Deoxyribonucleic Acid Cleavage Specificity of a Series of Acridine- and Acodazole–Iron Porphyrins as Functional Bleomycin Models†

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ABSTRACT: A series of metalloporphyrins linked through basic chains to certain DNA interactive groups has been synthesized. Several of these agents reproduce the characteristic properties of the antitumor glycopeptide bleomycin, including the oxygen-mediated scission of DNA in the presence of thiols, antitiobic activity under aerobic conditions, and activity against human and animal tumor models. Initial screening by scission of PM2-CCC-DNA identified six of the compounds, including those bearing acridine and acodazole intercalating groups, as the most active. The specificity of the oxygen-mediated scission of a 139 base pair *Hin*dIII/*Nci*I restriction fragment of pBR322 by these six selected agents was then determined and compared with the action of pancreatic DNase by densitometric scans. All six of these compounds produce uniform base and sequence neutral cleavage of the restriction fragment at each base site. The six active compounds bear either of two types of intercalators, 6-chloro-2-methoxyacridine or acodazole, and with linkages to the ferric binding domain of $-NH(CH_2)_2-$, $-NH(CH_2)_3-$, $-NH(CH_2)_4-$, or $-NH(CH_2)_3NH(CH_2)_3-$ and either porphyrin or deuteroporphyrin moieties. Comparison of the K_{assoc} values for binding to calf thymus DNA suggests that the enhanced binding observed with the linker $-NH(CH_2)_3NH(CH_2)_3-$ contributes to the efficiency of sequence neutral DNA scission and may be a factor in the relative anticancer activities of these agents. The iron porphyrins give no evidence of the production of base propenals in DNA degradation, and the autoradiograms clearly indicate that a phosphate group is attached to the 5′ end of the oligomer. The scission is partially suppressible by catalase and superoxide dismutase. These properties are in contrast to the behavior of bleomycin and suggest a mechanism of action that differs in details from that of the glycopeptide. The smooth and efficient sequence neutral scission of DNA by the metalloporphyrins suggests their application to footprinting procedures.

The glycopeptide antitumor antibiotic bleomycin is used widely in the clinical treatment of human malignancies including squamous cell carcinoma and testicular tumors (Hecht, 1979; Povirk, 1983; Friedman, 1978). Bleomycin appears to act by a unique mechanism involving site-selective binding to double-stranded DNA and oxygen-mediated scission of the strands catalyzed by the hexacoordinated iron binding domain thus brought into proximity with the target (Figure 1a) (Sausville et al., 1978; Kuramochi et al., 1981; Lown & Sim, 1977).

Functional bleomycin models (Figure 1b), in which the operational properties of the natural product were retained, were designed, synthesized, and tested (Lown et al., 1982, 1984b). The prototype structures hemin–spermine–acridines (Figure 1b) reproduce the essential features of the natural product in producing oxygen-mediated DNA scission in the presence of a reducing agent, e.g., DTT[1] or 2-ME, and at concentrations comparable with that employed with bleomycin itself (Lown et al., 1982, 1984b). Certain of these agents exhibit antibacterial activity specifically under aerobic conditions and anticancer activity against some animal and human tumor models (Lown et al., 1982, 1984b).

It was of interest to examine in greater detail the interaction of these new agents with DNA. Accordingly, we report the study of iron porphyrins bearing different DNA binding groups and bearing both one and two of such groups (Figure 2) and their scission of PM2-CCC-DNA in the presence of reducing agents and oxygen. In addition, we report the specificity of the oxygen-mediated degradation of selected examples of these agents using a *Hin*dIII/*Nci*I restriction fragment of pBR322 DNA and a preliminary examination of the mechanism of the scission process. As a result of this study, certain of the iron porphyrins investigated would appear to have significant potential for "footprinting" studies (Galas & Schmitz, 1978, Schmitz & Galas, 1977; Van Dyke et al., 1982; Dabrowiak, 1983; Goodisman & Dabrowiak, 1985).

Materials and Methods

Materials

Chemicals. Deuterohemin monomethyl ester (Caughey et al., 1966; Lown & Joshua, 1982) was prepared by refluxing

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[1] Abbreviations: NMR, nuclear magnetic resonance; MS, mass spectrometry; FAB, fast atomic bombardment; TLC, thin-layer chromatography; IR, infrared spectroscopy; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; DMF, dimethylformamide; CCC, covalently closed circular; OC, open circular; Me_2SO, dimethyl sulfoxide; FT, Fourier transform; EM, electron microscopy.

