Maduropeptin: An Antitumor Chromoprotein with Selective Protease Activity and DNA Cleaving Properties

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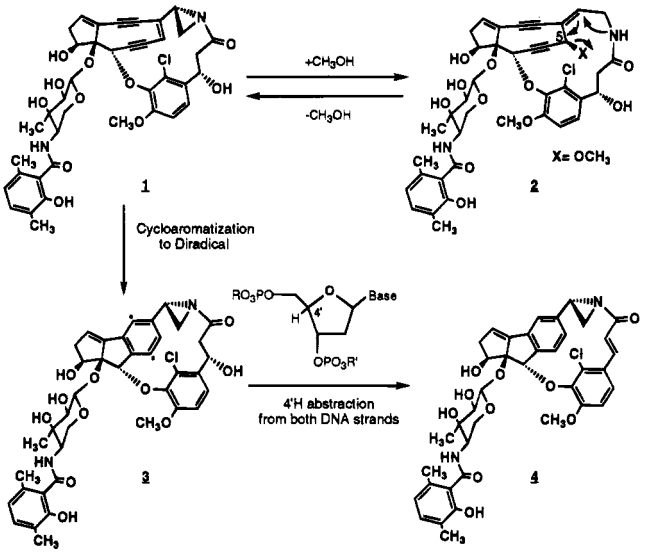
ABSTRACT: Maduropeptin (MDP) is a recently isolated antitumor antibiotic, consisting of an enediyne-containing chromophore embedded in a highly acidic protein. This holoantibiotic damages duplex DNA *in vitro*, producing a mixture of single- and double-strand breaks at selected sites. The chromophore, isolated as the methanol adduct from the protein-containing holoantibiotic, exhibits the same selectivity and cleavage chemistry as the chromoprotein complex. Preliminary evidence suggests that the primary DNA breaks involve 4′-H abstraction from the deoxyribose sugars at the cleavage sites. Unlike most other enediyne antitumor antibiotics, DNA strand scission is not bioreductively induced by MDP or the methanol adduct of the chromophore. This was also observed for the C1027 chromophore. DNA cleavage is inhibited in the presence of certain cations (Ca^{2+}, Mg^{2+}) as was observed with the kedarcidin chromophore. ^1H NMR spectroscopy studies on the methanol adduct of the maduropeptin chromophore in the presence of calcium chloride provide clues regarding its activation and give insight as to the regions of the chromophore important for DNA binding. Our results suggest that the solvent artifact of the chromophore may in essence be a prodrug and it regenerates the parent chromophore as in the holoantibiotic prior to cleaving DNA. As with kedarcidin and neocarzinostatin, maduropeptin exhibits a high affinity for histones, *in vitro*, cleaving them to low molecular mass peptides. Histone H1, the most opposite in net charge, is cleaved most readily. This latter activity may serve to disrupt the chromatin superstructure *in vivo*, prior to exposing the DNA to the chromophore.

Maduropeptin (MDP)[1] is a novel antitumor antibiotic, recently isolated from the broth filtrate of *Actinomadura madurae* (Hanada et al., 1991). MDP consists of a 1:1 complex of a labile nine-membered enediyne ring, possessing potent antibacterial and antitumor properties and an acidic stabilizing protein (32 kDa) (Marquardt H., personal communication). MDP apoprotein shows no homology to the related enediyne-containing chromoproteins neocarzinostatin (NCS)[1] (Koide et al. 1980), macromomycin (Chimura et al., 1968), actinoxanthin (Khokhlov et al., 1969), C-1027 (Otani et al., 1988), or kedarcidin (KDC)[1] (Hofstead et al., 1992). In contrast, the chromophore **1** (Schroeder et al., 1994) seems to be biogenetically related to the family of enediyne chromophores that have been characterized to date (Goldberg, 1991; Lee et al., 1991; Leet et al., 1993; Otani et al., 1988). However, unlike other nine-membered enediyne chromophores, the MDP chromophore does not appear to be accessible in a surface binding pocket and a new process to extract the chromophore from the protein had to be developed (Schroeder et al., 1994). Conditions necessary to denature the protein and release the MDP chromophore led to the isolation of solvent artifacts where nucleophilic addition to the double bond of the enediyne (at C-5) occured (Scheme 1, **2**). These solvent artifacts were crucial in the determination of the structure and the mechanism of action of the highly labile parent compound **1**. Similarly to other natural enediynes, methanol adduct **2** exhibits good antitumor and antibacterial activities. However, these activities are 100-fold less than those of **1**.

