Predictability of Designing Specific Binding Interactions for DNA Minor Groove Ligands from NMR Spectroscopy and Molecular Modeling: A Copper(II)-Activated DNA Cleaver Based on Hoechst 33258

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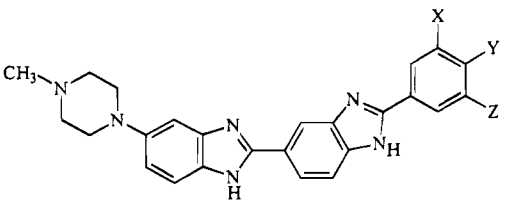
Received September 20, 1995; Revised Manuscript Received November 6, 1995[⊗]

ABSTRACT: Analogs of Hoechst 33258 have been designed and synthesized to incorporate a 3,4-catecholic or a 3,4,5-trihydroxyphenyl function in place of the 4-phenolic group of the template molecule. Molecular design was based on the three-dimensional models of the solution structures of Hoechst 33258 and its *m*-hydroxy isomer which had been derived from high-field NMR spectroscopic analysis. The predicted solution structure of the catecholic analog as its minor groove complex with duplex d(CGCGAATTCGCG)$_2$ was confirmed by direct high-resolution NMR spectroscopy combined with molecular dynamics, using NMR-derived distance restraints. While the 3,4-catecholic analog was unable to cleave DNA in the presence of Cu(II) ions, the 3,4,5-trihydroxyphenyl analog of Hoechst 33258 was found to be an effective cleaver of DNA at low concentration when activated by copper(II) ions, a difference ascribed to the inaccessibility for copper chelation of the 3,4-dihydroxy site in its minor groove complex with DNA.

While the field of rational drug design based on molecular graphics is established for enzymes and proteins, the situation for nucleic acids is relatively speaking in its infancy. The basis for rational ligand design must be an objective assessment of the predictive power of current science. Even for enzyme inhibitor design, a small change in the structure of an inhibitor can lead to major changes in the details of binding to the active site. This is lucidly illustrated for dihydrofolate reductase by the 180° twist found in the pteridine ring binding conformation for dihydrofolate compared to methotrexate (Matthews, 1978; Fontecilla-Camps, 1979), and the observation that simply replacing one sulfur atom of the disulfide bridge of GSSG by $-CH_2-$ (to give a $-CH_2S-$ bridge) leads to a major twist in the conformation accepted by the active site of glutathione reductase (Embrey et al., 1994). Experience in rationally designing ligands for DNA is considerably less than for proteins. One major question which our group has been among the first to address is the *predictability* of the process. Recently, from our NMR spectroscopic data on the solution structure of the minor groove ligand, Hoechst 33258 (**1**), with the duplex d(CGCGAATTCGCG)$_2$ (Parkinson et al., 1990), we predicted (Ebrahimi et al., 1992) that moving the *para* phenolic hydroxyl group to a *meta* position (to give *meta*-Hoechst, **2**) would lead to additional specific hydrogen bond interactions with the C=O group of C^9 and the NH_2 group of $G^{4'}$. High-field NMR spectroscopic analysis by ourselves (Parkinson et al., 1994) and others (Leupin et al., 1994) has borne out this prediction. In contrast to **1**, for which the phenolic OH group is free to rotate in the minor groove (Embrey et al., 1993; Parkinson et al., 1992), the phenolic ring is conformationally fixed for **2**, with the OH group directed into the minor groove (Parkinson et al., 1994) and located suitably for the predicted hydrogen bonds to form.



	X	Y	Z
1	H	OH	H
2	OH	H	H
3	OH	OH	H
4	OH	OH	OH

It is possible to test whether predicted interactions are present in macromolecule/ligand complexes in a variety of ways, not only on the basis of structural techniques but also on the basis of predicted chemical reactivities. Analysis of the *meta*-Hoechst/d(CGCGAATTCGCG)$_2$ model (Parkinson et al., 1994) shows that the phenolic OH group may act as both a H-bond donor (to C^9) and acceptor (from $G^{4'}$). A 3,4-dihydroxy analog (**3**) would be expected to behave similarly, but the introduction of the catechol and related functions introduces the possibility of a chemical test of binding orientation in larger, natural stretches of DNA, the potential for Cu^{2+} ion stimulated free radical production, and consequent DNA strand scission. Moreover, the radical-generating site is predicted to lie intimately juxtaposed to the minor groove cleavage target of DNA.

