New Insights into Sequence Recognition Process of Esperamicin A_1 and Calicheamicin γ_1^I: Origin of Their Selectivities and "Induced Fit" Mechanism†

Motonari Uesugi and Yukio Sugiura*

Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan

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ABSTRACT: This study addresses the DNA sequence recognition event of the enediyne antibiotics esperamicin A_1 and calicheamicin γ_1^I by the use of synthetic DNA oligomers, salt effects, and circular dichroism studies. The results reported here provide several important insights: (1) esperamicin A_1 requires a purine/pyrimidine trimer in host DNA for favorable interaction, (2) the sequence selectivity of esperamicin C is an origin of esperamicin A_1 and calicheamicin γ_1^I selectivities, (3) in the target recognition by esperamicin C, its total structure and hydrophobicity are important, and (4) the binding of hydrophobic esperamicin to DNA duplex induces dehydration and conformational change of the host DNA. The specific sequence recognition process of esperamicin/calicheamicin has been discussed.

Enediyne antitumor antibiotics show novel molecular structures, a fascinating action mode, and potent DNA cleaving activity (Nicolaou & Dai, 1991; Sugiura et al., 1990, 1991). Their discovery sparks a great deal of excitement in the research areas of chemistry, biology, and medicine. Esperamicins fall within this class of antibiotics. The structure of esperamicin A_1 consists of four subunits: a highly strained bicyclo[7.3.1]tridecadiyne ring system, an allylic trisulfide, deoxyfucose–anthranilate ring, and a trisaccharide ring (Golik et al., 1987a,b; Figure 1). The antibiotic causes a sequence-dependent DNA cleavage upon triggering with a thiol compound (Long et al., 1989; Sugiura et al., 1989), via a 1,4-benzene diradical produced in the enediyne sector (Zein et al., 1989b; DeVoss et al., 1990a; Lockhart et al., 1981). The cleavage preferences are particularly observed for pyrimidines in short oligopyrimidine tracts and for 3′-shifted nucleotides in the opposite oligopurine tracts. The 3′-staggered asymmetric cleavage pattern suggests double-strand scission across the minor groove of the DNA helix, and this is consistent with reported inhibition of the DNA cleaving efficiency of esperamicin A_1 by netropsin (Sugiura et al., 1989).

Calicheamicins are also a subclass of enediyne antibiotics (Lee et al., 1987a,b: Figure 1). Even though their similarities to esperamicins in structure are quite striking, calicheamicin γ_1^I exhibits somewhat different selectivity and is believed to be more sequence-specific than esperamicin A_1 (Zein et al., 1988). The cleavage patterns by calicheamicin γ_1^I are typical for double-strand scission across the minor groove, in which the third 5′ nucleotide of a pyrimidine-rich tetramer and a 3′ nucleotide shifted by three positions in the opposite purine-rich strand are attacked. Furthermore, it was shown that the majority of the cleavage within the TCCT/AGGA recognition sequence is initiated by hydrogen abstraction from the C-5′ position and hydrogen transfer to the C-4 position of the cycloaromatized calicheamicin γ_1^I molecule (DeVoss et al., 1990b).

Several efforts have been made to understand how esperamicin A_1 and calicheamicin γ_1^I recognize specific sites of duplex DNA. Zein et al. (1989a) demonstrated that the terminal rhamnose sugar and the ethylamino sugar on calicheamicin γ_1^I can be removed without affecting cleavage specificity. Our laboratory previously reported that removal of the deoxyfucose–anthranilate moiety from esperamicin A_1 (esperamicin C), or even further removal of the thiomethylhexapyranose moiety (esperamicin D), did not affect significantly the sequence selectivity observed in the parent compound, although esperamicin D lowered the DNA cleavage efficiency (Sugiura et al., 1989). Walker et al. (1992) investigated the DNA-cleaving properties of calicheamicin T, which is almost identical to esperamicin D, and they found that calicheamicin T shows no significant site selectivity. The conflicting results in the esperamicin D and calicheamicin T experiments make it difficult to understand the sequence recognition events of esperamicin A_1 and calicheamicin γ_1^I. Recently, footprinting experiments with synthetic oligosaccharide domain mimics were carried out to clarify whether the carbohydrate domain, independent of the aglycon, binds to a DNA duplex substrate (Aiyar et al., 1992; Nicolaou et al., 1992b). The results revealed that some, but not all, of the binding specificity of calicheamicin/esperamicin resides in its carbohydrate domain.

