0026-895X/88/030265-07$02.00/0

MOLECULAR PHARMACOLOGY, 33:265-271

Characteristics of the Interaction of Anthrapyrazole Anticancer Agents with Deoxyribonucleic Acids: Structural Requirements for DNA Binding, Intercalation, and Photosensitization

JOHN A. HARTLEY, KRZYSZTOF RESZKA, E. T. ZUO, W. DAVID WILSON, A. RICHARD MORGAN, and J. WILLIAM LOWN

Departments of Chemistry (J.A.H., K.R., J.W.L.) and Biochemistry (A.R.M.), University of Alberta, Edmonton, Alberta, T6G 2G2, Canada, and Department of Chemistry, Georgia State University, Atlanta, Georgia 30303 (E.T.Z., W.D.W.)

Received October 1, 1987; Accepted December 17, 1987

SUMMARY

The binding constants for interaction of several novel anthra[1,9-*cd*]pyrazol-6(2H)-ones (anthrapyrazoles) with DNA have been determined by an ethidium displacement method. The apparent binding constants range from $<2 \times 10^6$ to 2.7×10^8 M^{-1}. The binding is influenced not only by the nature of the side chains but also by the number and position of hydroxyl groups on the chromophore. Unwinding angles, determined by a topoisomerase I assay, ranged from 0° to 29.2°. The deshydroxy compound **1** gave the highest unwinding angle, and both substitution of hydroxyl groups in the chromophore and alterations in the side chains decrease the unwinding angle, consistent with a decreased or partial intercalation. Representative anthrapyrazoles cause an increase in sonicated DNA viscosity as expected for intercalators. Spectrophotometric examination of the binding of compound **1** to DNAs of different base composition show that the apparent binding to GC is approximately 3 times that of AT, a result which was paralleled by thermal denaturation studies. Certain of the anthrapyrazoles exhibit marked visible light photosensitization and induce DNA single-strand breakage upon illumination in the presence of NADH. The essential structural requirement for photosensitizing properties with these agents was the absence of hydroxyl groups in the chromophore. By employing ^{32}P-labeled DNA of known sequence, it was possible to examine the anthrapyrazole 1-photosensitized cleavage of DNA at the individual base level employing denaturing polyacrylamide sequencing gels. Smooth sequence neutral photosensitized cleavage of DNA is observed analogous to hydroxyl radical "footprinting."

The new class of DNA complexers, the anthra[1,9-*cd*]pyrazol-6(2H)-ones (anthrapyrazoles, Fig. 1), was synthesized with the rationale that appropriate chromophore modifications of the anthracenedione nucleus of agents such as the clinically useful mitoxantrone (1) might diminish cardiotoxicity by reducing the potential to form semiquinone free radicals (2). These novel agents demonstrated high levels of activity against a broad range of murine tumors *in vivo* including the P388 leukemia, mammary adenocarcinoma 16, and the B-16 melanoma (3–5).

The anthrapyrazoles bind strongly to DNA, are potent inhibitors of DNA synthesis, and cause the formation of DNA single-strand breaks in cells as determined by alkaline elution studies (3). They also induced far less superoxide dismutase-sensitive oxygen consumption than did doxorubicin in a rat liver microsomal system, which may be indicative of reduced cardiotoxicity (3). In addition, as a result of their change in light absorption characteristics compared to mitoxantrone, certain of these agents are effective photosensitizers (6). The production of both the superoxide radical and hydrogen peroxide in aerated aqueous solutions containing NADH or ascorbic acid was demonstrated for anthrapyrazole **1** (lacking the chromophore hydroxyl group), but only following illumination (7, 8). The anthrapyrazole-photosensitized formation of single-strand breaks in DNA has also been demonstrated for this compound (9).

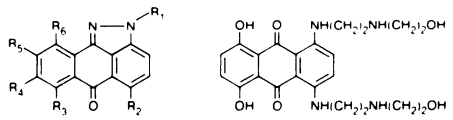
It is generally assumed that the mode of action of these agents, at least in part, is dependent on their interaction with DNA. The present study describes the characteristics of this binding and an examination of (a) the substituent dependence of these agents to both bind and intercalate efficiently into DNA and (b) the structural requirements for photosensitization.

Materials and Methods

Compounds. The anthrapyrazoles used in this study were kindly supplied by Dr. H. D. H. Showalter of Warner-Lambert Company, Ann

This investigation was supported by grants (to J. W. L.) from the National Cancer Institute of Canada and the Natural Sciences and Engineering Research Council of Canada, Grant A-2305 (to A. R. M.) from the Medical Research Council of Canada, and Grant GM 30267 (to W. D. W.) from the National Institutes of Health.

ABBREVIATIONS: EDTA, ethylenediaminetetraacetic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; CCC, covalently closed circular; OC, open circular; GC, guanine:cytosine base pair; AT, adenine:thymidine base pair.



Anthrapyrazole

Mitoxantrone

Compound	R_1	R_2	R_3	R_4	R_5	R_6
1	$(CH_2)_2NH(CH_2)_2OH$	$NH(CH_2)_2NH(CH_2)_2OH$	H	H	H	H
2	H	$NH(CH_2)_2NH(CH_2)_2OH$	H	H	H	H
3	$(CH_2)_2NH(CH_2)_2OH$	$NHCH_3$	H	H	H	H
4	$(CH_2)_2NH(CH_2)_2OH$	$NH(CH_2)_2NH(CH_2)_2OH$	OH	H	H	H
5	$(CH_2)_2NH(CH_2)_2OH$	$NH(CH_2)_2NH(CH_2)_2OH$	H	H	H	OH
6	$(CH_2)_2NH(CH_2)_2OH$	$NH(CH_2)_2NH(CH_2)_2OH$	OH	H	H	OH
7	CH_2CH_2OH	$NH(CH_2)_2NH(CH_2)_2OH$	OH	H	H	OH
8	$(CH_2)_2NH(CH_2)_2OH$	$NH(CH_2)_2NH_2$	OH	H	H	OH
9	$(CH_2)_2NH(CH_2)_2OH$	$NH(CH_2)_2NH(CH_2)_4NH(CH_2)_2NH_2$	OH	H	H	OH
10	$(CH_2)_2NH(CH_2)_2OH$	N⌒N-Me	OH	H	H	OH
11	$(CH_2)_2NH(CH_2)_2OH$	$NH(CH_2)_2NH(CH_2)_2OH$	OH	OH	H	OH
12	$(CH_2)_2NH(CH_2)_2OH$	$NH(CH_2)_2NH(CH_2)_2OH$	OH	H	OH	OH
13	$(CH_2)_2NH(CH_2)_2OH$	$NH(CH_2)_2NHCH_3$	OH	OH	H	OH
14	CH_3	H	$NH(CH_2)_2NH\text{-}(CH_2)_2OH$	H	H	H

Fig. 1. Structures of the anthrapyrazoles used in this study.

