Structural Characterization of a Calicheamicin–DNA Complex by NMR

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Abstract: The enediynes, including neocarzinostatin, calicheamicin, esperamicin, and dyneamicin, are an important class of antitumor antibiotics that cleave DNA. In spite of intense interest in the enediynes as potential drugs, there is no detailed structural information about how any of these compounds interacts with DNA. We report the first NMR studies of a complex between an enediyne, calicheamicin γ_1, and DNA. Calicheamicin γ_1 cleaves DNA in a double-stranded fashion at oligopyrimidine/oligopurine sequences. The molecular basis for the selective recognition of pyrimidine/purine runs is not well understood. Using NMR we have shown that calicheamicin γ_1 binds to the non-self-complementary DNA duplex, d[GTGACCTG]–d[CAGGTCAC], where ACCT is the recognition sequence. The DNA distorts upon binding to accommodate the drug. The distortion is largest at the CpC step of the recognition sequence and appears to be associated with a widening of the minor groove. A preliminary analysis of the data indicates that the drug itself does not distort much upon binding. It is proposed that binding selectivity reflects the ability of oligopyrimidine sequences to distort to accommodate the more rigid drug.

Introduction

Neocarzinostatin, calicheamicin, esperamicin, and dynemicin are members of the enediyne family of antitumor agents that cleave DNA.[1-3] In spite of intense interest in the enediynes as potential drugs, there is no detailed structural information about how any of them interacts with DNA. We report the first NMR studies of a complex between an enediyne, calicheamicin γ_1, and DNA.

Calicheamicin γ_1 (Figure 1) is an extraordinarily potent antitumor agent that cleaves DNA at oligopyrimidine/oligopurine runs, including TCCT, ACCT, CTCT, TCCC, and TTTT.[2-4] The mechanism of cleavage involves activation of the aglycon with exogenous thiol followed by cyclization of the enediyne to produce a 1,4-diyl radical (Scheme I).[3] If the drug is activated in the presence of DNA, the diradical abstracts a hydrogen atom from each strand of the DNA backbone, initiating strand scission. Cleavage studies on deuterium-labeled dodecamers have shown that a 5′ hydrogen (deuterium) atom is abstracted from the second nucleotide within the recognition sequence, while a 4′ hydrogen (deuterium) atom is abstracted from the nucleotide three base pairs in the 3′ direction on the flanking strand.[3,5] The locations of the abstracted deuteriums in the rearrangement product indicate that the drug is oriented in the minor groove with the oligosaccharide tail in the 3′ direction of the pyrimidine strand relative to the aglycon.[5] There is evidence that the cleavage selectivity reflects binding selectivity,[4,5] and both the aglycon and the oligosaccharide tail are critical for the selectivity.[6] However, the molecular basis for recognition of pyrimidine/purine runs is not well understood.

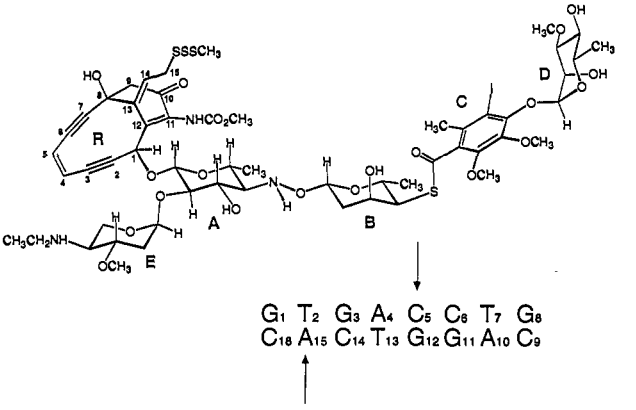

Figure 1. Calicheamicin γ_1 and the sequence of the DNA octamer used in the reported NMR studies. The expected cleavage sites are indicated.

We have undertaken NMR studies on calicheamicin γ_1 bound to DNA. We have found that calicheamicin γ_1 is stable in the presence of DNA as long as exogenous thiol is excluded. It binds to the non-self-complementary DNA duplex, d[GTGACCTG]–d[CAGGTCAC], where ACCT is the recognition sequence. The DNA within the recognition sequence distorts upon binding to accommodate the drug. The pyrimidine strand appears to distort more than the purine strand, with the largest conformational change at the CpC step. A preliminary analysis of the NMR data indicates that the drug itself does not distort much upon binding. On the basis of the NMR results, we propose that the

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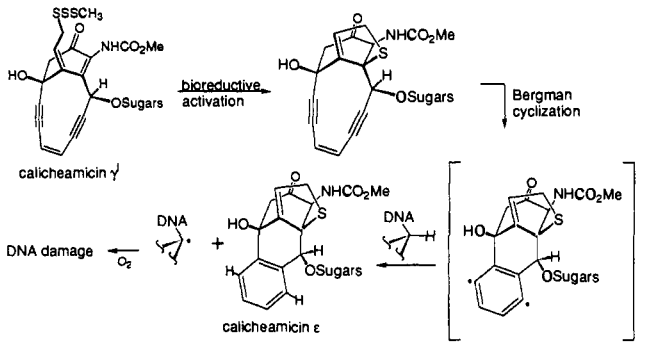
Scheme I. Mechanism of DNA Damage by Calicheamicin γ_1



binding selectivity reflects the ability of oligopyrimidine sequences to distort in the appropriate manner to accommodate the more rigid drug.

Experimental Procedures

Purified calicheamicin γ_1 was a gift from Lederle Laboratories. The octanucleotides d[GTGACCTG] and d[CAGGTCAC] were synthesized on a 10-μmol scale at the Princeton Synthesis Facility. Following dialysis to remove TEA salts, the strands were lyophilized and dissolved in 0.22 mL of NMR buffer (10 mM sodium phosphate, pH 7.0/70 mM NaCl/0.05 mM EDTA). Absorbances were measured and the concentrations were determined from the calculated extinction coefficients (75.1×10^3 for GTGACCTG and 73.9×10^3 for CACTGGAC).[7] Equimolar amounts of the two strands were mixed and the volume of the sample was brought to 0.5 mL with NMR buffer (containing sodium 3-(trimethylsilyl)-1-propanesulfonate as an internal reference). The concentration of DNA duplex in 0.5 mL was 3.3 mM. After annealing and repeated lyophilization from D_2O, the sample was dissolved in 1.0 mL of D_2O and either 0.5 or 1.0 equiv of calicheamicin was added in ~0.5 mL of CD_3OD. The mixture was transferred to an amber NMR tube where the volume was reduced to 0.5 mL by evaporation under argon. A precipitate forms in the tube during sample preparation.

