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Biochemistry

DNA intercalation and cleavage of an antitumor antibiotic dynemicin that contains anthracycline and enediyne cores

(hybrid antibiotic/nucleotide specificity/minor groove/action mechanism)

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ABSTRACT **Dynemicin is a hybrid containing anthraquinone and enediyne cores, which contribute to binding and cleavage of DNA, respectively. DNA strand scission by the antitumor antibiotic is significantly enhanced by the addition of NADPH or thiol compounds. The preferential cutting site of dynemicin is on the 3′ side of purine bases (i.e., 5′-GC, -GT, and -AG) and is clearly different from the cutting sites of esperamicin and calicheamicin. The double-stranded and the stem regions of single-stranded DNAs are preferentially cleaved by dynemicin. Therefore, dynemicin may be a useful reagent for probing secondary structures of DNA. Pretreatment of DNA with Adriamycin and actinomycin D alters the cutting mode of dynemicin. Dynemicin-mediated DNA breakage is strongly inhibited by pretreatment of the DNA with distamycin A and anthramycin, suggesting that dynemicin interacts with the minor groove of the DNA helix. Intercalation of the anthraquinone core into the DNA followed by the attack of the phenyl diradical formed from the enediyne core is considered as a possible mechanism of action of dynemicin.**

Dynemicin, isolated from the fermentation broth of *Micromonospora chersina*, possesses potent cytotoxicity and *in vivo* antitumor activity (1). This antibiotic is a hybrid molecule of two typical chemotypes of antitumor agent, enediyne and anthraquinone (Fig. 1). Esperamicin, calicheamicin, and neocarzinostatin, which produce DNA strand breaks, belong to the family of enediyne antitumor antibiotics (2–5). DNA cleavage by esperamicin and calicheamicin appears to involve rearrangement of the enediyne unit, a phenylene diradical that can abstract hydrogen atoms from the sugar phosphate backbone of DNA. On the other hand, daunomycin, Adriamycin, and aclacinomycin belong to the class of anthracycline antitumor antibiotics and can intercalate into DNA through the planar aromatic rings (6, 7). Indeed, an x-ray diffraction study (8) demonstrated that two daunomycin molecules intercalate between the GC base pairs in the self-complementary hexanucleotide CGTACG. Evidently, dynemicin possesses these two structural features in one molecule and hence we were interested in clarifying the mechanism through which dynemicin cleaves DNA.

MATERIALS AND METHODS

Drugs and Chemicals. Dynemicin was isolated from the fermentation broth of *Micromonospora chersin* and purified as described (1). Esperamicin A_1 and anthramycin were a generous gift of T. W. Doyle (Bristol-Myers) and L. H. Hurley (University of Texas), respectively. Adriamycin and distamycin A were offered by F. Arcamone (Farmitalia). Plasmid pBR322 DNA was isolated from *Escherichia coli* C600, and restriction enzymes, such as *Eco*RI, *Sal* I, and *Dra* II, were obtained from Takara Shuzo (Kyoto, Japan). Stem-and-loop structure G4 DNA obtained from phage R199/G4ori replicative form DNA was a kind gift of T. Komano (Kyoto University, Japan). Ethidium bromide, actinomycin D, and NADPH were purchased from Sigma. All other chemicals used were of commercial reagent grade.

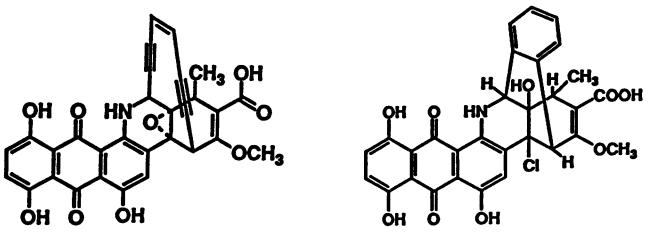

FIG. 1. Chemical structures of dynemicin (*Left*) and its aromatized derivative (*Right*). This aromatic compound was obtained by treating dynemicin with HCl and its structure was identified by NMR and x-ray crystallographic methods.

