The Purine 2-Amino Group as a Critical Recognition Element for Specific DNA Cleavage by Bleomycin and Calicheamicin

Christian Bailly† and Michael J. Waring*

Contribution from the Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, England

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Abstract: The influence of the 2-amino group of guanine on antibiotic-mediated cleavage has been studied using DNA in which that group has been removed from guanine, added to adenine, or both. A homologous series of 160 base pair fragments of DNA containing inosine and/or 2,6-diaminopurine residues in place of guanosine and/or adenine residues respectively were synthesized by the polymerase chain reaction and subjected to sequence-specific cleavage by the iron−bleomycin complex or calicheamicin γ_1^I. Although the 2-amino group is not absolutely required it constitutes a key structural element which directs sequence-specific cleavage of DNA. For bleomycin, relocating it created new cleavage sites at pyrimidine residues lying 3′ to 2,6-diaminopurine residues. For calicheamicin, the presence of a purine 2-amino group adjacent to the cutting site potentiated the cleavage reaction. Sequence recognition by bleomycin seems to rely on direct interaction with guanine whereas DNA conformation/flexibility appears more important for calicheamicin.

A large number of potent anticancer drugs owe their efficacy to their ability to promote DNA degradation. Such is the case for the bleomycins and the calicheamicins (Figure 1), two families of glycoconjugate antibiotics capable of inducing DNA strand breakage via free radical mediated mechanisms.[1−3] Cleavage of DNA by bleomycin is dependent on the participation of a redox-active metal ion and a source of oxygen[4−6] whereas calicheamicin and other enediyne compounds require a thiol cofactor.[7−9] In both cases the cleavage reaction proceeds via an attack on deoxyribose by highly reactive species produced upon chemical activation of the drug, but the molecular mechanisms of free radical generation and of DNA cleavage are different. The bleomycin−Fe^{II} complex combines with O_2 to produce a reactive oxygenated metallobleomycin species which is capable of abstracting a hydrogen atom from the deoxyribose ring.[2] Bleomycin generates mainly single strand breaks whereas calicheamicin produces almost exclusively double strand breaks.[10] The chemistry of calicheamicin and other enediyne compounds has been examined in detail in recent years: a reducing agent (e.g. glutathione, dithiothreitol) acts as a nucleophile to initiate the reaction of the trisulfide moiety with the enediyne system which then undergoes a Bergman cyclization reaction leading to a DNA-damaging benzenoid diradical.[3] A feature common to both agents is the high sequence-selectivity of the DNA cleaving reaction: pyrimidine residues 3′ to a guanine residue (i.e. GpC and GpT sequences) are preferentially cleaved by bleomycin[11−15] whereas calicheamicin selectively attacks a pyrimidine residue embedded

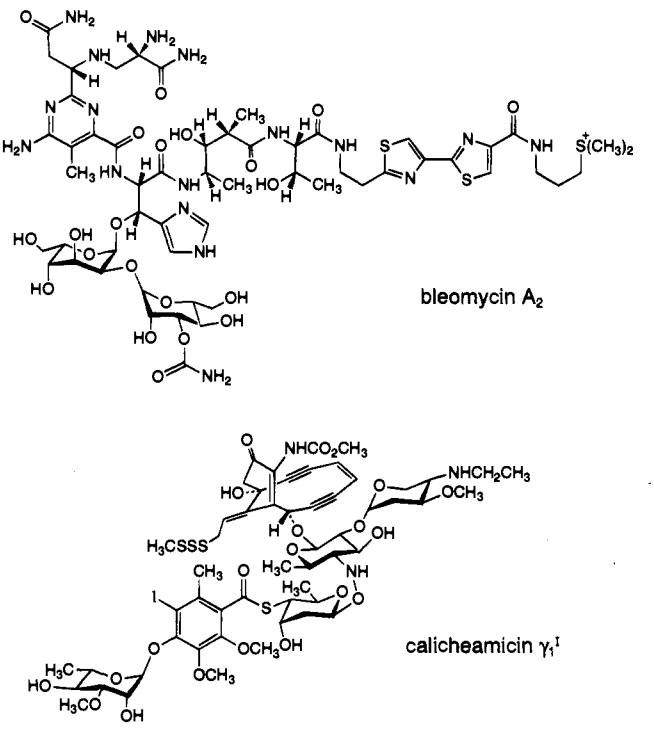

Figure 1. Structures of bleomycin A_2 and calicheamicin γ_1^I.

† Present address: Institut de Recherches sur le Cancer, INSERM Unité 124, Place de Verdun, 59045 Lille cedex, France. FAX (+33) 20 52 70 83.