This study compares DNA cleavage patterns between several esperamicin derivatives and calicheamicin by employing DNA oligomer substrates. The DNA oligomers were constructed in a systematic fashion as shown in Figure 2. The report is the first time that such a direct comparison has been made. In addition, circular dichroism studies showed that the complexing action of esperamicin is accompanied by reorganization and dehydration of the host DNA duplex. This study provides new insights into the sequence recognition process of the esperamicin/calicheamicin antibiotics.

MATERIALS AND METHODS

Materials. Esperamicins A_1, C, and D were a kind gift from Dr. T. W. Doyle (Bristol-Myers Squibb), and calicheamicin γ_1^I was generously provided by Dr. G. A. Ellestad (Lederle Laboratories). Plasmid pBR322 DNA was isolated from *Escherichia coli* C600. Bacterial alkaline phosphatase, *E. coli* DNA polymerase I large fragment, polynucleotide kinase, and restriction enzymes were purchased from Takara Shuzo (Kyoto, Japan). Distilled water was purified through

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* Author to whom correspondence should be addressed.

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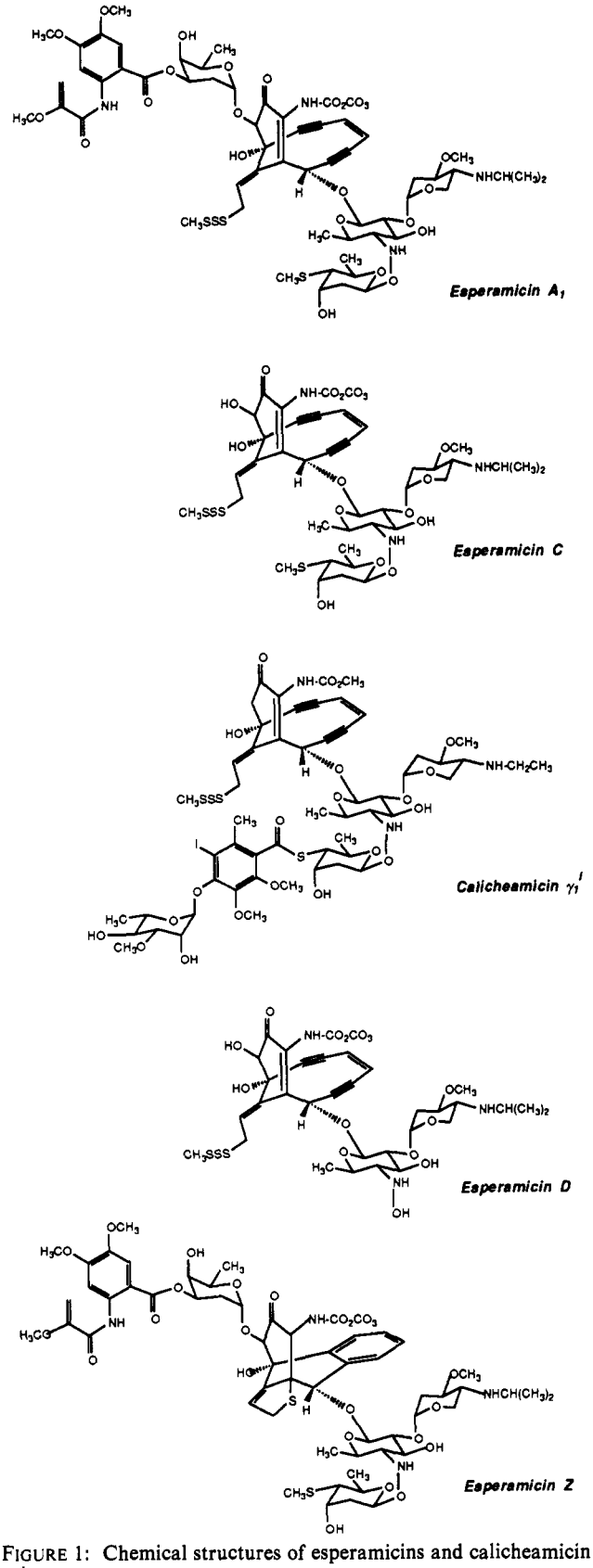


FIGURE 1: Chemical structures of esperamicins and calicheamicin γ_1^I.

a Sybron Nanopure II System. All other chemicals used were of commercial reagent grade.

