Interplay of Hydrogen Abstraction and Radical Repair in the Generation of Single- and Double-Strand DNA Damage by the Esperamicins

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Abstract: The source of hydrogens for the quenching of the phenylene diradical produced by esperamicin A (espA) and by esperamicin C (espC) in the presence of specifically deuteriated, double-stranded DNA (dsDNA) has been determined. Remarkably, both espA and espC were quenched at both positions of the diphenylene radical by exclusive abstraction of hydrogen from dsDNA. EspC, a predominantly ds-cleaver, afforded results consistent with 4′- and 5′-hydrogen transfer. EspA, a predominantly ss-cleaver, revealed no 4′-hydrogen transfer; however, results were consistent with 5′-hydrogen transfer and with the recently proposed 1′-hydrogen transfer (Yu, L.; Golik, J.; Harrison, R.; Dedon, P. *J. Am. Chem. Soc.* **1994**, *116*, 9733−9738). For espA, insufficient double strand DNA damage was produced to account for the role of DNA as the exclusive hydrogen source. In order to resolve this discrepancy, several reductants were used to activate espA and espC. The results indicated that a substantial portion of radical lesions produced in DNA by the esperamicins is subjected to repair by hydrogen transfer from the reductant. The efficiency of repair depended on the structural features of the reductant. The findings demonstrate that caution must be exercised when evaluating the propensity of DNA cleavers for ss- and ds-cleavage in the presence of reductants.

The esperamicins belong to the family of enediyne antitumor antibiotics.[1,2] Esperamicin A (espA; Figure 1) is one of the most potent antitumor agents having an IC_{50} of 300 pg/mL against the HCT116 human colon carcinoma cell line and an IC_{50} in the ng/mL range for many other cell lines.[3,4] EspA contains an unusual bicyclo [7.3.1] ring system with an allylic trisulfide attached to the bridging atom, a 1,5-diyne-3-ene within the 10-membered ring, and an α,β-unsaturated ketone in which the double bond is located at the bridge head of the bicyclic system.[5−7] In addition, espA contains two carbohydrate groups at C-4 and C-12. The C-4 substituent is an unusual 2-deoxy-L-fucosyl (methoxyacrylyl)anthranilate group. In mild acid, the glycosidic linkage to the fucosyl anthranilate is cleaved, producing esperamicin C (espC; Figure 1). The C-12 substituent is a hydroxylamine-linked trisaccharide containing thio- and amino-sugars. This substituent is structurally similar to the C-8 trisaccharide in calicheamicin γ1, a related enediyne antibiotic.[8] Thus, espC and calicheamicin are close structural analogs with respect to the enediyne core and to monosubstitution with the trisaccharide. EspA can be viewed, therefore, as an additionally glycosylated enediyne.

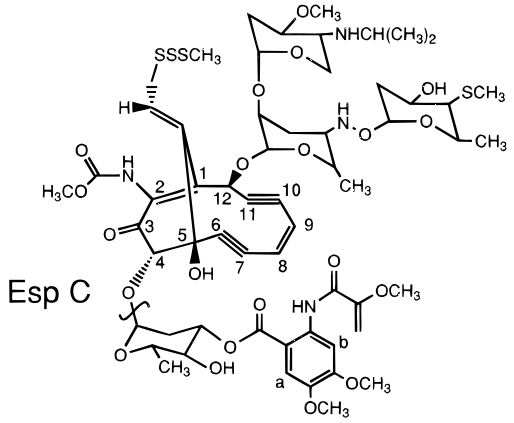

Figure 1. Esperamicin A and its analog esperamicin C. Esperamicin C is produced from acid hydrolysis of esperamicin A.

In the presence of a reducing agent, the methyl trisulfide group of both espA and espC is reduced to a thiolate anion (Scheme 1). An intramolecular conjugate addition of the thiolate across the α,β-unsaturated ketone results in saturation of the bridgehead double bond. The change in hybridization facilitates

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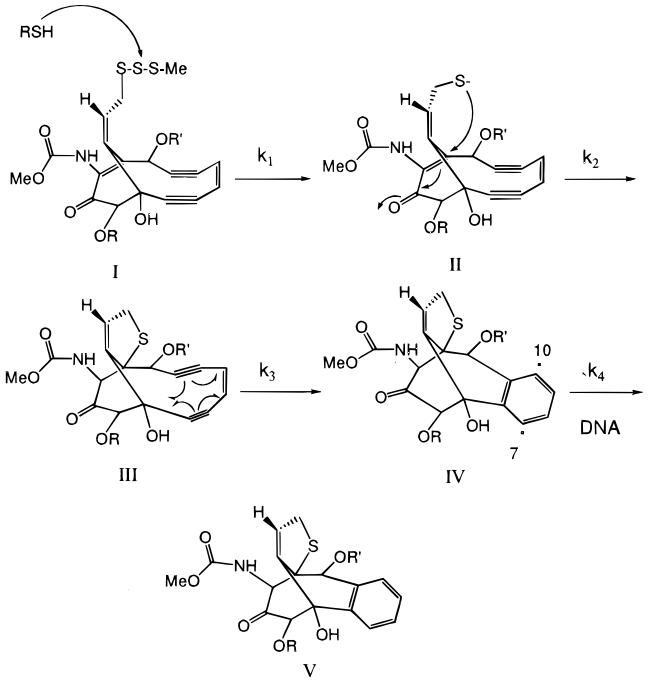
Scheme 1. Proposed Mode of Action for the Esperamicins



a Bergman cyclization of the enediyne core to generate a transient phenylene diradical that is responsible for DNA strand cleavage by hydrogen abstraction from the deoxyribose backbone. The cyclized form of espA has been named espZ[4] and, for convenience, we refer to the cyclized form of espC as espY. EspC effects predominantly double-strand (ds) cleavage of DNA, while espA performs predominantly single-strand (ss) cleavage.[9] Mechanisms of ds cleavage by other enediynes have been studied by several laboratories using specifically deuteriated oligonucleotides.[10−13]

The esperamicins bind in the minor groove of DNA with the enediyne group oriented perpendicular to the line of the groove and positioned to perform hydrogen atom abstraction from sugars on both strands of the DNA.[14,15] Analysis of cleavage chemistry by us using high resolution gel electrophoresis revealed that espC produced fragmentation products consistent with both 4′- and 5′-hydrogen abstraction from DNA, while espA generated fragmentation products indicative of 5′-chemistry only with no 4′-chemistry occurring.[16] In addition, no isotope effects were found upon substitution of the 4′- or 5′-hydrogens by deuterium. These results were consistent with the propensity of espC for ds-cleavage and of espA for ss-cleavage. More recent reports suggest that the methoxyacrylylanthranilate group of espA intercalates into dsDNA from the minor groove and causes a switch in the chemistry of bistranded DNA cleavage.[17,18] In support of this model, Yu et al. observed hydrogen transfer to the drug that was consistent with 1′-hydrogen abstraction and resulted in low levels of ds cleavage.[17] Thus, the discrepancies between espA and espC have remained unresolved.