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[‡] University of Edinburgh.
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[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1995.

0006-2960/95/0434-16240$09.00/0

To develop these aspects we have synthesized the catechol equivalent of **1** and **2**, which we shall refer to as cat-Hoechst (**3**) and the 3,4,5-trihydroxy analog (**4**, trihydroxy-Hoechst). We now report high-field NMR spectroscopic characterization of the solution structure of the cat-Hoechst (**3**)/d(CGCGAATTCGCG)$_2$ complex and preliminary analysis of the cleavage potential of **3** and **4** toward duplex plasmid DNA.

EXPERIMENTAL PROCEDURES

Cat-Hoechst (**3**) was synthesized as described for **2** (Ebrahimi et al., 1995) by replacing ethyl *m*-hydroxybenzimidate with ethyl 3,4-dihydroxybenzimidate in the last step. Ethyl 3,4-dihydroxybenzimidate hydrochloride was synthesized as follows. To a solution of 3,4-dihydroxybenzonitrile (9.075 g, 67.2 mmol), anhydrous toluene (30 mL) and superdry ethanol (18 mL, 0.313 mol), cooled over ice−salt, was added gradually gaseous HCl in several portions (to give a total of 10 g), keeping the temperature below 10 °C, to give two layers, the lower dark and the upper colorless. The stoppered flask was stored at 4 °C. After 5 days crystallization was almost complete, and the crystals were isolated and washed (dry ether) to give 11.5 g of a dark green compound which was stored over NaOH pellets and $CaCl_2$ under vacuum. The cooled filtrate gave a second crop (1.61 g) of product (total yield 90.7%): mp 182−183 °C dec; δ_H [270 MHz, $(CD_3)_2SO$] 1.45 (t, 3H, CH_3, J = 6.9 Hz), 4.56 (q, 2H, CH_2, J = 6.9 Hz), 6.90 (dd, 1H, Ar-H, J = 8.6, 1.3 Hz), 7.49 (d, 1H, Ar-H, J = 1.3 Hz), 7.57 (dd, 1H, Ar-H, J = 8.6, 1.0 Hz), 10.85 (br s, 1H), 11.51 (br s, 1H, OH); mass spectrum [*m/z* (EI), relative intensity] 183 [$(M + 2)^+$, 2.6%], 182 [$(M + 1)^+$, 21.7%], 181 (M^+, 20.67%), 166 ($M - CH_3$, 2.9%), 153 ($M - CH_2{=}CH_2$, 46%), 137 (M − 45, 100%), 136 ($M - OC_2H_5$, 35.27%), 121 (3%), 107, 91, 80, 63, 52, 39; FAB mass calculated for $C_9H_{12}NO_3$ 182.0317, found 182.0815 (1.1 ppm error); 184 (M + H + 2, 1.22), 183 (M + H + 1, 11.45), 182 (M + H, 100), 166, 154 (M − 28, 8.3), 136 (M − 46, 11.9).

The synthesis of 2-[2-(3,4-dihydroxyphenyl)-6-benzimidazolyl]-6-(4-methyl-1-piperazinyl)benzimidazole (3,4-dihydroxy-Hoechst) (3) from ethyl 3,4-dihydroxybenzimidate hydrochloride followed the published procedure (Ebrahimi et al., 1995) to yield product (**3**) as a dark brown solid, which was purified by chromatography over silica H (1:1 MeOH:EtOAc solvent linearly changing to 100% MeOH) with isolation of fractions corresponding to the slow moving yellow spot (TLC, UV visualization) running before the slowest of the red spots. The yellow powder obtained on solvent removal was further purified by preparative TLC (silica gel plates, 1:1 MeOH:EtOAc) to give the product as a yellow powder: mp >250 °C; δ_H (270 MHz, D_2O + DCl), 2.74 (s, 3H, N-CH_3), 2.83 [m, 4H, 2(N-CH_2)], 3.38 [m, 4H, 2(N-CH_2)], 6.25 (d, 1H, Ar-H, J = 8.9 Hz), 6.42 (s, 1H, Ar-H), 6.46 (s, 1H, Ar-H), 6.60 (d, 1H, ar-H, J = 8.2 Hz), 6.70 (d, 1H, Ar-H, J = 9.2 Hz), 6.98 (s, 1H, Ar-H), 7.01 (s, 1H, Ar-H), 7.17 (br s, 2H, Ar-H); FAB mass spectrometry (*m/z*, relative intensity) 433 [$(M + H)^+ + 2$, 4%] (calculated 4.27%), 442 [$(M + H^+) + 1$, 30%] (calculated 29.97%), 441 [$(M + H)^+$, 100%]; mass measurement, $C_{25}H_{24}N_6O_2$ calculated 441.20374, measured 441.2031 (1.4 ppm error).

