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Nucleotide-specific cleavage and minor-groove interaction of DNA with esperamicin antitumor antibiotics

(DNA–drug interaction/*Eco*RI site/distamycin)

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ABSTRACT **The cleavage of DNA by esperamicin is greatly accelerated in the presence of thiol compounds. Oxygen and active oxygen-radical scavengers have no significant influence upon DNA strand breakage by esperamicin. The preferential cutting sites of esperamicin are at thymidylate residues, and the frequency of bases attacked (T > C > A > G) is different from that of calicheamicin (C >> T > A = G), neocarzinostatin (T > A > C > G), or bleomycin (C > T > A > G). Esperamicin preferentially attacks at T and C bases in oligopyrimidine sequences such as 5′-CTC-3′, 5′-TTC-3′, and 5′-TTT-3′. In contrast to the preferred sites of cleavage by bleomycin, 5′-GT-3′ and 5′-GC-3′, the preferred sites of esperamicin-mediated DNA degradation are 5′-TG-3′ and 5′-CG-3′ sequences. The nucleotide-specific cleavage mode of esperamicin is significantly affected by pretreatment of DNA with netropsin and distamycin A, suggesting that interaction of esperamicin occurs through the minor groove of B-DNA. This is further supported by the asymmetric cleavage pattern to the 3′ side on the opposite strand of the DNA. The roles of the fucose–anthranilate moiety and the trisaccharide side chain of esperamicin in DNA binding and base recognition are discussed.**

Esperamicin and calicheamicin are members of a class of potent antitumor antibiotics produced by cultures of *Actinomadura verrucosospora* (1) and *Micromonospora echinospora* (2). The heart of these antibiotic molecules is a bicyclo[7.3.1]tridecane system that combines a methyl trusulfide and double and triple bonds in a 3-ene-1,5-diyne relationship. The unique structures of the antibiotics appear to contribute to their extreme potency as antitumor agents. Esperamicin preferentially inhibits DNA synthesis (3) in the mouse leukemia cell line L1210. Of special interest in this regard is a proposal that the esperamicin/calicheamicin antibiotics may belong to a new class of DNA-damaging agents (1–5).

It is well known that bleomycin attacks DNA deoxyribose through the intermediacy of a reactive, reduced form of oxygen and cleaves preferentially at guanine–pyrimidine (5′ → 3′) sequences, in particular GC sites (6, 7). Recently, a minor-groove interaction model of metallobleomycin in the DNA helix was developed by computer-constructed model building (8). In contrast, neocarzinostatin is itself converted into a diradical that directly attacks the deoxyribose of mainly thymidylate residues in DNA (9–14). Herein, we report that esperamicin clearly exhibits sequence-specific DNA breakage and minor-groove interaction. The present results were also compared with those for bleomycin and neocarzinostatin. The chemical structures of several esperamicin analogs used in this study are given in Fig. 1.