One- and two-dimensional proton NMR experiments in D_2O were recorded on a JEOL GSX/GX 500-MHz spectrometer. Phase-sensitive NOESY data sets at drug:DNA ratios of 0.5:1 (25 °C, 50 ms) and 1:1 (15 °C, 90 and 200 ms) were acquired by using the method of States *et al.*, with 2048 complex data points in the t_2 dimension and 300–500 data points in the t_1 dimension.[8] To obtain a sufficient signal-to-noise ratio in the complex, 80 scans per t_1 increment were required. The bandwidth was 4700 Hz, with a relaxation delay of 2 s and a presaturation pulse on the residual HOD signal. Following acquisition, the data were transferred to a Silicon Graphics 310VGX computer and processed with the FELIX program.[9] In general, the data were apodized with a sine-bell squared function in both dimensions and zero-filled in the t_1 dimension to 2048 points prior to Fourier transformation. For the 2D exchange (NOESY) experiment on the 0.5:1 complex, a window function which minimizes truncation effects without affecting crosspeak volumes was used.[10] Specifically, the t_2 dimension was multiplied by 1.0 for the first 1248 points and then by a sine-bell squared function which dropped smoothly to 0 at 2048 points. The t_1 dimension was zero-filled to 2048 complex points and multiplied by 1.0 for the first 270 points and then by a sine-bell squared function which decreased smoothly to 0 at 370 points.

Homonuclear correlation spectra (DQF-COSY) were recorded in the phase-sensitive mode in D_2O solution. In the t_1 dimension 370 increments were collected with a sweep width of 4700 Hz at 15 °C or 4900 Hz at 21 °C and 2048 points in the t_2 dimension. The repetition delay was 2 s and 96 scans were collected for each t_1 increment. Phase-sensitive TOCSY spectra were recorded with use of the MLEV-17 spin-lock pulse (50 and 65 ms mixing times).[11] The bandwidth was 4700 Hz and the relaxation delay was 2 s. Data sets were acquired with 4096 points in the t_2 dimension and 370 t_1 increments. The data were apodized with a sine-bell squared function in both dimensions and zero-filled in the t_1 dimension prior to transformation. Subsequently, both dimensions were baseline corrected.

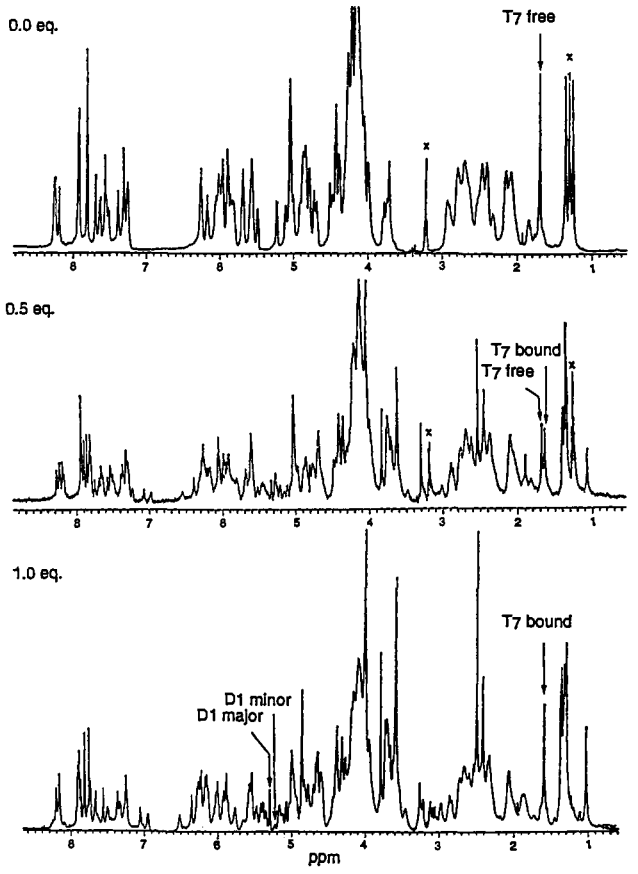


Figure 2. 1D ^1H NMR spectra in D_2O at 15 °C of the DNA duplex, $d[G_1T_2G_3A_4C_5C_6T_7G_8]$–$d[C_9A_{10}G_{11}G_{12}T_{13}C_{14}A_{15}C_{16}]$, in the presence of 0, 0.5, and 1.0 equiv of calicheamicin. X denotes impurities.

Results

When we initiated these studies it was not clear if it would be possible to use NMR to determine the structure of the calicheamicin γ_1–DNA complex and, if so, whether this complex would shed light on how the activated drug (intermediates **1** or **2**, Scheme I) recognizes DNA. We carried out an initial set of experiments to determine whether calicheamicin γ_1 can form a stable complex with a small DNA duplex that contains a putative recognition site. The duplex chosen, $d[G_1T_2G_3A_4C_5C_6T_7G_8]$–$d[C_9A_{10}G_{11}G_{12}T_{13}C_{14}A_{15}C_{16}]$, was designed to have an ACCT recognition site, two additional base pairs at the 5′ side of the recognition sequence, and a GC base pair at each end for stability. Although the precise cleavage sites on this small duplex have not been determined, cleavage studies on duplexes containing related sequences suggest that C5 H5′ (in the recognition sequence) and A15 H4′ (in the flanking sequence on the opposite strand) should be the principal sites of hydrogen atom abstraction (Figure 1).[3-5]

One-dimensional 500-MHz ^1H NMR spectra of the DNA duplex alone and in the presence of 0.5 and 1.0 equiv of calicheamicin γ_1 are shown in Figure 2. In the spectrum of the free duplex the base proton resonances (H2, H6, and H8) are located between 7.0 and 8.4 ppm while the H5 cytosine and sugar H1′ proton resonances are located between 5.1 and 6.5 ppm. The

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Table I. ^{1}H Chemical Shifts (ppm) of the Non-exchangeable DNA Resonances in the Calicheamicin–d[GTGACCTG]–d[CAGGTCAC] Complex