Preparation and Labeling of DNA Oligomer. Self-complementary oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer and purified by HPLC. Each 5'-end was labeled with polynucleotide kinase and [γ-^{32}P]-ATP. The labeled DNA was purified on a denaturing 15% polyacrylamide gel.

(I) 5'- CAGGACGCGTCCT

(II) 5'- CCGGACGCGTCCG

(III) 5'- CAAAACGCGTTTT

FIGURE 2: Sequences and numbering of the DNA oligomers used in this study.

Drug Reaction. A standard reaction sample contained 0.2 μM esperamicin A_1, 2 μM esperamicin C, 40 μM esperamicin D, or 0.2 μM calicheamicin γ_1^I with 1 mM dithiothreitol and 3 pmol of the end-labeled DNA in a total volume of 20 μL buffered to pH 7.5 with 20 mM Tris-HCl. The cleavage reaction was allowed to proceed at 4 °C for 30 min. To stop the reaction, ice-cold ethanol was added to the sample.

Analysis on a High-Resolution DNA Sequencing Gel. Each lyophilized sample was dissolved in 5 μL of formamide containing 0.01% bromophenol blue and loaded into a 15% polyacrylamide gel containing 7 M urea in TBE buffer (89 mM Tris–borate and 2 mM Na_2EDTA, pH 8). Electrophoresis was performed at 2000 V for 2 h. DNA sequencing was carried out by the Maxam–Gilbert method (Maxam & Gilbert, 1980). The autoradiograms were then scanned with a laser densitometer (LKB Model 2222 Ultro-Scan XL).

Assay for DNA Cutting Efficiency. A reaction sample contained 0.8 μg of pBR322 plasmid DNA and esperamicin C (0.2 μM) with 0.1 M NaCl, 20 mM Tris-HCl (pH 7.5), and various concentrations of inorganic salts. NaCl (0.1 M) was used to minimize the ion-exchange effect of DNA (Collins & Washabaugh, 1986). The cleavage reaction was initiated by addition of dithiothreitol (1 mM) and allowed to proceed at 20 °C for 5 min. After ethanol precipitation, each DNA sample was dissolved in 20 μL of loading buffer containing 0.05% bromophenol blue and 10% glycerol and then heated at 65 °C for 1 min. Electrophoresis was performed by using a 1% agarose gel containing ethidium bromide (0.5 μg/mL) in TBE buffer. The gels were photographed with Polaroid 665 film. The resulting forms of the plasmid were quantitated by measuring the intensities of the DNA bands on the negative films.

Measurements of Circular Dichroism Spectra. Circular dichroism spectra were obtained by a Jasco J-720 spectropolarimeter. Titrations were carried out at 15 °C by incrementally adding 4 mM esperamicin Z solution to 1 mL of 10% methanolic solution containing 0.1 M NaCl, 20 mM Tris-HCl (pH 7.5), and 2.9 μM DNA oligomer I. Samples were placed in a thermally jacketed quartz cuvette. Each spectrum was averaged over 10 accumulations and corrected for buffer and drug baseline.