Scheme 1: Proposed Mechanism of Action of Maduropeptin Chromophore **1** and the Methanol Adduct **2**



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[1] Abbreviations: DNA, deoxyribonucleic acid; KDC, kedarcidin; MDP, maduropeptin; NCS, neocarzinostatin; NMR, nuclear magnetic resonance; form I, supercoiled covalently closed circular DNA; form II, open circular DNA; form III, linear DNA; (+ strand) open circular single-strand DNA; M−G markers, Maxam−Gilbert chemically produced markers; CC_{50}, concentration at which 50% of DNA is cleaved.

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To date, the 10-membered ring enediynes—calicheamicin, esperamicin, and dynemicin—have been isolated without associated apoproteins whereas the 9-membered enediynes have always been isolated with a noncovalently bound apoprotein. It has been suggested that the antitumor and antimicrobial activities of the enediynes are primarily due to their ability to cleave DNA (Langley et al., 1994; Ellestad et al., 1994; Zein et al., 1993a); Despite great structural diversity, the majority of the enediynes cleave DNA via a benzenoid diradical that abstracts hydrogen atoms from the DNA deoxyribose sugars (Goldberg et al., 1994; Zein et al., 1989a,b; De Voss et al., 1990). In addition, it was demonstrated that the NCS and the KDC proteins possess selective proteolytic activity against histones (Zein et al., 1993b).

In this report, the mechanism of action of the most recently isolated chromoprotein, MDP, is discussed. Evidence is presented that MDP chromophore **1** and the methanol adduct **2** cut DNA at selected sites and that **2** exhibits the same DNA site selectivity and cleavage chemistry as the parent compound **1**. NMR spectroscopy studies in the presence of calcium chloride suggest the identity of the chromophore functionalities that are important in drug activation and in the initial binding to DNA. As with kedarcidin, these functionalities, i.e., the β-hydroxy amide and the *o*-hydroxybenzamide, are reminiscent of siderophore chelation sites (Telford et al., 1994). Of great interest to us is that the solvent artifacts of the chromophore, such as **2**, may in essence be prodrugs and regenerate the parent chromophore **1** as in the holoantibiotic prior to cleaving DNA (Scheme 1).

In this report, we also demonstrate that MDP cleaves histone H1 preferentially, strengthening the hypothesis that the protein component of the neocarzinostatin, kedarcidin and now maduropeptin may provide a "targeted delivery" of the highly cytotoxic chromophores to the chromatin (Zein et al., 1993b).

MATERIALS AND METHODS

Chemicals. The MDP chromoprotein and **2** (Scheme 1) as described previously (Hanada et al., 1991; Schroeder et al., 1994) were obtained from the Division of Chemistry, Bristol-Myers Squibb, Wallingford, CT. Reagents were obtained as follows: reagent grade chemicals from Aldrich, Boehringer Manheim, BRL, Novagen, and Sigma; DNA plasmids, the restriction enzymes, the klenow fragment of DNA polymerase I, the calf thymus histones, and the protease inhibitors from Boehringer Manheim, New England Biolabs, Stratagene, and Gibco BRL. The pAP 1-3 (Dedon & Goldberg, 1992) was generously provided by Peter C. Dedon and the pBKS+ by Hans-Georg Simon. The salmon sperm DNA was purchased from Sigma, sonicated, and ethanol precipitated prior to use. Netropsin was a gift from Lederle Labs. The radiochemicals were purchased from New England Nuclear/Dupont and Amersham.

Cleavage of Form I and (+ Strand) ΦX174 DNA. MDP or **2** (1 μL of a 10× solution) was incubated with 0.2 μg of form I or (+ strand) ΦX174 DNA in 50 mM tris-HCl, pH 7.2−8.2 or 10:90 dimethyl sulfoxide (DMSO)−tris respectively, at 37 °C for various lengths of time, in a total reaction volume of 10 μL. The different forms of DNA were separated on a 1% agarose gel after a 3 h electrophoresis at 60 V with subsequent ethidium bromide staining. The stained gels were photographed, and the intensity of the DNA bands was assessed by linear scanning microdensitometry (Sambrook et al., 1989).