	H6/H8	H2	H5	CH_3	H1′	H2′	H2″	H3′	H4′
G1[a]	7.92 (–0.02)				5.94 (0.00)	2.56 (+0.03)	2.75 (+0.01)	4.80 (+0.01)	4.21 (+0.05)
T2	7.27 (+0.02)			1.32 (+0.01)	5.79 (+0.01)	2.06 (+0.03)	2.42 (+0.03)	4.87 (+0.02)	4.18 (+0.03)
G3	7.89 (+0.01)				**5.37 (+0.19)**	2.67 (+0.02)	2.69 (+0.08)	4.99 (+0.03)	4.30 (+0.06)
A4	8.19 (–0.01)	7.85 (–0.06)			**6.54 (–0.31)**	2.69 (–0.02)	**3.12 (–0.21)**	5.05 (–0.03)	**4.34 (+0.16)**
C5	**6.98 (+0.26)**		5.10 (+0.10)		**5.89 (–0.11)**	**1.75 (+0.30)**	**2.87 (–0.45)**	4.84 (–0.08)	4.14 (+0.06)
C6	**7.35 (+0.14)**		5.41 (+0.05)		**5.61 (+0.27)**	**1.91 (+0.15)**	**2.63 (–0.20)**	**4.61 (+0.16)**	**4.61 (–0.46)**
T7	7.39 (–0.03)			1.62 (+0.05)	**6.05 (–0.21)**	**1.87 (+0.13)**	2.44 (–0.07)	**4.63 (+0.23)**	4.15 (+0.10)
G8	7.93 (–0.01)				6.17 (–0.02)	2.61 (+0.03)	2.32 (+0.05)	4.70 (0.00)	4.19 (+0.01)
C9[a]	7.69 (–0.07)		5.91 (–0.04)		5.56 (–0.03)	1.92 (–0.10)	2.35 (–0.06)	4.67 (+0.01)	4.03 (0.00)
A10	8.19 (+0.04)	7.78 (+0.01)			**6.29 (–0.32)**	2.70 (+0.06)	**3.08 (–0.18)**	5.02 (0.00)	4.42 (–0.04)
G11	7.58 (+0.09)				**5.50 (+0.17)**	**2.49 (+0.12)**	**2.52 (+0.15)**	4.96 (+0.02)	4.42 (0.00)
G12	**7.76 (–0.22)**				**6.15 (–0.21)**	2.47 (+0.01)	2.75 (–0.01)	**5.02 (–0.20)**	4.43 (–0.01)
T13[a]	**7.07 (+0.21)**			**1.05 (+0.18)**	**6.20 (–0.17)**	2.01 (+0.10)	2.52 (–0.01)	**4.73 (+0.12)**	**3.15 (+1.00)**
C14	7.52 (+0.01)		5.59 (+0.07)		5.56 (–0.01)	2.11 (–0.05)	2.36 (+0.02)	4.85 (–0.01)	4.04 (+0.09)
A15	8.23 (+0.02)	7.84 (–0.06)			6.26 (–0.03)	2.68 (0.00)	2.89 (–0.02)	5.01 (0.00)	4.41 (+0.02)
C16	7.26 (+0.03)		5.18 (–0.09)		6.02 (–0.02)	2.08 (+0.04)	2.09 (–0.03)	4.46 (–0.01)	3.97 (+0.01)

[a] H5′ resonances for these nucleotides were also assigned: G1H5's = 3.71, 3.73 ppm; C9H5's = 3.71, 3.70 ppm; T13H5's = 3.26, 3.58 ppm. Shift changes upon complexation are shown in parentheses: difference = δ free duplex – δ complex. A negative value indicates a downfield shift upon complex formation. Absolute chemical shift changes >0.10 ppm are listed in bold type. Samples of the complex may have contained residual CD_3OD. However, a comparison of the chemical shifts for the assigned free DNA resonances in the 0.5:1 drug–DNA complex and the free DNA resonances in the duplex show that the shift changes due to this difference in sample preparation are small (<0.05 ppm).

region between 1.8 and 3.0 contains the ribose 2-deoxy protons and the region between 3.7 and 5.1 ppm contains the H3′, H4′, H5′, and H5″ protons. The methyl resonances of the three thymines are located at 1.67, 1.33, and 1.23 ppm.[12] Upon adding 0.5 equiv of calicheamicin, many of the DNA resonances double. This doubling, which is most clearly seen with the resolved thymine methyl resonance near 1.7 ppm (T7, Figure 2), indicates that there are now two discrete sets of DNA resonances, corresponding to the free and bound DNA octamers. Thus, the drug is in slow exchange between DNA octamers on the chemical shift time scale. The sharpness of the resonance lines for the complex suggests tight binding to a specific site on the duplex. The rate constant for dissociation of calicheamicin from the DNA duplex at 25 °C was measured to be 3.1 ± 1.1 s^{-1} from the ratio of the crosspeak volume to the diagonal peak volume for several exchanging DNA resonances in a 2D exchange experiment on the 0.5:1 calicheamicin–DNA complex.[13-15] No significant decomposition of the sample was observed during the course of the 2D exchange experiment. In fact, in the absence of thiol the drug–DNA complex is stable for several months in buffer at 5 °C. The slow off-rate and the stability of the drug–DNA complex mean that detailed 2D NMR studies of the drug–DNA interaction are possible.

The spectrum of the 1:1 drug–DNA complex in D_2O is shown in the bottom of Figure 2. All the free DNA resonances disappear upon the addition of a full equivalent of drug. However, it is worth pointing out that small crosspeaks can be observed near the diagonal for some of the better resolved drug and DNA resonances in the NOESY of the 1:1 complex. These crosspeaks evidently represent exchange events between a major and minor drug–DNA binding mode.[15] The resolved major and minor resonances for the D1 anomeric proton in calicheamicin are indicated in the one-dimensional spectrum of the 1:1 complex (Figure 2c). The minor complex is present in approximately 8-fold lower concentration than the major complex. More information about the minor binding mode is not available due to the low concentration of the minor complex and the crowded diagonal, which permits identification of only a few exchange crosspeaks. However, the observation of a minor complex is consistent with cleavage results showing that calicheamicin frequently cuts at more than one nucleotide in the vicinity of a recognition sequence.[3] Studies on other sequences may shed additional light on the minor binding mode. This paper concerns the major binding mode only.

Assignment of DNA Resonances in the 1:1 Complex. The resonances in the free duplex and the complex were assigned from NOESY and COSY spectra following well-described procedures for assigning non-exchangeable resonances in right-handed DNA structures.[12,16] The proton chemical shifts for the non-exchangeable DNA resonances in the complex at 15 °C are reported in Table I.[17] The chemical shift changes upon complexation are also reported. The largest changes occur in the putative binding region.

Analysis of DNA Conformation. Distance connectivities (NOE crosspeaks) are useful for making sequential assignments in DNA duplexes and also provide information on DNA conformation.[16] Expanded NOESY contour plots showing distance connectivities between the base H8/H6 and sugar H1′ protons in the complex are shown in Figure 3. The NOESY spectra were acquired at 15 °C to minimize exchange effects on crosspeak intensity in the 1:1 complex as much as possible. In the complex each base proton (purine H8 or pyrimidine H6) exhibits NOEs to its own and to the 5′ flanking sugar H1′ protons, and a chain can be traced from G1–G8 (dashed lines) and from C9–C16 (solid lines). The DNA thus remains in a right-handed conformation upon binding

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(17) Six of the eight imino protons have also been assigned in the complex: $T_2 = A_{15}$ 13.71 ppm; $G_3 = C_{14}$ 12.68 ppm ($\Delta\delta$ = –0.18 ppm); $A_4 = T_{13}$ 14.10 ppm ($\Delta\delta$ = –0.5 ppm); $C_5 = G_{12}$ 12.85 ppm ($\Delta\delta$ = –0.12 ppm); $C_6 = G_{12}$ 13.35 ppm ($\Delta\delta$ = –0.61); $T_7 = A_{10}$ 13.37 ppm. The number in parentheses indicates the change in chemical shift upon binding calicheamicin ($\Delta\delta$ = δ free DNA – δ complexed DNA). The T_2 and T_7 iminos cannot be identified in the duplex because they exchange too rapidly. Binding of calicheamicin stabilizes the duplex, making it possible to identify all but the terminal imino protons.