For the synthesis of 2-[2-(3,4,5-trihydroxyphenyl)-6-benzimidazolyl]-6-(4-methyl-1-piperazinyl)benzimidazole (3,4,5-trihydroxy-Hoechst) (**4**), a suspension of 2-(3,4-diaminophenyl)-6-(4-methyl-1-piperazinyl)benzimidazole (640 mg, 2 mmol) and 3,4,5-trihydroxybenzaldehyde (314.5 mg, 2 mmol) in nitrobenzene (15 mL) was gradually heated up to 140 °C on an oil bath with the color changing to brown and then darkening and then stirred at 140 °C for 24 h using a condenser and $CaCl_2$ tube. The cooled dark solution was triturated with ether and the precipitate filtered off and washed with ether to give a dark brown solid, a hot methanolic solution of which was decolorized with charcoal, filtered, and evaporated to give a yellow-red precipitate, which was purified by the method used for cat-Hoechst (**3**): R_f = 0.15 in methanol; δ_H [270 MHz, $(CD_3)_2SO$], 2.25 (s, 3H, N-CH_3), 3.13 (br s, 4H, N-CH_2), 3.43 (br s, 4H, N-CH_2), 6.93 (br s, 2H, Ar-H), 7.18 (s, 1H, Ar-H), 7.47 (d, 1H, Ar-H, J = 8 Hz), 7.56 (m, 1H, Ar-H), 7.97 (m, 1H, Ar-H), 8.22 (d, 1H, Ar-H, J = 8 Hz), 8.32 (s, 1H, Ar-H), 9.00 (d, 1H, Ar-H, J = 8 Hz), 12.55 (br s), 12.75 (br s); FAB mass spectrum (*m/z*, relative intensity); 457 [$(M + H)^+$, 100%]; accurate mass, calculated for $C_{25}H_{25}N_6O_3$ 457.1988, measured 457.1987 (error ≈ 0.2 ppm).

The oligonucleotide [d(CGCGAATTCGCG)$_2$] and sample preparations for NMR experiments were as described previously (Parkinson et al., 1994).

Creation of the 1:1 cat-Hoechst/DNA complex was monitored by ^{1}H NMR spectroscopy by assessing the thymine methyl resonances of the oligonucleotide. A 1:1 ratio of ligand to oligonucleotide was judged to exist when the two thymine methyl resonances of the free DNA (δ 1.59, 2 × T^8CH_3; δ 1.28, 2 × T^7CH_3) had been completely replaced by four separate thymine methyl resonances (δ 1.432, T^8CH_3; δ 1.409, $T^{8'}CH_3$; δ 1.345, T^7CH_3; δ 1.26, $T^{7'}$-CH_3). Standard pulse sequences were used to acquire sets of two-dimensional NMR data for resonance assignment and for structural analysis including DQFCOSY, TOCSY, and NOESY data according to the details described for the *meta*-Hoechst/DNA complex (Parkinson et al., 1994). Under these solution conditions, the cat-Hoechst/DNA complex behaved in a closely similar manner to the *meta*-Hoechst/DNA complex. All NMR data were accumulated on a Varian VXR600S (Unity) 600 MHz NMR spectrometer on a dual (inverse) ^{1}H (X) probe head. Data were processed using the VNMR 4.3 and Tripos Sybyl 6.1 software package incorporating the Triad NMR module.

