Molecular Recognition between Oligopeptides and Nucleic Acids: Influence of van der Waals Contacts in Determining the 3'-Terminus of DNA Sequences Read by Monocationic Lexitropsins

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Abstract: The binding specificity and dynamic properties of a "truncated" lexitropsin **5**, which has one methylene group at the carboxyl-terminus, and a "normal" lexitropsin **8**, which has two methylene groups at the C-terminus, to the self-complementary decadeoxyribonucleotide d-$[CGCAATTGCG]_2$ are deduced by high-field ^1H NMR studies. Selective chemical shift changes of AH8(5), TH6(6,7), and GH8(8) locate the "truncated" lexitropsin **5** on the ATTG sequence. In contrast the most marked chemical shift changes for the "normal" lexitropsin **8**:DNA complex are observed between AH8(4) and TH6(7), which indicates that the ligand **8** is bound to the AATT site on the DNA. Intermolecular NOEs between the lexitropsin amide, formyl, pyrrole, and prochiral methylene protons and the imino protons GH8(8) and AH2(4,5) confirmed the location and orientation of the lexitropsins on the DNA. On the basis of the NOE results, models for the 1:1 complex of lexitropsin **5** and **8** with the decadeoxyribonucleotide are constructed. The conformations of the DNA in both complexes, deduced from NOE measurements, are in the B-form, which is similar to that of the free DNA. These results clearly show that the van der Waals interactions between the methylene group at the C-terminus of lexitropsins similar to **8** are responsible for reading the 3'-terminal AT base pair. The rate of exchange of **5** between the two equivalent ATTG sites is $\approx 185\ s^{-1}$ at 294 K with $\Delta G^{\ddagger} \approx 58 \pm 5$ kJ mol^{-1}, whereas the exchange of **8** between the two equivalent AATT sites is fast ($>180\ s^{-1}$) even at 277 K. The concentration effects on the exchange of lexitropsins **5** and **8** between the two equivalent sites on the DNA indicate that they proceed predominantly via an intermolecular mechanism and intramolecular process, respectively.

The sequence specific binding of proteins and polypeptides to nucleic acids is a fundamental process in the molecular biology of gene control and expression.[1] Consequently the determination of the structural and dynamic components of such sequence specific molecular recognition is of considerable current interest. In this regard the antitumor antibiotic oligopeptides netropsin and distamycin have received much attention as models of groove and sequence specific DNA binding ligands.[2] Biochemical evidence suggests that these agents exert their biological activities by blocking the template function of DNA by binding to $(AT)_n$ sequences in the minor groove of double helical DNA.[3] The firm and sequence specific binding of these agents to DNA is a net result of hydrogen bonding,[2a,4] electrostatic interaction,[5] and van der Waals contacts[2a,4] between the drug and DNA.

Recently we reported the complete ^1H NMR analysis of the 1:1 complex of a prototype lexitropsin (or information reading oligopeptide) G^+A-Py-Im-CH_2CH_2-Am^+[6] (in which G^+A, Py, Im, and Am^+ represent the guanidiniumacetyl, pyrrole, imidazole, and amidinium groups, respectively) and the decadeoxyribonucleotide d-$[CGCAATTGCG]_2$.[7] These initial studies provided a basis for structural comparison of the strict A.T specific binding of the neutral products netropsin and distamycin[2] and the radically altered sequence recognizing capabilities of the newer lexitropsin.[2a,7] The structural properties of the prototype lexitropsin were comparable with those of the complex of netropsin and the dodecamer d-$[CGCGAATTCGCG]_2$ deduced from X-ray diffraction[3] and ^1H NMR studies,[8] although, as expected, the rates of exchange differed.

The minor and major grooves of DNA have distinct electrostatic properties depending on the base composition. The deepest negative potential well is located in the minor groove in AT rich runs.[5,9] Therefore dicationic lexitropsins or other ligands will have a bias for AT rich sites regardless of the sequence specificity imposed by hydrogen bonding. Accordingly, the guanidiniumacetyl moiety at the N-terminus of netropsin analogues was replaced by an *N*-formyl group giving monocationic lexitropsins.[7,11] In this regard, the ligand formyl-Im-Im-CH_2CH_2-Am^+ is shown by DNase I footprinting on the Hind III/NciI fragment from the pBR322 DNA to be specific for the sequence 5'-CCGT, indicating that the AT bias of netropsin has been overcome.[10] Independent ^1H NMR studies of this lexitropsin when challenged with the sequence d-$[CATGGCCATG]_2$ indicate that it binds preferentially to the 5'-CCAT sequence.[11] Therefore, replacement of the pyrrole group(s) with hydrogen bond accepting heterocycles, such as imidazole, evidently affords compounds that can recognize GC sites by hydrogen bond formation to G-(C2)-NH_2.[2a,4]

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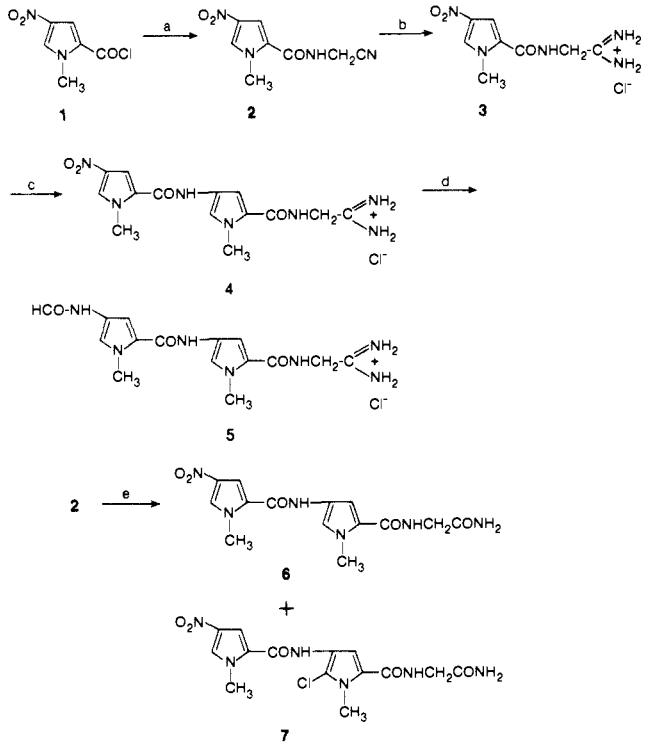
Scheme I[a]



[a] Reaction conditions: (a) H_2NCH_2CN, i-Pr_2EtN; (b) HCl in MeOH, then NH_3 in MeOH; (c) H_2, Pd/C then add **1**; (d) H_2, Pd/C then $CH_3COOCOH$; (e) $SnCl_2$, then NaOH, then add **1**.