Cleavage Selectivity with 5′- and 3′-End-Labeled Restriction Fragments. MDP or **2** (1 μL of a 10× solution; final concentrations 50 μg/mL or 10^{-6} M in MDP and 0.1 mg/mL or 10^{-4} M in **2**) was individually incubated with carrier salmon sperm DNA at 5 μg/mL and end-labeled DNA (~15 000 counts/min) in a total volume of 10 μL. The reactions were carried out at 37 °C for 15 h in tris (pH 7.4) or 10:90 DMSO−tris, lyophilized, dissolved in denaturing loading dye, and analyzed on a 12% polyacrylamide sequencing gel at 1200 V for 3, 6, or 9 h. Cleavage sites were deduced by comparison to the M−G marker lanes (G, AG, C, TC) (Maxam & Gilbert, 1980). The 5′-end-labeled restriction fragments studied were the following: pBKS+ *Ava*II, *Apa*LI (307 bp); pAP 1-3 *Ava*II, *Apa*LI (270 bp); pBR322 *Hind*III, *Eco*RV (158 bp); pBR322 *Eco*RV, *Hind*III (158 bp); pUC 18 *Eco*RI, *Pvu*II (180 and 143 bp); pBR322 *Sal*I, *Bam*HI (276 bp). The 3′-end-labeled restriction fragments studied were the following: pBKS+ *Pvu*I, *Apa*LI (297 bp); pBKS+ *Pst*I, *Pvu*II (272 bp); pUC 18 *Pst*I, *Pvu*II (215 bp); pBR322 *Hind*III, *Eco*RV; pBR322 *Sal*I, *Bam*HI.

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 as discussed previously for KDC (Zein et al., 1993a).

Cleavage in the Presence of Salts, Superoxide Dismutase, and Catalase. When the cleavage was performed in the presence of salts or additional enzymes, conditions identical to those mentioned above were used except for the addition of 1 μL of a 10× solution of $CaCl_2$, $MgCl_2$, superoxide dismutase, and catalase (Zein et al., 1993a). These reactions were carried using **2** and the pBR322 *Sal*I−*Bam*H1 5′-end-labeled restriction fragment.

Netropsin Competition Experiments. The pBR322 *Sal*I/*Bam*H1 5′-end-labeled fragment was treated with netropsin at 50 μg/mL in the presence of β-mercaptoethanol for 15 min in 10:90 DMSO−tris. The solution was then lyophilized, redissolved in buffer, and treated with **2** as described above.

Base and Sodium Borohydride Treatment of the Cleavage Products. Base treatment of the restriction fragment cleavage products was carried by heating the cleavage products in 0.1 M piperidine at 90 °C for 30 min. Cleavage of abasic sites involved treatment with 100 mM putrescine for 1 h at 37 °C. Treatment with hydrazine (100 mM) was carried out at room temperature for 1 h. Sodium borohydride treatment consisted of reacting 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 (Dedon & Goldberg, 1992).

High-Resolution ^1H NMR Spectroscopy Experiments. Two identical NMR samples, 1.72 mM in **2**, were prepared in [^{2}H]DMSO. To one sample were added increasing amounts of a solution of $CaCl_2$ (in ^2H_2O) to achieve concentrations of 0, 10, 30, 70, 150, and 310 mM. To the control sample were added equivalent amounts of ^2H_2O to account for dilution effects. NMR spectra of both samples were recorded for each concentration at 500.13 MHz on a Bruker AM-500 equipped with a 5 mm broad-banded probe. Each spectrum

was acquired using 32K data points, 128 scans using a 45° observation pulse. Spectra were referenced to the solvent ([^{2}H]DMSO, "100% deuterated" MSD Isotopes) at 2.49 ppm.

Histone Cleavage by MDP. MDP (1 μL of an aqueous 0.5 mg/mL; 10 μM solution, 10×) was incubated with each of the five calf thymus histones H1, H2A, H2B, H3, and H4 (1 μL of an aqueous 10 mg/mL solution) at 37 °C in a total volume of 10 μL. The reactions were carried out overnight in 50 mM tris. The samples were then heated in a denaturing dye for 1 min and analyzed on a 17% SDS−polyacrylamide gel. The protein bands were visualized with Coomassie blue.

Incubation with Various Protease Inhibitors. The protease inhibitors leupeptin, antipain, aprotinin, pepstatin, DFP, and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), a water-soluble alternative to PMSF (Pefabloc), were added at a final concentration of 20 μg/mL, 50 μg/mL, 20 μg/mL, 20 μg/mL, 575 mM, and 1 mM, respectively, to the MDP/H1 reaction mixture described above.

RESULTS

Cleavage of ΦX174 Plasmid DNA. Incubation of MDP or **2** at 37 °C, pH 7.4, with form I ΦX174 DNA overnight resulted in the conversion of form I DNA to form II and form III. A total of 50% of form I DNA was consumed at CC_{50}^1 of 4×10^{-8} M in MDP and CC_{50}^1 of 5×10^{-6} M in **2**. Around the CC_{50}, the ratio of form III to form II for both drugs varied between 0.3 and 0.4. A pH-dependent study using **2** (pH was varied between 7.2 and 7.8) at the CC_{50} showed enhanced activity as the pH was increased. Neither chromophore had any effect on single-strand ΦX174 (+ strand), even at relatively high concentrations of drugs (results not shown).