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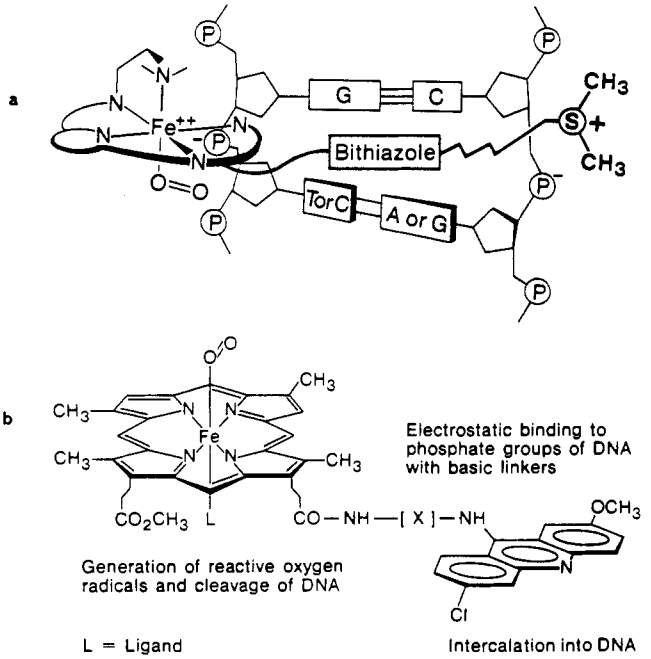
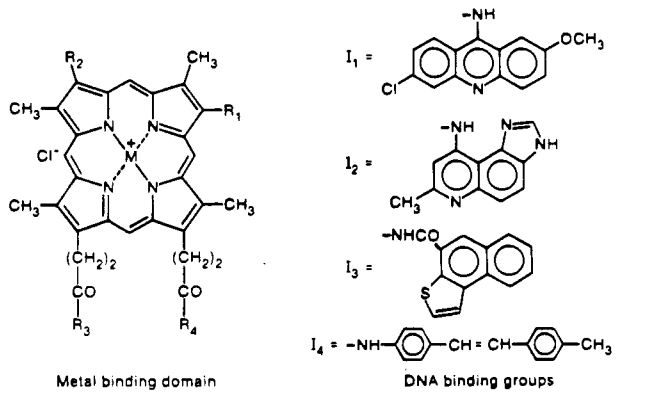


FIGURE 1: (a) Depiction of suggested mode of action of the antitumor antibiotic bleomycin in site-specific binding to DNA and strand scission initiated by stereochemically directed hydrogen atom abstraction at the deoxy sugar moiety. (b) Depiction of a possible mode of action of representative anticancer hemin–acridine–bleomycin models involving intercalative binding to double-helical DNA and iron–oxygen-mediated strand scission.



1	$R_1 = R_2 = H$,	$R_3 = OCH_3$,	$R_4 = -NH(CH_2)_2-I_1$
2	$R_1 = R_2 = H$,	$R_3 = OCH_3$,	$R_4 = -NH(CH_2)_3-I_1$
3	$R_1 = R_2 = H$,	$R_3 = OCH_3$,	$R_4 = -NH(CH_2)_3NH(CH_2)_3-I_1$
4	$R_1 = R_2 = H$,	$R_3 = R_4 = -NH(CH_2)_4I_1$	
5	$R_1 = R_2 = H$,	$R_3 = R_4 = -NH(CH_2)_4I_2$	
6	$R_1 = R_2 = -CH=CH_2$,	$R_3 = R_4 = -NH(CH_2)_4I_2$	
7	$R_1 = R_2 = H$,	$R_3 = R_4 = -NHC_6H_4I_2$	
8	$R_1 = R_2 = H$,	$R_3 = R_4 = -NH(CH_2)_6I_3$	
9	$R_1 = R_2 = H$,	$R_3 = R_4 = -NH(CH_2)_{10}I_3$	
10	$R_1 = R_2 = H$,	$R_3 = R_4 = -NH(CH_2)_{12}I_3$	
11	$R_1 = R_2 = H$,	$R_3 = R_4 = I_4$	
12	$R_1 = R_2 = -CH=CH_2$,	$R_3 = R_4 = -NHC_6H_4I_2$	
13	$R_1 = R_2 = -CH=CH_2$,	$R_3 = R_4 = -NH(CH_2)_6I_3$	
14	$R_1 = R_2 = -CH=CH_2$,	$R_3 = R_4 = -NH(CH_2)_{12}I_3$	
15	$R_1 = R_2 = H$,	$R_3 = R_4 = -NH(CH_2)_6I_3$	

For 1-14, M = Fe and for 15, M = Mn

FIGURE 2: Structures of metalloporphyrin-linked DNA binding agents used in the oxygen-mediated DNA scission and sequencing studies.

deuteroporphyrin monoethyl ester with an equivalent of ferric chloride in DMF for about 1 h (Adler et al., 1970) and then purified by column chromatography over alumina with 15% MeOH in $CHCl_3$ as eluent. Deuterohemin and deuteroporphyrin were prepared from hemin by the method of Caughey et al. (1966). Heating of 6,9-dichloro-2-methoxyacridine with a large excess of the linking diamine or triamine at 80 °C for 2 h gave the corresponding 9-substituted aminoacridines, which were purified as their hydrochlorides (Lown & Joshua, 1982). The aminostilbene was prepared according to a literature procedure (Calvin & Buckles, 1940; Walden & Kernbaum, 1890).

Condensation of the chromophores 7-chloro-2-methoxy-10-aminoacridine, 9-amino-7-methylimidazo[4,5-*f*]quinoline, and naphtho[2,1-*b*]thiophene-3-carboxylic acid and the aminostilbene with the appropriate α,ω-diamines was effected with carbonyldiimidazole or by thermal means following literature procedures (Spencer et al., 1975; Snyder et al., 1977; Lown & Joshua, 1982). The resulting diamine-linked chromophores were then condensed with deuterohemin monomethyl ester, deuterohemin, or hemin as appropriate, in the presence of carbonyldiimidazole and *N*-ethylmorpholine following previously described procedures (Lown & Joshua, 1982; Lown et al., 1984b) to afford compounds **1–15**. [Full details of the synthesis of these compounds together with complete spectral and analytical characterization is available as supplementary material; see paragraph at end of paper regarding supplementary material.]