In this report, we address the fate of each radical formed by espA and espC. Direct physical evidence for hydrogen abstraction from the 4′-position of the DNA sugar phosphate backbone by espC is presented, as well as evidence for exclusive hydrogen abstraction from DNA for both espA and espC. In addition, we attempt to resolve the conundrum of ss- versus ds-DNA cleavage with exclusive bistranded hydrogen abstraction by probing for thiol-specific repair mechanisms.

Materials and Methods

Materials. EspA and espC were provided by Bala Krishnan and Jerzy Golik of Bristol Myers Squibb. Double-stranded circular ϕX174, Taq DNA polymerase, *N*-(2-Hydroxyethyl)piperazine-*N*′-ethanesulfonic Acid (HEPES), tris(hydroxymethyl)aminomethane (Tris), and electrophoresis grade agarose were obtained from Gibco-BRL. Thin layer chromatography (TLC) was performed on Whatman silica gel plates (250 μm). Deuterium oxide (D_2O; 99.97% d) and d_{12}-disodium ethylenediamine tetraacetate (EDTA; 97% d) were obtained from Cambridge Isotope Laboratories. Deuteriated algae extract, Celtone-D (97% d), was purchased from Martek Biosciences. Methyl thiolglyocolate (MTG), tri-*n*-butylphosphine (TnBP), hydrazine, piperidine, d_8-Tris (98% d), d_3-sodium acetate (NaAc; 98% d), DCl (99% d), d_4-methanol (99.9%, d), d_6-ethanol (99.9% d), and all NMR solvents (99.9%+ d) were obtained from Aldrich. HPLC grade CD_3OH and $CHCl_3$ were also obtained from Aldrich. All other reagents were obtained from Sigma Chemical Co.

Kinetic data was obtained on a Bruker AMX500 MHz NMR spectrometer, while all other spectra were obtained with a Varian Unity 500 MHz spectrometer. Mass spectrometry was performed by electron spray ionization (ESI) using a Finnigan-MAT TSQ700 Triplequadrupole with a Finnigan Electrospray interface.

Low Temperature Cyclization in the Absence of DNA. Low temperature experiments were performed in the NMR tube. EspA (0.5 mg, 0.75 mM) or espC (0.5 mg, 1.08 mM) was reacted with tri-*n*-butylphosphine (6 equiv) at −48 °C in 0.5 mL of d_4-methanol. The reaction was then warmed to −12 °C, and the progress of the reaction was monitored by NMR for the next 9 h. Data was saved in 20 different files every 27.2 min. Each file contained 520 scans (20.8 min; acquisition time, 2.4 s/scan) and 160 nonacquisition scans (6.4 min, no data collected).

Kinetic data was calculated from the time dependent disappearance of the doublet corresponding to H-9 (espA, 6.15 ppm and espC, 6.10 ppm), as well as the doublet corresponding to H-8 (espA, 5.95 ppm and espC, 5.90 ppm).

Integrations were calculated for each 20.8 min acquisition spectrum. For each data set, the time was assigned at 10.4 min into each 20.8 min acquisition ($t = {}^1/_2$). For example, the first time point is at 10.4 min, even though data was averaged over 20.8 min. The second time point is at 37.6 min 10.4 min into the second data set plus the 27.2 min between data sets. The data was fit by nonlinear regression (MacNlin, courtesy of Nancy Thornberry, MRL). The slope (k) represented the first-order rate constant used to calculate the Gibbs free energy ($\Delta G^{\ddagger} = -RT \ln(k)$), where k = first-order rate constants given in Table 1, $R = 8.314$ cal/mol K and $T = 261$ K.

Preparation of 4′-Deuteriated DNA. The nucleotide triphosphates (dNTPs) were prepared by synthesis from the 4′-deuteriated ribose.[19,20] The deuterium content at the 4′-position was >95% as evidenced by

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^{1}H NMR. A 1300 bp fragment of pBR322 was then PCR amplified with the 4′-deuteriated dNTPs using the primers [5′-GCT AGA GTA AGT AGT T-3′] and [5′-ATG CGT CCG GCG TAG A-3′]. PCR conditions were varied from standard conditions in order to acquire sufficient yields of DNA for NMR experiments. Each reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 mM 4′-[^{2}H]dATP, 4′-[^{2}H]dGTP, 4′-[^{2}H]dCTP, and 4′-[^{2}H]dTTP, 1 mM of each primer, 5 ng of target DNA and 50 units of Taq polymerase in a final volume of 100 μL. Reactions were cycled 30 times as follows: 1 min at 94 °C, 2 min at 42 °C, and 5 min at 74 °C. The PCR product was purified by ethanol precipitation. The DNA was resuspended in a standard TE buffer [10 mM Tris-HCl (pH 7.6) and 1 mM EDTA], pooled with the other PCR reactions, and applied to a Centricon Microconcentrator spin column (Amicon), through which any residual impurities (dNTP′s or primers) were removed. The purified PCR product was confirmed to be 1300 bp by agarose gel electrophoresis and the concentration determined by UV spectroscopy at 260 nM. The yield for each PCR reaction was 0.05−0.07 mg.

Preparation of Perdeuteriated DNA. The deuteriated M9 minimal media at 5× concentration contained the following: 0.25 M $NaHPO_4$, 0.110 M KH_2PO_4, 43 mM NaCl, and 10 mM NH_4Cl in D_2O (99.97% d). *Escherichia coli* BL21(DE3) cells were adapted for growth on deuteriated minimal media as previously described[21] and transformed with the pBlueK$^+$ plasmid. The pBlueK$^+$ plasmid was chosen due to its high copy number (10−15 per cell). Transformations were performed according to standard protocol,[22] with the exception that all media was prepared in D_2O rather than H_2O. After a growth period of 48 h at 37 °C, five colonies were detected to contain ampicillin resistance (conferred by the pBlueK$^+$ plasmid). A single colony was inoculated into deuteriated media containing 1× M9 minimal media, 2% d_3-acetate (98% d), 2% Celtone-D (97% d), and 5 μg/mL ampicillin in 1L of D_2O (99.97% d). Cells were incubated at 37 °C for 72 h at which point they reached a stationary phase ($OD_{600} = 0.7$). The cells were harvested, and the plasmid DNA was isolated by Qiagen midi prep with yields of approximately 0.5 mg/L, as determined by UV spectroscopy.

