Double-Strand Cleavage of DNA by a Monofunctional Transition Metal Cleavage Agent

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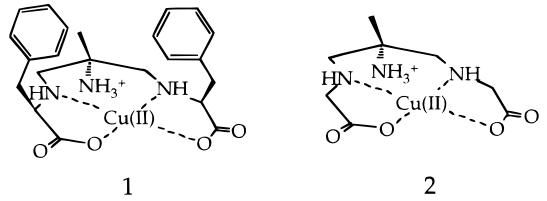
Abstract: A copper-based transition metal complex has been designed which performs double-stranded cleavage of DNA in a nonrandom fashion. The complex, ((2*S*,8*R*)-5-amino-2,8-dibenzyl-5-methyl-3,7-diazanonanedioate)copper-(II), presents an ammonium group on one side of the metal equatorial coordination plane to the DNA backbone phosphate groups, while the aromatic phenylalanine-derived side chains are constrained to the opposite side of the coordination plane toward the DNA groove. This structure was designed to bind at locations where phosphate groups are in proximity to accessible hydrophobic regions of the DNA. We have estimated single-strand break to double-strand break ratios for DNA strand scission by this complex under a variety of activation conditions, and they are substantially lower than that predicted by statistical models for a random DNA linearization process. This means that more double-strand breaks are produced per single strand break than can be accounted for by random coincident single-strand breaks. We have also investigated the formation of abasic sites, and found that at least as many abasic sites can be cleaved to linear DNA as are linearized in the initial cleavage reaction. We interpret this to mean that the complex binds both at the intact DNA surface for strand scission, and binds at nicked sites on the DNA (where the charged end groups of the nick are likely to be proximate to the accessible hydrophobic interior) for reactivation and complementary strand scission. Insofar as double-strand cleavage may be more potent biologically than single-strand cleavage as a source of lethal DNA lesions, the recognition characteristics of this complex may aid in the design of chemotherapeutic agents.

Introduction

Among the strategies available for chemotherapeutic treatment of cancer is the use of naturally occurring antitumor antibiotics.[1] As a group, these complex molecules create a variety of different types of DNA lesions, ranging from DNA cross-linking to strand scission. The antitumor agents whose mode of action entails DNA strand scission fall into separate categories by mechanism: those which generate a difunctional carbon-based biradical cleaving moiety (enediynes)[2] and those which form activated oxygen species (bleomycins).[3] Both types of antibiotics can create both single-strand breaks (ssb) and double-strand breaks (dsb) in duplex DNA.[4] Double-strand breaks are thought to be more significant biologically than are ssb's as sources of cell lethality, because they apparently are less readily repaired by DNA repair mechanisms.[5,6]

Because of the biological and clinical importance of double-strand cleavage as a mode of cell lethality, we have investigated the design of a synthetic transition metal-based DNA cleavage agent which performs double-stranded DNA cleavage in a nonrandom fashion. A monofunctional transition metal-based double-stranded cleavage agent must effect four events: nick formation, binding at the nicked site, activation, and complementary strand scission to form linear DNA. The design of a molecule which can effect these activities must therefore include a reactivatable metal center and recognition elements that bind both to duplex DNA and at nicked sites. We have begun our study using square-planar copper-containing metal complexes that have pendant ammonium groups with amino acid side chains as recognition elements. Our initial attempt employs a ligand framework (5-amino-5-methyl-3,7-diazanonane-1,9-dioate) that fixes a positively charged group above the square-planar coordination plane of the molecule, with hydrophobic amino acid side chains constrained to the opposite side of the coordination plane.

Chart 1. Structures of Complexes **1** and **2**



1 2

The rationale behind this molecular architecture is that the positively charged ammonium group can interact with phosphates on the DNA backbone, orienting the amino acid side chains toward the bottom of the groove, for interaction with the hydrophobic interior. For our initial complexes, we have used phenylalanine as the amino acid side chain group in one version of the molecule, ((2*S*,8*R*)-5-amino-2,8-dibenzyl-5-methyl-3,7-diazanonanedioate)copper(II) (**1**), and glycine, (5-amino-5-methyl-3,7-diazanonanedioate)copper(II) (**2**), in the other, as a control for the effects of the hydrophobic side chain groups (Chart 1). The hydrophobic side chains of **1** are available for interactions with DNA bases; hydrophobic interactions are expected to be significant for binding of **1** to the intact DNA

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(1) Neidle, S.; Waring, M., Eds. *Molecular Aspects of Anticancer Drug−DNA Interactions*; CRC Press: Boca Raton, FL, 1993−4; Vols. 1 and 2.

(2) Goldberg, I. H. *Molecular Aspects of Anticancer Drug−DNA Interactions*; Neidle, S., Waring, M., Eds.; CRC Press: Boca Raton, FL, 1993; Vol. 1, p 243.

(3) Natrajan, A.; Hecht, S. M. *Molecular Aspects of Anticancer Drug−DNA Interactions*; Neidle, S., Waring, M., Eds.; CRC Press: Boca Raton, FL, 1994; Vol. 2, p 197.

(4) Dedon, P. C.; Goldberg, I. H. *Chem. Res. Toxicol.* **1992**, *5*, 311.

(5) Povirk, L. F. *Molecular Aspects of Anti-cancer Drug Action;* Neidle, S., Waring, M., Eds.; Verlag-Chemie: Weinheim, 1983; p 157.

(6) Povirk, L. F. *Mutat. Res.* **1991**, *257*, 127.