Arbor, MI. Their synthesis, purification, and properties have been described previously (2). Ethidium bromide was purchased from Sigma Chemical Company, St. Louis, MO. Mitoxantrone was supplied by Dr. K. C. Murdock of Lederle Laboratories, Pearl River, NY.

Buffers. The test solution for the ethidium binding assay was 5 mM Tris, 0.5 mM EDTA, 0.5 μg/ml ethidium bromide, pH 8. Tris-HCl buffer for the determination of DNA unwinding angles contained 10 mM Tris-HCl, 2 mM EDTA, 0.1 M KCl, 0.1 mg/ml gelatin at pH 8. The 0.1× SSC buffer used in the determination of thermal melting contained 15 mM NaCl, 1.5 mM sodium citrate, pH 7. PIPES 10 buffer contained 10 mM PIPES, 1 mM EDTA, 100 mM NaCl adjusted to pH 7.0. PIPES buffers at higher ionic strength were prepared by increasing the NaCl concentration.

DNA samples. Calf thymus, *Micrococcus lysodeikticus*, and *Clostridium perfringens* DNAs were obtained from Sigma Chemical Company. DNA polymer samples and pBR322 were from Pharmacia PL Biochemicals. DNA samples were dissolved overnight at 4° in 0.1 × SSC buffer. Phage PM2 CCC-DNA was prepared as described previously (10). Sonicated calf thymus DNA was prepared as described in detail previously (11). All sonications were achieved using a Heat Systems Ultrasonics W375B pulsed sonicator.

Enzymes. Calf thymus DNA topoisomerase I was prepared as described previously (10). The enzyme relaxed PM2-CCC-DNA completely, giving a drop in fluorescence of 33 ± 3% in the pH 12 ethidium bromide assay (12), which corresponds to a superhelical density of −0.126, given an average ethidium unwinding angle of 28° per intercalated cation (13).

Estimation of drug-DNA binding constants. Drug-DNA binding constants were estimated as described previously (12). To 2 ml of Tris-EDTA buffer, pH 8, containing 1.3 μM ethidium bromide, calf thymus DNA was added to give a final concentration of 1.35 μM. The fluorescence was measured after equilibrium for a few minutes, using a Turner model 430 spectrofluorometer (Turner Amsco Instruments, Carpinteria, CA) equipped with a 150-W xenon lamp, at an excitation wavelength of 525 nm and an emission wavelength of 600 nm. Aliquots of concentrated drug solutions were added and the fluorescence was measured. Controls were performed to show that the drugs themselves did not interfere with the fluorescence measurements at the levels employed. From a plot of the decreased fluorescence of the ethidium-DNA complex with increasing dose of drug, the concentration of drug needed to reduce the fluorescence by 50% was determined and used to calculate a relative binding constant for the drug, given the binding constant of ethidium to be 10^{-7} M^{-1} under similar conditions (14).

Determination of DNA unwinding angles using topoisomerase I. Varying concentrations of drug (0–16 μl of a 0.1 mM stock solution) were added to 0.24 mM PM2-CCC-DNA in 10 mM Tris-HCl buffer (10 mM Tris-HCl, 2 mM EDTA, 0.1 M KCl, 0.1 mg/ml gelatin, pH 8) in a total volume of 33 μl. When topoisomerase was present, 1 μl of a stock solution was added to give a final concentration known to completely relax the DNA in 30 min under the reaction conditions. Samples were incubated at 22°. Aliquots (15-μl) were taken at 1 and 2 hr and added to 2 ml of pH 12 ethidium assay solution (12). The fluorescence was measured both before and after heat denaturation at 96° for 2 min. Fluorescence was plotted against the drug-to-DNA base pair ratio and the unwinding angle was calculated from the crossover point of the curves in the presence and absence of topoisomerase I (12).

Viscosity assay. Viscometric measurements were made in Cannon-Ubbelhohde semimicrodilution viscometers using electronic timing as previously described (15). Sonicated calf thymus DNA in PIPES 10 buffer at a concentration of 6–7 × 10^{-5} M was used for measuring length increases in linear DNA with increasing drug concentrations.

Spectrophotometric method. Absorbance measurements were made on a Cary 219 spectrophotometer interfaced to an Apple IIe microcomputer through a bidirectional digital communications port. Cell holders were thermostated by using Neolab or Haake circulating water baths. To remove some of the random error, for each absorbance measurement the microcomputer calculated the average of 100 acquired absorbance readings at the preselected wavelength for the compound under study. These averaged absorbance values were converted by the microcomputer to υ (mol of compound bound/mol of DNA base pairs) and free ligand concentrations using the free and bound extinction coefficients for the compound (16). At the end of the titration, the computer plotted the digitized data which were in the fraction-bound range, 0.2–0.8. Any binding results outside of this range are subject to large systematic errors due to experimental inaccuracies in extinction coefficients. The computer then calculated nonlinear least squares best fit k (equilibrium constant) and n (base pairs per binding site) values from the site exclusion method of McGhee and von Hippel (17). Dimerization constants were also determined spectrophotometrically as previously described (18) and binding isotherms were corrected for dimerization, where necessary, before fitting to the site exclusion model.

Thermal denaturation studies. Solutions of DNA from *C. perfringens* (31% GC), calf thymus (45% GC), *M. lysodeikticus* (72% GC), and polydA·polydT were dissolved in 0.1× SSC buffer at 150 μM. Samples were heated at a rate of 1°/min in the absence and presence of drug at a drug/DNA base pair ratio of 0.05. The melting temperature midpoint (T_m) was determined from the absorbance profile produced on the Gilford 250 spectrophotometer equipped with a thermal programmer.