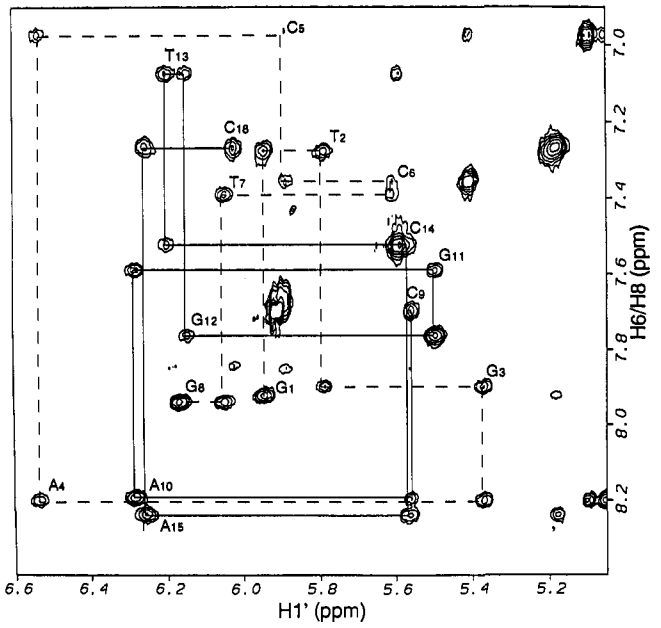
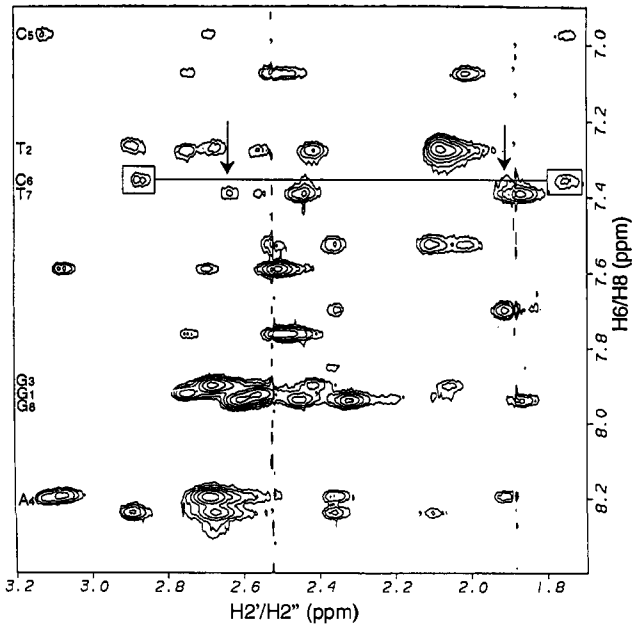


Figure 3. Expansion of the 200-ms NOESY contour plot in D_2O at 15 °C showing the base H8/H6 to anomeric H1′ proton distance connectivities for the 1:1 drug–DNA complex. The dashed line shows sequential distance connectivities for G1–G8 and the solid lines shows sequential connectivities for C9–C16.

calicheamicin. Several of the NOE crosspeaks are weaker than expected for B form DNA (note, for example, C5H6 to A4H1′ and to C5H1′; C6H6 to C5H1′ and to C6H1′; T7H6 to C6H1″). Exchange processes affect NOE intensities and probably account for some of the anomalous crosspeak volumes in the NOESY spectrum, making it difficult to relate crosspeak size to structure. Other NMR data show, however, that the conformation of the DNA distorts upon binding calicheamicin.

For example, the DQF-COSY spectrum of the drug–DNA complex indicates a conformational change in the C6 ribose sugar in the recognition sequence. The relative intensities of the crosspeaks in a DQF-COSY spectrum are proportional to the coupling constants. The coupling constants of the ribose sugars in a DNA duplex provide information on sugar pucker, which in turn is related to the overall conformation of the duplex.[18] In typical B DNA duplexes in solution, the crosspeaks between H1′ and H2′ are larger than those between H1′ and H2″ (*i.e.*, the $^3J_{H1'-H2'}$ coupling constants are larger than the $^3J_{H1'-H2''}$ coupling constants) while the crosspeaks for H2′–H3′ are present but those for H2″–H3′ are not.[16f-h] Inspection of the DQF-COSY spectrum for the calicheamicin–DNA complex shows that all the ribose sugars except C6 fall in the range of conformations expected for a B form duplex in solution. In the case of C6, however, the crosspeak for H1′–H2″ is present but that for H1′–H2′ is not (Figure 4b). Moreover, both H2′ and H2″ show COSY crosspeaks to the H3′ resonance (data not shown).[19] Since in the free duplex the coupling constants for C6 (and all the other ribose sugars) fall in the expected range, the conformation of the C6 ribose sugar changes significantly upon binding calicheamicin.

More evidence for an unusual conformation in the vicinity of the CpC step of the recognition sequence in the complex can be inferred from the NOE data for the base H8/H6 to 2-deoxy



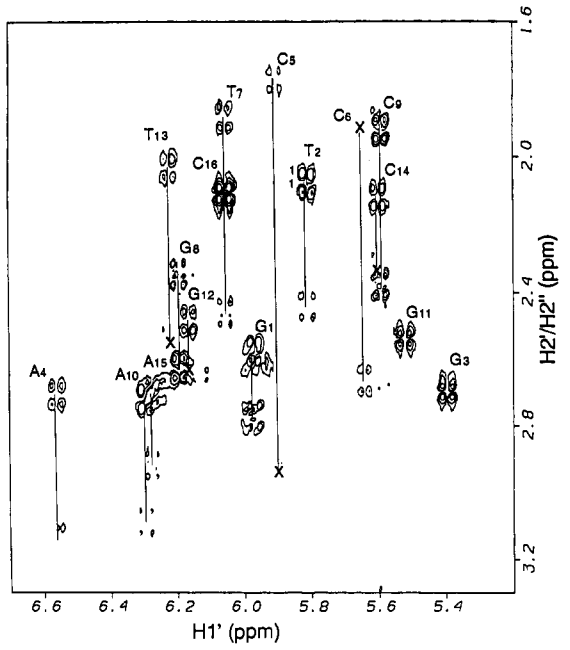


Figure 4. (a, bottom) Expansion of the DQF-COSY plot of the 1:1 drug–DNA complex in D_2O at 21 °C showing the H1′ to H2′/H2″ region. Missing crosspeaks are indicated with an X. A DQF-COSY spectrum was also acquired at 15 °C and there are no differences in the crosspeak patterns. (b, top) Expansion of the 200-ms NOESY contour plot of the 1:1 drug–DNA complex in D_2O at 15 °C showing the base H8/H6 to 2-deoxy proton distance connectivities. The base H8/H6 resonance assignments for the GTGACCTG strand are indicated on the left of the spectrum. The boxed crosspeaks are C5H2″ to C6H6 (left box) and C5H2′ to C6H6 (right box). The arrows point to the expected locations of crosspeaks for C6H2″ to C6H6 (left arrow) and C6H2′ to C6H6 (right arrow).