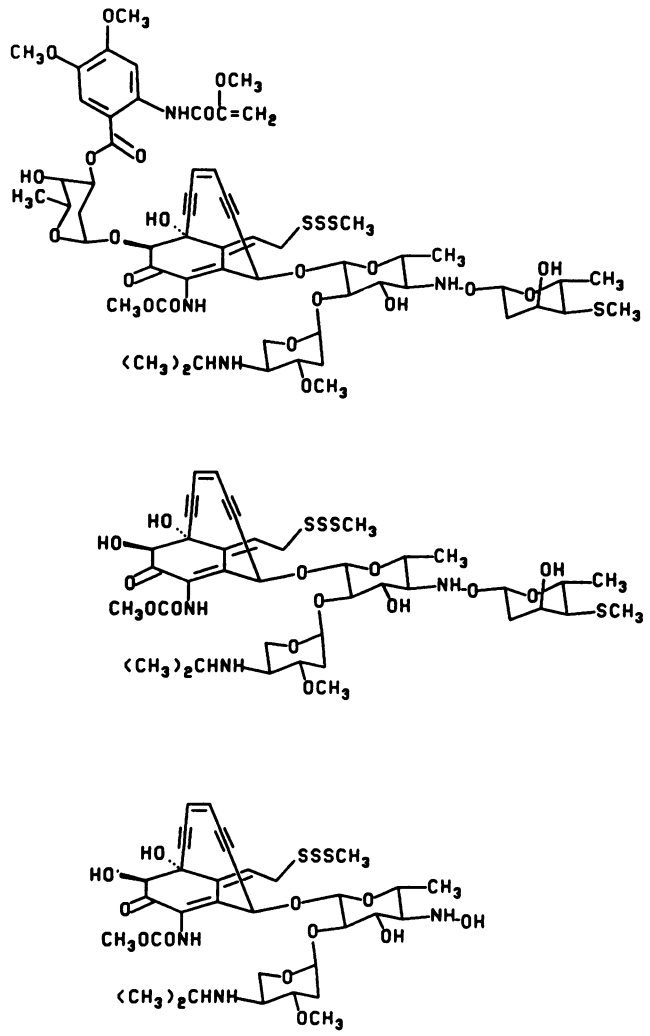

FIG. 1. Chemical structures of esperamicin A_1 (*Top*), esperamicin C (*Middle*), and esperamicin D (*Bottom*).

MATERIALS AND METHODS

Drugs and Chemicals. Esperamicins A_1, C, and D, distamycin A, and the *Acc* I–*Acc* II DNA fragment of plasmid pUC19 were gifts of Bristol-Myers, F. Arcamone (Farmitalia), and T. Komano (Kyoto University, Kyoto, Japan), respectively.

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*Eco*RI restriction endonuclease was obtained from Takara Shuzo (Kyoto, Japan). Netropsin and actinomycin D were purchased from Sigma. All other chemicals used were of commercial reagent grade.

DNA Cleavage Reaction. The reaction samples (total volume, 20 μl) contained 0.6 μg of pBR322 plasmid DNA, 1 mM deferoxamine, 10 mM EDTA (adjusted to pH 8.0), and 10 mM Tris/HCl buffer (pH 7.5). Deferoxamine and EDTA were included to avoid the influence of contaminating metal ions such as iron. The cleavage reaction was initiated by addition of dithiothreitol (0.1 mM), and the samples were incubated at 37°C. The reactions were stopped by addition of cold ethanol (70 μl) and 3 mM sodium acetate, and then the samples were immediately chilled at −70°C in a dry ice/ethanol bath. Each lyophilized sample was dissolved in 25 μl of loading buffer containing 0.05% bromophenol blue and 10% glycerol and heated at 60°C for 1 min before electrophoresis. Electrophoresis was performed using 1% agarose gel containing ethidium bromide (0.5 μg/ml).

Nucleotide Sequence Analysis. The reaction samples (total volume, 20 μl) contained the 5′-end-labeled 322-base pair DNA fragment (pUC19 *Acc* I–*Acc* II), sonicated calf thymus DNA (225 μM base), 1 mM deferoxamine, 10 mM EDTA, and 10 mM Tris/HCl buffer (pH 7.5). The nucleotide sequence cleavages were initiated by addition of esperamicin (50 μM) and diothiothreitol (0.5 mM), and then the samples were incubated at 37°C for 15 min. Cold ethanol was added to the sample solutions in order to stop the reaction. Electrophoresis was performed in a 10% polyacrylamide/7 M urea slab gel at 1500 V for 4 hr. DNA sequencing was carried out by the Maxam–Gilbert method (15).

RESULTS

DNA Cleavage by Esperamicin A_1. Fig. 2 shows typical gel electrophoretic patterns for esperamicin-mediated strand scission of covalently closed, supercoiled (form I) pBR322 DNA. In the presence of dithiothreitol, esperamicin markedly stimulated DNA breakage to form open-circular (form II) and linear (form III) DNAs even at the reaction time of 1 min. At 10–120 min, extensive fragmentation of DNA was clearly observed (Fig. 2, lanes 5–8). Fig. 3 displays the effect of reducing agents on the DNA cleavage by esperamicin A_1. Sodium borohydride, NADPH, and L-ascorbic acid had no significant effect on the DNA cleavage reaction. Only the sulfur-containing reducing agents had an effect, clearly accelerating the cleavage process.

