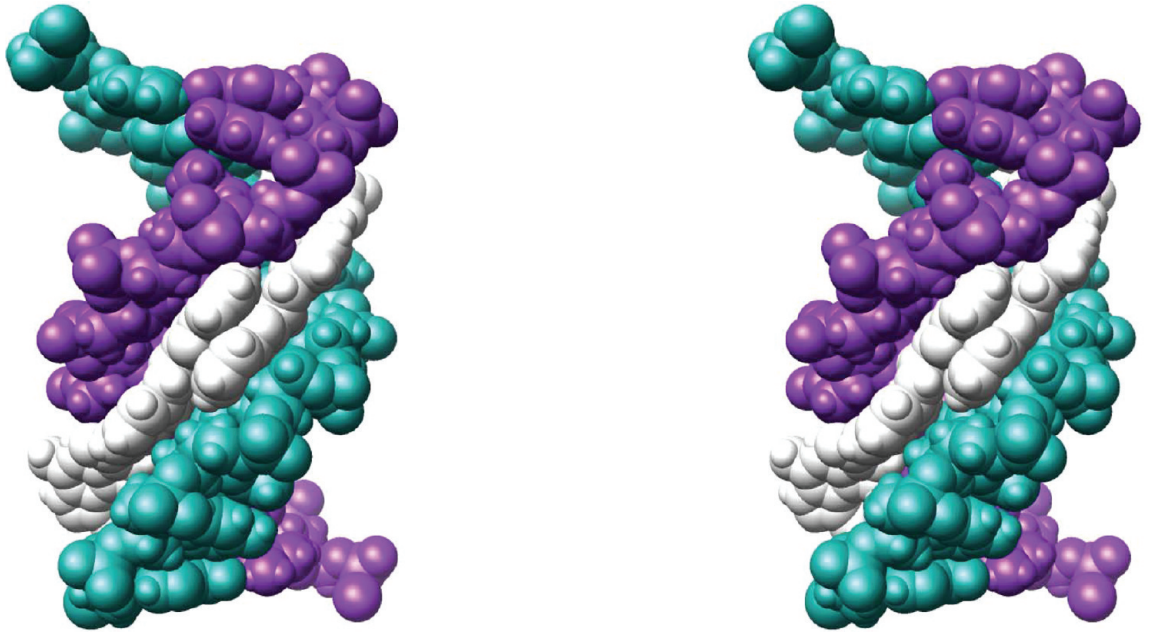

Figure 4. Stereoview of the 5′-d(CGATTAATCG)$_2$–adozelesin Watson–Crick adduct. DNA strands are colored purple and green, while the adozelesin molecules are colored white.

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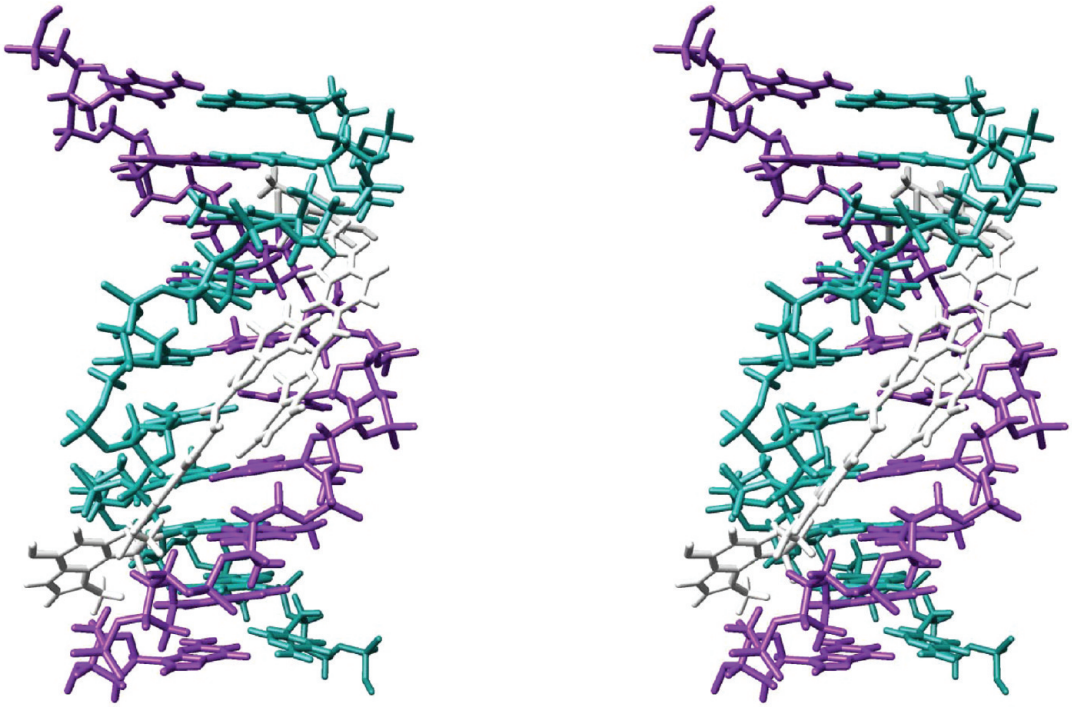