Nature and Sequence of Cleavage Sites. The nature and the DNA site selectivity were examined by the reaction of the MDP chromophore or **2** with six 5′- and five 3′-end-labeled pBKS+, pBR322, and pUC18 restriction fragments in the presence of salmon sperm DNA. The cleavage products were determined by comparison with the M−G standards on sequencing gels. The results indicate that MDP and **2** exhibit identical cleavage patterns. They both cleave DNA in a sequence-dependent manner. A prominent cleavage site that is observed is 5′-TC*T*T/3′-*A*GAA; it is interesting to note that 5′-TTCT is not cleaved nor is 3′-AAGA (Figures 1 and 3). Another preferred site is 5′-TC*TC*/3′-*A*GAG (Figure 3); cleavage at this site was dependent upon the flanking sequence. Similar results were noted for 5′-TTTT/3′-AAAA in which case the scission site and magnitude of cleavage within this tetramer varied with the flanking sequences (Figures 1 and 2). In the case of these primary cleavage sites, scission on one strand was always accompanied with scission on the complementary strand having equal intensity and with a two-nucleotide 3′-stagger (Figures 1−3). Secondary cleavage sites were also observed; examples of such sites are 5′-NTTT (Figure 2); 5′-TTAT; 5′-TCCT; 5′-ATCT, 5′-TCAT (Figure 2); 5′-TACT (Figure 1), 5′-TCAC, and their complementary sequences. The magnitude of cleavage and the chemistry at these sites (i.e., number of bands produced, their relative intensities, and their electrophoretic mobilities relative to the M−G markers) were dependent on the DNA sequence beyond the immediate neighboring DNA bases.

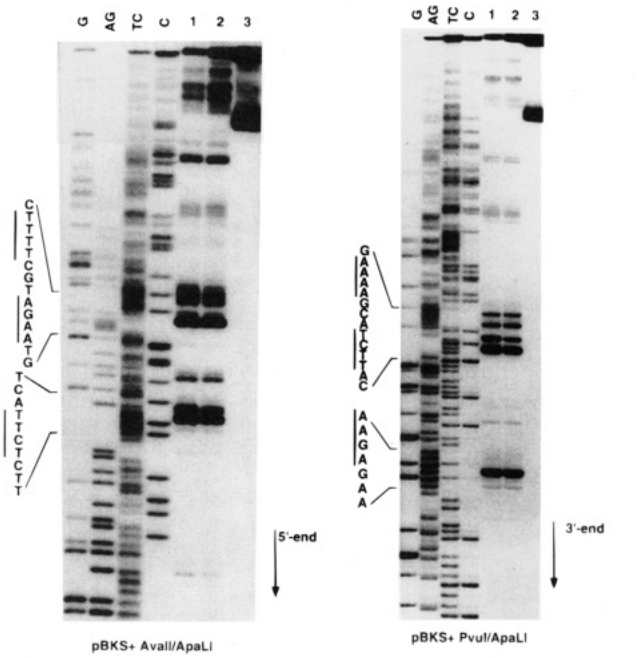


FIGURE 1: Autoradiogram of the reaction products of MDP and **2** with the 5′-end-labeled pBKS+ *Ava*II−*Apa*LI and its complementary, the 3′-end-labeled pBKS+ *Pvu*I−*Apa*LI. Lanes G, AG, TC, and C are Maxam−Gilbert lanes; lanes 1 and 2 are MDP and **2** at 0.05 and 0.1 mg/mL, respectively; lane 3 is the control.

Characterization of the Cleavage Chemistry at the Preferred Sites. In order to begin to understand the cleavage chemistry at the preferred sites, cutting reactions in the presence of MDP and **2** using the 5′-end-labeled pBKS+ *Ava*II, *Apa*LI, and the pAp 1-3 *Ava*II, *Apa*LI restriction fragments were carried out. These fragments contained the 5′-TC*T*T, the 3′-*A*GAA, the 5′-CTT*T*G, and the 3′-G*AA*AAC sites (Figures 1 and 2). When the DNA was 5′-end-labeled, the reactions yielded fragments that migrated similarly to or slightly ahead of the M−G standards (e.g., Figure 2, site 5′-CTT*T*G). Base and sodium borohydride treatment did not affect the mobility of the fragments, which matched the chemical standards when the DNA was 3′-end-labeled. To characterize further the cleavage chemistry, two complementary 16-mers were synthesized, individually 5′-end-labeled, annealed with the nonlabeled complementary strand, and reacted with **2**. These oligomers contained a preferred cleavage site determined from studies on the pBR322 *Eco*RV−*Hind*III fragment—the 5′-TC*TC*/3′-*A*GAG tetramer (Figure 3). The reaction products were treated with putrescine and hydrazine. In the case of both oligomers, putrescine treatment caused an increase in the intensity of the observed cleavage products. Hydrazine treatment resulted in the formation of DNA fragments that migrated slower than the M−G standard, i.e., than the phosphate-ended fragment.