Detection of photosensitized DNA scission. Reaction mixtures (40 μl) contained 20 μM PM2 DNA, 10 mM Tris-HCl, 1 mM EDTA, and 100 mM KCl, pH 7.6. NADH was added where appropriate to a final concentration of 1 mM. Drugs were added in the dark from 0.1 mM stock solutions in distilled water. Samples were illuminated for 15 min where appropriate with light of wavelength 400 ≲ λ ≲ 540 nm from a 300-W projector lamp equipped with a combination of broad band filters. The incident fluence rate was ~10 W/m^2 as measured using a YSI Radiometer model 65A. Following illumination, aliquots of 2 μl of 10% SDS were added to control and illuminated samples, and 5 μl of a sample solvent containing 50% glycerol and 0.25% bromophenol blue tracking dye.

Samples were loaded onto 1% agarose gels and run at 4 V/cm for 6 hr on a horizontal submerged gel electrophoresis system (Bethesda Research Laboratories, Rockville, MD). The agarose electrophoresis running buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.1) contained 0.1% SDS in order to dissociate intercalator molecules from the DNA. Gels were stained with 0.5 μg/ml ethidium bromide after the SDS had been removed by several washes with water. After staining for 1 hr, the gels were destained for several hours, illuminated with ultraviolet light, and photographed. Quantitation of bands was achieved by microdensitometry of the negative produced from the gel photograph using an Ultrascan XL Laser Densitometer (LKB Products, Sweden).

Photosensitized DNA cleavage specificity. *Hind III* digested pBR322 DNA was ^{32}P-labeled at its 5′-ends and further digested with

EcoRI using established procedures (19). Reactions were identical to those described above for the detection of photosensitized DNA scission except that the reaction mixture contained ~10,000 cpm of ^{32}P-labeled DNA. Following illumination, samples were frozen, lyophilized, and taken up in 2 μl of sample solvent containing 80% formamide, 1 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cylanol. Samples were heated at 90° for 1 min and chilled on ice prior to loading onto a 0.4-mm thick, 55-cm long 6% acrylamide, 5 M urea polyacrylamide sequencing gel. Samples were run at 2500 V, 55° for approximately 1.5 hr on an LKB Macraphor Electrophoresis unit. The gel was transferred onto filter paper and placed in intimate contact with Kodak XAR X-ray film and stored at −70° for 24 hr. After development the film was scanned using an LKB Ultroscan XL Laser densitometer.

Results

Drug-DNA binding constants. The binding constants of the drugs to DNA were estimated and compared by measuring the reduction in fluorescence of an ethidium-DNA complex as a result of competitive displacement (12). The drug concentration which produced 50% inhibition of fluorescence was assumed to be inversely proportional to the binding constant.

The apparent binding constants for the series of anthrapyrazoles **1–14** is shown in Table 1. None of the drugs tested interfered with the fluorescent measurement at the levels used, and all displaced ethidium from the DNA with apparent binding constants ranging from $<2 \times 10^6$ to 2.7×10^8 M^{-1}. With the exception of compound **8**, all of the anthrapyrazoles had lower binding constants than mitoxantrone. The binding was seen to be influenced not only by the nature of the side chains R_1 and R_2, but also by the number and position of hydroxyl groups on the chromophore at positions R_3–R_6. When R_1 and R_2 were kept constant, the placing of a hydroxyl group at R_6 (compound **5**) resulted in a reduction in apparent binding constant by a factor of 2 over the deshydroxy compound **1**. In contrast, a single hydroxyl group at R_3 (compound **4**) increased the binding, and hydroxyl substitutions at both R_3 and R_6 (compound **6**) resulted in a greater than 2-fold enhanced binding constant. Trihydroxy-substituted compounds **11** and **12** similarly showed an increased binding compared to compound **1**.

Altering the nature of the side chains R_1 and R_2 also affected the apparent binding constant. When R_1 was replaced by hydrogen (compound **2**), the binding was reduced 20-fold. Similarly, the shortened side chain at R_1 in compound **7** resulted in a 5-fold reduction. Alterations in R_2 could either slightly enhance the binding (compounds **8** and **10**), or decrease it (compounds **9**, **13**, and **3**). In the case of compound **3**, the effect was dramatic, the shortened side chain resulting in a 35-fold reduction in binding constant. Compound **14**, in which the parent side chain is placed on the chromophore at R_3, still maintained a high degree of binding similar to that of the deshydroxy compound **1**.

TABLE 1

Relative DNA binding constants for the anthrapyrazoles as determined by the ethidium displacement assay

Compound	DNA binding constant
	M^{-1}
1	7.4×10^7
2	4.0×10^6
3	$<2.0 \times 10^6$
4	1.0×10^8
5	3.7×10^7
6	2.0×10^8
7	4.1×10^7
8	2.7×10^8
9	7.6×10^7
10	2.4×10^8
11	1.9×10^8
12	8.0×10^7
13	8.7×10^7
14	6.5×10^7
Mitoxantrone	2.6×10^8

Determination of DNA unwinding angles using topoisomerase I. Topoisomerase I will relax negatively supercoiled CCC-DNA in the absence of drug and the topological winding number α will increase. However, at a particular level of intercalating drug, sufficient to relax the DNA, the topoisomerase will have no effect on α. As the level of drug is increased the enzyme will relax positive supercoils and α will decrease. Thus, a plot of intercalated ethidium fluorescence can be used to determine the level of drug that can just relax the DNA by measuring the crossover point of curves in the presence and absence of topoisomerase.

Such a plot is illustrated in Fig. 2A. Compound **1** gave a well defined crossover point. In the absence of drug the fluorescence for the reaction containing topoisomerase relative to that without showed the expected decrease of 33 ± 3%. For compound **1** the crossover corresponded to an unwinding angle of 29.2°, indicating an efficient intercalative binding similar in magnitude to ethidium.

