Microgonotropens and Their Interactions with DNA. 2.[1] Quantitative Evaluation of Equilibrium Constants for 1:1 and 2:1 Binding of Dien-Microgonotropen-a, -b, and -c as Well as Distamycin and Hoechst 33258 to d(GGCGCAAATTTGGCGG)/d(CCGCCAAATTTGCGCC)

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Received August 25, 1992

Abstract: A quantitative methodology has been introduced to determine equilibrium constants for minor groove binding by double-stranded DNA oligomers. The method is dependent upon the fact that Hoechst 33258 (Ht) fluoresces when bound in the minor groove of B-DNA while lexitropsins and dien-microgonotropens do not. Equilibrium constants were determined from competitive binding experiments with Ht at 35 °C. Equilibrium constants for the 1:1 and 1:2 complexing of the double-stranded DNA hexadecamer d(GGCGCAAATTTGGCGG)/d(CCGCCAAATTTGCGCC) with dien-microgonotropen-a, -b, and -c (**5a**, **5b**, and **5c**) have been compared to the same constants for the complexing of lexitropsins **2** and distamycin (Dm) as well as Ht. The following equilibrium constants were determined: K_{Ht1} = [DNA:Ht]/[DNA][Ht]; K_{Ht2} = [DNA:Ht_2]/[DNA:Ht][Ht]; K_{L1} = [DNA:L]/[DNA][L]; K_{L2} = [DNA:L_2]/[DNA:L][L]; K_{HtL} = [DNA:Ht:L]/[DNA:Ht][L]; and K_{LHt} = [DNA:Ht:L]/[DNA:L][Ht]. Anticooperativity for complexing of **2** is marked by K_{L2} being an order of magnitude less than K_{L1}. The first and second bindings of **2** to the hexadecamer are between 1 and 4 orders of magnitude weaker than the comparable bindings of **5a**, **5b**, **5c**, Dm, or Ht. For the latter, all second association constants (K_{Ht2}, K_{HtL}, K_{L2}, and K_{LHt}) are larger than the first association constants by ~1–3 orders of magnitude, indicating positive cooperativity. Although for **5a**, **5b**, **5c**, Dm, or Ht the equilibrium constants for stepwise complexation of one and two L or Ht species varied, the calculated equilibrium constants for formation of DNA:L_2 or DNA:Ht_2 species ($K_{L1}K_{L2}$ or $K_{Ht1}K_{Ht2}$) were similar [(1–20) × 10^{16} M^{-2}] and 10^4 greater than the comparable constant for **2**. The order of affinity is **5a** ~ **5b** ~ **5c** > Ht > Dm ≫ **2**. Replacement of the triamine substituents of **5a**, **5b**, and **5c** with a methyl group provides **2**. Thus it can be seen that the triamine substituents contribute substantially to double-stranded DNA (dsDNA) complexation of **5a**, **5b**, and **5c**. The temperature dependence of Ht binding to the hexadecamer between 20 and 40 °C shows a critical temperature at ~32 °C. Cooperativity for Ht binding to the hexadecamer duplex is 6 orders of magnitude greater below 30 °C (log K_{Ht1} = 4.4, log K_{Ht2} = 12.3) than above 30 °C (log K_{Ht1} = 7.5, log K_{Ht2} = 9.3) even though log $K_{Ht1}K_{Ht2}$ is essentially unchanged. This is attributed to a marked conformational change in the DNA:Ht_1 species. In the special cases of **5a**, **5b**, and **5c**, a 38% quenching of the fluorescence of Ht in the DNA:Ht:L mixed complexes was observed. This has been shown to be due to static quenching by the $(CH_2)_nN\{(CH_2)_3N(CH_3)_2\}_2$ substituent (n = 3, 4, 5, for **5a,b,c**, respectively).

Introduction

The study of sequence-selective agents interacting with double-stranded DNA (dsDNA) has become increasingly important during the last decade. Peptide motifs (i.e., the SPKK repeat, the zinc finger, and the helix-turn-helix)[6] and the third strand of the DNA triple helix[7] have attracted considerable attention. Many small organic molecules, often based on natural products, have also been investigated for their ability to selectively bind in the minor groove of B-DNA. These molecules include, but are not limited to, the calicheamicins,[8] (+)-CC-1065,[9] Hoechst 33258,[10] and the lexitropsins[11] (amide-linked pyrrolic analogues of distamycin and netropsin). Exciting prospects for functionalization of these molecules range from enhancing binding strength, efficiency, and selectivity to modifications that can alter DNA conformation, chemically modify DNA, and perform site-selective cleavage.[12]

We have introduced the name microgonotropen for minor groove binding agents substituted in such a manner that the substituent reaches out of the minor groove to interact with the phosphodiester backbone or the major groove.[4] The planar crescent shape of distamycin (Dm) and its abundance of hydrogen-

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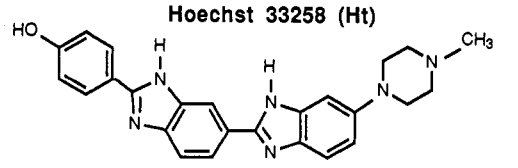
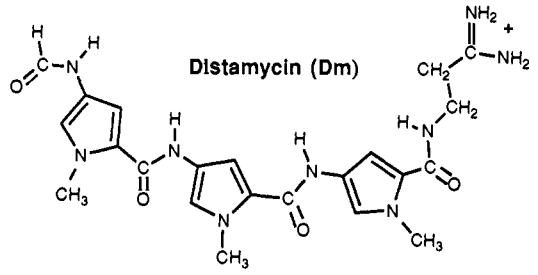
Chart I

Hoechst 33258 (Ht)



Distamycin (Dm)



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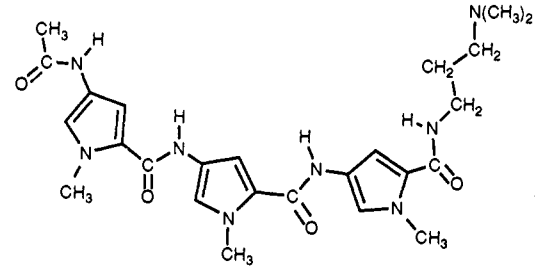

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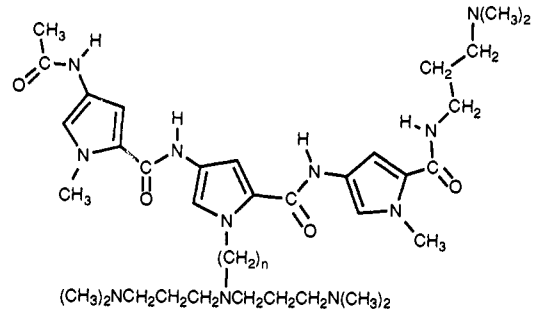

5a, n=3; **5b**, n=4; **5c**, n=5

bond donors make Dm ideally suited to "slide" into the natural curve of the minor groove of A+T-rich B-DNA. Once bound in the minor groove, the 1-methyl substituents of the 4-amino-1-methyl-2-pyrrolecarboxylic acid residues are directed outward from the minor groove.[13] This presents the interesting notion of replacing one or more of the pyrrole *N*-methyl groups of a distamycin-like analogue to obtain microgonotropen products.[2] The synthesis and binding selectivity of the first members in a series of these compounds (**5a**, **5b**, and **5c** of Chart I; the numbering of the analogues remains the same as that in the previous paper in this issue) have been described.[4]

A number of techniques have been offered to determine equilibrium constants for ligands binding to dsDNA.[14] Present useful techniques have not allowed the determination of even approximate association constants for agents binding cooperatively to oligomeric DNA.[15] In addition, calculation of association constants in biochemical systems has generally employed one or another reciprocal plotting technique in search of linear relationships (i.e., Scatchard and Lineweaver–Burk plots[16]). The use of such procedures is restrictive, and in more complex systems, plots of data points are noticeably curved and cannot be fit to a single straight line.[14a,b] This can lead to serious errors when attempting to interpret data.[17] However, with the aid of high-speed modern computers and data-fitting programs, plots of total concentration (which is readily known) vs some observable parameter can now be fit to complex, nonlinear curves, thus obviating the need for reciprocal plotting. This allows iterative fitting of the experimental points such that constants for single or multiple equilibria can be solved for and accurately dissected.