RESULTS

Cleavage of Oligomer I Containing a 5'-AGGA/TCCT Tract. Oligomer I, which contains a consensus binding sequence of calicheamicin γ_1^I, TCCT/ACCA, was labeled with ^{32}P at the 5'-terminus and subjected to DNA cleavage reactions with esperamicins or calicheamicin γ_1^I. Figure 3 shows the direct comparison of their DNA cleavage patterns. The damaged sites are clearly different from one another. The calicheamicin γ_1^I cleavage feature confirms the results already reported by the Lederle group (Zein et al., 1989) with another oligomer. Calicheamicin γ_1^I (lane 5) cleaved oligomer I at G-7 and C-11, which are shifted from each position to

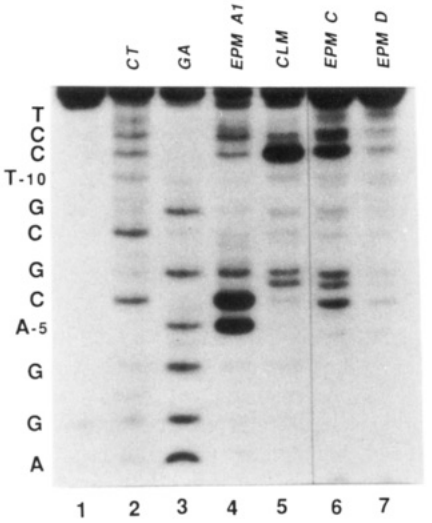


FIGURE 3: Comparison of DNA cleavage by esperamicins and calicheamicin γ_1^I. The 5′-labeled oligomer I was incubated with esperamicin A_1 (EPM A1, lane 4), calicheamicin γ_1^I (CLM, lane 5), esperamicin C (EPM C, lane 6), or esperamicin D (EPM D, lane 7), and subjected to 15% gel electrophoresis. Lane 1 shows intact DNA alone. Lanes 2 and 3 are the Maxam–Gilbert sequencing ladders for C + T and G + A.

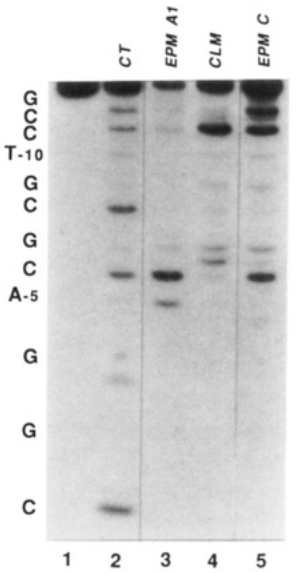


FIGURE 4: Comparison of DNA cleavage by esperamicins and calicheamicin γ_1^I. The 5′-labeled oligomer II was incubated with esperamicin A_1 (EPM A1, lane 3), calicheamicin γ_1^I (CLM, lane 4), or esperamicin C (EPM C, lane 5), and subjected to 15% gel electrophoresis. Lane 1 shows intact DNA alone. Lane 2 is the Maxam–Gilbert sequencing ladders for C + T.

the 3′-direction. The 3′-shifted asymmetric pattern indicates its double-strand scission across the minor groove of double-helical DNA, because in the minor groove the proximal deoxyriboses on opposite strands are shifted to the 3′-side (Dervan, 1986). The doublet band at G-7 suggests that two kinds of product are formed at the position. The slower migrating band of the doublet exhibited electrophoretic mobility identical to that of the Maxam–Gilbert marker, indicating 3′-phosphate termini. The faster moving band is presumed to bear 3′-phosphoglycolate termini, because the 3′-phosphoglycolate product induced by Fe(II)–elsamicin A (Uesugi et al., 1991; Uesugi & Sugiura, 1992) gave the identically migrating band (data not shown). The product is well-known as one derived from 4′-hydrogen abstraction in the deoxyribose backbone (Stubbe & Kozarich, 1987). Very recently, Hangeland et al. (1992) reported deuterium transfer experiments which unequivocally show that the 4′-hydrogen atom of the targeted nucleotide on the AGGA-containing strand is abstracted by calicheamicin.

Esperamicin A_1 (lane 4) gave two strong bands at A-5 and C-6 situated in 3′-flanking positions of purine trimers. Only faint bands were also detected at C-11 and C-12 in the opposite strand. The cleavage pattern of esperamicin C (lane 6), a truncated derivative lacking the deoxyfucose–anthranilate moiety from esperamicin A_1, was close to a fusion of calicheamicin γ_1^I and esperamicin A_1 patterns.

