New Dimeric Analogues of Ethidium: Synthesis, Interaction with DNA, and Antitumor Activity

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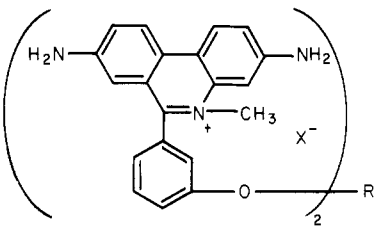
Three new dimeric analogues of ethidium cation in which the monomeric moieties are linked at the 3′ positions by α,ω-diethers of varying length and composition have been synthesized. The circular dichroism spectra of all three compounds indicate that they double intercalate, and their effects on the thermal helix–coil transition profile of poly(dA-dT) show extremely high affinity for helical DNA, with details of the binding interaction depending on the length and composition of the connecting chain. The ability of the compounds to inhibit nucleic acid synthesis in L1210 cell culture also differed significantly, as did their antitumor effects against P388 leukemia and B16 melanoma. Compound **2**, with 10 methylene groups in the connecting chain, is 5–20 times as potent as ethidium against murine P388 leukemia. These results clearly illustrate the advantage gained by incorporating a weak antitumor agent in a double-intercalating analogue.

Several antitumor agents with diverse chemical structures—for example, actinomycins,[1] anthracyclines,[2] and ellipticines[3]—have a common property of intercalative binding to DNA. While intercalative DNA binding probably is not the only factor responsible for their antitumor efficacy,[4] it is no doubt a significant factor. A more thorough study of the biological effects of intercalating compounds is necessary to understand the mode of action of existing antitumor agents and to design improved agents.

One fruitful approach to the understanding of intercalation is the study of dimeric compounds that can intercalate at two sites simultaneously. Recently, several classes of such compounds, including acridines,[5] pyridocarbazoles,[6,7] anthracyclines,[8] chloroquines,[9] quinaldines,[10] and phenanthridines,[11,12] have been studied. The affinity of these compounds for DNA has been found, with few exceptions, to be so great that it is difficult to measure accurately.[6,12,13] Linkage of monomers with little or no antitumor activity sometimes produces dimers with significant activity.[7,14]

Our approach to double intercalating analogues of ethidium (**4a**) cation is to connect the two halves through the meta, or 3′, position of the 6-phenyl ring. This position has the advantage that carbons 1′, 2′, and 3′ of the phenyl substituent form the first three atoms in the link between the two intercalating phenanthridinium moieties. These atoms also extend in the desired direction and are part of a known intercalator.

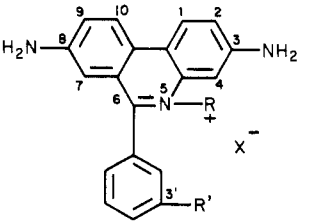
We first linked two ethidium analogues using amide functions to attach a methylene chain between the two 3′ positions.[12] These compounds showed the expected high DNA affinity, but they were relatively inactive as inhibitors of nucleic acid synthesis in L1210 cells. This paper reports the synthesis and evaluation of three compounds in which the two ethidium analogues are linked through ether functions: **1** with seven methylene groups between the two



1, R = $-(CH_2)_7-$
2, R = $-(CH_2)_{10}-$
3, R = $-(CH_2)_2O(CH_2)_2-$

ether oxygens, **2** with ten methylene groups between the two ether oxygens, and **3** in which an additional ether link is incorporated within the methylene chain. These compounds have been found to intercalate with the involvement of both phenanthridinium units and to have much improved biological properties relative to the amido compounds.

A monomeric analogue, **4b**, of ethidium, **4a**, was prepared to assay the effect of the 3′-alkoxy substitution. No



4a (ethidium), R = C_2H_5; R′ = H
b, R = CH_3; R′ = OCH_3

appreciable differences between **4a** and **4b** were found in in vitro assays.