It was also deduced from the latter NMR study that the reading of a 3′-terminal AT base pair is presumably dictated by the van der Waals interactions between the methylenes at the C-terminus of the lexitropsin and the DNA. These methylene groups would enter into steric contact with the guanine-NH_2 moiety of a GC base pair, thereby preventing the binding of the C-terminus of netropsin analogues to a GC site and forcing the recognition of an AT pair by default. In order to test this interpretation and to examine the influence of the methylenes at the C-terminus in the molecular recognition of netropsin analogues to DNA, we have designed a "truncated" lexitropsin **5** (with only one methylene group at the C-terminus) for this study. This investigation is supported by comparison with the ^1H NMR analysis of lexitropsin **8**,[12] which has the "normal" ethylene group at the C-terminus. Thus in this paper, we report the structure and dynamics of the 1:1 complexes of lexitropsin **5** and d-[CGCAATTGCG] (complex A), as well as ligand **8** and the same decadeoxyribonucleotide (complex B).

Synthesis

The initial approach to the synthesis of **5** based on literature methods[13a] proved to be unsatisfactory owing to the quite different chemical properties of amidoacetonitrile compared with amidopropionitrile in the natural antibiotics distamycin and netropsin. Arcamone et al. found the nitro group in **2** to be resistant to hydrogenation and employed an unusually large proportion of Pd/C catalyst (1:2 catalyst:compound) and a long reaction time (4 h) to afford a modest yield (53%) of the product of coupling with the acyl chloride **1**. We avoided the inconvenience of poisoning of the catalyst by the amidoacetonitrile in **2** by first converting the nitrile group to an amidine moiety in **3** by a Pinner reaction (see Scheme I).[6] The nitro group in **3** was then readily reduced catalytically and coupled with the acyl chloride **1** to give the dipeptide **4**. Catalytic reduction of the nitro group and reaction with acetic formic anhydride afforded the desired final product **5** in 62% yield.[13b]

Experimental Section

Melting points were determined on a Fisher-Johns apparatus and are uncorrected. The IR spectra were recorded on a Nicolet 7199 FT spectrophotometer, and only the principal peaks are reported.

The ^1H NMR spectra were recorded on Bruker WH-200 and WH-400 spectrometers. FAB (fast atom bombardment) mass spectra were determined on Associated Electrical Industries (AEI) MS-9 and MS-50 focusing high-resolution mass spectrometers. Kiselgel 60 (230–400 mesh) of E. Merck was used for flash chromatography and precoated sheets of silica gel 60F-254 of E. Merck were used for TLC. TLC system: (i) covalent peptidic compounds was chloroform–methanol 9:1; (ii) ionic compounds was methanol with some AcOH.

1-Methyl-4-nitropyrrole-2-carboxamidoacetonitrile (2). Aminoacetonitrile hydrochloride (3.3 g, 35.5 mmol) and diisopropylethylamine (13 mL, 74.7 mmol) were dissolved in anhydrous acetonitrile (60 mL) and cooled to −20 °C. The acyl chloride **1**[12] (6.65 g, 35.3 mmol) in acetonitrile (40 mL) was added. The mixture was filtered through charcoal and the solvent was removed by evaporation. After addition of water the pure compound **2** was collected to give 7.06 g (96% yield): mp 185–186 °C (lit.[13a] mp 188–190 °C); ^1H NMR (DMSO-d_6) δ 3.90 (s, 3 H), 4.25 (d, 2 H), 7.47 (d, 1 H), 8.15 (d, 1 H), 9.07 (t, 1 H); IR ν_{max} (Nujol) 1312, 1375, 1475, 1495, 1525, 1542, 1655, 2248, 3130, 3380 cm^{-1}; mass spectrum, m/z for $C_8H_8N_4O_3$ 208.0595 (−0.5 ppm).

1-Methyl-4-nitropyrrole-2-carboxamidoacetamidine Hydrochloride (3). Compound **2** (1.5 g, 7.2 mmol) was treated with methanol in the presence of HCl at room temperature for 4 h. The product partially precipitated. The solvent was evaporated and the residue was treated with dry ammonia in methanol for 2 h. After evaporation of the solvent the residue was washed with hot acetonitrile to give pure **3** (1.57 g, 83% yield): mp 257 °C dec without melting; ^1H NMR (DMSO-d_6) 3.90 (s, 3 H), 4.13 (d, 2 H), 7.60 (d, 1 H), 8.10 (br s) overlapping with 8.20 (d, 5 H), 9.20 (t, 1 H); IR ν_{max} (Nujol) 1375, 1465, 1495, 1530, 1555, 1642, 1680, 3090, 3120, 3240, 3370 cm^{-1}; mass spectrum, FAB m/z 226 $(M - Cl)^+$. Anal. Calcd for $C_8H_{12}ClN_5O_3$: C, 36.7; H, 4.6; Cl, 13.5; N, 26.7. Found: C, 36.4; H, 4.9; Cl, 13.9; N, 27.0.

1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamidoacetamidine Hydrochloride (4). Compound **3** (930 mg, 3.55 mmol) in methanol (10 mL) was reduced over 200 mg of 5% Pd/C. The catalyst was removed by filtration and the solvent evaporated. The residue was dissolved in water, the solution was cooled to 0 °C, Et-i-Pr_2N (626 μL, 3.6 mmol) was added followed by the acyl chloride **1** (680 mg, 3.6 mmol) dissolved in 5 mL of toluene, and the mixture was shaken for 0.5 h. A yellow precipitate was formed. Toluene was evaporated under reduced pressure and the solid collected. The latter was dissolved in hot water and some impurities were removed by filtration. The filtrate was evaporated to dryness and after addition of ethanol a yellow solid was collected to give pure **4** (1.09 g, 80% yield): mp ~300 °C dec; ^1H NMR (DMSO-d_6) δ 3.84 (s, 3 H), 3.98 (s, 3 H), 4.12 (d, 2 H), 7.02 (d, 1 H), 7.32 (d, 1 H), 7.65 (d, 1 H), 8.20 (d, 1 H), 8.62 (t, 1 H), 8.94 (s, 4 H), 10.40 (s, 1 H); IR ν_{max} (Nujol) 1315, 1445, 1502, 1595, 1645, 1665, 1701, 3120, 3270, 3380, 3420 cm^{-1}; mass spectrum, FAB m/z 348 $(M - Cl)^+$. Anal. Calcd for $C_{14}H_{18}ClN_7O_4$: C, 43.8; H, 4.7; Cl, 9.2; N, 25.5. Found: C, 43.7; H, 4.9; Cl, 9.5; N, 25.2.