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in a short homopyrimidine • homopurine tract. Double-stranded lesions produced by calicheamicin γ_1^I (the leading compound in the series) occur with a tetranucleotide specificity mainly at TCCT • AGGA, with other minor sites at short runs of pyrimidines, e.g. TCCG, TCCC, TCTC.[16-18]

The pattern of cleavage is believed to result from prior sequence-selective binding of the antibiotics to DNA.[19-27,28-36] Although the exact modes of binding remain controversial, molecular modeling and experimental studies have suggested that both antibiotics bind within the minor groove of the DNA helix where the 2-amino group of guanine may constitute a critical sequence recognition element. Models have been proposed which involve, for bleomycin, hydrogen bonding between one of its bithiazole nitrogens and the guanosine 2-amino group[37-38] and, for calicheamicin, interaction between the polarizable iodine atom on its benzoate core and the exocyclic amino group of the 5′ guanine in the sequence AGGA.[39,40] The bleomycin model appears consistent with footprinting studies on bithiazole-netropsin hybrid ligands,[41] prompting the inference that interaction of the antibiotic with the 2-amino group of guanine must somehow be responsible for the sequence specificity of DNA cleavage by the iron–bleomycin complex. By contrast, footprinting studies on calicheamicin γ_1^I suggest that interaction with guanine is likely to be a minor factor in determining the sites of cleavage.[42]

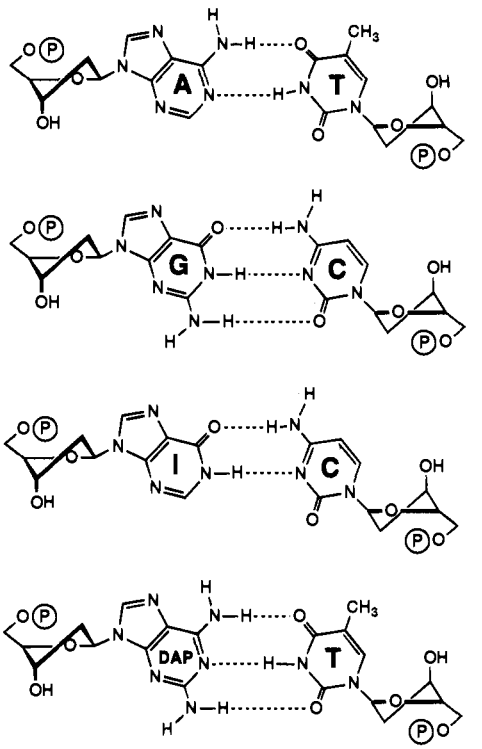

Figure 2. Structures of hydrogen-bonded purine–pyrimidine base pairs. Broken lines represent hydrogen bonds. I represents inosine; DAP represents 2,6-diaminopurine (2-aminoadenine).

To examine directly the influence of this substituent on DNA cleavage by the two antibiotics we have observed the reaction using DNA in which the purine 2-amino group has been removed, added, or shifted. Removal amounts to converting guanosine nucleotides to inosines (Figure 2). Addition can be accomplished by a modification such that the 2-amino group is present on all purines: this involves replacing the adenines with 2,6-diaminopurines (DAP, Figure 2) while retaining it on guanines. Shifting the 2-amino group can be accomplished by relocating it on to the A • T base pairs (A → DAP substitution) while at the same time removing it from the G • C pairs (G → I substitution). Therefore, by comparing the patterns of cleavage with each of these DNA fragments we can observe the precise effect of the purine 2-amino group on the sequence-specific cleavage of DNA by bleomycin–Fe^{II} complex and calicheamicin.

Results

A series of 160 base pair *tyr*T(A93) DNA fragments[43] containing normal or modified nucleotides were synthesized using the polymerase chain reaction (PCR), labeled on one or another of the complementary strands, and then subjected to a standard cleavage reaction in the presence of each antibiotic.

Cleavage by Bleomycin. The cleavage pattern generated with the bleomycin–Fe^{II} complex varies markedly depending on whether the 2-amino group is present on guanine or adenine

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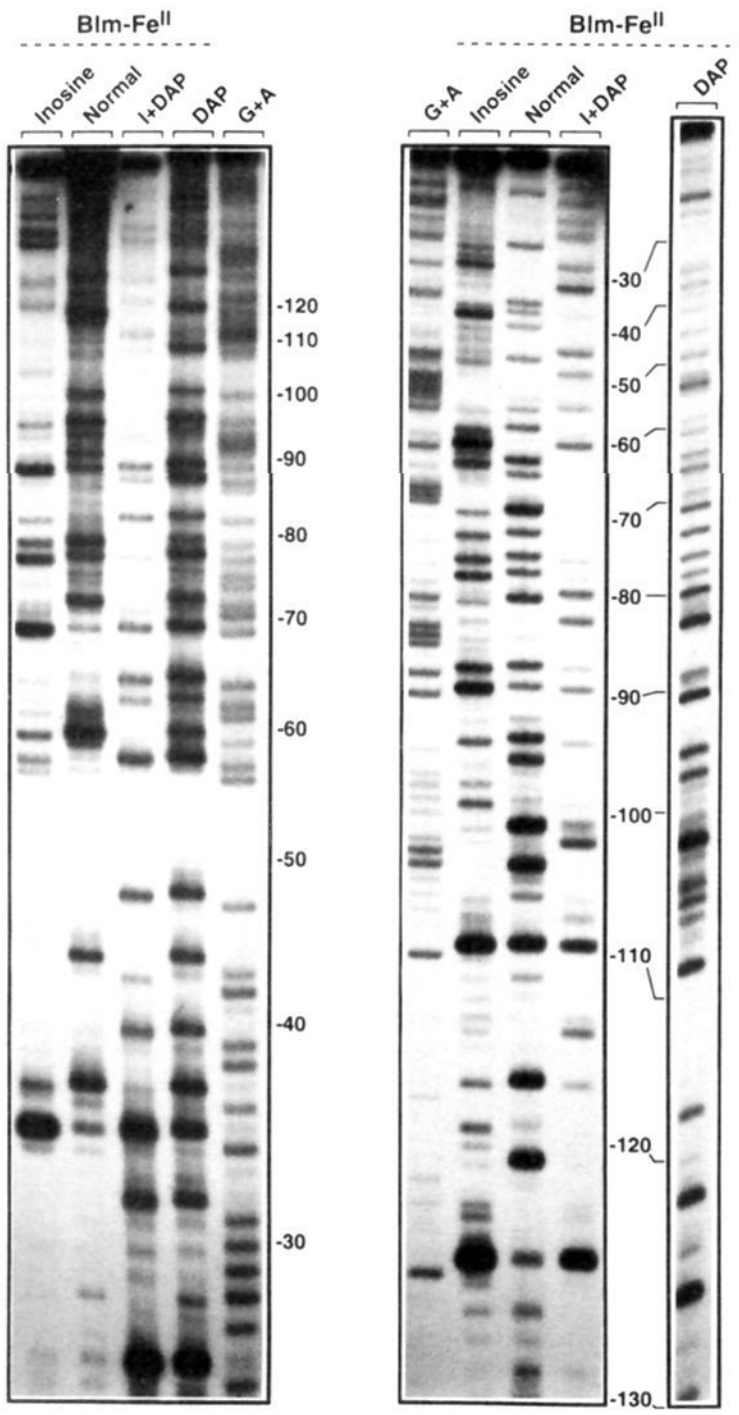