Chemistry. The synthetic route for the target compounds 1–4 is shown in Scheme I. The phenanthridine

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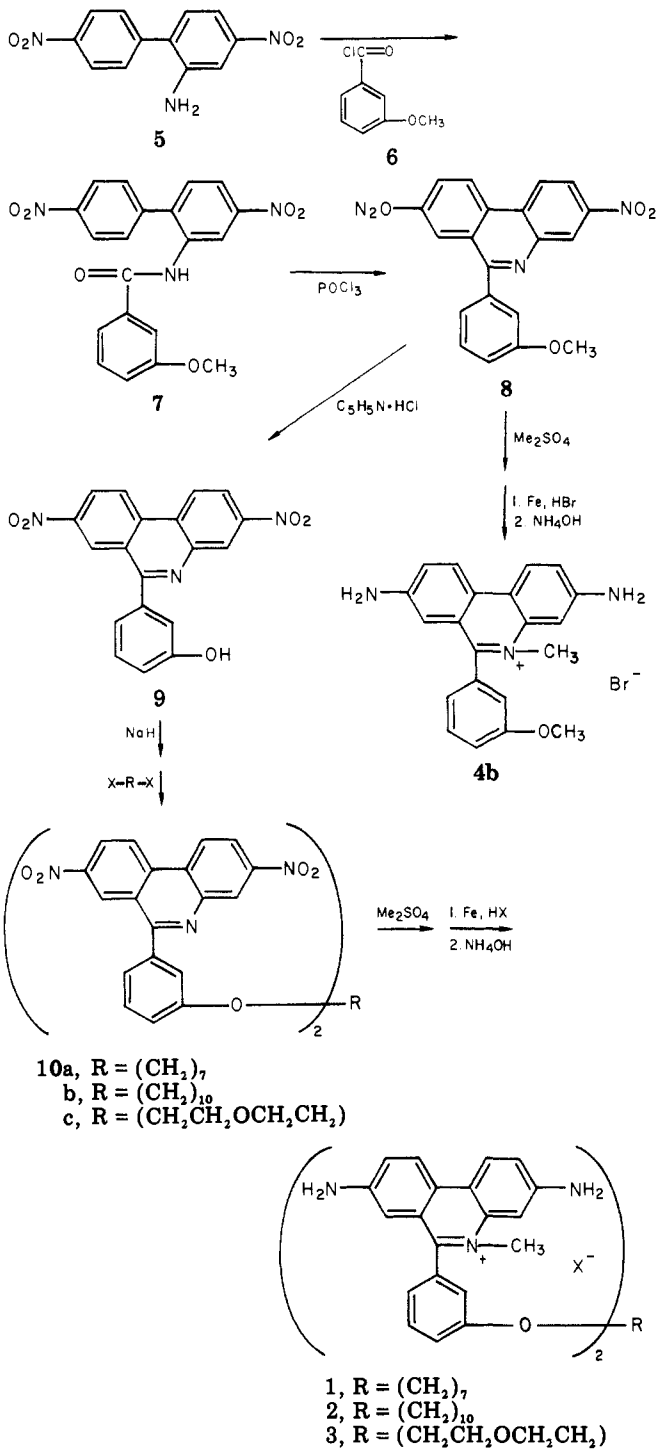
Scheme I



10a, R = $(CH_2)_7$
b, R = $(CH_2)_{10}$
c, R = $(CH_2CH_2OCH_2CH_2)$

1, R = $(CH_2)_7$
2, R = $(CH_2)_{10}$
3, R = $(CH_2CH_2OCH_2CH_2)$

8 was generated by reported procedures,[15] condensing 5[16] with 6 to give amide 7, which was cyclized in refluxing nitrobenzene by the addition of a small amount of $POCl_3$. Demethylation[17] of 8 was accomplished by fusion with pyridinium chloride, to give 9 in almost theoretical yield. Treatment[18] of 9 in DMF solution with NaH, followed by condensation with the appropriate dihalide, yielded 10.