A new quantitative procedure for the determination of the equilibrium constants for the complexation of minor groove binders by dsDNA is described. On the basis of fluorescence changes upon addition of Hoechst 33258 and subsequent nonlinear curve fitting, this procedure is straightforward and expeditious. The equilibrium constants for the formation of 1:1 and 2:1 complexes of the agents **5a**, **5b**, **5c**, **2**, Dm, and Ht with d(GGCGCAAATTTGGCGG)/d(CCGCCAAATTTGCGCC) are compared at 35 °C. The temperature dependence of the equilibrium constants for dsDNA hexadecamer complexation with one and two Ht molecules reveals a sharp temperature-dependent change in the conformation of the 1:1 complex.

Experimental Section

Materials. The synthesis and characterization of **2**, **5a**, **5b**, and **5c** are described elsewhere.[4] Bis[3-(dimethylamino)propyl]methylamine was prepared by a known method.[18] Hoechst 33258 (Aldrich) and distamycin (Sigma) were used without further purification. Stock solutions (in distilled deionized H_2O) of each agent were stored on ice for the duration of the experiment and were maintained frozen at −20 °C between experiments. All other reagents were from standard sources and used without further purification except buffers, which were filtered through sterile 0.45-μm Nalgene disposable filters.

The hexadecameric oligonucleotides d(GGCGCAAATTTGGCGG) and d(CCGCCAAATTTGCGCC) (UCSF Biomolecular Resource Facility) were annealed by heating a solution containing equimolar concentrations of both oligomers to 95 °C for 5 min in 0.01 M potassium phosphate buffer, pH 7.5, 0.1 M NaCl and then allowing the sample to slowly cool to 30 °C over the course of 100 min. The resultant double-stranded DNA was precipitated with the addition of 2 volumes of ice-cold 100% ethanol, washed with 70% ice-cold ethanol, and vacuum-dried before resuspension in 0.01 M potassium phosphate buffer, pH 7.0, 0.01 M NaCl (μ = 0.028). The hexadecamer concentration was determined spectrophotometrically ($\epsilon_{260} = 3.08 \times 10^5\ M^{-1}\ cm^{-1}$ duplex hexadecamer) using quartz cuvettes in an OLIS modified Cary-14 recording spectrophotometer.

Oligonucleotide Melt Analysis. Absorbance changes at 260 nm were monitored with a Perkin-Elmer 553 UV/vis spectrophotometer while the duplex d(GGCGCAAATTTGGCGG)/d(CCGCCAAATTTGCGCC) (5.0×10^{-7} M in double strands, 0.01 M potassium phosphate (pH 7.0), 0.01 M NaCl) was heated from 28 to 68 °C. The temperature was controlled by circulating water from a HAAKE circulating bath through a water-jacketed cuvette holder in the spectrophotometer. Data points were collected every 0.4–1 °C.

Fluorescence Methods. In all fluorescent titrations, solutions were buffered with 0.01 M potassium phosphate (pH 7.0), 0.01 M NaCl in distilled deionized H_2O (μ = 0.028). The final concentration of duplex hexadecamer was always 5.0×10^{-9} M. Ligand concentrations varied from 8.0×10^{-9} to 1.4×10^{-8} M for **5a**, **5b**, **5c**, and distamycin and from 5.0×10^{-8} to 1.0×10^{-7} M for **2**. Buffered solutions (2.8 mL) containing dsDNA ± ligand were titrated with a 3.5×10^{-6} M solution of Hoechst 33258 in distilled deionized H_2O until a final concentration of 1.0×10^{-7} M was reached. All titration volumes were measured with Gilson Pipetman microliter pipets and disposable pipet tips. The solutions were excited at 354 nm, and fluorescence emissions were measured at 450 nm using the mean value of triplicate data collections with a thermostated (35 °C, unless explicitly stated otherwise) Perkin-Elmer LS-50 fluores-

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cence spectrophotometer. The samples were continuously stirred in matched quartz cuvettes (1-cm path length) and allowed at least 4 min to equilibrate between each titrant addition. The cuvettes were washed exhaustively with 10% HNO_3 before being used for the next set of titrations in order to limit nonspecific background fluorescence due to the adsorption of the previous experiment's hexadecamer and Hoechst 33258 to the cuvette. Background fluorescence intensity (buffered solution of hexadecamer before the addition of any Hoechst 33258) was subtracted from each titration point to provide the corrected fluorescence intensity, *F*. These corrected fluorescence intensity data points were fit to theoretical curves with one or two different iterative nonlinear least-squares computer routines and plotted in the Kaleidagraph 2.1.2 program (Abelbeck Software) on a Macintosh II computer.

Quenching experiments were performed with the same reagents and under the same conditions described above. A 3.5×10^{-6} M solution of bis[3-(dimethylamino)propyl]methylamine, essentially the polyamine moiety of **5a**, **5b**, and **5c**, was used to titrate two different solutions of DNA complexes until a final concentration of 5.0×10^{-8} M in polyamine was reached. In the first experiment, fluorescence intensity was followed as the polyamine was added to a solution containing a 1:1:1 ratio of 5.0×10^{-9} M hexadecamer, Ht, and **5c**. A fluorescent solution of 5.0×10^{-9} M hexadecamer and 1.0×10^{-8} M Ht (1:2 ratio) was used in the second titration with the polyamine.

Results

Our initial study involved an attempt to apply a literature procedure,[12e] based on UV/visible spectrophotometry, to determine the equilibrium constants for the binding of **2**, **5a**, **5b**, **5c**, and Dm to the hexadecameric duplex d(GGCGCAAATTTGGCGG)/d(CCGCCAAATTTGCGCC). This procedure proved unsatisfactory, since one of the two broad UV peaks (230 nm) for these tripyrrole peptides overlaps with the DNA absorbance maximum at 260 nm and the other tripyrrole peptide absorbance (300 nm) interferes with the viewing of the absorbance peak at 320 nm due to DNA complex formation. One important piece of information was extracted from this study—the absorbance in the DNA–ligand complex region increased in a linear manner from 0 to 2 equiv of tripyrrole (*vide infra*).