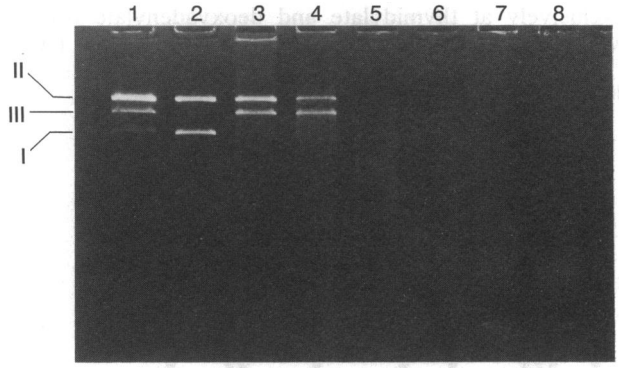

FIG. 2. Agarose gel electrophoretic patterns of pBR322 DNA after treatment with esperamicin A_1 (10 μM) in the absence (lane 1) or presence (lanes 3–8) of dithiothreitol (0.1 mM). The reaction samples were incubated at 37°C for 1 min (lane 3), 5 min (lane 4), 10 min (lane 5), 30 min (lane 6), 60 min (lane 7), or 120 min (lanes 1 and 8). Lane 2 shows intact DNA alone. Positions of form I (covalently closed, supercoiled), form II (circular, not supercoiled), and form III (linear) DNAs.

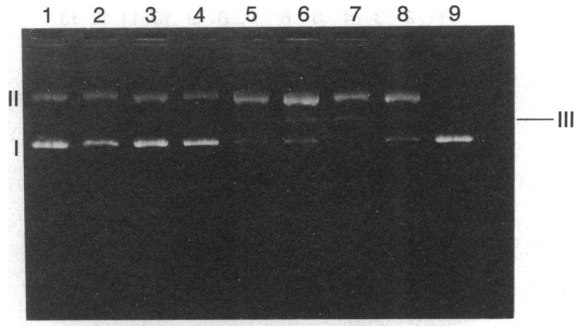

FIG. 3. Agarose gel electrophoretic patterns of pBR322 DNA after treatment with esperamicin A_1 (10 μM) in the presence of various reducing agents at 37°C for 10 min. The samples contained the following reductants (0.1 mM): lane 1, none; lane 2, sodium borohydride; lane 3, NADPH; lane 4, L-ascorbic acid; lane 5, sodium dithionite; lane 6, glutathione; lane 7, L-cysteine; and lane 8, dithiothreitol. Lane 9 shows intact DNA alone.

Effects of Oxygen and Radical Scavengers. Deaeration was achieved by purging the sample Tunberg cuvette with argon gas and then evacuating for 5 min. Fig. 4 shows that oxygen had a negligible effect on the primary DNA strand breakage by esperamicin A_1. Under the same experimental conditions, the DNA cleavage activity of the peplomycin–iron complex was markedly inhibited by deaeration (lanes 6 and 7). It is well known that bleomycin antibiotics require iron and oxygen as cofactors for strong DNA cutting (16). Further, we checked the effect of some active oxygen-radical scavengers. The breakage of DNA by esperamicin was not affected appreciably by addition of superoxide dismutase (0.1 mg/ml, 300 units/ml), catalase (10 μg/ml, 450 units/ml), mannitol (1 mM), KI (1 mM), 1,4-diazabicyclo[2.2.2]octane (1 mM), or Tiron (1,2-dihydroxy-3,5-benzenedisulfonate, 1 mM). These results strongly indicate that oxygen and active oxygen-radical species do not participate in DNA strand scission by esperamicin antibiotics.