Effect of Various Factors on DNA Cleavage. As was observed with kedarcidin, competition experiments with netropsin showed that some of the cleavage sites of **2** are modified or eliminated. Exclusion of oxygen inhibited strand scission, while the use of excess superoxide dismutase or catalase in the reaction mixture did not alter most of the DNA damage due to **2**. Addition of 10 mM $CaCl_2$ or $MgCl_2$ to the reaction mixtures resulted in at least 90% inhibition of the DNA cleavage (results not shown). Similar observations with kedarcidin prompted NMR spectroscopy studies on **2**

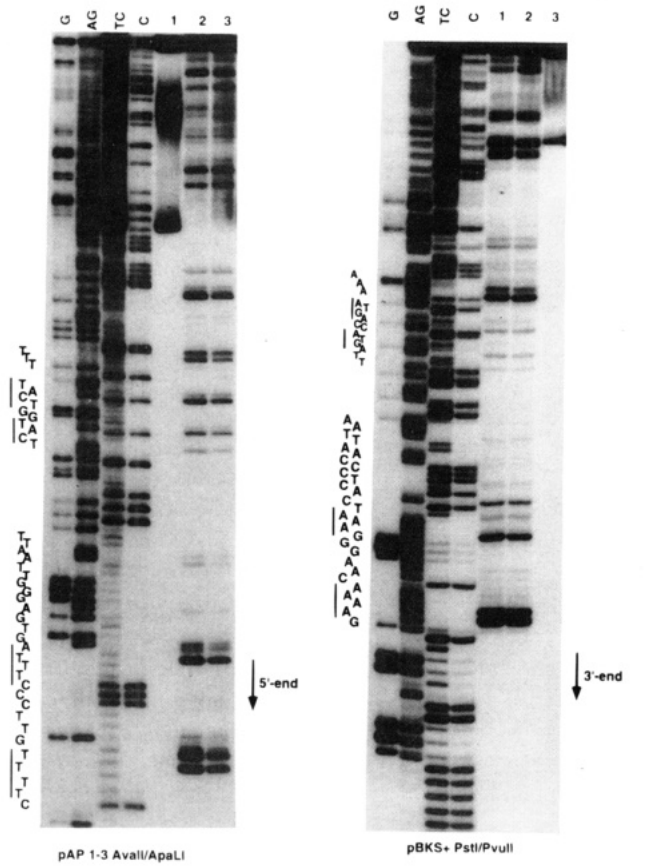


FIGURE 2: Autoradiogram of the reaction products of MDP and **2** with the 5′-end-labeled pAP 1-3 *Ava*II−*Apa*LI (A) and its complementary, the 3′-end-labeled pBKS+ *Pst*I−*Pvu*II (B). Lanes G, AG, TC, and C are Maxam−Gilbert lanes; in (A) lane 1 is the control, lanes 2 and 3 are MDP and **2** at 0.05 and 0.1 mg/mL, respectively; in (B) lanes 1 and 2 are MDP and **2** at 0.05 and 0.1 mg/mL, respectively; lane 3 is the control.

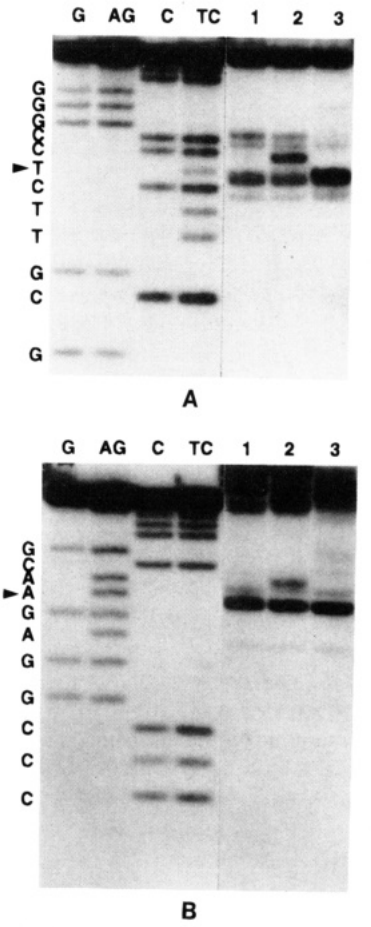
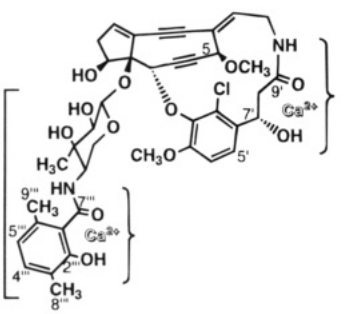


