Synthesis and Antitumor Properties of an Anthraquinone Bisubstituted by the Copper Chelating Peptide Gly-Gly-L-His

Elisabeth Morier-Teissier,* Nezha Boitte, Nicole Helbecque, Jean-Luc Bernier, Nicole Pommery,† Jean-Loup Duvalet,‡ Charles Fournier,‡ Bernard Hecquet,‡ Jean-Pierre Catteau,§ and Jean-Pierre Hénichart

Inserm U 16, Place de Verdun, 59045 Lille, France, Laboratoire de Toxicologie, Faculté de Pharmacie, 3, rue du Professeur Laguesse, B.P. 83, 59006 Lille, Cedex, France, Laboratoire de Pharmacodynamie Clinique, Centre Oscar Lambret, B.P. 307, 59020 Lille, Cedex, France, and Laboratoire de Chimie Organique Physique, USTL, 59655 Villeneuve d'Ascq, France

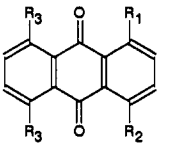
Received September 30, 1992

A new molecule 4 [(GGH-DAE)$_2$DHQ] associating the 1,4,5,8-tetrahydroxyanthraquinone ring (DHQ) of the antitumor drug mitoxantrone (2), two diaminoethylene chains (DAE), and the metal-chelating peptide Gly-Gly-His (GGH) has been synthesized. Such a molecule presents characteristics able to induce antitumor activity: compound 4 intercalates into DNA as measured by ΔT_m, fluorescence quenching, and viscometry; ESR studies demonstrate that several types of Cu complexes are formed depending on pH; and the production of free radicals, as evidenced by spin-trapping, is enhanced by 4. *In vitro*, in leukemia cells L1210 and mammary cells MCF7, 4 is slightly less cytostatic than mitoxantrone, but substantially less toxic. *In vivo*, in leukemia P388 on mice, a T/C value of 230 is obtained at 25 mg/kg, higher than the one of mitoxantrone, which is toxic at the same dose.

The discovery of antitumor activity of 1,4-bis((aminoalkyl)amino)anthracene-9,10-diones such as ametantrone (1) and mitoxantrone (2) (Figure 1)[1-5] has led to numerous physicochemical and pharmacological studies on the tumoricidal mechanisms of these chemotypes.[6] Although the mechanism of action of the antitumor activity of the anthracene-9,10-diones 1 and 2 is probably multimodal in nature, a number of studies have indicated that an intercalative interaction with DNA may be a major cellular event.[7,8] The planar tricyclic system is known to intercalate into DNA base pairs and interfere in the transcription and replication processes of the cell.[9,10]

The DNA binding affinity (quantified as a binding affinity constant) and the dissociation rate constant for the DNA–ligand complex have been evaluated. Drug-DNA binding constants for 1, 2, and related congeners with calf thymus DNA show a large sensitivity to the position and number of the OH substitutions and the nature of the charged side chain.[7] Among the numerous structure–activity relationships, we have concentrated on the following: 5,8-dihydroxylation of the aromatic nucleus substantially increases activity; the [(aminoethyl)amino]-ethanol side chain is quite unique in the sense that minor structural modifications resulted in compounds with diminished antineoplastic activity. Another important point is that the nitrogen atom in the center of the side chain plays an important role and must be separated from the NH on the nucleus by two carbons. A suitable high hydrophilicity is more important than mere steric characteristics.[3]

The antitumor activity of molecules such as 1 and 2 could also be partially due to the free radical intermediates formed during reductive or oxidative metabolic activation, leading to destructive free oxygen radicals, responsible for the cleavage of DNA.[11-14] Indeed, substituted anthraquinones bear a quinoid moiety. *In vivo*, enzymatic



1, R_3 = H; R_1 = R_2 = -NH-$(CH_2)_2$-NH-$(CH_2)_2$-OH
2, R_3 = OH; R_1 = R_2 = -NH-$(CH_2)_2$-NH-$(CH_2)_2$-OH
3, R_3 = OH; R_1 = OH; R_2 = -Gly-L-His-L-Lys-OH
4, R_3 = OH; R_1 = R_2 = H-Gly-Gly-L-His-DAE-

Figure 1. Structures of anthraquinonic derivatives.

one-electron reduction of a quinone gives a semiquinone radical, while two-electron reduction gives a hydroquinone. The semiquinone radical is capable of transferring an electron to molecular oxygen, to form the superoxide anion radical $O_2^{\bullet-}$, which is not a particularly reactive species. This superoxide anion is converted to hydrogen peroxide (H_2O_2) by a disproportionation process. Damage is done when H_2O_2 comes into contact with the reduced form of certain metal ions, e.g. iron or copper and generates, by a Fenton reaction, the highly reactive hydroxyl radical, which is responsible for deleterious effects such as oxidation of polyunsaturated fatty acids, degradation of protein, and DNA damage.[15-19]

In the course of a program aimed at providing new antineoplastic drugs, we have designed synthetic models including an anthraquinone chromophore and a metal-chelating peptide. The two moieties may act synergistically to yield ultimately a hydroxyl radical with damaging properties toward DNA. The first chosen metal-chelating entity was the peptide, Gly-L-His-L-Lys, a growth factor, and a metal carrier with a high affinity for copper and iron.[20-23] The adopted method of synthesis led to the monosubstituted compound 3. Its intercalative properties were weak: in the normal range for monosubstituted anthraquinones, but the peptide part of the molecule was actually able to form a complex with copper and in the presence of H_2O_2 and ascorbate to produce a OH$^\bullet$ radical responsible for a high DNA cleavage. The best results were obtained when a complex involving the peptide and the oxygen of the quinone was formed. The concept of

* Address correspondence to E. Morier-Teissier. Present address: Serlia and INSERM U 325, Institut Pasteur, 1, rue du Professeur Calmette, 59019 Lille, Cedex, France.
† Laboratoire de Toxicologie.
‡ Laboratoire de Pharmacodynamie Clinique.
§ Laboratoire de Chimie Organique Physique.