The lysed cell debris pellet obtained during the Qiagen preparation was saved, and the chromosomal DNA was isolated in order to obtain larger quantities of perdeuteriated DNA. Each pellet (about 1 g) was resuspended in 2.5 mL of 10 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.5), 1% SDS, and 0.1 mg/mL proteinase K (Gibco BRL), mixed gently, and incubated at 65 °C for 10 min. The mixture was cooled to room temperature and extracted with 2.5 mL of phenol saturated with Tris (obtained from Boehringer-Mannheim). The layers were separated by centrifugation at 4000*g* for 10 min at 4 °C. The upper (aqueous) phase was recovered and extracted a second time with phenol saturated with $CHCl_3$. The phases were separated by centrifugation at 4000*g* for 10 min at 4 °C. The DNA was recovered by ethanol precipitation in 0.3 M sodium acetate. The yield of chromosomal DNA was 4 mg/L of growth as determined by UV spectroscopy at 260 nM.

Cyclization of the Esperamicins with DNA. EspA or espC was cyclized in the presence of protio salmon sperm DNA in deuterio buffer. The DNA (4 mg) was dissolved in D_2O and the solvent removed by vacuum. This process was repeated several times to remove any residual H_2O and exchangeable protons.[23] The DNA was preincubated with espA (0.8 mg; 150 μM) or espC (0.5 mg; 130 μM) for 30 min at 4 °C in 40 mM d_8-Tris-DCl (pD 7.4), 10 mM d_{12}-EDTA and 20% C_2D_5OD in a final volume of 4 mL of D_2O. The reaction was initiated with 14 mM MTG and allowed to proceed at 4 °C for 16 h. The DNA was removed by ethanol precipitation in 0.2 M NaCl. The ethanol supernatant containing the cyclized esperamicin was concentrated and loaded onto a preparative TLC plate. The plate was developed in 9:1/$CHCl_3$:CH_3OH (v/v). Cyclized espA (espZ) migrated with an $R_f = 0.7$, and cyclized espC (espY) migrated at an $R_f = 0.4$. Bands were isolated and extracted in 2 mL of 100% CH_3OH. Solvent was removed by vacuum. The yield for espZ was approximately 60% as quantitated by UV spectroscopy of the cyclized product and the published extinction coefficient[5,24] ($\epsilon = 25900$ at 253 nM), as well as the weight of recovered material. The yield for espY was approximately 50% as measured by the weight of recovered material.

In addition, espA was reacted with perdeuteriated bacterial chromosomal DNA (4 mg) under protio buffer conditions. The DNA was preincubated with espA (0.8 mg; 150 μM) for 30 min at 4 °C in 40 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 20% C_2H_5OH in a final volume of 4 mL of H_2O. The reaction was initiated with 14 mM MTG and allowed to proceed at 4 °C for 16 h. Purification was identical to the procedure described above, and the yield for espZ was about 60%.

Similarly, espA or espC was reacted with the 4′-deuteriated PCR fragment (4 mg). The DNA was preincubated with espA (0.8 mg; 150 μM) or espC (0.5 mg; 130 μM) for 30 min at 4 °C in 40 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 20% C_2H_5OH in a final volume of 4 mL of H_2O. The reaction was initiated with 14 mM MTG and allowed to proceed at 4 °C for 16 h. Purification was identical to the procedure described above with similar yields.

Plasmid Topoisomer Studies. Each reaction contained 10 pmol ϕX174 (7.5 nM), 50 mM HEPES−KOH (pH 7.6), and 1 mM EDTA in a final volume of 30 μL. The concentration of espA was 4.7 nM while the concentration of espC was 0.4 μM.[9] The concentration for espA was determined from the published extinction coefficient,[5,24] while the concentration for espC was deduced from dissolving a weighed amount of espC in ethanol. The reactions were initiated by the addition of 5 mM of each reductant (prepared fresh that day) and allowed to proceed for 10 min at room temperature. The reaction was then divided in three parts: one remained as a control, one was treated with 100 mM hydrazine at 25 °C for 1 h, and one was treated with 100 mM piperidine at 37 °C for 1 h. Control reactions were maintained at 25 °C. Each reaction was then quenched with 5 μL of gel loading buffer (0.1% bromophenyl blue, 65% glycerol, 50 mM EDTA, and 1% SDS) on ice and applied directly onto an 0.8% agarose gel that was cast in 16 mM HEPES−KOH (pH 7.6), 16 mM NaAc, and 0.5 mM EDTA (pH 8.0). Gels were electrophoresed for 1 h at 12 V/cm in the same buffer, with constant exchange of buffer between anode and cathode. Gels were then stained with ethidium bromide (5 μg/mL) for 30 min. Form II (nicked), form III (linear), and form I (supercoiled) DNA were quantitated by fluorimager analysis (Molecular Dynamics) where fluorescence intensity was measured for each band and divided by the total for that lane. Because of its supercoiled nature, form I DNA was less efficiently stained by the ethidium bromide under saturating conditions than forms II and III.[25] A correction factor of 0.8 was determined by comparing the fluorescence intensity of equal quantities of form I and form II ϕX174 DNA. In addition, about 15% of the ϕX174 DNA was already converted to form II. This amount was subtracted when calculating the final amount of form I DNA converted to form II by the esperamicins.

Results

Low Temperature Cyclization of EspA and EspC. Low temperature NMR kinetic analyses have been reported for the cyclization of calicheamicin[26] and neocarzinostatin[27] in the absence of DNA. These studies have demonstrated the existence of discrete intermediates, established the rate constant for Bergman cyclization (k_3, Scheme 1), and provided an estimate for the half-life of these compounds under physiological conditions. Similar analyses of the kinetics of the cyclization of espA and espC cyclization were undertaken here. Tri-*n*-butylphosphine (TnBP) was employed to ensure attack at the central sulfur of the trisulfide.[28] In addition, TnBP contains no aromatic protons which would interfere with the NMR analysis

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Table 1. Comparison of Kinetic Data Generated for EspA and EspC with That of Other Enediynes

	EspA	EspC	calicheamicin[a]	neocarzinostatin[b]
k (10^{-4} s^{-1})	0.95	0.70	5.0	1.0
$\Delta G^{\ddagger}$ (kcal/mol)	20.1 (±0.5)	21 (±1)	19.3 (±0.2)	18.0 (±0.1)

[a] Data collected at −11 °C in d_4-methanol (6 equiv of nBu_3P).[26] [b] Data collected at −38 °C in 9:1 tetrahydrofuran-d_8:CD_3CO_2H (30 equiv of $CH_3O_2CCH_2SH$).[27]

of the aromatic peaks generated by cyclization of the esperamicins. EspA or C treated with tri-*n*-butyl phosphine at −48 °C revealed an immediate shift of the H-12 methine proton resonance at 6.18 to 5.5 ppm. This shift is attributed to Michael addition of the thiolate anion, generated subsequent to TnBP reduction across the α,β-unsaturated ketone as seen in Scheme 1 (**II** to **III**). The intermediate **III** is stable for several hours. However, when the reaction mixture is warmed to −12 °C, a first-order conversion of intermediate **III** to **V** occurs. It is possible to monitor the progress of the reaction by following the disappearance of the resonances corresponding to the doublets for H-9 (6.0 ppm) and H-8 (6.15 ppm).