The procedure which we have employed takes advantage of the dramatic increase in the fluorescence of Hoechst 33258 (Ht) upon its binding in the minor groove of B-DNA.[14c] With the low concentrations of dsDNA and ligand employed, the meager fluorescence of the tripyrrole peptide Dm[19] and, hence, the related compounds **5a**, **5b**, **5c**, and **2** is not influenced by binding to dsDNA. This finding allows the use of competitive binding of Ht with the tripyrrole ligands to determine all equilibrium constants. In practice, the concentration of the duplex hexadecamer was held constant at 5.0×10^{-9} M in 2.8 mL of a solution of 0.01 M phosphate buffer (pH 7.0) and 0.01 M NaCl (μ = 0.028). Titrations of these hexadecamer solutions containing 8.0×10^{-9} to 1.4×10^{-8} M **5a**, **5b**, **5c**, or Dm with a solution of 3.5×10^{-6} M in Ht (2.5×10^{-9} to 1.2×10^{-7} M final Ht concentrations) were carried out in quartz fluorescence cuvettes at 35 °C (unless stated otherwise). Significantly higher concentrations of **2** were required for its titration. A melting temperature of 56 °C (data not shown) verified the predicted integrity of the hexadecameric duplex under the conditions of the titrations. In the following pages, we describe the derivation of appropriate equations by which the equilibrium constants for complexing to a single dsDNA binding site can be iterated *via* the computer fitting of the data points of corrected fluorescence (*F*) vs [Ht] at given concentrations of L (=**5a**, **5b**, **5c**, Dm, or **2**).

Hoechst 33258 (Ht) association constants with the hexadecamer d(GGCGCAAATTTGGCGG)/d(CCGCCAAATTTGCGCC) were determined by spectrofluorometric assay for the fluorescent DNA:Ht and DNA:Ht_2 complexes (Scheme I). Evidence for the formation of a 1:2 hexadecamer to Ht complex stems from the observation that the fluorescence emissions saturate

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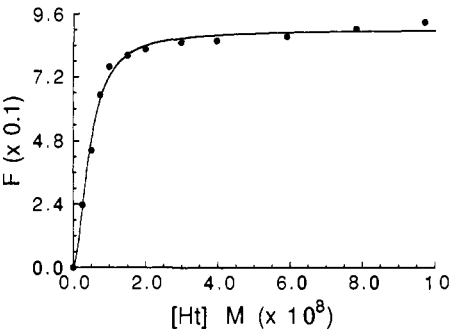


Figure 1. Plot of fluorescence (*F*, in arbitrary units) vs Hoechst 33258 (Ht) concentration at pH 7.0 and 35 °C in the presence of 5.0×10^{-9} M hexadecamer duplex. The data points shown represent mean values of two titrations of the hexadecamer with Ht. The line which fits the points was computer generated by use of eq 5.

Scheme I

$$\text{DNA} \underset{}{\overset{K_{Ht1}[Ht]}{\rightleftharpoons}} \text{DNA:Ht} \overset{K_{Ht2}[Ht]}{\rightleftharpoons} \text{DNA:Ht}_2$$

when the Ht concentration is twice that of the hexadecamer (Figure 1) and that the fluorescence intensity increases linearly upon increase of [Ht] up to twice the hexadecamer concentration. Iteration *a posteriori* for the fluorescent contribution of each Ht species to the total fluorescence determined that DNA:Ht contributes half as much fluorescence as DNA:Ht_2 (eq 1). The

$$\sum\Phi = \Phi_1[\text{DNA:Ht}] + \Phi_2[\text{DNA:Ht}_2] \qquad (1)$$

$$\text{where } \Phi_2 = 2\Phi_1$$

constant $\sum\Phi$ is defined as the fluorescence of 5.0×10^{-9} M hexadecameric duplex d(GGCGCAAATTTGGCGG)/d(CCGCCAAATTTGCGCC) saturated with Ht. The equilibrium constants of eqs 2a and 2b follow from Scheme I and, when

$$K_{Ht1} = [\text{DNA:Ht}]/[\text{DNA}][\text{Ht}] \qquad (2a)$$

$$K_{Ht2} = [\text{DNA:Ht}_2]/[\text{DNA:Ht}][\text{Ht}] \qquad (2b)$$

combined with the material balance of the total DNA concentration (DNA_T) in eq 3a, provide eqs 3b and 3c. Upon

$$\text{DNA}_T = [\text{DNA}] + [\text{DNA:Ht}] + [\text{DNA:Ht}_2] \qquad (3a)$$

$$= [\text{DNA:Ht}]\{1/K_{Ht1}[\text{Ht}] + 1 + K_{Ht2}[\text{Ht}]\} \qquad (3b)$$

$$= [\text{DNA:Ht}_2]\{1/K_{Ht1}K_{Ht2}[\text{Ht}]^2 + 1/K_{Ht2}[\text{Ht}] + 1\} \qquad (3c)$$

rearrangement, eqs 3b and 3c yield the concentration of each fluorescent species in terms of DNA_T (eqs 4a and 4b, respectively).

$$[\text{DNA:Ht}] = \text{DNA}_T\frac{K_{Ht1}[\text{Ht}]}{1 + K_{Ht1}[\text{Ht}] + K_{Ht1}K_{Ht2}[\text{Ht}]^2} \qquad (4a)$$

$$[\text{DNA:Ht}_2] = \text{DNA}_T\frac{K_{Ht1}K_{Ht2}[\text{Ht}]^2}{1 + K_{Ht1}[\text{Ht}] + K_{Ht1}K_{Ht2}[\text{Ht}]^2} \qquad (4b)$$

Combining eq 1 with eqs 4a and 4b affords eq 5, which relates

$$F = \sum\Phi K_{Ht1}[\text{Ht}]\frac{0.5 + K_{Ht2}[\text{Ht}]}{1 + K_{Ht1}[\text{Ht}] + K_{Ht1}K_{Ht2}[\text{Ht}]^2} \qquad (5)$$

the dependence of the fluorescence (*F*) on the concentration of

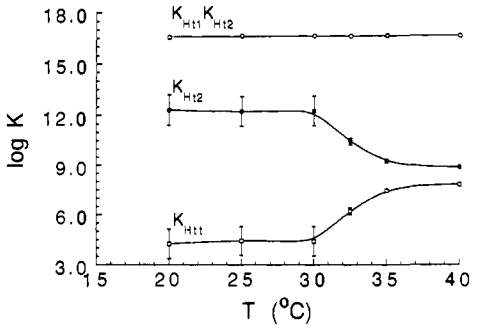


Figure 2. The temperature dependence of the logarithm of the various equilibrium constants for the complexing of Hoechst 33258 by 5.0×10^{-9} M hexadecamer duplex at pH 7.0. The lines connecting the data points for K_{Ht1} and K_{Ht2} are interpolations only. The error bars indicate calculated standard deviations for computer-generated fits of the data points at that temperature with two different iterative nonlinear least-squares routines. The line through the $K_{Ht1}K_{Ht2}$ data points is a linear least-squares fit (correlation coefficient $R = 0.92$).

