# Characterization of a Covalent Monoadduct of Neocarzinostatin Chromophore at a DNA Bulge†

Lizzy S. Kappen and Irving H. Goldberg*

*Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115*

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ABSTRACT: Neocarzinostatin chromophore (NCS-Chrom) induces highly efficient site-specific strand cleavage at the bulge of a folded single-stranded 31-mer DNA in the presence of oxygen [Kappen, L. S., and Goldberg, I. H. (1993) *Science 261*, 1319−1321]. Under anaerobic conditions, the major product is a material having gel mobility slower than that of the parent 31-mer. In order to characterize this product, it was stabilized by reduction with borane/pyridine, labeled with $^{32}P$ at its 5′ or 3′ end, and subjected to chemical cleavage dependent on base elimination or modification, and the cleavage products were analyzed on a sequencing gel. A cleavage pattern comparable to that of the 31-mer was obtained until the bases on either side of $T_{22}$ at the bulge. Cleaved fragments inclusive of $T_{22}$ from the 5′ or the 3′ end had retarded and anomalous mobilities and appeared as a smear of bands closer to the starting material, presumably due to the presence of the covalently bound drug. Pyrimidine-specific agents such as hydrazine and potassium permanganate, but not the DNA sugar-specific probe thiol-activated NCS-Chrom, induced strand cleavage at $T_{22}$. Mass spectral analysis of the presumed adduct isolated from anaerobic reactions containing NCS-Chrom and a bulge duplex substrate made up of a 10-mer and an 8-mer showed that the adduct contains one molecule of the drug and one molecule of the 10-mer. Taken together, the results show that (i) drug adduction is at $T_{22}$ on the full-length substrate; (ii) the pyrimidine ring is accessible to base-specific chemical modifications, hence, presumably free of the drug; (iii) it is most likely that drug adduction is via its C6 position to the 5′ carbon of $T_{22}$, based on the current results and the known chemistry of the hydrogen abstraction by the drug in the presence or absence of oxygen; (iv) there is no involvement of the neighboring bases by way of inter- or intrastrand cross-linking; and (v) the product is a monoadduct.

The main target of the anticancer antibiotic neocarzinostatin is DNA. Its biologically active component is an enediyne chromophore (NCS-Chrom) (**1**, Scheme 1) that normally requires a thiol activator and duplex DNA for its substrate (reviewed in ref 1). NCS-Chrom induces strand cleavage and a variety of other lesions following its binding to the minor groove and its conversion to a diradical species upon nucleophilic attack by thiol at C12 (*2*). Strand cleavage is initiated by abstraction of hydrogen atoms at the 5′, 4′, or 1′ positions of the DNA sugar, generating carbon-centered radicals that in the presence of oxygen lead to strand breakage. In the absence of oxygen, NCS-Chrom forms covalent adducts on the DNA sugar (*3*).

While NCS-Chrom is a prototype of several other structurally related enediyne antibiotics that share common mechanisms in DNA damage, unlike any of them or other drugs, it is unique in its ability to induce highly efficient site-specific cleavage selectively at a bulge in DNA (*4*−*6*). The bulge-specific damage does not require a thiol activator. Drug activation, in this case, is by a novel mechanism (Scheme 1), which involves a base-catalyzed, intramolecular Michael addition of the phenol enolate at C1″ to C12 to give cumulene **2**, which by a Bergman-type rearrangement is converted to the biradical **3** (*7*, *8*). The abstraction by the diradical of the C-5′ hydrogen atom from the deoxyribose of the target nucleotide ultimately results, in the presence of oxygen, in a strand break having a $PO_4$ at the 3′ end and a nucleoside 5′ aldehyde at the 5′ terminus. In addition, the generation of a new drug product **5**, dependent on a competent bulged substrate and concomitant with strand cleavage, implicates the bulged DNA in the drug activation process by excluding radical-quenching solvent from the binding pocket and possibly by promoting a conformational change in the active drug intermediate so as to enable intramolecular carbon−carbon bond formation (**3a**) (*4*, *7*). The structure of a complex of a bulge DNA substrate and a stable analogue of the proposed NCS-Chrom diradical (**4**) has been elucidated recently (*9*).

In the absence of oxygen, there is no significant strand cleavage at the bulge; instead a product having mobility slower than that of the starting material and in quantity nearly equal to strand cleavage was found to be the main product in DNA bulges (*4*, *5*, *10*) and also in DNA−RNA hybrid bulges (*11*). This material, presumably a monoadduct or an interstrand cross-link or a mixture of both, can be important in the cytotoxic action of the drug, considering that bulged structures play a crucial role in many biological processes (*12*−*15*) and that in tumors under hypoxic conditions the adduct is likely to be the main lesion.