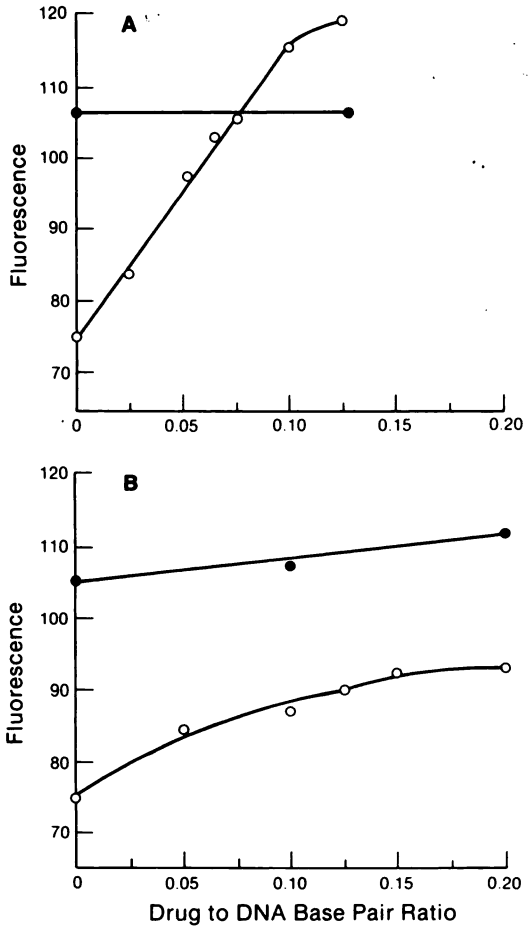


Fig. 2. Unwinding angle assays of compound **1** (A) and compound **12** (B), employing calf thymus topoisomerase I and PM2-CCC-DNA. Fluorescence was measured in arbitrary units. Reactions were carried out as in Materials and Methods either with (○) or without (●) topoisomerase.

The corresponding unwinding angles for the other anthrapyrazoles are seen in Table 2. With the exception of the three trihydroxy-substituted compounds **11**, **12**, and **13**, all of the agents tested showed unwinding angles between 16.9° and 29.2°. The deshydroxy compound **1** gave the highest unwinding angle, and both substitutions of hydroxyl groups at positions R_3–R_6, and alterations in the side chains R_1 and R_2, resulted in a decreased value of the unwinding angle consistent with a decreased, or partial, intercalation.

In contrast to the apparent binding constant, the intercalative ability of the drugs, as determined by the unwinding angle, was affected more by the presence of hydroxyl groups on the chromophore than by marked changes in the R_1 and R_2 side chain, e.g., compounds **2** and **3**, which show that the largest decreases in apparent binding constant were still efficient intercalating agents, giving unwinding angles of 24° and 22.8°, respectively, compared to 29.2° for the parent compound **1**. However, the compounds containing three hydroxyl groups (**11**–**13**), which have relatively high binding constants, did not produce crossover points, and, therefore, no value for an unwinding angle could be determined. This is illustrated in Fig. 2B for compound **12**. Thus, it would appear that exterior DNA binding, rather than intercalation, is the major mode of binding of these latter agents.

As a control, the unwinding angle of ethidium was also determined under the same conditions. An unwinding angle of 28.3° corresponded to previously published data (13). Similarly, the value of 17° obtained for mitoxantrone corresponds to that published previously (11), indicating that, with the exception of the trihydroxy compounds, the anthrapyrazoles are more efficient intercalators than mitoxantrone.

Viscosity assay. Compounds **1**, **4**, and **6**, in which R_1 and R_2 are identical and which differ only in the number of hydroxyl groups on the chromophore, were compared using the viscometric titration assay (Fig. 3). All three compounds caused an increase in sonicated DNA viscosity as expected for intercalators. The three compounds gave quite similar titration curves, the viscosity increases being slightly higher than that observed previously for mitoxantrone (11).

Spectrophotometric assay. Shifts in the spectrum of compound **1** induced by the addition of DNA are shown in Fig. 4. Other anthrapyrazoles show similar large hypochromicity and relatively small shifts to longer wavelengths on binding to DNA. Shifts of this type with different DNA samples were used to evaluate binding constants and base pair interaction specificity. Typical binding isotherms are shown in the form of Scatchard plots in Fig. 5. Best fit k and n values used to construct the solid lines in the figure were determined using the equation (17):

$$\upsilon/C = k[1 - n\upsilon][(1 - n\upsilon)/[1 - (n - 1)\upsilon]]^{n-1}$$

Compound **1** binds strongly to all three DNA samples at this

TABLE 2
DNA unwinding angles of the anthrapyrazoles

Compound	Unwinding angle
	deg
1	29.2
2	24.0
3	22.8
4	23.8
5	18.5
6	21.0
7	17.6
8	20.5
9	21.6
10	16.9
11	0
12	0
13	0
14	19.6
Mitoxantrone	17.0

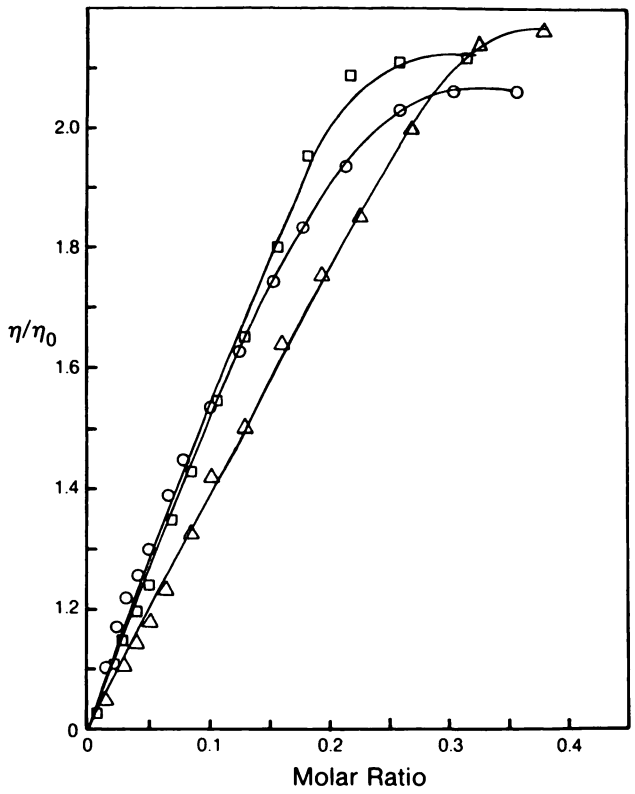


Fig. 3. Viscometric titrations of calf thymus DNA with compounds **1** (△), **4** (□), and **6** (○). Titrations were in PIPES 10 buffer at 30°.