Biochemicals. Bacteriophage PM2 supercoiled DNA, plasmid pBR322 DNA, and calf thymus DNA (type 1, Na^+ salt) were obtained from Boehringer Mannheim Biochemicals, Bethesda Research Laboratories, and Sigma, respectively. The enzymes *Nci*I, *Hin*dIII, and AMV reverse transcriptase were purchased from Bethesda Research Laboratories. Bovine pancreatic DNase I (type DN-CS) was obtained from Sigma in a lyophilized form and dissolved in 50% glycerol–50 mM Tris-HCl, pH 7.0. [α-^{32}P]dATP was purchased from New England Nuclear. Bleomycin (clinical blenoxane) was a gift from the Bristol-Myers Co., Syracuse, NY.

Purification of DNA. pBR322 plasmid DNA was subjected to extensive dialysis against 50 mM Tris-HCl buffer, pH 7.0, containing 20 mM EDTA, followed by dialysis against 50 mM Tris-HCl–1 mM EDTA, pH 7.0. Bacteriophage PM2-DNA was treated similarly. Calf thymus DNA was allowed to dissolve in 20 mM Tris-HCl buffer, pH 8.0. Deproteinization was subsequently performed by extraction with buffered phenol (pH 8.0) and then with a 24:1 (v/v) chloroform–isoamyl alcohol mixture and finally with ether, as outlined previously (Maniatis et al., 1982). This purification procedure was repeated until the ratio of absorbances of DNA at 260 and 280 mm was greater than 1.85. The DNA solution was brought to 0.3 M NaOAc by the addition of 3 M NaOAc, and the entire contents then received two equivalent volumes of 95% ethanol. The mixture was frozen (in a centrifuge tube) on dry ice and subsequently centrifuged for 2 h at 10000 rpm. The DNA pellet was lyophilized and resuspended in the 50 mM Tris-HCl–20 mM EDTA buffer and subjected to dialysis as described above.

Calculation of DNA concentration was performed with an extinction coefficient of 13 100 cm^{-1} M^{-1} (260 nm) per DNA base pair (Maniatis et al., 1982; Wells et al., 1970). The assumed molecular weight of one base pair in solution was 650.

Preparation of Radiolabeled DNA. For autoradiographic purposes, a 139 base pair restriction fragment of pBR322 was employed. The restriction fragment was prepared by digesting 20 μg of pBR322 in a total volume of 40 μL at 37 °C for 2 h. The digest solution was comprised of 7 μL of a 4.5 mM base pair solution of the plasmid, 2 μL of 0.1 M β-mercaptoethanol, 4 μL of a buffer containing 500 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 100 mM $MgCl_2$, 2 μL of *Hin*dIII (10

units/μL), 2 μL of *Nci*1 (10 units/μL), and 23 μL of deionized water.

Thereupon, 5 μL of reverse transcriptase (2.5 units/mL) was added to the mixture, and the entire contents were incubated for a further 2 h at 37 °C with 150 μCi of lyophilized [α-^{32}P]dATP. Afterward, 20 μL of a solution containing 70% glycerol, 0.5% sodium dodecyl sulfate, and 0.025% each of bromphenol blue and xylene cyanol was added to the digested plasmid solution and the mixture electrophoresed for 6 h at 600 V on an 8% polyacrylamide [29:1 acrylamide–bis(acrylamide)] gel, 40 cm in length and 3 mm in thickness. The gel was subjected to autoradiography with Kodak XAR-5 X-ray film, and the appropriate band corresponding to the 139-mer was excised from the gel and the DNA eluted (by agitation on a shaker bath over a 48-h period) into 20 mM Tris-HCl buffer, pH 8.0. The DNA was collected in the supernatant upon filtration through a glass wool plug (Maniatis et al., 1982) and was thereupon precipitated with ethanol, centrifuged, lyophilized, and resuspended in 50 mM Tris-HCl–1 mM EDTA buffer, pH 7.0, as described earlier.

Methods

Iron Porphyrin Mediated Scission of PM2-CCC-DNA. The DNA cleavage ability of the metalloporphyrins was qualitatively ascertained by digestion of supercoiled DNA by the chelates in the presence of a reducing agent. All digestion reactions were performed in 50 mM Tris-HCl buffer–1 mM EDTA, pH 7.0. Each digestion reaction comprised a total volume of 15 μL containing 5 μL of 50 μM base pair PM2-DNA, 5 μL of a 30% DMF buffer solution containing the metalloporphyrin, and 5 μL of 30 mM dithiothreitol solution. The reactions were allowed to proceed at 37 °C for 1 h at various porphyrin to DNA base pair ratios, r_t. The final concentration of DMF in the mixture was never allowed to exceed 10% even though DMF is a poor denaturant for DNA (Bussan et al., 1974). The reactions were terminated by the addition of 10 μL of solution comprising 70% glycerol, 0.025% each of bromphenol blue and xylene cyanol, 50 mM *n*-butyl isocyanide, and 50 mM *N*-methylimidazole.

Electrophoresis of digested PM2 superhelical DNA was performed on a 0.8% agarose slab gel in a horizontal electrophoresis system (Bethesda Research Laboratories). Electrophoresis proceeded at 1 V/cm for 14 h in a buffer containing 80 mM Tris–phosphate and 2 mM EDTA at pH 8.0. The agarose gel was subsequently allowed to soak for 1 h in 1 L of deionized water containing 100 μL of a 10 mg/mL solution of ethidium bromide. The stained gel was illuminated from beneath with a flat-topped UV light source and photographed by a Polaroid 545 Land camera using Polaroid type 57 (positive, 3000 ASA) or type 55 (positive and negative, 50 ASA) film.