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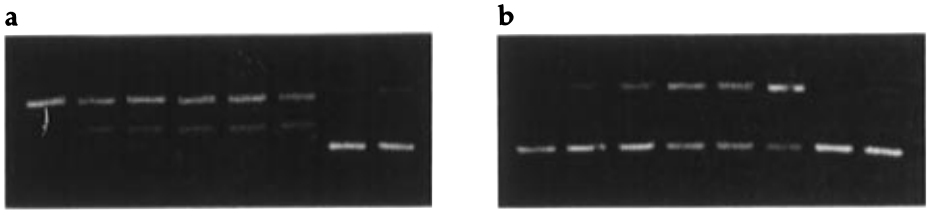


Figure 1. pUC18 DNA scission chemistry of **1** (a) and **2** (b) as a function of time as shown by agarose gel electrophoresis. Cleavage conditions: 10 μM base pairs, 123 μM metal complex, 150 μM sodium ascorbate, 150 μM H_2O_2, 22.5 °C, 20 mM Tris, 2.5 mM sodium acetate buffer, pH 7.5. Reaction was quenched by the addition of 10 mM EDTA buffer after the required reaction time. Reaction times at room temperature for lanes 1−6 [both (a) and (b)]: 3, 15, 30, 60, 90, and 120 min. Lane 7, DNA alone; lane 8, DNA + metal complex only (120 min incubation each).

surface and to the nicked site, where the hydrophobic interior may be more accessible than in intact DNA.

Experimental Section

UV−vis spectra were recorded on an SLM-Aminco Milton-Roy 3000 diode array spectrophotometer. Infrared spectra were recorded as KBr pellets using a Midac M1200 FTIR spectrophotometer. pH titrations were performed using a Fisher Scientific pH meter. All materials were reagent grade and were used without further purification unless otherwise noted.

Synthesis. Complexes $\mathbf{1}^{+}Cl^{-}\cdot DMF\cdot H_2O$ and $\mathbf{2}^{+}Cl^{-}\cdot H_2O$ were synthesized by literature procedures.[7−9]

Electrophoresis. Agarose gel electrophoresis was performed using 1% agarose gels under standard conditions. Samples, usually 20 μL, were run on horizontal gels using 100 mM Tris, 100 mM borate, and 2 mM Na_2EDTA (pH 8) as buffer. Electrophoresis was continued typically for 60 min at 12 V/cm. After electrophoresis, gels were ethidium stained and destained, and then visualized under UV light.

DNA Scission Conditions. Scission reactions were performed in Tris (20 mM)−acetate (2 mM) buffer (pH 7.5) or 20 mM phosphate buffer (pH 7.0) in either a 20 or 25 μL volume using 10 μM base pairs pUC18 plasmid DNA. Reaction was quenched by the addition of loading buffer (final EDTA concentration was 0.02 M) after the required reaction time.

For determination of AP site formation,[10] activation conditions in 20 mM pH 7.5 phosphate buffer were as follows: 20 μM DNA base pairs; 200 μM metal complex; either 250 μM or 1 mM sodium ascorbate/H_2O_2. Phosphate buffer was used to avoid AP site formation by the amine group of Tris.[11] The formation of AP sites was estimated as follows. The cleavage reaction was allowed to proceed for varying lengths of time, and then the reaction mixture was diluted (1:1) with either pH 12 or 7 buffer containing EDTA (1 mM) to quench the cleavage reaction. The pH of the alkaline solution was estimated to be 10.4 from a parallel high-volume reaction with calf thymus DNA under identical reaction conditions. The reaction was then incubated at room temperature for 2 h, followed by electrophoresis. The total duplex DNA quantitated for the high pH reactions was similar to that found for the neutral reactions, suggesting that the alkaline conditions were nondenaturing. A control lane of cleaved but unincubated DNA was used to normalize the AP lanes.

Densitometric quantitation was performed using a Macintosh Quadra 950 equipped with NIH Image software.[12] Supercoiled plasmid DNA values were corrected by a factor of 1.3, based on average literature estimates of lowered binding of ethidium to this structure.[13,14]

Hydroxy Radical Assay. Hydroxy radical production by **1** and **2** was assayed by a standard method with 2-deoxy-D-ribose followed by quantitation of the product with 2-thiobarbituric acid.[15] Metal complex (102 μM) was reacted with 2-deoxy-D-ribose (1.0 mM) in the presence of 188 μM concentrations of H_2O_2 and sodium ascorbate in a 4 mL volume of 20 mM phosphate buffer (pH 7.0). A high ribose concentration was employed to maximize trapping of hydroxy radical. Parallel runs containing hydroxy radical quenchers DMSO or thiourea (1 mM) were run under identical conditions. Reaction solutions were quenched with EDTA (2.4 mM final concentration) and stored on ice. Each aliquot was then reacted with 2-thiobarbituric acid at 95 °C for 15 min. The chromophore concentration was measured by fluorescence intensity at 553 nm (532-nm excitation). Fluorescence intensity was converted to concentration with a standard fluorescence curve constructed using known concentrations of the chromophore, synthesized from malondialdehyde and 2-thiobarbituric acid.[16] Blank reactions lacking cleavage agent were included in each run, and the blank intensity was subtracted from each experimental point.