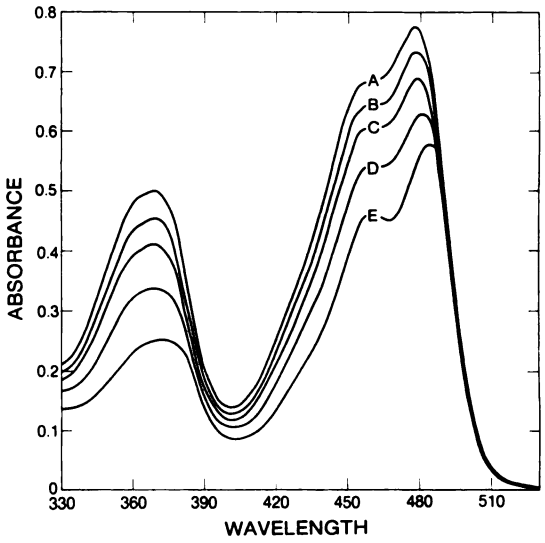


Fig. 4. Spectra for compound **1** at 6.3 × 10^{-5} M in a 10-cm quartz cuvette in PIPES buffer with 0.1 M added NaCl. Drug-to-DNA base pair ratios are: A, 0; B, 1.53; C, 0.79; D, 0.39; and E, 0.15.

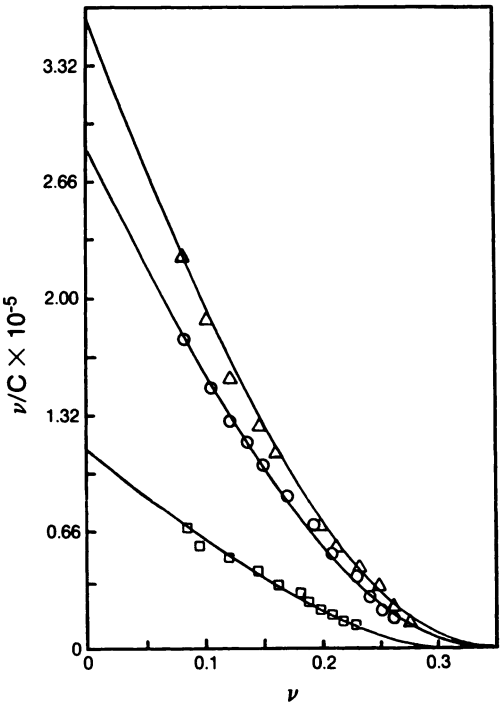


Fig. 5. Scatchard plots for the binding of compound **1** to calf thymus DNA (△), poly d(G-C)$_2$ (○), and poly d(A-T)$_2$ (□). The *symbols* represent experimental points which have been corrected for dimerization of this compound. The *solid lines* represent nonlinear least squares best fit results to the data using the site exclusion model of McGhee and von Hippel (17).

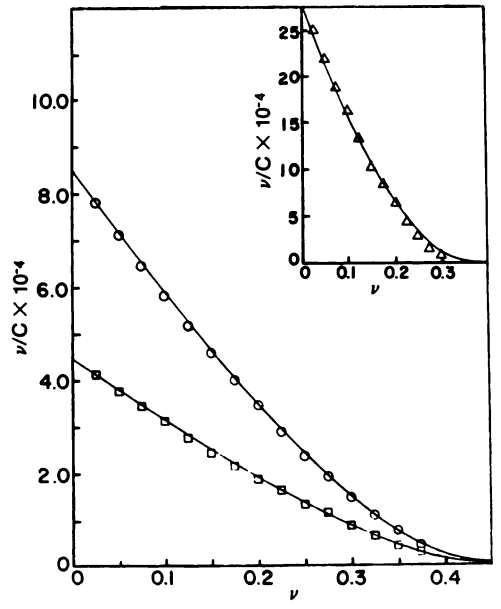


Fig. 6. Scatchard plots for the binding of compound **1** to calf thymus DNA at different ionic strengths: PIPES buffer with 0.1 M NaCl (△), 0.2 M NaCl (○), and 0.3 M NaCl (□). Fitting was as in Fig. 4.

salt concentration. The apparent binding to GC is approximately 3 times that of AT.

Binding of compound **1** to calf thymus DNA was also investigated as a function of ionic strength, and typical isotherms at different salt concentrations are shown in Fig. 6. A plot of log K versus log [Na^+] for these results had a slope of −1.9. The condensation theory (20, 21) of polyelectrolytes predicts that the slope of a plot such as the one shown in Fig. 6 should have a slope of approximately (0.24 ± 0.82 m') where m' is the number of ion pairs formed between the drug and DNA phosphate groups (16). Clearly m' is 2 for the interaction of compound **1** with DNA, indicating that both of the asymmetric catonic groups of compound **1** can form a strong electrostatic interaction with the DNA helix when the anthrapyrazole is intercalated.

Thermal denaturation studies. Thermal denaturation studies were carried out on selected anthrapyrazoles in which R_5 and R_6 remained constant and R_3–R_6 contained zero, one, two, and three hydroxyl groups. It can be seen in Fig. 7 that, at a drug/DNA base pair ratio of 0.05, all of the agents produced an elevation in the T_m of the DNAs. The effect was most striking in the case of deshydroxy compound **1** and was smallest with the trihydroxy compound **11**. The elevation in T_m thus appeared to parallel the intercalative ability of the agents as determined by the unwinding angle, rather than their overall ability to bind to the DNA. In addition, all the agents showed a small but significant increase in the T_m with increasing GC content of the DNA, indicating a preference for such sequences. The exception was compound **4**, which showed an elevated T_m with *C. perfringens* (31% GC) and calf thymus (45% GC) DNAs over poly dA·poly dT but reproducibly showed a reduction in the T_m with *M. lysodeikticus* DNA (72% GC).

Anthrapyrazole-photosensitized DNA scission. CCC-DNA represents a sensitive substrate for detecting single- and double-strand breaks in DNA. A single-strand break converts the double-stranded CCC DNA into an OC which migrates more slowly on electrophoresis in 1% agarose gels. Double-strand breaks generate linear molecules which migrate intermediately between the OC and CCC forms.