1-Methyl-4-(1-methyl-4-(formylamino)pyrrole-2-carboxamido)-pyrrole-2-carboxamidoacetamidine Hydrochloride (5). Compound **4** (200 mg, 0.52 mmol) was hydrogenated in the presence of 50 mg of 5% Pd/C in water. The catalyst was removed by filtration and the water evaporated under reduced pressure. The residue was treated with acetic formic anhydride. After evaporation of the solvent (acetic acid and an excess of anhydride), methanol was added and pure compound **5** was collected (124 mg, 62% yield): mp 295 °C dec; ^1H NMR (DMSO-d_6) δ 3.83 and 3.86 (2 s, 6 H), 4.10 (d, 2 H), 6.92 (d, 1 H), 7.02 (d, 1 H), 7.20 (d, 1 H), 7.28 (d, 1 H), 8.12 (d, 1 H), 8.54 (t, 1 H), 8.90 (d, 4 H), 10.00 (s, 1 H), 10.16 (d, 1 H); UV λ_{max} (Nujol) (H_2O) 240 nm (ϵ 1.75×10^4), 298 nm (ϵ 2.12×10^5); IR ν_{max} 1387, 1445, 1465, 1530, 1585, 1645, 1708, 3040, 3140, 3250, 3350 cm^{-1}; mass spectrum, FAB m/z 346 $(M - Cl)$. Anal. Calcd for $C_{15}H_{20}ClN_7O_3$: C, 47.2; H, 5.3; Cl, 9.3; N, 25.7. Found: C, 47.5; H, 5.5; Cl, 9.0; N, 25.4.

Reduction of Compound 2 with Stannous Chloride and Coupling with Acyl Chloride 1. Compound **2** (31.2 mg, 1.5 mmol) was added to $SnCl_2 \cdot 2H_2O$ (6.75 g, 30 mmol) in 4 mL of concentrated hydrochloric acid at room temperature. The mixture was made alkaline with sodium hydroxide solution upon cooling and was extracted with a mixture of chloroform and acetonitrile (1:1). The extracts were condensed to 25 mL

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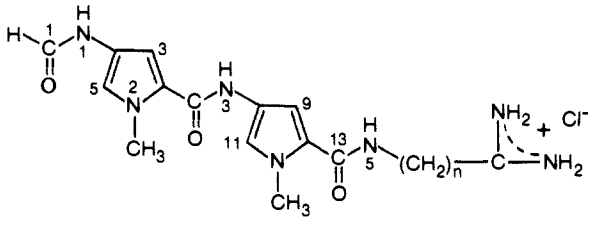
(13) (a) Arcamone, R.; Penco, S.; Monache, F. D. *Gazz. Chim. Ital.* **1969**, *99*, 620. (b) Our expectation that catalytic reduction could be replaced by reduction with $SnCl_2$ in HCl, as we had found for the case of the amidopropionitrile derivatives,[6] was not fulfilled. Although the reduction proceeds readily the nitrile group is simultaneously hydrolyzed affording, after coupling with the acyl chloride **1**, the amides **6** and **7**. In the latter compound simultaneous ring chlorination occurs via a reaction that we have interpreted recently.[6]

Chart I. Structures and Numbering Systems for Lexitropsins **5** and **8** and the Decadeoxyribonucleotide.



5, n=1
8, n=2

5'-	C_1	G_2	C_3	A_4	A_5	T_6	T_7	G_8	C_9	G_{10}	
	I	II	III	IV	V	V	IV	III	II	I	
	G_{10}	C_9	G_8	T_7	T_6	A_5	A_4	C_3	G_2	C_1	-5'

and *i*-Pr_2EtN (267 μL, 1.5 mmol) was added followed by acyl chloride **1** (282 mg, 1.5 mmol). After a time a yellow precipitate was collected. It was washed with water and triturated with hot acetonitrile to give pure compound **6** (122 mg), mp 298 °C dec. From the acetonitrile extract compound **7** was isolated (10 mg), mp 280 °C dec.

1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamidoacetamide (6): ^1H NMR (DMSO-d_6) δ 3.71 (d, 2 H), 3.81 (s, 3 H), 3.98 (s, 3 H), 6.90 (d, 1 H), 7.00 (br s, 1 H), 7.28 (d, 1 H), 7.31 (br s, 1 H), 7.60 (d, 1 H), 8.20 (d) and 8.22 (t, 2 H), 10.30 (s, 1 H); IR δ_{max} (Nujol) 1305, 1380, 1410, 1455, 1490, 1530, 1565, 1632, 1655, 1680, 3130, 3170, 3300, 3400 cm^{-1}; mass spectrum, *m/z* for $C_{14}H_{16}N_6O_5$ 348.1181 (−0.3 ppm).

1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)-5-chloropyrrole-2-carboxamidoacetamide (7): ^1H NMR (DMSO-d_6) δ 3.75 (d, 2 H), 3.82 (s, 3 H), 3.92 (s, 3 H), 7.02 (2s overlapped, 2 H), 7.30 (br s, 1 H), 7.70 (d, 1 H), (8.23 d, 1 H), 8.33 (t, 1 H), 9.88 (s, 1 H); IR δ_{max} (Nujol) 1305, 3375, 1410, 1460, 1515, 1540, 1595, 1632, 1690, 3095, 3120, 3280, 3325, 3432, 3443 cm^{-1}; mass spectrum, *m/z* for $C_{14}H_{15}N_6O_5{}^{35}Cl$ 382.0785 (−1.8 ppm), for $C_{14}H_{15}N_6O_5{}^{37}Cl$ 384.0767 (1.0 ppm).

Lexitropsin **8**, 1-methyl-4-[1-methyl-4-(formylamino)pyrrole-2-carboxamido]pyrrole-2-carboxamidopropionamidine hydrochloride, was prepared with use of the previously published procedure.[12]

The NMR sample of the 1:1 complex of **5** and the decadeoxyribonucleotide d-$[CGCAATTGCG]_2$ (complex A) was prepared by dissolving the DNA (9.60 mg) in a 99.8% D_2O solution (0.4 mL) containing 30 mM potassium phosphate (pH 7.2), 5 mM sodium chloride, and 0.1 mM EDTA. The sample was lyophilized twice from 99.8% D_2O and finally redissolved in 99.996% D_2O (0.4 mL). A fresh stock solution of lexitropsin **1** in the same buffer solution was prepared before the titration experiment. For detecting the exchangeable ^1H NMR signals, the sample was made up in a 9:1 (v/v) H_2O:D_2O solution. The ^1H NMR sample of the 1:1 complex of lexitropsin **8** and d-$[CGCAATTGCG]_2$ (complex B) was prepared with use of the same procedure as shown above.