Results

DNA Scission Chemistry. In order to assess the competence of **1** and **2** for DNA strand scission, each complex was incubated with pUC18 plasmid DNA under identical reaction conditions, using ascorbate/H_2O_2 activation.[17−21] As shown in Figure 1, both complexes are competent to effect DNA strand scission. The difference between the cleavage agents is, however, that under the conditions used, **2** converts supercoiled plasmid to a mixture of supercoiled and nicked DNA, while **1** evidences a much higher DNA cleavage efficiency, producing a mixture of nicked and linear DNA, with no supercoiled plasmid remaining. In addition, the appearance of a well-defined electrophoresis band for linear DNA by **1** suggests that the production of linear DNA by **1** is due to a nonrandom strand scission event, since random cutting is expected to produce a smear of fragments.

Densitometric quantitation of the gel electrophoresis results presented in Figure 1a is shown in Figure 2. Complex **1** rapidly converts supercoiled DNA to nicked DNA in $\ll$15 min of reaction while complex **2** produces ~50% nicked DNA after 120 min of reaction (Figure 1b). Furthermore, complex **1** (in a slower process) converts nicked DNA cleanly to linear DNA in a ~1:2 ratio of linear to nicked DNA, a reaction which **2** does not perform under the conditions used. The maximum

(7) Comba, P.; Curtis, N. F.; Lawrance, G. A.; Sargeson, A. M.; Skelton, B. W.; White, A. H. *Inorg. Chem.* **1986**, *25*, 4260.
(8) Comba, P.; Hambley, T. W.; Lawrance, G. A.; Martin, L. L.; Renold, P.; Varnagy, K. *J. Chem. Soc., Dalton Trans.* **1991**, 277.
(9) Balla, J.; Bernhardt, P. V.; Buglyo, P.; Comba, P.; Hambley, T. W.; Schmidlin, R.; Stebler, S.; Varnagy, K. *J. Chem. Soc., Dalton Trans.* **1993**, 1143.
(10) Lloyd, R. S.; Haidle, C. W.; Hewitt, R. R. *Cancer Res.* **1978**, *38*, 3191.
(11) Strauss, B.; Hill, T. *Biochim. Biophys. Acta* **1970**, *213*, 14.
(12) The NIH Image program (written by Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd.,Springfield, VA 22161, part number PB93−504868) is public domain software.
(13) Hertzberg, R. P.; Dervan, P. B. *J. Am. Chem. Soc.* **1982**, *104*, 313.
(14) Mirabelli, C. K.; Huang, C.-H.; Crooke, S. T. *Cancer Res.* **1980**, *40*, 4173.
(15) Halliwell, B.; Gutteridge, J. M. C. *CRC Handbook of Methods for Oxygen Radical Research*; Greenwald, R. A., Ed.; CRC Press, Inc.: Boca Raton, FL, 1985; p 177.
(16) Burger, R. M.; Berkowitz, A. R.; Peisach, J.; Horwitz, S. B. *J. Biol. Chem.* **1980**, *255*, 11832.
(17) Chiou, S. H.; Chang, W. C.; Jou. Y. S.; Chung, H. M. M.; Lo, T. B. *J. Biochem. (Tokyo)* **1985**, *98*, 1723.
(18) Mack, D. P.; Iverson, B. L.; Dervan, P. B. *J. Am. Chem. Soc.* **1988**, *110*, 7572.
(19) Mack, D. P.; Dervan, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 4604.
(20) Mack, D. P.; Dervan, P. B. *Biochemistry*, **1992**, *31*, 9399.
(21) Shullenberger, D. F.; Eason, P. D.; Long, E. C. *J. Am. Chem. Soc.* **1993**, *115*, 11038.

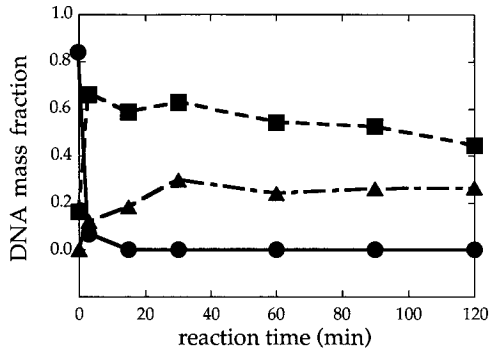


Figure 2. DNA composition as a function of reaction time with **1** (quantitation of Figure 1): (●) fraction of supercoiled plasmid; (▲) fraction of linear DNA; (■) fraction of nicked DNA. Fractions are calculated based on the total intensity of the DNA control lane, with the nicked fractions corrected for nicked DNA present prior to reaction.

amount of form III linear DNA produced by cleavage with **1** is reached after ca. 30 min of reaction.

The comparison of **1** and **2** in Figure 1 illustrates some significant features of the DNA scission chemistry of each. First, it shows that both **1** and **2** are competent to effect cleavage chemistry. **1** nicks plasmid DNA much more efficiently than **2** and unlike **2** can produce well-defined electrophoresis bands of linear DNA. The difference in cleavage behavior between **1** and **2** suggests that neither complex releases copper(I) on reduction, since there would be no ligand dependence on DNA scission if this were the case. Finally, the differences in cleavage characteristics of **1** *vs* **2** appear to be due to the phenylalanine side chains of **1**, since the molecules are matched in all other respects. The aromatic side chains appear to play a role in binding related to both nicking and linearizing plasmid DNA, since **1** is more efficient than **2** at both of these activities.