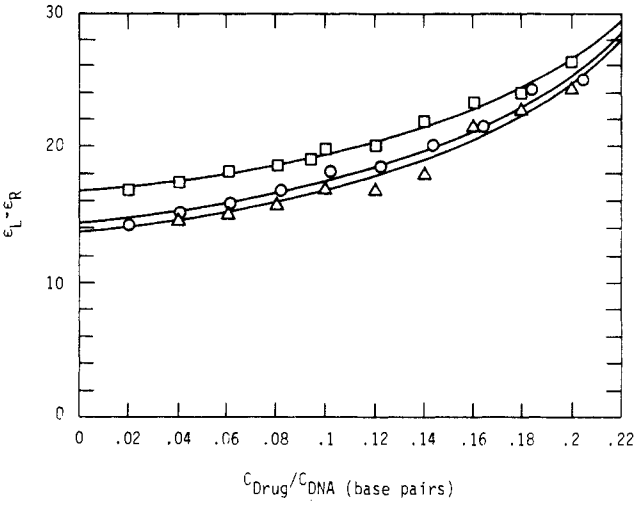


Figure 1. Induced circular dichroism of drug–DNA complexes at 310 nm vs. ratio of concentrations of drug to DNA (base pairs) in 0.01 M phosphate buffer (pH 7): 1, △; 2, ○; 3, □. C_{drug} = 0.018–0.032 mM, C_{DNA} = 0.91–0.16 mM. Solid lines calculated according to ref 19 with $n = 4$. 1: $\Delta\epsilon_0 = 13.5$, $\Delta\epsilon_{ex} = 22$, $R^2 = 0.965$. 2: $\Delta\epsilon_0 = 14.3$, $\Delta\epsilon_{ex} = 22$, $R^2 = 0.977$. 3: $\Delta\epsilon_0 = 16.6$, $\Delta\epsilon_{ex} = 20$; $R^2 = 0.988$.

Quaternization of 10 with Me_2SO_4 in DMF gave a syrup that was dissolved in ethanol and clarified by filtration. Concentration of the ethanol solution and chilling yielded an orange solid that was used directly in the final step. Reduction of the quaternary salt of 10 to the final product was accompanied by the formation of several byproducts, detected by TLC. Purification was accomplished on a Sephadex LH-20 column; in some cases, repetitive treatment was required. This technique probably involves both gel filtration and adsorption; the column retained some material, indicated by the pink color that never could be removed completely. The structures of the final products were established by ^1H NMR spectra and elemental analyses. The synthesis of 1 from 10a, a typical procedure for the preparation of the desired products, is described under Experimental Section. Properties of the products and intermediates are presented in Table I.

Results and Discussion

Interaction with DNA. The new compounds were assayed for double intercalation by the effect of DNA on their CD spectra and by their effect on the helix–coil transition temperature of poly(dA-dT).

The induced CD of bisethidium analogues is an indicator of double intercalation.[19] Association with DNA induces a band in the CD of ethidium analogues at about 310 nm, which is due to exciton interactions between bound drug molecules. In a monomer, the intensity of the exciton band, $\Delta\epsilon_{310}$, vanishes as the ratio of drug to DNA goes to zero. With a dimeric intercalator, the interaction between the two connected ethidium analogues persists as this ratio is decreased and $\Delta\epsilon_{310}$ approaches a nonzero limit. For the three ether-linked bisethidium analogues, $\Delta\epsilon_{310}$ is shown as a function of the ratio of drug/DNA (base pairs), ν, in Figure 1. Clearly there is substantial intensity as ν approaches 0 for each compound.

The dependence of $\Delta\epsilon_{310}$ on ν can be used to estimate the number, n, of base pairs blocked by a bound double intercalator. In each case in Figure 1, the best agreement between theory and experiment is obtained if we assume

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Table I. Physical and Analytical Data for Phenanthridinium Salts and Intermediates

compd	% yield	mp,[a] °C	recrystn solvent	TLC,[b] R_f	formula	anal.
7	95	166-167,[c] 178-179	C_2H_5OH	0.28 (A) 0.80 (D)	$C_{20}H_{15}N_3O_6$	C, H, N
8	66	237-241	DMF	0.48 (A) 0.90 (D)	$C_{20}H_{13}N_3O_5$	C, H, N
4	24	175-185[d]	i-C_3H_7OH	0.51 (B),[e] 0.49 (C)	$C_{21}H_{20}N_3OBr \cdot C_3H_7OH \cdot 0.2H_2O$	C, H, N, Br
9[f]	95	333-337[g]	DMF	0.60 (D)	$C_{19}H_{11}N_3O_5$	C, H, N.
10a	70	251-256	DMF	0.90 (D)	$C_{45}H_{34}N_6O_{10} \cdot H_2O$	C, H, N
10b	65	214-218	DMF	0.9 (D)		
10c	65	265-271	DMF	0.72 (D)		
1	43[h]	214-221[d]		0.38 (B),[e] 0.18 (C)	$C_{47}H_{48}N_6O_2Br_2 \cdot 2H_2O$	C, H, N, Br
2	10[i]	205-212[d]		0.45 (B)[e]	$C_{50}H_{54}N_6O_2Cl_2 \cdot 3.5H_2O$	C, H, N, Cl
3	14[j]	220-238[d]		0.30 (B)[e]	$C_{44}H_{42}N_6O_3Br_2 \cdot 2.25H_2O$	C, H, Br; N[k]