Nucleotide Sequence-Specific Cleavage. Fig. 5 presents a typical autoradiographic result showing breakage of a 5′-end-labeled 322-base-pair DNA fragment by esperamicin A_1 in the presence of dithiothreitol. Esperamicin preferentially attacked at pyrimidine bases, in particular thymine (lanes 4 and 11). Preincubation of the DNA fragment with netropsin (lanes 5 and 6), distamycin A (lanes 7 and 8), or actinomycin D (lanes 9 and 10) gave major alterations in the pattern of DNA cleavage by esperamicin. Netropsin and distamycin A clearly inhibited cutting at 5′-TTTT-3′ and 5′-AAAA-3′ se-

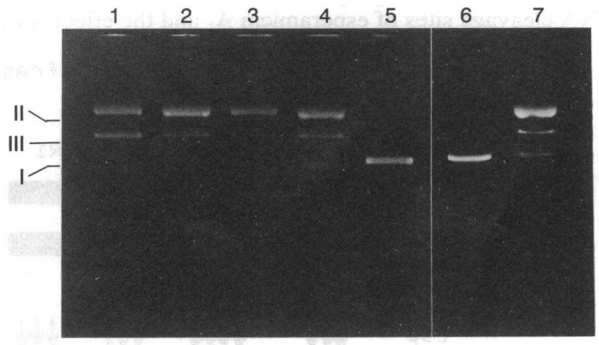

FIG. 4. Agarose gel electrophoretic patterns of pBR322 DNA after treatment with esperamicin A_1 at 20°C for 5 min under anaerobic (lanes 1 and 3) and aerobic (lanes 2 and 4) conditions. Lanes 1 and 2, treatment with esperamicin (10 μM) alone; lanes 3 and 4, treatment with esperamicin (1 μM) plus dithiothreitol (0.1 mM); lane 5, untreated DNA; lanes 6 and 7, treatment with peplomycin–iron complex (20 μM) at 0°C for 20 min under anaerobic (lane 6) and aerobic (lane 7) conditions.

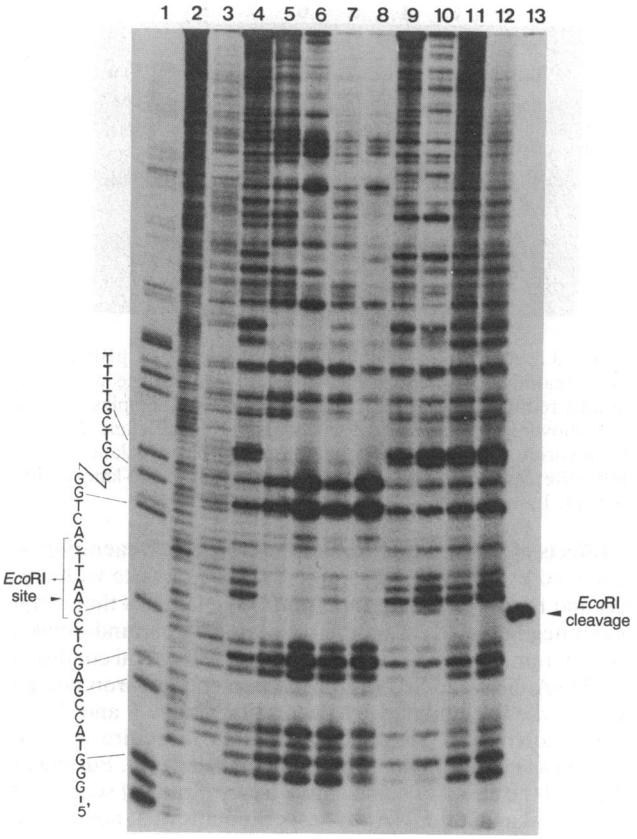

FIG. 5. Autoradiograms of strand scission by esperamicin A_1 for native DNA fragment (lanes 4 and 11) and for DNA fragment pretreated with netropsin (lanes 5 and 6), distamycin A (lanes 7 and 8), actinomycin D (lanes 9 and 10), or *Eco*RI (lane 12). After the preincubation with these drugs {drug/nucleotide ratios of 0.05 (lanes 5, 7, and 9) and 0.25 (lanes 6, 8, and 10)} at 37°C for 30 min, the DNA samples were cleaved by esperamicin (50 μM) in the presence of dithiothreitol (0.5 mM) at 37°C for 15 min. Lane 13, digestion of native DNA fragment with *Eco*RI (14 units). Lanes 1–3, products of Maxam–Gilbert sequencing reactions (G, A > C, and C + T, respectively).

quences. Actinomycin D protected certain 5′-TG-3′ and 5′-CG-3′ sites from cleavage. In contrast, preincubation of DNA with *Eco*RI (14 units) induced no changes in the mode of DNA cleavage by esperamicin (lane 12). The restriction enzyme cleaved the DNA fragment at the *Eco*RI site in the presence of 7 mM $MgCl_2$ (lane 13). Fig. 6 summarizes the DNA cleavage sites of esperamicin A_1 and the effects of the various drug pretreatments on the cleavage pattern. The cleavage data for the other strand (3′-^{32}P-labeled DNA fragment) are also added in Fig. 6.