Investigation of DNA Linearization by 1. The observation that complex **1** produces a well-defined electrophoresis band for linear DNA from supercoiled DNA suggests that **1** mediates reaction at nicked sites to produce proximate cuts on the complementary strand to form linear DNA. In order to assess whether the observed linearization truly represents a nonrandom linearization process, the statistical test of Povirk *et al.* that has been used to assay bleomycin[22,23] and calicheamicin[24] double-strand cleavage was applied. This test assumes a Poisson distribution of strand cuts, and calculates the average number of dsb's per molecule, n_2, from the fraction of linear DNA after strand scission chemistry, and the total average number of single- plus double-strand breaks ($n_1 + n_2$), from the fraction of remaining supercoiled DNA after reaction.[25] The values for n_1 and n_2 are found for a range of cleavage conditions. The resulting values are then compared to the Freifelder−Trumbo relationship,[26] which describes the number of dsb's expected from a process of random ssb formation.

In order to determine n_1 and n_2 for our system, a variety of combinations of activating agent concentration, metal complex concentrations, and reaction times were used. Varied conditions were required, since the calculation of n_1 and n_2 requires both the quantitation of the amount of linear DNA formed during a reaction and the amount of supercoiled DNA remaining. Since the kinetics of nicking of **1** are fast relative to the rate of linearization, under conditions of rapid reaction, a large pool of nicked DNA forms with little measurable supercoiled DNA remaining, or if reaction conditions are adjusted for low rates of reaction, supercoiled DNA remains but little linear DNA is formed. Thus, reaction concentrations and times must be balanced to lead to formation of linear DNA while supercoiled DNA still remains so that the required fractions of DNA can be obtained.

Table 1. Statistical Efficiency of Single-Strand and Double-Strand Break Formation by **1** as a Function of Reaction Conditions[a]

trial	[**1**] (μM)	[H_2O_2], [ascorbate] (μM)	reaction time (min)	n_1	n_2	n_1/n_2
1	50	188	10.0	1.24	0.19	6.5
2	123	150	3.0	1.88	0.24	7.8
3	82	1000	0.6	1.83	0.16	11.4
4	16	1000	4.0	2.12	0.18	11.8
5	102	150	2.0	0.97	0.05	19.4
6	100	150	20.0	2.39	0.12	19.9
7	50	150	20.0	1.09	0.05	21.8
8	123	150	2.0	1.11	0.05	22.2
9	150	50	60.0	2.60	0.11	23.6
10	200	50	20.0	1.23	0.04	30.7

[a] 10 μM base pairs pUC18 DNA, 20 mM phosphate buffer, pH 7.0, 23 °C.

Table 1 shows the statistical results of DNA linearization experiments performed under a variety of reaction conditions. Using the maximum n_1 value from Table 1 (which provides the most conservative estimate of random dsb formation), the Freifelder−Trumbo relation suggests that approximately 120 ssb's are required per dsb if the process is completely random. Under all reaction conditions tried, $n_1/n_2 \ll 120$ (Table 1), suggesting a nonrandom process for all combinations of reaction conditions. Comparable values, in the range of 5−20, have been observed for iron bleomycin, depending on the DNA and analytic method used.[27] The statistical data in Table 1 for strand scission by complex **1** show that under a variety of scission conditions, linear DNA is formed with greater efficiency than can be accounted for by proximate random cuts on opposing strands. Table 1 also demonstrates that there is no simple relationship between the ultimate efficiency of dsb formation, as indicated by the n_1/n_2 value, and the experimental variables. Rather, a complex kinetic relationship appears to exist between metal complex and activating agent concentrations and reaction duration. A few broad generalizations can be made, however. Low concentrations of cleavage agent require higher concentrations of activating agent or longer reaction times or both for efficient formation of dsb's (trials 1, 3, 4). In addition, under constant activating conditions and reaction times, increases in cleavage agent concentration lead to increased amounts of both ssb's and dsb's, resulting in similar n_1/n_2 ratios (trials 6, 7 and 5, 8). Finally, the most efficient dsb formation (lowest n_1/n_2 ratio) tends to occur with higher concentrations of activating agent and shorter reaction times (trials 1−4 *vs* trials 9 and 10), although this generalization is clearly affected by the cleavage agent concentration (trial 1 *vs* 4).

Cleavage as a Function of Metal Complex Concentration. In an attempt to quantify the formation of ssb and dsb sites as a function of the concentration of **1**, we conducted cleavage studies by varying concentrations of agent using constant

(22) Povirk, L. F.; Wubker, W.; Kohnlein, W.; Hutchinson, F. *Nucleic Acids Res.* **1977**, *4*, 3573.

(23) Povirk, L. F.; Houlgrave, C. W. *Biochemistry* **1988**, *27*, 3850.

(24) Drak, J.; Iwasawa, N.; Danishefsky, S.; Crothers, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7464.

(25) The fraction of linear DNA after scission chemistry, $f_{III} = n_2 \exp(-n_2)$. The supercoiled fraction remaining after treatment is $f_I = \exp[-(n_1 + n_2)]$ (ref 22). The Freifelder−Trumbo relation is $n_2 = n_1^2(2h + 1)/4L$, where h is the maximum separation in base pairs between two cuts on complementary strands that produces a linear DNA molecule ($h = 16$), and L is the number of phosphoester bonds per DNA strand in the plasmid ($L = 2686$, [pUC18]) (ref 26).

(26) Freifelder, D.; Trumbo, B. *Biopolymers* **1969**, *7*, 681.

(27) Absalon, M. J.; Kozarich, J. W.; Stubbe, J. *Biochemistry* **1995**, *34*, 2065.