Figure 3. Autoradiographs showing cleavage of normal and modified DNA by the bleomycin−Fe^{II} complex (5 μM). The left and right panels refer respectively to the labeled Watson and Crick strands of *tyr* T-(A93) DNA containing the four natural nucleotides (normal DNA), inosine residues in place of guanosine (Inosine DNA), diaminopurine residues in place of adenine (DAP DNA), or inosine plus DAP residues in place of guanosine and adenine respectively (I+DAP DNA). Chemical identities of the cleavage products were assigned by reference to formic acid−piperidine markers specific for purine residues (lanes G+A), corrected for the expected 1−1.5 band shift due to 5′ end-labeling.[51,52] The G+A track shown in the right pair of panels was determined with the I+DAP-substituted DNA so it is strictly an I+DAP track; it is characterized by much stronger bands at the DAP residues than the inosines, which helps to confirm the correctness of the sequence and the faithful incorporation of I in place of G and DAP in place of A. The scales on the sides of the autoradiographs correspond to the standard numbering of the *tyr* T(A93) sequence as represented in Figure 4.

residues or both (Figure 3). The differences between normal and modified DNA are summarized in the histograms shown in Figure 4. With normal DNA, the iron−bleomycin complex cuts most strongly at GpC and GpT sequences (the underlined nucleotide indicates the reactive site). Some ApC and GpA sites, plus occasional ApT steps, are cleaved as well. When every purine residue bears a 2-amino group (DAP DNA) cleavage can be observed at almost all GpC, GpT, DAPpC, and DAPpT sites, i.e. at nearly all 5′-purine−pyrimidine (RpY) dinucleotide steps. Yet DNA fragments completely lacking the 2-amino group remain susceptible to cleavage by bleomycin. Inosine DNA is cut best at 5′-ApC sites, with weaker cleavage at certain IpC and ApT sequences. With Inosine DNA the majority of the strong cutting sites have a T residue at position 2 so it seems that 5′-TRYR sequences, particularly TACI, provide the preferred sites for cleavage by the antibiotic.[44] With the doubly substituted DNA containing I·C and DAP·T base pairs, the cleavage occurs principally at DAPpC and DAPpT sites. Occasional IpC sites are weakly cut by the iron−bleomycin complex.

Cleavage by Calicheamicin. Calicheamicin γ_1^I cuts normal DNA at a restricted number of sites such as 5′-TTCA, TTCT, TTAC, TCCC, and TTTT (Figure 5). As expected, the cuts appear displaced asymmetrically toward the 3′ ends of the complementary strands (Figure 6) due to binding of the antibiotic in the minor groove. Calicheamicin γ_1^I is known to cleave normal DNA via a pair of cuts separated by 2−3 nucleotides in a 3′ direction such that at least one of those cuts occurs preferentially at certain sequences.[28−36] Normal DNA is generally less well cleaved than modified DNAs under identical conditions. The difference is emphasized in Figure 7 which shows the results of a comparative concentration-dependence study using normal and doubly-substituted DNAs. Several sites of reaction with the two DNAs occur in much the same places but the intensity at a given antibiotic concentration is much stronger with the I+DAP DNA. This may in part reflect the fact that the readable portion of the *tyr*T(A93) DNA autoradiogram lacks certain calicheamicin-favored sequences such as 5′-TCCT, GCCT, TCCG, or TCTC. Figure 5 reveals additionally that cleavage of inosine DNA appears much more uniform: as well as strong reaction at a few canonical sites such as TTTT or TTCA there is significant non-specific cleavage indicating that removal of the 2-amino group is directly responsible for diminished sequence recognition by the enediyne compound. The results obtained with DAP and I+DAP DNA provide clues as regards the need for a calicheamicin−guanine interaction to promote a high level of DNA breakage. Cleavage at the underlined pyrimidine residue in the sequences TTCA·TGAA and TTCT·AGAA (positions 45 and 52, respectively) is abolished when the adjacent G·C pair is replaced by an I·C pair. Conversely, there are numerous sequences cleaved weakly or not at all in normal DNA which become exquisitely susceptible to calicheamicin attack when a 2-amino group is introduced on to the adjacent base pair. Such is the case at positions 33, 113, 44, and 60 (TTAC·GTAA, CCTT·AAGG, GTTC·GAAC, and TAAC·GTTA, respectively). At the latter three sites the I+DAP DNA is strongly cleaved whereas the DAP DNA remains insensitive, as observed with normal DNA. It is also noteworthy that although calicheamicin γ_1^I can cut homopyrimidine·homopurine sequences lacking a G·C base pair (such as those at positions 49 and 67, TTTT·AAAA and TTTA·TAAA, respectively) the extent of cleavage at such sites is considerably enhanced when a 2-amino group is introduced on to the purine residues of the complementary strand (A → DAP substitution).