In this paper, we show that the main product in the anaerobic reaction of a bulge DNA substrate with NCS-Chrom is a covalent monoadduct, the drug most likely being linked via its C6 position to the 5′ carbon of the target nucleotide at the bulge target site.

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* To whom correspondence should be addressed.

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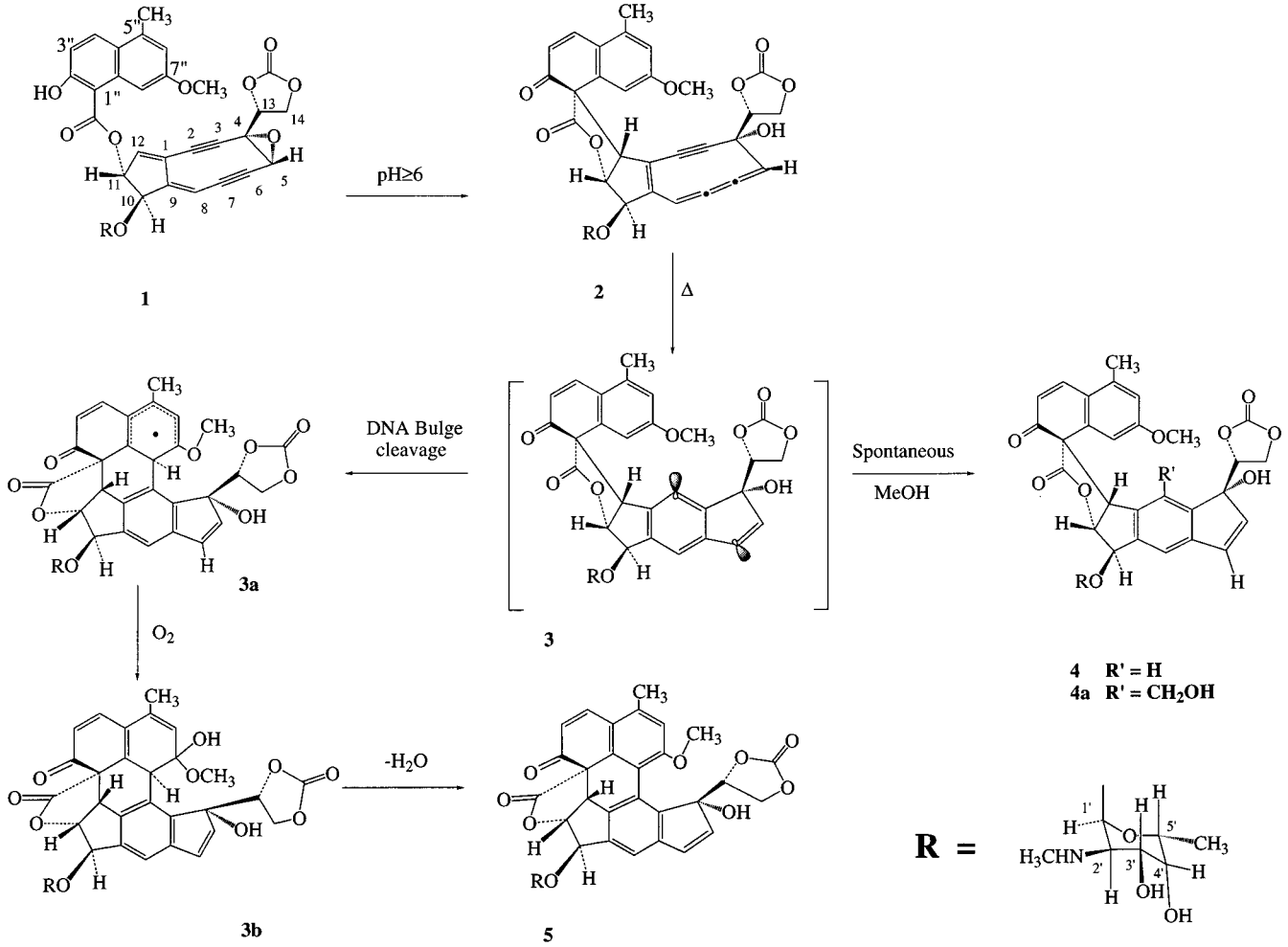
Scheme 1: Proposed Mechanism for Intramolecular Activation of NCS-Chrom at Basic pH in the Absence or Presence of Bulged DNA




## MATERIALS AND METHODS

*Nucleic Acid Substrates*. Oligodeoxyribonucleotides were purchased from Chemgenes or Midland Certified Reagent Company. Radioactive materials and enzymes were from New England Nuclear and New England Biolabs, respectively. Oligomers were 5′- or 3′-end labeled with $^{32}P$ using [$\gamma$-$^{32}P$]ATP and cordycepin 5′-triphosphate, respectively, by standard procedures (*16*). The labeled oligomers were purified by electrophoresis on a 15% sequencing gel. Neocarzinostatin powder (holo NCS) was purchased from Kayaku Antibiotics (Tokyo).