If the quenching of the diradical **IV** is irreversible and much faster than its formation, then the rate of cyclization observed is equivalent to the rate of formation of the diradical.[26] With this assumption, a first-order rate constant and the $\Delta G^{\ddagger}$ of cyclization can be estimated (Table 1). The kinetics of the reaction with espA and with espC are identical within experimental error and are similar to the values measured for other enediynes. Thus, the differences in the dsDNA cleaving capabilities of espA and espC are the result of binding to DNA rather than any differences in the kinetics of cyclization.

Subsequent product analyses of the cyclized esperamicins by thin layer chromatography revealed differences in their modes of radical quenching. In the case of espC, espY could be recovered in >50% yields comparable to previously reported recoveries for calicheamicin[26] and neocarzinostatin.[27] For espA, however, espZ was generally isolated in <10% yield consistent with previous work on the conversion of espA to espZ in organic solvents.[4] Several other minor products were also recovered which revealed loss of the anthranilate group by NMR. These results suggest that the diradical derived from espA has additional mode(s) of quenching available to it which could involve intramolecular hydrogen transfer from the fucosyl (methoxyacrylyl)anthranilate group to the C-7 radical. Generation of a new radical in this moiety could ultimately result in its partial or complete cleavage affording products other than espZ. The absence of the fucosyl (methoxyacrylyl)anthranilate group in espC precludes this pathway and affords espY in good yields by intermolecular hydrogen transfer from solvent. Significantly, espZ is isolated in excellent yields (∼60%) when espA is cyclized in the presence of DNA, suggesting that binding to DNA prevents intramolecular radical quenching which can destroy the molecule (vide infra). Since the kinetics reported here rely on the rate of disappearance of **III** as a measure of cyclization, an irreversible process, subsequent alternative mechanisms of quenching of **IV** to multiple products will not affect the rates of cyclization or comparisons of espA and espC.

Establishing the Source of Hydrogen for Quenching the Esperamicins. In order to understand the basis for the mechanistic differences between espA and espC, a series of experiments was undertaken to determine the hydrogen source responsible for quenching the phenylene diradical for both enediynes. In the first experiment, espA was cyclized in the presence of protio DNA in a completely deuteriated buffer containing d_6-Tris-DCl, d_{12}-EDTA, and 20% CD_3CD_2OD (for solubility of espA) in D_2O. The final deuterium content in the solvent was >99.9%. Methyl thioglycolate (MTG; protio) was used as the reductant. The resulting cyclized product, espZ, was purified by thin layer chromatography (∼60% yield). The ^1H NMR spectrum of the espZ derived from this experiment is shown in Figure 2A. Singlets at 8.5 and 7.6 ppm correspond to the protons of the fucosyl anthranilate group (a and b, Figure 1). The resonances of these protons were essentially unchanged from those in the starting material (espA) and were useful for monitoring the integrity of the molecule during cyclization and purification. Doublets at 7.22 and 7.76 ppm correspond to the protons at positions H-10 and H-7, respectively. H-10 and H-7 were assigned by a 2D ROESY NMR experiment wherein H-10 exhibited an NOE crosspeak to the H-12 methine proton (5.15 ppm). Triplets at 7.28 and 7.34 ppm correspond to positions H-9 and H-8, respectively. Integration of the peaks corresponding to H-10 and H-7 revealed one proton at each position, establishing that no deuterium was incorporated at either the C-7 or the C-10 radical. Clearly, the hydrogens which are incorporated into the espZ are not derived from the solvent.

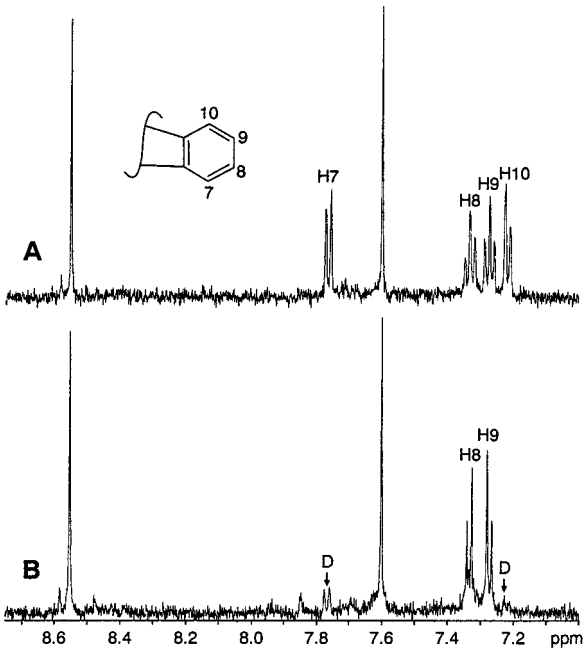

Figure 2. (A) NMR spectrum of espZ formed by cyclization of espA with protio DNA in a deuterio buffer**.** Spectra for espZ were obtained in CD_3CN for optimal clarification of the aromatic peaks. Singlets at 8.5 and 7.6 ppm correspond to the protons on the fucosyl anthranilate group (a and b as seen in Figure 1). Doublets at 7.22 and 7.76 ppm correspond to the protons at positions H-10 and H-7, respectively. Triplets at 7.28 and 7.34 ppm correspond to positions H-9 and H-8, respectively. (B) NMR spectra of espZ formed by cyclization of espA with perdeuteriated DNA in a protio buffer**.** The usual doublets at 7.22 and 7.76 ppm, corresponding to positions of the C-10 and C-7 radical centers, revealed deuterium incorporated. Furthermore, the triplets at 7.28 and 7.34 ppm were reduced to doublets.

Three possible sources of nonexchangeable protium for quenching the phenylene diradical are present in the preceding experiment: (i) the espA molecule itself, (ii) the reductant, MTG, and (iii) the DNA. If espA were capable of quenching one of the radical positions by intramolecular hydrogen transfer, the resulting remote radical generated in the molecule could undergo two possible fates: (i) further reaction leading to modification or decomposition of espZ[29] and/or (ii) repair of the radical by deuterium transfer from reductant affording espZ

(28) Harpp, D. N.; Smith, R. A. *J. Am. Chem. Soc.* **1982**, *104*, 6045−6053.