DNA Cleavage by Various Esperamicins. Fig. 7 shows nucleotide sequence cleavages by esperamicins A_1, C, and D. In addition to esperamicin A_1, esperamicin C exhibited potent DNA breakage activity. In contrast, the activity of esperamicin D was much less. The nucleotide sequence-specific cleavage patterns of these three esperamicins are very similar. Esperamicins C and D also preferentially attacked at pyrimidine bases in the T- and C-rich regions. This result provides information on the role of various portions of the esperamicin molecule in DNA cleavage activity and base recognition.

DISCUSSION

Characteristics of DNA Cleavage by Esperamicin. In the presence of a sulfhydryl compound, the DNA-cutting activity of esperamicin A_1 increased remarkably. The reaction appears to be initiated by reduction of the trisulfide with thiol compounds. Indeed, such easy reduction of simple trisulfides or selenotrisulfides with thiols such as cysteine and glutathione has been reported (17, 18). The results under anaerobic conditions or in the presence of active oxygen-radical scavengers strongly suggest that DNA degradation by esperamicin is primarily independent of oxygen. In contrast, dioxygen is an essential cofactor in bleomycin-mediated DNA cleavage (16). It has been established that malondialdehyde detected during bleomycin-mediated DNA degradation is produced from a product containing a nucleic acid base and a 3-carbon fragment derived from the deoxyribose ring and that the DNA cleavage by bleomycin is due to oxidative attack at the C-4′ position of deoxyribose (6, 21). A preliminary assay using thiobarbituric acid revealed high production of a malondialdehyde-like compound during esperamicin-mediated DNA degradation, as well as during bleomycin-mediated cleavage (data not shown). During DNA degradation by the *o*-phenanthroline–Cu^+ complex, which has been proposed to attack at the C-1′ position of deoxyribose, the formation of thiobarbituric acid reaction products is remarkably low (22). Recent studies have postulated that neocarzinostatin (23) and calicheamicin (5) attack the C-5′ position of deoxyribose. Therefore, DNA strand breakage by esperamicin may be due to oxidative attack at the C-4′ and/or C-5′ position of deoxyribose. To establish the detailed mechanism of DNA degradation by esperamicin, further product analysis is needed.

Nucleotide Specificity of Esperamicin-Mediated DNA Degradation. DNA damage by neocarzinostatin occurs almost exclusively at thymidylate and deoxyadenylate residues, with cleavage occurring at all thymidylate residues (10, 11,