region. An expansion of the NOESY spectrum showing distance connectivities between the base and sugar H2′ and H2″ protons in the complex is shown in Figure 4b. Although overlap in this region of the spectrum prevents an analysis of many of the distance connectivities, the crosspeaks from C6H6 to the 2-deoxy region are well-resolved. The NOE intensities for C6H6 are rather weak overall; however, it is clear that *both* the interresidue crosspeaks from C6H6 to the 5′ flanking 2-deoxy protons are larger than either of the intraresidue crosspeaks from C6H6 to its own 2-deoxy protons. Since H8/H6 protons in B form DNA

(18) (a) Dickerson, R. E. *Structure & Methods, Vol. 3: DNA & RNA*; Sarma, R. H., Sarma, M. H., Eds.; Adenine Press: New York, 1990; pp 1–36. (b) Kollman, P. A.; Rao, S. N. *Structure & Expression, Vol. 2: DNA and its Drug Complexes*; Sarma, R. H., Sarma, M. H., Eds.; Adenine Press: New York, 1987; pp 229–235. (c) Drew, H. R.; McCall, M. J.; Calladine, C. R. *Annu. Rev. Cell Biol.* **1988**, *4*, 1.

(19) The H2′ and H2″ protons were assigned based on the relative sizes of their NOE cross peaks to H1′: regardless of the DNA conformation, H1′ is always closer to H2″ than to H2′ and therefore has a larger NOESY cross peak to it.[12,16]

Table II. ^{1}H Chemical Shifts (ppm) of Calicheamicin γ_1 in the Complex

	H1	H2(a)[a]	H2(e)[a]	H3	H4	H5(a)[a]	H5(e)[a]	CH_3	NCH_2CH_3	NCH_2CH_3	$OCH_3(m)$[a]	$OCH_3(o)$[a]
A	4.64	3.49		4.35	2.35	4.03		1.33				
B	5.16	1.89	2.36	4.34	3.72	4.12		1.38				
C								2.43			4.03	3.82
D	5.33		4.67	4.04	3.63	4.25		1.39			3.61	
E	5.45	1.64	2.76	4.06	3.30	4.06	4.13		3.01	1.36	3.61	
	H1	H4[b]	H5[b]	H9a	H9b	H14	H15a	H15b	SCH_3			
R	6.40	6.26	6.19	3.26	2.86	6.30	3.95	3.65	2.52			

[a] The parentheses specify the resonance in question when there is more than one possibility: a = axial, e = equatorial, referring to protons on a single carbon; o = ortho, m = meta, referring to the relationship of the methoxy group to the D ring glycosidic linkage. [b] The assignments of vinyl protons on the aglycon are tentative and may be reversed.

typically have much larger NOEs to their own H2′ protons than to the preceding H2′ proton, this anomalous pattern provides strong evidence for a shift *away* from a B form conformation in this part of the DNA duplex.[16,20]

Further characterization of the distortion induced in the recognition sequence upon binding calicheamicin will require a more quantitative analysis of NMR data in conjunction with molecular dynamics. However, it is worth pointing out that the unusual spectral features seen in the CpC step—coupling constants indicative of a C3′endo sugar conformation and a strong sequential $H2'_i$–$H1'_{i+1}$ NOE relative to the intranucleotide $H2'_i$–$H1'_{i+1}$ NOE—have been seen in the DNA complexes formed with the antitumor agent chromomycin A_3.[20] Chromomycin A_3 induces a very large conformational change in DNA when it binds and the spectral features observed are characteristic of A form DNA. When calicheamicin binds, some of these same features are observed. Unlike with chromomycin, however, the conformational changes are assymmetric with respect to the two DNA strands. The pyrimidine strand has a few features characteristic of A form DNA in the CpC step while the purine strand is closer to standard B form. Taken together, the data are consistent with a distorted B form duplex, with the binding of calicheamicin altering the conformation of the pyrimidine strand more than the conformation of the purine strand. Intermolecular NOEs (*vide infra*) put the B sugar and part of the C ring of the calicheamicin oligosaccharide very close to the C6 ribose sugar, and the steric demands of calicheamicin may lead to the observed conformational adjustments.

Resonance Assignments and Conformational Analysis of Calicheamicin γ_1. The proton resonances of calicheamicin both free and bound to DNA were assigned by using NOESY, DQF-COSY, and TOCSY data and the assignments for the bound drug are given in Table II.

The bound conformation of the drug was evaluated from DQF-COSY and NOESY spectra. COSY crosspeak intensities were inspected to determine sugar ring conformation, while NOESY crosspeak intensities were evaluated to define the orientation of the residues with respect to each other and to the floor of the minor groove. The results indicate that the bound conformation of the calicheamicin oligosaccharide is similar to the solution conformation in many respects. For example, the A ring of calicheamicin is in the $^4C_1$ (chair) conformation in solution; all the vicinal coupling constants are large and the corresponding COSY crosspeaks are intense. When the drug is bound to the DNA, the relative intensities of the COSY crosspeaks are similar to what they are in solution, indicating that the conformation does not change significantly. The relative COSY crosspeak intensities for the other sugars are also similar in solution and bound to DNA. Thus, each sugar ring maintains its preferred conformation upon binding. We have previously argued that the hexose rings in oligosaccharides that function as DNA binders are unlikely to undergo large conformational changes upon binding for energetic reasons.[21,22] The NMR data on calicheamicin as well as that on chromomycin[20] suggest that it is reasonable to assume, in the absence of experimental data to the contrary, that the hexose sugars in other DNA binding oligosaccharides do not undergo dramatic conformational changes upon binding.[22]

Table III. Interresidue NOEs in the Calicheamicin–DNA Complex

residue 1	proton	residue 2	proton	size[a]
aglycon	R1	A sugar	A1	m
	R4 (A15H1′)[c]		A6	w
	R5		A6	w
	R1	E sugar	E5a (E3)[d]	m
	R1		E5e (C5H4′)[e]	m
A sugar	A5	B sugar	B1	w
	A2	E sugar	E1	s
	A6		B1	m
B sugar	B3	C ring	CH3	w
C ring	OCH3(o)[b]	D sugar	D1	w
	OCH3(o)[b]		D6	m

[a] s, strong; m, medium; w, weak. [b] Ortho refers to the relationship of the methoxy group to the D ring glycosidic linkage. [c–e] A few NOEs from the drug cannot be unambiguously assigned because of resonance overlap: (c) the downfield vinyl resonance overlaps with A15H1′; (d) the E5a and E3 resonances overlap in the complex; NOEs from R1 to both resonances are observed in the free drug; (e) the E5e resonance overlaps with C5H4′.