Fig. 8 shows a typical agarose gel of PM2 DNA. In the absence of drug and in the dark (Fig. 8, lane *1*), the DNA is essentially all of the superhelical CCC form. Illumination with light for 15 min does not increase the amount of OC DNA (Fig. 8, lane *2*). Three deshydroxy anthrapyrazoles (compounds **1**, **2**, and **3**) and three containing hydroxyl groups at *R*3 and R6 (compounds **6**, **7**, and **10**) were compared for their ability to induce single-strand breaks in DNA, in the absence and presence of light. All samples contained 1 mM NADH as electron donor. None of the compounds tested produced any DNA damage in the dark (Fig. 8, lanes *3–8*). Following 15 min of illumination, however, the three deshydroxy compounds induced a significant level of single-strand breaks into the DNA

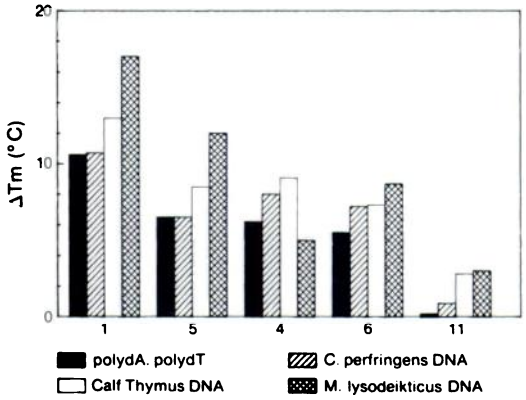


Fig. 7. Increase in the T_m for compounds **1**, **4**, **5**, **6**, and **11** at a drug/DNA base pair ratio of 0.05 on polydA·polydT, *C. perfringens* DNA (31% GC), calf thymus DNA (45% GC), and *M. lysodeikticus* DNA (72% GC) in 0.1 × SSC buffer.

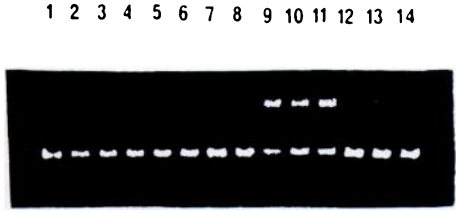


Fig. 8. Anthrapyrazole-photosensitized cleavage of PM2 DNA. Agarose gels (1%) of PM2 DNA in the CCC and OC forms are shown. All reactions contained 1 mM NADH, 20 μM PM2 DNA, and 20 μM drug where appropriate. Lanes *1* and *2*, no drug; lanes *3* and *9*, compound **1**; lanes *4* and *10*, compound **2**; lanes *5* and *11*, compound **3**; lanes *6* and *12*, compound **6**; lanes *7* and *13*, compound **7**; and lanes *8* and *14*, compound **10**. For lanes *1* and *3–8*, samples were in the dark; for lanes *2* and *9–14*, samples were illuminated for 15 min.

such that >50% of the DNA was in the relaxed OC form (Fig. 8, lanes *9–11*). The extent of damage was not affected by the marked changes in the side chains *R*1 and *R*2 in compounds **2** and **3** compared with the parent compound **1**. No damage was observed with the three hydroxyl-substituted anthrapyrazoles (compounds **6**, **7**, and **10**) even after 15 min of illumination (Fig. 8, lanes *12–14*).

Photosensitized DNA cleavage specificity. Using ^{32}P-labeled DNA of known sequence, it was possible to examine the anthrapyrazole **1**-photosensitized cleavage of DNA at the individual base level employing denaturing polyacrylamide sequencing gels. In Fig. 9, autoradiogram densitometric traces are seen of anthrapyrazole **1**-treated DNA following 0, 8, and 16 min of illumination. Photosensitized cleavage of the DNA is observed at every base along the sequence, and no sequence preference for the cleavage is apparent.

Discussion

The present paper describes some of the aspects of the interaction between a number of examples from the novel class of DNA binders, the anthrapyrazoles, and DNA. This class of compounds was synthesized with the rationale that appropriate chromophore modification of the anthracenediones related to mitoxantrone might provide agents with diminished cardiotoxicity because of a lower tendency to generate reactive oxygen-derived free radicals (2). It has been possible in the present study to compare a number of related compounds in order to determine the structural requirements for efficient DNA binding, intercalation into DNA, sequence specificity, and photosensitization.

The anthrapyrazoles bear the requisite planar aromatic chromophore recognized as an essential requirement for intercalation (22). In molecules such as mitoxantrone, however, the presence of the two extended side chains is assumed to preclude the incorporation of all parts of the molecule, which has led to various suggestions for alternative modes of binding (23, 24) and would account for the value of 17° observed for the unwinding angle of this drug, consistent with a partial intercalation. The essential features of the intercalation model for mitoxantrone into GC base pairs, i.e., binding from the major groove with alignment of the chromophore perpendicular to the base pair axis, originally deduced by NMR (25), has recently been confirmed by X-ray diffraction analysis (26).

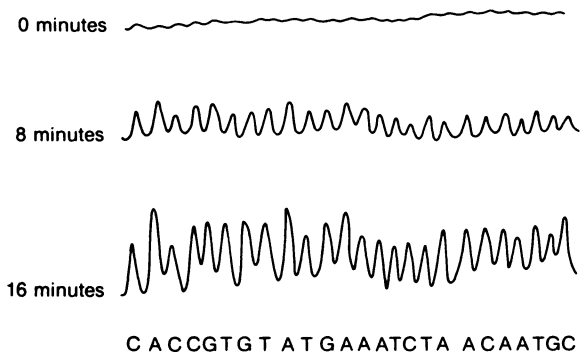


Fig. 9. Anthrapyrazole-photosensitized cleavage of pBR322 DNA. Smoothed densitometric traces of sequencing gel autoradiograms of pBR322 DNA following treatment with compound **1** and illumination for the times indicated. The corresponding base sequence of the DNA is also indicated.

In the present study, however, the presence of extended side chains on the anthrapyrazole structure does not necessarily inhibit the ability of the drug to intercalate efficiently. In fact, the deshydroxy compound **1**, which contains the same side chains as mitoxantrone, is seen to be a very efficient intercalator, similar in magnitude to ethidium (13). But the presence of hydroxyl groups on the chromophore appears to interfere with the intercalative ability of the drug since the observed unwinding angle decreases with increasing numbers of hydroxyl substitutions. This trend is also true in the case of ametantrone, the deshydroxy analogue of mitoxantrone, which gives an unwinding angle of 23.7°, considerably higher than that of mitoxantrone.[1] The increased value for the unwinding angle of the deshydroxy anthrapyrazole (29.2°) compared with ametantrone (23.7°) may be due to the more rigid planar structure of the anthrapyrazole imposed by the fourth ring in the molecule.