^1H NMR spectra were obtained at 21 °C with Bruker WM360 and WH400 cryospectrometers, both of which are interfaced with Aspect 2000 computers. The COSY[14] and NOESY[15a] spectra were symmetrized but not apodized. The COSY, NOESY, and 1D-NOE difference[16] experiments were performed in the same way as described in our previous studies.[17] The conditions used for the NMR experiments are given in the figure legends. The NOE experiments performed on complex A and shown in Figure 4 were run at 37 °C so as to minimize spin diffusion. For detecting the exchangeable proton NMR signals, the binomial 1-3-3-1 pulse sequence[18a] was used to suppress the intense HDO signal. Complete assignments for the exchangeable and nonexchangeable ^1H NMR resonances of d-$[CGCAATTGCG]_2$ have been reported.[7]

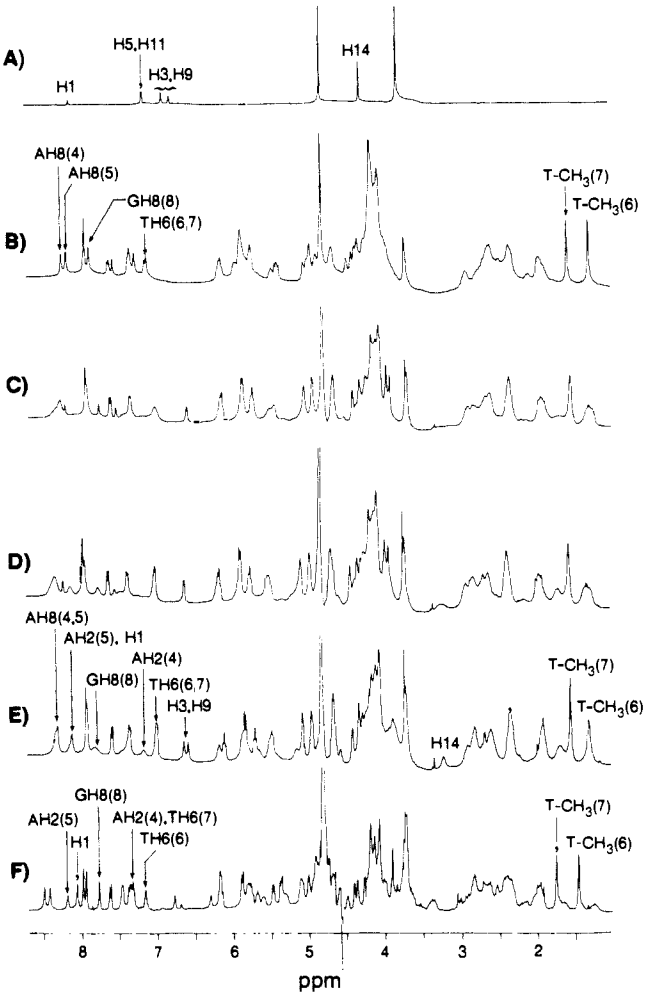


Figure 1. Titration of lexitropsin **5** to d-$[CGCAATTGCG]_2$. Parts A and B correspond to the free lexitropsin and decadeoxyribonucleotide, respectively. Parts C, D, and E represent the titration of 0.3, 0.7, and 1.0 molar equiv of **5** to the DNA. The NMR spectrum for the 1:1 complex of **8** to the decadeoxyribonucleotide is shown in part F.

The computer-generated diagrams of complex A and B were obtained on a Zenith Z-158 microcomputer (Zenith Data Systems, MI) using the molecular graphics program PC model (Academic Press Inc., Toronto) and Arnott's coordinates for B-DNA.[18b]

Results and Discussion

The structures and numbering systems for the self-complementary decadeoxyribonucleotide d-$[C_1G_2C_3A_4A_5T_6T_7G_8C_9G_{10}]_2$ and the oligopeptides **5** and **8** are given in Chart I. The imino protons of the decadeoxyribonucleotide are designated by Roman numerals.

Titration of Lexitropsin 5 to the DNA. The results of the titration of **1** to the oligonucleotide d-$[CGCAATTGCG]_2$ are given in Figure 1C–E, together with the reference spectra of free lexitropsin **1** (Figure 1A), free DNA (Figure 1B), and complex B (Figure 1F).

With increasing proportions of **5** (Figure 1C–E), the ^1H NMR signals undergo chemical shift changes and the lines are broadened. The broadening of the NMR signals is presumably due to the increase in the rotational correlation time[19] and the exchange process of ligand **5** on the DNA. The change in the chemical shifts of selected DNA resonances could be due to the chemical anisotropy of the pyrrole moieties or minor conformational changes of the DNA backbone as a result of drug binding. The absence of exchange signals in the spectrum of complexes A and B (parts E and F of Figure 1, respectively) suggests that the exchange processes are fast on the NMR time scale at 294 K. The dynamics

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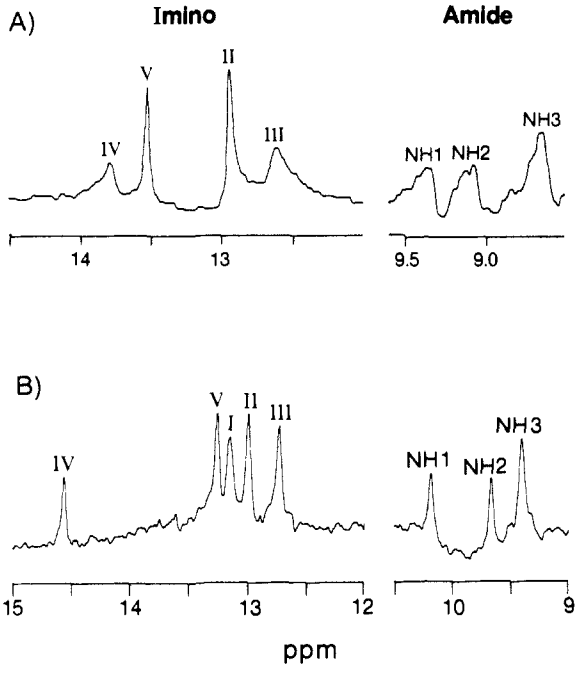

Figure 2. One-dimensional imino and amide proton NMR spectra for complex A at 21 °C (part A) and complex B (part B) at 4 °C. Experimental conditions: sweep width 8000 Hz, and 1000 FIDs were collected for each experiment.

and mechanism of the exchange processes are discussed below.