Figure 5. Stereoview of the 5′-d(CGATTAATCG)$_2$−adozelesin Hoogsteen adduct. Slight distortion can be seen at the central Hoogsteen base pairs, but the disruption to the surrounding bases is minimal.

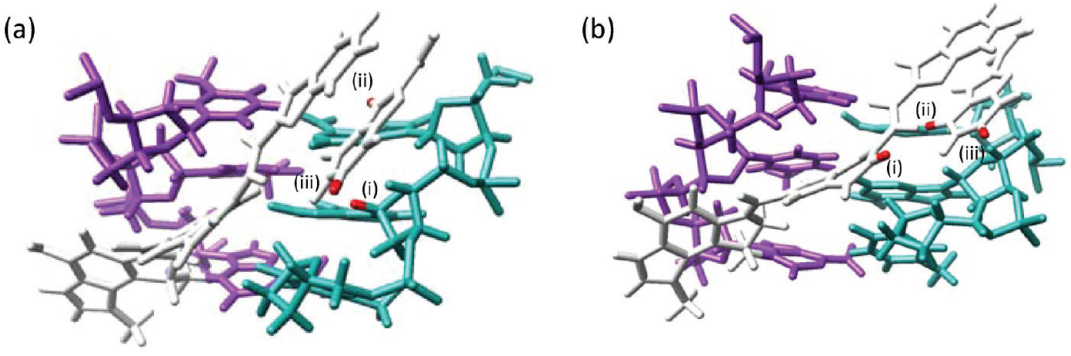

Figure 6. Sections of the 5′-d(CGATTAATCG)$_2$−adozelesin Watson−Crick adduct central region. (a) A7 H1′ (i), Ado H3′2 (ii), and Ado H7′2 (iii) are colored red. These NOE connectivities (along with others not shown) show close association between the ligand and modified strand. (b) Ado H6′1 (i), Ado H4′2 (ii), and Ado H6′2 (iii) are colored red. NOE connectivities between these protons confirm overlap of the benzofuran subunits.

established that, like in the first adduct, the adozelesin benzofuran C ring residues were π-stacked and located in a similar position within the minor groove. Previous studies by Seaman and Hurley[26,27] with the CPI dimer bizelesin provided possible identities for the second adozelesin adduct. Within this bizelesin study, two adducts were produced, neither of which was the expected Watson−Crick duplex adduct. The first adduct contained an open base-pair arrangement (a mixture of three related exchanging structures) and the second a Hoogsteen base-paired adduct in which the central two bases were rearranged into a Hoogsteen conformation. The open base-pair arrangements observed in the first adduct resulted in relatively free movement of the "open" bases and exchange among three related structures. These three structures could be identified using ROESY spectroscopy. Similar NMR experiments with the adozelesin adduct provided no evidence of molecular movement or conformation exchange, and hence, the open base pair model was rejected. Close examination of the NMR data for the Hoogsteen bizelesin adduct, however, revealed striking similarities to this new adozelesin adduct, and we were able to confirm that the second adduct formed was the Hoogsteen base-paired version of the expected Watson−Crick adduct. The ROESY data also indicate that there is no exchange between the two duplex adducts (at least on the NMR time scale). However, previous gel electrophoresis studies with (+)-CC-1065, bizelesin, and adozelesin have shown that the duplex adducts formed are reversible and migration is possible between duplexes and binding sequences.[10] These studies would suggest that the two duplex adducts observed within this study should be in slow equilibrium, and the almost equivalent concentrations would indicate approximately equal stability at the temperature studied. The adducts were studied for up to 3 weeks before the level of breakdown products became an issue; during this period, no changes in the relative quantities of the two adducts were observed. Unfortunately, the study