Discussion

These results lead to the following conclusions: (i) Adding a 2-amino group on to adenine residues (A → DAP substitution) is sufficient to create new cleavage sites for both antibiotics. (ii) Removing the 2-amino group from guanine residues (G →

(44) The base adjacent to the cleaved dinucleotide can markedly affect the extent of damage. A pyrimidine on the 5′ side increases cleavage efficiency while a purine decreases cleavage. The base on the 3′ side of the dinucleotide can also modify the cleavage intensity to a lesser extent.[45]

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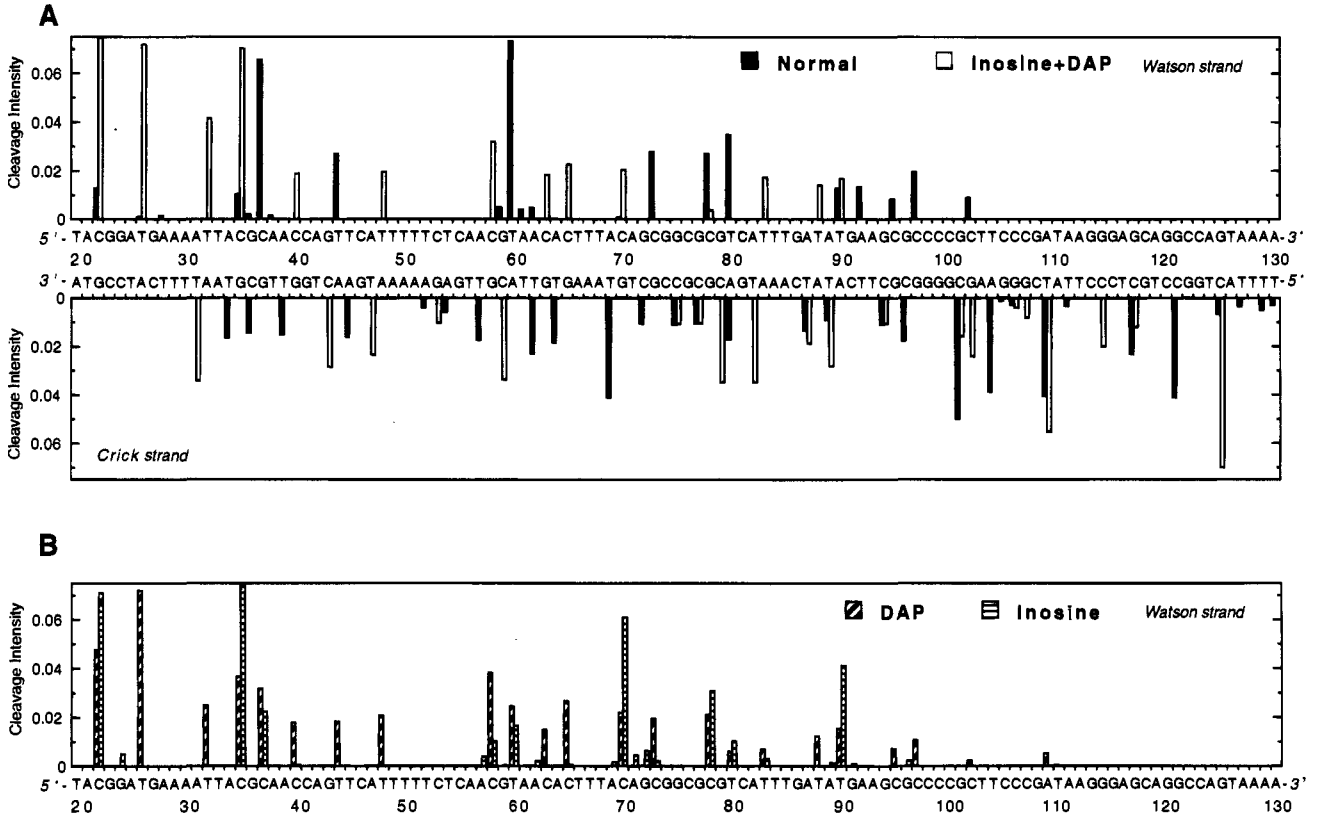

Figure 4. Susceptibility of normal and modified DNA to cleavage by bleomycin. In the modified nucleic acids adenine and/or guanosine residues are replaced by diaminopurine and/or inosine residues, respectively. The relative cleavage intensity (in arbitrary units) at a given bond is expressed as a fraction of the total cleavage of all the phosphodiester bonds within the sequence. Quantitative analysis was limited to regions where peaks were sufficiently well resolved to permit unambiguous analysis. Data are compiled from quantitative analysis of three sequencing gels (including the gel shown in Figure 3) and must be considered as a set of averaged values.