0022-2623/93/1836-2084$04.00/0

copper chelator–peptide anthraquinone appeared to work when strict conditions were applied to produce free radicals and to induce DNA breakage.[24]

The aim of the present study was to design a new synthetic model named $(GGH\text{-}DAE)_2DHQ$ (**4**) consisting of the 5,8-dihydroxyanthraquinone-9,10-dione chromophore of the mitoxantrone, substituted by two side chains to assume a good affinity for DNA and a metal-chelating peptide to cleave it. The side chains begin with the spacer $NH_2\text{-}(CH_2)_2\text{-}NH_2$,[25] both nitrogen atoms of which are separated by two carbons as is the case in the mitoxantrone. On the terminal amino function, a tripeptide Gly-Gly-L-His[26-32] which has already been used as a cleavage and metal-chelating agent, is fixed. It is worth mentioning that such a molecule may function as oxidases which contain a copper-chelating moiety associated with a covalently bound hydroquinone cofactor.[33]

The present report shows to what extent this new molecule is able to have the binding and copper chelating properties, the cytotoxicity against leukemia L1210 and carcinoma MCF7 cells and the antitumor activity against leukemia P388 in mice, of the parent drugs.

Chemistry

The method of preparation of the (substituted-alkyl)-aminoanthraquinones was based on that of Greenhalgh and Hughes,[34] involving condensation of 5,8-dihydroxyleucoquinizarine with an excess of the appropriate amine, followed by air oxidation of the dihydro intermediates.[2-3,34] In a preceding project,[24] Gly was directly bound to the anthraquinone ring, but whatever the conditions used, the product **3** was merely monosubstituted, as shown by the presence of three OH signals in NMR relative to a NH_2 or CH_2 function. To obtain a 1,4-disubstituted compound, an (aminoethyl)amino DAE spacer, without any carboxylic function, was then chosen.

5,8-Dihydroxyquinizarine was synthesized without isomers by a Friedel and Crafts reaction between 3,6-dimethoxyphthalic anhydride and hydroquinone in the presence of $AlCl_3$.[35,36] The structure was assessed by comparison of our results with literature data UV spectrum,[37] IR spectrum,[38] and NMR.[39] Its leucoderivative was obtained by reduction in the presence of zinc and AcOH, and its properties were compared with literature data.[1,36,40]

According to Greenhalgh and Hughes,[34] the direct reaction of 5,8-dihydroxyleucoquinizarine with ethylenediamine led, by cyclization of the side chain in position 2, to a major product identified as 6-[(2-aminoethyl)amino]-8,11-dihydroxy-1,2,3,4-tetrahydronaphtho[2,3-*f*]quinoxaline-7,12-dione, even when the reaction was carried at 20 °C.[34]

To avoid this cyclization, during the condensation step, one amino function of ethylenediamine was protected by the t-BOC group, easily eliminated in the presence of TFA.[25] Our major component **6** was then identified as 1,4-bis[(2-aminoethyl)amino]-5,8-dihydroxyanthraquinone on the basis of UV, NMR, and mass spectra. A minor compound had the UV spectrum of the cyclized compound described by Greenhalgh and Hughes.[34]

Finally, the amino group was coupled with the preformed protected tripeptide Z-Gly-Gly-L-His-OH[41] using conventional coupling agents: dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (HOBt). The Z protecting group was cleaved by HBr–acetic acid (AcOH) to give the

Scheme I. Synthesis of **4**

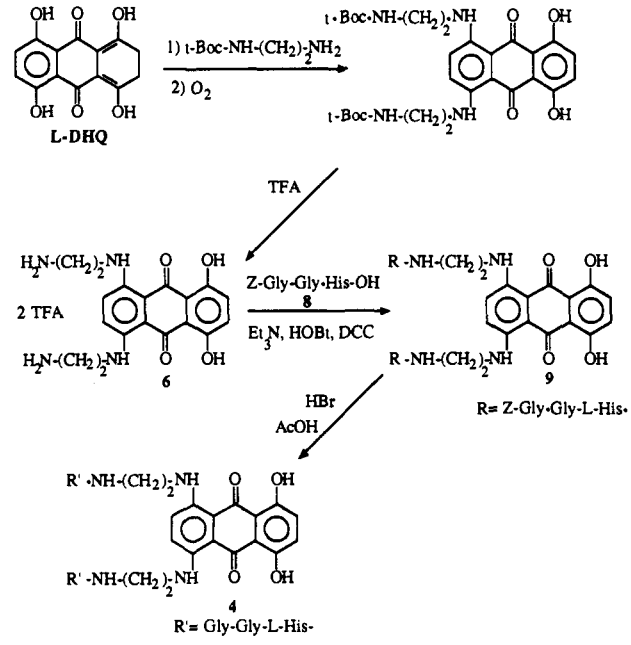


Table I. DNA Binding Parameters of **4** and Related Anthraquinones

compd	ΔT_m (°C)	Q_{50} (μmol)	lengthening (Å)	unwinding (deg)
2	15.9	0.1	3.26	26.5
3	11.3	3		
4	17.1	0.23	2.45	10.97

1,4-bis((2-((glycylglycyl-L-histidyl)amino)ethyl)amino)-5,8-dihydroxyanthracene-9,10-dione, dihydrobromide $(GGH\text{-}DAE)_2DHQ$ (**4**) (Scheme I).

DNA Interaction

DHAQ, GHK-DHQ, and other compounds containing the same tripeptide GHK caused precipitation of DNA. However, in the presence of **4**, decrease of absorption in the UV–visible spectrum was noticed only with the highest concentrations. The interaction of compound **4** with DNA was studied using three experimental techniques including thermal denaturation of DNA, fluorescence quenching in the presence of DNA, and viscometry (Table I).

Thermal Denaturation of DNA. The ΔT_m measurement technique provides information about the interaction of drugs with the double helix. It has been shown that a typical effect of DNA intercalating drugs is to stabilize the double-helical DNA against heat denaturation. Thus thermal denaturation of calf thymus DNA (% GC 42) has been evaluated in the absence (T_m value of 67.5 °C) and in the presence of **4**. An elevation of + 17.1 °C in the T_m of the DNA indicates a stabilization of the DNA double helix. This value is of the same order of magnitude as that of mitoxantrone (ΔT_m = 15.9 °C)[42] and higher than that of the monosubstituted anthraquinone GHK-DHQ (ΔT_m = 11.3 °C).[24] The thermal denaturation results reported here clearly demonstrate the role of the anthraquinonic ring in the stabilization of the DNA helix.