A quantitative analysis of the NOESY data in conjunction with molecular modeling will be necessary to define precisely the interresidue torsion angles. However, the relative orientation of the residues is available from a qualitative analysis of the interresidue and intermolecular NOEs (Tables III and IV). Certain NOEs, including some NOEs from the aglycon, could not be unambiguously assigned because of resonance overlap. Since in these instances both possible assignments are compatible with the same model, the NOEs are listed in the tables with the ambiguities noted. In general, a comparison of the NOEs observed in solution and in the bound drug suggests that the drug binds in a conformation close to the predominant solution conformation, consistent with our proposal that the drug is relatively rigid.[23] As discussed below, we believe that the drug's limited flexibility is related to the mechanism of sequence selective recognition.

The Calicheamicin–DNA Complex. A schematic representation showing the location of the A, B, C, and D rings in calicheamicin with respect to the DNA is shown in Figure 5. NOEs between the A sugar of calicheamicin and both the A15 and C5 sugars of the DNA show that the A ring spans the minor groove. The

(20) (a) Gao, X.; Patel, D. J. *Biochemistry* **1989**, *28*, 751. (b) Gao, X.; Patel, D. J. *Q. Rev. Biophys.* **1989**, *22*, 93. (c) Banville, D. L.; Keniry, M. A.; Kam, M.; Shafer, R. H. *Biochemistry* **1990**, *29*, 6521.

(21) Walker, S.; Valentine, K. G.; Kahne, D. *J. Am. Chem. Soc.* **1990**, *112*, 6429.

(22) Sugar rings also do not usually distort when binding to proteins. One exception involves sugars bound to lysozyme, whose function is to stabilize the transition state for hydrolysis. See: (a) Phillips, D. C. *Scientific American* **1966**, *215*, 78. (b) Strynadka, N. C. J.; James, M. N. G. *J. Mol. Biol.* **1991**, *220*, 401.

(23) Minor adjustments in the drug conformation upon binding are not ruled out (and indeed may be likely).[32b] Nevertheless, the general shape of the drug is maintained. In addition to more typical linkages found in oligosaccharides, calicheamicin contains some unusual linkages such as the N–O bond and the thioester linkage to the aromatic ring. These linkages play a role in determining the overall shape of the molecule and cannot be readily distorted. See: (a) Reference 21. (b) Walker, S.; Yang, D.; Gange, D.; Kahne, D. *J. Am. Chem. Soc.* **1991**, *113*, 4716.

Table IV. Intermolecular NOEs in the Calicheamicin–DNA Complex[a]

calicheamicin residue[c–f]		d($G_1T_2G_3A_4C_5C_6T_7G_8$)–d($C_9A_{10}G_{11}G_{12}T_{13}C_{14}A_{15}C_{16}$)			
		DNA residue	H1′	H4′	H5′,H5″
A sugar[c]	A1	C5		w	
	A3	C5	w		
	A6	A15		s	
B sugar[d]	B2a	C5	w		
	B2e	C5	w		
	B3	C6	w		
	B4	C6	w		
	B4	C6		m	
	B6	C6		m	
C ring[e]	CH3	C6	s		
	OCH3(meta)[b]	T13		m	
D sugar[f]	D1	T13		w	m
	D2	G12		w	
	OCH3	G12		m	

[a] s, strong; m, medium; w, weak. [b] Meta refers to the relationship of the methoxy group to the D ring glycosidic linkage. [c–f] Intermolecular NOEs from the aglycon R1, R4, and R5 cannot be unambiguously assigned because of resonance overlap: (*c*) the A sugar of calicheamicin spans the minor groove, contacting the A15 and C5 backbone sugars on opposite strands of the DNA; (*d*) the b sugar contacts the C5 and C6 sugars on the pyrimidine strand; (*e*) the C ring contacts the C6 and T13 sugars on opposite strands of the DNA; (*f*) the D sugar contacts the G12 and T13 sugars on the purine strand.

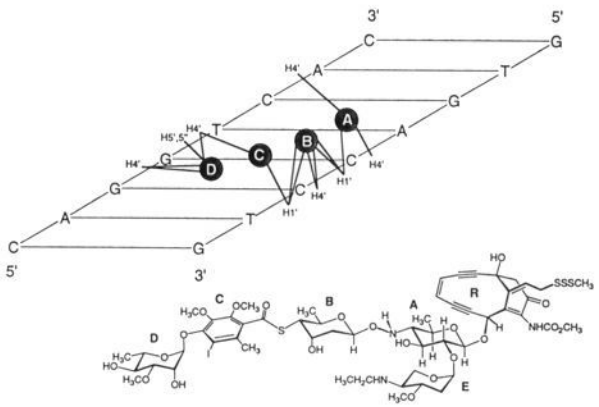


Figure 5. Schematic showing the intermolecular NOEs. Shaded circles represent the approximate positions of the calicheamicin rings with respect to the minor groove of the DNA octamer. Each solid line emanating from a shaded circle represents a single NOE between a proton on that ring and the indicated DNA proton. Table IV lists all of the NOEs in the above figure, including the specific drug protons involved. The E ring and the aglycon are omitted from the figure because no intermolecular NOEs could be unambiguously identified for either. Their positions can be deduced from interresidue NOEs to the A ring.

B sugar of calicheamicin contacts both the C5 and C6 ribose sugars of the DNA. No contacts to the purine strand can be detected, suggesting that the B sugar is closer to the pyrimidine strand. However, because of resonance overlap we cannot rule out the possibility that there may be some contacts. The methyl group on the C ring of calicheamicin has a large NOE to C6H1′ on the DNA, indicating that both the methyl and the iodine point in toward the floor of the minor groove, as has been proposed by Schreiber.[24] The iodine is positioned so that it can contact the G11 amino group but not the G12 amino group. There is also an NOE between one of the methoxy substituents on the aromatic C ring and T13H4′. This methoxy substituent has been assigned as *meta* to the D ring glycosidic oxygen since the *other* methoxy substituent has an NOE to the D ring C6 methyl. T13H4′ shifts 1.00 ppm upfield upon complexation, suggesting that it is positioned in the shielding current of the aromatic C ring. This interpretation is consistent with the observed NOEs from the C ring to the DNA. The D sugar of calicheamicin shows NOEs to both the G12 and T13 ribose sugars but none to the pyrimidine strand, suggesting that it lies closer to the purine strand (although again, resonance overlap may prevent the observation of NOEs to the pyrimidine strand). The observed NOEs from D1 to the T13 H5's and from the D3 methoxy to G12 H4′ position the C6 methyl substituent in the D ring so that it faces out of the minor groove.