The plasmid DNA used was pMA802. This plasmid contains the 649 bp *Eco*RI−*Hind*III insert of pMT702 cloned into the expression vector pMAC5−14 (Andrews et al., 1991). Cleavage conditions were based on conditions developed for Cu(II)−RSH cleavage of DNA (Reed & Douglas, 1989, 1991), and Chelex-treated water was used to prepare solutions. In a typical cleavage experiment, plasmid DNA (1 μg) was incubated for 30 min in 10 mM sodium phosphate buffer, pH 8.0, with an appropriate concentration of compounds **1**−**4** (typically up to 250 μM) with the addition or absence of Cu^{2+} (up to 150 μM) added as an aliquot (1 μL) from a stock solution in water (to maintain solubility, it is preferable to add the Cu^{2+} solution to the DNA). At appropriate times the reaction was stopped by loading the incubation mixtures onto agarose (1%) gels for electrophoresis (60 mA, 1 h) in TAE buffer. The relative amounts of supercoiled (sc), open circular (oc), and linear DNA were quantified by ethidium bromide staining (at 0.5 μg/mL^{-1} ethidium bromide) and densitometry (Bio-Rad

densitometer). The film used to photograph the gels (Polaroid type 665) was shown to have a linear response in the range of DNA quantities used. Since supercoiled DNA is restricted in its ability to bind ethidium bromide relative to open circular and linear forms, it was necessary to correct values obtained for supercoiled compared to open circular DNA by using the ratio 1.28:1 (Reed & Douglas, 1991). The electrophoretic positions of open circular and linear DNA were confirmed by partial and complete *Eco*RI restriction digestion, respectively.

RESULTS AND DISCUSSION

One-dimensional titration of a solution of d(CGCGAATTCGCG)$_2$ with increasing amounts of **3** was monitored by following the resonances of the thymine methyl groups (Figure 1). These data are the precise counterparts of those found for Hoechst 33258 (Parkinson et al., 1990) and *meta*-Hoechst (Parkinson et al., 1994) bound to d(CGCGAATTCGCG)$_2$, showing a tightly bound complex.

The two-dimensional NOESY data for all nonexchangeable protons (data acquired from a sample dissolved in 99% D_2O), when fully assigned for the complex, revealed that the region correlating spatial relationships between the ligand and the DNA duplex was almost identical to that for data acquired on the *meta*-Hoechst/DNA complex (Parkinson et al., 1994). Besides some very minor differences in chemical shifts between the sets of data, the most obvious difference was the absence of an H4 resonance in the NMR data for the cat-Hoechst/d(CGCGAATTCGCG)$_2$ complex, where H4 of **2** has been replaced in **3** by an OH group. NOE correlations between H4 and the DNA backbone, present for the *meta*-Hoechst/DNA complex, were therefore not observed for the cat-Hoechst/DNA complex.

Analysis of the intermolecular NOEs between the catecholic ring and the DNA duplex showed that H2 of **3** was orientated toward the floor of the minor groove with NOEs to $A^{6'}H2$ and $A^{5'}H2$. NOEs from protons on the convex edge of **3** were only seen to oligonucleotide 4′ and 5′ protons (e.g., H6 to $C^{9}5''H$), indicating that they are directed toward the bulk solvent [as was the case for **2** with this oligonucleotide (Parkinson et al., 1994)].

Interproton distance limits were calculated from the NOESY data using a full-relaxation matrix approach. Twenty picoseconds of restrained molecular dynamics simulations of the complex with these restraints was run using several random number seeds. The oligonucleotide base pairs were fixed using distance constraints between hydrogen-bonded partners in the Watson−Crick base pairs. The average structure over all runs was energy- minimized with 512 steps of conjugate gradient minimization. A structure consistent with the NMR spectroscopic/molecular dynamics analysis is shown as Figure 2. The catecholic site was found to lie with its 3-hydroxy group directed into the minor groove, as was found for *meta*-Hoechst (Parkinson et al., 1994).