Iron Porphyrin Induced Strand Scission of a HindIII/NciI Restriction Fragment from pBR322 DNA. The specificity of the DNase I activity in the hydrolysis of the 139-mer was referenced against G-specific and C-specific chemical degradation as outlined by Maxam and Gilbert (1980). The nuclease activity of the metalloporphyrins was then compared to the cleavage specificity of the bovine pancreatic 5′-endonuclease DNase I (Moore, 1981). Enzymatic digestion of the radiolabeled 139-mer was performed in the presence of calf thymus DNA, which enabled accurate control of the extent of digestion of the radiolabeled restriction fragment. The enzyme stock was diluted to a concentration such that degradation of the 139-mer was statistically confined to the "one-hit" limit (Lane et al., 1983). The activated enzyme was prepared, as required by the addition of 2 μL of a suitable stock solution (of the enzyme in 50% glycerol) to 398 μL of a buffer comprising 50 mM Tris-HCl, 32 mM $MgCl_2$, and 8 mM $CaCl_2$, pH 7.0.

Enzymatic digestion of the 139-mer entailed the combination of 2 μL of calf thymus DNA (770 μM base pair in 50 mM Tris-HCl buffer, pH 7.0), 2 μL of 139-mer (~7.7 μM base pair in similar buffer), 2 μL of buffer, and 2 μL of the activated enzyme complex. The 8-μL mixture was allowed to digest for 10 min at 37 °C and was subsequently terminated by the addition of 10 μL of a solution containing 70% urea, 20 mM EDTA, and 0.025% each of bromphenol blue and xylene cyanol maintained at 80 °C.

Porphyrins successfully screened for nuclease activity in plasmid DNA were employed in the digestion of radiolabeled 139-mer. This entailed the incubation of 2 μL of 770 μM base pair calf thymus DNA, 2 μL of ~7.7 μM base pair 139-mer, 2 μL of a 40% DMF buffer solution containing the porphyrin, and 2 μL of a 40 mM dithiothreitol solution. Incubation proceeded at 37 °C for 1 h and was stopped by the addition of 10 μL of a solution containing 70% urea, 50 mM *n*-butyl isocyanide, 50 mM *N*-methylimidazole, and 0.025% each of bromphenol blue and xylene cyanol, maintained at 80 °C. Prior to electrophoresis, each of the solutions was heated for 5 min at 90 °C.

Electrophoresis of the digested radiolabeled 139-mer was performed on a Hoeffer Model 1800 sequencing apparatus, using a 12% polyacrylamide [29:1 acrylamide–bis(acrylamide)] denaturing (50% urea) (Ansorge & Barker, 1984) field gradient gel with tapered Teflon spacers of width 0.3 mm (top) to 0.6 mm (bottom) to partly correct for nonlinearity of DNA migration through the gel matrix (Olsson et al., 1984; Ansorge & Labeit, 1984). Electrophoresis proceeded at 50 V/cm and ~70 °C for 2 h in a buffer composed of 90 mM Tris–borate and 2 mM EDTA, pH 8.3.

The gel was subsequently soaked in a 10% acetic acid– 5% methanol solution for 30 min, transferred to a filter paper backing (Bio-Rad), and evaporated to dryness on a Bio-Rad Model 1125B slab dryer. The dried gel was placed in intimate contact with Kodak XAR-5 X-ray film and stored at −20 °C for periods between 2 and 8 days depending on the specific radioactivity of the 139-mer. After development, the film was subjected to densitometric analysis, as described elsewhere (Dabrowiak et al., 1986).

Propenal Assay of Degraded DNA. The analysis for the base propenals, which are typical end products of Fe(III)–bleomycin-digested DNA, was conducted according to published procedures (Giloni et al., 1981). Thus, 30 μL of calf thymus (DNA (1.50 mM base pair), 30 μL of metalloporphyrin [or Fe(III)–bleomycin] solution (75.0 μM), and 30 μL of 5 mM reducing reagent were incubated for 4 h at 37° °C. Thereupon, 900 μL of a 0.6% solution of thiobarbituric acid was added to the digest and the entire mixture incubated for 30 min at 90 °C. Solutions were subsequently assayed optically for absorption at 532 nm (pink) as outlined earlier (Giloni et al., 1981) and compared to identical digests incubated with Fe(III)–bleomycin.

RESULTS

Iron Porphyrin Mediated Strand Scission of PM2-CCC-DNA. An initial examination of the oxygen-mediated DNA scission capabilities of the metalloporphyrins was carried out in the presence of dithiothreitol (DTT) as reducing agent on PM2-CCC-DNA. Analysis of the products was carried out with agarose gel electrophoresis, the results of which are summarized in Figures 3 and 4. The metalloporphyrins **1**–**6** exhibit nuclease activity under these particular experimental

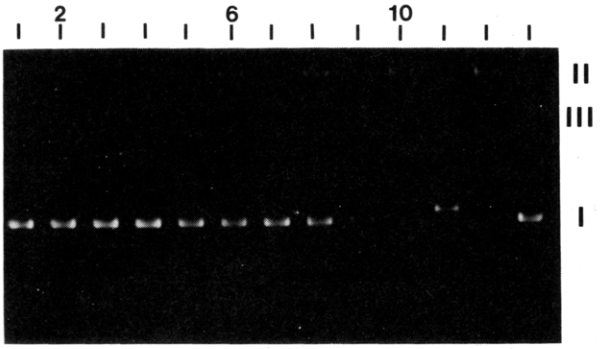


FIGURE 3: Digestion of PM2-DNA by Fe(III) porphyrins. Conditions are as described under Materials and Methods; r_t = 0.2. (Lanes 1 and 13) PM2 alone; (lane 2) DTT control; (lane 3) deuteroheme control; (lane 4) deuteroheme + DTT; (lane 5) **1** control; (lane 6) **1** + DTT; (lane 7) **2** control, (lane 8) **2** + DTT; (lane 9) **3** control; (lane 10) **3** + DTT; (lane 11) **4** control; (lane 12) **4** + DTT.