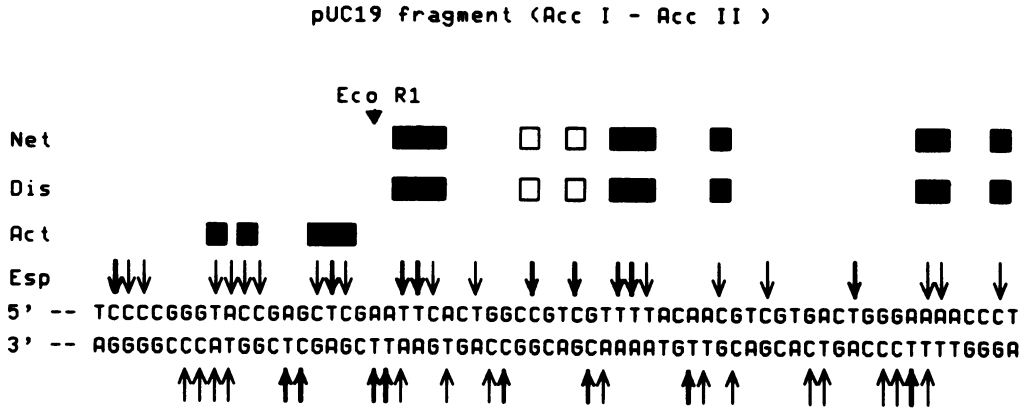

FIG. 6. DNA cleavage sites of esperamicin A_1 (Esp) and changes induced by pretreatment of DNA with netropsin (Net) distamycin A (Dis), or actinomycin D (Act). Arrows indicate the breakage sites; arrow thickness indicates the relative intensity of the band on the autoradiogram. Solid boxes represent inhibition of DNA cleavage; open boxes represent enhanced DNA cleavage. Solid triangle shows the *Eco*RI cleavage site.

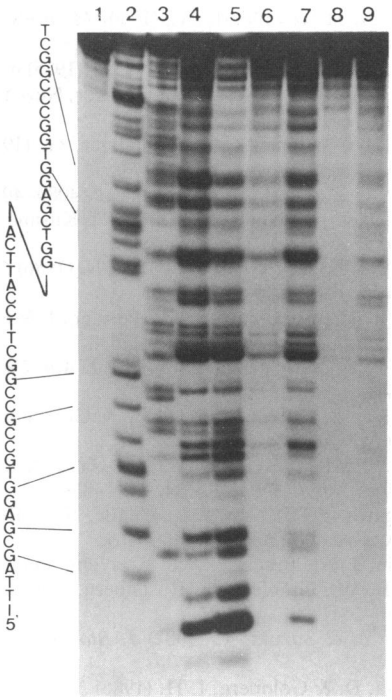

FIG. 7. Autoradiograms of DNA cleavage by esperamicin A_1 (lanes 4 and 5), esperamicin C (lanes 6 and 7), or esperamicin D (lanes 8 and 9). DNA samples were treated with esperamicin at 0.2 μM (lanes 4 and 6), 2 μM (lanes 5, 7, and 8), or 20 μM (lane 9) in the presence of dithiothreitol (10 mM) at 37°C for 30 min. Lane 1, untreated DNA. Lanes 2 and 3, products of Maxam–Gilbert sequencing reactions (G and C + T, respectively).

19, 20). Although the most preferred cutting site for esperamicin is also at thymidylate residues, the order of bases attacked (T > C > A > G) in the esperamicin reaction is clearly different from that (T > A > C > G) in the neocarzinostatin reaction. DNA cleavage by esperamicin is highly specific for pyrimidine bases. In addition, esperamicin preferentially attacks at T and C in oligopyrimidine regions such as 5′-CTC-3′, 5′-TTC-3′, and 5′-TTT-3′ and in 5′-TG-3′ and 5′-CG-3′ sequences. Neocarzinostatin has no clear specificity for neighboring nucleotides, although in certain instances thymidylate residues in 5′-TG-3′ sequences are favorable sites for strand scission (10). Calicheamicin γ_1 also preferentially attacks at C and T in oligopyrimidine regions such as 5′-TCC-3′ and 5-CTC-3′ (5). Similar specific cleavage at pyrimidine bases has been observed in the DNA strand scission by bleomycin (7). In the case of bleomycin antibiotics, the frequency of bases attacked is C > T > A > G and the preferred cutting sites are at 5′-GC-3′ and 5′-GT-3′ sequences.