Fluorescence Studies. The fluorescence of ethidium is markedly enhanced when bound to DNA. Addition of a second DNA binding ligand with comparable or higher affinity to such DNA–ethidium complexes provides a reduction of fluorescence caused by displacement of ethidium from DNA. The percentage of fluorescence decrease observed with added drugs and an initial ethidium

Table II. ESR Parameters of Cu(II) and 4 Complexes

	5			8		
pH	A//	g//	complex	A//	g//	complex
C1	125	2.296	2N	130	2.296	2N
C2				215	2.188	4N

binding ratio D/P (molar dye concentration over molar mononucleotide concentration) of 0.1 are described as quenching values Q.[43] Q_{50} were obtained from the curves and compared for different anthraquinones including mitoxantrone. The model DHQ-GHK (Q_{50} = 3) appears to be 30 times less active than the mitoxantrone (Q_{50} = 0.10), whereas the disubstitution by a spacer bound to the tripeptide Gly-Gly-L-His-OH in the 1,4-positions affords a good improvement of the affinity (Q_{50} = 0.23). Quenching is a useful tool to estimate binding constants, but it has been assumed that this method is a sensitive measure of nonintercalative as well as intercalative binding.

Viscometry. Viscometry is a classical technique to appreciate helical lengthening and DNA unwinding and therefore is essential to characterize a classical intercalation.

Helix extension studies is of valuable interest to give an insight into the mode of insertion into DNA. Experiments designed to measure the helix extension produced by binding of compounds 4 and 2 were performed essentially by the method of Cohen and Eisenberg.[44,45] The length increase on adding 4 to sonicated DNA at pH 7.0 is characterized by a slope of 0.72 (lengthening of 2.45 Å), near to that of mitoxantrone (slope 0.96, lengthening 3.26 Å) or to that of daunorubicin (3.4 Å).[46] Such values correspond to a monointercalative process.

Unwinding of base pairs at and around the binding site is a necessary consequence of intercalation. The degree of angular unwinding measured by the reversal of negative supercoiling in a covalently closed circular double-stranded DNA is conventionally calculated relative to the 29° value for ethidium. The change in the superhelicity of the circular DNA, related to intercalation, is followed by the variation of the viscosity of the complex 4/DNA with increasing quantities of this product. The unwinding angle calculated from the 26° angle created by the ethidium bromide is 10.97°. This value is smaller than that obtained with ethidium bromide (26°) or with mitoxantrone (26.5°)[47] but agrees with values found for mono- (10.6°) or disubstituted (14.2°) anthraquinones.[48] The unwinding angle of 10.97° for DNA in the presence of 4 is low, but such low unwinding angles have been reported for adriamycin (12°)[49] or daunorubicin (10°)[46] and reflect either strong influences changing the interaction between the intercalated ligand and the two adjacent base pairs or the existence of more than one type of DNA binding site available.

In any case, viscometry results agree well with those obtained by thermal denaturation and fluorescence studies and can be thought to reflect partial intercalation.

ESR of Cu^{2+} Complex with 4

ESR spectra were recorded for two pH values where histidine was either protonated (pH 5) or not (pH 8). The ratio Cu/P was 1.8, since there were two peptidic moieties able to complex copper. Spectra of the complexes were completely different from those observed in the presence of free copper. The difference was more evident at pH 8.

Axially symetric A// and g// factors are summarized in the Table II. The g// and A// values allow to determine the nature and number of ligands for Cu(II), when compared to values described in the Peisach's plots[50] with peptides comprising several copper complexing nitrogen atoms. At an acidic pH of 5, only one species C1 involving 2 nitrogens was observed. On the contrary, at the higher pH of 8, a supplementary complex C2 involving a coupling of Cu^{2+} with four nitrogen ligands was detected.

Table III. Increase with Time of the Free Radical Production of 4 by Spin Trapping in the Presence of PBN (0.08 M Ethanolic Solution at pH 9)

time (min)	area (arbitrary units)	time (min)	area (arbitrary units)
5	36	15	136.6
10	84.6		

Study of Free Radical Production by ESR

The free radical production was detected using the spin-trapping technique. It was studied by pH 9 by addition of NaOH. OH^- anions favored the formation of semiquinone radicals by nucleophilic attack on the anthraquinone nucleus, replacing the role of reductive agents. Phenyl-*N*-*tert*-butylnitrone (PBN) was used as spin trap and the production of free radicals was evaluated from integration of the curves and comparison between the areas. The formation of an $OH^\bullet$ radical adduct with PBN spin trap, well characterized by the corresponding ESR spectrum: triplet of doublet with a g factor of 2.006, a_N = 15.25 G and a_β = 3G. These values are identical to those found by Harbour et al.[51]

Control experiments made on PBN, H_2O_2, or Cu^{2+} used separately did not produce the same ESR signals. In the presence of PBN, Cu^{2+}, and H_2O_2, a spectrum characteristic of PBN-OH is obtained. The peak area, proportional to the free radical amount produced is maximum after 10 min and equal to 12.5 arbitrary units. In the presence of 4, spectra show a higher yield of free radicals, which varies with time, as shown in the Table III. The free radical production is 10 times higher in the presence of 4 and increase with time. Mitoxantrone (2) poorly produced free radicals whereas DHQ-GHK 3 gave rise to substantial free radical production, pointing out the role of the peptidic moiety.

Biological Results

Cytotoxicity. Mitoxantrone (2) and the prepared analogue 4 were first evaluated as inhibitors of the growth of L1210 *in vitro*. Both compounds show antileukemic activity. $(GGH\text{-}DAE)_2DHQ$ (4) appears less potent than mitoxantrone and displays lower inhibitory activity (ID_{50} = 5×10^{-8} M for 4 and 7×10^{-9} M for 2). Cytotoxicity was measured for both compounds (LD_{50} = 7×10^{-8} M for 4 and 7×10^{-9} M for 2).

Activities against MCF7 cell lines were further evaluated. Mean percentage reductions in cell growth were comparable (ID_{50} values were respectively 8×10^{-7} M for 4 and 2.7×10^{-7} M for 2).

Antitumor Activity. Compound 4 was tested against P388 leukemia in mice by administration at D1, D5, and D9 after tumor inoculation. Table IV indicates the therapeutic effects obtained for several doses and compared to those obtained with mitoxantrone at optimal dosing. The maximal effect of 4 was obtained at a dose of 25 mg/kg. The compound caused a 130% increase in lifespan (T/C = 230%), and 3/7 mice were cured. A

Table IV. T/C (%) Using the Median Survival Time of Drug-Treated (T) and Control (C) Groups after Administration of 4 (5, 10, and 25 mg/kg D1, D5, D9) and Mitoxantrone (2) (1.6 mg/kg D1, D5, D9)

compd	dose (mg/kg)	days of administration	T/C
2	1.6	1, 5, 9	190
4	5	1, 5, 9	140
4	10	1, 5, 9	150
4	25	1, 5, 9	230

decrease in dose resulted in moderate activity (T/C = 150% for 10 mg/kg and 140% for 5 mg/kg).