No NOES between the aglycon or the E sugar to the DNA can be identified unambiguously, and these residues are therefore not shown in the schematic. However, the orientation of the aglycon with respect to the rest of the drug is defined by several interresidue NOEs. Its position in the DNA is defined by the position of the rest of the oligosccharide tail. When the drug is docked into DNA with both the interresidue and intermolecular NOE constraints satisfied, the C3 and C6 positions of the aglycon (Figure 1) are close to the A15 and C5 ribose sugars, respectively. A15H4′ and C5H5′ are the expected hydrogen atom abstraction sites based on the recognition sequence, so the NMR data are consistent with the cleavage data. We have generated an initial model for the calicheamicin–DNA complex by docking calicheamicin into B form DNA in an orientation that satisfies the qualitative NOE constraints (Figure 6). The model shows the bound conformation of calicheamicin and the location of calicheamicin with respect to the recognition sequence. Note that the E ring protrudes out of the groove and the E ring ethylamino group probably forms a salt bridge to the C5 phosphate.[25] The model has not been refined yet and there are a number of unfavorable steric contacts between calicheamicin and the DNA, many of them between the B sugar of calicheamicin and the CpC step of the recognition sequence. It is possible that the DNA distorts to relieve these unfavorable steric interactions when calicheamicin binds.

Discussion

The molecular basis for the cleavage selectivity of calicheamicin γ_1 has been the subject of a great deal of speculation. Interest was aroused following reports that calicheamicin recognizes TCCT and a limited number of closely related GC-containing oligopyrimidine sequences.[3] Few small molecules display this level of discrimination in their interactions with DNA. Several proposals regarding the origins of the cleavage specificity were immediately put forth. Hawley *et al.* suggested that selective cleavage is due to sequence selective binding by the oligosaccharide-aryl tail to the TCCT recognition site.[24] In the model, the sequence selectivity was attributed to the interaction of particular functional groups on calicheamicin with a particular array of functional groups in the TCCT sequence. Hawley *et al.* proposed that much of the selectivity derives from favorable interactions between the aryl iodide on calicheamicin and the guanine N2 amino groups protruding in the minor groove. Alternatively, Zein *et al.* proposed that selective cleavage is due to a shape selective binding interaction between calicheamicin (with the aglycon playing an especially important role) and the DNA.[25] Zein *et al.* suggested that calicheamicin senses inherent groove conformation and that TCCT sequences and other closely related sequences have a conformation that provides a complementary fit to the drug.

Further studies on the cleavage selectivity of calicheamicin γ_1 in our laboratory showed that it recognizes TTTT sites as well as (and sometimes in preference to) GC-containing pyrimidine/purine tracts.[4] This finding ruled out the possibility that the aryl iodide plays a principal role in sequence recognition *by interacting with guanine amino groups* in the minor groove. The focus changed from trying to understand why calicheamicin recognizes

(24) Hawley, R. C.; Kiessling, L. L.; Schreiber, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1105.

(25) Zein, N.; Poncin, M.; Nilakantin, R.; Ellestad, G. A. *Science* **1989**, *244*, 697.

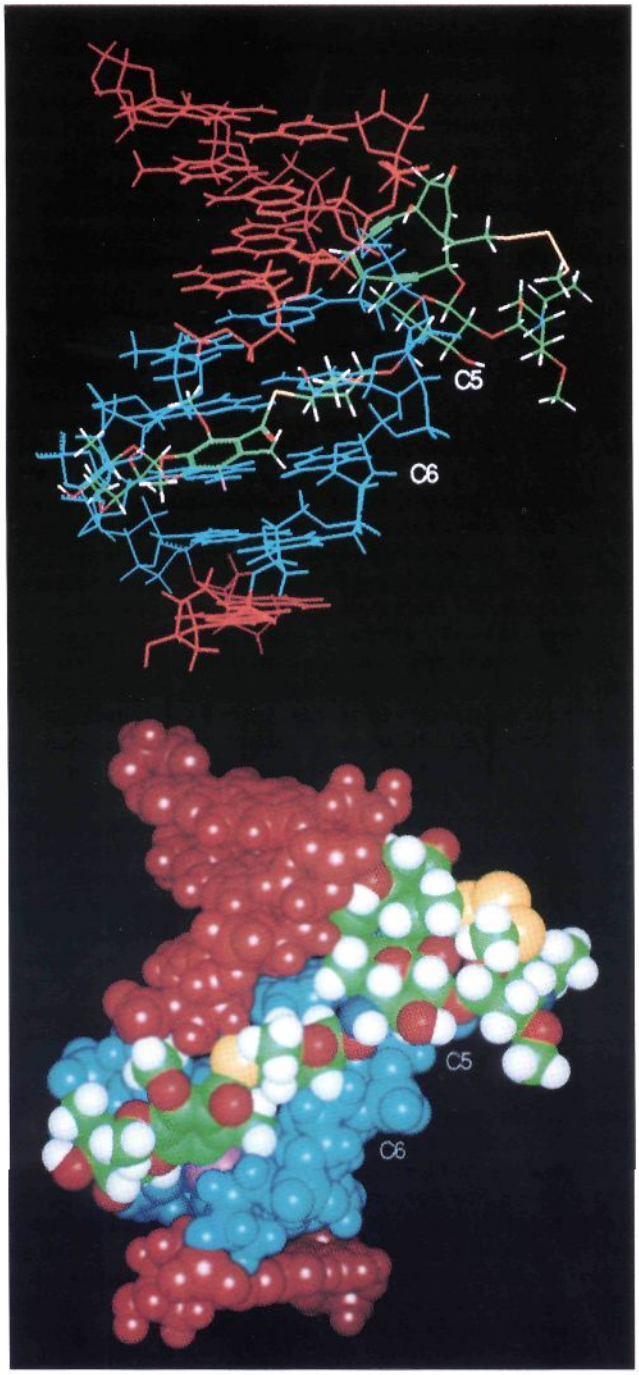


Figure 6. (a, top) Model for the drug–DNA complex generated by using the InsightII molecular graphics package. The drug has been docked into the minor groove of a B form octamer in a manner that satisfies all the drug–drug and drug–DNA NOE constraints. The recognition sequence is shown in blue. Although the model shows the orientation of the drug in the groove, there are some unfavorable steric interactions in the model between the drug and the DNA. It is possible that these are relieved by adjustments in the DNA conformation (see text). (b, bottom) Space-filling model of the complex shown in part a.

GC-containing pyrimidine sequences to understanding why it recognizes pyrimidine/purine tracts in general.