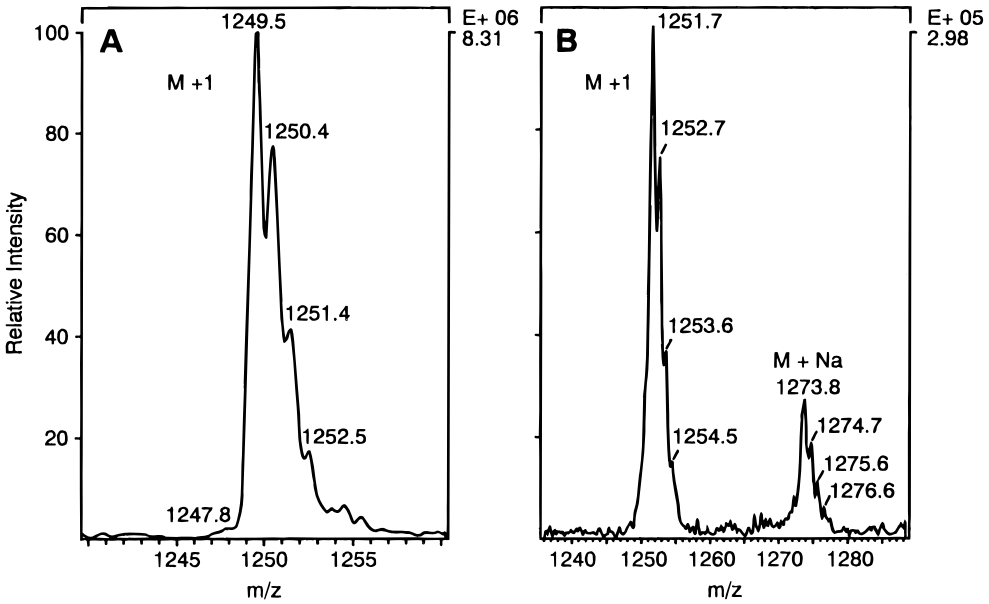

Figure 3. Ion clusters of espZ resulting from cyclization of espA under two experimental conditions: (A) EspA cyclized with salmon sperm DNA in deuterio buffer; (B) espA cyclized with perdeuteriated DNA in a protio buffer. Mass spectrometry was performed by ESI. Samples of espZ were dissolved in acetonitrile:methanol (80:20) with 0.01% TFA and infused at 5 μL/min. Data was averaged over 0.5 min. The molecular ion (M + 1, 1249.5) was consistent with the calculated molecular weight of 1248. Deuterium incorporation at both radical positions results in a change by 2 mass units (M + 1, 1251.7).

containing deuterium at a position remote from the initial site of diradical generation. Since the majority of the cyclized espA was recovered as intact espZ, decomposition via a remote radical center does not constitute a major pathway. In addition, a complete analysis of the ^{1}H NMR of the espZ did not show any obvious deuterium incorporation at the other positions in the molecule (data not shown). Finally, deuterium incorporation into drug was definitively excluded by ESI-MS analysis (Figure 3). The ion cluster of espZ derived from protio DNA/deuterio-buffer (Figure 3A) showed no change from controls generated in completely protio conditions, negating the possibility of intramolecular abstraction followed by repair.

Having excluded solvent, buffer, and the espA itself as possible hydrogen sources, a distinction between the reductant MTG and the DNA hydrogen pools remains. The reductant was excluded as a hydrogen source by cyclization of espA in the presence of perdeuteriated DNA in a protio buffer (Figure 2B). In this experiment DNA was the only possible deuterium source. Proton NMR analysis revealed >90% deuterium incorporation at positions H-10 and H-7, proving that, in the presence of DNA, both hydrogens were abstracted from DNA: there was no internal quenching or no abstraction from the reductant. These results were confirmed by MS analysis which revealed a change in the molecular weight of espZ by +2 (Figure 3B). Thus, for espA, it appeared that most, if not all of the individual phenylene diradicals are quenched by direct transfer of nonexchangeable hydrogens from DNA. Therefore, any hypothesis attempting to explain the predominance of ss-cleavage by espA must account for this observation.

EspC was also cyclized under similar sets of conditions in order to ascertain which hydrogen source quenched the phenylene diradical. In the first experiment, espC was cyclized in the presence of protio DNA in a completely deuteriated buffer containing d_6-Tris-DCl, d_{12}-EDTA, and 20% CD_3CD_2OD (for solubility) in D_2O. The ^{1}H NMR spectrum of the resulting cyclized product, espY, is shown in Figure 4A. The doublets corresponding to H-10 and H-7 appear at 7.35 and 7.62 ppm,

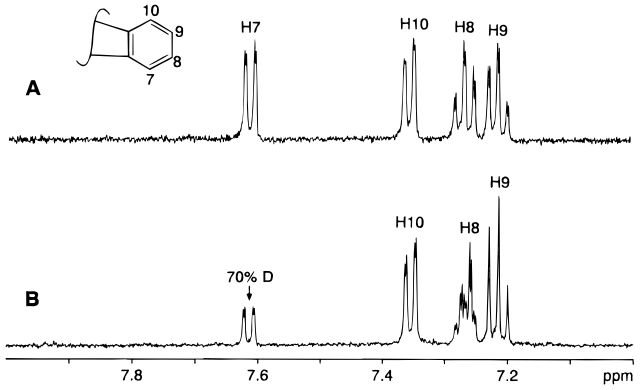

Figure 4. (A) NMR spectrum of espY formed by cyclization of espC in the presence of protio DNA in a deuterio buffer. Spectra were obtained in CD_3OD for optimal clarification of aromatic protons. The doublets corresponding to H-10 and H-7 appear at 7.35 and 7.62 ppm, respectively, and the triplets corresponding to H-9 and H-8 appear at 7.22 and 7.26 ppm, respectively. (B) NMR spectrum of espY resulting from cyclization of espC in a protio buffer with 4′-deuteriated PCR fragment**.** NMR integration revealed 70% deuterium incorporation at the doublet corresponding to position H-7, 7.62 ppm. The triplet at 7.26 ppm, corresponding to position H-8, is rendered more complex by partial reduction to a doublet, and the signal at 7.22 ppm (H-9) was simplified by loss of meta-coupling.

respectively, and the triplets corresponding to H-9 and H-8 appear at 7.22 and 7.26 ppm, respectively. Distinction between H-10 and H-7 was again resolved by a 2D ROESY NMR experiment. Integration of the peaks corresponding to H-10 and H-7 revealed one proton at each position, indicative of no deuterium incorporation at the location of either radical center. Therefore, the hydrogens which are incorporated into the cyclized product are not derived from the solvent.