When **1** or **2** was incubated with pMA802 plasmid DNA in the absence or presence of Cu^{2+} ions (up to 150 μM), we could find no evidence of DNA scission. The molecular model (see Figure 2) indicates that if **3** were already bound to DNA, Cu^{2+} ions could not enter the chelating arms of the catechol unit. If the Cu^{2+} complex of **3** were preformed, the Cu^{II}−catecholic site would compromise the closely contoured fit of the Hoechst framework to the minor groove

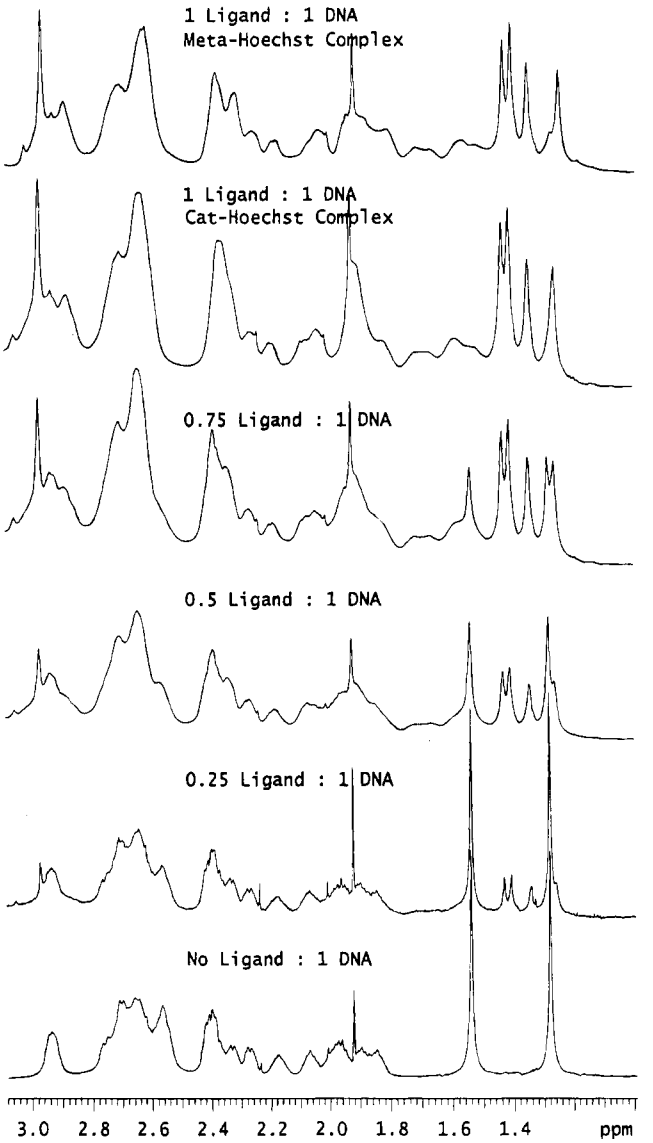

FIGURE 1: 600 MHz ^{1}H NMR spectra of the formation of the 1:1 cat-Hoechst/d(CGCGAATTCGCG)$_2$ complex in D_2O. Aliquots of a concentrated solution of cat-Hoechst were added stepwise to a solution of the free DNA dissolved in 700 μL of buffered solvent [10 mM phosphate, 100 mM NaCl, 1 mM TSP standard, pH 7.51 (uncorrected meter reading)]. A one-dimensional ^{1}H NMR spectrum was accumulated after each addition of ligand in order to monitor complex formation. Comparison of the same region of the 1:1 *meta*-Hoechst complex ^{1}H NMR data at 600 MHz is shown at the top of the figure, for which the solution conditions were identical to those used for the cat-Hoechst complex.

in this region. The model indicated that if the Hoechst molecule were to be oriented with its catecholic unit directed *out* of the minor groove, a Cu^{2+} complex could readily form and DNA scission might reasonably be expected. To achieve this, we added an additional OH group to the phenolic ring to give **4** in which the 3-OH group would hold the phenolic ring tightly into the minor groove through interactions with C9 and G4′ [as found here for **3** and previously (Parkinson et al., 1994) for **2**]. This would leave an *exo*-directed catecholic site, the 4,5-dihydroxyphenyl, to effect Cu^{2+} cleavage (Figure 3). Figure 4 shows the dependence on trihydroxy-Hoechst (**4**) concentration of the cleavage of supercoiled DNA by 150 μM $CuSO_4$. There was no detectable scission in the absence of added Cu^{2+} for 4 (250 μM) incubated with plasmid DNA (data not shown).