FIGURE 3: Autoradiograms of the reaction products of chromophore **2** with (A) the 5′-end-labeled 16-mer 5′-GGGCGT{TCTC}CGGGCC annealed with the nonlabeled complementary strand and (B) the 5′-end-labeled complementary strand 5′-GGCCCG{GAGA}ACG-CCC annealed with the nonlabeled 16-mer represented in (A). Lane 1 is **2** at 1 mg/mL; lanes 2 and 3 represent the reaction products from lane 1 treated with hydrazine and putrescine, respectively.

in the presence of $CaCl_2$ in order to locate the chelation sites on the chromophore.

NMR Spectroscopy Studies in the Presence of Calcium Chloride. NMR studies showed that, upon addition of increasing amounts of $CaCl_2$ to **2**, protons in two regions of the molecule were affected (Figure 4 and **5**).



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At low concentrations (beginning at 10 mM) of $CaCl_2$, two protons (H-5′ at 7.25 ppm and H-8′ at 2.71 ppm) were shifted downfield, suggesting the localization of a Ca^{2+} ion in the region of C-9′ and C-7′, i.e., in the β-hydroxy amide portion of the atoms that bridge the core rings. In addition, with increasing concentrations of $CaCl_2$, the protons at 6.97, 6.58, 2.19, and 2.10 ppm (H-4‴, H-5‴, and two aromatic methyls 9‴ and 8‴, respectively) were broadened, consistent with Ca^{2+} ion chelation at the 2‴ phenol and 7‴ carbonyl sites, i.e., in the region of the terminal *o*-hydroxybenzamide (Figure 4 and **5**).

MDP Reaction with Calf Thymus Histones. Reaction of MDP with total calf thymus histones caused the formation of low molecular weight peptides. Incubation of the individual calf thymus histones (H1, H2A, H2B, H3, H4) with MDP at 50 μg/mL showed that H1 is the most susceptible to proteolysis (Figure 5). Increasing the concentration of MDP resulted in cleavage of the other histones in the following order: H1 ≫ H2B, H3, H2A > H4. A concentration-dependence study demonstrated that 50% of H1 was consumed at an MDP concentration of 10 μg/mL. A time course experiment with histone H1 revealed the formation of low molecular weight bands within 3 h of incubation. The effect of chromophore alone on histones was not examined due to our limited supply of **2**. Unlike KDC, addition of the protease inhibitors leupeptin, antipain, aprotinin, AEBSF, and DFP, at the concentrations described above, had no effect on the MDP proteolytic activity (results not shown).

DISCUSSION

Experiments with supercoiled ΦX174 DNA show that MDP and **2** cause double- and single-strand breaks as evidenced by the formation of linear and open-circular forms

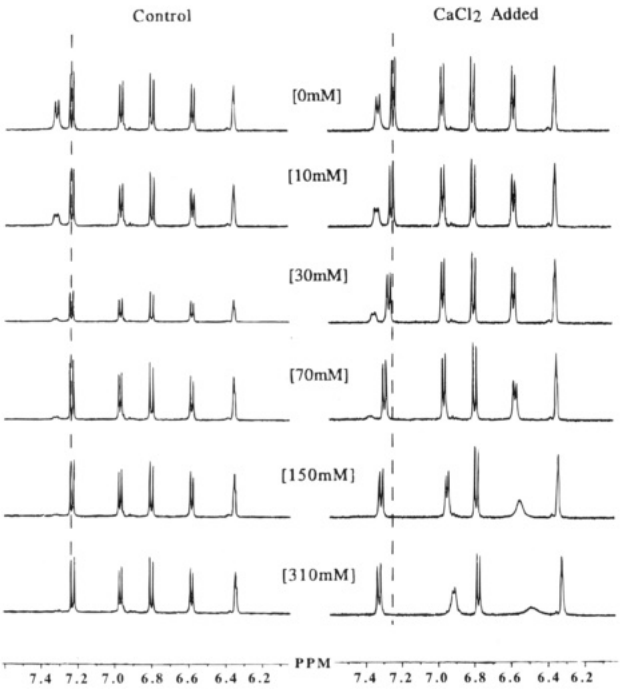

FIGURE 4: Downfield ^1H NMR spectra of **2** with increasing amounts of ^2H_2O (control) or $CaCl_2$ in ^2H_2O. Spectra for the upfield region are not shown.