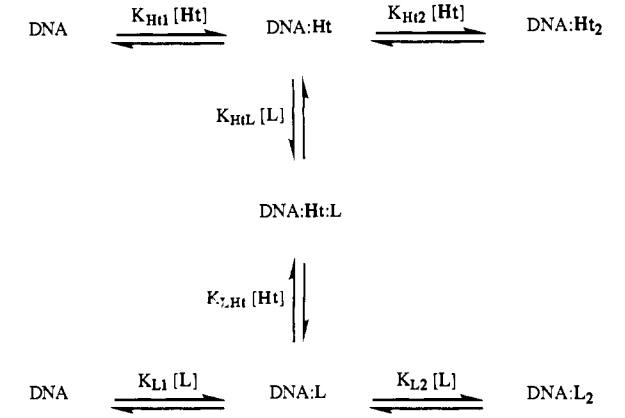
Ht. The theoretical curve that best fits eq 5 to the experimental points is shown in Figure 1 as a plot of F vs [Ht]. From the best solutions to this equation, the logarithms of the first and second association constants for Ht binding to the hexadecamer (log K_{Ht1} and log K_{Ht2}) were determined to be 7.5 and 9.3, respectively, while $\sum\Phi$ was calculated as 90 (arbitrary fluorescence units).

The temperature dependence of the associations of the hexadecamer and Hoechst 33258 (Ht) is depicted in plots of log K vs temperature (°C) in Figure 2 (where $K = K_{Ht1}$, K_{Ht2}, or $K_{Ht1}K_{Ht2}$). In the plots where $K = K_{Ht1}$ or K_{Ht2} there are three distinct regions: (1) a linear region with a slope of 0 below 30 °C characterized by log $K_{Ht1} = 4.4$ and log $K_{Ht2} = 12.3$; (2) a sharp change (within 5 °C) in slope with a critical temperature at ~32 °C where log $K_{Ht1} = 6.2$ and log $K_{Ht2} = 10.4$; and (3) a second region with a slope of 0 above 35 °C in which log $K_{Ht1} = 7.5$ and log $K_{Ht2} = 9.3$. Throughout this temperature range, the value of log $K_{Ht1}K_{Ht2}$ remains constant at 16.8. The maximum fluorescence decreases in a linear manner as the temperature is varied from 20 to 40 °C (data not shown).

The association constants for the binding of 2 and Dm with the oligomer d(GGCGCAAATTTGGCGG)/d(CCGCCAAATTTGCGCC) were determined by the competition of the fluorescent dye Ht with **2** and with Dm for the A_3T_3 minor groove binding site. The association of Ht with the hexadecamer was monitored by the increase in fluorescence intensity as the prebound nonfluorescent ligands **2** and Dm were displaced. It is reasonable to assume that if two Ht molecules can bind to one duplex hexadecamer molecule (Scheme I), then two molecules of L (=**2** or Dm) should be able to bind in a like manner. In addition, during the course of titration with Ht, the possibility of the hexadecamer simultaneously binding both an L and an Ht molecule must be considered as depicted in Scheme II. These assumptions are supported by the following observations. The plots of F vs Ht in the presence of **2** and Dm reveal an approximately linear increase in fluorescence that breaks to a plateau shortly after two Ht molecules per hexadecamer have been added (Figure 3). At this point, the fluorescence slowly increases until it reaches the same maximum ($\sum\Phi$) found at saturation of dsDNA by Ht in the absence of **2** or Dm. Subsequent calculations are based on the equilibria in Scheme II.

Scheme II and the equations derived from it follow lines of logic similar to those of Scheme I and eqs 1 through 5 for Ht alone. The values of $\sum\Phi$, K_{Ht1}, and K_{Ht2} determined for the maximum fluorescence and association constants of Ht alone complexed to 5.0×10^{-9} M hexadecamer duplex were used in the calculations of all other association constants of this study. Under the conditions of these experiments, the molecules represented by L (=**2** or Dm) and the complexes DNA:L and DNA:L_2 do not fluoresce (data not shown), but the mixed species, DNA:Ht:L,

Scheme II



does appear to fluoresce to the same extent as DNA:Ht (eq 6).

$$\sum\Phi = \Phi_1[\text{DNA:Ht}] + \Phi_2[\text{DNA:Ht}_2] + \Phi_3[\text{DNA:Ht:L}] \quad (6)$$

$$\text{where } \Phi_2 = 2\Phi_1 = 2\Phi_3$$

As with Ht alone (in eqs 2–4), expressions can be derived for each fluorescent species in terms of DNA_T from the material balance and from the individual equilibrium expressions (eqs 7a–7d). The sum of the quantum yields (eq 6) then relates each

$$\text{DNA}_T = [\text{DNA}] + [\text{DNA:Ht}] + [\text{DNA:Ht}_2] + [\text{DNA:Ht:L}] + [\text{DNA:L}] + [\text{DNA:L}_2] \quad (7a)$$

$$[\text{DNA:Ht}] = \text{DNA}_T\{K_{Ht1}K_{LHt}[\text{Ht}]\}/\{K_{LHt} + K_{LHt}K_{Ht1}[\text{Ht}] + K_{LHt}K_{Ht1}K_{Ht2}[\text{Ht}]^2 + K_{LHt}K_{Ht1}K_{HtL}[\text{L}][\text{Ht}] + K_{LHt}K_{L1}[\text{L}] + K_{Ht1}K_{HtL}K_{L2}[\text{L}]^2\} \quad (7b)$$

$$[\text{DNA:Ht}_2] = \text{DNA}_T\{K_{Ht1}K_{Ht2}K_{LHt}[\text{Ht}]^2\}/\{K_{LHt} + K_{LHt}K_{Ht1}[\text{Ht}] + K_{LHt}K_{Ht1}K_{Ht2}[\text{Ht}]^2 + K_{LHt}K_{Ht1}K_{HtL}[\text{L}][\text{Ht}] + K_{LHt}K_{L1}[\text{L}] + K_{Ht1}K_{HtL}K_{L2}[\text{L}]^2\} \quad (7c)$$

$$[\text{DNA:Ht:L}] = \text{DNA}_T\{K_{Ht1}K_{HtL}K_{LHt}[\text{Ht}][\text{L}]\}/\{K_{LHt} + K_{LHt}K_{Ht1}[\text{Ht}] + K_{LHt}K_{Ht1}K_{Ht2}[\text{Ht}]^2 + K_{LHt}K_{Ht1}K_{HtL}[\text{L}][\text{Ht}] + K_{LHt}K_{L1}[\text{L}] + K_{Ht1}K_{HtL}K_{L2}[\text{L}]^2\} \quad (7d)$$

equation to the fluorescence at any given value of [Ht] and [L]. This expression further simplifies with the observation in Scheme II that the relationship of eq 8 must pertain such that elimination

$$K_{Ht1}K_{HtL} = K_{L1}K_{LHt} \quad (8)$$

of K_{LHt} yields eq 9. This latter substitution considerably in-

$$F = \sum\Phi K_{Ht1}[\text{Ht}](0.5 + K_{Ht2}[\text{Ht}] + 0.5K_{HtL}[\text{L}])/\{1 + K_{Ht1}[\text{Ht}] + K_{Ht1}K_{Ht2}[\text{Ht}]^2 + K_{Ht1}K_{HtL}[\text{Ht}][\text{L}] + K_{L1}[\text{L}] + K_{L1}K_{L2}[\text{L}]^2\} \quad (9)$$

creases the rate of convergence and, most importantly, eliminates potential local minima into which the iterative process can fall when there are redundant constants. It is then a simple matter