I substitution) does not abolish but reduces the DNA cleavage by bleomycin and greatly diminishes the sequence-specificity of calicheamicin γ_1^I. Therefore the 2-amino group is a powerful determinant but not absolutely necessary for DNA breakage by both bleomycin and calicheamicin. (iii) Shifting the 2-amino group from guanine to adenine residues (A → DAP plus G → I substitutions) produces a complete redistribution of the drug-mediated cleavage sites, showing that both antibiotics are extremely sensitive to the relocation of the purine 2-amino group in DNA.

Thus the 2-amino group of guanine constitutes a key structural element in the mechanism of DNA cleavage by the iron–bleomycin complex as well as by calicheamicin γ_1^I. The results strongly suggest that upon binding to specific sequences the antibiotic molecule engages in contact with the 2-amino group of guanine exposed in the minor groove, consistent with proposed models.[37–41] In particular, the results reported here agree fully with recent NMR studies on the interaction of bleomycin–Zn and bleomycin–Co complexes with the oligonucleotides d(CGCTAGCG)$_2$ and d(CCAGGCCTGG)$_2$, respectively.[38,46] In both cases, the bleomycin molecule is engaged in direct contact with the 2-amino group of guanine, either via one of its bithiazole ring nitrogens (Zn–bleomycin) or via the methyl group of the pyrimidinyl moiety (Co–bleomycin). For calicheamicin, our observations are entirely consistent with the recent NMR experiments on the interaction between d(GGAGCGC)·d(GCGCTCC) and the neocarzinostatin chromophore, another enediyne system, showing that the guanine exocyclic amino group plays a critical role in stabilizing the binding of this drug to double-stranded DNA.[47,48] However, it is clear that other aspects of DNA structure contribute to sequence recognition, especially by calicheamicin. Base substitutions remote from the cutting site can significantly affect the extent of cleavage by bleomycin,[45] and it has been suggested that the flexibility/deformability of the pyrimidine strand is exploited by calicheamicin γ_1^I to distort the DNA structure so that the drug can fit within the minor groove.[49–52] The fact that the inosine-containing DNA, expected to be a good deal more flexible than normal DNA, provides an acceptable substrate for bleomycin and calicheamicin γ_1^I cleavage is in accord with these ideas. Our data vindicate the hypothesis[49–53] that both DNA structure and interaction with guanine are involved in determining sequence-specific cleavage of DNA by bleomycin and calicheamicin.

Experimental Section

Antibiotics, Chemicals, and Biochemicals. Blenoxane (a mixture of 60% bleomycin A$_2$, 30% bleomycin B$_2$, and 10% other bleomycins)

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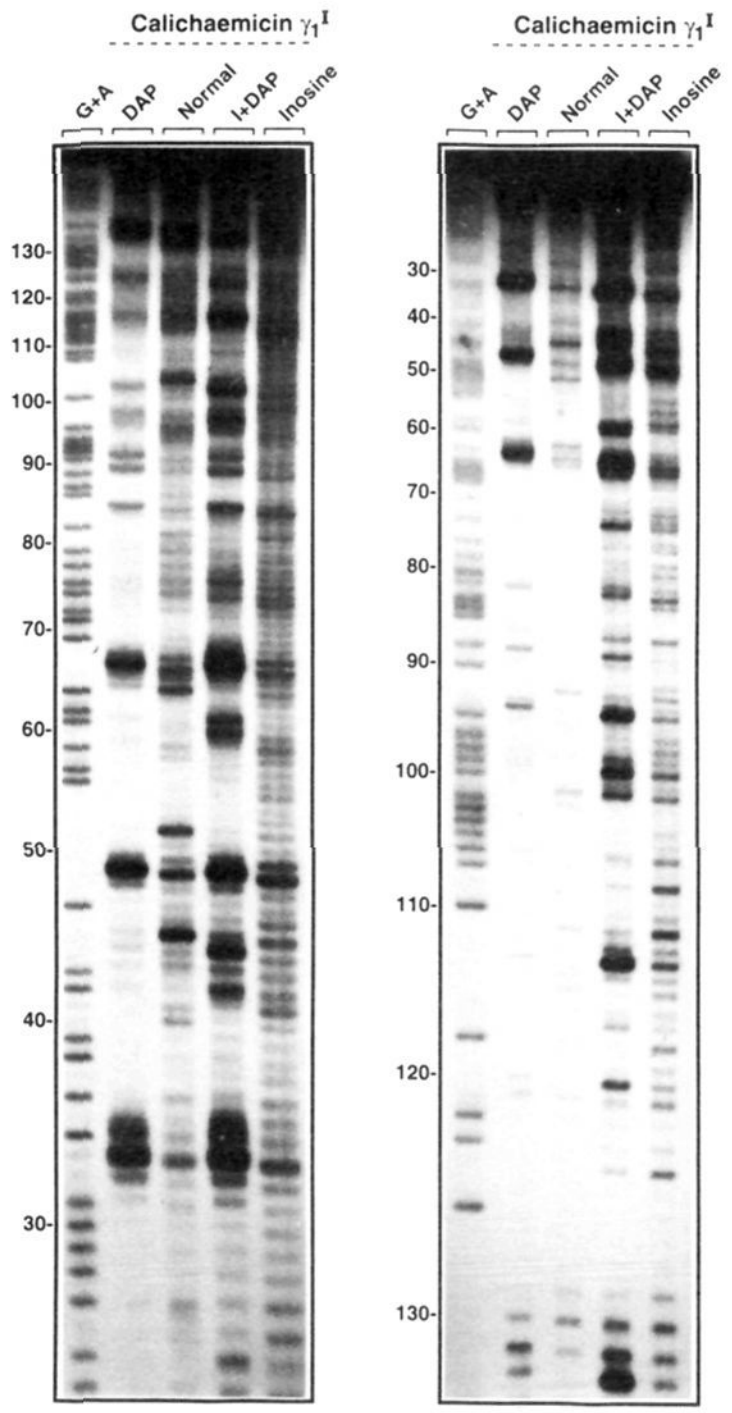