*Anaerobic Drug Reaction*. NCS-Chrom was extracted from the holoantibiotic by cold methanol containing 0.5 M acetic acid by a procedure similar to that described (*17*). The chromophore was stored at −70 °C protected against light. Anaerobic reactions were performed in a vessel equipped with a side arm as described (*18*). This procedure permits the removal of oxygen from the reaction vessel under conditions where the drug will be stabilized by binding to the substrate, but the damage is minimal or not at all until it is initiated by raising the pH. The substrate oligomers were placed in the main chamber in sodium acetate, pH 5.0, and Tris-HCl, pH 9.0, in the side arm. The vessel was cooled on ice prior to the addition of NCS-Chrom to the mixture in the main chamber. The contents of the flask were frozen and evacuated. Evacuation was repeated four times with intermittent thawing and freezing. The contents of the two

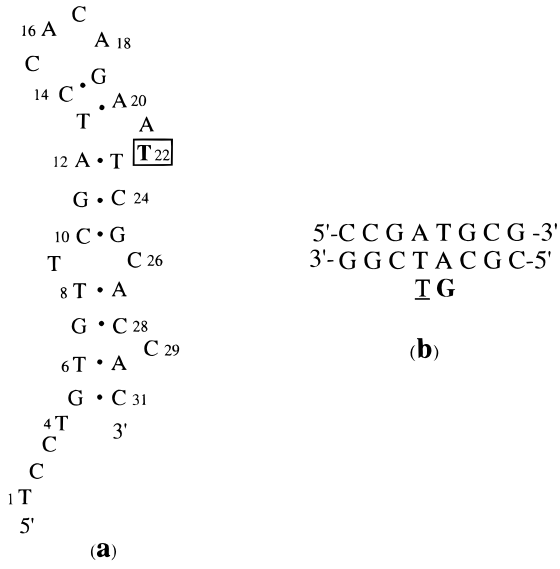



FIGURE 1: Sequences of the bulge DNA substrates: (a) 31-mer, the site of attack ($T_{22}$) is within the box; (b) 10-mer + 8-mer bulge duplex, the underlined T is the target residue.

chambers were then mixed, and the reaction was allowed to proceed in the dark on ice for 1 h. The standard reaction contained 15 mM sodium acetate, pH 5.0; 100 mM Tris-HCl, pH 9.1; 7.2 $\mu$M hairpin 31-mer oligodeoxyribonucleotide containing a two-nucleotide bulge (Figure 1a) with

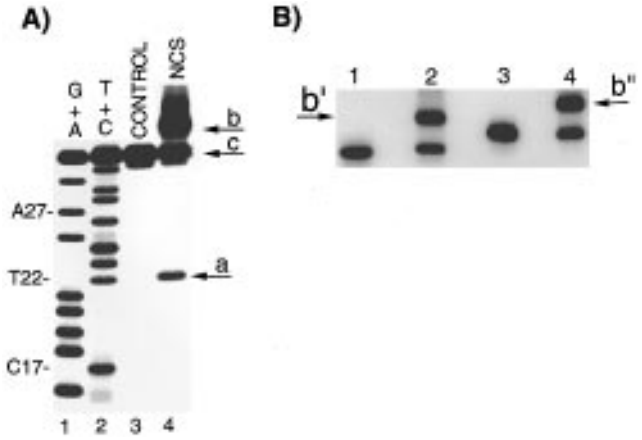



FIGURE 2: NCS-Chrom adduct formation at a DNA bulge under anaerobic conditions. (A) 5′-$^{32}$P end labeled 31-mer (Figure 1a) was treated with NCS-Chrom and analyzed on a sequencing gel as described in Materials and Methods. G+A and T+C are Maxam−Gilbert markers for the control 31-mer. Arrow b indicates the adduct. NCS-Chrom is indicated as NCS. (B) Reactions similar to those in Figure panel A having 5′-$^{32}$P end label (lane 1, control; lane 2, NCS-Chrom) or 3′-$^{32}$P end label (lane 3, control; lane 4, NCS-Chrom) were reduced prior to gel analysis. Arrows b′ and b″ indicate the reduced adducts. b″ has a slower mobility than b′ due to the presence of the cordycepin end label.

appropriate $^{32}$P end labels; and 68 $\mu$M NCS-Chrom. In the case of the bulge duplex substrate (Figure 1b), a mixture of non-radioactive 10-mer (78 $\mu$M) and the 8-mer, the latter in 1.5-fold excess, was placed in the main chamber and treated with NCS-Chrom (102 $\mu$M). Maximum methanol concentration from drug addition was 10%.