Table I. Mean Values of the Association and Quenching Constants for Ht and the Ligands **2**, **5a**, **5b**, **5c**, and **Dm** Binding To d(GGCGCAAATTTGGCGG)/d(CCGCCAAATTTGCGCC) (in H_2O, 10 mM phosphate buffer, pH 7.0, and 10 mM NaCl at 35 °C)[a]

ligand	log K_{L1}	log K_{L2}	log $K_{L1}K_{L2}$	log K_{HtL}	log K_{LHt}	Q'
Dm[b]	7.1 ± 0.0	8.9 ± 0.2	16.0	8.8 ± 0.1	9.3 ± 0.1	
2[c]	6.8 ± 0.1	5.8 ± 0.2	12.6	3.4 ± 0.3	4.2 ± 0.2	
5a[b]	8.1 ± 0.3	9.2 ± 0.4	17.3	10.0 ± 0.1	9.5 ± 0.2	0.62 ± 0.19
5b[b]	7.2 ± 0.1	10.1 ± 0.2	17.3	10.0 ± 0.1	10.4 ± 0.1	0.62 ± 0.094
5c[b]	8.2 ± 0.1	9.0 ± 0.1	17.2	9.9 ± 0.0	9.4 ± 0.1	0.60 ± 0.069

[a] For **Ht**, log K_{Ht1} = 7.5 ± 0.0088 and log K_{Ht2} = 9.3 ± 0.013. These constants were calculated from the mean values calculated from two different computer programs for two titrations of the hexadecamer with **Ht**. [b] The standard deviations, σ_n, are from the mean values at 8.0 × 10^{-9}, 1.0 × 10^{-8}, 1.2 × 10^{-8}, and 1.4 × 10^{-8} M ligand. [c] The standard deviations, σ_n, are from the mean values at 5.0 × 10^{-8} and 1.0 × 10^{-7} M **2**.

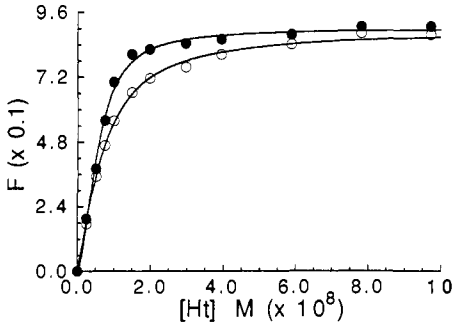


Figure 3. Representative plots of fluorescence (F, in arbitrary units) vs Hoechst 33258 (Ht) concentration at pH 7.0 and 35 °C for 1.0 × 10^{-8} M Dm (- -O- -) and 5.0 × 10^{-8} M **2** (- -●- -) in the presence of 5.0 × 10^{-9} M hexadecamer duplex. The lines which fit the points were computer generated by use of eq 9 and the data in Table I. Additional plots at different concentrations of Dm and **2** have been omitted, since their extreme similarity to the above data would confuse the data presented and would not add significant additional information.

to calculate K_{LHt} from the computer-iterated constants and eq 8. Representative computer optimized fits of the data points (F vs [Ht] titrant) to eq 9 at constant concentrations of **2** and Dm are provided in Figure 3. As can be seen in this figure, the fluorescence rapidly increases in an essentially linear manner from 0 to ~4 molar equiv of Ht to hexadecamer. At this point, there is a break in the direction of the slope from a positive value to zero. The mean calculated values of log K_{L1}, log K_{L2}, log K_{HtL}, and log K_{LHt} for **2** and Dm are included in Table I.

Equilibrium constants for association of the dien-microgonotropens (5a, 5b, and 5c) with the hexadecamer d(GGCGCAAATTTGGCGG)/d(CCGCCAAATTTGCGCC) were determined in a manner analogous to that used for those of **2** and Dm. While the treatment described in the previous section works well in the cases of **2** and Dm, which have what appear to be constant fluorescent maxima (denoted by $\Sigma\Phi$), a complexity is present in the Ht titration of the dien-microgonotropens. In the titration of the hexadecamer with Ht in the presence of **5a**, **5b**, or **5c**, the fluorescence intensity increases rapidly through the first 1 or 2 equiv of Ht to hexadecamer added. The plots of F vs [Ht] then change to show gentler slopes which have not leveled off to a maximum fluorescence by the highest concentrations of Ht used [20-fold excess of Ht over hexadecamer (Figure 4a–c)]. Clearly, the characteristic that distinguishes the hexadecameric complexation of Ht, **2**, and Dm from **5a**, **5b**, and **5c** is the dien $[(CH_2)_nN\{(CH_2)_3N(CH_3)_2\}_2]$ substituent on **5a**, **5b**, and **5c**.

Two experiments were conducted to determine if the triamine side chain of **5a**,**b**,**c** quenches the fluorescence of Ht when in the presence of the complexes DNA:Ht:L and DNA:Ht_2. Aliquots of a $CH_3N\{(CH_2)_3N(CH_3)_2\}_2$ solution were added to a solution containing the hexadecameric duplex plus Ht in the ratio of 1:2. Fluorescence intensity was found to be independent of $[CH_3N\{(CH_2)_3N(CH_3)_2\}_2]$ (Figure 5). The titration with $CH_3N\{(CH_2)_3N(CH_3)_2\}_2$ was repeated with a solution containing a 1:1:1 ratio of hexadecamer, Ht, and **5c**. Again, the fluorescence intensity was found to be independent of $[CH_3N\{(CH_2)_3N(CH_3)_2\}_2]$ (Figure 5). This shows that the polyamine moiety alone, or as

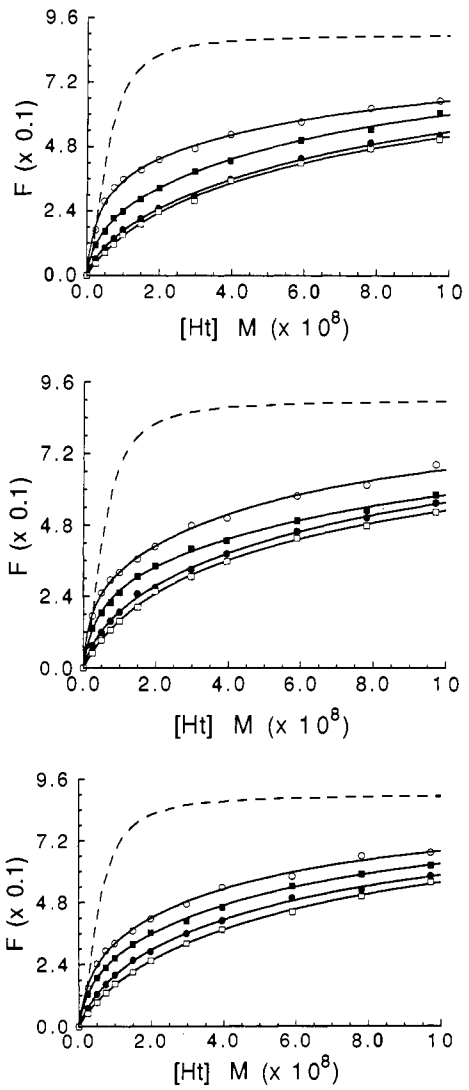


Figure 4. Plots of fluorescence (F, in arbitrary units) vs Hoechst 33258 (Ht) concentration at pH 7.0 and 35 °C for (a, top) **5a**, (b, middle) **5b**, and (c, bottom) **5c** at 8.0 × 10^{-9} M (- -O- -), 1.0 × 10^{-8} M (- -■- -), 1.2 × 10^{-8} M (- -●- -), and 1.4 × 10^{-8} M (- -□- -) in the presence of 5.0 × 10^{-9} M hexadecamer duplex. The lines which fit the points were computer generated by use of eq 10 and the data in Table I. The theoretical curve for 5.0 × 10^{-8} M **2** (- - -) is presented with each set of curves and data points as a reference.

the dien substituent of the dien-microgonotropens **5a**,**b**,**c** does not behave as a *biomolecular quencher* of fluorescence (at least over the concentration range of this study).