Assignment of the Imino Protons: Location and Orientation of Lexitropsin 5 on the Decadeoxyribonucleotide d-[CGCAATTGCG]$_2$. The DNA imino and ligand amide proton NMR spectrum of complex A, given in Figure 2A, was recorded in a 9:1 (v/v) H_2O:D_2O solution at 294 K. The spectrum in Figure 2A reveals four imino proton signals (12.61 (s, br), 12.94 (s), 13.52 (s), and 13.79 (s br)) and three amide proton peaks (9.40, 9.10, and 8.65 ppm, all singlets). The imino proton signals were assigned by NOE-difference measurements (data not shown).[20a] Saturation of the signal at 13.52 ppm gave only an NOE peak at 13.52 ppm, thus, it can be ascribed to imino proton V. Since the imino proton signals for AT base pairs are always found at lower field than GC sites,[20b] the signal at 13.79 ppm is assigned to imino proton IV. On the basis of the NOE observed between proton IV and the signal at 12.61 ppm, the latter peak is assigned to proton III. Therefore the remaining imino proton signal at 12.94 ppm can be assigned to proton IV. Imino proton I is not observable at 21 °C, and this could be due to fraying of the termini which is prevalent under these conditions.

The broad imino ^{1}H NMR signals for III and IV, due to the exchange of lexitropsin **5** on the two equivalent sites on the DNA, suggest that the lexitropsin is located on the T(7) and G(8) bases. These results provide the first indication that ligand **5** is bound to the 5′-ATTG-3′ sequence. Variable-temperature imino ^{1}H NMR studies showed that the broad signals for III and IV sharpened as the temperature was raised. These results provide evidence for the exchange of **5** on the two equivalent sites of the DNA. Imino proton II, in the DNA duplex, exhibits exchange to the medium at a rate greater than 200 s^{-1} at 50 °C and as a result the signal is broad.[21] The three remaining imino protons (III, IV, and V) are broad at 60 °C, therefore this cooperative effect provides evidence that lexitropsin **5** is bound to the ATTG sequence.

The imino proton NOE results are also useful for determining the orientation of the lexitropsin on the DNA. Saturation of imino proton V gave NOEs at 8.11 for lexitropsin H1, 9.40 for amide NH1, and 9.10 for NH3. Furthermore, irradiation of proton IV produced NOEs at 6.63, 6.58, and 8.65 ppm for H3, H9, and NH5, respectively. Finally an NOE signal at 8.65 ppm for NH5 is observed when proton III is irradiated. These results confirm the location and orientation of the N- to C-termini of **5** on the 5′-ATTG-3′ sequence in the decadeoxyribonucleotide. A summary of the ^{1}H NMR signals for lexitropsin **5** in its free and complex forms is given in Table I.

Table I. ^{1}H NMR Chemical Shift Assignments of the Nonexchangeable Protons of Lexitropsins **5** and **8** in Complexes A and B, respectively, at 21 °C

proton	lexitropsin	complex A		lexitropsin **8**	complex B	
		δ	$\Delta\delta$		δ	$\Delta\delta$
H1	8.10	8.11	0.01	8.11	8.18	0.07
H3	6.87	6.63	−0.24	6.92	6.68	−0.24
H5	7.14	7.36	0.22	7.18	7.45[a]	0.27
H9	6.77	6.58	−0.19	6.92	6.77	−0.15
H13	7.14	7.36	0.22	7.18	7.46[a]	0.28
H14	4.28	3.23	−1.05	2.62	3.01	0.39
H15				3.51	3.39	−0.12
N-CH_3(6)	3.78	3.89[a]	0.11	3.78[b]	3.72[b]	0.05
N-CH_3(12)	3.78	3.93[a]	0.15	3.80[b]	3.90[b]	−0.10

[a,b] Assignments can be interchanged.

Binding of lexitropsins to DNA stabilizes the DNA double helix,[7,11,22] and as a result, all the imino proton signals of the ligand:DNA complex can be detected at room temperature. Since the imino proton signal for I in complex A can only be detected at 4 °C (12.88 ppm), the kinetic stabilization of the DNA duplex is low compared to lexitropsin complexes examined in our previous studies.[7,11] The imino proton signals for IV, V, III, and II at 4 °C are located at 13.76, 13.51, 12.98, and 12.58 ppm, respectively. The signals for protons III and IV are very broad at 4 °C due to the slow exchange of **5** on the two equivalent sites on the DNA. Thermal denaturation studies by determination of UV hyperchromicity at 260 nm of complex A and free decadeoxyribonucleotide, at 265 mM sodium chloride and pH 7.0, were performed (data not shown). The T_m values for complex A and free DNA are 58 and 55 °C, respectively. These results suggest that the duplex DNA is only slightly stabilized by minor groove binding of **5**.

Assignment of the Nonexchangeable Proton Resonances in Complex A: Confirmation of the Location and Orientation of Lexitropsin 5 and the Conformation of the DNA. The nonexchangeable ^{1}H NMR signals for complex A were assigned by using a combination of NOE difference,[16] COSY,[14] and NOESY[15] techniques. The results from the COSY experiment of complex A are depicted in Figure 3. The COSY spectrum reveals two cytosine H5,H6 cross peaks. The lower field higher intensity CH5, CH6 correlation at 7.58 ppm is assigned to C(1), as will be discussed later. The higher field overlapping CH5,CH6 cross peak at 7.36 ppm is due to C(3,9). The intensity of the CH5,CH6 cross peak is sensitive to the local mobility of the cytosine residue.[23] Therefore, binding of a drug on or near the cytosine residue in question will reduce the local mobility and thereby lower the intensity of the CH5,CH6 cross peak in the COSY spectrum. Since the CH6 signals for C(3) and C(9) are superimposed, and the lexitropsin has been shown to reside on the ATTG sequence, it is likely that the cross peak at 7.36 ppm is mainly due to C(9). This indicates that the CH5,CH6 correlation of C(3) is diminished as a result of the binding of **5** on the sequence [$A_5T_6T_7G_8$.$C_3A_4A_5T_6$].

The location and orientation of lexitropsin **5** on the DNA is further corroborated by the following NOE studies. The results from the NOESY experiment are limited since very few cross peaks were observed.[15b] This is probably due to the increase in correlation time and cross-relaxation between many protons.[24a]

(20) (a) All results listed as data not shown are available on request from the authors. (b) Debart, F.; Rayner, B.; Imbach, J.-L.; Chang, D. K.; Lown, J. W. *J. Biomol. Struct. Dyn.* **1986**, *4*, 343.
(21) Patel, D. J.; Pardi, A.; Itakura, K. *Science* **1982**, *216*, 581.
(22) Marky, L. A.; Snyder, J. G.; Remeta, D. P.; Breslauer, K. J. In *Biomolecular* Stereodynamics; Sarma, R. H., Ed.; Academic Press: New York, 1983; Vol. 1, p 487.
(23) Borah, B.; Roy, S.; Zon, G.; Cohen, J. S. *Biochem. Biophys. Res. Commun.* **1985**, *133*, 380.