Sequence Selectivity of Esperamicin D. Esperamicin D lacks both the deoxyfucose–anthranilate and thiomethyl-hexapyranose moieties from esperamicin A_1. Lane 7 of Figure 3 shows a DNA cleavage pattern of esperamicin D when oligomer I was used as a substrate. Although minor enhancements occurred at almost the same position as observed in the esperamicin C lane, esperamicin D appears to be far less specific than esperamicin C. Indeed, DNA affinity of esperamicin D is much weaker than (about $^1/_{50}$) that of esperamicin C (Long et al., 1989).

Cleavage of Oligomer II and a Longer DNA Fragment. In oligomer II, A-2 and T-13 of oligomer I were replaced by C-2 and G-13 to form a single target of tri(purine/pyrimidine) tract, 5′-GGA/CCT (Figure 2). Esperamicin A_1 (lane 3 of Figure 4) cleaved the oligomer specifically at the C-6 nucleotide 3′-adjacent to the 5′-GGA box. Calicheamicin γ_1^I (lane 4) recognized the 5′-CGGA/GCCT tract and caused the 3′-shifted double-strand scission at the expected sites. In the cleavage patterns, esperamicin C (lane 5) was evidently a fusion of calicheamicin γ_1^I and esperamicin A_1.

These cleavage features were confirmed by the experiment in which a 190-bp DNA fragment containing the same target sequence (*Bam*HI/*Sph*I fragment from pBR322 DNA) was employed as a substrate (data not shown). Similarly, the single damaged site in the esperamicin A_1 reaction, the 3′-shifted double-strand cutting pattern in calicheamicin γ_1^I cleavage, and the combined breakage pattern by esperamicin C were detected. Therefore, these cutting features are not peculiar to the short DNA oligomer II.

Cleavage of Oligomer III. To test whether GC base pairs in oligo(purine)·oligo(pyrimidine) tract affects the recognition properties of the drugs, we constructed an oligomer with a 5′-AAAA/TTTT tract (Figure 2) and examined the cleavage patterns by these enediyne compounds. Figure 5 reveals that esperamicin A_1 (lane 2), calicheamicin γ_1^I (lane 3), and esperamicin C (lane 4) recognized the AAAA/TTTT tract and cleaved the oligomer at positions similar to those in the oligomer I experiments, even though the intensity patterns were different, especially in the T-12 cleavage by calicheamicin. The result indicates that the replacement of GC base pairs by AT base pairs in the target site has no significant effect on target recognition of these drugs, although it affects their cleavage intensities. This fact is surprising because AAAA/TTTT sequences have significantly different minor-groove width and local conformations from GC-containing sequences (Nelson et al., 1987; Travers, 1989).

Effects of Salts on DNA Cutting/Binding Efficiency of Esperamicin C. Ding and Ellestad (1991) reported the hydrophobic contribution to the calicheamicin/DNA association on the basis of the effect of various inorganic salts on the cleavage rate of DNA. We tried a similar approach for esperamicin C. As shown in Figure 6, its DNA cleavage rate was affected by different salt species in the same rank order as the Hofmeister series (Collins & Washabaugh, 1985). The rank decreased in the order $CH_3COO^- > Br^- > ClO_4^-$ for

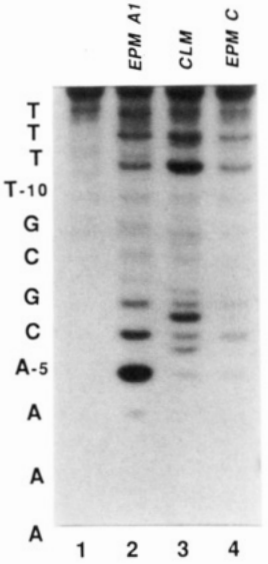


FIGURE 5: Comparison of DNA cleavage by esperamicins and calicheamicin γ_1^I. The 5′-labeled oligomer III was incubated with esperamicin A_1 (EPM A1, lane 2), calicheamicin γ_1^I (CLM, lane 3), or esperamicin C (EPM C, lane 4), and subjected to 15% gel electrophoresis. Lane 1 shows intact DNA alone.