According to the mean survival time, 4 showed an antitumoral activity at least as great as the one of mitoxantrone (230% vs 190%). But comparison of survival curves does not establish a significant difference. It is also to be noted that activity was obtained with a dose larger for 4 (25 mg/kg) than for mitoxantrone (1.6 mg/kg).

No weight loss could be observed in mice after 4 administration, even with the largest dose. It can thus be concluded that, in the range of the tested doses, the activity of 4 is not associated with immediate toxicity.

Discussion

The inherent goal of this research was to explore the effect on biological activity of the 1,4-bisubstitution of 5,8-dihydroxyanthracenediones chemotypes by a peptidic chain linked to an (aminoethyl)amino function, instead of the well-known [(aminoethyl)amino]ethanol moiety. In mice, 4 is less toxic than mitoxantrone, and no immediate side-effect can be observed up to 25 mg/kg. 4 is at least as active as mitoxantrone, at equitoxic dose, against P388 in mice. Nevertheless activity needs higher dosing with 4. The T/C value reaches 230 at a dose of 25 mg/kg which is generally obtained only with the more toxic mitoxantrone or with compounds bearing at least one [(aminoethyl)-amino]ethanol moiety (T/C = 244 at 25 mg/kg)[52] (T/C = 300 at 10 and 20 mg/kg),[53] the presence of one side arm similar to the one of 2 being necessary to lead such effectiveness.

Good biological efficacy required an ethylenediamine substituent at both the 1- and 4-positions; 5,8-dihydroxylation of the aromatic nucleus substantially increased activity. Compound 4 contains the moiety $NHCH_2CH_2N$ attached to the aromatic ring, which is believed to be required for the antitumor activity,[4] but here it ends with a peptidic chain quite different from the classical aminoethanol moiety. Moreover, the nitrogen atom following the ethyl chain plays an important role in antileukemic activity.[3] In the present case, this nitrogen is not included in a secondary amine but in an amide, which prevents the cyclization of the side chain to a piperazine ring.[34]

It might be speculated that the antitumor activities expressed by the anthracenediones of the type studied here are based on two molecular portions of the molecules. The anthracenedione skeleton acts as the DNA intercalant and the side chains at positions 1 and 4 add several roles: solubility, production of free radicals, and stabilization of the intercalation.

Evidence for an interaction of the anthraquinone chromophore with DNA results from data given by techniques such as ΔT_m and fluorescence. Viscometry allows us to determine that 4 acts as a monointercalant, but that more than one type of DNA binding site is available. So, the importance of the side chain in the binding process is fundamental.

It appears that the terminal N atoms must be fully basic, in order to bind to phosphoric acid residues of a DNA chain, since the precursor of 4 protected on the terminal NH_2 function lacks *in vitro* activity.

A suitably high hydrophilicity with a hydrophobic–lipophilic balance is important. The former compound with the peptidic moiety Gly-His-Lys showed excellent *in vitro* activity but was inactive *in vivo*. This could reflect a rapid degradation of the peptide part *in vivo* or a poor cell penetration power of the molecule. In contrast, 4 with the Gly-Gly-His peptide showed not only *in vitro* but also *in vivo* activity.

Redox cyclings, as reflected in half-wave reduction potentials, do not correlate with activity: compounds 3 and 4 would have almost identical half-wave potentials since the side chains would have limited effect on this property, but changing the peptidic moiety can change the capacity to complex Cu^{2+} ions and to produce free radicals.

Further investigations such as redox activity, free radical production, oxidase activity, and interaction with topoisomerase II enzyme are planned to characterize the properties of this new compound. Metabolic studies are required to evaluate the potential interest of 4 for human tumor treatment, but this encouraging result will prompt us to prepare other compounds in this series.

Experimental Section

The purity of all compounds has been assessed by TLC, HPLC, ^{1}H-NMR, and mass spectroscopy. Kieselgel 60 (70–230 mesh) (Merck) was used for columns developed with mixtures of hexane, CH_2Cl_2, or MeOH. TLC was carried out using silica gel 60F-254 (Merck) (0.25-mm-thick) precoated UV-sensitive plates in the following solvents: A, CH_2Cl_2–MeOH (4:1); B, BuOH–Pyr–AcOH–H_2O (30:20:6:24); C, THF–AcOH–H_2O (8:1:2). Spots were visualized by inspection under visible or UV light at 254 nm and after exposure to vaporized I_2. A Waters Associates gradient system (linear gradient) with a manual injection and UV detector (λ = 250 and 599 nm) was used. A Waters Novapack (150 × 3.9) CD ODS column (5-μm particle size) was eluted with a mixture of CH_3CN (0–50%) in a 0.1 M pH 2.5 KH_2PO_4–H_3PO_4 buffer, flow rate 1 mL/min. Melting points were determined in capillary tubes and are uncorrected. Absorption spectra were recorded on Uvikon-Kontron 930 spectrophotometer in water or MeOH. The IR spectra were recorded on a Perkin-Elmer 1310 spectrophotometer in KBr pellets, and only the principal sharply defined peaks are given. ^{1}H-NMR spectra were recorded on a Brucker WP 80 SY or on a Brucker AM 400 WB spectrophotometer. Chemical shifts were reported in ppm from tetramethylsilane as an internal standard and were given in δ units. FAB mass spectra were determined on a Kratos MS-50 RF mass spectrophotometer arranged in an EBE geometry. The sample was bombarded using a beam of xenon with a kinetic energy of 7 keV. The mass spectrometer was operated at 8-kV accelerating voltage with a mass resolution of 3000. In the case of 5,8-hydroxylated compounds a better mass peak was obtained after acetylation.

1,4-Bis[[(t-Boc-amino)ethyl]amino]-5,8-dihydroxyanthracene-9,10-dione (5). Freshly distilled t-Boc-ethylenediamine[27] (4.27 g, 26.6 mmol) in MeOH was deaerated by stirring for 15 min as a stream of N_2 was bubbled through it. After the gradual addition of 730 mg (2.66 mmol) of 5,8-dihydroxyleucoquinizarine,[1,36,40] the color gradually changed from yellow to green and the solution was heated under reflux during 3 h. Heating without nitrogen allows a mild oxidation of the solution which turned to purple and the end of oxidation was monitored by UV spectroscopy. Methanol was removed *in vacuo*, and the residual dark paste was column chromatographed over silica; impurities were eliminated successively with CH_2Cl_2–hexane and CH_2Cl_2 and the pure product with CH_2Cl_2–AcOEt (95:5) (27% yield): mp 204 °C; TLC R_f A = 0.9; IR ν_{max} 3340, 1680 cm^{-1}; UV λ_{max} (MeOH) 619, 672 nm; ^{1}H NMR (DMSO-d_6) 13.5 (s exch, 2H),

10.4 (broad, 2H), 7.5 (s, 2H), 7.2 (s, 2H), 3.4 (m, 8H), 1.3 (s, 18H); mass spectrum, 557 M^+.