When the side chains *R*1 and *R*2 are shortened (e.g., compounds **2** and **3**), a modification which markedly decreases the apparent overall DNA binding constant of the anthrapyrazoles, the intercalative ability of the drugs is maintained as determined by the unwinding angle. Thus, it would appear that, at least in the case of these agents, the side chains *per se* are not interfering in the intercalative binding. The trihydroxy compounds **11**, **12**, and **13** bind strongly to DNA but do not appear to intercalate since no unwinding angle could be obtained. Previously, these compounds, in contrast to the other anthrapyrazoles, were observed not to produce the protein-linked single- and double-strand DNA breaks observed with other intercalators (3, 27). This was attributed either to the instability of these drugs in media or to their inability to traverse the cell membrane, but could equally be due to their inability to intercalate into the DNA as observed in the present study.

Both the spectrophotometric results and the thermal melting data indicate a slight preference of the anthrapyrazoles for GC-rich sequences. This has been observed previously for mitoxantrone using spectroscopic binding studies (11), electron microscopy studies (28), and by NMR (29). The sequence recognition is evidently subtle, however, since the DNase footprinting technique applied to mitoxantrons reveals no marked sequence preference (30).

We have recently shown that anthrapyrazole **1** is an effective

[1] J. A. Hartley, K. Reszka, E. T. Zuo, W. D. Wilson, A. R. Morgan, and J. W. Lown, unpublished observation.

photosensitizer (6), a property not shared to any significant extent by mitoxantrone or ametantrone.[2] The anthrapyrazole 1-photosensitized formation of single-strand breaks in DNA was also demonstrated (9), and the presence of hydroxyl groups on the chromophore was shown to remove the photosensitizing properties. This is confirmed in the present study on a larger group of drugs containing the same chromophore and is extended to demonstrate that even marked modifications in the side chains *R*1 and *R*2 do not affect the photosensitizing properties. The positions of the hydroxyl groups on the chromophore are such that they can form intramolecular hydrogen bonding which can facilitate the radiationless deactivation of singlet and triplet excited states (31–34). This could explain why only the non-hydroxylated anthrapyrazoles produce photosensitized DNA damage.

The mechanism of photosensitized DNA damage by anthrapyrazole 1 was discussed in detail previously, and the involvement of hydroxyl radicals in the photosensitized production of single-strand breaks in DNA was indicated (9). The production of such radicals has been shown to induce breaks in DNA (35), presumably by attacking the deoxyribose sugars on the surface of the DNA (36) and the resulting deoxyribose-centered radicals, causing secondary reactions which break the DNA backbone (37). Such a mechanism of strand break production is consistent with the non-sequence-specific cleavage observed in the present study and is similar to the hydroxy radical "footprinting" observed previously (35). Another mechanism of photosensitized cleavage may also occur with the anthrapyrazoles under anoxic conditions, however, involving NADH as a cofactor (9).

In conclusion, the present structure-activity relationship for a number of the anthrapyrazole anticancer agents indicates the structural requirements of this class of drugs with significant clinical potential for efficient binding to DNA, intercalation, and photosensitization. An understanding of the substituent dependence and clinical consequences of such properties should allow for the design of agents with enhanced anticancer activity, reduced toxicity, and perhaps with an application in photodynamic therapy.