Assay for DNA Cleavage Activity. Analysis of drug (5 μM)-induced damage to supercoiled, covalently closed, circular (form I) pBR322 DNA was performed in the presence of NADPH, dithiothreitol, 4-hydroxythiophenol, or $NaBH_4$ (compound used was added at 5 μM–100 mM) for 30 min–24 hr at 37°C, followed by agarose gel electrophoresis to separate the various DNA products—namely, nicked relaxed circular DNA (form II) and linearized DNA (form III). DNA bands were visualized by using ethidium bromide binding and UV illumination.

Nucleotide Sequence Analysis. The reaction samples (total volume, 50 μl) contained a 5′-end-labeled 70-base-pair (bp) (*Eco*RI–*Dde* I) fragment or a 128-bp (*Sal* I–*Dra* II) pBR322 DNA fragment, sonicated calf thymus carrier DNA (20 μg/ml), and 10 mM Tris·HCl buffer (pH 7.5). Nucleotide sequence cleavage was initiated by addition of dynemicin (5–50 μM) and NADPH (5 mM) or dithiothreitol (5 mM), and then the samples were incubated at 37°C for 5 hr. Cleavage experiments for the complementary strand (3′-^{32}P-labeled DNA) were carried out by using the *Sal* I–*Dra* II pBR322 DNA fragment. Ice-cold ethanol was added to the samples to stop the reaction. After preincubation of the DNA fragment with distamycin A and some intercalators such as ethidium bromide and Adriamycin at 37°C for 30 min, the dynemicin-induced DNA cleavage was investigated and compared with the nucleotide sequence cleavage of intact DNA with dynemicin. DNAs modified with aflatoxin B_1 (9), *cis*-diammine-dichloroplatinum (10), and anthramycin (11) were prepared. Electrophoresis was performed on a 10% polyacrylamide/7 M urea slab gel, and DNA sequencing was carried out by the Maxam–Gilbert method (12). The autoradiograms were scanned with a laser densitometer (LKB model 2222 Ultro-Scan XL).

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RESULTS

Sequence-Specific DNA Cleavage by Dynemicin. Agarose gel electrophoretic results for dynemicin-mediated strand scission demonstrated that NADPH, 4-hydroxythiophenol, or dithiothreitol stimulated DNA cleavage by dynemicin. Whereas ascorbic acid, sodium dithionite, or sodium borohydride had only a weak effect on dynemicin-mediated DNA breakage. With 4-hydroxythiophenol or dithiothreitol, form I DNA was converted to form II and form III DNAs within 30 min. Although the cleavage reaction was slow in the NADPH system, extensive fragmentation of DNA was clearly observed after 5 hr. Cleavage data for both strands (DNA fragments ^{32}P-labeled at both ends) in the dynemicin–NADPH system are presented in Fig. 2. In this experiment, the *Sal* I–*Dra* II fragment (128 bp) was used as DNA substrate. As shown in the histogram (Fig. 3), (*i*) dynemicin preferentially attacks the 3′ side of purine bases such as 5′-AG, 5′-GC, and 5′-AT and (*ii*) among the four bases guanine is a relatively favorable cutting site for dynemicin. In addition, the drug appears to cause typical double-strand cuts at the sequence

3′-GATGATGACC

5′-CTACTACTGG.

Alteration of Dynemicin-Induced DNA Cleavage by Pretreatment of DNA with Groove Binders and Intercalators. Fig. 4 shows DNA fragments generated by dynemicin after pretreatment with ethidium bromide, proflavine, Adriamycin, actinomycin D, anthramycin, and distamycin A. These patterns were compared with the standard nucleotide-specific cleavage products for intact DNA in the dynemicin–NADPH system. Inhibition of the dynemicin-induced DNA cleavage by Adriamycin was considerably greater than by ethidium bromide or proflavine. Actinomycin D also masked some dynemicin cutting sites such as 5′-GC. In addition, as in lane 6, cleavage at certain nucleotides was appreciably enhanced