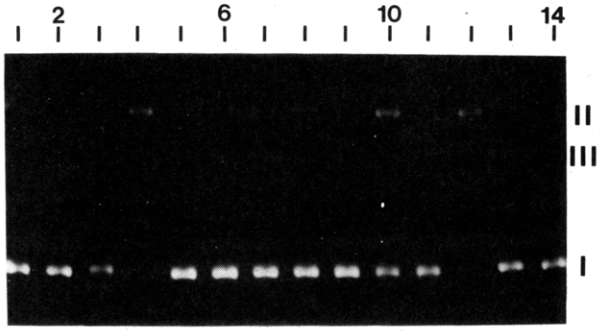


FIGURE 4: Digestion of PM2-DNA by Fe(III) porphyrins. Conditions are as described under Materials and Methods; r_t = 5. (Lane 1) PM2 alone; (lane 2) DTT control; (lane 3) **3** control; (lane 4) **3** + DTT; (lane 5) **7** control; (lane 6) **7** + DTT; (lane 7) **8** control; (lane 8) **8** + DTT; (lane 9) **5** control; (lane 10) **5** + DTT; (lane 11) **6** control; (lane **12**) **6** + DTT; (lane 13) **9** control; (lane 14) **9** + DTT.

conditions whereas in separate controls the chromophores I_1, I_2, I_3, and I_4 bearing the amine linkers but without attached metalloporphyrins were inactive. A single nick in the supercoiled CCC-PM2 allows a relaxation in the in-built tension in the helical structure (Campbell, 1976) producing the OC form, which travels less rapidly through the agarose matrix (Serwer & Allen, 1984). The remaining metalloporphyrins were found to have limited solubility in the DMF buffer solution used in the assay, and this may be the reason for the lack of strand scission activity in the cases of **7**–**15**. It was determined that the strict requirements for DNA scission by agents such as **1**–**6** were a reducible metal bound to the porphyrin (removal of the metal or replacement of Fe by Zn destroys activity), a suitable reducing agent (DTT or 2-ME), and access to molecular oxygen (Lown & Joshua, 1982; Lown et al., 1984b). The compounds also exhibit a slight enhancement of the efficiency of oxygen-mediated DNA scission with lowering pH in the range 6.0–8.0 (Lown et al., 1982, 1984b).

It may be noted from Figure 4 that the metalloporphyrins **3** and **4** are bound to the DNA even during electrophoresis. Compound **4** retards the migration of CCC-DNA but not the OC or linear forms. The change in electrophoretic mobility of CCC-DNA in the presence of two of the porphyrins is probably due to the fact that these porphyrins remain bound to the DNA during agarose gel electrophoresis. This implies that the DNA "off-rates" for these porphyrins are longer than for the others studied. Consideration of the structures of **3** and **4** suggests that off-rates can be lengthened by bis intercalation and/or monointercalation with a positive charge in

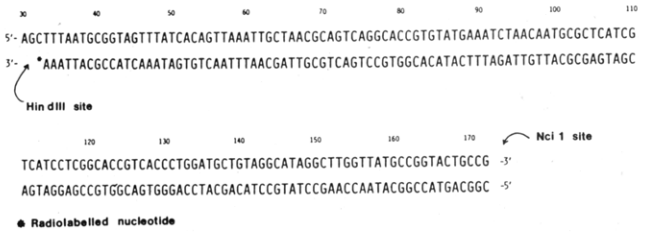


FIGURE 5: A listing of the sequence of the 139 base pair restriction fragment of pBR322 used in the sequencing reactions.

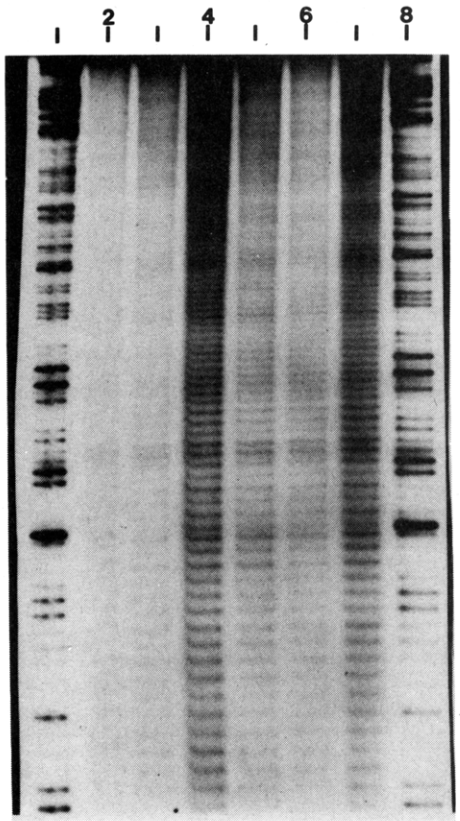


FIGURE 6: An autoradiogram representing digested 139-mer after electrophoresis. The lane numbers and DNA-cleavage agents were as follows: 1 and 8, DNase I; 2, **1**; 3, **2**; 4, **3**; 5, **4**; 6, **5**; 7, **6**. The ratio of metalloporphyrin to DNA base pairs r_t was 0.10.

the linker for the chromophore.