Figure 5. Cleavage of normal and modified DNA by calicheamicin γ_1^I (0.35 μg/mL). The left and right panels refer to the 5′-end labeled Watson and Crick strands of *tyr*T(A93) DNA, respectively. Other details as for Figure 4.

was obtained from Lunbeck. Stock solutions were prepared in 10 mM Tris−HCl buffer containing 10 mM NaCl (pH 7.0), divided into 250 μM aliquots, and stored at −20 °C. Calicheamicin γ_1^I from Cyanamid was dissolved in ethanol to furnish a stock solution at 1.75 μg/mL. Ammonium persulfate, tris base, acrylamide, bis-acrylamide, ultrapure urea, boric acid, tetramethylethylenediamine, and dimethyl sulfate were from BDH. Formic acid, piperidine, and formamide were from Aldrich. Photographic requisites were from Kodak. Bromophenol blue and xylene cyanol were from Serva. Unlabeled deoxynucleoside triphosphates, including dITP, were purchased from Pharmacia. The nucleoside triphosphate labeled with [^{32}P] (γ-ATP; 6000 Ci/mmol) was obtained from NEN Dupont. 2,6-Diaminopurine deoxyribonucleoside triphosphate was obtained by phosphorylation of the corresponding nucleoside (Sigma) according to published procedures.[54,55] Restriction endonucleases *Eco*RI and *Ava*I (Boehringer), *Taq* polymerase (Promega), DNase I (Sigma), and T4 polynucleotide kinase (Pharmacia) were used according to the supplier's recommended protocol in the activity buffer provided. The primers, 5′-AATTCCGGTTACCTTTAATC and 5′-TCGGGAACCCCCACCACGGG having a 5′-OH or 5′-NH_2 terminal group, were synthesized at the Laboratory of Molecular Biology, Medical Research Council, Cambridge. Checks were carried out to ensure that the primers blocked with a 5′-NH_2 group were free from contaminants and not labeled by the kinase. All other chemicals were analytical grade reagents, and all solutions were prepared using doubly deionized, Millipore filtered water.

Preparation, Purification, and Labeling of DNA Fragments Containing Natural and Modified Nucleotides. Plasmid pKMp27[43]- was isolated from *E. coli* by a standard sodium dodecyl sulfate-sodium hydroxide lysis procedure and purified by banding in CsCl−ethidium bromide gradients. Ethidium was removed by several 2-propanol extractions followed by exhaustive dialysis against Tris-EDTA buffer. The purified plasmid was then precipitated and resuspended in appropriate buffer prior to digestion by the restriction enzymes. The 160 base pair *tyrT*(A93) fragment used as a template was isolated from the plasmid by digestion with restriction enzymes *Eco*RI and *Ava*I. It is worth mentioning that this template DNA bore a 5′-phosphate due to the action of *Eco*RI and thus only the newly synthesized DNA (with normal or modified nucleotides) can be labeled by the kinase.

(a) Polymerase Chain Reaction (PCR). The protocol used to incorporate inosine and/or 2,6-diaminopurine residues into DNA is comparable to those previously used to incorporate 7-deazapurine or inosine residues with only a few minor modifications.[56−59] PCR reaction mixtures contained 10 ng of *tyrT*(A93) template, 1 μM each of the appropriate pair of primers (one with a 5′-OH and one with a 5′-NH_2 terminal group) required to allow 5′-phosphorylation of the desired strand, 250 μM of each dNTP (dTTP, dCTP plus dATP or dDTP and dGTP or dITP according to the desired DNA), and 5 units of *Taq* polymerase in a volume of 50 μl containing 50 mM KCl, 10 mM Tris−HCl, pH 8.3, 0.1% Triton X-100, and 1.5 mM $MgCl_2$. To prevent unwanted primer-template annealing before the cycles began, the reactions were heated to 60 °C before adding the *Taq* polymerase.[60] Finally, paraffin oil was added to each reaction to prevent evaporation. After an initial denaturing step of 3 min at 94 °C, 20 amplification cycles were performed, with each cycle consisting of the following segments: 94 °C for 1 min, 37 °C for 2 min, and 72 °C for 10 min. After the last cycle, the extension segment was continued for an additional 10 min at 72 °C, followed by a 5-min segment at 55 °C and a 5-min segment at 37 °C. The purpose of these final segments was to maximize annealing of full-length product and to minimize annealing of unused primer to full-length product. The reaction mixtures were then extracted with chloroform to remove the paraffin oil, and parallel reactions were pooled. Several extractions with water-saturated *n*-butanol were performed to reduce the volume prior to loading the samples on to a 6% non-denaturing polyacrylamide gel. After electrophoresis for about 1 h, a thin section of the gel was stained with ethidium bromide so as to locate the band of DNA under UV light. The same band of DNA free of ethidium was excised, crushed, and soaked in elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate) overnight at 37 °C. This suspension was filtered through a Millipore 0.22 μm filter and the DNA was precipitated with ethanol. Following washing with 70% ethanol and vacuum drying of the precipitate, the purified DNA was resuspended in the kinase buffer.