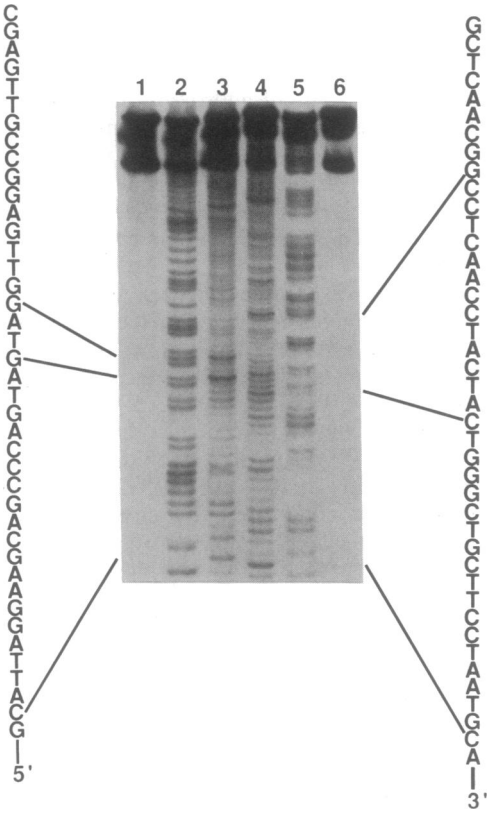

FIG. 2. Strand scission of 5′-end-labeled (lanes 1–3) and 3′-end-labeled (lanes 4–6) DNA sequences by dynemicin. Lanes 3 and 4 show DNA cleavage patterns by dynemicin (50 μM) and NADPH (5 mM) at 37°C for 5 hr. Lanes 1 and 6 show intact DNA. Lanes 2 and 5 indicate the Maxam–Gilbert sequencing reaction for G+A.

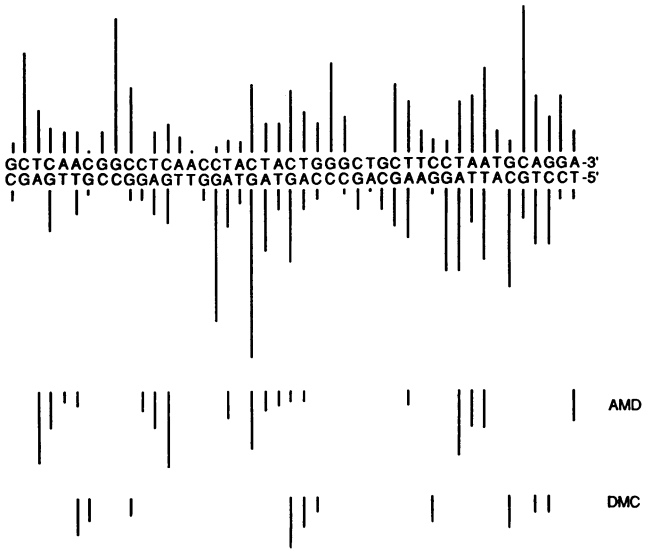

FIG. 3. Histograms of DNA-cutting sites by dynemicin for intact DNA and actinomycin D (AMD)- or distamycin A (DMC)-pretreated DNA. Relative DNA cleavage frequencies were obtained from densitometric scans of autoradiograms from gels.