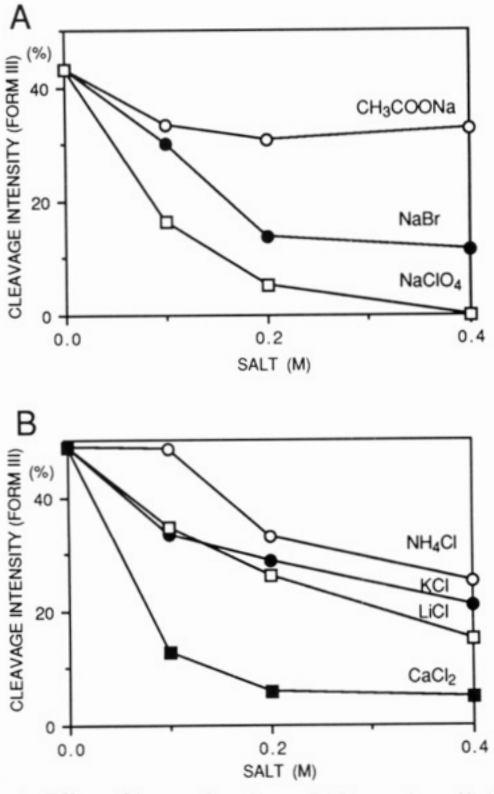
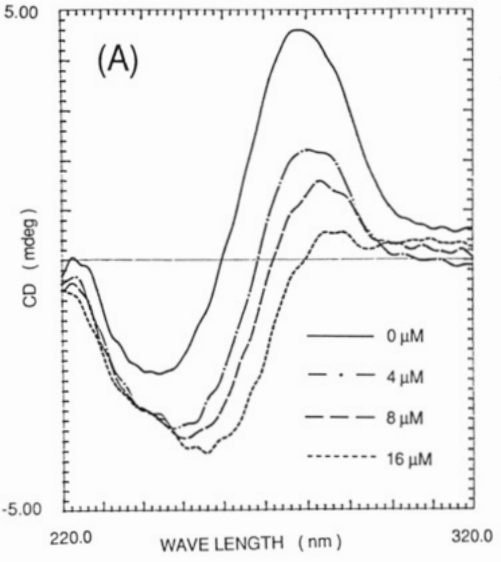


FIGURE 6: Effect of inorganic salts on DNA cutting efficiency of esperamicin C. Plasmid pBR322 was damaged by esperamicin C with increasing concentrations of salts (0–0.4 M). The reaction conditions and quantitation are described under Materials and Methods. (A) Comparison among anions. (B) Comparison among cations.

anions and $NH_4^+ > K^+ > Li^+ > Ca^{2+}$ for cations. These results are considered as evidence for hydrophobic interactions (Dill, 1990), because lower rank (less hydrated) salts tend to shield the lipophilic surfaces of the drug and the groove walls of DNA and then inhibit hydrophobic binding of the drug to host DNA.

Circular Dichroism Studies of DNA–Esperamicin Z Complex. Circular dichroism (CD) is one of the most sensitive techniques for monitoring conformational changes of DNA. Figure 7A illustrates typical CD spectra for native oligomer I and for its complexes with aromatized esperamicin Z. The long-wavelength spectrum of oligomer I in 10% methanol solution is characteristic of B-form DNA (Saenger, 1984). The esperamicin Z-induced negative CD intensities and red shifts indicate an obvious change of DNA conformation brought by the association with the drug. These CD changes can be explained in light of the hydration state of DNA helix, since the greatly decreased positive band around 275 nm implies a structural change to a less hydrated DNA duplex (Bokma et al., 1987; Girod et al., 1973). Similar spectral changes of the DNA duplex are induced by concentrated salt solutions (Hanlon et al., 1975; Wolf & Hanlon, 1975; Ivanov et al., 1973), or observed in mixtures of water and organic solvents such as alcohol (Girod et al., 1973; Bokma et al., 1987) and ethylene glycol (Green & Mahler, 1971). Indeed, oligomer I in 50% ethanolic and 80% glycolic solutions exhibited a CD spectrum very close to that of 8 μM esperamicin