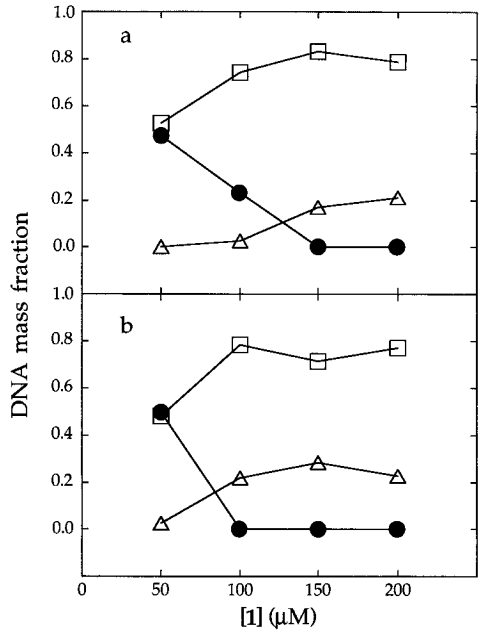


Figure 3. Study of DNA cleavage as a function of concentration of **1** under two different sets of activating conditions. Cleavage conditions: (a) 100 μM H_2O_2−sodium ascorbate; (b) 188 μM H_2O_2−sodium ascorbate. All reactions were 5 min in duration using 10 μM base pairs pUC18 DNA and 20 mM potassium phosphate buffer at pH 7.0 and 23 °C: (●) supercoiled form I DNA; (□) nicked form II DNA; (△) linear form III DNA.

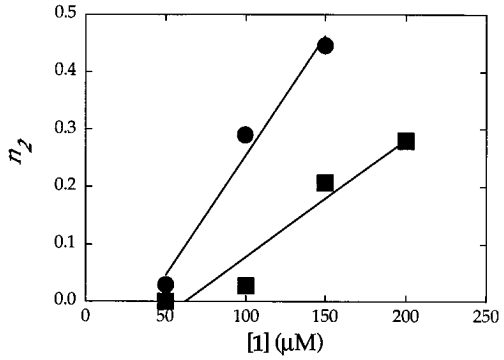


Figure 4. n_2 as a function of concentration of **1**: (■) 100 μM H_2O_2−sodium ascorbate activation conditions; (●) 188 μM H_2O_2−sodium ascorbate activation conditions.

reaction time (5 min) and activation conditions. These results are presented in Figure 3. At the concentrations used, the levels of both nicked and linear DNA appear to reach a plateau at high cleavage agent concentration. This occurs under both sets of activating conditions used. At lower metal complex concentrations, the product distribution clearly depends on cleavage agent concentration. As expected, the plateau region is reached more rapidly at higher activating agent concentrations. The absence of either supercoiled or linear DNA in most of the final product mixtures upon which Figure 3 is based precludes the calculation of the n_1/n_2 ratios, so these data cannot be used for a direct assessment of the relationship between dsb and ssb formation and cleavage agent concentration. Since n_2 is dependent only on the amount of form III DNA present, it can be determined as a function of metal complex concentration (Figure 4). As can be seen from Figure 4, there is a linear relationship between n_2 and cleavage agent concentration over the range studied.

Formation of AP (Apurinic/Apyrimidinic) Sites. In order to quantitate the formation of AP sites during strand scission chemistry, strand scission was performed with **1** in phosphate buffer for varying reaction times. The cleavage reaction was then quenched and incubated at room temperature either under neutral pH conditions or nondenaturing alkaline conditions (pH ~10.4), followed by electrophoresis and quantitation. Table 2 presents the fraction of AP sites formed (based on the total cleaved DNA) as a function of cleavage reaction time. As can be seen, the fraction of linear DNA after incubation under alkaline conditions rises by a factor of 2 to 3 as compared to the fraction after incubation at neutral pH. This suggests that for every dsb formed during the cleavage reaction there are one to two additional alkaline labile sites that are appropriately positioned to form linear DNA upon exposure to hydrolytic conditions. Such sites may either be supercoiled DNA which contains two closely opposing AP sites, or nicked circular DNA, which contains an AP site proximate to the nick on the complementary strand. In the case of bleomycin, the latter form (an AP site proximate to a nick) contributes almost exclusively to the formation of linear DNA on base hydrolysis.[23]

Table 2. AP Site Formation by **1** As Indicated by Base Treatment of Cleaved Plasmid[a]

reaction time (min)	$[H_2O_2]$, [ascorbate]	fraction of linear DNA: after cleavage	fraction of linear DNA: neutral pH incubation	fraction of linear DNA: high pH incubation
5.0	250 μM	0.030	0.042	0.126
5.0	1 mM	0.088	0.071	0.146
10.0	250 μM	0.116	0.112	0.240

[a] As a fraction of total cleavage product DNA based on quantitation prior to pH incubation. See Experimental Section for reaction conditions.

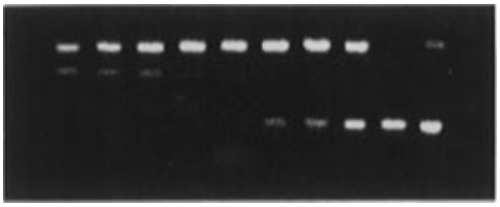


Figure 5. Ionic strength dependence of pUC18 cleavage by **1**. Lanes 1−8: 0, 10, 20, 50, 100, 300, 500, and 800 mM NaCl added to cleavage buffer. Cleavage conditions: 10 μM DNA base pairs, 123 μM metal complex, 150 μM sodium ascorbate, 150 μM H_2O_2, 22.5 °C, 20 mM Tris, 2.5 mM sodium acetate buffer, pH 7.5. Reaction was quenched by the addition of 10 mM EDTA buffer after 1 h of reaction time. Lane 9, DNA alone; lane 10, DNA + metal complex only.