Interaction of Esperamicin with the Minor Groove of DNA. As clearly shown in Fig. 5, nucleotide-specific cleavage by esperamicin A_1 is significantly affected by pretreatment of DNA with netropsin and distamycin A. These drugs are typical minor-groove binders and have A·T binding specificity (24). Indeed, among the original cutting sites of esperamicin, the A+T-rich regions (5′-AATTCA-3′, 5′-TTTTA-3′, and 5′-AAAA-3′) are strongly protected by netropsin and distamycin A. In contrast, these drugs enhance the cutting at two cytosines between 5′-AATTCA-3′ and 5′-TTTTA-3′ sequences, suggesting some changes of local DNA structure with the binding of netropsin or distamycin A. Certain cleavage sites such as 5′-GCTCG-3′ and 5′-CCG-3′ are also protected by the binding of actinomycin D, which is known to intercalate through C·G base pairs in the minor groove of DNA (25). *Eco*RI endonuclease specifically recognizes the double-stranded sequence 5′-GAATTC-3′ in the major groove of DNA (26). The enzyme cuts at the *Eco*RI site in the presence of Mg^{2+} (Fig. 5, lane 13). However, the binding of *Eco*RI gives no alterations in the cleavage pattern of esperamicin (Fig. 5, lane 12). These results indicate that an interaction of esperamicin occurs via the minor groove of B-DNA. This is further supported by the asymmetric cleavage pattern staggered by 3 base pairs to the 3′ side of DNA helix (Fig. 6) and suggests an orientation of the diyne–ene perpendicular to the plane of the base pairs in the helix. Pretreatment with distamycin A results in a large change in the sequence-specific DNA cleavage mode of bleomycin, which binds in the minor groove of B-DNA (8, 27). In the bleomycin–DNA interaction, the 2-amino group of guanine adjacent to the 5′ side of the cleaved pyrimidine is a key element of the specific 5′-GC-3′ or 5′-GT-3′ recognition (27). Neocarzinostatin has also been proposed to intercalate in the minor groove of B-DNA (28).

Structure–Activity Relationship of Esperamicins in DNA Cleavage. Esperamicin C lacks both the 2-deoxy-L-fucose and the aromatic ring moieties from esperamicin A_1, and esperamicin D is similar to esperamicin C but also lacks the thiomethylhexapyranose moiety. Esperamicin C showed high cleavage activity similar to that of esperamicin A_1, albeit at a 10-fold greater concentration. In addition, the sequence-specific breakage pattern of esperamicin C resembled very closely that of esperamicin A_1. This result suggests that the fucose–anthranilate moiety does not contribute signficantly to base recognition by the esperamicin antibiotics, although it may have some effect on binding. The strong antitumor activity of esperamicin A_1 (4) suggests that the fucose–anthranilate group may favor increased uptake of the antibiotic by cells. Esperamicin D showed almost the same sequence-specific cleavage mode, although its DNA cleavage activity was considerably lower than those of esperamicins A_1 and C. This result indicates that the trisaccharide side chain of esperamicin plays an important role in the binding of esperamicin to DNA, while the diyne–ene moiety appears to be the key functional group for sequence-specific cleavage by the esperamicin antibiotics. The mechanism by which esperamicin degrades DNA has been suggested to involve bioreductive cleavage of the allylic trisulfide and Michael addition of the resultant thiolate to the neighboring bridgehead olefin (4, 5, 29), followed by diyne–ene cyclization to give a phenylene diradical. Therefore, the first step of DNA breakage in the minor groove may be abstraction of the C-4′ or C-5′ proton from deoxyribose units of thymidylate and deoxycytidylate residues by the phenylene diradical. With regard to the question of C-4′ vs. C-5′ oxidation, precise analysis of the cleavage products of esperamicin will be necessary.

In conclusion, esperamicin A_1 shows potent DNA-cleavage activity in the presence of sulfhydryl-containing compounds such as dithiothreitol, glutathione, and cysteine. In the esperamicin-mediated DNA degradation, thymidylate and deoxycytidylate residues in oligopyrimidine regions (5′-CTC-3′, 5′-TTC-3′, and 5′-TTT-3′ sequences) are preferred cleavage sites. The frequency of bases attacked (T > C > A > G) is different from that of calicheamicin (C >> T > A = G), neocarzinostatin (T > A > C > G), or bleomycin (C > T > A > G). The binding of a typical A·T-specific minor-groove binder (netropsin or distamycin A) to DNA strongly inhibits esperamicin-mediated cleavage at 5′-TTC-3′, 5′-TTT-3′, and 5′-AAA-3′ sequences. On the other hand, the binding of *Eco*RI gives no changes in the sequence-specific cleavage mode of esperamicin. The results suggest that esperamicin interacts with the minor groove of B-DNA. In addition, the trisaccharide side chain appears to be important for binding of esperamicin to DNA.

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