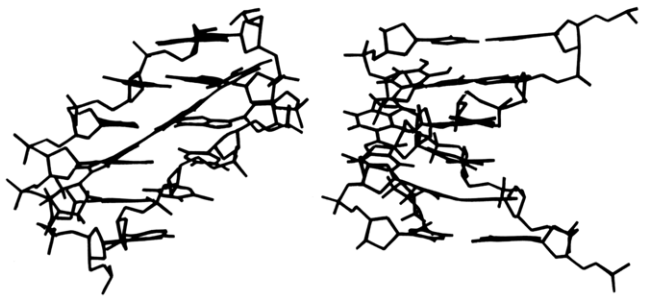

FIGURE 2: Orthogonal views of cat-Hoechst (**3**) bound in the minor groove of duplex d(CGCGAATTCGCG)$_2$. The left-hand view looks down into the minor groove showing the ligands with its piperazinyl group at the lower left and the catecholic site at the upper right, with its 3-hydroxy group clearly directed into the groove bottom. The right-hand view shows the ligand wending its way up the minor groove, on the left edge of the duplex, with its catecholic site at the top between the top two base pairs. The structures were calculated using NMR-derived distance restraints and the TRIAD software of Tripos SYBYL 6.1.

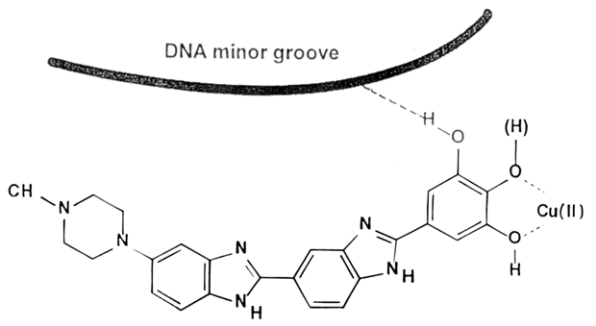

FIGURE 3: Orientation in the minor groove of the putative Cu(II) complex of trihydroxy-Hoechst (**4**) responsible for strand cleavage.

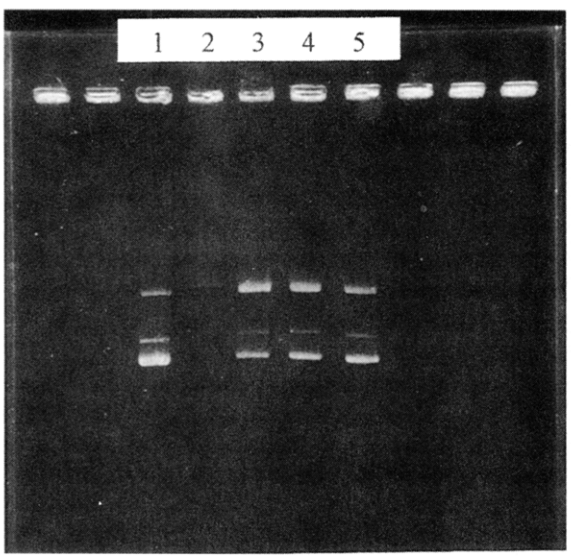

FIGURE 4: Agarose gel electrophoresis of the cleavage of pMA802 DNA by 3,4,5-trihydroxy-Hoechst (**4**). Lane 1 was for a control sample of plasmid DNA alone in buffer with neither Cu(II) nor **4** present. Incubation mixtures contained plasmid DNA and Cu(II) in pH 8.0 phosphate buffer with the following additions of **4** for the lanes indicated: lane 2, 8 μM; lane 3, 4 μM; lane 4, 2 μM; lane 5, 1 μM.