Ionic Strength Dependence of Strand Scission Chemistry. Figure 5 shows the results of an ionic strength study in which pUC18 DNA is treated with complex **1** under conditions of increasing ionic strength through added NaCl. Two separate ionic strength dependencies for nicking *vs* linearization of plasmid DNA are apparent. The process of linearization is significantly more sensitive to increasing ionic strength than is nicking. Virtually no linear DNA forms above 100 mM added NaCl, while substantial amounts of nicking continue to occur. The process of DNA nicking becomes inhibited at extremely high ionic strengths, but does not stop even at 800 mM added NaCl. The pattern in these data may reflect two separate binding events that have different electrostatic components, or the lack of linear DNA at higher ionic strengths may be a kinetic consequence of a lower degree of nicking at higher ionic strengths, which would deplete the substrate pool for formation of linear DNA. Quantitation of the total amount of reacted DNA (nicked + linear) per lane (normalized to the supercoiled DNA) gives an average value of 0.83 ± 0.04 over all lanes. This value with its low standard deviation suggests that the same number of strand scission events occurs regardless of the ionic strength

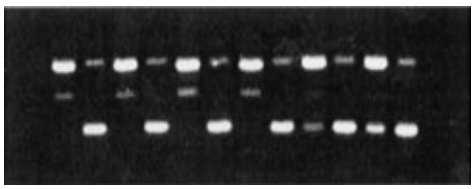


Figure 6. DNA strand scission by **1** as a function of pH. Each pair of lanes shows a scission experiment with an accompanying metal control lane at identical pH; lanes 1−2, pH 5.60; lanes 3−4, pH 5.80; lanes 5−6, pH 7.19; lanes 7−8, pH 7.51; lanes 9−10, pH 8.42; lanes 11−12, pH 8.60. Cleavage conditions as for Figure 1.

of the solution. This implies that the ionic strength-dependent changes are due to binding differences.

Further confirmation of an electrostatic binding component comes from a study of DNA scission *vs* pH, as shown in Figure 6. In the low to neutral pH range, where the pendant amine of complex **1** is certainly protonated, the linearization process is observed. At pH values above this range, deprotonation of the pendant amine begins, and linearization declines; however, substantial nicking is still seen. The formation of significant amounts of nicked DNA in the high pH region, where we believe the complex is deprotonated (leading to a total charge on the deprotonated complex of 0), suggests that the electrostatic contribution to the binding event associated with nicking is minor compared to hydrophobic and other contributions. This would lead to a relative insensitivity of nicking to ionic strength, as is observed (Figure 5). Thus, the data in Figure 5 may represent a situation in which the formation of large amounts of nicked DNA even at high ionic strength reflects a weak electrostatic contribution to binding and not a strong electrostatic interaction. This would explain why the binding event associated with linearization, which supposedly occurs at a negatively charged nick gap, appears to have greater sensitivity to ionic strength than the binding event associated with nicking in Figure 5.

Investigation of the Active Species. Copper, in the presence of oxygen donor species, is thought to be able to form different oxidative intermediates, depending on the specific complex and conditions. A nondiffusable copper−oxene intermediate has been invoked in some cleavage reactions,[28] while in others, Fenton-type chemistry, which involves release of diffusable hydroxy radical, has been suggested. In model reactions run under conditions identical to those used in DNA cleavage, using 2-deoxy-D-ribose as a hydroxy radical trap, both complexes **1** and **2** produce trapping products which are spectroscopically quantified as the 2-thiobarbituric acid adduct (Figure 7).[15] We have used relatively high concentrations of 2-deoxy-D-ribose (1.0 mM) for quantitation of radical production. The data in Figure 7 also show that in the presence of known hydroxy radical quenchers (either DMSO or thiourea), little or no product is formed over the time course of the reaction. The formation of product along with the quenching data are taken as evidence that the cleavage mechanism of both **1** and **2** involves the formation of freely diffusable hydroxy radical from a Fenton mechanism, and not a metal−oxene species. Further, complex **1** is less efficient than **2** at the production of hydroxy radicals, by a factor of about 2. This may be a consequence of the fact that **2** has two *trans* coordination sites available for reaction, while **1** has only a single coordination site, since the phenylalanine side chains provide little steric clearance.

Discussion

The evidence presented here suggests that the structure of complex **1** facilitates binding of the complex at nicked sites on

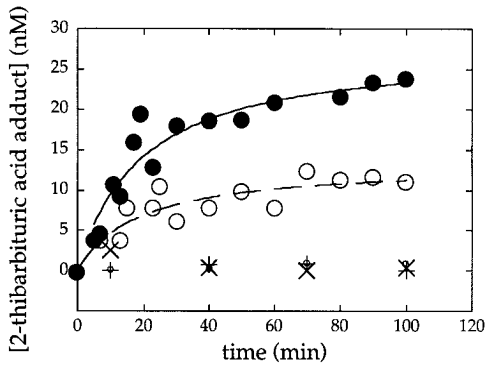


Figure 7. Production of $OH^{\bullet}$ by **1** and **2** as assayed by the 2-deoxy-D-ribose/2-thiobarbituric acid system. (○) reaction of **1** with 2-deoxy-D-ribose; (●) reaction of **2** with 2-deoxy-D-ribose; (×), reaction of **1** with 2-deoxy-D-ribose in the presence of 1 mM thiourea; (+) reaction of **1** with 2-deoxy-D-ribose in the presence of 1 mM DMSO; (⊙) reaction of **2** with 2-deoxy-D-ribose in the presence of 1 mM thiourea.