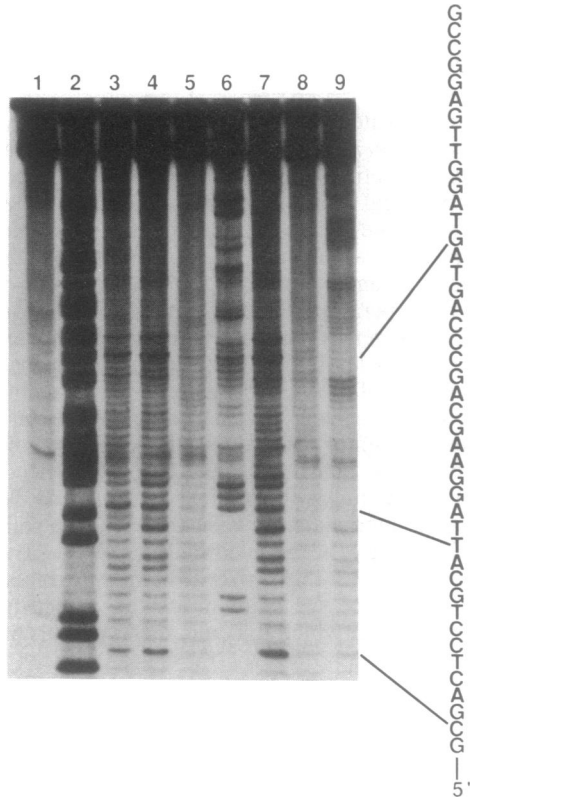

FIG. 4. DNA-cutting modes by dynemicin after pretreatment with ethidium bromide (lane 3), proflavine (lane 4), Adriamycin (lane 5), actinomycin D (lane 6), anthramycin (lane 8), or distamycin A (lane 9). After the pretreatment of the DNA fragment with these compounds (50 μM) at 37°C for 30 min, the DNA cleavage reactions were carried out by dynemicin (50 μM) and NADPH (5 mM) at 37°C for 5 hr. Lane 7 shows dynemicin-induced DNA cleavage for intact DNA. Lanes 1 and 2 show intact DNA and the Maxam–Gilbert sequencing reaction for G+A, respectively.