The selective retardation of the CCC-DNA but not OC or linear DNA suggests unwinding of the supercoiled structure, as has been observed for other porphyrin structures (Fiel & Munson, 1980; Carvlin & Fiel, 1983). Compound **3** appears to compact the supercoiled structure as evidenced by the "bleeding" of CCC-DNA in the **3** control lane. Similar marked compaction of DNA has been observed in electron microscopy studies of the intercalation of the anticancer drugs mitoxantrone and bisantrene and related structures with PM2-DNA and pBR322 DNA (Lown et al., 1984a). The reduced intensity of the PM2-CCC-DNA bands in both **3** and **4** controls is not due to autocutting but rather to the inaccessibility of DNA to ethidium, presumably owing to the occupation of binding sites by bound metalloporphyrin (Morgan et al., 1979).

Iron Porphyrin Mediated Strand Scission of a HindIII/NciI Restriction Fragment of pBR322 DNA. Comparative studies with DNase I and the porphyrins **1**–**6** revealed that, while the enzyme cleaved the pBR322 restriction fragment (Figure 5) in a sequence-dependent manner, the latter compounds produced strand scission with no apparent specificity, see Figures 6 and 7. Microdensitometric scanning of the

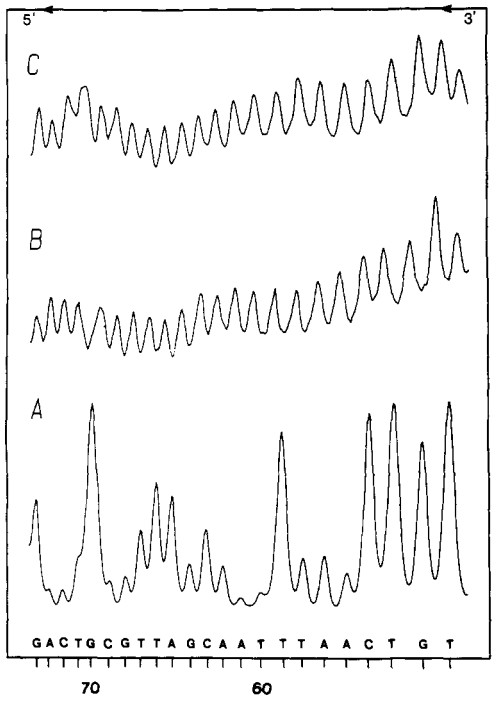


FIGURE 7: Densitometric scans of a select region in the lanes represented in Figure 8: (A) lane 1, DNase I; (B) lane 4, **3**; (C) lane 7, **6**.

autoradiogram showed, however, that the iron porphyrin induced cleavage of the fragment is slightly enhanced at approximately 10 base pair intervals, Figure 7. Inspection of individual bands revealed that porphyrin degradation of DNA produces single well-formed bands having apparent electrophoretic mobilities identical with their DNase I produced counterparts. Measurement of the amount of porphyrin-induced DNA cleavage by densitometry revealed that the efficiency of breakage by the compounds studied was in the order **3** > **6** > **4** > **1**, **2**, and **5**.

Discussion

The first three compounds active in the PM2-CCC-DNA scission assay, **1**–**3**, bind to double-helical DNA as monointercalators. The next three active compounds, **4**–**6**, bear two potential DNA-interactive groups, and while they appear to bind intercalatively, they may, or may not, act as bis intercalators. For this reason, comparison of the effects of DNA binding on cleavage efficiency is restricted to compounds **1**–**3**. These metalloporphyrin–acridines bind to double-helical calf thymus DNA with K_{assoc} ($\times 10^6$ L·mol^{-1}) at pH 7.0 and 37 °C of (**1**) 3.2, (**2**) 3.9, and (**3**) 9.2 (Lown & Joshua, 1982). The values for K_{assoc} ($\times 10^6$ L·mol^{-1}) under similar conditions for the corresponding metal-free (MF) porphyrin–acridines are (**1**-MF) 1.9, (**2**-MF) 1.6, and (**3**-MF) 3.0 while neither the porphyrin nor metalloporphyrin counterparts lacking the DNA interactive acridine moieties bind to calf thymus DNA under these conditions. These data suggest that the acridine moiety is essential for DNA binding and scission, that binding is enhanced by the positive charge on the metal-bearing domain, and that the linker $-NH(CH_2)_3NH(CH_2)_3-$ also enhances binding significantly compared with methylene chains. In addition, the value of K_{assoc} ($\times 10^6$ L·mol^{-1}) of 4.5 for compound **4** under similar conditions (Lown et al., 1984b) confirms that the longer and more basic linker present in **3** favors stronger binding, possibly to the phosphate groups in DNA, as is found with bleomycin and tallysomycin (Lown & Sim, 1977; Hecht, 1979) despite the presence of two potential intercalating groups in **4**. Binding constants for compounds **5** and **6** could not be determined accurately owing to the strong intrinsic fluorescence of the acodazole moiety, which interferes with the assay procedure (Morgan et al., 1979).