[a] On a Kofler-type hot stage. [b] Spots detected as UV absorbing, unless otherwise noted. Solvent systems: A, $CHCl_3$; B, MeOH/0.1 NHCl (10:1); C, BuOH/HOAc/H_2O (4:1:1); D, $CHCl_3$/CH_3OH (50:1). [c] Two melting points observed. [d] Amorphous; melting point difficult to determine on very dark red solid. [e] Bright orange-red fluorescent spot. [f] Reference 17. [g] Amorphous product changing to crystalline form at 290-310 °C and then melting at 333-336 °C. [h] Based on 10a. [i] Based on 10b. Losses suffered during removal of an impurity that was only slightly less soluble than 2 resulted in greatly decreased yields. A MeOH-H_2O (2:1) solution of the chloride salt of 2 [obtained by treatment of the bromide salt with Dowex 1-X (Cl^- form) resin] was allowed to stand overnight, and then the supernatant liquid was decanted from the sticky precipitate; several such decantations yielded a solution of pure 2. [j] Based on 10c. After quaternization of 10c with Me_2SO_4 and removal of DMF, the syrupy residue was triturated with dilute HBr to give an orange-brown solid that was extracted several times with methanol. Concentration yielded a solid that was further purified on a dry column (Woelm, activity III silica, developing with $CHCl_3$-MeOH, 100:1). The orange band (R_f about 0.3-0.5) was cut out and extracted to yield an orange product, almost homogeneous, R_f 0.9 on TLC (D), that was subjected to reduction. [k] N: calcd, 9.30; found, 8.80.

that all the drug is bound as long as saturation of the DNA binding sites is not reached, and each drug occupies four base pairs when bound. The solid lines in Figure 1 were calculated on this basis, with the CD of isolated and interacting bound drugs fit by least squares.

The sensitivity of the theoretical CD curves to the parameter n decreases rapidly as n increases. Therefore, these results do not rule out the possibility that n may be larger than 4 for some of these molecules. However, the value 4 is the minimum value expected for a double intercalator with "neighbor exclusion" applicable to both intercalated chromophores.

The effect of the ether-linked compounds on the thermal helix–coil transition curve of poly(dA-dT) is shown in Figure 2. Quantitative analysis of these curves is beyond the scope of this paper.[20] However, qualitatively, these curves can be explained as follows. The poly(dA-dT) passes from the helical form to a random coil form ("melts") when the free energy of the helical and coil forms are roughly equal. The magnitude of the free-energy difference between coil and helix decreases with temperature because of the negative enthalpy of transition from coil to helix. The homogeneity of poly(dA-dT) and the cooperative nature of the transition make this transition occur over a very narrow range of temperature.

An intercalator bound to the poly(dA-dT) provides additional free energy of stabilization for the helical form relative to the coil form. Because of this additional stabilization of the helix, equality of helix and coil free energy occurs at a higher temperature.[21] However, the helix–coil transition curve is not simply shifted along the temperature axis. Ligand binding affects the shape of the curve as well.

Below saturation of the binding sites, the ligand (drug) is not homogeneously distributed over the poly(dA-dT) lattice. Regions with the least bound ligand will begin to melt first, with the liberated ligands moving to the remaining helical sections. Thus, as the transition proceeds, the average stabilization per base pair increases, and the transition is much less sharp. In the case of the double