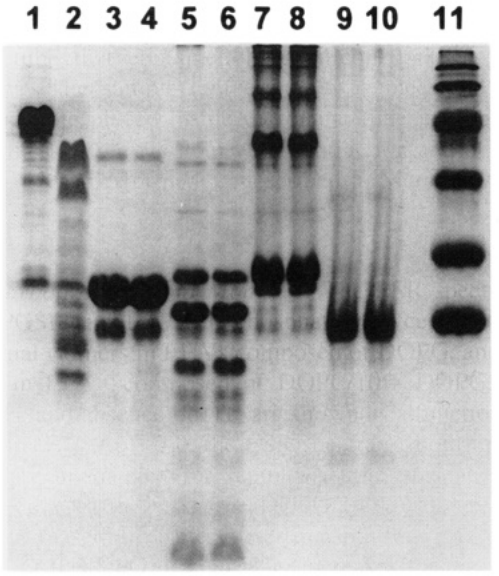

FIGURE 5: A 17% SDS−polyacrylamide gel of the reaction of maduropeptin with calf thymus histones. Reaction conditions: 50 mM Tris-HCl pH 7.5, 1 mg/mL individual histones, 50 μg/mL MDP, in a total volume of 10 μL, 37 °C overnight. The control reactions were carried out under identical conditions except that MDP was replaced by pure water. Lanes 1, 3, 5, 7, and 9 are control reactions with H1, H2A, H2B, H3, and H4, respectively; lanes 2, 4, 6, 8, and 10 are reactions of MDP with H1, H2A, H2B, H3, and H4, respectively, lane 11 is the molecular weight protein standards from 200 to 14.3 kDa.

of DNA. **2** is 100-fold less potent than MDP, consistent with the nature of the poor-leaving group in **2**, i.e., OCH_3 at C-5. Optimal cutting was observed in basic conditions (pH 8), suggesting a base-assisted deprotonation of the amide NH and consistent with the proposed mechanism of action of **2** (Scheme 1). At physiological conditions, binding site catalysis may be necessary to assist in the formation of **1**, and this might only happen at selected binding sites, accounting in part for the diminished potency of **2** as compared to **1**. Single-strand ΦX174 was unaffected by both compounds, indicating their affinity for the geometry of duplex DNA, as has been observed for all enediyne chromophores thus far studied.

Cleavage studies on various labeled restriction fragments indicate that both MDP and **2** cleave DNA in a sequence−dependent fashion and that they share the same DNA site selectivity. These observations are compatible with Scheme 1, where we propose that, for both holoantibiotic and methanol adduct **2**, the same highly reactive enediyne species **3** is responsible for DNA cutting. The primary cleavage sites observed in our studies, 5′-TC*T*T/3′-*A*GAA, 5′-TC*T*C/3′-*A*GAG, and 5′-TTTT/3′-AAAA, suggest that MDP and **2** exhibit a preference toward polypyrimidine/polypurine regions of the DNA. Similar observations were reported for calicheamicin γ_1^1, kedarcidin, and C-1027 (Zein et al., 1988; Walker et al., 1992; Xu et al., 1994). It is likely that these regions are flexible areas of the DNA that can accommodate the chromophores, as opposed to the more "rigid polyC/polyG" tracts, which are not cleaved by any of the above-mentioned compounds (Walker et al., 1993).

In the case of the above-mentioned primary sites, the DNA breaks observed on both strands are of similar intensities and occur with a two-nucleotide 3′-stagger. Given the fact that the cleavage experiments are run under single-hit kinetics, our results suggest that the DNA damage at those sites could result from bistranded lesions. Preliminary evidence on the cleavage chemistry at those sites was obtained by comparing the electrophoretic mobilities of the cleaved 5′- and 3′-end-labeled restriction fragments with those of the M−G markers. On the 3′-end-labeled DNA, cleavage resulted for the most part in fragments that migrated identically to the M−G markers, indicating oligonucleotides terminating with 5′-phosphates. However, on the 5′-end-labeled DNA, the cleaved fragments migrated similarly or slightly faster than the M−G markers. These observations are reminiscent of bleomycin and calicheamicin γ_1^1 chemistry where 4′-H abstraction has been shown to generate in part 3′-phosphoglycolate and 5′-hydroxyl termini at the site of bond cleavage. Hydrogen abstraction at this position could either lead to strand scission or to the generation of abasic sites (Stubbe & Kozarich, 1987; Kozarich et al., 1989). Further evidence for 4′-H abstraction was obtained from studies using the two complementary 16-mers containing the 5′-TC*T*C/3′-*A*GAG tetramer. For both oligomers, putrescine treatment caused an increase in 3′-phosphate-ended fragments, consistent with the presence of an alkali-labile abasic site. In addition, hydrazine treatment resulted in DNA oligomers that migrate slower than the M−G standard (i.e., than the phosphate-ended fragments). As shown with bleomycin (Sugiyama et al., 1988), such oligomers end with 3′-phosphopyridazine residues. These residues were shown to arise from the reaction of hydrazine with a product of 4′-H chemistry, i.e., the 4′-hydroxylated abasic site.