1,4-Bis[(Aminoethyl)amino]-5,8-dihydroxyanthracene-9,-10-dione, Bis(trifluoroacetate) (6). The t-Boc protecting group was removed by treatment of 5 (0.7 mmol) with 10 mL of trifluoroacetic acid at room temperature for 30 min. The acid was evaporated *in vacuo* to give an oil; methanol was added to the oil and evaporated to dryness several times to give a blue solid (100% yield): mp >270 °C; UV λ_{max} (MeOH) 613 (15 550), 665 nm (19 200); TLC R_f C = 0.15; ^1H NMR (DMSO-d_6) 13.5 (s exch, 2H), 10.4 (broad, 2H), 8 (broad, 4H), 7.6 (s, 2H), 7.2 (s, 2H), 3.8 (m, 4H), 3.1 (m, 4H); mass spectrum, 357 M^+.

Z-glycyl-glycyl-L-histidine Methyl Ester (7). To a solution of Z-Gly-Gly-OH (5.32 g, 20 mmol), *N*-hydroxybenzotriazole (HOBt) (3.12 g, 20 mmol), and *N,N'*-dicyclohexylcarbodiimide (DCC) (4.12 g, 20 mmol) in dry dimethylformamide (DMF) (100 mL) stirred at 0 °C for 1 h, was added a solution of L-His-OMe, 2HCl (4.84 g, 20 mmol) and Et_3N (4 g, 40 mmol) in 50 mL of DMF. The reaction mixture was stirred at 0 °C for 1 h more and then at room temperature overnight. *N,N'*-Dicyclohexylurea (DCU) was removed by filtration and the solvent evaporated to dryness *in vacuo*. The crude oil was dissolved in water and acidified to pH 2 by HCl, and impurities such as HOBt, DCU, and Z-Gly-Gly-OH precipitated. The filtrate was cooled and brought to pH 8–9 by NaOH, and 7 precipitated (78% yield): mp 170 °C; TLC R_f C = 0.8; ^1H NMR (DMSO-d_6) 8.25 (t exch, 1H), 7.97 (d exch, 1H), 7.55 (s, 1H), 7.36 (s, 5H), 6.82 (s, 1H), 5.04 (s, 2H), 4.48 (m, 1H), 3.70 (d, 2H), 3.59 (d, 2H), 3.32 (s, 3H), 2.86 (m, 2H).

Z-glycyl-glycyl-L-histidine (8). 7 (2 g, 4.8 mmol) in 100 mL of MeOH and 5 mL of 1 N NaOH in water was left at room temperature for 1 h. After TLC analysis, the solution was acidified to pH 3–4 by the addition of 1 M HCl and evaporated to dryness. The residue was dissolved in MeOH to separate the insoluble NaCl, dried, and used without further purification: hygroscopic white powder (100% yield); mp 178 °C; TLC R_f C = 0.6; IR no more bond at 1750 cm^{-1} (CO ester); ^{H} NMR (DMSO-d_6) disappearance of the peak corresponding to OCH_3, other peaks as above; mass spectrum, 404 M^+.

1,4-Bis((2-((Z-glycyl-glycyl-L-histidyl)amino)ethyl)amino)-5,8-dihydroxyanthracene-9,10-dione (9). To a solution of 8 (320 mg, 0.79 mmol), HOBt (200 mg, 1.20 mmol), and DCC (250 mg, 1.20 mmol) in dry DMF (50 mL) stirred at 0 °C for 1 h were added 70 mg (0.19 mmol) of 6 and Et_3N (40 mg, 0.38 mmol). The reaction mixture was treated and purified as described for 7. The crude product was purified on silica gel with CH_2Cl_2–MeOH (7:3) as eluent: blue powder (55% yield); mp >270 °C; TLC R_f C = 0.7; UV λ_{max} (MeOH) 617, 670 nm; ^1H NMR (DMSO-d_6) 13.6 (s, 2H), 11.7 (s exch, 2H), 10.6 (t exch, 2H), 8 (m exch, 6H), 7.65 (s, 2H), 7.50 (m, 2H), 7.30 (s broad, 10H), 7.20 (s, 2H), 6.80 (s, 2H), 5.1 (s, 4H), 4.45 (m, 2H), 4.10 (d, 4H), 3.60 (m, 4H), 3.45 (d, 4H), 3.10 (m, 4H), 2.80 (d, 4H); mass spectrum, 1127 M^+; mass spectrum of the acetylated compound, 1211 M^+.

1,4-Bis((2-((glycylglycyl-L-histidyl)amino)ethyl)amino)-5,8-dihydroxyanthracene-9,10-dione, Dibromhydrate (4). The Z protecting group of 9 was removed by treatment of 0.100 g (0.088 mmol) with 10 mL of acetic acid saturated with HBr at room temperature for 15 min. The acetic solution was evaporated *in vacuo* to give a blue solid; ethanol was added, and the solution was evaporated to dryness several times to eliminate the acids. The blue product was dissolved in water (5 mL), washed with AcOEt (2 × 5 mL), and lyophilized to give 0.073 g (0.072 mmol) of a blue hygroscopic powder (80% yield): HPLC t_R = 32 min; mp >270 °C dec; UV λ_{max} (MeOH) 615 (14 350), 665 nm (18 000); ^1H NMR (DMSO-d_6) 14 (d exch, 4H), 13.5 (s exch 2H), 10.6 (t exch, 2H), 9.1 (s, 2H), 8.6 (s, 2H), 8.3 (m, 2H), 8 (m exch, 4H), 7.65 (s, 2H), 7.15 (s, 2H), 6.90 (s, 2H), 4.45 (m, 2H), 4.30 (d, 4H), 3.80 (m, 4H), 3.60 (d, 4H), 3.30 (m, 4H), 2.90 (m, 4H); mass spectrum, 859 M^+. Anal. ($C_{38}H_{46}O_{10}N_{14}$·2HBr) C, H, N.