However, in the presence of 150 μM Cu^{2+} progressive, concentration-dependent cleavage occurs (Figure 4). The staining intensity of the linear form of DNA in lanes 3−5 (all of which had been exposed to the Hoechst minor groove binder **4** prior to ethidium staining (at 0.5 μg/mL) is weaker than in lane 1 (for which ethidium did not have to compete with the stronger binding **4** and for which there could be no complication of fluorescence quenching from the simultaneous binding of the two fluorophores). The data were corrected for this factor. Densitometry of the gel indicated that the percentage of linear DNA detected did not change with increasing concentration of trihydroxy-Hoechst (**4**) over the concentration range of 1−4 μM, whereas the ratio of supercoiled to open circular form changed from 4.53 in the absence of **4** to 1.72, 1.10, and 0.67 in the presence of 1, 2, and 4 μM trihydroxy-Hoechst (**4**), respectively. Thus, the kinetic process is cleanly described by initial single-stranded cutting of the supercoiled form to give oc DNA, followed by cleavage of oc DNA to give linear DNA when two single-strand cuts on opposite strands occur sufficiently close to one another. The two processes of sc-to-oc and oc-to-linear DNA are cleanly time-resolved for this cutter. The change in ratio of supercoiled to open circular form on going from control lane 1 with no minor groove cutter present to lane 5 with 1 μM **4** is from 4.53 to 1.72 so that even at 30 min efficient cleavage has taken place. This may be related to the extremely tight binding of these minor groove ligands and to the intimate juxtaposition of the Cu(II) site for radical generation and the deoxyribose backbone along the edge of the minor groove. Cleavage of supercoiled DNA by **4** under the influence of Cu(II) was also shown to be time-dependent.

Cu(II)-induced cleavage of DNA has been reported for several systems. A benzene metabolite (1,2,4-benzenetriol) has been reported to damage human DNA, possibly by autoxidation in the presence of copper ions, but the species responsible for the damage is not yet clear (Kawanishi et al., 1989; Rao & Pandya, 1989). Hydroquinone shows copper ion-dependent DNA strand cleavage with other metal ions [Fe(III), Mn(II), Cd(II), Zn(II)] being considerably less active (Li & Trush, 1993). Partial protection was afforded by singlet oxygen scavengers but not hydroxyl scavengers. Other polyhydroxy compounds which lead to Cu(II)-promoted DNA damage include flavanols and procyanidins, again by a mechanism largely specific to Cu(II) (Shirahata et al., 1989). These classes of copper-dependent damage to DNA mostly involve oxygen radical or active oxygen species effecting radical abstraction reactions at the deoxyribose backbone, but Cu(II) in the presence of hydrogen peroxide causes damage to the bases themselves also (Aruoma et al., 1991). It is possible for both types of Cu(II)-induced effects to occur in the same system as we have found for the Cu(II)−thiol DNA cleavage system (Reed & Douglas, 1991; John & Douglas, 1993), and it will be of interest to define the detailed mechanism of DNA strand scission for the trihydroxy-Hoechst/Cu(II) system given the very intimate nature of the ligand binding to the minor groove for this cleaver, based on the currently available high-resolution structure that we have determined for the 3,4-dihydoxy-Hoechst/DNA complex. Study of such an intimate cleavage complex has been possible for some other situations, the most notable being Cu−phenanthroline (Pan et al., 1994). Other cleavage systems show varying degrees of proximity between the radical-generator site and the DNA site of radical abstraction, e.g., Fe−EDTA directed to DNA by tethering onto intercalators (Hertzberg & Dervan, 1984), oligonucleotides (Boutorin et al., 1984), or both (Boidot-Forget et al., 1988) and cobalt, ruthenium, and rhodium complexes (Kirshenbaum et al., 1988; Müller et al., 1987). In principle, one can separate the binding and cleavage components of

the action of **4** on DNA. By using metal complexes which do not cleave DNA [e.g., Mn(II)] a range of EPR and NMR spectroscopic approaches to define structural aspects of the radical-generator site becomes accessible. This avenue is likely to be successful given the very high binding constants of the DNA complexes of Hoechst 33258 [approximately 10^8 M^{-1} (Loontiens et al., 1990) and its analogs (F. G. Loontiens, S. Sadat-Ebrahimi, and K. T. Douglas, unpublished data)]. The experimentally demonstrated close contact of the phenolic units of **1**−**3** with the minor groove of DNA (Parkinson et al., 1990; Parkinson et al., 1994; Leupin et al., 1994) means that the radicals once generated will have to diffuse only short distances to induce cleavage and the details of the cleavage mechanism should be readily probed.

ACKNOWLEDGMENT

We are grateful to the EPSRC for time on the 600 MHz NMR instrument at Edinburgh (K.T.D.), to the Wellcome Trust for computing facilities, and to the Iranian Government (S.S.E.).

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