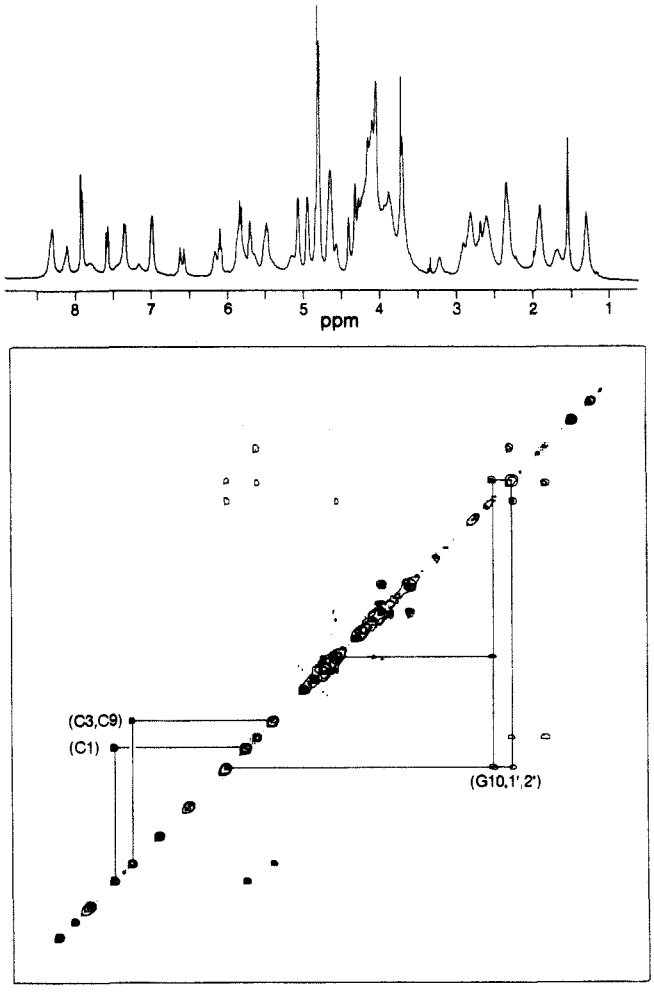

Figure 3. COSY spectrum for the 1:1 complex of lexitropsin **5** and d-[CGCAATTGCG]$_2$ at 21 °C. Experimental conditions: initial t_1 = 3 μs, Δt_1 = 0.33 ms, spectral width = 3012 Hz, F_2 = 1 K, and 16 FIDs were collected for each of the 256 experiments.

To overcome this problem, the 1D-NOE difference experiments were performed at 37 °C in which the ^{1}H NMR lines are sharper. The results of the NOE difference experiments are depicted in Figure 4, together with the reference spectrum[24b] (Figure 4A). Adenine-H8 signals are found at lower field than all other base protons.[25] Irradiation of the low field signal at 8.35 ppm for AH8(4,5), as shown in Figure 4B, gave NOEs at 2.82 ppm for AH2'1(4,5), 2.94 for AH2'2(4,5), 5.08 for AH3'(4,5), 5.90 for AH1'(4), and 6.19 for AH1'(5). In addition, NOE peaks are observed at 1.33 ppm for T-CH_3(6), 7.00 ppm for TH6(6), and 7.36 ppm for CH6(3). These results confirm the assignment of the signal at 8.35 ppm to AH8(4,5). The pattern of NOE intensities of H2' ≫ H1' ≥ H3', when the signal for AH8(4,5) is saturated, indicates that the DNA duplex in complex A adopts a conformation that belongs to the B family.[26a] This is similar to the conformation of the free decadeoxyribonucleotide under

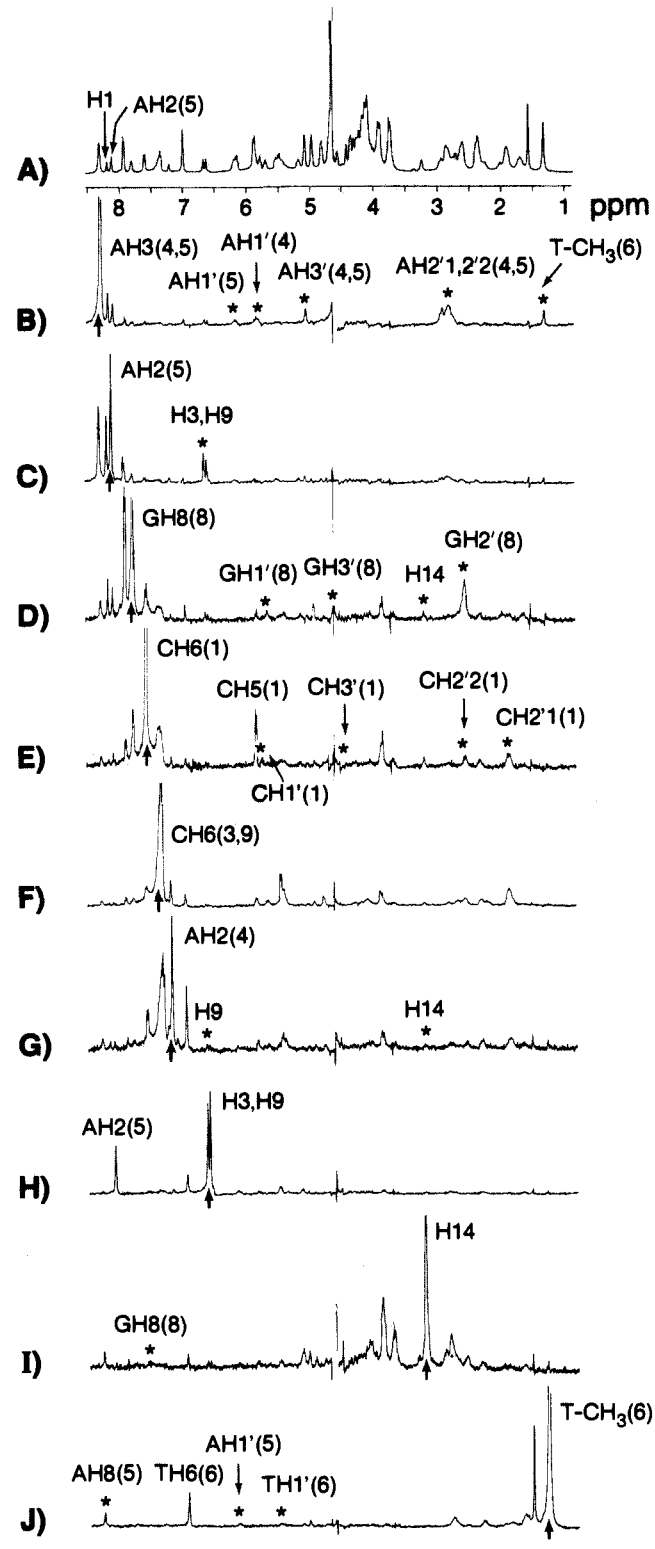

Figure 4. NOE difference spectra for complex A at 37 °C. Sweep width = 4000 Hz, irradiation time for NOE buildup = 0.6 s, and 800 FIDs were collected for each measurement. The arrows indicate the peaks saturated, and asterisks show the NOEs observed.