To account for the lessened emission of the DNA:Ht:L complex (with L = **5a**, **5b**, or **5c**) compared to the DNA:Ht and DNA:Ht_2 complexes, we attenuated the fluorescence quantum yield of the species DNA:Ht:L by multiplying the term Φ_3 by Q' (eq 10). The best value of Q' determined by computer iteration is ~0.62. Equation 10 allows the calculation of the mean values of log K_{L1}, log K_{L2}, log K_{HtL}, log K_{LHt}, and Q' for **5a**, **5b**, and **5c** (Table I).

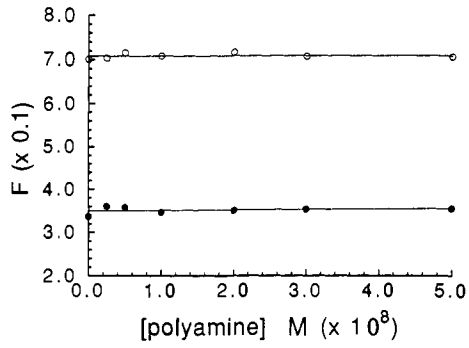


Figure 5. Plot of fluorescence (F, in arbitrary units) vs bis[3-(dimethylamino)propyl]methylamine concentration at pH 7.0 and 35 °C for solutions of 5.0×10^{-9} M hexadecamer duplex, Ht, and **5c** (- -●- -) and of 5.0×10^{-9} M DNA and 1.0×10^{-8} M Ht (- -○- -).

$$F = \sum \Phi K_{Ht1}[Ht](0.5 + K_{Ht2}[Ht] + 0.5K_{HtL}[L]Q')/\{1 + K_{Ht1}[Ht] + K_{Ht1}K_{Ht2}[Ht]^2 + K_{Ht1}K_{HtL}[Ht][L] + K_{L1}[L] + K_{L1}K_{L2}[L]^2\} \quad (10)$$

The fitting of eq 10 to the experimental points of the plots of F vs [Ht] titrant for the hexadecamer in the presence of **5a**, **5b**, and **5c** is shown in Figure 4.

Discussion

The complementary hexadecamers d(GGCGCAAATTTGGCGG) and d(CCGCCAAATTTGCGCC) were chosen for their A+T-rich tracts that make the interior of the constructed dsDNA very similar to previously studied lexitropsin-binding sites[13,15,20,21] and for the G+C-rich termini, which help stabilize the duplex to its 56 °C melting temperature. The end sequences of each hexadecamer were specifically designed to be noncomplementary in order to prevent hairpin structures from being formed.[22] If hairpin structures were to form, the actual concentration of dsDNA would not be known and accurate binding constants could not be determined.

Ethidium bromide fluorescence increases by 21-fold when it intercalates between the base pairs of dsDNA.[23] The release of ethidium bromide upon competitive binding with a minor groove binding agent results in a decrease in the fluorescence. Its ease of use in binding competition experiments with DNA has made ethidium bromide popular for this purpose.[12c,24] There are major disadvantages, however, in the use of this technique. Decrease in fluorescence is generally quantified as the C_{50} value, the concentration of competitive binder at which the fluorescence decreases to 50% of its maximum. A C_{50} value often has little meaning outside the context of a given set of experiments. Only in special cases, when the second agent binds noncompetitively with linear displacement of prebound ethidium bromide, can the C_{50} data permit the accurate determination of the DNA-bound agent concentration. This is a requirement for the accurate measurement of binding constants by methods such as Scatchard plots.[12c,25] Since ethidium bromide is an intercalation agent, its complexation by DNA alters the latter's three-dimensional structure. An additional shortcoming in the use of ethidium bromide release to determine binding constants with DNA is that the ethidium bromide molecules in the vicinity of complexation may be displaced but the nearby sites will remain intercalated with ethidium bromide such that the decrease in fluorescence is not marked. This is not ideal. Problems with the use of UV/visible spectrophotometry in determining equilibrium constants for the complexation of tripyrrole peptides to double-stranded oligomers are provided in the Results.

Equilibrium Binding Constants of Hoechst 33258 (Ht) at 35 °C. In order to study the association of a sequence-selective minor groove binding agent *via* a competition method, a suitable compound which fluoresces upon binding in the minor groove at a similar or the same sequence should be chosen. Instead of using the agent of interest as the competitor, as in the ethidium bromide experiments, the fluorescent compound could be a competitor titrant. This allows an increase in the observable (fluorescence in this case) to be related to the concentration of the competitor compound. Although distamycin has been reported to have fluorescence properties when complexed to calf thymus and poly(dA-dT) dsDNA,[19] the hexadecamer complexes DNA:L and DNA:L_2 did not fluoresce (where L = **2**, **5a**, **5b**, **5c**, or distamycin). The minor groove binder Hoechst 33258 is quite fluorescent when bound to DNA. Suzuki demonstrated the utility of Hoechst 33258 displacement in binding studies of the repeating peptide motif SPKK with salmon sperm dsDNA.[26] For A+T-rich dsDNA tracts, Hoechst 33258 is ideal for use as a fluorescent competitor.

We have found only one noteworthy report in the literature of Hoechst 33258 binding cooperatively to dsDNA.[27] In that case, the oligomer $d(CTTTTGCAAAAG)_2$ had two distinct and symmetric A+T-rich sites. A dsDNA:Ht_2 complex was detected before 1 equiv of Ht had been added to the DNA, indicating that binding of two Ht was occurring in a positively cooperative manner. The ^1H NMR procedure employed allowed determination that one Ht was present in each of the two sites but did not allow the determination of either of the association constants. In the present study, multiple binding is obvious with the observation of fluorescence intensity saturation at a ratio of 1:2 hexadecamer duplex to Ht (Figure 1). Cooperativity is evident from the values of the first and second association constants determined from the titration data and eq 5 (Table I). Because the hexadecamer in the present study has only one A+T-rich region, both Ht molecules must bind to a single site. Our determined equilibrium constants for complexation of Ht to hexadecameric dsDNA ($\log K_{Ht1} = 7.5$ and $\log K_{Ht2} = 9.3$) are reasonable. The average of the logarithms of the first and second association constants, 8.4, compares favorably with the previously reported value of ~8.6 per binding site for Ht with oligomeric dsDNA and poly(dA-dT)·poly(dA-dT)[14c] and is a little higher than another reported "average" value of ~7.8 per binding site for poly(dA-dT)·poly(dA-dT).[12e]