*Reduction and Isolation of the Adduct.* In order to stabilize the products, the reaction mixture was reduced with borane−pyridine (Aldrich) at room temperature for 20 h by the addition of a 1 M stock solution in 2-propanol (final concentration, 80 mM). DNA was recovered by ethanol precipitation, and the pellets were dissolved in 80% formamide and 1mM EDTA for analysis on a 15% DNA sequencing gel. Adduct bands were excised from the sequencing gels and soaked overnight at room temperature in 0.3 M sodium acetate/0.1 mM EDTA. The product in the gel eluate was recovered by ethanol precipitation.

The 10-mer adducts were purified by reverse-phase HPLC using an analytical Beckman C18 column and a linear gradient (1 mL/min) with solvents (A) $H_2O$ vs (B) 70% $CH_3CN$ in $H_2O$ both containing 50 mM triethylamine acetate pH, 7.0. The absorbance (254 nm) and fluorescence (excitation 400; emission 550) of the fractions were monitored. The fractions containing the peaks of interest (10-mer, 17 min; adduct, 23.2 min) were dried in a speed vac concentrator. The dry pellets were redissolved in $H_2O$ and dried again.

*Chemical Cleavage of the Isolated Adduct.* Chemical cleavage reactions specific for (T+C) was done using hydrazine (15 min, 37 °C) as described by Maxam and Gilbert (*19*). Cleavage reactions specific for G+A and T using formic acid and potassium permanganate, respectively, were carried out by published procedures (*20*). T reaction (37 $\mu$L) contained 38 mM ammonium bicarbonate, pH 7.0, and 0.19 mM $KMnO_4$. Incubation was at 37 °C for the times indicated in the figure legends. The reaction was terminated by the addition of allyl alcohol (14 $\mu$L) followed by freezing and drying in a speed vac concentrator. The pellets were suspended in 15 $\mu$L of $H_2O$ and dried two times before piperidine treatment. Two microgram of sonicated calf thymus DNA was added as carrier to all the reactions.

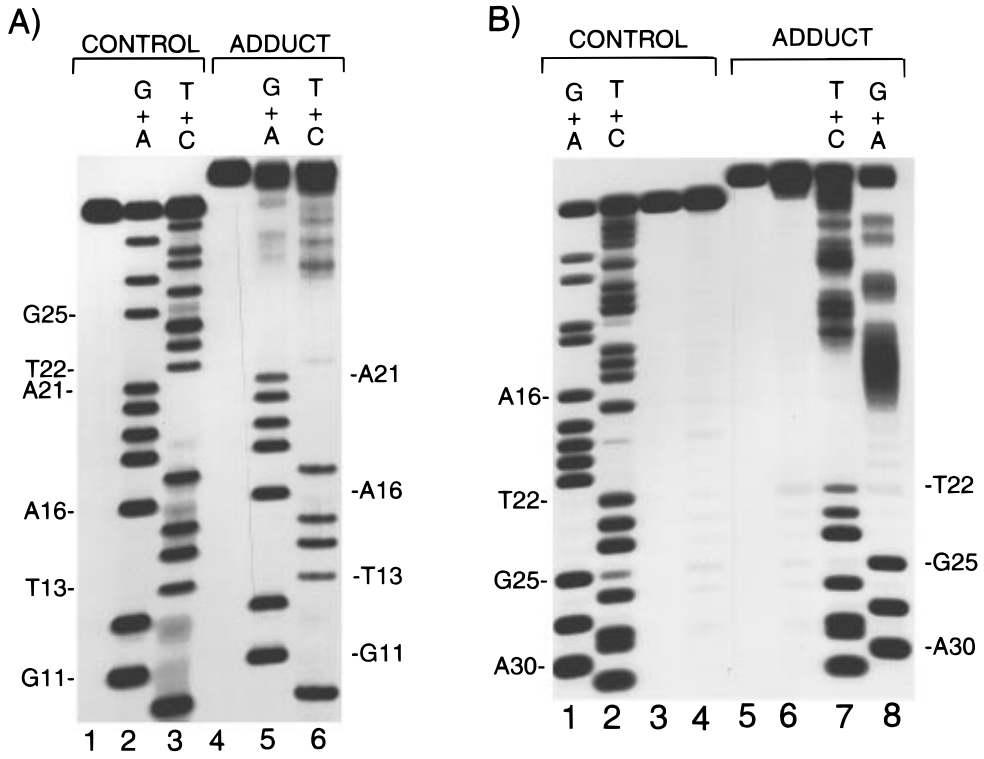



FIGURE 3: Chemical cleavage and sequencing of the NCS-Chrom−DNA adduct. (A) 5′-$^{32}$P end labeled control 31-mer and the adduct (Figure 2B, arrow b′) were subjected to G+A- and T+C-specific cleavage reactions as described in Materials and Methods. Lanes 1 and 4 contain 31-mer and the adduct, respectively, not treated with the cleaving agents. (B) Reactions similar to those in panel A contained 31-mer and the adduct having 3′-$^{32}$P end label (Figure 2B, arrow b″). Lane 3, 31-mer; lane 4, 31-mer heated in piperidine; lane 5, adduct; lane 6, adduct heated in piperidine.