As with the espA experiments described above, three possible sources of nonexchangeable hydrogens for quenching the phenylene diradical are present for espC: (i) the espC molecule itself, (ii) the reductant, MTG, and (iii) the DNA. EspC is excluded as a source of hydrogens on the basis of the excellent yields of espY (~50%) that were recovered as intact espY,

(29) Langley, D. R.; Golik, J.; Krishnan, B.; Doyle, T. W.; Beveridge, D. L. *J. Am. Chem. Soc.* **1994**, *116*, 15−29.

precluding a destructive intramolecular hydrogen tranfer as a major pathway; a complete analysis of the ^{1}H NMR of the espY which did not show any obvious deuterium incorporation at the other positions in the molecule (data not shown); and an ESI-MS analysis which reveals that the ion cluster of espY (M + 1 = 856.4 mu) derived from protio DNA/deuterio-buffer showed no deuterium incorporation (data not shown).

The nonexchangeable hydrogens of the reductant, MTG, are also a possible hydrogen source. This source is excluded by previous studies showing that espC functions predominantly as a double strand cleaver, with a single molecule of espC responsible for two lesions on opposite strands of dsDNA.[9,16,17] In addition, similar experiments performed on calicheamicin γ1, the structural analog of espC, have reported dsDNA as the sole hydrogen source for quenching of the phenylene diradical.[30] Therefore, espC likely abstracts hydrogens exclusively from dsDNA. Furthermore, high resolution gel electrophoresis of the fragmentation products produced by the espC has suggested that 5′- and 4′-hydrogen abstraction predominate.[16] To test this hypothesis we synthesized [4′-^{2}H] dNTPs and incorporated them into DNA. Because the esperamicins are considerably less sequence selective than calicheamicin,[14,31] it was necessary to deuteriate DNA at the 4′-position for all four nucleotides.

EspC was reacted with [4′-^{2}H]DNA in a protio buffer. The resulting espY was analyzed by ^{1}H NMR and results are shown in Figure 4B. Integration of the spectrum revealed 70% deuterium incorporation at the doublet corresponding to position H-7, 7.62 ppm. Furthermore, the triplet at 7.26 ppm, corresponding to position H-8 was rendered more complex by partial reduction to a doublet, and the signal at 7.22 ppm (H-9) was simplified by loss of the meta-coupling. The integration for both H-9 and H-8 remained the same. These results provided direct physical evidence for abstraction of 4′-hydrogen from DNA by the C-7 radical to generate espY. Deuterium incorporation into espY was also confirmed by MS (M + 1 = 857.5 mu).

High resolution gel electrophoresis of the fragmentation products generated when espA interacts with DNA is consistent with 5′-hydrogen abstraction alone,[16] although more recent reports have suggested limited (23%) 1′-hydrogen abstraction.[17] The absence of 4′-hydrogen abstraction with espA was verified by its reaction with [4′-^{2}H]DNA. The resulting espZ revealed no deuterium incorporation at either position C-10 or C-7 by ^{1}H NMR, and the spectrum remained identical to that already shown in Figure 2A. These findings are consistent with the previous studies and can be accommodated by a change in the regiochemistry of hydrogen abstraction from 4′ -position of DNA by the C-7 of espC to the 1′-position by espA.

Plasmid Topoisomer Studies. Previous studies by us[16] and others[9] have been interpreted to indicate that espA, unlike espC, is not involved in ds-cleavage. These results contrast with a recent report, using glutathione as the reductant, suggesting that ~23% of DNA damage by espA was double stranded.[17] Even under these specific conditions, 75% of the cleavage with espA would be anticipated to result from quenching C-7 by reductant or solvent. In contrast to these expectations, however, we have shown above that all of the hydrogens in espZ are derived from DNA. A possible explanation for this paradox is that the C-7 radical of espA always abstracts hydrogen from DNA, but the resulting DNA radical is quenched by another hydrogen donor prior to trapping by oxygen and cleavage.

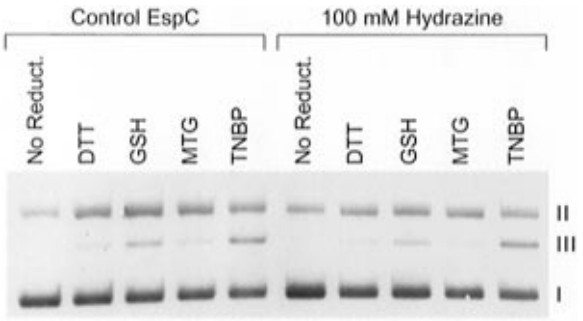

Figure 5. Gel depicting treatment of supercoiled ϕX174 with espC, using several different reductants. Cyclization was initiated as described in Materials and Methods and allowed to proceed for 10 min. Reactions products were then treated an additional 60 min at 25 °C with 100 mM hydrazine. Each reaction contained 7.5 nM ϕX174 and 0.4 μM espC. Form II (nicked), form III (linear), and form I (supercoiled) DNA were quantitated by fluorimager analysis.

An excellent candidate for hydrogen donor in this radical repair process is the exogenous thiol and might explain the discrepancies in ds-cleavage noted above for espA. Previous work on the radioprotection of pBR322 by thiols[32] has demonstrated that the net charge of the thiols mediates their ability to scavenge hydroxyl radicals and repair DNA radical centers. Therefore, GSH used by Yu et al.,[17] which has a net charge of −1 at physiological pH would be less efficient at repairing radical centers than DTT, used previously by us,[16] or MTG which has a net charge of 0. Additional steric constraints imposed by the DNA−drug complex could also favor the smaller thiols, DTT and MTG. Furthermore, TnBP, which is not efficient in hydrogen transfer should exhibit very little repair of DNA radicals and allow full expression of ds-cleavage.