Temperature Dependence of Equilibrium Constants for Complexation of Hoechst 33258 (Ht). Several interesting features can be seen in Figure 2. At temperatures greater than 35 °C, the first and second equilibrium binding constants for Ht binding to the hexadecamer differ by approximately 2 orders of magnitude. An essentially symmetric and dramatic change in the equilibrium binding constants can be seen between 35 and 30 °C in which K_{Ht1} decreases and K_{Ht2} increases. The temperature-independent association constants below 30 °C indicate continuing positive cooperativity but at a much greater degree than the cooperativity found above 35 °C. This suggests some temperature-dependent conformational change. An explanation becomes apparent from the observation that $\log K_{Ht1}K_{Ht2}$ is constant from 20 to 40 °C even though the values of $\log K_{Ht1}$ and $\log K_{Ht2}$ exhibit substantial changes. From eq 11, the value of the product $K_{Ht1}K_{Ht2}$ will be

$$K_{Ht1}K_{Ht2} = [DNA:Ht_2]/[DNA][Ht]^2 \quad (11)$$

(20) Pelton, J. C.; Wemmer, D. E. *J. Am. Chem. Soc.* **1990**, *112*, 1393.
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(22) (a) Wu, T.; Orgel, L. E. *J. Am. Chem. Soc.* **1992**, *114*, 317. (b) Chen, X.; Burrows, C. J.; Rokita, S. E. *J. Am. Chem. Soc.* **1992**, *114*, 322.
(23) LePecq, J.-B.; Paoletti, C. *J. Mol. Biol.* **1967**, *27*, 87.
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(25) Baguley, B. C.; Denny, W. A.; Atwell, G. J.; Cain, B. F. *J. Med. Chem.* **1981**, *24*, 170.
(26) Suzuki, M. *EMBO J.* **1989**, *8*, 797.
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a constant if the conformations of DNA and DNA:Ht_2 either are temperature independent or change in an identical manner with temperature. This means that the temperature dependence of K_{Ht1} and K_{Ht2} reflects a change in conformation of the DNA:Ht_1 species.

Equilibrium Binding Constants for Compound 2 and Distamycin (Dm) at 35 °C. As can be seen in Table I, the first and second equilibrium constants for association of Dm and the hexadecameric dsDNA are similar in relative magnitude to the comparable constants for the binding of Ht to the same binding site. The average of the logarithms of the first and second association constants $[(\log K_{L1} + \log K_{L2})/2]$ compares favorably (8.0) with a previously reported calorimetrically determined "average" value of 8.14 for the complexation of Dm to the homopolymer duplex poly(dA)·poly(dT).[28] The association constants for the mixed fluorescence species, K_{HtL} and K_{LHt} of Scheme II, are similar in magnitude to K_{L2} and K_{Ht2}, respectively. This indicates that once a single Dm or Ht molecule is bound in the minor groove, the addition of either a second Dm or Ht molecule is more facile than that of the first (positive cooperativity to form the DNA:Ht_2, DNA:L_2, or DNA:Ht:L complex). In the formation of complexes containing both Ht and Dm, the equilibrium constant for the formation of the mixed complex is dependent upon which species, Ht vs Dm, binds first. Thus, the value of K_{HtL} when L is distamycin (6×10^8 M^{-1}) is smaller than K_{LHt} (2×10^9 M^{-1}). From ^1H NMR titration studies, Pelton and Wemmer presented evidence that a 1:2 d(CGCAAATTTGCG)$_2$ to distamycin complex formed prior to the addition of one full equivalent of distamycin.[20] This can now be explained by the large difference in log K_{L1} (7.1) and log K_{L2} (8.9) for Dm found in the present study. Since the Ht constants K_{Ht1} and K_{Ht2} and the Dm constants K_{L1} and K_{L2} are alike, we suspect that a DNA:Ht_2 complex should also be seen in the ^1H NMR spectrum with an appropriate A_3T_3 dsDNA binding site.

Due to the low affinity of **2** for dsDNA, higher concentrations (as compared to those of **5a**, **5b**, **5c**, and Dm) were required in order to compete effectively with the Hoechst titrant. The value of K_{L1} for **2** is only slightly lower than that for Dm, but K_{L2} for **2** is over 3 orders of magnitude lower than K_{L2} for Dm. Unlike the cases of Ht and Dm, binding of **2** reflects anticooperativity, since the association of the first molecule decreases the affinity for the binding of the second ($K_{L2} \ll K_{L1}$). The overall binding ability is given by log $K_{L1}K_{L2}$. This value is smaller for **2** than for Dm mainly due to the fact that $K_{L2} \ll K_{L1}$ for **2**. The structural changes in the conversion of Dm → **2** are (i) the replacement of a positively charged amidine by a $CH_2N(CH_3)_2$ substituent [which should be protonated at the pH value of this study (pH 7.0)][2] and (ii) substitution of an acetyl for a formyl group at the amino terminus. It is known that the amidine substituent of Dm forms bifurcated hydrogen bonds to the nucleotide base acceptors on the floor of the minor groove.[13] Presumably, the minor groove does not accommodate the protonated $CH_2N(CH_3)_2$ substituent as well as it accommodates the amidine. In the following paper in this issue, we describe the structure of a dodecamer duplex:**5c** complex. This may be a special case, but it is probably worth noting here that the $CH_2N(CH_3)_2$ moiety of **5c** is located outside or on the outer edge of the minor groove.[5]

Equilibrium Binding Constants of Dien-Microgonotropens at 35 °C. The binding of **5b** to our hexadecamer exhibits greater cooperativity than does that of **5a** or **5c**. The first association constants for **5a** and **5c** are roughly 1 order of magnitude greater than the K_{L1} for Dm while the second association constant is nearly the same as K_{L2} for Dm. Hence, the average binding constants $[(\log K_{L1} + \log K_{L2})/2]$ for **5a** and **5c** are slightly higher than that for Dm (Table I). While the logarithms of K_{L1} and K_{L2} for both **5a** and **5c** ranged from 8.1 to 9.2, the logarithms of the first and second association constants for **5b** were 7.2 and 10.1, respectively. With **5b**, the nearly 3 orders of magnitude that separates K_{L1} and K_{L2} indicates a very strong positive cooperativity effect. The relatively weak first binding of **5b** is comparable to the initial binding of **2**. This is compensated, however, by the very high second association constant. Clearly, the equilibrium complexation of **5a** and **5c** differs from that of **5b**.

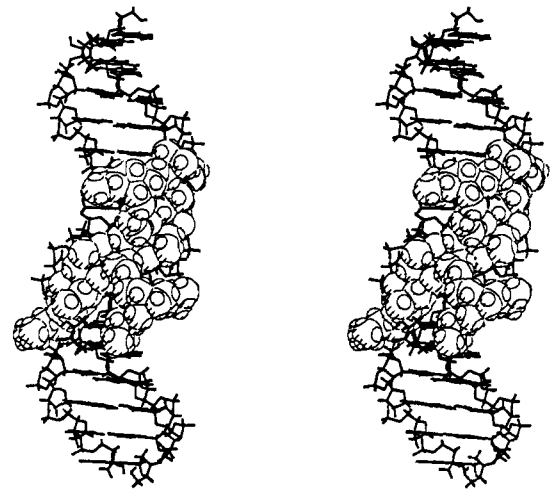

Figure 6. Stereoview of a plausible model of two molecules of **5c** binding to a single A_3T_3 binding site of the hexadecamer. The model was generated on a Silicon Graphics (Mountain View, CA) Iris 4D/340GTX workstation in the graphics program QUANTA version 3.2.3 (Polygen/Molecular Simulations, Waltham, MA). A QUANTA-generated hexadecamer d(GGCGCAAATTTGGCGG)/d(CCGCCAAATTTGCGCC) was overlapped onto Wemmer's NMR structure of a 1:2 complex of d(CGCAAATTTGCG)$_2$ and distamycin.[20] Next, two molecules of **5c** [with the conformation established in the following paper of this issue for the 1:1 d(CGCAAATTTGCG)$_2$:**5c** complex] were overlappped with the distamycin molecules in Wemmer's structure. The dodecamer and distamycins were then removed to yield the structure shown here. The tripyrrole peptide portions of the two **5c** molecules are antiparallel, and the dien polyamine portion extends to the major groove with the amino functions juxtaposed to phosphodiester linkages.