(b) DNA Labeling and Purification. The purified PCR products were 5′-end labeled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase according to a standard procedure for labeling blunt-ended DNA fragments.[61] After completion the labeled DNA was again purified by 6% polyacrylamide gel electrophoresis and extracted from the gel as described above. Finally, the labeled DNA was resuspended in 10 mM Tris−HCl buffer at pH 7.0 containing 10 mM NaCl.

Cleavage of DNA by the Bleomycin−Fe^{II} Complex and Calicheamicin γ_1^I. In a typical experiment, the freshly prepared bleomycin−Fe^{II} complex (4 μL) was added to 6 μL of 5′-end labeled DNA (~1 nM) in 10 mM Tris−HCl buffer at pH 7.0 containing 10 mM NaCl. The equimolar bleomycin−Fe complex consisted of 2 μL of a 25 μM

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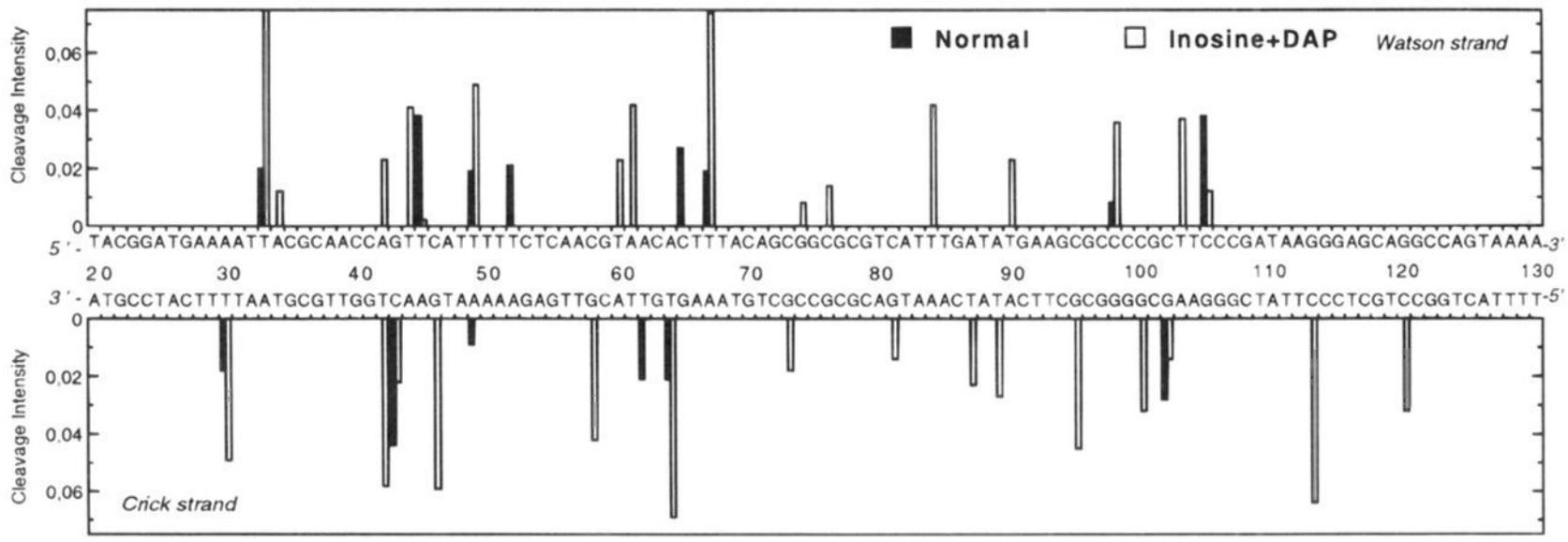

Figure 6. Susceptibility of normal and I+DAP DNA to cleavage by calicheamicin γ_1^I. Details as in Figure 4.