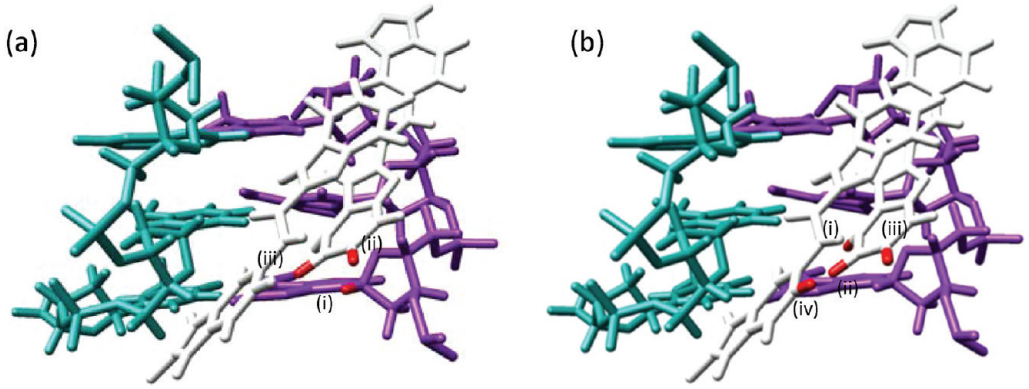

Figure 7. Sections of the 5′-d(CGATTAATCG)$_2$—adozelesin Hoogsteen adduct central region. (a) A7 H1′ (i) and Ado H5′2 (ii) and H6′2 (iii) are colored red. These NOE connectivities (along with others not shown) show close association between the ligand and modified strand. (b) Ado H4′2 (i), Ado H5′2 (ii), Ado H6′2 (iii), and Ado H6′1 (iv) are colored red. The NOE connectivity between these protons confirms overlap of the benzofuran subunits.

of the relative kinetic and/or thermodynamic stability of these adducts is not possible because of the inherent heat instability of the resulting CPI adducts and depurination and strand breakage.

This NMR study provides a new model for the binding of the CPI antitumor antibiotics that could also apply to many other covalent and noncovalent minor groove ligands. Previous studies with noncovalent anticancer antibiotics based on netropsin and distamycin have shown stacked conformations; however, this study suggests that stacked adducts may be common in other DNA covalent-binding compounds. For many years, new minor groove interactive ligands have been designed to be flat to allow insertion into the "narrow" minor groove.[31−34] We now propose that this planarity may also allow two molecules to "stack" effectively, creating pseudo-DNA cross-linkers and opening new possibilities in anticancer drug design and optimization of ligands. The study also provides further evidence of the dynamic nature of drug—DNA interactions and how molecules can manipulate the DNA and also be manipulated by the DNA to accommodate binding.

■ ASSOCIATED CONTENT

Supporting Information. ^{1}H NMR one-dimensional spectra for the duplex and duplex adducts, additional expansions of the ^{1}H NMR, 2D NOESY spectrum for the duplex adducts, and cartoon representations of the NOE interactions given in Tables 2 and 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

CPI, cyclopropapyrroloindole; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE-correlated spectroscopy; rMD, restrained molecular dynamics; rMM, restrained molecular mechanics; FID, free induction decay; DMF, dimethylformamide; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography.

■ REFERENCES

(1) Hanka, L. J., Dietz, A., Gerpheide, S. A., Kuentzel, S. L., and Martin, D. G. (1978) CC-1065 (NSC-298223), a new anti-tumor antibiotic: Production, *in vitro* biological-activity, microbiological assays and taxonomy of producing microorganism. *J. Antibiot. 31*, 1211–1217.

(2) Martin, D. G., Biles, C., Gerpheide, S. A., Hanka, L. J., Krueger, W. C., McGovren, J. P., Miszak, S. A., Neil, G. L., Stewart, J. C., and Visser, J. (1981) CC-1065 (NSC-298223), a potent new anti-tumor agent: Improved production and isolation, characterization and anti-tumor activity. *J. Antibiot. 34*, 1119–1125.