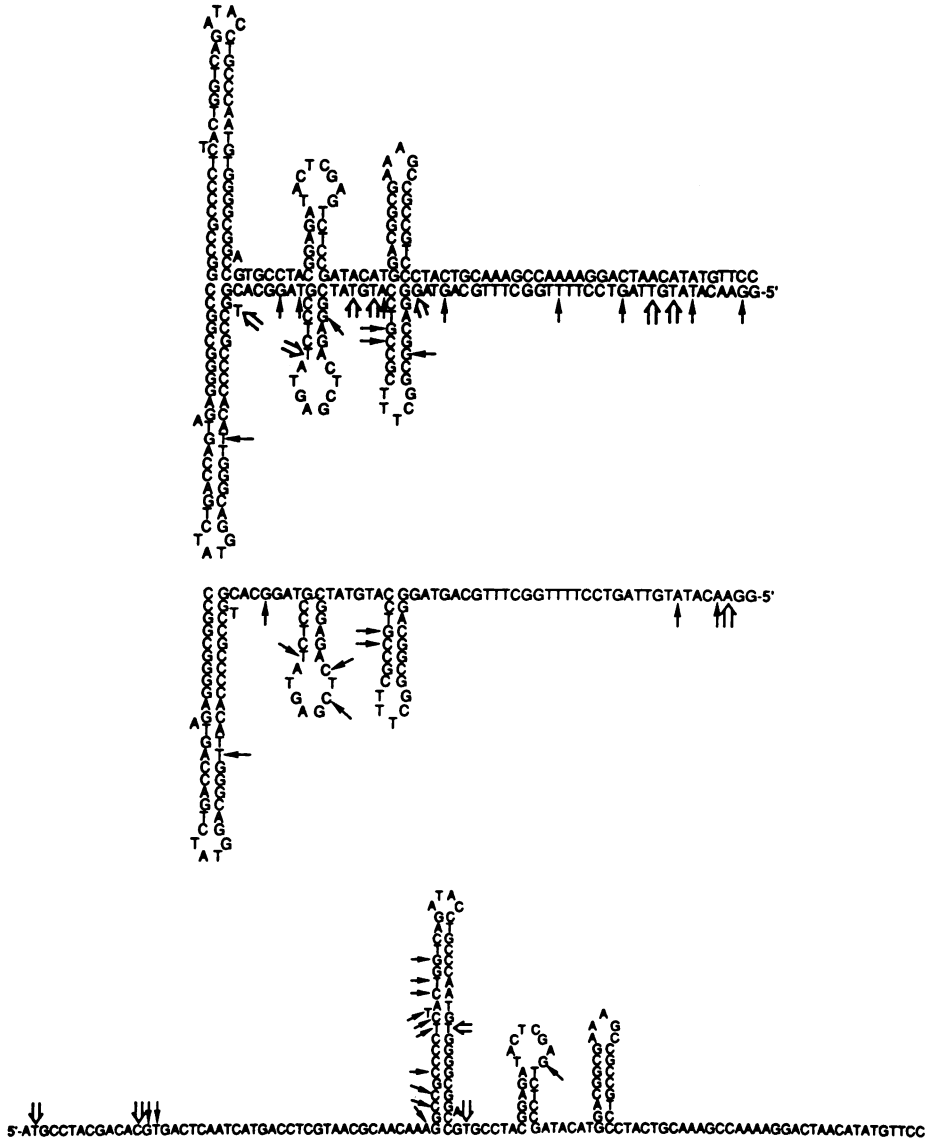

FIG. 5. Cleavage sites of G4 gene F/G space DNA fragments by dynemicin. Arrows indicate the cleavage sites and their thicknesses reflect the relative intensity of the band on the autoradiograms.

by pretreatment with actinomycin D. Preincubation of the DNA fragment with distamycin A, which is a typical minor groove binder, caused strong inhibition of DNA strand scission by dynemicin near A+T-rich regions. The effects of actinomycin D and distamycin A on the dynemicin-induced DNA cleavage are summarized in Fig. 3. In another DNA fragment (*Eco*RI–*Dde* I), the original cutting sites in A+T-rich regions, such as TTTATC-5′ and CTGTAA-5′, were also depressed by pretreatment with distamycin A, an AT-preferential minor groove binder. Furthermore, modification of the guanine 2-amino group with anthramycin inhibited DNA cleavage by dynemicin. Inhibition of anthramycin (lane 8) was approximately equivalent to that of Adriamycin (lane 5). In contrast, similar covalent attachment of aflatoxin B_1 to the N-7 of guanine gave no obvious inhibition or alteration of the dynemicin-induced DNA cleavage. Similarly, modification at the N-7 of guanine with *cis*-diamminedichloroplatinum also did not significantly affect DNA strand scission by dynemicin (data not shown). It should be noted that the 2-amino group and the N-7 atom of guanine are situated in the minor and major grooves of the DNA duplex, respectively.

Cleavage of Stem-and-Loop Structures in DNA by Dynemicin. To investigate the dynemicin cleavage of DNA that is able to form secondary and tertiary structures, we used single-stranded and double-stranded DNA substrates derived from the phage G4 origin of complementary-strand synthesis. Fig. 5 summarizes sequence-specific dynemicin cleavage of the single-stranded and double-stranded DNA substrates. Some of the preferred cutting sites in double-stranded DNA were clearly resistant to cleavage in single-stranded DNA. In single-stranded DNA, the preferred sites of dynemicin-induced DNA cleavage lay primarily on the inverted repeat sequences. Most of the strong cutting sites by dynemicin for these substrates were at the dinucleotide sequences 5′-AT and 5′-GT. A certain bulge thymine was also a preferred cleavage site in double-stranded DNA.

DISCUSSION

Dynemicin has potent DNA breakage activity in the presence of thiol compounds, as do esperamicin and calicheamicin (2–5). In addition, one characteristic of DNA cleavage mediated by dynemicin that is shared with esperamicin and calicheamicin is cleavage induction by NADPH. In analogy with other enediyne antibiotics such as esperamicin, dynemicin was irreversibly inactivated by preincubation with dithiothreitol or 4-hydroxythiophenol. Indeed, a dynemicin derivative (Fig. 1), which was prepared by treatment of