Cleavage of the $^{32}$P-labeled 31-mer adduct or its control by thiol-activated NCS-Chrom in an aerobic reaction was carried out, after annealing with an excess of its complementary strand, by heating the mixture to 70 °C and slow cooling to room temperature. The annealing mixture contained $^{32}$P-labeled adduct or control 31-mer, unlabeled 31-mer (266 $\mu$M in nucleotide), twice as much of the complementary 31-mer, and 40 mM Tris-HCl, pH 8.0. In the final reaction, the components of the annealed mixture were diluted 1.6-fold by the addition of the rest of the reactants and $H_2O$. The mixture was cooled on ice before the addition of NCS-Chrom, followed by 2-mercaptoethanol (final concentration 10 mM). Reaction was allowed to proceed on ice for 1 h. NCS-Chrom concentrations are given in the figure legends. In experiments using 3′-$^{32}$P end labeled substrate, the cleavage products were heated in 0.1 M NaOH for 30 min at 90 °C to convert the nucleoside aldehyde at the 5′ end of the break to $PO_4$ as described (*21*).

*Product Analysis*. In order to determine strand cleavage, portions of the reaction mixture were dried, and the pellets were dissolved in 80% formamide containing 1 mM EDTA and marker dyes and analyzed on a 15% sequencing gel. Quantitation of the gel band intensities was done on a Molecular Dynamics phosphor imager. The 10-mer adduct, isolated from anaerobic reactions by HPLC, was subjected to mass spectral analysis by matrix-assisted laser desorption ionization time-of-flight (MALDI) mass spectrometry (Perseptive Biosystems).

## RESULTS AND DISCUSSION

*Adduct Stability*. Previous work has shown that a single-stranded 31-mer DNA, which in its folded, hairpin form contains a two-base bulge (Figure 1a), is an excellent substrate for NCS-Chrom in the base-catalyzed reaction. Site-specific strand cleavage occurs exclusively at $T_{22}$ in the bulge in the presence of oxygen (*4, 5*). Under anaerobic conditions, instead of strand cleavage (arrow a) as the major DNA damage product, a material amounting to 58% of the total radioactivity and having a mobility slower than that of the substrate is the predominant product (Figure 2A, lane 4, arrow b). The isolated product (arrow b), presumably a covalent drug−DNA monoadduct, interstrand cross-link, or a mixture of both, on heating with piperidine partially (20%) breaks down to generate a band at $T_{22}$ (data not shown). Reduction of this material prior to piperidine treatment protects it against degradation. The reduction may have protected the major product itself or a minor product having an abasic site. On the other hand, material isolated from the band corresponding in mobility to the parent 31-mer in the unreduced drug reaction (arrow c) does not show any cleavage on piperidine treatment (data not shown). It should be pointed out that the unreduced product, on heating in 0.1 M NaOH or piperidine, revealed on high-resolution gel analysis multiple bands with mobilities very close to that of the parent adduct itself, possibly due to the partial degradation of the bound drug. In experiments involving isolated drug adduct, borane−pyridine reduction of the reaction mixture preceded isolation (Figure 2B).

*Chemical Cleavage and Sequencing of the Adduct.* In order to locate the site of drug adduction on the DNA, the modified DNA was sequenced by chemical cleavage based on elimination or modification of the DNA bases, followed by piperidine treatment to induce strand cleavage (*19*). Analysis of the cleavage products from 5′-$^{32}$P end labeled 31-mer and adduct on a sequencing gel reveals that in the G+A-specific cleavage, dependent on depurination, the ladder of discrete fragments generated from the adduct (Figure 3A, lane 5) matches that of the control (lane 2) only up until and including $A_{21}$. After a considerable mobility void to the 3′ side of $A_{21}$, smeared bands appear closer to the starting material. In the hydrazine-dependent T+C cleavage, which results from an initial modification of the pyrimidine ring, the $T_{22}$ band in the adduct is very faint (Figure 3A, lane 6). A $T_{23}$ band is not found at its expected position, and its cleaved fragment is presumably included in the group of bands with much slower mobilities. A similar analysis carried out from the other end with 3′-$^{32}$P end labeled adduct (Figure 3B) also shows that the normal cleavage pattern stops at $T_{22}$ (lane 7) and that the $A_{21}$ band and others 5′ to it move anomalously slower (lane 8). As was found with the 5′-end labeled substrate, the band representing cleavage at $T_{22}$ is at the normal position but is relatively weak, possibly due to the hindrance of the attacking hydrazine by the adducted drug. The mobility gap and the slower moving fuzzy bands resulting from chemical treatments of both the 5′-end labeled and 3′-end labeled adduct substrates are consistent with the continued presence of the adducted drug on these oligonucleotide-cleaved fragments. These experiments allow us to localize the adduct to $T_{22}$.