(3) Lin, C. H., Beale, J. M., and Hurley, L. H. (1991) Structure of the (+)-CC-1065-DNA adduct: Critical role of ordered water-molecules and implications for involvement of phosphate catalysis in the covalent reaction. *Biochemistry 30*, 3597–3602.

(4) Mc Govren, J. P., Clarke, G. L., Pratt, E. A., and Dekoning, T. F. (1984) Preliminary toxicity studies with the DNA-binding antibiotic, CC-1065. *J. Antibiot. 37*, 63–70.

(5) Neil, G. L., Clarke, G. L., and McGovren, J. P. (1981) Anti-tumor activity and acute toxicity of CC-1065 (NSC-298223) in the mouse. *Proc. Am. Assoc. Cancer Res. 22*, 1244.

(6) Fleming, G. F., Ratain, M. J., O'Brien, S. M., Vogelzang, N. J., and Earhart, R. H. (1992) Phase-I study of adozelesin administered by 24-h continuous intravenous-infusion. *Proc. Am. Assoc. Cancer Res. 33*, 265.

(7) Burris, H., Earhart, R., Kuhn, J., Shaffer, D., Smith, L., Weiss, G., Kasunic, D., Radbury, G., Campbell, L., and Von Hoff, D. D. (1992) Phase I study with the DNA sequence-specific agent adozelesin. *Proc. Am. Assoc. Cancer Res. 33*, 520.

(8) Reynolds, V. L., Molineux, I. J., Keplan, D. J., Swenson, D. H., and Hurley, L. H. (1985) Reaction of the antitumor antibiotic CC-1065 with DNA: Location of the site of thermally induced strand breakage and analysis of DNA-sequence specificity. *Biochemistry 24*, 6228–6237.

(9) Cameron, L., and Thompson, A. S. (2000) Determination of the structural role of the linking moieties in the DNA binding of adozelesin. *Biochemistry 39*, 5004–5012.

(10) Hurley, L. H., and Draves, P. H. (1993) in *Molecular Aspect of Anticancer Drug-DNA Interactions* (Neidle, S., and Waring, M. J., Eds.) Vol. I, pp 89−133, Macmillan Press Ltd., Basingstoke, England.

(11) Gait, M. J., Ed. (1984) *Oligonucleotide Synthesis: A Practical Approach*, IRL Press, Oxford, England.

(12) Thompson, A. S., and Hurley, L. H. (1995) Monoalkylation and cross-linking of DNA by cyclopropapyrroloindoles entraps bent and straight forms of A-tracts. *J. Am. Chem. Soc. 117*, 2371–2372.

(13) Sklenar, V., and Bax, A. (1987) Spin echo water suppression for the generation of pure phase two-dimensional NMR spectra. *J. Magn. Reson. 74*, 469–479.

(14) Blake, P. R., and Summers, M. F. (1990) NOESY-1−1-Echo spectroscopy with eliminated radiation damping. *J. Magn. Reson. 86*, 622–624.

(15) Bax, A., Griffey, H., and Hawkins, B. (1983) Correlation of proton and nitrogen-15 chemical shifts by multiple quantum NMR. *J. Magn. Reson. 55*, 301–315.

(16) Purcel, W. P., and Singer, J. A. (1967) Brief review and table of semiempirical parameters used in the Hückel molecular orbital method. *J. Chem. Eng. Data 12*, 235–246.

(17) Gasteiger, J., and Marsili, M. (1980) Iterative partial equalization of orbital electronegativity: A rapid access to atomic charges. *Tetrahedron 36*, 3219–3228.

(18) Young, M. A., Jayaram, B., and Beveridge, D. L. (1997) Intrusion of counterions into the spine of hydration in the minor groove of B-DNA: Fractional occupancy of electronegative pockets. *J. Am. Chem. Soc. 119*, 59–69.

(19) Blanco, M. (1991) Molecular silverware. I. General solutions to excluded volume constrained problems. *J. Comput. Chem. 12*, 237–247.

(20) Hansen, M., and Hurley, L. H. (1995) Altromycin B threads the DNA helix interacting with both the major and the minor grooves to position itself for site-directed alkylation of guanine N7. *J. Am. Chem. Soc. 117*, 2421–2429.

(21) Antonow, D., Barata, T., Jenkins, T. C., Parkinson, D. N., Howard, P. W., Thurston, D. E., and Zloh, M. (2008) Solution structure of a 2:1 C2-(2-naphthyl) pyrrolo[2,1-c][1,4]benzodiazepine DNA adduct: Molecular basis for unexpectedly high DNA helix stabilization. *Biochemistry 47*, 11818–11829.

