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Circular Dichroism Studies of Calicheamicin-DNA Interaction: Evidence for Calicheamicin-Induced DNA Conformational Change

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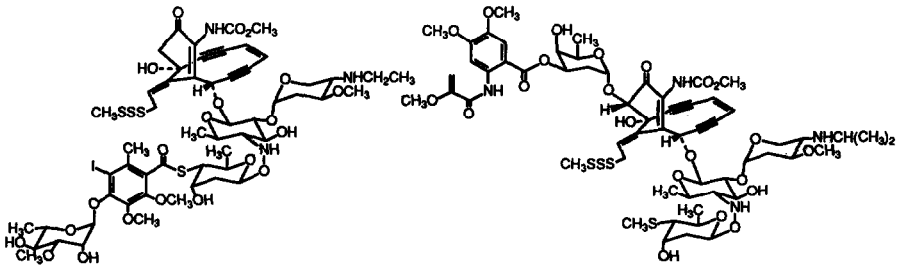
Abstract: Evidence from circular dichroism studies suggests that the binding of calicheamicin to DNA induces an optically detectable conformational change of B-form DNA. CD titration of a dodecamer containing a TCCT binding/cleavage site with calicheamicin γ_1^I and the aromatized calicheamicin ε brings about a decrease primarily in the ellipticity of the positive 270 nm signal. Binding constants for calicheamicin γ_1^I and ε with this dodecamer were estimated to be 9×10^5 and 5×10^4 M^{-1}, respectively, based on analyses of the CD changes.

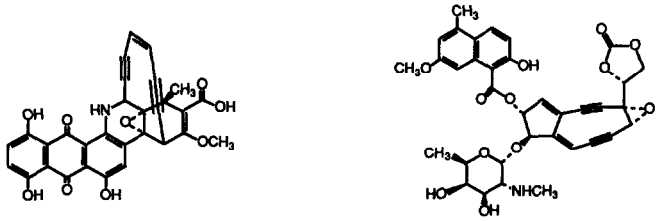
INTRODUCTION

Calicheamicin γ_1^I (**1**)[1], along with esperamicin A_1 (**2**)[2] are two 10-membered ring enediyne-containing antitumor agents which are some 1000 times more active than the clinically used anthracycline, adriamycin. Other members of this structurally and biologically remarkable class of fermentation products include dynemicin (**3**)[3], neocarzinostatin chromophore (**4**)[4], the oldest member, and two recently isolated compounds, C-1027 chromophore (**5**)[5] and kedarcidin chromophore (**6**)[6]. Neocarzinostatin, C-1027 and kedarcidin chromophores are enediynes in 9-membered rings and are co-produced with stabilizing proteins which exhibit protease activity.[7] All of these enediyne antitumor antibiotics are potent DNA cleaving agents. The chemistry of the DNA cleavage involves a Bergman cycloaromatization which is triggered, in the case of calicheamicin and esperamicin, by a reductive cleavage of the allylic trisulfide moiety (Scheme 1).[8]

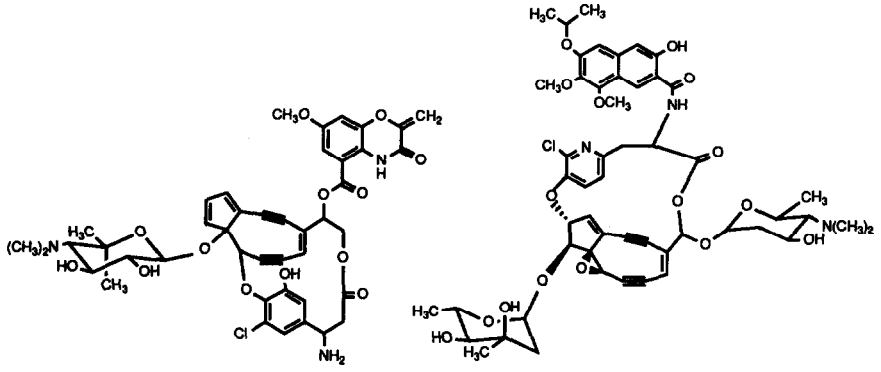
The transient p-benzyne intermediate (**7**) abstracts proximal hydrogen atoms from the deoxyribose sugars, which initiates oxidative strand cleavage. The aromatized end product, calicheamicin ε, (**8**) binds but does not cleave DNA. Calicheamicin γ_1^I, the principal component of a mixture of metabolites obtained from fermentations of *Microminospora echinospora*, ssp. *calichensis*, is of special interest because it exhibits unusual DNA cleavage specificity for a molecule of only 1367 Daltons. It targets the minor groove of duplex DNA homopyrimidine/purine domains of four to six base pairs including sequences containing GC base pairs.[9] Specific hydrogen atom abstraction takes place at 5'(S) of the deoxyribose of the 5'C of a TCCT-containing dodecamer and at the 4' carbon of the deoxyribose two nucleotides to the 3' side of the complementary AGGA sequence.[10] It has recently been shown to cleave TTTT sequences in a restriction fragment which lacks the originally observed specific recognition sequences of TCCT or CTCT.[11] Calicheamicin binds in the minor groove with the thiobenzoate carbohydrate tail segment directed to the 3'-



1 Calicheamicin γ_1^I **2** Esperamicin A_1



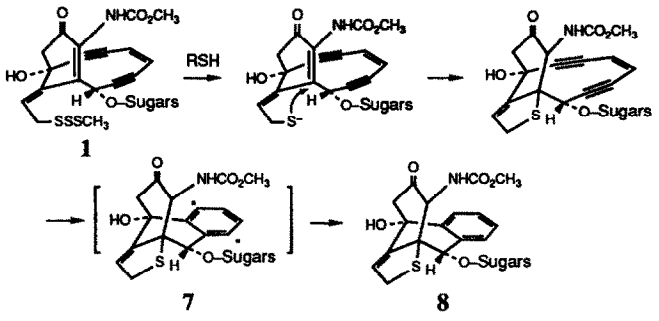
3 Dynemicin **4** Neocarzinostatin Chromophore



5 C-1027 Chromophore **6** Kedarcidin Chromophore

side of the TCCT sequence. Recent footprinting studies have shown that both the aglycone and the carbohydrate tail units are required for the observed sequence selectivity.[12]

The molecular basis for calicheamicin binding/cleavage specificity has been the subject of much speculation since the molecule bears no resemblance to the classical minor groove peptide binders such as netropsin and distamycin and related analogues. Walker et al., have suggested that the cleavage specificity is not simply an inherent groove conformation dictated by a particular sequence but more likely a result of a drug-induced fit.[11] It is likely that certain sequences would be more amenable to induced conformational change on calicheamicin binding than others.



1 7 8

Scheme 1

Circular dichroism (CD) is an extremely sensitive spectroscopic technique to monitor conformational changes of DNA on interaction with external ligands.[13a] The CD of DNA arises from the interaction of heterocyclic bases with each other and the sugar-phosphates.[13,d,e] Several years ago we observed in a preliminary study that the calicheamicin ε interaction with sonicated calf thymus DNA caused a reduction of the DNA dichroic absorption, primarily at 270 nm.[9b] It was proposed that this was due to a tightening of the DNA duplex caused by association between the drug and DNA based on the assumption that this change was in a way similar to that observed for high salt solutions and ethanol. In order to obtain a more definitive understanding as to what kind of conformational change the DNA undergoes upon binding with calicheamicin we have now extended these studies with a 12-mer, 5'-d(CCCGGTCCTAAG) containing only one specific TCCT cleavage site. The specificity of hydrogen atom abstraction in this dodecamer has been well characterized by deuterium transfer experiments.[10b] Analysis of the CD change of this dodecamer has also permitted the estimation of the relative binding affinities of the enediyne and aromatized forms.

RESULTS

The CD spectrum of the 12-mer used in this study is shown in Figure 1 and is typical of B-form DNA. Titration with calicheamicin ε caused a decrease in the positive CD at 266 nm and a somewhat lesser reduction in the negative amplitude at 240 nm after subtraction of the small inherent CD of calicheamicin ε. An isoelliptic point is observed at 251 nm and suggests that a two-state transition can be used to describe the conformational change in the DNA. Figure 2 shows the binding curve obtained from monitoring the CD change at 271 nm and at 219 nm. Calicheamicin ε has a small inherent CD throughout the spectrum except at 271 nm where the CD of the unbound ε is zero. Thus at 219 nm it was necessary to subtract the contribution of the drug with the assumption that the binding-induced CD of the drug is negligible. Binding constants of 6.9 and 5.3 $x10^4$ M^{-1} were derived from a nonlinear least-squares fit of the binding isotherms (KaleidaGraph) for 271 and 219 nm, respectively. The curve fit suggested one binding site per duplex dodecamer. Analysis of the same data by Hill plots gave values of 4.3 and 3.0 $x10^4$ M^{-1}. The fact that these K_a estimates are similar suggests that our assumptions are reasonable.

We also carried out CD titration experiments with calicheamicin γ_1^I and the same TCCT-containing dodecamer. These experiments, however, were more difficult to analyze because of the large inherent CD of

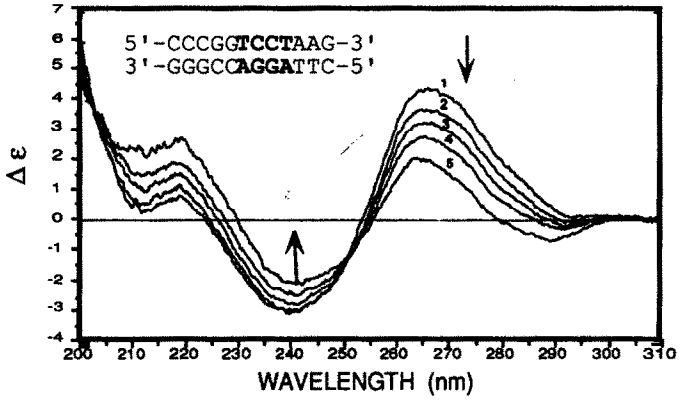


Figure 1. CD spectra of the dodecamer 5'-d(CCCGGTCCTAAG) (23μM of duplex) in the absence (1); and presence of calicheamicin ε at 11 μM (2) ; 23 μM (3) ; 46 μM (4) ; 131 μM (5). The concentration-scaled CD contribution of the drug has been subtracted from the recorded spectra.

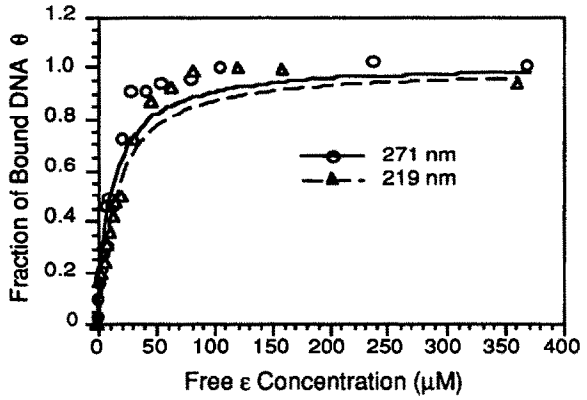


Figure 2. Binding isotherms of calicheamicin ε interaction with the TCCT dodecamer. Increasing concentrations of the drug were titrated into duplex DNA and the resultant CD intensity changes at 219 nm (triangle) and 271 nm (circle) were used to calculate the fractional DNA saturation values. The solid line represents the theoretical binding curve generated by KaleidaGraph. Analysis of the binding curves provide K_a values of 5.3 and 6.9 (x 10^4 M^{-1}) at 219 nm and 271 nm respectively. The χ^2 was 0.14 at 219 nm and 0.08 at 271 nm. The correlation coefficient was 0.97 at both wavelengths.

γ_1^I. Figure 3 shows the CD spectra of dodecamer-γ_1^I complex, additive spectrum of the dodecamer and γ_1^I at the same concentrations assuming no interaction, and the difference spectrum. In the difference spectrum the negative band at 266 nm and the positive band at 240 nm clearly demonstrate a decrease in rotational strength of these DNA CD extremums. A binding constant of $9x10^5$ M^{-1} for the γ_1^I/DNA association was derived from the CD intensity change at 236 nm where the CD contribution of the unbound drug was negligible (figure 4). This association constant is in good agreement with a previous estimate obtained from

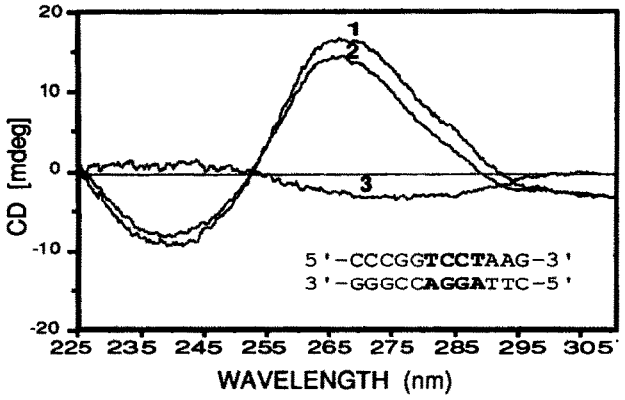


Figure 3. CD spectral changes due to the interaction of calicheamicin γ_1^I with the tcct dodecamer. The sum of the CD spectra of 3 μM of dodecamer and 2.4 μM of calicheamicin γ_1^I assuming no interaction (1); the measured spectrum of the dodecamer-γ_1^I complex at these indicated concentrations (2); difference spectrum obtained by the subtraction of curve 2 from curve 1 (3).

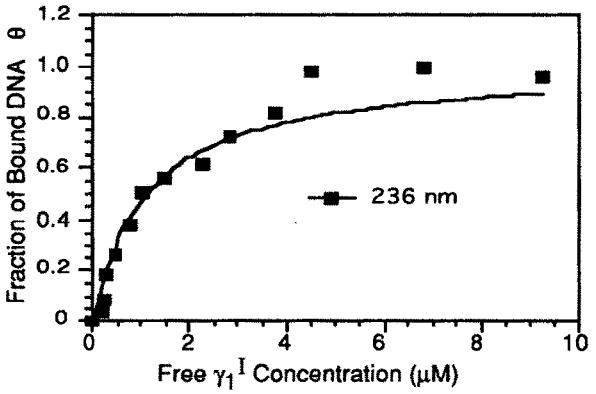


Figure 4. Binding isotherm of calicheamicin γ_1^I interaction with the TCCT dodecamer. Increasing concentrations of the drug were titrated with dodecamer duplex and the fractional DNA saturation values were calculated from the CD intensity changes at 236 nm. The solid line represents the theoretical binding curve generated by KaleidaGraph. Curve fit analyses of the binding isotherm provided a K_a value of 9×10^5 M^{-1}. The χ^2 value was 0.08 and correlation coefficient 0.97.

gel cleavage experiments with pBR322 DNA.[14a] However, it is one to two orders of magnitude less than that reported by Danishefsky and Crothers (10^7 to 10^8).[14b] The reason for this discrepancy is unknown but probably reflects the difference in methodology and possibly DNA substrates.

DISCUSSION AND CONCLUSIONS

It is known that DNA can adopt several basic structures denoted by A, B, C and Z as well as forms intermediate to these depending on sequence, solvent, and ionic strength. The B-form contains a wide and shallow major groove with a narrow and deeper minor groove. As shown in figure 1, curve 1, the CD spectrum of B-form DNA is characterized by a rather symmetrical exciton split with a positive signal around 270-280 nm, a negative signal at 240 nm and a crossover at about 260 nm depending upon the sequence. An intensely positive 187 nm band is also present and is believed to be very sensitive to base-base interactions. In contrast, the A-form groove dimensions are reversed with a shallow and wider minor groove and a narrow and deeper major groove. A-DNA CD does not show the more symmetrical exciton split characteristic of B-DNA. Instead, a strong positive signal is observed around 270 nm, a weakly negative band near 240 nm, and a strongly negative band at 210 nm. Z-DNA is a left handed helical form and its CD is generally the opposite that of B-form DNA with a negative band at 270-290 nm and positive band at around 240 nm. The C-DNA CD spectrum is characterized by a more or less conservative B-form spectrum in which both the positive and negative extremums are reduced from that of the B-form.[13d] The structural basis for this form is not fully understood.

The observed decrease in intensity of the positive CD band caused by calicheamicin is strongly reminiscent of a DNA structure brought about by a reduction in the degree of hydration caused by temperature, alcohols, and high salt.[13,15,16] Furthermore, certain DNA binding proteins have been shown to cause a similar decrease of the positive extremum in the CD spectrum of DNA on binding to the regulatory protein.[17] Some DNA binding proteins, on the other hand, cause an increase in the positive extremum. The reduction in the positive CD band has been attributed to an increase in the winding angle of the DNA helix with a concomitant decrease in the twist angle which results in a slight decrease in the number of base pairs per helical turn.[13d,15,16,17] Indeed, changes in the rotational strength of the positive CD band above 260 nm are used as a diagnostic measure of winding angle changes within B-form DNA.[16] Our results are clearly not compatible with a drug-induced transition to an A or Z form. We interpret the decrease in amplitude of the positive CD band as a small alteration of B-form DNA.[12a,13d,18]

Based on these CD titrations, the binding affinity of calicheamicin γ_1^I to this dodecamer is about an order of magnitude greater than that of ε (9×10^5 vs 5×10^4) which corresponds to a difference in binding energy of 1.4 Kcal. The increased DNA binding affinity of calicheamicin γ_1^I provides important evidence that the enediyne segment of the aglycone does make a significant contribution to the overall binding energy as previously suggested.[11,12]