dynemicin with HCl causing aromatization of the enediyne structure, had no DNA cleavage activity under the experimental conditions used with dynemicin. This result strongly indicates that the enediyne chromophore significantly contributes to the present DNA strand scission. Presumably, a phenylene diradical produced by cyclization of the diyne-ene is the active form of dynemicin, similar to the mechanism proposed for compounds in this class of antitumor agents responsible for potent DNA breakage (2, 4, 13, 14). For dynemicin, opening the epoxide appears to be the key in activation to the diaryl radical form of dynemicin—namely, the DNA-cleaving intermediate. A more reasonable mechanism for the epoxide ring opening reaction requires the conversion of dynemicin by a one-electron reduction or two sequential one-electron reductions to the hydroquinone. The quinone methide formed can undergo nucleophilic attack by water or act as nucleophile and become protonated giving the hydroquinonediol or the quinone alcohol, respectively (15, 16). The compounds can then undergo the Bergman reaction and aromatize (Fig. 6). The blocking of DNA damage by high levels of thiols and the detection of free radical signals in a preliminary ESR study suggest a radical mechanism for the action of dynemicin. However, dynemicin-mediated strand scission frequently occurs at guanines and adenines. The bases adenine and guanine are particularly susceptible to alkylation that could result in cleavage at these sites without invoking the presence of radicals. Perhaps dynemicin takes advantage of both alkylation and hydrogen atom abstraction to effect DNA cleavage. In the presence of thiol cofactors, dynemicin at concentrations as low as 0.5 μM causes DNA cleavage in supercoiled pBR322 DNA. Calicheamicin and esperamicin appear to be more efficient at generating DNA strand breaks.

Although the DNA damage by dynemicin is not particularly base-specific, dynemicin does preferentially attack bases adjacent to the 3′ side of purines such as 5′-GC, 5′-GT, 5′-AT, and 5′-AG. Among the four bases, guanine is a favored cutting site for dynemicin. It is of interest that this base is the most resistant to cleavage by esperamicin (3), calicheamicin (4), and neocarzinostatin (5). The cleavage mode of dynemicin clearly differs from that of esperamicin (3) and calicheamicin (4), which show preferential cytosine and thymine cutting in oligopyrimidine regions. As shown in the autoradiograms (Figs. 2 and 4), the radioactive bands produced by dynemicin were found to be electrophoretically identical to the Maxam–Gilbert products (12), suggesting the presence of 3′-phosphates at the present DNA breaks. When the cleavage products were treated with base, the products coincidently migrated with the sequencing markers in this DNA fragment. It is also observed that dynemicin occasionally caused strong strand breaks 3 bp apart in the two DNA strands (for example, the 5′-CTACTACTGG and its complement 3′-GATGATGACC). This cleavage pattern is well-known for DNA damage by calicheamicin (4) and esperamicin (3). This cutting mode is characteristic of double-stranded DNA breakage. Furthermore, the asymmetric cleavage pattern on the 3′ side of opposite strands suggests interaction of dynemicin with the minor groove of the DNA helix (17). Indeed, the nucleotide cleavage pattern generated by dynemicin is significantly affected by pretreatment of the DNA with distamycin A and anthramycin, strongly supporting an interaction between the minor groove of DNA and dynemicin. Distamycin A is a well-known minor groove binder and anthramycin is a typical modifier of the guanine 2-amino group. In contrast, the covalent attachment of aflatoxin B_1 or *cis*-diamminedichloroplatinum to the guanine N-7 atom, which is situated in the major groove of DNA, does not generate changes in the dynemicin cleavage pattern. On the other hand, Adriamycin and actinomycin D strongly inhibited dynemicin-induced DNA cleavage. This suggests that these drugs disturb sites of dynemicin–DNA interaction by intercalating between purine bases. In the *Eco*RI–*Dde* I and *Sal* I–*Dra* II pBR322 DNA fragments, repeated DNase I cleaving inhibition analyses did not show a large number of distinct footprints for DNA complexed to dynemicin but did show certain weak footprints of approximately 2 bp, such as 5′-GC and 5′-AT. The sequence-preferred DNA binding sites of several anthraquinone–base intercalating drugs, including mitoxantrone, which were revealed by DNase I footprinting have been reported to be 5′-purine-pyrimidine sites, such as 5′-GC, -AC, and -AT (18). On the basis of the present results and a similar anthraquinone core in dynemicin, therefore, this drug is likely to bind to DNA by intercalating or stacking with at least one purine base.