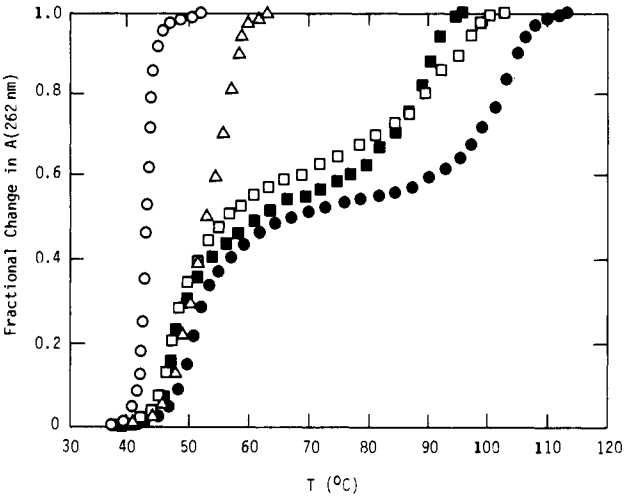

Figure 2. Helix–coil transition curves of complexes with poly(dA-dT): poly(dA-dT) alone, ○; with 1, ●; with 2, □; with 3, ■; with 4, △. Concentrations in 0.01 M phosphate buffer (pH 7) with 5% $(CH_3)_2SO$: poly(dA-dT) (base pairs), 1.7×10^{-6} M; 1-3, 1.7×10^{-6} M; 4, 3.4×10^{-6} M.

intercalators, the curves actually become biphasic, as the nearly saturated helical sections have very high transition temperatures because of the large free energy of binding of these ligands.

It is clear from the helix–coil transition curves that the various double intercalating ligands all stabilize the poly(dA-dT) double helix much more than does the monomer but to different extents under the same conditions of temperature and concentration.

Biological Results. The biological activity data for the ether-linked bisethidium analogues are summarized and compared with that for ethidium chloride in Table II. The first two columns show the concentrations necessary to inhibit 50% of RNA and DNA synthesis (IC_{50}) in cultured L1210 cells. These data clearly demonstrate a strong dependence of inhibitory potency on the nature of the connecting chain. The two compounds with a simple diether linking chain, 1 and 2, are significantly more potent inhibitors of nucleic acid synthesis than ethidium or the

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Table II. Biological Test Data for Ether-Linked Bisethidium Analogues

compd	inhibn of nucleic acid synth in L1210 cells: [a,b] IC_{50}, μM		antitumor act. in mice [c]			
			leukemia P388, qd 1-9		melanoma B16, qd 1-9	
	RNA	DNA	dose, [d] μmol/kg	T/C, [e] %	dose, [d] μmol/kg	T/C, [e] %
ethidium chloride	11 ± 1	31 ± 6	17.9	149 (24) [f]	34.2	135 [h]
			8.9	150 (18) [f]	17.1	112 [h]
			4.5	135 (10) [f]	8.6	108 [h]
1	0.6 ± 0.1	12 ± 2	0.22	120 (40)	0.17	138 [h]
			0.11	126 (13)	0.16	134 [h]
			0.05	120 (5)	0.09	136 (4)
					0.04	116 (14)
2	0.3 ± 0.1	1.7 ± 0.1	1.7	158 (33) [g]		
			0.86	160 (15)		
			0.43	156 (24)		
			0.21	141 [h]		
3	16 ± 3	187 ± 9	3.4	152 (39)	2.2	142 (7) [g]
			1.7	156 (28)	1.1	127 (20)
			0.85	132 (37)	0.55	109 (3)
			0.43	124 (20)		
4b	10 ± 1	25 ± 2				

[a] Determined by the method of G. Tong, W. W. Lee, D. R. Black, and D. W. Henry, *J. Med. Chem.*, 19, 395 (1975), except that the drugs were initially dissolved in a volume of Me_2SO that gave a final concentration of Me_2SO of 1%. [b] IC_{50} is the concentration of drug necessary to reduce by 50% the incorporation of tritiated thymidine in the DNA or tritiated uridine in the RNA of actively growing L1210 cells. [c] Assays conducted under the auspices, and according to the protocols, of the NCI. [d] At higher doses, there were no surviving animals on toxicity evaluation day (day 5). [e] Ratio of average survival time of treated mice to untreated mice × 100. Average of two test results, except where noted. Values of T/C > 120 signify activity. Range of T/C values are shown in parentheses. [f] Average of three test results. [g] Dose schedule qd 1-5. [h] One test result.

monomer analogue. The IC_{50} for RNA and DNA synthesis inhibition for **2** (0.3 and 1.7 μM, respectively) are comparable to values observed for the antibiotic daunorubicin (0.33 and 0.66) in this test. On the other hand, the triether-linked compound **3** is significantly less potent than the monomeric species. It should be noted, however, that although **3** differs from **1** and **2** by the presence of the central ether function, it also has the shortest linking chain (seven atoms) of the three compounds.