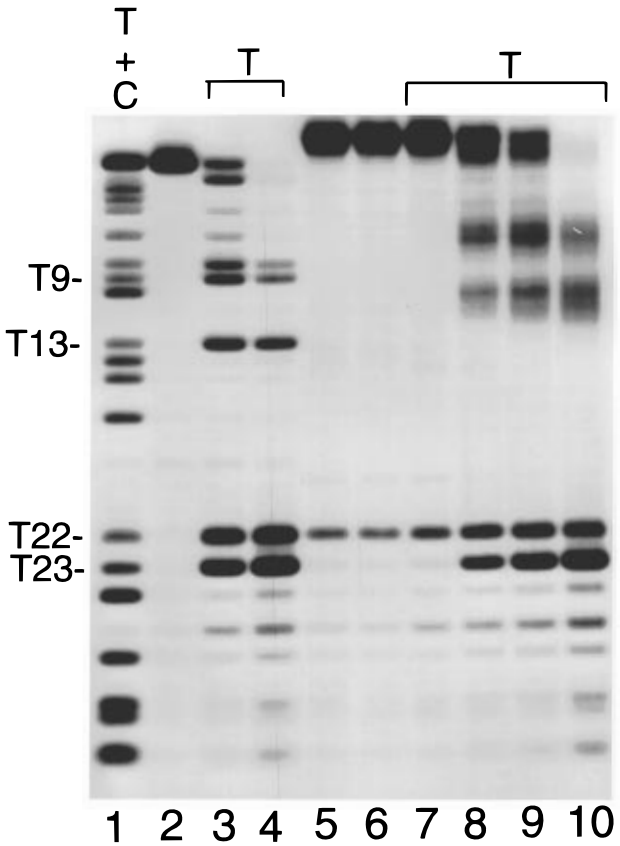



FIGURE 4: T-specific cleavage in NCS-Chrom adduct. 3′-$^{32}$P end labeled 31-mer and the adduct were reacted with $KMnO_4$ under conditions given in Materials and Methods. Lanes 1−4, 31-mer; lanes 5−10, adduct. Incubation times (min) with $KMnO_4$ were as follows: lane 3, 12; lane 4, 30; lane 7, 0; lane 8, 6; lane 9, 12; lane 10, 30. No $KMnO_4$ was added to the samples in lanes 2, 5, and 6 but were incubated for lane 2, 30; lane 5, 0; and lane 6, 30. All samples including those not reacted with $KMnO_4$ were heated in piperidine.