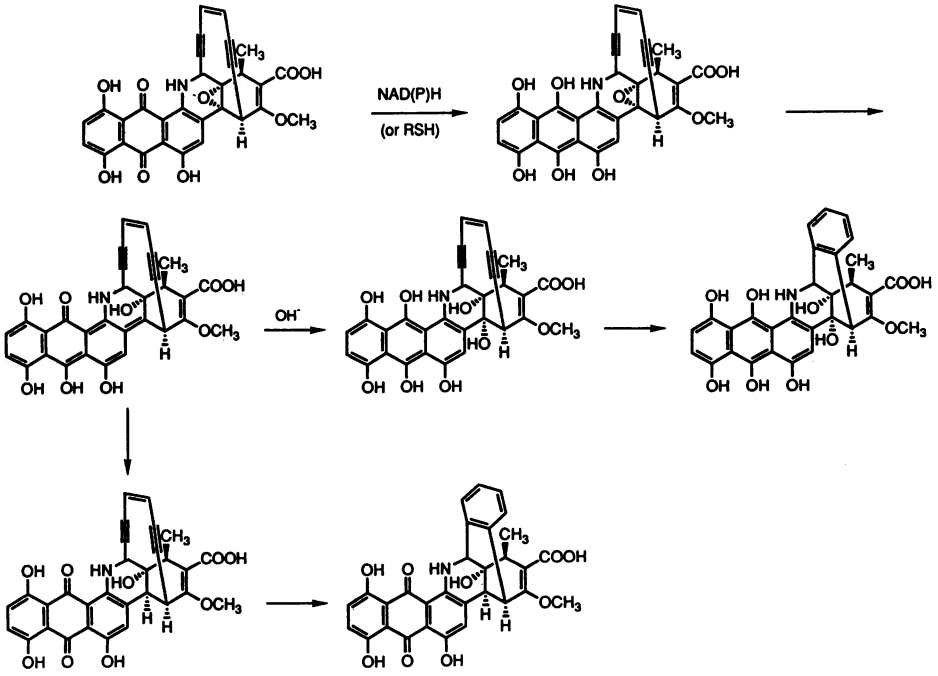

FIG. 6. Model for the mechanism of DNA breakage by dynemicin.

Fig. 5 shows possible secondary structures near the G4 origin of complementary-strand synthesis (19) and some interesting features of dynemicin cutting, although the secondary structure of DNA is not unequivocally clarified. Most of the preferred cleavage sites are in stem regions of the single-stranded DNA, indicating that dynemicin does not bind efficiently to single-stranded DNA. The loop regions in the double-stranded and single-stranded DNAs were also resistant to the dynemicin-induced cleavage, although the single-stranded DNA was cleaved at certain bases that lie outside the inverted repeat sequences. This suggests that the site preferred by dynemicin may form a stem-and-loop structure. Therefore, dynemicin appears to be a probe for stem-and-loop structures. It should be noted that dynemicin preferentially cleaved DNA at the predicted stem regions [inverted repeat DNA sequences that potentially form stem-and-loop structure have often been found at regulatory sites such as operator and transcription termination regions and DNA replication origin (20–22)].

In conclusion, dynemicin is a unique hybrid of an anthraquinone and a 1,5-diyn-3-ene that shows potent DNA cleavage activity in the presence of NADPH and dithiothreitol. Preferred cleavage sites in dynemicin-mediated DNA degradation are at bases adjacent to the 3′ side of purines such as 5′-GC, 5′-GT, 5′-AT, and 5′-AG, and the present nucleotide sequence cleavage mode clearly differs from those of other enediyne antibiotics, such as esperamicin and calicheamicin. The strong inhibition of the dynemicin-induced DNA cutting by distamycin A and anthramycin indicates a minor groove interaction of B-DNA with dynemicin. In addition, dynemicin appears to bind intercalatively to DNA through its anthraquinone core. Indeed, the double-stranded DNA and the stem regions of single-stranded DNA are good substrates for the dynemicin-induced DNA strand scission. The proposed mechanism of action most likely involves phenyl diradical formation similar to that involved in DNA strand cleavage by esperamicin, calicheamicin, and neocarzinostatin.

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