A related difference in the dien-microgonotropens' (**5a**, **5c** vs **5b**) interactions with DNA is seen in the electrophoretic mobilities of a series of DNA restriction fragments, as reported in the previous paper in this issue.[4] The DNA fragments demonstrate a linear decrease in mobilities with increasing concentration of **5a** and **5c**. At low concentrations of **5b**, the change in the apparent length of the DNA fragments with an increase in [**5b**] is more marked than are the cases with an increase in either [**5a**] or [**5c**]. At higher concentrations of **5b**, the increase in apparent DNA length begins to level off, as if reaching a saturation in **5b**'s ability to change the electrophoretic mobilities of DNA. The differences in the apparent sizes of DNA fragments with increasing concentration of dien-microgonotropens can be explained by differences in the binding behaviors of **5a**, **5c** and **5b** discussed above. The small degree of cooperativity seen in the DNA binding of **5a** and **5c** facilitates a linear increase in apparent DNA length with increasing concentrations of these ligands. The large positive cooperativity in the binding of **5b** to the duplex hexadecamer indicates that DNA-binding sites should quickly saturate with **5b**.

The value of log $K_{L1}K_{L2}$ for **5b** is very similar to those of **5a**, **5c**, Dm, and Ht (Table I). The increased affinity of **5a**, **5b**, and **5c** for our hexadecameric dsDNA relative to Ht, Dm, and in particular **2** is due to the protonated polyamine moiety $(CH_2)_nN\{(CH_2)_3N(CH_3)_2\}_2$, which can reach up and out of the minor groove to firmly complex the phosphate groups. A model structure of two molecules of **5c** in the minor groove of the hexadecamer is provided in Figure 6.

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Fluorescence Quenching of Ht by Dien-Microgonotropens. Two main types of energy quenching commonly occur in solution: kinetic and static. In kinetic quenching, a donor in an excited state and a potential acceptor of energy collide with energy transfer to the acceptor rather than with energy being emitted as a photon as the excited state decays.[29] If the donor and acceptor are held together in some manner and then the donor is excited, static quenching can occur by the direct transfer of energy to the acceptor.[29] Within each of these two broad classes of quenching, knowledge of various subsets is continuing to expand. Studies of DNA interactions with small organic molecules tend to focus on static quenching as determined through the observation of fluorescence emissions of an excited chromophore and through flash photolysis techniques. Brun and Harriman measured electron transfer from the DNA intercalators ethidium bromide and acridine orange to *N,N'*-dimethyl-2,7-diazapyrenium dichloride (DAP^{2+}) with laser flash photolysis techniques.[30] They were able to demonstrate that intervening DNA base pairs could attenuate the coupling of electron transfer between intercalating donor and acceptor molecules. Porphyrins have been observed to quench the fluorescence of DNA intercalated with ethidium bromide while at distances up to 25–30 Å from the ethidium bromide binding site. The evidence indicates that energy transfer through the DNA matrix permits "extensive electronic communication between bound drug molecules" without photon emission.[31] In fluorescence emission studies, Kumar and Asuncion reported observations of the excitation of the absorption bands in A+T-rich DNA (but not from G:C base pairs) leading to energy transfer directly from DNA bases to intercalated 9-anthracenemethylamine hydrochloride (AMAC).[32] A mechanism different from those described above appears to apply in our situation with **5a**, **5b**, and **5c**.

The results seen in Figure 5 show that there is no kinetic quenching by the polyamine $CH_3N\{(CH_2)_3N(CH_3)_2\}_2$ over the concentrations employed (2×10^{-9} to 5×10^{-8} M), such that the polyamine moieties of **5a,b,c** could not be involved in *kinetic* fluorescence quenching in the binding titrations with Ht. The quenching must, therefore, be static, intercomplex quenching. The binding of **5a,b,c** in the minor groove adjacent to a photoexcited **Ht**† molecule allows an intracomplex quenching by the dien polyamine moiety of the dien-microgonotropens. The effective molarity of the polyamine in relation to Ht† is quite high, and quenching is efficient. Amine free bases are known to be effective quenching agents,[33] and at the pH employed (7.0) at least one of the amines in the $(CH_2)_nN\{(CH_2)_3N(CH_3)_2\}_2$ group (pK_a 6.8, 9.6, and 12.2)[2] of **5a**, **5b**, or **5c** will be partially deprotonated. As expected for static quenching, the quenching term (Q') in eq 10 is not dependent upon concentration over the concentration range examined. Furthermore, the mean value of Q' for each of the compounds is almost exactly the same (Table I). Therefore, the quenching efficiencies of **5a,b,c** are not dependent on the length (n = 3, 4, or 5) of the $(CH_2)_n$ linker (Table I). Since Q' indicates the fraction that the mixed complex hexadecamer:Ht:L fluoresces relative to the hexadecamer:Ht complex, subtracting the value of Q' from 1 and multiplying it by 100 yields the percent quenching that is occuring in the DNA:Ht:L complex. For **5a**, **5b**, and **5c**, this value is ~38%.

Conclusions on Cooperative Binding. Our investigation represents the first of its kind in which equilibrium constants have been determined for the complexation of both one and two minor groove binding agents to a short duplex DNA oligomer with a single binding site [d(GGCGCAAATTTGGCGG)/d(CCGCCAAATTTGCGCC)]. Previous investigators have used spectroscopies and other techniques to note that 1:2 dsDNA to distamycin complexes are formed and are present in solution under conditions where cooperativity in the binding of distamycin (Dm) must be presumed.[12,14b,15a,20,27,35] We have been able to confirm that Dm does indeed bind cooperatively to a single minor groove binding site and that Hoechst 33258 (Ht) can also bind cooperatively to the same sequence. The first and second association constants for Dm and Ht have been determined as have the equilibrium binding constants for the new dien-microgonotropens **5a**, **5b**, and **5c** and the previously described[35] but uncharacterized lexitropsin **2**. The calculated equilibrium constants for the formation of the DNA:L_2 or DNA:Ht_2 species ($K_{L1}K_{L2}$ or $K_{Ht1}K_{Ht2}$) decrease in the order **5a** ~ **5b** ~ **5c** > Ht > Dm ≫ **2**. The temperature dependencies of the first and second equilibrium constants for Ht indicate a critical temperature of ~32 °C for a marked change in the conformation of the hexadecameric dsDNA 1:1 complex with Ht.

Crystals of oligomeric dsDNA with Dm or Ht have been prepared for structure elucidations by vapor diffusion cocrystallization of the agent and oligomeric dsDNA.[13,36] By X-ray crystallography, the structures were 1:1 complexes only. This finding may be due to the lessened solubility and greater crystallinity of the 1:1 complex or to the restricted motion of the oligomer in the crystalline state, which would disfavor the formation of 1:2 oligomer to agent complexes.

Acknowledgment. This work was supported by grants from the Office of Naval Research and the National Science Foundation.

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