The compounds used in this study possess an iron porphyrin moiety appended to one or two groups capable of interacting with DNA. Since the macrocyclic portion of the compounds can be activated to yield a flux of DNA-damaging radicals, the materials are structurally similar to the synthetic DNA-cleaving agent methidiumpropylEDTA–iron MPE (Hertzberg & Dervan, 1984). The latter compound possesses an iron–EDTA moiety appended to the DNA intercalator ethidium bromide. The fact that all of the compounds studied here produce the same cleavage pattern indicates that a variety of DNA binding/linker combinations can be used without altering the sequence neutrality of the cleavage agent. Interestingly, this behavior does not appear to extend to other appended porphyrins. For example, iron porphyrins appended to the potent mutagen 2-amino-6-methyldipurido[1,2-*a*:3′,2′-*d*]imidazole (Hashimoto et al., 1984) have been reported to cleave DNA in a sequence-dependent manner similar to that of the anticancer agent bleomycin.

Although a detailed analysis of the mechanism by which the porphyrins induce strand scission was not undertaken, a few observations pertaining to the nature of the breakage chemistry are possible. A proposed mechanism of cleavage of the deoxyribose sugar in DNA by bleomycin–Fe(II)–O_2 envisages the formation of base propenals as one of the DNA degradation products (Giloni et al., 1981). The formation of a pink chromogen from enals and thiobarbituric acid contributed to the identification of base propenals in bleomycin–Fe(II)–DNA digests. Although the barbituric acid colorimetric test worked well in a control for DNA digested by bleomycin–Fe(II), no pink chromogen formed upon incubation of thiobarbituric acid with DNA digested by the porphyrin Fe(II) chelates **1**–**6**. Thus, it appears that, in contrast to bleomycin, base propenals are not the ultimate products of DNA degradation in this instance and is suggestive of either a different mechanism of strand scission or further oxidation of the base propenals. That the latter may be the case is indicated by the effects of certain cell-protective enzymes on the porphyrin-mediated strand scission process. The oxygen-mediated DNA scission exhibited by the hemin-linked acridines and acodazoles is partially suppressed by added catalase and/or superoxide dismutase (Lown et al., 1982, 1984b) in contrast to the case of the bleomycin–Fe(II)–O_2–DNA complex (Burger et al., 1979; Sausville et al., 1978). This indicates the intermediacy in the former case of H_2O_2 and $O_2^{\cdot -}$ species, respectively, which could cause further oxidation of the base propenals.

Action of bleomycin–Fe(II)–O_2 on DNA is known to leave a 5′-phosphate group and a glycolic acid residue esterified to the 3′-position of the two sugars adjacent to the site of DNA breakage (Giloni et al., 1981). While the nature of porphyrin-induced terminal chemistry was not directly addressed in the study, the fact that the shortest porphyrin-produced oligomers (12-mers) comigrate with their DNAse I produced counterparts suggests that strand scission leaves a phosphate group attached to the 5′-position of the sugar adjacent to the site of cleavage. Studies with short segments of DNA and DNase I have revealed that differences in terminal chemistry can give rise to significant differences in apparent electrophoretic mobilities of DNA in polyacrylamide gel matrices (Tapper & Clayton, 1981). The absence of satellite bands in the cleavage pattern further indicates that the porphyrin-

produced 5′-end chemistry is "clean" and that only a single oligomer of given length is produced in the degradation process.

An initial iron–oxygen complex is expected in the hemin group (Figure 1b) by analogy with the reversible binding of oxygen to hemoglobin (Walsh, 1979). The additional ligand L in the sixth coordinate position may be solvent, or in the case of the unbound compound, this may be accommodated by intramolecular participation of a nitrogen ligand. The iron porphyrins give spectral evidence of self-association in solution (Lown et al., 1984b). The analogy between the suggested mode of action of the synthetic agents and bleomycin or hemoglobin is strengthened by the observed inhibition by benzylisonitrile (Lown et al., 1982, 1984b). The latter, by preferentially binding to the iron in **3**, prevents formation of the ferryl–oxygen intermediate exactly as in the case of ethylisonitrile with bleomycin (Burger et al., 1979) and in the poisoning of hemoglobin (Walsh, 1979).

The relevance of the synthetic functional metalloporphyrins of the type **1–14** for bleomycin action is emphasized by the observation of biological activity. A ferroporphyrin–acridine strongly inhibits *Escherichia coli* growing in aerobic conditions in contrast to anaerobic conditions, which is also characteristic of bleomycin (Hecht, 1979). This result emphasizes the participation of oxygen in the intermediate involved in the cytotoxic event. In addition, of the compounds described in this paper, whereas **1–3** all exhibit growth inhibitory activity ($ID_{50} < 1.0\ \mu g \cdot mL^{-1}$) against murine L1210 leukemia cells in culture, compound **3** also gives a 22% increase in life span against P388 leukemia in mice, while the metal-free counterpart of **3** is inactive (Lown et al., 1984b). Thus, although it is recognized that other pharmacological factors including cellular uptake must be taken into account, the superior DNA binding and scission of **3** in the presence of reductants and oxygen may contribute to its anticancer properties.

The potential of compounds **1–6** as useful footprinting probes for studying the binding specificities of equilibrium binding drugs has not gone unnoticed. The observation that the compounds cleave DNA in a sequence neutral manner offers a distinct advantage over enzymatic probes such as DNase I. Since the latter is sensitive to twist angle, which is in turn dependent on sequence (Dickerson & Drew, 1981; Drew, 1984; Drew & Travers, 1984), enzyme-catalyzed DNA hydrolysis yields an "uneven" cleavage pattern, e.g., Figure 7A, making it difficult to evaluate binding phenomena at all points on the DNA lattice. The sequence neutrality of the metalloporphyrins and the fact they apparently yield "clean" 5′-end chemistry are desirable qualitites of useful footprinting probes.