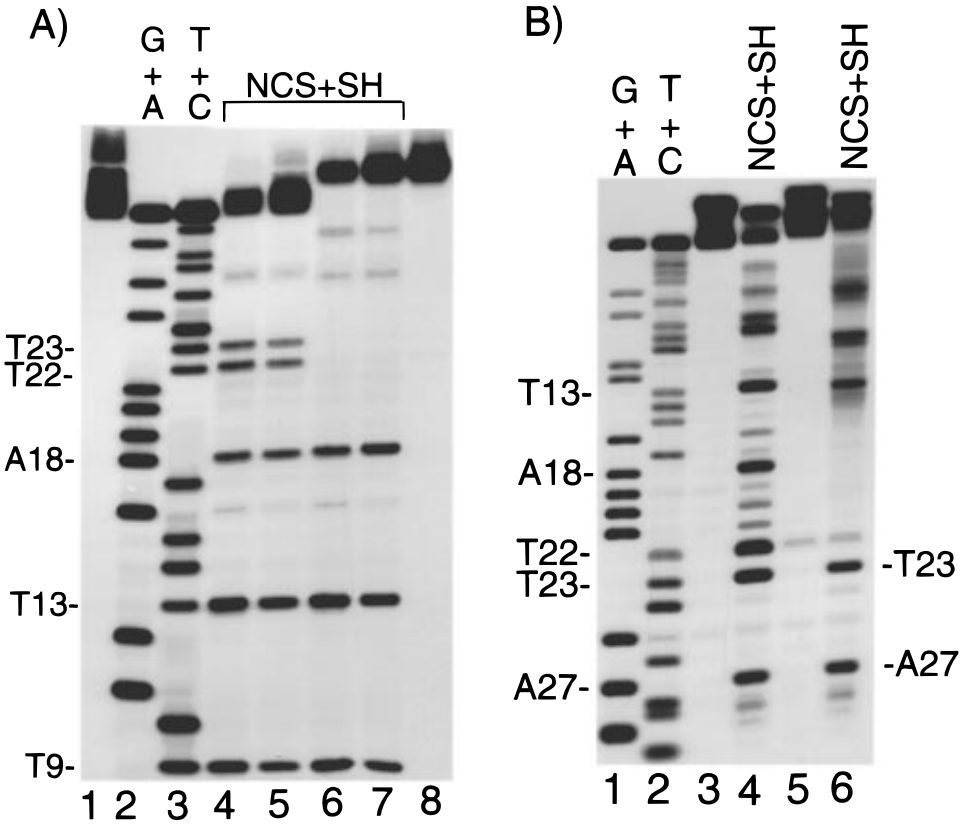



FIGURE 5: Cleavage of 31-mer and the isolated adduct by thiol-activated NCS-Chrom in an aerobic reaction. (A) 5′-$^{32}$P end labeled 31-mer and the adduct in their duplex form were treated with NCS-Chrom in the presence of a thiol (NCS+SH) as described in Materials and Methods. Lane 1, control 31-mer; lanes 2 and 3, Maxam−Gilbert markers for the control; lanes 4 and 5, 31-mer treated with NCS-Chrom 64 and 26 $\mu$M, respectively; lanes 6 and 7, adduct treated with NCS-Chrom 64 and 26 $\mu$M, respectively; and lane 8, adduct. (B) Reactions were similar to those in panel A except 3′-$^{32}$P end labeled 31-mer and adduct were used. Following the cleavage reaction, the reaction mixture was heated in alkali to convert the nucleoside aldehyde at the 5′ ends of the breaks to $PO_4$ (*21*). G+A and T+C are markers for the control 31-mer. Lanes 3 and 4, control 31-mer; lanes 5 and 6, adduct.

$KMnO_4$ induces T-specific cleavage (T ≫ C > G,A) by glycolization of the 5−6 double bond in pyrimidines followed by oxidation to carboxylic acid and/or aldehyde products and ring opening (*22*). The data presented in Figure 4 using 3′-$^{32}$P end labeled substrate shows that in the control 31-mer $KMnO_4$ induces cleavage selectively at every T residue (lane 3). $KMnO_4$ also produces from the adduct cleaved fragments at the expected position for $T_{22}$ and $T_{23}$, but all the other T sites generate bands with retarded mobilities.

Taken together, the chemical cleavage results show that (i) the drug adduction is at $T_{22}$ on the full-length substrate; (ii) the pyrimidine ring is accessible to base-specific chemical modifications, hence presumably free of the drug; (iii) the most likely position for drug adduction is the 5′ carbon of $T_{22}$, based on the known chemistry of the hydrogen abstraction by the drug in the presence or absence of oxygen (*18*) and on previous work with thiol-activated NCS-Chrom (*23, 24*); and (iv) there is no involvement of the neighboring bases by way of inter- or intrastrand cross-linking, since a normal cleavage pattern is obtained for bases on either side of $T_{22}$.

*Thiol-Activated NCS-Chrom as a Probe.* The cleaving agents used in the preceding experiments attack DNA bases. In order to further explore the nature of the adduct, we used, as a probe, thiol-activated NCS-Chrom, which is known to cleave duplex DNA preferentially at T and A residues (T > A > C ≫ G) (*25*) by attacking DNA deoxyribose in an oxygen-dependent reaction (*1*). The breaks, mainly initiated by abstraction of a hydrogen atom at the 5′ carbon, have a phosphate at their 3′ end and a nucleoside aldehyde at the 5′ end, the latter being converted to $PO_4$ on alkali treatment (*21, 26*). In the experiments presented in Figure 5A, 5′-$^{32}$P end labeled control 31-mer and its isolated adduct were annealed to the complementary strand to form a Watson−Crick duplex and then treated with NCS-Chrom in the presence of a thiol and oxygen. As expected, cleavage occurs mainly at T and A residues in the control (lanes 4 and 5), whereas in the adduct the cleavage pattern matches that of the control at A and T residues only till $A_{18}$ (lanes 6 and 7). In the adduct, both $T_{22}$ and $T_{23}$ bands are absent at the expected positions because a fragment resulting from cleavage at $T_{23}$ will contain the adducted drug at $T_{22}$ and, hence, will have altered mobility. On the otherhand, the fragment at the 3′ end of the break will be free of the bound drug and should have the same mobility as that of its counterpart in the control. This is the result obtained with the 3′-$^{32}$P end-labeled adduct (Figure 5B) where the $T_{23}$ band is strong (lane 6) and coincident with that in the normal 31-mer (lane 4). The lack of reactivity at $T_{22}$ to thiol-activated NCS-Chrom is consistent with drug adduction at the sugar of $T_{22}$.