comparable conditions.[7] Similarly, saturation of CH6(1) (Figure 4E) and CH6(3,9) (Figure 4F) produced NOE peaks for their sugar protons with the patterns of NOE intensities consistent with those of a B-DNA.[26b] Furthermore, the presence of NOE between T-CH_3(6) and AH1'(5) as shown in Figure 4J confirms the B conformation of the DNA.[27] NOEs are also observed between

(24) (a) Kalk, A.; Berendsen, H. C. J. *J. Magn. Reson.* **1976**, *24*, 343. (b) All of the observed NOEs were assigned but some of them are not labeled so as to avoid crowding in Figure 4. The additional assignments are available for the interested reader, upon request, to the authors.

(25) Frechet, D.; Cheng, D. M.; Kan, L. S.; Ts'O, P. O. P. *Biochemistry* **1983**, *22*, 5194.

(26) (a) Gronenborn, A. M.; Clore, G. M. *Prog. Nucl. Mag. Reson. Spectrosc.* **1985**, *17*, 1. (b) Consideration was given to the existence of single stranded hairpin loops in solution. However, no additional sets of signals corresponding to such species were observed in the solutions of either complexes. Moreover if single strand hairpin loops were present the ^{1}H signals would not be as sharp and well-separated as they are for complex A at 37 °C (Figure 4) or complex B at 21 °C (Figure 1F). Since the signals are sharp and well-separated, all the resonances were assigned unambiguously and correspond to the criteria of Gronenborn et al.[26a] for a DNA belonging to the B family of conformations.

(27) Hare, D. R.; Wemmer, D. E.; Chou, S. H.; Drobyn, G.; Reid, B. R. *J. Mol. Biol.* **1983**, *171*, 319.

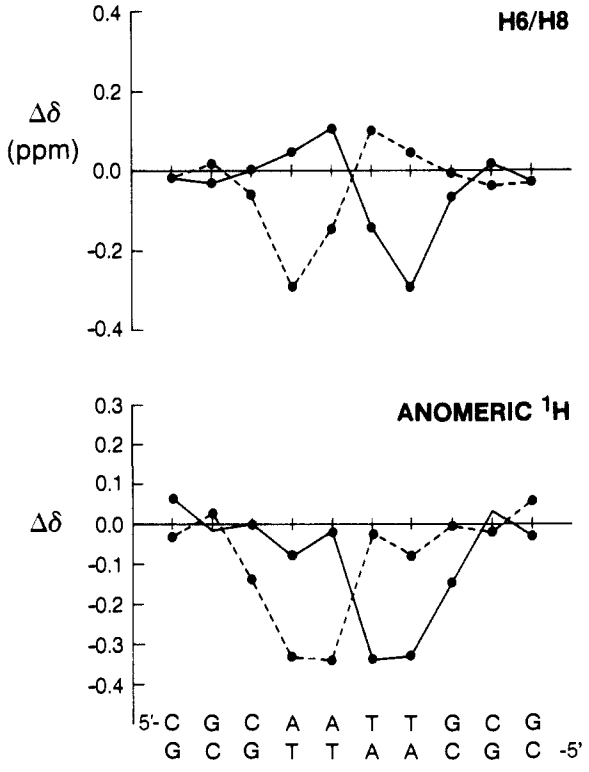

Figure 5. Graphical presentation of the lexitropsin-induced chemical shift changes for selected protons in the DNA sequence for complex A. Positive values show that the resonances in the 1:1 complex are at lower field than that in the free DNA. Both strands of the DNA are shown at the bottom, and the lexitropsin-induced chemical shift changes ($\Delta\delta$) for each of the two strands are drawn.

the peak at 7.36 ppm and the two pyrrole-CH_3 groups at 3.89 and 3.93 ppm, thus indicating the H5 and H13 of **5** are located at 7.36 ppm (Figure 4F).

Saturation of the AH2(5) signal at 8.13 ppm gave NOEs at 6.62 and 6.67 ppm for the pyrrole protons (H9 and H3) of the concave face of lexitropsin 5 (Figure 4C). Subsequent irradiation of the resonance at 7.82 ppm for GH8(8) produced an NOE peak at 3.23 ppm for H14 of **5** (Figure 4G). Furthermore, saturation of H3 and H9 (Figure 4H) and H14 (Figure 4I) of **5** gave NOEs that are consistent with the above results. These NOE difference measurements confirm the location and orientation of the nitrogen to carboxyl terminii of the lexitropsin on the 5'-ATTG-3' sequence of the DNA, as well as the B conformation of the duplex DNA in complex A.

Sequential analysis of the 1D-NOE and COSY results permits assignments of the nonexchangeable and imino protons of the DNA in complex A. A summary of these assignments as well as those for the lexitropsin are given in Table II together with those of the free DNA and **5** and the lexitropsin-induced chemical shift changes ($\Delta\delta$). The chemical shifts for the aromatic and anomeric protons from A(4) to G(8) are most affected (Table II). It is clear from the graphs of lexitropsin-induced chemical shift changes of individual protons versus the DNA sequence, as shown in Figure 5, that lexitropsin **5** is located on the ATTG sequence.

From the nonselective inversion-recovery experiments of complex A, measured at 21 °C (data not shown), two slowly relaxing signals are observed at 8.11 and 7.20 ppm. It has been shown that in B-DNA the sugar-base orientation is *anti*, and as a result the adenine-H2 signals have the longest spin–lattice relaxation time.[28] The presence of NOEs between the peak at 8.11 ppm and 1.33 for T-CH_3(6) (Figure 4C) and that at 7.20 ppm and 5.48 for CH5(3) (Figure 4G) permits the assignment of the signals 8.11 and 7.20 ppm to AH2(5) and AH2(4), respectively.

(28) Kearns, D. R. *C. R. C. Crit. Rev. Biochem.* **1984**, *15*, 237.