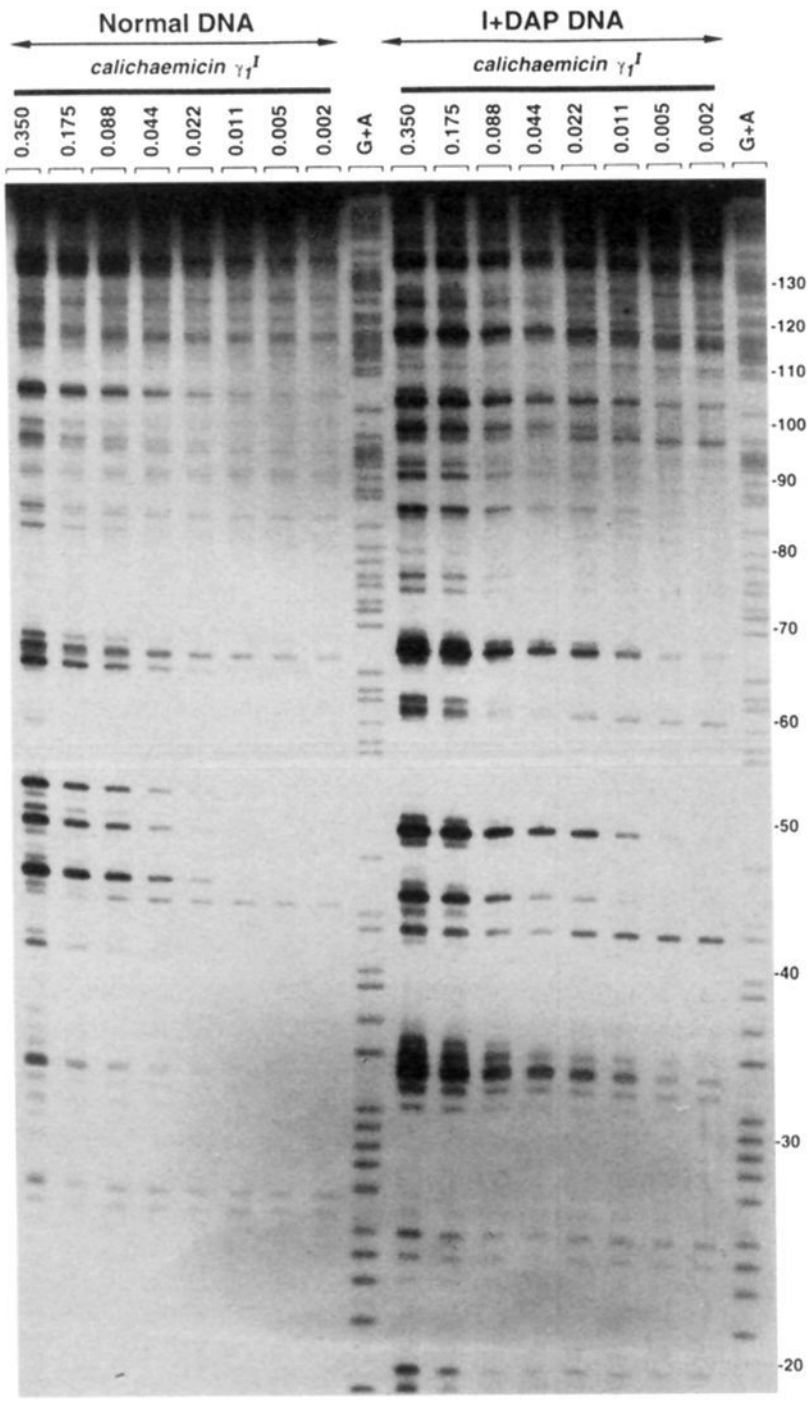

Figure 7. Cleavage of normal and inosine plus DAP-containing DNA in the presence of increasing concentrations of calicheamicin γ_1^I. The reactions were conducted in accordance with the procedure described in the Experimental Section. The calicheamicin concentration in (μg/mL) is indicated at the top of each lane.

solution of blenoxane and 2 μL of a 25 μM solution of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ mixed just prior to the experiment. After incubation at room temperature for periods varying from a few seconds to 1 min the cleavage reaction was stopped by freezing. Samples were lyophilized, resuspended in 50 μL of water, and lyophilized again. The cleavage products were resuspended in 4 μL of formamide−dye solution and resolved on a denaturing polyacrylamide gel as described below.

Two microliters of a 1.75-μg/mL stock solution of calicheamicin γ_1^I in ethanol were incubated with 7 μL of DNA (~1 nM) in 10 mM Tris−HCl buffer at pH 7.0 containing 10 mM NaCl. Solutions of calicheamicin were prepared by serially diluting the master stock solution into ethanol. The final ethanol content in the reaction mixture was 20%. An equivalent volume of ethanol was added to the control tubes. The DNA-calichaemicin γ_1^I solutions were equilibrated for 10 min prior to the initiation of the cleavage chemistry. At the chosen time, 1 μL of 1 mM dithiothreitol was added and the reaction allowed to proceed for 5 min at room temperature. After precipitation with ethanol, the DNA sample was resuspended in 4 μL of formamide−dye solution and the products resolved on a denaturing polyacrylamide gel. Samples were heated at 90 °C for 4 min and chilled in ice for 4 min prior to electrophoresis.

Electrophoresis, Autoradiography, and Quantitation by Storage Phosphor Imaging. DNA cleavage products were resolved by polyacrylamide gel electrophoresis under denaturing conditions (0.3 mm thick, 8% acrylamide containing 8 M urea) capable of resolving DNA fragments differing in length by one nucleotide. Electrophoresis was continued until the bromophenol blue marker had run out of the gel (about 2.5 h at 60 W, 1600 V in TBE buffer, BRL sequencer model S2). Gels were soaked in 10% acetic acid for 15 min, transferred to Whatman 3MM paper, dried under vacuum at 80 °C, and examined by autoradiography using either a phosphorimager or X-ray films (Fuji R-X) exposed at −70 °C with an intensifying screen usually for 24 h. For quantitative analysis, a Molecular Dynamics 425E PhosphorImager was used to collect data from storage screens exposed to the dried gels overnight at room temperature. Base line corrected scans were analyzed by integrating all the densities between two selected boundaries using ImageQuant version 3.3 software. Each resolved band was assigned to a particular bond within the *tyr*T(A93) fragment by comparison of its position relative to sequencing standards generated by treatment of the DNA with formic acid followed by piperidine-induced cleavage at the purine residues (G+A track).

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