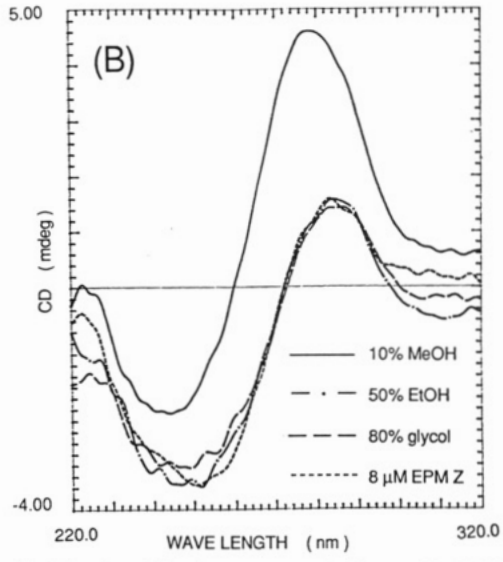


FIGURE 7: Circular dichroism spectra of oligomer I. (A) Various amounts of esperamicin Z were added to a 10% methanolic solution (1 mL) containing 2.9 μM oligomer I, 20 mM Tris-HCl (pH 7.5), and 0.1 M NaCl. (B) Circular dichroism spectrum of oligomer I (2.9 μM) in 50% ethanolic solution, in 10% methanolic solution, in 80% ethylene glycolic solution, and in 10% methanolic solution with 8 μM esperamicin Z. Each sample contained 20 mM Tris-HCl (pH 7.5) and 0.1 M NaCl.

Z solution (Figure 7B). The observation indicates that binding of hydrophobic esperamicin Z into the minor groove of the DNA duplex displaces hydrating water molecules and induces a conformational change of the host DNA duplex.

DISCUSSION

Esperamicin A_1 Requires a Purine/Pyrimidine Trimer for Favorable Interaction. We previously reported that esperamicin A_1 preferentially attacks at nucleotides near an oligo(purine)·oligo(pyrimidine) tract (Sugiura et al., 1989). It was obscure, however, how long a tract is needed for efficient DNA damage. Figure 3 reveals that esperamicin A_1 cleaves the nucleotide 3′-adjacent to purine trimers, 5′-AGGN and 5′-GGAN. This observation allows us to propose that a purine/pyrimidine trimer is important for DNA/esperamicin A_1 association. Cleavage data from oligomer II and longer DNA fragments, containing a single target of three base pairs (5′-GGA/TCC), support this interpretation. The results with oligomer III are also consistent with the proposal.

The Selectivities of Esperamicin A_1 and Calicheamicin γ_1^I Are Based on That of Esperamicin C. Esperamicin C, which lacks a deoxyfucose–anthranilate moiety from esperamicin A_1, exhibits mixed sequence specificity of esperamicin A_1 and calicheamicin γ_1^I cleavages. This observation indicates that the sequence selectivity of esperamicin C is an origin for esperamicin A_1 and calicheamicin γ_1^I specificities, even though deoxyfucose–anthranilate of esperamicin A_1 and methoxylated mannose–thiobenzoate of calicheamicin γ_1^I play a role as well in modifying the fundamental specificity of esperamicin C to create the higher selectivities. We must determine the key recognition process of esperamicin C before fully understanding the homo(purine/pyrimidine) discrimination of the esperamicin/calicheamicin antibiotics.

Total Structure and Hydrophobicity of Esperamicin C Participate in Its Recognition Event. Since esperamicin D has low DNA affinity and little sequence selectivity in contrast to esperamicin C, the thiomethylhexapyranose moiety is important for the DNA binding and sequence recognition event. However, it is unlikely that the single sugar moiety is solely responsible for determining the affinity and selectivity, because the single sugar is too small to bind tightly within the minor groove of the DNA helix and to discriminate specific recognition sequences. Very recently, Aiyar et al. (1992) showed that the trisaccharide domain of esperamicin C inhibited calicheamicin γ_1^I-mediated cleavage only to a very small extent. Therefore, the total structure of esperamicin C (the trisaccharide plus the aglycon) appears to be significant.