The variety of sequences cleaved suggests that calicheamicin senses DNA conformation rather than sequence *per se*. However, since GC-containing sequences tend to have wider minor grooves and present a very different microenvironment than TTTT tracts,[26] it does not seem likely that calicheamicin senses "inherent" groove structure. We have proposed the binding event involves some degree of induced fit.[4]

We are trying to understand the cleavage specificity of calicheamicin γ_1 by studying how it binds to DNA. Although the compound that effects DNA cleavage is an unstable rearrangement product of calicheamicin γ_1 and there is a formal possibility that it binds to DNA differently from the parent compound,[27] the NMR results presented above argue against this. Calicheamicin γ_1 binds to the putative recognition sequence in a manner that positions the aglycon near the presumed hydrogen atom abstraction sites (the A15 and C5 ribose sugars). This suggests that NMR studies on the binding of the parent compound, calicheamicin γ_1, are relevant to understanding the recognition event that leads to selective cleavage. The NMR results on the drug–DNA complex show that when calicheamicin binds, the DNA undergoes a conformational change. For example, the C6 ribose sugar undergoes a large change toward a conformation commonly associated with A form DNA (*i.e.*, toward C3′-endo) while the sugars on the complementary strand remain in a more standard B-like conformation (*i.e.*, toward C2′-endo). The nature of the changes suggests that the minor groove must open up to accommodate the drug.[20] It is interesting that the distortion is most pronounced in the pyrimidine strand. We propose that the cleavage selectivity for oligopyrimidine sequences correlates with the ability of such sequences to distort to accommodate the drug.[28] One hypothesis is that the ability to distort appropriately is related to the fact that the energy available from stacking interactions is very different along the two DNA strands of a pyrimidine/purine tract. For example, the pyrimidine strand may come partially unstacked more readily and/or increased overlap of the purines can help pay the cost of opening up the minor groove. There is some evidence that pyrimidine/purine runs have some conformational peculiarities that are related to the differential overlap of the bases in the two strands.[29] Studies on other recognition sequences are currently underway to see if calicheamicin binding induces similar conformational changes in other pyrimidine/purine runs (*e.g.*, TTTT). These studies are critical for evaluating our proposal that sequence-dependent DNA flexibility is the basis for site-selective recognition (*vide infra*).

The NMR study also shows the location of the drug in the minor groove, and a preliminary analysis of the NOEs suggests that the conformation of the drug does not distort significantly upon binding. We believe that the drug's rigidity is intimately related to the proposed mechanism for site-selective recognition. As von Hippel and Berg have noted with regard to protein–DNA recognition, "Certain DNA sequences may be easier to distort to fit the binding site of the protein and some base pair choices can therefore be important for specificity even if they do not contribute any specific interactions. In this way, sequence-dependent DNA flexibility can increase binding specificity. However, to be effective, this mechanism also requires a "stiff" protein; otherwise, the conformational distortions will take place in the protein rather than in the DNA."[30,31]

We point out that this mechanism also implies that portions of a DNA binder, whether it be a protein or a drug like calicheamicin, can be important in specificity not because they

(26) (a) Yoon, C.; Prive, G. G.; Goodsell, D. S.; Dickerson, R. E. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 6332. (b) Yanagi, K.; Prive, G. G.; Dickerson, R. E. *J. Mol. Biol.* **1991**, *217*, 201. (c) Tullius, T. D.; Price, M. A. *Biochemistry* **1993**, *32*, 127 and references therein.

(27) De Voss, J. J.; Hangeland, J. J.; Townsend, C. A. *J. Am. Chem. Soc.* **1990**, *112*, 4554.

(28) Aiyar *et al.* have shown that binding of the oligosaccharide-aryl tail alters the probability of DNA cleavage at adjacent bases by nonspecific cleaving agents; they have proposed that binding of the oligosaccharide causes alterations in minor groove width that extend *beyond* the immediate binding site.[6a] However, there may be other explanations for the observed effects. See: Goodisman, J.; Dabrowiak, J. C. *Biochemistry* **1992**, *31*, 1058.

(29) (a) Drew, H. R.; Travers, A. A. *Cell* **1984**, *37*, 491. (b) Nickol, J. M.; Felsenfeld, G. *Cell* **1983**, *35*, 467. (c) Schon, E.; Evans, T.; Welsh, J.; Efstratiadis, A. *Cell* **1983**, *35*, 837. (d) Calladine, C. R.; Drew, H. R. *J. Mol. Biol.* **1984**, *178*, 773.

(30) von Hippel, P. H.; Berg, O. G. *Protein–Nucleic Acid Interactions*; Saenger, W., Heinemann, U., Eds.; CRC Press: Boca Raton, FL, 1989; pp 1–18.

make specific contacts to particular base pairs, but because they influence the overall shape of the binder.[21] In this regard, the cleavage studies on derivatives of calicheamicin may be better understood by considering the drug as a whole rather than a collection of parts that each have a preference for particular base pairs. For example, it was found that the aglycon of calicheamicin cleaves DNA with no specificity and it was concluded that the oligosaccharide tail therefore determines the selectivity.[6a] It was subsequently shown that a derivative of calicheamicin containing the A and E sugars binds weakly in the minor groove but still cleaves with almost no selectivity.[4] Adding the B sugar increases the binding affinity significantly and imparts a preference for short oligopyrimidine tracts (*e.g.*, 5′-CTC, 5′-TTT, 5′-TTC) and 5′-TG and 5′-CG sequences.[32] Adding the aryl-rhamnose moiety further increases the affinity and the selectivity for oligopyrimidine sequences. The initial explanations for the increased selectivity imparted by the aromatic ring focused on specific contacts between substituents on the thiobenzoate ring and particular base pairs (specifically, GC base pairs). However, subsequent data on the cleavage selectivity of calicheamicin made this explanation untenable. There is now mounting evidence that the site selectivity is due not to specific contacts but to the ability of the DNA to distort to accommodate a relatively "stiff" drug without a prohibitive energy cost. This should focus attention on understanding how the substituents and various linkages in calicheamicin play a role in influencing the shape and flexibility of the drug and how that in turn relates to selective binding.

(31) See also: (a) Travers, A. A. *Annu. Rev. Biochem.* **1989**, *58*, 427. (b) Wang, A. H.-J.; Liaw, Y.-C.; Robinson, H.; Gao, Y.-G. *Molecular Basis of Specificity in Nucleic Acid–Drug Interactions*; Pullman, B., Jortner, J., Eds.; Kluwer, Dorderecht: The Netherlands, 1990; pp 1–21.

(32) This conclusion is based on results with esperamicin C.[3e,f]

Conclusion

The work reported above have established the feasibility and relevance of NMR studies of calicheamicin γ_1 bound to DNA and has led to the development of an initial model for the complex. On the basis of the preferred binding sites and our initial NMR study, we have suggested that selective recognition is due to sequence-dependent DNA flexibility, *i.e.*, the ability of pyrimidine/purine sequences to distort to accommodate a relatively inflexible drug. The next step is to refine the structure with the aid of molecular dynamics. Further studies on calicheamicin bound to other sequences are underway and will allow us to test and refine the proposed mechanism for DNA recognition. Ultimately, a combination of NMR studies and biochemical data should lead to a good understanding of how the drug discriminates between different sites on the DNA.

Acknowledgment. This work was supported by the National Institutes of Health. We thank Drs. George Ellestad and Wei-Dong Ding of Lederle Laboratories for generously providing us with calicheamicin γ_1.