Even though preliminary, our results suggest that, in the case of the observed primary cleavage sites, MDP and **2** cause DNA breaks by abstracting hydrogen from the C-4′ site on the deoxyribose sugars.

The nature (DS versus SS), the chemistry (position of the hydrogen atom on the targeted deoxyribose sugar), and the magnitude of cleavage at the secondary sites were found to be contingent upon the DNA sequence beyond the immediate neighboring DNA bases. Such is the case of 5′-NTTT, 5′-

TTAT, 5′-TCCT, 5′-ATCT, 5′-TCAT, 5′-TACT, 5′-TCAC, and their complementary sequences. These observations clearly indicate the importance of the DNA microstructure at the cleavage sites and suggest that certain stretches of DNA are more "accommodating" than others. Conclusive evidence as to the chromophore cleavage chemistry awaits more detailed studies such as those described for calicheamicin (Zein et al., 1989a,b; De Voss et al., 1990), bleomycin (Rabow et al., 1990a,b) and neocarzinostatin (Goldberg & Kappen, 1994).

That preincubation with netropsin, a known minor groove binder, alters the chromophore cleavage pattern suggests that the chromophore acts in the minor groove of DNA. As shown for other enediynes, the results obtained under anaerobic conditions and in the presence of excess superoxide dismutase and catalase along with the specificity of the cleavage argue for a mechanism involving a nondiffusible, carbon-centered radical **3**, as depicted in Scheme 1. It is interesting to note that the nine-membered enediyne chromophores MDP, NCS, and C-1027 cleave DS DNA with a two-nucleotide stagger. In contrast, the DS cuts in the case of the 10-membered enediynes, calicheamicin and esperamicin, occur with a three-nucleotide stagger.

The inhibition of DNA cleavage in the presence of $MgCl_2$ and $CaCl_2$ and the NMR studies on **2** in the presence of $CaCl_2$ provided clues regarding the activation of the chromophore and gave insight as to the chromophore regions important in DNA binding. The NMR results suggest the chelation of a calcium cation in the β-hydroxy amide portion of the atoms that bridge the core rings. Such chelation would decrease the nucleophilicity of the amide nitrogen in **2**, thus hindering aziridine formation and the concurrent loss of methanol via the intramolecular ring contraction step to form parent enediyne **1** (Scheme 1) and ultimately the DNA-damaging intermediate **3** as well. Under these conditions, DNA cleavage cannot occur. The NMR data also show the localization of Ca^{2+} near the terminus of the sugar side chain. This observation suggests the involvement of the *o*-hydroxybenzamide moiety in DNA binding since in the chelated form the chromophore cannot associate with DNA and thus this may be the reason DNA cleavage does not occur upon addition of $CaCl_2$. As with kedarcidin, these siderophore-like chelation sites (Telford et al., 1994) obviously play a role in the interaction of the chromophore with DNA and add to the unique chemical features of these chromophores.

As was shown for KDC and NCS, MDP exhibits proteolytic activity. Although we have not yet determined the requirements for the activity or specificity, MDP is also selective in the proteins it cleaves *in vitro*. Among the proteins tested, histone H1 is a favored substrate. Even though preliminary, our results suggest that the nature of the MDP proteolytic activity is different from that of KDC and NCS. Also, the cleavage sites on histone H1 are unique to each of the three chromoproteins mentioned (Zein et al., 1995).

In conclusion, we have shown that MDP is one of a growing class of enediyne-containing chromoproteins that exhibit multiple functions: a selective DNA-cleaving chromophore and a protease-like apoprotein that stabilizes and solubilizes the chromophore. The potent cytotoxicity of enediyne chromophores suggests that the preferred DNA scission sites are biologically critical DNA sequences. In addition, even though MDP apoprotein shows no homology to the KDC and NCS class of proteins [Hanada et al. (1991); Hans Marquardt, personal communication], our data strengthen the hypothesis that the protein component of the neocarzinostatin, kedarcidin, and now maduropeptin may provide a "targeted delivery" of the highly cytotoxic chromophores to the chromatin (Zein et al., 1993b). This "two-pronged" attack could explain the high potency of these natural products.

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