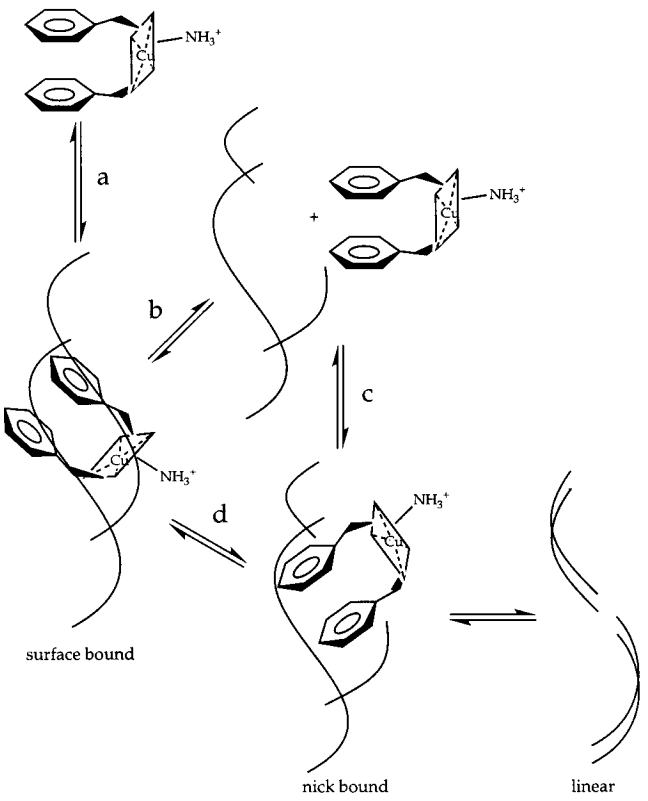
DNA, in addition to binding at the intact DNA surface. The highest yields of linear DNA are found in product mixtures of ∼1:2 linear to nicked DNA (Figure 2); since base-hydrolyzable sites form in a 1:1 ratio to dsb sites, complex **1** can produce a total amount of complementary lesions in $\sim^2/_3$ of the DNA. We attribute the efficiency of dsb formation exhibited by complex **1** to its charged and aromatic recognition elements, as indicated by the comparison with **2**. The greater efficiency of **1** over **2** at both nicking and linearization cannot be explained by any greater efficiency of **1** over **2** in the production of active species, since the opposite is the case. The simplest explanation for the difference between the two complexes is that **1** binds more efficiently than **2** to all DNA structures involved in this chemistry. It is also worthwhile to note that "free" copper(II) and iron(II) under very similar reaction conditions efficiently nick but produce no quantifiable amount of form III DNA.[13,29]

Nicked DNA in general has a structure very close to that of B-DNA, with perturbations due to the nick only occurring at the nick position (on both strands) and at the flanking base pairs.[30,31] Changes of approximately 1 Å are estimated to occur in this region.[30] The site is likely to have 3′- and 5′-phosphate and 3′-phosphoglycolate end groups at the nick, as these have been shown to be product termini of hydroxy radical-mediated DNA strand scission.[32] The nick gap probably allows greater access to the hydrophobic interior than normally found along the DNA surface. Given that a nick provides a negatively charged gap in close proximity to an accessible region of the DNA interior, the structure of **1** may be an excellent initial paradigm for a nick-binding molecular architecture.

Depending on reaction conditions, moderate to efficient double-strand cleavage is achieved by **1** (Table 1). Given the diversity of results in Table 1, it is possible that more than one mechanistic regime is traversed under the conditions surveyed. In attempting to assess the mechanistic picture for DNA cleavage by **1**, it appears that three relevant points emerge from the data. First, nick formation by **1** is faster than the linearization rate, leading to formation of a large pool of nicked DNA (Figure 2). Next, there appears to be a linear relationship between n_2 and cleavage agent concentration (Figure 4). Finally, **1** appears to cleave by production of hydroxy radical, possibly through a Fenton-type mechanism (Figure 7). Any mechanism

(28) Sigman, D. S.; Chen, C. B. *ACS Symp. Ser.* **1989**, *402*, 24.
(29) Baker, A. D.; Morgan, R. J.; Strekas, T. C. *J. Chem. Soc., Chem. Commun.* **1992**, 1099.
(30) Snowden-Ifft, E. A.; Wemmer, D. E. *Biochemistry* **1990**, *29*, 6017.
(31) Pieters, J. M. L.; Mans, R. M. W.; van den Elst, H.; van der Marel, G. A.; van Boom, J. A.; Altona, C. *Nucleic Acid Res.* **1989**, *17*, 4551.
(32) Hertzberg, R. P.; Dervan, P. B. *Biochemistry* **1984**, *23*, 3934.

Scheme 1



based on these observations has to account for activation, binding at the intact DNA surface, and binding at nick sites. With regard to the formation of double-strand breaks, the issue of significance concerns binding at nicks. Two limiting scenarios can be envisioned: either complex **1** nicks intact DNA and returns to bulk solution, followed by subsequent binding at a nick site either in activated form or with activation after binding (Scheme 1, a → b → c), or alternately, **1** remains bound at the nicked site after nick formation while reactivation (Scheme 1, a → d) and subsequent complementary strand scission occurs. An intermediate scenario is also possible: **1** may remain electrostatically bound to the DNA surface after nick formation (but not specifically at the nick), followed by reactivation, nick binding, and scission chemistry.