The antitumor effect of these compounds is indicated by the results also shown in Table II. Against leukemia P388 in mice, compound **2** shows greater efficacy (T/C = 160%) than ethidium (T/C = 150%) but at 5- and 20-fold lower dose levels than ethidium. The antitumor potency (effective dose level) and efficacy of compound **2** in this test are comparable to those of the clinically useful anthracycline antibiotics daunorubicin (T/C = 160 ± 27 at 1.4 μmol/kg) and adriamycin (T/C = 197 ± 26 at 1.3 μmol/kg), both on a qd 1–9 dose schedule. (Note that these data are not strictly comparable, as they were obtained using different dose schedules. However, of the ethidium analogues, the more potent, more active **2** was tested in the schedule with fewer doses over a shorter time span (qd 1–5). If these results were projected to the qd 1–9 schedule, the difference between **2** and ethidium should be at least as great.) Once again, the effect of varying the connecting chain is apparent, with compound **3** being more potent than ethidium but less so than **2**, while compound **1** shows only borderline antitumor activity but at extremely low doses.

Against B16 melanoma, however, both **1** and **3** show activities comparable to that of ethidium but at much lower dose levels; **1** is 20–40 times as potent as ethidium, while **3** is 15 times as potent.

Conclusion

These three ether-linked dimeric analogues of ethidium all appear to double intercalate with DNA, based on studies of the DNA complexes including helix–coil transition temperatures and CD spectra.

From the helix–coil transition curves it appears that the details of the interaction with DNA depend on the length and perhaps on the nature of the linking chain. This dependence is reflected in the biological activity of the compounds.

Compound **2** as an active and potent agent against P388 leukemia is far superior to ethidium and can be compared favorably with the clinically useful anthracycline antibiotics in the same test. The chemical structure of **2** is radically different from anthracyclines, and the pattern of side effects would also be expected to be different.

Thus, the poor antitumor activity of ethidium cation has been greatly improved by combining two of the residues in a double-intercalating analogue.

Experimental Section

Melting points, uncorrected, were determined on a Kofler-type hot stage. Solutions in organic solvents were dried over anhydrous $MgSO_4$. Solutions were concentrated under reduced pressure on a rotary evaporator. Thin-layer chromatography was accomplished on silica gel (0.25 mm GF, Analtech) plates; spots were detected under UV light. Spectra were determined on the following instruments: NMR on a Varian EM 390 or XL-100-15 spectrometer equipped with Fourier transform in solutions as noted with Me_4Si (δ 0.0) internal reference and signals described as s (singlet), d (doublet), t (triplet) and m (multiplet); CD, on a Durrum-Jasco ORD/UV spectrophotometer equipped with a Sproul Scientific SS-20 CD modification.

Calf thymus DNA was purchased from Worthington Biochemical Corp., and poly(dA-dT) was from P-L Biochemicals. Thermal denaturation studies and L1210 nucleic acid synthesis assays were carried out as described previously.[22] Poly(dA-dT) was used because, with it, the transition temperature ranges were within reasonable limits. The transition for native DNA complexed with **1** is not complete until about 130 °C, whereas with poly(dA-dT) the highest temperature required was 110 °C.

1,7-Bis[3-(3,8-dinitrophenanthridinyl)phenoxy]heptane (10a). Glassware was dried at 100 °C overnight and moisture excluded from the reaction flask. A mixture of 284 mg (0.79 mmol) of **9** and 34 mg of 57% NaH in an oil dispersion (0.8 mmol of NaH) in 6 mL of dry DMF was stirred until gas evolution ceased. A solution of 105 mg (0.40 mmol) of 1,7-dibromoheptane was added, and the dark brown solution was stirred at room temperature for

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6 h, with a solid separating after a short time. The yellow solid was collected and washed with a small amount of DMF and then with ethanol, yielding 225 mg (70%) of **10a**: mp 250–254 °C; TLC ($CHCl_3$–MeOH, 50:1) 0.8. A small amount was recrystallized from DMF to give an analytically pure sample, mp 251–256 °C. Anal. ($C_{45}H_{34}N_6O_{10}\cdot H_2O$) C, H, N.

1,7-Bis[3-(3,8-diamino-5-methylphenanthridinium-6-yl)-phenoxy]heptane Dibromide (1). To a solution of 964 mg (1.17 mmol) of **10a** (dried iv, 24 h) in 20 mL of freshly distilled DMF, at 150 °C under N_2, was added 6 mL of Me_2SO_4, causing separation of a small amount of yellow solid, which quickly dissolved; heating under N_2 was continued for 2 h. The light brown solution was concentrated to a syrup, which was dissolved in 25 mL of hot ethanol; upon addition of 50 mL of H_2O, an orange-red oil separated and then solidified. This material was separated and extracted several times with a total of 300 mL of boiling ethanol. The filtered ethanol solution was concentrated to about one-third its volume and cooled with separation of 716 mg of an orange-red solid. Further concentration of the mother liquors yielded 210 mg of solid.