DNA Solutions. Calf thymus DNA (type I, highly polymerized, MW 400 000, Sigma Chemical Co.) was used throughout the experiments without purification. Concentrations of the DNA were determined spectroscopically from their extinction coefficients (ϵ_{260}, M^{-1} cm^{-1}).[54]

Fluorescence quenching was determined employing 20 μM DNA in 0.01 M ionic strength buffer (9.3 mM NaCl, 2 mM NaOAc buffer, pH 5, plus 0.1 mM EDTA).[43]

For ΔT_m determinations, 2×10^{-4} M DNA was prepared in 0.1 SSC buffer (0.15 M NaCl/0.015 M sodium citrate, pH 7.0). Six curves were scanned with P/DNA = 0, 0.2, 0.4, 0.6, 0.8, and 1.

Thermal Denaturation of DNA. "Melting" temperatures were measured by using an Uvikon-Kontron 810/820 spectrophotometer coupled to an Uvikon Recorder 21 and an Uvikon Thermoprinter 48. Samples were placed in a thermostatically controlled cell-holder (10-mm pathlength). The cuvette was heated by circulating water from a Haake unit set. The temperature inside the cuvette was monitored by using a thermocouple in contact with the solution. The absorbance at 260 nm was measured over the range 20–95 °C with a heating rate of 1 °C/min. The "melting" temperature (T_m) was taken to be the midpoint of the hyperchromic transition.

Fluorescence Quenching. *Q* values for quenching were determined employing 3 mL of 20 μM DNA containing 3 μL of ethidium bromide in such a way that there was minimal ethidium displacement and maximum drug-induced quenching.[43] All measurements were made in 10-mm-pathlength quartz cuvette at 20 °C on a Jobin-Yvon JY3 spectrofluorimeter equipped with an *X–Y* recorder (excitation at 546 nm and measurement at 593 nm); 10^{-3} M drugs in water were added by fraction of 3 μL. The *Q* value is defined as the drug concentration which reduced the fluorescence of initially DNA-bound ethidium by 50%.

Viscometry. Helical-lengthening measurements were made by using a Ubbelohde semimicrodilution viscometer. The temperature was maintained at 20 ± 0.01 °C in a thermostatically controlled water bath. Flow times were electronically measured to an accuracy of 0.1 s (Schott ABS/G type detector). Calf thymus DNA was reduced to rod-like species with a French press, and experiments were done in 0.01 SHE buffer (9.4 mM NaCl/2 mM HEPES/10 μM EDTA buffer, pH 7.0) as described by Wakelin and Waring.[55] Solutions were filtered through 0.45 μm Millipore filters before measurements.

Unwinding studies using closed circular DNA (plasmid pBr322 containing fragments of adenovirus) were performed essentially as described by Saucier et al.[46] and Revet et al.[56] on the same apparatus described above. The viscometer contained 2.0 mL of a 150 μM solution of DNA. Drugs were added in increments of 5–10 μL from a stock solution (c = 150 μM). Flow times were measured with an accuracy of 0.1 s. Ethidium bromide was used as reference inducing an unwinding angle of 26°, according to Wang.[57]

ESR Measurements. ESR spectra were recorded on a Varian E-109 X band spectrometer under the following conditions.

For Free Radicals Production. A 100-kHz high-frequency modulation with a maximum amplitude of 1 G was used with a 10-mW microwave power. The sample solutions were examined in flat quartz cell inserted in an E-238 cavity operating in the TM_{100} mode. Phenyl-*N*-*tert*-butylnitrone (PBN) was used to detect the production of $OH^•$ radicals. The reaction mixture for spin-trapping experiments consisted of different ratios of a 10^{-2} M $(GGH\text{-}DAE)_2DHQ$ aqueous solution, H_2O_2, and Cu^{2+} aqueous solutions. An ethanolic solution of PBN (8.10^{-2} M) was used as spin trap.

For Cu(II) Complexes. The ESR spectra were performed at 77 K with a maximum modulation amplitude of 8 G in a dual cavity operating in the TE_{104} mode. Samples were prepared from 10^{-2} M drugs aqueous solutions in a 1/10 ratio against $CuSO_4$ and adjusted to pH 9. Addition of a small amount of glycerol led to the formation of good glasses at 77 K. The samples were frozen in liquid nitrogen into 4-mm-diameter cylindrical quartz tubes. The *g* factor measurements were related to the Varian "strong pitch", g = 2.0028.

Cell Cultures. L1210 (mouse lymphocytic leukemia) and MCF7 (human breast adenocarcinoma) cells were grown in RPMI 1640 and Dulbecco's modified Eagle's medium (Gibco), respectively, supplemented with 10% (v/v) foetal calf serum, and the assays were performed in the same medium.

Cultures utilized to assess drugs effects were in exponential growth phase, and the cytotoxic activity was determined over a 3-day period. L1210 was originally seeded at 1.5×10^5 cells/mL in 25-cm^2 flasks. MCF7 were incubated in 6-well plates at a

density of 10^5 cells/well. Both compounds, mitoxantrone (2) or $(GGH\text{-}DAE)_2DHQ$ (4), were added to attain final concentrations ranging from 10^{-9} to 10^{-4} M.

Cell growth and viability were determined with trypan blue staining. The growth inhibition data were then used to calculate the ID_{50} values (the drug concentration required to inhibit cell growth by 50% of control).

***In Vivo* Efficacy Studies.** Experiments involving P388 leukemia were initiated by implanting 10^6 cells ip to DBA2 mice at day 0 (D0). Each drug-treated group consisted of 7 mice, and the nontreated control group consisted of 10 mice. Dose levels of 5, 10, and 25 mg/kg of 4 were administered ip at D1, D5, and D9. In order to compare 4 with a known drug of the same class, a group of seven mice was treated with mitoxantrone (1.6 mg/kg, D1, D5, and D9). Experiments were terminated on D45. Mice were weighted daily. Antitumor activity was evaluated by calculating a T/C (%) using the median survival time of drug-treated (T) and control (C) groups. Comparison of efficacy was performed using log-rank test (limit of significance 0.05).

Acknowledgment. This research was supported by grants from the Association pour la Recherche sur le Cancer JPH-69-32. The authors wish to thank Dr. John Surtees for reviewing the English text.