(22) Auffinger, P., Louisemay, S., and Westhof, E. (1995) Multiple molecular-dynamics simulations of the anticodon loop of tRNA(Asp) in aqueous-solution with counterions. *J. Am. Chem. Soc. 117*, 6720–6726.

(23) Walker, G. S., Fagerness, P. E., Farley, K. A., and Mizsak, S. A. (1997) The complete H-1 and C-13 chemical shift assignments of a cyclopropylpyrroloindole analog: Adozelesin. *J. Heterocycl. Chem. 34*, 295–299.

(24) Thompson, A. S., and Hurley, L. H. (1995) NMR and distance restrained molecular dynamics of the bizelesin-[d(CGTTTTTACG/GCAAAAATGC)] cross-linked adduct. *J. Mol. Biol. 252*, 86–107.

(25) Thompson, A. S., Fan, J.-Y., Hansen, M. R., and Hurley, L. H. (1995) NMR and restrained molecular dynamics of the seven base-pair cross-linked adduct of bizelesin with [d(CGTTAGTTACG/GCAATCAATGC)]. *Biochemistry 34*, 11005–11016.

(26) Seaman, F. C., and Hurley, L. H. (1993) Interstrand cross-linking by bizelesin produces Watson-Crick to Hoogsteen base-pairing transition region in $d(CGTAATTACG)_2$. *Biochemistry 32*, 12577–12585.

(27) Seaman, F. C., and Hurley, L. (1996) Manipulative interplay of the interstrand cross-linker bizelesin with $d(TAATTA)_2$ to achieve sequence recognition of DNA. *J. Am. Chem. Soc. 118*, 3434–3443.

(28) Wang, A. H.-J., Ughetto, G., Quigley, G.-J., Hakoshima, T., van der Marel, G., van Boom, J. H., and Rich, A. (1984) The molecular-structure of a DNA Triostin-A complex. *Science 225*, 1115–1121.

(29) Wang, A. H.-J., Ughetto, G., Quigley, G.-J., and Rich, A. (1986) Interactions of quinoxaline antibiotic and DNA: The molecular-structure of a Triostin A-*d*(GCGTACGC) complex. *J. Biomol. Struct. Dyn. 4*, 319–342.

(30) Blaskó, A., and Bruice, T. C. (1993) Stoichiometry and structure of complexes of DNA oligomers with microgonotropens and distamycin by ^{1}H-NMR spectroscopy and molecular modeling. *Proc. Natl. Acad. Sci. U.S.A. 90*, 10018–10022.

(31) Tiberghien, A. C., Evans, D. A., Kiakos, K., Martina, C. R. H., Hartley, J. A., Thurston, D. E., and Howard, P. W. (2008) An asymmetric C8/C8′-tripyrrole-linked sequence-selective pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) dimer DNA interstrand cross-linking agent spanning 11 DNA base pairs. *Bioorg. Med. Chem. Lett. 18* (6), 2073–2077.

(32) Rettig, M., Langel, W., Kamal, A., and Weisz, K. (2010) NMR structural studies on the covalent DNA binding of a pyrrolobenzodiazepine-naphthalimide conjugate. *Org. Biomol. Chem. 8* (14), 3179–3187.

(33) Antonow, D., Barata, T., Jenkins, T. C., Parkinson, G. N., Howard, P. W., Thurston, D. E., and Zloh, M. (2008) Solution structure of a 2:1 C2-(2-naphthyl) pyrrolo[2,1-*c*][1,4]benzodiazepine DNA adduct: Molecular basis for unexpectedly high DNA helix stabilization. *Biochemistry 47* (45), 11818–11829.

(34) Wells, G., Martin, C. R. H., Howard, P. W., Sands, Z. A., Laughton, C. A., Tiberghien, A., Woo, C. K., Masterson, L. A., Stephenson, M. J., Hartley, J. A., Jenkins, T. C., Shnyder, S. D., Loadman, P. M., Waring, M. J., and Thurston, D. E. (2006) Design, synthesis, and biophysical and biological evaluation of a series of pyrrolobenzodiazepine-poly(N-methylpyrrole) conjugates. *J. Med. Chem. 49*, 5442–5461.