Without further characterization, this material was subjected to reduction of the nitro groups; 485 mg was mixed with 32 mL of 60% ethanol, 0.2 mL of 48% HBr, and 500 mg of Fe powder. The mixture was heated under reflux for 1.5 h and then filtered hot. The pH of the dark red filtrate was adjusted to 8.5 with NH_4OH. After the solution was filtered through Celite, concentrated to about 10 mL, and chilled overnight, a dark red solid product, 100 mg, separated. Retreatment of the solids that had been collected by filtration from the reduction reaction mixture, by mixing with aqueous ethanol, HBr, and Fe, refluxing for 3 h, and working up as previously described, yielded an additional 126 mg of product. These combined materials were purified on a Sephadex LH-20 column (2.5 × 30 cm) using methanol solvent, collecting 2-mL fractions. Examination by TLC showed that the initial fractions contained largely impurities; subsequent fractions, still slightly impure, were combined and repurified on the same column. Concentration of like fractions yielded a slightly sticky residue that solidified on trituration with acetone, yielding 235 mg of 1 (43%, based on **10a**) of dark red amorphous solid: mp 214–221 °C (?); TLC (MeOH–0.1 N HCl, 10:1) R_f 0.38; TLC (BuOH–HOAc–H_2O, 4:1:1) R_f 0.18, bright orange-red fluorescent spot under UV light; NMR (CD_3OD) δ 8.40 (t, 4, H_1, H_{10}), 7.0–7.8 (m, 14, Ar protons), 6.58 (d, 2, H_7), 4.0–4.15 (s + m, 10, *N*-Me, OCH_2), 1.3–1.9 [m, 10, $(CH_2)_5$]. Anal. ($C_{47}H_{48}N_6O_2Br_2\cdot 2H_2O$) C, H, N, Br.

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Anticoccidial Derivatives of 6-Azauracil. 4. A 1000-fold Enhancement of Potency by Phenyl Sulfide and Phenyl Sulfone Side Chains[1]

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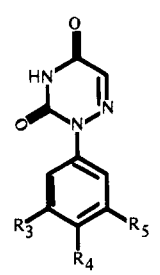
We report further progress in exploiting our earlier discovery that the anticoccidial activity of 6-azauracil increases markedly when appropriately substituted benzyl or phenyl groups are attached at N-1. With guidance from previous structure–activity relationships and a multiple linear regression analysis, 6-azauracils containing phenyl sulfone or phenyl sulfide side chains were prepared. These prevented a broad spectrum of coccidial infections in chickens at minimum inhibitory concentrations by weight in feed as low as 0.25 ppm, a 4000-fold increase in potency over 6-azauracil, and had shorter plasma half-lives than earlier potent analogues. Sulfides were more potent than sulfones, although they were oxidized rapidly to sulfones in vivo.

This paper is part of a series of publications on anticoccidial derivatives of 6-azauracil. Earlier papers in the series described how the anticoccidial activity of 6-azauracil can be markedly increased by attaching at the "ribosylation position", N-1, certain meta-substituted benzyl or phenyl groups.[2-4] The lower potencies of **2–6** relative to **1** seemed to indicate that either electron-donating or electronegative groups at the para position in the side chain had an undesirable effect on activity. On the other hand, it was recognized very early that activity was enhanced by a strong para-oriented, exocyclic-vectored dipole (as in **1**), which increases the acidity of the imide hydrogen,[2] and **7** was equipotent with **1**, suggesting that further variations in the physical–chemical properties of R_4 should be tried.



	R_3	R_4	R_5
1	Cl	H	Cl
2	Cl	CH_3	Cl
3	Cl	OCH_3	Cl
4	Cl	SCH_3	Cl
5	H	OC_6H_5	H
6	Cl	SO_2CH_3	Cl
7	Cl	Cl	Cl
8	H	COC_6H_5	H

The difficulty of correlating the group electropolarity (σ), group lipophilicity (π), and group dipole moment (μ)

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