This hypothesis was tested under standard reaction conditions for the esperamicins using GSH, DTT, MTG, and TnBP as reductants. EspC, due to its intrinsically high propensity for ds-cleavage, provides the clearest illustration of the effect of reductants on the relative amounts of ds-cleavage and ss-cleavage (Figure 5 and Table 2). EspC was incubated with supercoiled ϕX174 DNA (form I) in the presence of the reductant, and the amounts of nicked (form II, ss-cleavage) and linear (form III, ds-cleavage) were analyzed on an agarose gel. For each thiol, the reaction was additionally treated with 100 mM hydrazine, in order to fully develop alkaline labile lesions.[17] Figure 5 provides a direct visualization of the results. Dramatic suppression of ds-cleavage (form III) relative to TnBP is clearly observed for DTT or MTG. Significantly more ds-cleavage is seen when GSH is used as the reductant. The quantitation of forms I-III is shown in Table 2 along with the calculated ratios of ds-cleavage to ss-cleavage (ds/ss). Hydrazine treatment increased the amount of ds-cleavage, but the trend in reductant efficiency was unchanged. TnBP, the least efficient at repairing DNA lesions, gave 76% ds-cleavage before and >90% ds-cleavage (ds/ss > 10) after hydrazine treatment. GSH gave 70% ds-cleavage before and >90% ds-cleavage after hydrazine treatment. However, the small, neutral thiols DTT and MTG show substantial reduction of ds-cleavage. For DTT, 28% and 63% ds-cleavage was found before and after hydrazine treatment, respectively; for MTG, 43% and 64% ds-cleavage. The concentration of thiols used (5 mM) effected optimal DNA cleavage and represented a compromise between efficiency of esperamicin activation and thiol-mediated repair. At lower concentrations (<2 mM) the analysis was complicated by an overall decrease in total DNA cleavage (data not shown).

(30) Zein, N.; McGahren, W. J.; Morton, G. O.; Ashroft, J.; Ellestad, G. A. *J. Am. Chem. Soc.* **1989**, *111*, 6888−6890.
(31) Lu, M.; Guo, Q.; Krishnan, B.; Golik, J.; Rosenberg, I. E.; Doyle, T. W.; Kallenback, N. R. *J. Biomol. Struct. Dyn.* **1991**, *9*, 285−298.
(32) Zheng, S.; Newton, G. L.; Ward, J. F.; Fahey, R. C. *Radiat. Res.* **1992**, *130*, 183−193.

Table 2. Relative Repair of EspC and EspA Induced DNA Damage by Reducing Agents

	no reduct	DTT	GSH	MTG	TnBP
		EspC[a]			
form I	0.85	0.64	0.59	0.62	0.51
form I-hyd	0.81	0.62	0.50	0.56	0.41
form II	0.15	0.30	0.23	0.28	0.23
form II-hyd	0.19	0.26	0.22	0.28	0.18
form III	0	0.06	0.18	0.10	0.26
form III-hyd	0	0.12	0.28	0.16	0.41
ratio ds/ss[b]	−	0.40	2.25	0.77	3.25
ratio ds/ss-hyd	−	1.71	9.30	1.78	>10
		EspA[a]			
form I	0.86	0.43	0.66	0.41	0.51
form I-hyd	0.88	0.41	0.55	0.39	0.35
form II	0.14	0.56	0.33	0.58	0.47
form II-hyd	0.12	0.53	0.38	0.53	0.58
form III	0	0.01	0.01	0.01	0.02
form III-hyd	0	0.06	0.07	0.05	0.07
ratio ds/ss[b]	−	0.02	0.05	0.02	0.06
ratio ds/ss-hyd	−	0.15	0.27	0.12	0.15

[a] Form I (supercoiled), form II (nicked), and form III (linear) ϕX174. [b] Ratio of ds/ss cleavage was calculated by dividing the fraction of form III by the fraction of form II. ϕX174 molecules already converted to form II prior to treatment with espC have been subtracted prior to calculating the ratio of ds/ss-DNA cleavage.

The results of a similar study with espA are also shown in Table 2. Compared to espC, the relative amounts of ds-cleavage were substantially lower for all reductants both before and after hydrazine treatment; however, GSH gave a maximum of 21% ds-cleavage after hydrazine treatment, while DTT and MTG gave 12% and 10% ds-cleavage, respectively. The results for TnBP were unexpected yielding a maximum of 13% ds-cleavage. Treatment with piperidine instead of hydrazine afforded similar results.

Discussion

Previous work on the fate of the diradical formed by the esperamicins in the presence of DNA has been confusing and, at times, contradictory. Dobrowiak and co-workers demonstrated by plasmid topoisomer studies that espA was predominantly a ss-DNA cleaver while espC was predominantly a ds-DNA cleaver.[9] High resolution gel electrophoresis studies by us[16] suggested that the C-10 radical of both espA and espC abstracted a 5′-hydrogen from DNA consistent with the deuterium transfer experiments done with calicheamicin,[10] the structural analog of espC. Our studies also suggested that the C-7 radical of espC performed 4′-hydrogen abstraction as judged by the formation of 3′-phosphoglycolate termini, an unambiguous product of 4′-chemistry.[16] Abstraction of the 4′-hydrogen has now been confirmed by the deuterium transfer experiment to espC from [4′-^{2}H]DNA reported here.

The fate of the C-7 radical of espA has remained unresolved. Limited 1′-hydrogen abstraction from DNA by espA has been proposed to account for ~23% ds-cleavage observed for espA in the presence of glutathione.[17] However, the bulk of the C-7 radical of espA appears to be quenched by processes that do not lead to the high levels of ds-cleavage observed by espC. This suggested that alternative sources of hydrogen other than DNA might quench espA. To resolve this issue we have have assessed the sources of hydrogen abstraction for espA.

Remarkably, our experiments clearly demonstrate that for espA the C-7 and C-10 hydrogens of the resulting espZ were derived exclusively from DNA. EspA is, therefore, quite similar to espC and calicheamicin in its ability to perform a highly efficient bistranded lesion in DNA. However, there was no 4′-hydrogen abstraction by either radical center of espA, as evidenced by lack of deuterium transfer from [4′-^{2}H]DNA. Thus, espA and espC differ significantly with respect to the specificity of hydrogen abstraction from DNA by the C-7 radical.

The possibility that a portion, at least, of hydrogen abstraction from DNA by the C-7 of espA is the result of 1′-chemistry is supported by several lines of evidence. DNA unwinding studies have indicated that the fucosyl anthranilate group of espA intercalates via the minor groove, the locale for 1′-, 4′-, and 5′-hydrogens.[17] NMR studies have supported this intercalation motif and have further suggested that the C-7 radical of espA is positioned to perform 1′-hydrogen abstraction.[18] Finally, direct tritium transfer from [1′-,2′-3H]thymidine in DNA to espA has been demonstrated and the resulting lesion has been shown to be alkali-labile consistent with 1′-chemistry although 2′-hydrogen abstraction cannot be rigorously excluded.[17] As mentioned earlier, 1′-abstraction opposite 5′-abstraction leading to ds-cleavage represented only ~20−25% of the total DNA lesions.