References

(1) Zee-Cheng, R. K. Y.; Cheng, C. C. Antineoplastic agents. Structure-activity relationship study of bis(substituted aminoalkylamino)-anthraquinones. *J. Med. Chem.* **1978**, *21*, 291–294.
(2) Zee-Cheng, R. K. Y.; Podrebarac, E. G.; Menon, C. S.; Cheng, C. C. Structural modification study of bis (substituted aminoalkylamino)anthraquinones. An evaluation of the relationship of the ((2-(2-hydroxyethyl)amino)ethyl)amino side chain with antineoplastic activity. *J. Med. Chem.* **1979**, *22*, 501–505.
(3) Murdock, K. C.; Child, R. G.; Fabio, P. F.; Angier, R. B.; Wallace, R. E.; Durr, F. E.; Citarella, R. V. Antitumor agents. 1,4-bis-((aminoalkyl)amino)-9,10-anthracenediones. *J. Med. Chem.* **1979**, *22*, 1024–1030.
(4) Cheng, C. C.; Zee-Cheng, R. K. Y. The design, synthesis and development of a new class of potent antineoplastic anthraquinones. *Prog. Med. Chem.* **1983**, *20*, 83–118.
(5) Zee-Cheng, R. K. Y.; Cheng, C. C. Structure activity relationship study of anthraquinones: 1,4-dihydroxy-5,8-bis((2-(2-hydroxyethoxy)ethyl)amino)-9,10-anthracenedione, an analog of an established antineoplastic agent. *J. Pharm. Sci.* **1982**, *71*, 708–709.
(6) Lown, J. W. Anthracycline and Anthracenedione-Based Anticancer Agents. In *Bioactive Reviews*; Elsevier: Amsterdam, Netherlands, 1988; Vol. 6, pp 1–753.
(7) Denny, W. A. DNA-intercalating ligands as anti-cancer drugs: prospects for future design. *Anti-Cancer Drug Design* **1989**, *4*, 241–263.
(8) Kohn, K. W. DNA as Target for Anticancer Drug Action. In *Anticancer Drugs*; Tapiero, Robert J., Lampidis, T. J., Eds.; Colloque Inserm/John Libbey Eurotext Ltd.: 1989; Vol. 191, pp 77–86.
(9) Johnson, R. K.; Zee-Cheng, R. K. Y.; Le, W. W.; Acton, E. M.; Henry, D. W.; Cheng, C. C. Experimental antitumor activity of aminoanthraquinones. *Cancer Treat. Rep.* **1979**, *63*, 425–439.
(10) Lown, J. W.; Morgan, A. R.; Yen, S. F.; Wang, Y. H.; Wilson, W. D. Characteristics of the binding of the anticancer agents mitoxantrone and ametantrone and related structures to deoxyribonucleic acids. *Biochemistry* **1985**, *24*, 4028–4035.
(11) Basra, J.; Brown, J. R.; Patterson, L. H. Free radical formation in-vitro by cytotoxic anthraquinone derivatives; comparison with doxorubicin. *Investigational New Drugs* **1984**, *2*, 117.
(12) Basra, J.; Wolf, C. R.; Brown, J. R.; Patterson, L. H. Evidence for human liver mediated free-radical formation by doxorubicin and mitoxantrone. *Anti-Cancer Drug Design* **1985**, *1*, 45–52.
(13) Sinha, B. K.; Motten, A. G.; Hank, K. W. The electrochemical reduction of 1,4-bis((2-(2-hydroxyethyl)-amino)ethylamino)anthracenedione and daunomycin: biochemical significance in superoxide formation. *Chem. Biol. Interact.* **1983**, *43*, 371–377.
(14) Sinha, B. K. Free radicals in anticancer drug pharmacology. *Chem. Biol. Interact.* **1989**, *69*, 293–317.
(15) Moore, H. W. Bioactivation as a model for drug design bioreductive alkylation. *Science* **1977**, *147*, 527–532.
(16) Favaudon, V. On the mechanism of reductive activation in the mode of action of some anticancer drugs. *Biochimie* **1982**, *64*, 457–475.
(17) Halliwell, B.; Gutteridge, J. M. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* **1984**, *219*, 1–14.
(18) Kappus, H. Overview of enzyme systems involved in bioreduction of drugs and in redox cycling. *Biochem. Pharmacol.* **1986**, *35*, 1–6.
(19) Kolodziejcyk, P.; Reszka, K.; Lown, J. W. Enzymatic oxidative activation and transformation of the antitumor agent mitoxantrone. *Free Rad. Biol. Med.* **1988**, *5*, 13–25.
(20) Pickart, L.; Thaler, M. M. Tripeptide in human serum which prolongs survival on normal cells and stimulates growth in neoplastic liver. *Nature (London) New Biol.* **1973**, *243*, 85–87.
(21) Pickart, L.; Thaler, L.; Thaler, M. M. A synthetic tripeptide which increases survival of normal liver cells, and stimulates growth in hepatoma cells. *Biochem. Biophys. Res. Commun.* **1973**, *54*, 562–566.
(22) Pickart, L.; Freedman, J. H.; Loker, W. J.; Peisach, J.; Perkins, C. M.; Stenkamp, R. E.; Weinstein, B. Growth-modulating plasma tripeptide may function by facilitating copper uptake into cells. *Nature (London)* **1980**, *288*, 715–717.
(23) Pickart, L.; Lovejoy, S. Biological activity of human plasma copper-binding growth factor glycyl-L-histidyl-L-lysine. *Methods Enzymol.* **1987**, *147*, 314–328.
(24) Morier-Teissier, E.; Bernier, J. L.; Lohez, M.; Catteau, J. P.; Hénichart, J. P. Free radical production and DNA cleavage by copper chelating peptide-anthraquinones. *Anti-Cancer Drug Design* **1990**, *5*, 291–305.
(25) Houssin, R.; Bernier, J. L.; Hénichart, J. P. A convenient and general method for the preparation of tert-butoxycarbonylaminoalkanenitriles and their conversion to mono-tert-butoxycarbonylalkanediamines. *Synthesis* **1988**, 259–261.
(26) Yokoyama, A.; Aiba, H.; Tanaka, H. Acid dissociation constants of some histidine-containing peptides and formation constants of their metal complexes. *Bull. Chem. Soc. Jpn.* **1974**, *47*, 112–117.
(27) Bossu, F. P.; Margerum, D. W. The stabilisation of trivalent nickel in deprotonated-peptide complexes. *J. Am. Chem. Soc.* **1976**, *98*, 4003–4004.
(28) Bossu, F. P.; Paniago, E. B.; Margerum, D. W.; Kirskey, S. T., Jr.; Kurtz, J. L. Trivalent nickel catalysis of the autoxydation of nickel (II)tetraglycine. *Inorg. Chem.* **1978**, *17*, 1034–1042.
(29) Graham Lappin, A.; Murray, C. K.; Margerum, D. W. Electron paramagnetic resonance studies of nickel(III)-oligopeptide complexes. *Inorg. Chem.* **1978**, *17*, 1630–1634.
(30) Sakurai, T.; Nakahara, A. Reaction nickel(II)-glycyl-glycyl-L-histidine complex with molecular oxygen and formation of decarboxylated complex. *Inorg. Chem. Acta* **1979**, *34*, L243–L244.
(31) Chiou, S. H. DNA- and protein scission activities of ascorbate in the presence of copper ion and a copper-peptide complex. *J. Biochem.* **1983**, *94*, 1259–1267.
(32) Mack, D. P.; Iverson, B. L.; Dervan, P. B. Design and chemical synthesis of a sequence-specific DNA cleaving protein. *J. Am. Chem. Soc.* **1988**, *110*, 7572–7574.
(33) Ito, N.; Phillips, S. E. V.; Stevens, C.; Ogel, Z. B.; Mc Pherson, M. J.; Keen, J. N.; Yadav, K. D. S.; Knowles, P. F. Novel thioether bond revealed by a 1.7 Å crystal structure of galactose oxidase. *Nature* **1991**, *350*, 87–90.
(34) Greenhalgh, C. W.; Hughes, N. The reaction of leucoquinizarines with alkylenediamines. *J. Chem. Soc.* **1968**, 1284–1288.
(35) Allen, C. F. H.; Frame, G. F.; Wilson, C. V. The structure of the so-called toluidine blue. *J. Org. Chem.* **1941**, *6*, 732–749.
(36) Houben-Weil. *Anthraquinone*; Georg Thieme Verlag: Stuttgart, 1979; Vol. 7/3c, pp 82–170.
(37) Bloom, H.; Briggs. L. H.; Cleverley, B. Physical properties of anthraquinone and its derivatives. Part I-infrared spectra. *J. Chem. Soc.* **1959**, 178–185.
(38) Brockmann, H.; Blodt, P. ε-Iso-Rhodomycinon. *Chem. Ber.* **1961**, *94*, 2174–2187.
(39) Song, G. Q.; Wu, J. A.; He, X. G. Studies on ^1H-NMR of hydroxyl groups of substituted hydroxy-anthraquinones. *Acta Chim. Sin.* **1985**, *43*, 145–149.
(40) Bruce, D. B.; Thomsom, R. H. Aromatic keto-enols. Part II. Some new 2,3-dihydro-1,4-naphtoquinones and anthraquinones. *J. Chem. Soc.* **1952**, 2759–2766.
(41) Yokoyama, A.; Aiba, H.; Tanaka, H. Acid dissociation constants of some histidine-containing peptides and formation constants of their metal complexes. *Bull. Chem. Soc. Jpn.* **1974**, *47*, 112–117.
(42) Collier, D. A.; Neidle, S. Synthesis, molecular modelling, DNA binding and antitumor properties of some substituted amidoanthraquinones. *J. Med. Chem.* **1988**, *31*, 847–857.
(43) Baguley, B. C.; Denny, W. A.; Atwell, G. J.; Cain, B. F. Potential antitumor agents. 34. Quantitative relationships between DNA binding and molecular structure for 9-anilinoacridines substituted in the anilino ring. *J. Med. Chem.* **1981**, *24*, 170–177.
(44) Cohen, G.; Eisenberg, H. Conformation studies on the sodium and cesium salts of calf thymus deoxyribonucleic acid. *Biopolymers* **1966**, *4*, 429–440.
(45) Cohen, G.; Eisenberg, H. Viscosity and sedimentation study of sonicated DNA-proflavine complexes. *Biopolymers* **1969**, *8*, 45–55.
(46) Saucier, J. M.; Festy, B.; Le Pecq, J. B. The change of the torsion of the DNA helix caused by intercalation. II. Measurement of the relative change of torsion induced by various intercalating drugs. *Biochimie* **1971**, *53*, 973–980.