On the negative side, the porphyrins appear to be relatively inefficient DNA-cleaving agents. Despite the long incubation time, 1 h and high amounts of probe relative to DNA, r_t of 0.1, only ~5% of the fragment (estimated from the autoradiogram) was cleaved by the most active porphyrin, **3**. Although direct comparisons were not made, compound **3** appears to be less efficient in DNA breakage than MPE (van Dyke & Dervan, 1983). This factor and the observation that structures **1–6** have limited solubility in water must be addressed if the compounds are to gain importance as new footprinting probes. Development of new more water-soluble DNA-cleaving agents along these lines and their application to footprinting will be reported in due course.

Supplementary Material Available

Full details of the synthesis of **1–15** together with their spectral and analytical characterization (7 pages). Ordering information is given on any current masthead page.

Registry No. **1**, 103501-26-4; **2**, 103501-13-9; **3**, 103501-14-0; **4**, 103501-15-1; **5**, 103501-27-5; **6**, 103501-16-2; **7**, 103501-17-3; **8**, 103501-18-4; **9**, 103501-19-5; **10**, 103501-20-8; **11**, 103501-21-9; **12**, 103501-22-0; **13**, 103501-23-1; **14**, 103501-24-2; **15**, 103501-25-3; $NH_2(CH_2)_4NH_2$, 110-60-1; $NH_2(CH_2)_6NH_2$, 124-09-4; $NH_2(CH_2)_{10}NH_2$, 646-25-3; $NH_2(CH_2)_{12}NH_2$, 2783-17-7; *p*-$MeC_6H_4CH{=}CHC_6H_4NH_2$-*p*, 97136-66-8; 7-methyl-9-chloroimidazo[4,5-*f*]quinoline, 55403-05-9; 9-(4-aminoanilino)-7-methylimidazo[4,5-*f*]quinoline, 103533-08-0; naphtho[2,1-*b*]thiophene-3-carboxylic acid, 37094-53-4; 6-amino-1-(naphtho[2,1-*b*]thiophene-3-carbonyl-3-yl)hexanamine, 103533-09-1; 10-amino-1-(naphtho[2,1-*b*]thiophene-3-carbonyl-3-yl)decanamine, 103533-10-4; 12-amino-1-(naphtho[2,1-*b*]thiophene-3-carbonyl-3-yl)dodecanamine, 103533-11-5; deuterohemin monomethyl ester, 85363-10-6; 2-methoxy-6-chloro-9-ethylenediamine–acridine dihydrochloride, 103533-12-6; 2-methoxy-6-chloro-9-propylenediamine–acridine dihydrochloride, 103533-13-7; 2-methoxy-6-chloro-9-propylenetriamine–acridine dihydrochloride, 103533-14-8; deuterohemin, 21007-21-6; 2-methoxy-6-chloro-9-butylenediamine–acridine, 7657-92-3; aminobutylaminoacodazole, 103533-15-9; hemin, 16009-13-5; bleomycin, 11056-06-7.

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Identification and Assignment of Base Pairs in Three Helical Stems of *Torulopsis utilis* Ribosomal 5S RNA and Its RNase T_1 and RNase T_2 Cleaved Fragments via 500-MHz Proton Homonuclear Overhauser Enhancements†

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ABSTRACT: Imino proton resonances in the downfield region (10–14 ppm) of the 500-MHz ^{1}H NMR spectrum of *Torulopsis utilis* 5S RNA are identified (A·U, G·C, or G·U) and assigned to base pairs in helices I, IV, and V via analysis of homonuclear Overhauser enhancements (NOE) from intact *T. utilis* 5S RNA, its RNase T_1 and RNase T_2 digested fragments, and a second yeast (*Saccharomyces cerevisiae*) 5S RNA whose nucleotide sequence differs at only six residues from that of *T. utilis* 5S RNA. The near-identical chemical shifts and NOE behavior of most of the common peaks from these four RNAs strongly suggest that helices I, IV, and V retain the same conformation after RNase digestion and that both *T. utilis* and *S. cerevisiae* 5S RNAs share a common secondary and tertiary structure. Of the four G·U base pairs identified in the intact 5S RNA, two are assigned to the terminal stem (helix I) and the other two to helices IV and V. Seven of the nine base pairs of the terminal stem have been assigned. Our experimental demonstration of a G·U base pair in helix V supports the 5S RNA secondary structural model of Luehrsen and Fox [Luehrsen, K. R., & Fox, G. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2150–2154]. Finally, the base-pair proton peak assigned to the terminal G·U in helix V of the RNase T_2 cleaved fragment is shifted downfield from that in the intact 5S RNA, suggesting that helices I and V may be coaxial in intact *T. utilis* 5S RNA.

5S RNA is found in the large subunit of all ribosomes except those of fungal and mammalian mitochondria (Thurlow et al., 1984) and is an essential component of ribosomal protein biosynthesis (Rohl & Nierhans, 1982; Brewer et al., 1983). Since its discovery (Rossett & Monier, 1963), 5S RNA has been studied intensively via genetic, enzymatic, chemical, and physical techniques in an effort to unravel its structure and the nature of its interactions with ribosomal proteins (Vandenberghe et al., 1985; Paleologue et al., 1985; Bohm et al., 1985; Kjems et al., 1985; Eigen et al., 1985; Curtiss &

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