A central question thus emerges from these studies: if espA is capable of exclusive hydrogen abstraction from DNA, then why is there significantly less ds-cleavage than with espC? Several possible explanations can be proposed: (i) the hydrogens could be derived from the same strand of DNA when ss-cleavage occurs; (ii) the hydrogens could have been abstracted from nonexchangeable positions on the nucleic acid bases rather than the 1′-position during ss-cleavage; (iii) espA undergoes intramolecular quenching and decomposition similar to the mode of quenching in the absence of DNA (vide supra); or iv) the radical lesions are susceptible to repair by exogenous reductant that is more efficient in the presence of espA than with espC. Derivation of the hydrogens from the same strand of DNA is highly unlikely, being inconsistent with the NMR solution structure of the espA/DNA complex[18] and with all proposed models of the interaction of the enediyne core of the esperamicins and calicheamicin with DNA. The nonexchangeable hydrogens of the nucleic acid bases are also unlikely on the basis of models;[18] however, if espA could abstract the 5-hydrogen of a cytidine, the result could be either repair or abstraction from the 1′- or 2′-position of the *adjacent* sugar. A similar mechanism has been demonstrated for 5-bromouracil.[33,34] The possibility of intramolecular quenching and decomposition cannot be rigorously excluded.[29] Although the resulting espZ is recovered in good yield (>50%), a substantial amount of material remains unaccounted for. Thus, the espZ collected for NMR analysis may have represented a single reaction pathway, i.e. ds-cleavage. Other ss-cleavage pathways may not lead to recoverable, cyclized products by our methods. However, that the amount of espZ is significantly greater than the amount of ds-cleavage by espA suggests that a major portion of espZ is derived from ss-cleavage events. The possibility of repair of radical lesions by exogenous reductant demands further scrutiny.

In the simplest model, each radical lesion may follow one of two possible pathways: reaction with molecular oxygen followed by multiple mechanistic manifolds leading to strand cleavage[16,35] or reaction with exogenous reductant resulting in hydrogen transfer with immediate repair.[32,36] Thus the fractional efficiency of DNA damage at a given radical lesion (eq 1) will be determined by the rate of radical oxidation ($k_{ox}[O_2]$) and the rate of radical repair by reductant (k_{red}[SH]). The rate constant

(33) Sugiyama, H.; Tsutsumi, Y.; Saito, I. *J. Am. Chem. Soc.* **1990**, *112*, 6720−6721.
(34) Sugiyama, H.; Tsutsumi, Y.; Fujimoto, K.; Saito, I. *J. Am. Chem. Soc.* **1993**, *115*, 4443−4448.
(35) Stubbe, J.; Kozarich, J. W. *Chem. Rev.* **1987**, *87*, 1107−1136.
(36) von Sonntag, C. *The Chemical Basis of Radiation Biology*; Taylor and Francis: London, 1987, pp 353−374.

for radical oxidation (k_{ox}) will be a function of the stability of the radical center and its accessibility to oxygen in the DNA−drug complex. The rate constant (k_{red}) for radical repair will also depend on the stability of the radical center as well as the electronic and steric structure of the reductant and its ability to access the radical center. These kinetic parameters would be expected to differ for each radical position (1′-, 4′-, and 5′-) and, perhaps, for the presence of espA or espC.

$$\text{cleavage fraction} = k_{ox}[O_2]/(k_{ox}[O_2] + k_{red}[SH]) \quad (1)$$

It follows that ds-cleavage is observed only at those bistranded radical lesions in which both lesions lead exclusively to oxidation and are not repaired. Repair of either radical by reduction will result in an ss-cleavage site. Therefore, quantitation of ds- vs ss-cleavage in the presence of different reductants can be used to test this hypothesis.

Comparison of the cleavage pattern of supercoiled ϕX174 by espC activated with a variety of reductants confirms that small, neutral thiols (such as DTT and MTG) were more efficient in radical repair yielding larger amounts of ss-cleavage, while the negatively charged and sterically larger GSH gave considerably more ds-cleavage. TnBP, an activator of espermicins and a non-hydrogen donor, afforded nearly complete ds-cleavage consistent with this explanation. The results with espC are in excellent agreement with a series of studies performed on the effects of thiol structure and charge on repair of DNA damaged by ionizing radiation.[32,37,38] While the results do not distinguish between the kinetics of repair between 4′- and 5′-radicals, the large amounts of ds-cleavage suggest that the kinetics of repair at both positions are comparable and slow relative to oxidation.

Similar studies with espA were not as clear-cut. Although GSH gave a marginally greater amount of ds-cleavage than DTT or MTG, TnBP did not afford the expected high degree of ds-cleavage suggesting that the 1′-radical may be particularly prone to repair mechanisms that are independent of exogenous thiols. Indeed, the presumed inaccessibility of the 1′-radical in the DNA−drug complex relative to the corresponding 4′-radical in espC suggests that substantial repair may be occuring by intramolecular processes. Overall, the switch to 1′-chemistry by the C-7 radical of espA, the structural differences between espA and espC, and the use of different reductants have a profound impact on the expression of bistranded radical damage. Minimally, the results provide a possible explanation for the discrepancy of our earlier work with DTT-activated espA in which we observed no significant 1′-chemistry (i.e., no ds-cleavage)[16] and later studies with GSH-activated espA in which we detected limited 1′-chemistry (i.e., limited ds-cleavage).[17]

The effects of thiol structure on DNA damage by the related antibiotic neocarzinostatin have been recently reported.[39] In this case, the drug is activated by conjugate addition of the thiol to form an indene bisradical. The process of activation, therefore, results in the formation of a distinct drug species for each thiol. Thus, the effects of thiol-mediated repair cannot be deconvoluted from the direct effects of the thiol on the activated drug. For the esperamicins, thiol-mediated trisulfide cleavage results in the same activated drug for each thiol. Therefore, the esperamicins provide a unique opportunity to assess the effects of thiol-mediated repair directly. Our findings demonstrate the caution must be exercised when assessing the efficiency of ds-cleavage by drug in the presence of reductants and making comparisons between structurally related drugs in the absence of a complete analysis of their individual chemistries.

We have demonstrated that espA and espC can both effect exclusive hydrogen abstraction from DNA but with different specificities for hydrogen abstraction. The manifestation of DNA damage, however, is a complex process which is clearly sensitive to a variety of parameters. Future studies will need to be performed to understand the mechanisms of repair at the various radical sites and how the nature of the lesion and of the drug, the structure and redox potential of the thiol and the reaction conditions impact the resulting DNA damage.

Acknowledgment. This work was supported by NIH Grant GM-34454 to J.S. and J.W.K. The authors thank Alice Marcy and Doug Wisniewski of MRL for providing the D_2O adapted *E. coli*, Paul Meechan of MRL for helpful discussions, and Yiu Fai Lam of the University of Maryland for his assistance with the low temperature NMR experiments.

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