(47) Kapuscinski, J.; Darzynciewicz, Z.; Traganos, F.; Melamed, M. R. Interaction of a new antitumor agent, 1,4-dihydroxy-5,8-bis((2-((2-hydroxyethyl)amino)ethyl)amino)-9,10-anthracenedione, with nucleic acids. *Biochem. Pharmacol.* **1981**, *30*, 231–240.
(48) Islam, S. A.; Neidle, S.; Gandecha, B. M.; Partridge, M.; Patterson, L. H.; Brown, J. R. Comparative computer graphics and solution studies of the DNA interaction of substituted anthraquinones based on Doxorubicin and Mitoxantrone. *J. Med. Chem.* **1985**, *28*, 857–864.
(49) Waring, M. J. DNA modification and cancer. *Annu. Rev. Biochem.* **1981**, *50*, 159–192.
(50) Peisach, J.; Blumberg, W. E. Structural implications derived from the analysis of EPR spectra of natural and artificial proteins. *Archiv. Biochem. Biophys.* **1974**, *165*, 691–6.
(51) Harbour, J. R.; Chow, V.; Bolton, J. R. An ESR study of the spin adducts of OH and OH_2 radicals with nitrones in the ultraviolet photolysis of aqueous hydrogen peroxide solutions. *Can. J. Chem.* **1974**, *52*, 3549–55.
(52) Krapcho, A. P.; Getahum, Z.; Avery, K. L.; Vargas, K. J.; Hacker, M. P.; Spinelli, S.; Pezzoni, G.; Manzotti, C. Synthesis and antitumor evaluations of symmetrically and unsymetrically substituted 1,4-bis((aminoalkyl)amino)anthracene-9,10-diones and 1,4-bis((aminoalkyl)amino)-5,8-dihydroxyanthracene-9,10-diones. *J. Med. Chem.* **1991**, *34*, 2373–2380.
(53) Stefanska, B.; Dzieduszycka, M.; Martelli, S.; Borowski, E. Synthesis and unsymmetrically substituted 1,4-bis((aminoalkyl)amino)anthracene-9,10-diones as potential antileukemic agents. *J. Med. Chem.* **1989**, *32*, 1724–1728.
(54) Mahler, H. R.; Kline, B.; Mehrotra, B. D. Some observations on the hypochromism of DNA. *J. Mol. Biol.* **1964**, *9*, 801–811.
(55) Wakelin, L. P. G.; Waring, M. J. The binding of echinomycin to DNA. *Biochem. J.* **1976**, *157*, 721–740.
(56) Revet, B.; Schmir, M.; Vinograd, J. Direct determination of the superhelix density of close circular DNA by viscometric titration. *Nature New Biol.* **1971**, *229*, 10–13.
(57) 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 gradients. *J. Mol. Biol.* **1974**, *89*, 783–801.