*Adduct Formation on a 10-mer Bulge Duplex.* An oligomer duplex, composed of two separate strands and having the potential to form a bulge, is a good substrate for cleavage by NCS-Chrom exclusively at the bulge (*5*). We used a bulge duplex composed of a 10-mer and an 8-mer (Figure 1b) for further study. In an anaerobic reaction, the adduct is the main product. In experiments using $^{32}$P end

label on the 10-mer or the 8-mer strand of the bulge duplex, it was found that adduct formation occurs only on the 10-mer strand but that the 8-mer is needed to form the bulge structure (data not shown). An adduct from this system was purified by HPLC for mass spectral analysis. In order to ensure that the isolated material is a drug adduct, it was labeled with $^{32}P$ at the 5′ or the 3′ end and subjected to chemical cleavage and gel analysis similar to those performed for the 31-mer adduct. The results (not shown) confirmed that the isolated material is the expected NCS-Chrom adduct on the T at the bulge. Further, analysis of the 10-mer adduct by MALDI mass spectrometry confirms that only one molecule of NCS-Chrom and one molecule of 10-mer is present in the adduct [10-mer: 3044.05 (calculated), 3044.94 (observed); 10-mer + drug: 3703.27 (calculated), 3703.43 (observed); drug: 659.22 (calculated), 658.49.(observed)].

## CONCLUDING REMARKS

In the present study, we used chemical cleavage combined with DNA sequencing to locate the site of NCS-Chrom adduction on the bulge DNA. Cleavage of the adduct-containing DNA strand (Figure 3) gives a normal cleavage pattern till the bases on either side of $T_{22}$ ($A_{21}$ and $T_{23}$), suggesting that the drug adduction is at $T_{22}$, which is also the same single site for oxygen-dependent strand cleavage (*4*, *5*). This is not surprising since the drug activation does not require oxygen and hydrogen atom abstraction from C5′ of DNA deoxyribose occurs under both aerobic and anaerobic reactions (*18*).

In order to account for the generation of only single-strand breaks at the bulge in the oxygen-dependent cleavage reaction, it was proposed that intramolecular quenching of the C2 radical, perhaps through the involvement of the DNA bulge structure, leaves a monofuctional species with a radical center at C6, which abstracts a 5′ hydrogen atom at the target T to generate a C5′ deoxyribose radical. In the aerobic reaction, further oxidative steps at C5′ will lead to strand cleavage. In the absence of oxygen, the radical at C5′ of the target T may react with the double bond at the C6 position of a drug product, such as shown in **3** in Scheme 1. It is likely that the linkage of NCS-Chrom to the DNA is via its C6 to the C5′ of the target nucleotide. Drug adduction to the carbon-centered radical at C5′ is analogous to the labile, covalent addition products formed with dioxygen or the nitroaromatic radiation sensitizer (and oxygen substitute) misonidazole. In the case of dioxygen, a peroxy radical species is believed to be formed, while with mosonidazole evidence has been obtained for the formation of a nitroxyl radical adduct intermediate at C5′ (*27*, *28*). In the latter case, as expected, under anaerobic conditions, misonidazole adduct formation prevents the formation of the drug DNA monoadduct (data not shown).

The finding that $T_{22}$ itself is amenable to attack by agents such as hydrazine and $KMnO_4$ (Figures 3 and 4) that initiate strand cleavage by reacting with the pyrimidine base is also consistent with covalent drug binding to the deoxyribose at $T_{22}$ and not to the base. This notion is also consistent with the absence of strand cleavage at $T_{22}$ (Figure 5) on treatment of the isolated adduct with thiol-activated NCS-Chrom, although steric hindrance by the adducted drug in the DNA minor groove is likely.

Unlike in the case of C1027, a closely related enedyine antibiotic which forms interstrand cross-links and monoadducts (*29*), the main product in the anaerobic reaction of NCS-Chrom at the DNA bulge is a monoadduct. By forming covalent adducts on DNA sugars (*23*, *24*), NCS-Chrom is also different from many other DNA-damaging agents, such as mitomycin C (*30*), *cis*-diamminedichlroplatinum(II) (*31*), psoralen (*32*, *33*), and benzo[*a*]pyrene (*34*), all of which form adducts on the DNA bases.

## ACKNOWLEDGMENT

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