We also presented here the hydrophobic contribution to the DNA/esperamicin C association. The DNA cleavage rates of esperamicin C were affected by different salt species in the same rank order as the Hofmeister series. These salt effects are generally considered as evidence for hydrophobic interactions (Dill, 1990). Taken together, the hydrophobicity of the total esperamicin C structure takes part in its DNA binding and recognizing event. It is wrong to assume that the sequence recognition involves contacts made only by the enediyne unit or by the carbohydrate side chain. It is due to a set of hydrophobic contacts made my the total structure of esperamicin C.

"Induced Fit" Process Explains Sequence Selectivity of Esperamicin/Calicheamicin. How do the esperamicin/calicheamicin antibiotics recognize the specific sites? It is unlikely that the antibiotics strictly recognize DNA minor-groove width, because they discriminate both AT-rich and GC-containing oligo(purine)·oligo(pyrimidine) tracts (Figures 3 and 5; Walker et al., 1992). X-ray crystallographic data and hydroxyl radical footprinting experiments indicate that DNA sequences including GC base pairs have significantly different minor-groove width and local conformations from DNA sequences containing sequential AT base pair (McNamera & Harrington, 1991; Yoon et al., 1988; Nelson et al., 1987; Drew et al., 1981; Drew & Dickerson, 1981; Coll et al., 1987; Burkhoff & Tullius, 1987, 1988).

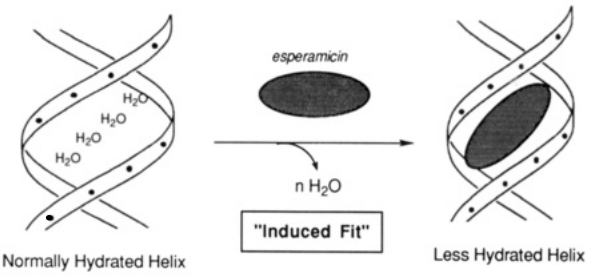


FIGURE 8: Schematic representation for the DNA binding process of esperamicin.

Our experiments demonstrate an "induced fit" mechanism in the recognition event of the enediyne drugs. The CD titration experiments of oligomer I with aromatized esperamicin Z show a reorganization of host DNA brought by the association with the drug. The binding of hydrophobic esperamicin reduces the degree of hydration in the minor groove and then induces a conformational change in the host DNA duplex (Figure 8). The conformational change appears to be a decrease in the number of base pairs per turn since intensity of the 275-nm CD band of DNA can be related directly to the change in the number of base pairs per turn (Baase & Johnson, 1979; Bokma et al., 1987). The reorganization of the host DNA prvides a minor-groove surface that is complementary to the drug molecule. In other words, the geometry of the dehydrated minor groove is important for the target recognition of esperamicin/calicheamicin rather than that of normally hydrated minor groove. This idea can reasonably explain the recognition of both AT-rich and GC-containing oligo(purine)·oligo(pyrimidine) tract, because in sequential AT regions a spine of water molecules running down the floor of the minor groove stabilized their particular conformation (Nelson et al., 1987; Privé et al., 1987, 1991).

A common feature of oligo(purine/pyrimidine) structures is purine–purine stacking in the oligo(purine) strand. The crystal structure of a homopolymeric run of six AT base pairs shows that purine–purine stacking is a very significant force in stabilizing homo(purine/pyrimidine) structure (Nelson et al., 1987). This feature seems to be compatible with GC-containing poly(purine/pyrimidine) structure (Zimmerman & Pheiffer, 1981; Drew & Travers, 1984). The purine–purine stacking geometry would correlate with the target recognition process. There is a possibility that the stacking helps the drug to displace hydrating water molecules and therefore homo(purine/pyrimidine) sites are more flexible for an induced fit by the drug.

Finally, this study provides important insights into sequence recognition process of the esperamicin/calicheamicin antibiotics. Many synthetic works have been inspired by their novel molecular architecture and fascinating mode of action (Nicolaou et al., 1992a; Nicolaou & Dai, 1991). An understanding of the DNA recognition process of the natural enediyne compounds will allow further developments in the area of drug design of the synthetic enediynes. The second-generation enediynes may find future application in cancer chemotherapy and biotechnology.

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