The above CD evidence for calicheamicin-induced alteration of B-form DNA to a structure similar to that of a dehydrated form is interesting. Previously we reported results which suggested that there is a significant hydrophobic factor involved in the binding of calicheamicin to DNA.[14] These findings were based on the effect of the Hofmeister series of salts on calicheamicin binding/cleavage as well as the nonlinear van't Hoff behavior of the drug/DNA association observed by examining the temperature dependence of binding/cleavage. Thus, the drug-induced conformational change in the DNA appears to be accompanied by dehydration of the DNA. In any event, the DNA alteration observed by CD supports the proposal by Walker

et. al.[11] that binding/cleavage specificity observed for calicheamicin relates to calicheamicin-induced DNA conformational changes.

NMR evidence for a conformational change of the DNA on binding to calicheamicin has recently been obtained by Kahne's group.[19] A study of a calicheamicin γ_1^I-oligonucleotide complex (d[GTGACCTG] containing an ACCT recognition site) has shown that the DNA is distorted upon binding to the drug with the largest change at the CC step. The bound drug, however, is apparently not conformationally distorted from that of the free drug. Calicheamicin γ_1^I was previously shown by this group to exist in an extended and substantially preorganized conformation with a pronounced curvature closely matching that of the DNA minor groove.[20]

CD changes similar to those reported here for calicheamicin have been reported recently by Uesugi and Sugiura for esperamicin.[21] These workers proposed that esperamicin DNA association also causes a DNA transition to a structure reminiscent of a dehydrated form. Such drug-induced alteration of the DNA appears to be important for both calicheamicin and esperamicin recognition at the observed binding/cleavage sites.

EXPERIMENTAL

The duplex dodecamer in this study is referred to as the 'TCCT' dodecamer and was synthesized using an Applied Biosystems DNA synthesizer. Dodecamers were purified by ion exchange HPLC (Bio-Rad DEAE). Concentration of the single-stranded DNA oligomers was determined spectrophotometrically. Equal concentrations of the single stranded oligomers were combined in 10 mM pH 7 phosphate buffer and 0.1 M NaCl and heated at 70 °C for five minutes. The DNA was then annealed by cooling slowly for several hours at ambient temperature. Calicheamicin ϵ and γ_1^I stock solutions were prepared in ethanol and their concentrations were calculated using molecular weights of 1291 and 1367.

CD spectra were recorded with a Jasco J-600 spectropolarimeter interfaced to and controlled by an HP Vectra computer. The instrument was calibrated with ammonium d-10-camphorsulfonate according to the vendor's procedure. Run parameters for the CD spectra were the following: band width, 1 nm; time constant, 1-8 s; scan speed, 5-20 nm/min. In a typical experiment, aliquots of the calicheamicin stock solution were combined with the DNA solution in the indicated buffer composition and 10% ethanol. The mixture was equilibrated for at least 5 minutes prior to recording the spectra. CD spectrum of the solvent blank was subtracted from each of these spectral scans. Calicheamicin ϵ /DNA spectra were recorded in a 1 mm path length rectangular demountable cell while calicheamicin γ_1^I/ DNA spectra were recorded in a 1 cm path length cylindrical CD cell. Molar ellipticity was calculated based upon the oligonucleotide concentration in nucleotides and cell path length in centimeters. For determination of binding constants, increasing concentrations of the drug were titrated into the DNA solution. DNA and drug concentrations were corrected for volume changes. DNA spectral changes were monitored at 219 nm and 271 nm for the titration of calicheamicin ϵ and 236 nm for γ_1^I. Fractional saturation of DNA, θ, is given by $(\Delta\epsilon-\Delta\epsilon_0)/(\Delta\epsilon_{max}-\Delta\epsilon_0)$[22] Concentrations of the free drug were determined using the computed θ values and the total DNA concentration. Fractional DNA saturation values and the free drug concentration [L] were fit by nonlinear least squares to equation 1, where K_a is the association constant for drug interaction with the oligomer

$$\theta = \frac{nK_a[L]}{1+K_a[L]} \qquad (1)$$

and **n** represents the number of interaction sites on the oligomer. KaleidaGraph software (version 2.1, Abelweck Software) was used to generate the binding isotherms with K_a and **n** as adjustable parameters. The goodness of fit of the experimental data to Equation 1 was judged both by χ^2 values and the correlation coefficients. The reported binding isotherms and the K_a values, are based upon fits with χ^2 values ≤ 0.15 and correlation coefficients ≥ 0.97.

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