The experimental distinction between these two limiting scenarios has generally been assessed either based on the dependence of n_2 alone on the cleavage agent concentration or on the dependence of the n_1/n_2 ratio on this concentration.[5,27,33] A linear relationship between n_2 and n_1/n_2 on cleavage agent concentration has been interpreted to mean that only a single molecule is involved in creating both lesions to afford double-strand cleavage.[5,27] In our study (Figure 3), as noted above, our system is not amenable to direct determination of the n_1/n_2 ratio over a cleavage agent concentration range using a single set of reaction conditions. However, there does appear to be a linear relationship between n_2 and the concentration of **1** over a 4-fold concentration range (Figure 4), suggesting consistency with the second mechanistic scenario. The linear dependence of dsb formation shown in Figure 4 also appears to rule out double-strand cleavage due to binding of any dimeric form of **1**.

The data also contain another indication of consistency with the second mechanistic type. Table 1 suggests a general correlation of high concentrations of activating agents with low n_1/n_2 ratios (i.e., efficient dsb formation). The second scenario requires the reactivation rate of **1** for cleavage to be fast compared to its off-rate from the nick binding site for efficient dsb formation. Higher activating agent concentrations should therefore favor the reactivation rate of bound **1** over the off-rate, to lead to greater dsb formation efficiency, as is observed. From these considerations, we conclude that the second mechanistic scenario is possible for dsb formation by **1**, especially where cleavage reactions are run in the higher range of activating agent concentrations. However, the first mechanistic scenario (direct equilibrium between bulk solution and nick binding sites) or an intermediate type of mechanism cannot be ruled out based on these data, and further study is required before any concrete conclusions can be established.

Our preliminary mechanistic assessment can therefore be stated as follows. The double-strand cleavage of DNA by **1** occurs with a greater rate of ssb formation than dsb formation. This likely results in part from a much larger number of sites available for nicking on form I DNA than for dsb formation on form II DNA, since there is probably very little sequence preference for nicking. In addition, there likely are differences in the binding constants at the intact *vs* nick sites (Figures 5 and 6). Nicking probably produces a negatively charged gap that **1** recognizes by electrostatic and hydrophobic interactions. The complementary lesions required for double-strand break formation may be effected by a single molecule of **1** which remains bound at the nick site after nick formation. However, we cannot currently rule out a mechanism in which **1** dissociates from the nick followed by binding of cleavage agent from bulk solution at the nick. In either case, **1** appears to be competent to recognize and bind to a nick binding site. The active species in strand scission chemistry appears to be hydroxy radical released by Fenton-type chemistry. The release of a diffusable species by **1** can account for how a nick-bound cleavage agent can effect damage at the complementary strand, leading either to complete strand scission and dsb formation or to formation of an AP site complementary to the nick.

Bleomycin is the paradigmatic transition-metal-mediated double-strand DNA cleavage agent. Bleomycin-mediated double-strand cleavage of DNA was noted in early bleomycin studies,[34−36] and recognition that dsb formation by bleomycin is not a random cleavage process began with the observation by Povirk *et al*. that bleomycin-induced double-strand scission occurred more commonly than could be accounted for by coincident random single-strand breaks.[22] In addition, an ionic strength dependence was observed for the formation of bleomycin-induced dsb's,[37] which was followed by the observation of enhanced cleavage opposite to a phosphate-charged gap (serving as a ssb model) to form a dsb.[38] In bleomycin-mediated DNA damage, the ssb/dsb ratio is independent of bleomycin concentration, supporting the idea that a single drug molecule effects double-strand cleavage.[27,39,40] Current mechanistic proposals for bleomycin-mediated double-strand cleavage invoke reactivation of nick-bound bleomycin by a C-4′ peroxyl radical.[39] While DNA double-strand cleavage by **1** exhibits a

(33) (a) Gonikberg, E. M.; Odintsova, S. P. *Mol. Biol. (Moscow)* **1986**, *20*, 1048. (b) Gonikberg, E. M.; Odintsova, S. P. *Mol. Biol. (Engl. Transl.)* **1987**, *20*, 853.

(34) Suzuki, H.; Nagai, K.; Yamaki, H.; Tanaka, N.; Umezawa, H. *J. Antibiotics (Tokyo)* **1969**, *22*, 446.

(35) Suzuki, H.; Nagai, K.; Akutzu, E.; Yamaki, H.; Tanaka, N.; Umezawa, H. *J. Antibiotics (Tokyo)* **1970**, *23*, 473.

(36) Haidle, C. W. *Mol. Pharmacol.* **1971**, *7*, 645.

(37) Lloyd, R. S.; Haidle, C. W.; Robberson, D. L. *Biochemistry* **1978**, *17*, 1890.

(38) Keller, T. J.; Oppenheimer, N. J. *J. Biol. Chem.* **1987**, *262*, 15144.

(39) Absalon, M. J.; Wu, W.; Kozarich, J. W.; Stubbe, J. *Biochemistry* **1995**, *34*, 2076.

(40) Steighner, R. J.; Povirk, L. F. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 8350.

number of these characteristics, there are significant mechanistic distinctions between **1** and bleomycin, since dsb formation by **1** depends on exogenous reactivation and a diffusable intermediate, neither of which are features of bleomycin dsb formation.[27,39]

The development of molecules which are able to achieve nonrandom double-strand cleavage may have application in the area of drug design for chemotherapeutic agents, since this type of DNA damage appears to be biologically more potent than single-strand damage. The identification of molecular structures which can efficiently perform double-strand cleavage of duplex DNA through sequential strand scission events, when combined with sequence-specific recognition groups, may therefore prove to be an important strategy in the design of antineoplastic agents.

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