Acknowledgments

J. A. H. thanks the Alberta Heritage Foundation for a Fellowship.

References

1. Smith, J. E. Mitoxantrone (Novantrone): a review of experimental and early clinical studies. *Cancer Treat. Rev.* **10**:103–114 (1983).
2. Showalter, H. D. H., J. L. Johnson, L. M. Werbel, W. R. Leopold, R. C. Jackson, and E. F. Elslager. 5-[(Aminoalkyl)amino]-substituted anthra(1,9-*cd*]pyrazol-6(2H)-ones as novel anticancer agents. Synthesis and biological evaluation. *J. Med. Chem.* **27**:253–258 (1984).
3. Showalter, H. D. H., D. W. Fray, W. R. Leopold, J. W. Lown, J. A. Plambeck, and K. Reszka. Design, biochemical pharmacology, electrochemistry and tumor biology of antitumor anthrapyrazoles. *Anti-Cancer Drug Design* **1**:73–85 (1986).
4. Showalter, H. D. H., J. L. Johnson, J. M. Hoftiezer, W. R. Turner, L. M. Werbel, W. R. Leopold, J. L. Shillis, R. C. Jackson, and E. F. Elslager. Anthrapyrazole anticancer agents. Synthesis and structure-activity relationships against murine leukemias. *J. Med. Chem.* **30**:121–131 (1987).
5. Leopold, W. R., J. M. Nelson, J. Plowman, and R. C. Jackson. The anthrapyrazoles, a new class of intercalating agents with high level, broad spectrum activity against murine tumors. *Cancer Res.* **45**:5532–5535 (1985).
6. Reszka, K., P. G. Tsoungas, and J. W. Lown. Photosensitization by antitumor agents. 1. Production of singlet oxygen during irradiation of anthrapyrazoles with visible light. *Photochem. Photobiol.* **43**:499–504 (1986).
7. Reszka, K., P. Kolodziejczyk, and J. W. Lown. Photosensitization by antitumor agents. 2. Anthrapyrazole photosensitized oxidation of ascorbic acid and 3,4-dihydrophenylalanine. *J. Free Rad. Biol. Med.* **2**:203–211 (1987).
8. Reszka, K., P. Kolodziejczyk, and J. W. Lown. Photosensitization by antitumor agents. 3. Spectroscopic evidence for superoxide and hydroxyl radical production by anthrapyrazole sensitized oxidation of NADH. *J. Free Rad. Biol. Med.* **2**:267–274 (1987).
9. Hartley, J. A., K. Reszka, and J. W. Lown. Photosensitization by antitumor agents. 4. Anthrapyrazole-photosensitized formation of single-strand breaks in DNA. *J. Free Rad. Biol. Med.*, in press.
10. Pulleyblank, D. E., and A. R. Morgan. Partial purification of "ω" protein from calf thymus. *Biochemistry* **14**:5205–5209 (1975).
11. Lown, J. W., A. R. Morgan, S. F. Yen, and W. D. Wilson. Characteristics of the binding of the anticancer agents mitoxantrone and ametantrone and related structures to DNA. *Biochemistry* **24**:4028–4035 (1985).
12. Morgan, A. R., J. S. Lee, D. E. Pulleyblank, N. L. Murray, and D. H. Evans. Review: Ethidium fluorescence assays. Part 1. Physicochemical studies. *Nucleic Acids Res.* **7**:547–569 (1979).
13. Wang, J. C. The degree of unwinding of the DNA helix by ethidium. I. Titration of twisted PM2 DNA molecules in alkaline cesium chloride density. *J. Mol. Biol.* **89**:783–801 (1974).
14. Le Pecq, J. B., and C. Paoletti. A fluorescent complex between ethidium bromide and nucleic acids. *J. Mol. Biol.* **27**:87–106 (1967).
15. Jones, R. L., A. C. Lanier, R. A. Keel, and W. D. Wilson. The effect of ionic strength on DNA-ligand unwinding angles for acridine and quinoline derivatives. *Nucleic Acid Res.* **8**:1613–1624 (1980).
16. Wilson, W. D., and I. G. Lopp. Analysis of cooperativity and ion effects in the interaction of quinacrine with DNA. *Biopolymers* **18**:3025–3041 (1979).
17. McGhee, J. D., and P. H. von Hippel. Theoretical aspects of DNA-protein interactions: cooperative and non-cooperative binding of large ligands to a one-dimensional homogeneous lattice. *J. Mol. Biol.* **86**:469–489 (1974).
18. Gough, A. N., R. L. Jones, and W. D. Wilson. Dimerization of coralyne and its propyl analogue and their association with DNA. *J. Med. Chem.* **22**:1551–1554 (1979).
19. Maniatis, T., E. F. Fritsch, and J. Sambrook. *Molecular Cloning—A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
20. Manning, G. S. The molecular theory of polyelectrolyte solutions with applications to the electrostatic properties of polynucleotides. *Q. Rev. Biophys.* **11**:179–246 (1978).
21. Record, M. T., Jr., C. F. Anderson, and T. M. Lohman. Thermodynamic analysis of ion effects of binding and conformational equilibria of proteins and nucleic acids: roles of ion association or release, screening, and ion effects on water. *Q. Rev. Biophys.* **11**:103–178 (1978).
22. Lerman, L. S. Structural consideration in the interaction of DNA and acridines. *J. Mol. Biol.* **3**:18–30 (1961).
23. Kapuscinski, J., Z. Darzynkiewicz, F. Traganos, and M. R. Melamed. Interactions of new antitumor agent, 1,4-dihydroxy-5,8-bis[2-hydroxyethyl)-amino]ethylamino]9,10-anthracenedione with DNA. *Biochem. Pharmacol.* **30**:231–240 (1981).
24. Foye, W. D., O. Vajragupta, and S. K. Sengupta. DNA-binding specificity and RNA polymerase inhibitory activity of bis(aminoalkyl)anthraquinones and bis(methylthio)vinyl quinolinium iodides. *J. Pharm. Sci.* **71**:253–257 (1982).
25. Lown, J. W., and C. C. Hanstock. High field ^{1}H-NMR analysis of the 1:1 intercalation complex of the antitumor agent mitoxantrone and the DNA duplex d[(CpGCpG)]$_2$. *J. Biomol. Struct. Dyn.* **2**:1097–1106 (1985).
26. Viswamitra, M. W., and B. Ramakrishnan. Mitoxantrone and ametantrone binding with dCpG-DNA sequence: the crystal interactions show close correlations between structure and activity. Fifth Conversation in Biomolecular Stereodynamics, Albany, NY, June 2–6 (1987). Proceedings, in press.
27. Fry, D. W., T. J. Bortizki, J. A. Besserer, and R. C. Jackson. *In vitro* DNA strand scission and inhibition of nucleic and synthesis in L1210 leukemia cells by a new class of DNA complexers, the anthra[q-*cd*]pyrazol-6(2H)-ones (anthrapyrazoles). *Biochem. Pharmacol.* **34**:3499–3508 (1985).
28. Lown, J. W., C. C. Hanstock, R. D. Bradley, and D. G. Scraba. Interactions of the antitumor agents mitoxantrone and bisantrene with DNAs studied by electron microscopy. *Mol. Pharmacol.* **25**:178–184 (1984).
29. Kotovych, G., J. W. Lown, and J. P. K. Tong. High field ^{1}H and ^{31}P NMR studies on the binding of the anticancer agent mitoxantrone to d([CpGApTpCpG)]$_2$. *J. Biomol. Struct. Dyn.* **4**:111–125 (1986).
30. Fox, K. R., M. J. Waring, J. R. Brown, and S. Neidle. DNA sequence preferences for the anticancer drug mitoxantrone and related anthraquinones revealed by DNase I footprinting. *FEBS Lett.* **202**:289–294 (1986).
31. Beckett, A., and G. Porter. Primary photochemical processes in aromatic molecules. X. Photochemistry of substituted benzophenones. *Trans. Faraday Soc.* **59**:2051–2057 (1963).
32. Lamola, A. A., and L. J. Sharp. Environmental effects on the excited states of *o*-hydroxy aromatic carbonyl compounds. *J. Phys. Chem.* **70**:2634–2638 (1966).
33. Mordzinski, A., and A. Grabowska. Intra-molecular proton transfer in excited benzoxazoles. *Chem. Phys. Lett.* **90**:122–127 (1982).
34. Werner, T. Triplet deactivation in benzotriazole-type ultraviolet stabilizers. *J. Phys. Chem.* **83**:320–325 (1979).
35. Tullius, T. D., and B. A. Dombroski. Hydroxyl radical "footprinting": high resolution information about DNA protein constants and application to λ repressor and Cro protein. *Proc. Natl. Acad. Sci. USA* **83**:5469–5473 (1986).
36. Hertzberg, R. P., and P. B. Dervan. Cleavage of DNA with methidium propyl-EDTA/iron(II). Reaction conditions and product analyses. *Biochemistry* **23**:3934–3945 (1984).
37. Wu, J. C., J. W. Kozarich, and J. Stubbe. The mechanism of free base formation from DNA by bleomycin. *J. Biol. Chem.* **258**:4694–4697 (1983).

[2] J. A. Hartley, K. Reszka, E. T. Zuo, W. D. Wilson, A. R. Morgan, and J. W. Lown, unpublished observations.

Send reprint requests to: Dr. J. William Lown, Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada.