

Kedarcidin chromophore: An enediyne that cleaves DNA in a sequence-specific manner

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ABSTRACT Kedarcidin chromophore is a 9-membered enediyne, recently isolated from an actinomycete strain. *In vivo* studies show this molecule to be extremely active against P388 leukemia and B16 melanoma. Cytotoxicity assays on the HCT116 colon carcinoma cell line result in an IC₅₀ value of 1 nM. *In vitro* experiments with ΦX174, pM2 DNA, and ³²P-end-labeled restriction fragments demonstrate that this chromophore binds and cleaves duplex DNA with a remarkable sequence selectivity producing single-strand breaks. The cleavage chemistry requires reducing agents and oxygen similar to the other naturally occurring enediynes. Certain cations (Ca²⁺ and Mg²⁺) prevent strand cleavage. High-resolution ¹H NMR studies on the chromophore in the presence of calcium chloride implicate the 2-hydroxynaphthoyl moiety in DNA binding. Interestingly, the kedarcidin chromophore appears structurally related to neocarzinostatin yet recognizes specific DNA sequences in a manner similar to calicheamicin γ₁, an enediyne with a significantly different structure. Moreover, kedarcidin and calicheamicin share a DNA preferred site, the TCCTNmer. These observations indicate that the individual structural features of these agents are not solely responsible for their DNA selectivity. Rather, a complementarity between their overall tertiary structure and the local conformation of the DNA at the binding sites must play a significant role in the recognition process.

The DNA binding and cleavage properties of the potent enediyne antitumor antibiotics have been the focus of intense study by a number of groups for the past several years. Kedarcidin chromoprotein is a recently identified member of this unusual family of microbial metabolites (1–3, ‡). *In vitro* cytotoxicity assays using the HCT116 colon carcinoma cell line showed the chromophore to possess potent cytotoxicity (IC₅₀, 1 nM) similar to that of Adriamycin (W.S. and N.Z., unpublished data). Moreover, the chromophore exhibited superior antitumor activity against murine P388 leukemia and B16 melanoma models (W. C. Rose, personal communication). As with neocarzinostatin (4–7), kedarcidin chromophore, a labile 9-membered enediyne, occurs as a component of a highly acidic chromoprotein (1–3). This is in contrast to esperamicin, calicheamicin, and dynemicin, agents that contain 10-membered enediyne rings and, to date, have been isolated without associated apoproteins (8–11). In this paper, we report that the kedarcidin chromophore interacts with duplex DNA and produces specific single-strand cuts. The cleavage chemistry requires reducing agents and oxygen similar to the other naturally occurring enediynes (4–7, 12–20). Interestingly, in contrast to calicheamicin and esperamicin, the addition of divalent cations, such as Ca²⁺ and Mg²⁺, inhibits DNA cleavage by the kedarcidin chromophore. High-resolution ¹H NMR experiments in the pres-

ence of CaCl₂ implicate the 2-hydroxynaphthoyl moiety of the kedarcidin chromophore in the binding to the DNA.

MATERIALS AND METHODS

Chemicals. Kedarcidin chromophore and esperamicin A₁, prepared as described (3, 7), were obtained from the Division of Chemistry, Bristol-Myers Squibb (Wallingford, CT). The DNA plasmids [ΦX174 replicative form I (RFI), replicative form II (RFII), and + strand; pM2; pBR322; and pUC18], the restriction enzymes, and the Klenow fragment of DNA polymerase I were purchased from Boehringer Mannheim, New England Biolabs, and GIBCO/BRL. The salmon sperm DNA was purchased from Sigma, sonicated, and ethanol-precipitated prior to use. Netropsin and calicheamicin γ₁ were a gift from Lederle Laboratories (Pearl River, NY). The radiochemicals were purchased from New England Nuclear/DuPont.

Preparation of Linearized (Form III) ΦX174. Linearized ΦX174 DNA (form III) was obtained by cleaving the covalently closed circular RFI DNA with the restriction enzyme *Pst* I. The digest was phenol-extracted and washed with chloroform prior to ethanol precipitation and washing. The DNA was quantitated by UV spectroscopy.

Determination of Single-Strand vs. Double-Strand Cleavage. The chromophore (1 μl of a 10× solution; see Fig. 1) was incubated with 0.16 μg of pure covalently closed circular (form I) pM2 DNA and 14 mM 2-mercaptoethanol in dimethyl sulfoxide (DMSO)/35 mM Tris-HCl, pH 7.5, 10:90 (vol/vol), (DMSO/Tris) at 37°C for various lengths of time, in a total reaction volume of 10 μl. When required, hydrazine and putrescine were added, each to 100 mM (final concentration), and the reaction mixture was incubated at 37°C for an additional hour (21). The different forms of DNA were separated on a 0.9% agarose gel after a 15-h electrophoresis at 30 V with subsequent ethidium bromide staining. The stained gels were photographed and the intensity of the DNA bands was assessed by linear scanning microdensitometry (22).

Comparison of the Cleavage of Different ΦX174 Forms by the Chromophore. All four forms (+ strand, RFI, RFII, and RFIII) of ΦX174 DNA were incubated overnight with the chromophore as described above.

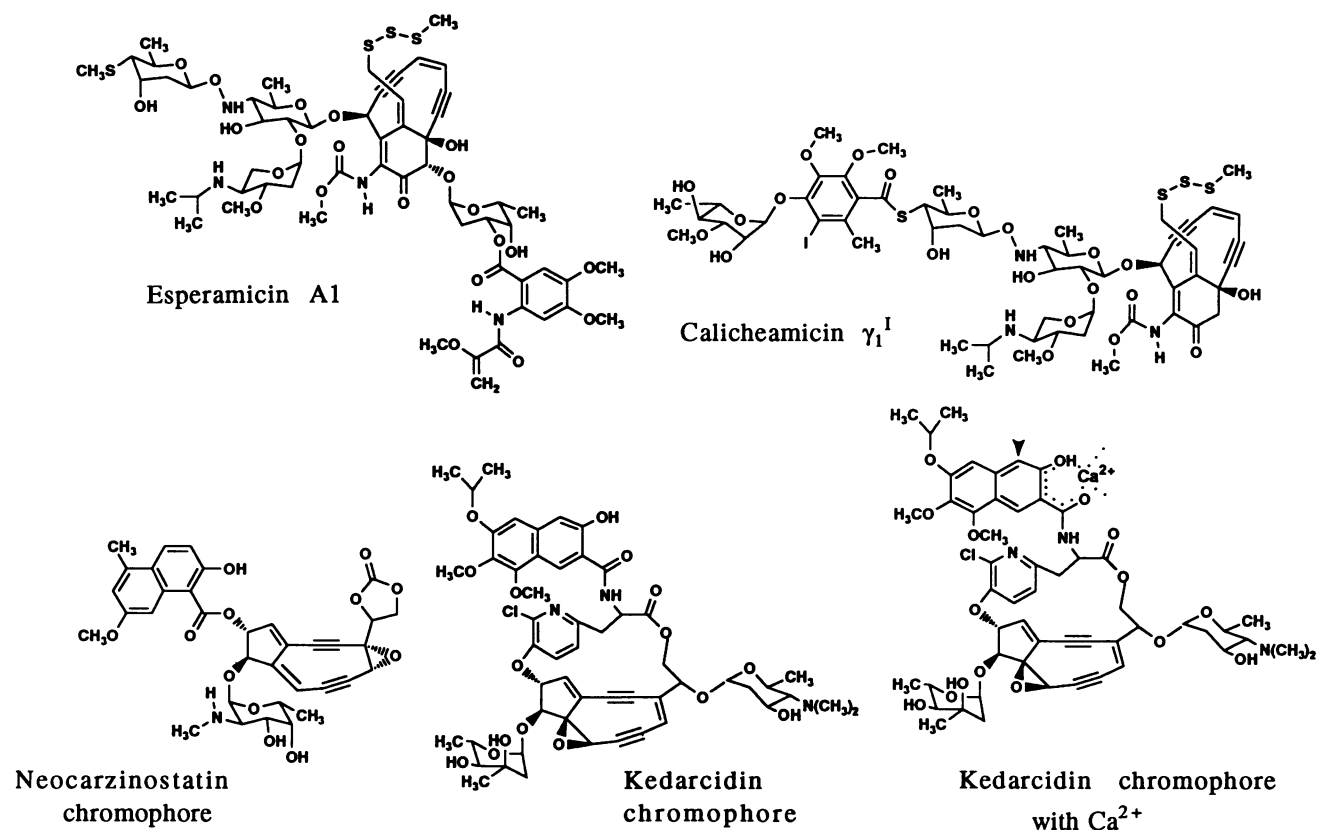
Preparation and Labeling of Restriction Fragments. Five restriction fragments were isolated and 5'-end-labeled by using [³²P]dATP and polynucleotide kinase. The fragments were a 275-bp pBR322 *Sal* I–*Bam*HI fragment, a 159-bp pBR322 *Hind*III–*Eco*RV fragment, a 159-bp pBR322 *Eco*RV–

Abbreviations: RF, replicative form; DMSO, dimethyl sulfoxide.

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HindIII fragment, a 180-bp pUC18 *EcoRI*-*Pvu* II fragment, and a 140-bp pUC18 *EcoRI*-*Pvu* II fragment. Two of these fragments, the 275-bp pBR322 *Sal*I-*Bam*HI fragment and the 159-bp pBR322 *Hind*III-*Eco*RV fragment, were also 3'-end-labeled using the Klenow fragment methodology with [α - ^{32}P]dCTP (22).

Cleavage Specificity with 5'- and 3'-End-Labeled Restriction Fragments. The compound (1 μl of a 10 \times solution) was incubated with carrier salmon sperm DNA at 5 $\mu\text{g}/\text{ml}$ and end-labeled DNA (≈ 6000 cpm) in presence of 14 mM 2-mercaptoethanol in a total volume of 10 μl . The reactions were carried out at 37 $^\circ\text{C}$ for 15 h in DMSO/Tris, lyophilized, dissolved in denaturing loading dye, and analyzed on a 12% polyacrylamide sequencing gel at 1200 V for 3 h. Cleavage sites were deduced by comparison to the Maxam-Gilbert chemical sequencing lanes (G, AG, C, and TC) (22, 23).

Cleavage Under Anaerobic Conditions. The reaction conditions were identical to those described earlier except that the final reaction volume was in this case 50 μl . The solution was deaerated several times prior to the addition of drug and 2-mercaptoethanol to the reaction mixture, by using argon and a Firestone valve. The incubation was carried out under a positive pressure of argon.

Cleavage in the Presence of Salts, Superoxide Dismutase, and Catalase. When the cleavage was performed in presence of salts, identical conditions to those mentioned above were carried except for the addition of 1 μl of a 10 \times solution of CaCl_2 , NaCl , MgCl_2 , superoxide dismutase, and catalase (see Fig. 3).

Base and Sodium Borohydride Treatment of the Cleavage Products. Base treatment of the cleavage products was carried by heating the cleavage products in 0.1 M NaOH or NH_4OH at 90 $^\circ\text{C}$ for 3 min. Sodium borohydride treatment consisted of incubating the cleavage products with 0.28 M NaBH_4 for 90 min. Base and borohydride treatments were preceded and followed by DNA precipitation and an ethanol wash.

Netropsin Competition Experiments. The DNA was treated with netropsin at 50 $\mu\text{g}/\text{ml}$ in presence of 2-mercaptoethanol for 15 min in DMSO/Tris. The solution was then lyophilized, redissolved in buffer, and treated as described above with kedarcidin chromophore.

High-Resolution Proton ^1H NMR Experiments. Two identical NMR samples of 3.6 mM kedarcidin in [^2H]DMSO were prepared. To one sample, increasing amounts of a solution of CaCl_2 in $^2\text{H}_2\text{O}$ were added to achieve concentrations of 0 mM, 8 mM, 16 mM, 58 mM, 125 mM, and 258 mM. To the control sample, equivalent amounts of $^2\text{H}_2\text{O}$ were added to account for dilution effects. NMR spectra of both samples were obtained at each concentration at 500.13 MHz on a Bruker AM-500 equipped with a 5-mm broad-banded probe. Each spectrum was acquired using 32,000 data points, 128 scans, and a 45 $^\circ$ observation pulse. Exponential multiplication with a line broadening of 0.1 Hz was used in data processing. Spectra were referenced to the solvent ([^2H]DMSO, "100% deuterated"; MSD Isotopes) at 2.49 ppm.

RESULTS

In Vitro Single-Strand Cleavage. Incubation of the kedarcidin chromophore at 37 $^\circ\text{C}$ with form I pM2 DNA in presence of 14 mM 2-mercaptoethanol overnight resulted in the conversion of form I DNA to mostly open circular DNA (form II). Some double-strand breaks (form III) were observed at higher concentrations. A plot of the consumption of form I and the formation of forms II and III as a function of drug concentration is shown in Fig. 1. The data show that, at 37 $^\circ\text{C}$, single-strand breaks accumulate on average four times more rapidly than double-strand breaks. Treatment of the cleavage products with 100 mM hydrazine and putrescine did not have any effect on the results. A time-dependent study at one concentration of chromophore shows that the chromophore cleaves the DNA within 5 min of incubation time. Omitting 2-mercaptoethanol from the reaction mixture greatly decreased the DNA cleavage. A temperature-dependent study

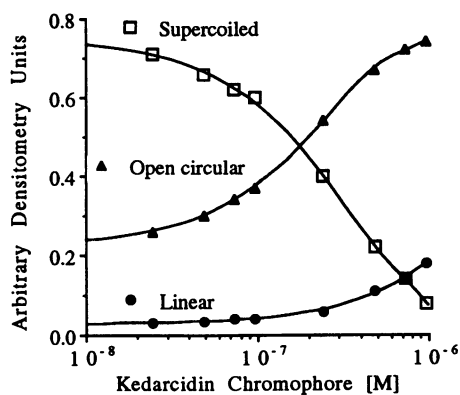


FIG. 1. Plot of the conversion of covalently closed circular pM2 DNA (form I) to open circular (form II) and linear (form III) DNA as a function of increasing kedarcidin chromophore concentrations. The ratio of single-strand to double-strand breaks was determined from densitometry studies to be 4.2 ± 0.85 (mean \pm SEM).

at 4°C and 37°C showed the rate of accumulation of both forms to be ≈ 5 times more rapid at 37°C than at 4°C.

Comparison Between Various Forms of DNA. In the presence of 2-mercaptoethanol, kedarcidin chromophore at concentrations as low as $0.075 \mu\text{M}$ caused single-strand breaks in supercoiled ΦX174 . A concentration of $2.5 \mu\text{M}$ ($33\times$ higher than for RFI) was required to see faint cutting of the open circular form. At that concentration the linearized DNA seemed to be unaffected. At $100 \mu\text{M}$, single-strand ΦX174 (+ strand) was unaffected by the drug.

DNA Cleavage Site Sequence Specificity. 5'-End-labeled studies. DNA cleavage studies in presence of 2-mercaptoethanol on five 5'-end-labeled pBR322 and pUC18 restriction fragments showed the chromophore to be highly sequence-specific (Figs. 2 and 3). A preferred site was the 3' nucleotide (N = T, C, G, or A) adjacent to the TCCT tetramer (N = T, C, G, or A) adjacent to the TCCT tetramer. Another favored site was TCGTN, where N was a C or a G. The 5-mer TCATN was less-preferred than TCCTN ($\leq 50\%$ of the TCCTN band), with the extent of cleavage dependent on the flanking sequences. Secondary sites were ACGCN and TCTAN ($< 50\%$ of the TCCTN band; Fig. 2). The electrophoretic mobilities of the drug-induced fragments appeared to be similar to those of the Maxam-Gilbert markers. Treatment of the cleavage products with base and sodium borohydride did not have any effect on the reaction products. These observations suggest fragments ending in 3'-phosphate or 3'-phosphoglycolate termini.

3'-End-labeled studies. Studies with the 3'-end-labeled pBR322 *Sal*I-*Bam*HI fragment also showed strong specificity for the TCCTN site as shown by a major band matching the chemically produced marker (Fig. 4). In addition, two faint and poorly resolved bands that migrate as though they were two nucleotides longer than the principal band were observed. NaOH treatment moved the slowest band c to where it matched the Maxam-Gilbert marker but the middle band b and the principal band a were unaffected. Sodium borohydride treatment of the cleavage products caused band

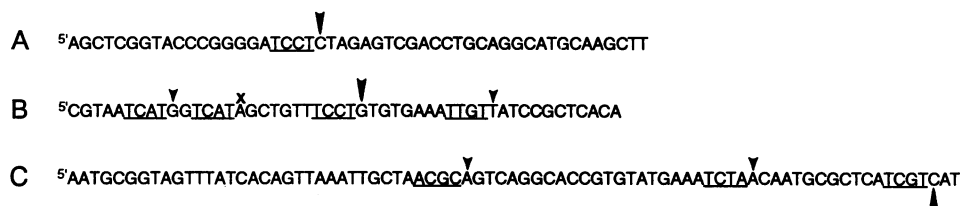


FIG. 2. Cleavage of three restriction fragments. The size of arrows approximates the relative extent of cleavage at each site. (A-C) Portions of the 140-bp PUC18 *Eco*RI-*Pvu* II fragment, the 180-bp PUC18 *Eco*RI-*Pvu* II fragment, and the 158-bp pBR322 *Hind*III-*Eco*RV fragment, respectively.

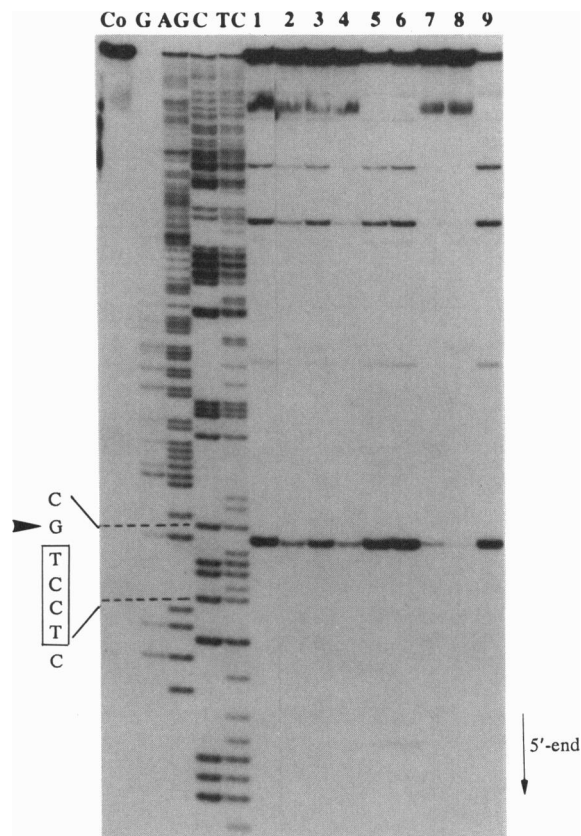


FIG. 3. Autoradiogram of the reaction products of kedarcidin chromophore with the 5'-end-labeled *Sal*I-*Bam*HI pBR322 fragment. Lanes: G, AG, C, and TC, Maxam-Gilbert lanes; 1, chromophore at $5 \mu\text{M}$; 2, under anaerobic conditions; 3, 4, and 7, with 50 mM NaCl , 10 mM MgCl_2 , and 10 mM CaCl_2 , respectively; 5 and 6, in presence of catalase and superoxide dismutase; 8, in absence of 2-mercaptoethanol; 9, chromophore at $50 \mu\text{M}$; Co, control lane.

c to move slightly faster leaving band a unaffected (Fig. 4). Bands b and c could be assigned to fragments ending in 5' aldehydes and perhaps acids, and band a might end in an intact phosphorylated terminus.

Effects of Various Factors on the Cleavage Reaction with a 5'-End-Labeled pBR322 *Sal*I-*Bam*HI Fragment. Omitting 2-mercaptoethanol from the reaction mixture inhibited the cutting. Cleavage was reduced drastically when oxygen was removed by purging with argon. Catalase and superoxide dismutase had no effect on the cleavage. Addition of 10 mM CaCl_2 and 10 mM MgCl_2 resulted in at least 90% inhibition of the cleavage whereas 50 mM NaCl did not alter the results much (Fig. 3). In contrast, addition of 100 mM CaCl_2 to a DNA/esperamicin A_1 and DNA/calicheamicin γ_1^I reaction mixtures had no effect on their cleaving properties. Competition experiments with netropsin, a known minor groove binder, showed that some of kedarcidin cleavage sites were modified or eliminated by netropsin.

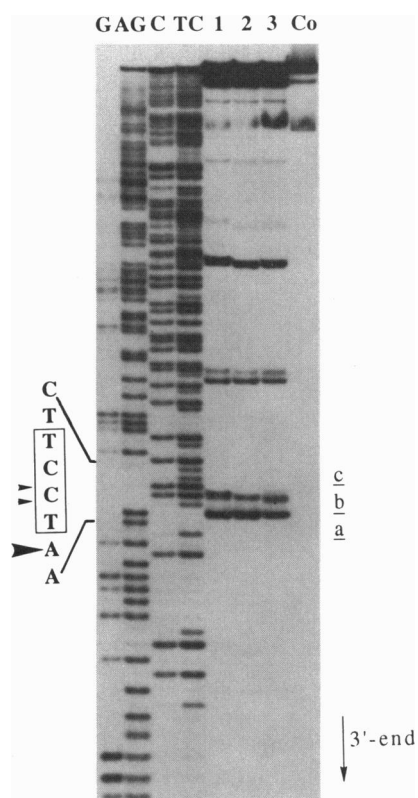


FIG. 4. Autoradiogram of the reaction products of kedarcidin chromophore with the 3'-end-labeled *Sal I*-*BamHI* pBR322 fragment. Lanes: G, AG, C, and TC, Maxam-Gilbert lanes; 1, chromophore at 5 μM ; 2 and 3, products of lane 1 treated with NaOH and NaBH₄, respectively, as described in text; Co, control lane.

NMR Studies in the Presence of Calcium Chloride. NMR studies showed that upon addition of increasing amounts of CaCl₂ to a kedarcidin chromophore solution only one proton was significantly affected. The singlet at 7.1 ppm shifted downfield whereas all other protons shifted slightly upfield relative to the control (Fig. 5). This suggested the localization of the Ca²⁺ ion in the region of the naphthoic acid moiety as shown.

DISCUSSION

A plot of the optical density data derived from agarose gel experiments with pM2 DNA as a function of increasing chromophore concentrations showed that, at low concentrations, single-strand breaks accumulate on average four times more rapidly than double-strand breaks, consistent with a single-strand cutting behavior (24). The comparative study between the cleavage of various forms of ΦX174 indicates that the single-strand form is not recognized by the kedarcidin chromophore and that supercoiled DNA appears to be a better substrate for the chromophore than the open circular and the linearized forms. Cleavage studies on 5'- and 3'-end-labeled restriction fragments showed this chromophore to be a remarkably site-specific cleaving agent. Competition experiments with netropsin suggest association of the chromophore in the minor groove of the DNA. The lack of effect of superoxide dismutase and catalase on the cleavage and the requirement of oxygen imply the cutting is due to a nondiffusible species, probably to one of the transiently produced radicals during the aromatization of the enediyne moiety. It is likely that reaction with the thiol at C-12 and epoxide ring opening triggers the cyclization of the enediyne to an activated diradical intermediate. This intermediate then abstracts hydrogen from the DNA causing strand breakage. The

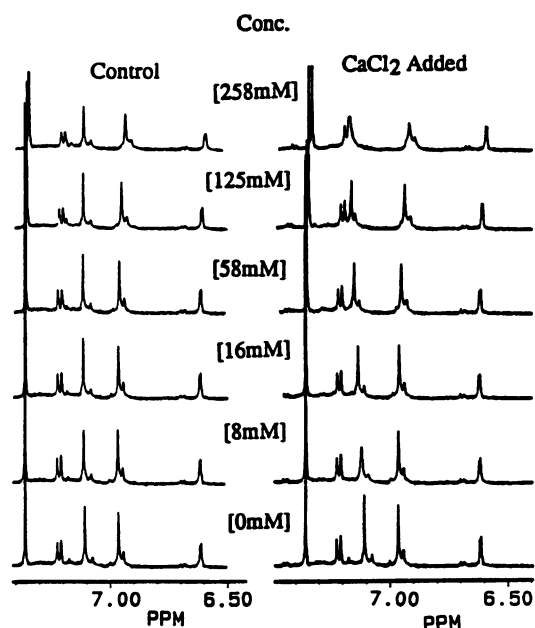


FIG. 5. Expanded NMR spectra of the kedarcidin chromophore with increasing amounts of ²H₂O (control) (A) or CaCl₂ in ²H₂O (B).

electrophoretic mobilities of the cleavage products obtained in the 5'- and 3'-end-labeled studies seem to suggest 4' hydrogen atom abstraction along with a small portion of 5' hydrogen abstraction from the targeted DNA deoxyribose sugars (4, 12-16, 25-34). However, a definitive interpretation of these results is not presently in hand.

The inhibition of DNA cleavage by Mg²⁺ and Ca²⁺ provided insight as to the chromophore regions important to DNA binding. The high-resolution ¹H NMR results show the chelation of Ca²⁺ at the naphthoate moiety reminiscent of siderophore chelation properties (35). In the chelated form, kedarcidin presumably cannot associate with the DNA binding site, thus preventing DNA cleavage. These results implicate the naphthoate moiety in DNA binding.

CONCLUSION

The kedarcidin chromophore appears structurally related to the neocarzinostatin chromophore yet unexpectedly recognizes specific DNA sequences in a manner similar to calicheamicin γ_1^I . That two disparate molecules (kedarcidin chromophore and calicheamicin γ_1^I) share a DNA recognition site (TCCTN) suggests that individual structural features are not solely responsible for the observed DNA selectivity of these agents. Rather, a complementarity between their overall tertiary structure and the local conformation/hydration of the DNA at the binding sites must play a significant role in the recognition process (36-49). These results point out the necessity of examining by x-ray crystallography and NMR spectroscopy the drug-DNA complexes and the microstructure of the DNA at the recognition sites.

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