Table II. ^{1}H NMR Chemical Shift (ppm) Assignments of Individual Protons of Complexes A and B at 21 °C in D_2O Solutions[a]

		complex A		complex B	
base	DNA	δ	$\Delta\delta$	δ	$\Delta\delta$
CH6(1)	7.60	7.58	−0.02	7.62	0.02
CH8(2)	7.94	7.91	−0.03	7.93	0.01
CH6(3)	7.37	7.36	−0.01	7.35	−0.02
AH8(4)	8.26	8.31	0.05	8.48	0.22
AH8(5)	8.20	8.31	0.11	8.41	0.21
TH6(6)	7.13	6.99	−0.14	7.15	0.02
TH7(7)	7.28	6.99	−0.29	7.31	0.03
GH8(8)	7.88	7.82	−0.06	7.83	−0.05
CH6(9)	7.36	7.38	0.02	7.33	−0.03
CH8(10)	7.95	7.93	−0.02	7.97	0.02

[a] Complete assignment of nonexchangeable and exchangeable protons for d-$[CGCAATTGCG]_2$ are given in ref 7.

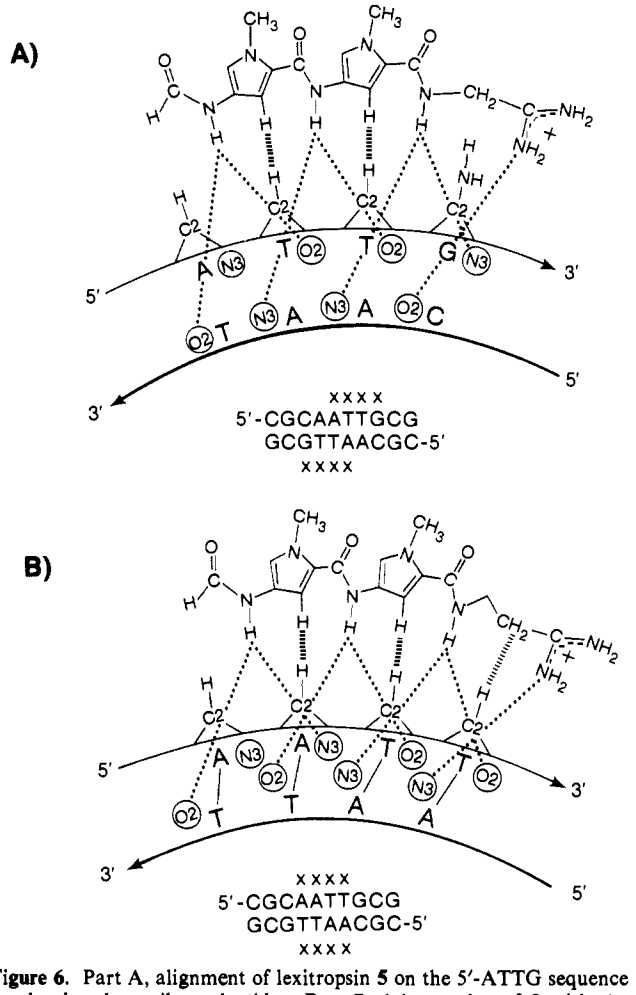

Figure 6. Part A, alignment of lexitropsin **5** on the 5'-ATTG sequence on the decadeoxyribonucleotide. Part B, 1:1 complex of **8** with the 5'-AATT sequence of the DNA. Dotted lines represent hydrogen bonding between the drug amide and amidinium protons and purine-N3 and pyrimidine-O2. Dashed lines represent the intermolecular van der Waals interactions.

The above results demonstrate unambiguously that lexitropsin **5**, which has only one methylene group or is "truncated" at the C-terminus, binds preferentially to the sequence ATTG rather than AATT. This investigation indicates that the second methylene group on the C-terminus, as in netropsin, which has van der Waals interactions with adenine-H2,[3,7,10,11] is responsible for reading the AT base pair at the 3' end of the DNA sequence. Therefore, removal of one of the methylene groups in netropsin analogues, such as lexitropsin **5**, eliminates the steric interactions between the methylene protons on the C-terminus of lexitropsins and the guanine-2-NH_2 moiety. Accordingly, the "truncated" lexitropsin **5** reads a terminal GC base pair. A model for the 1:1

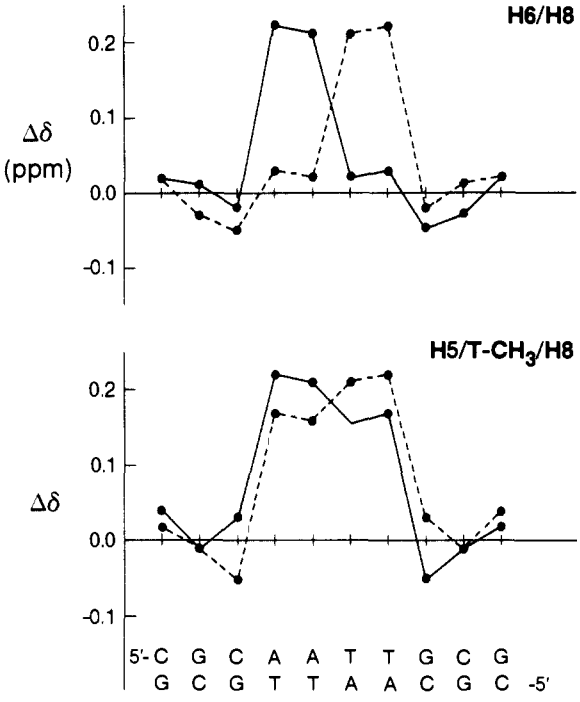

Figure 7. Graphical representation of the lexitropsin-induced chemical shift changes for selected protons in the DNA sequence for complex B.

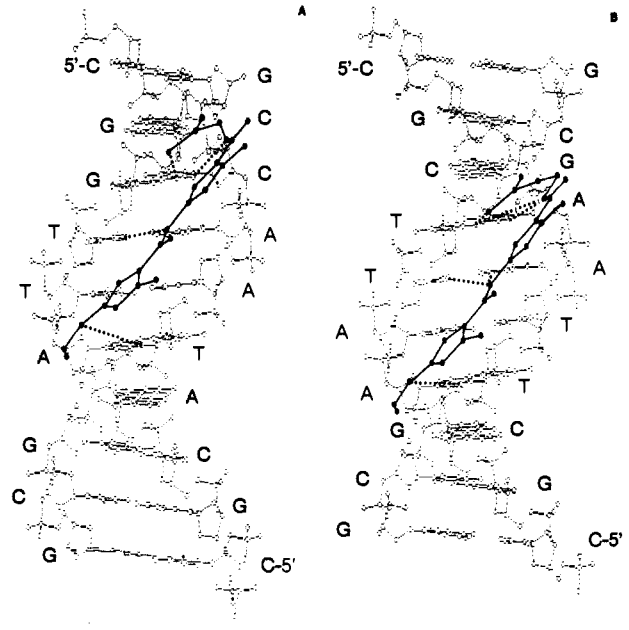

Figure 8. (A) Computer-generated depiction of complex A. The dotted lines indicate hydrogen bonds between the lexitropsin and DNA. (B) Computer-generated diagram for complex B.

complex of lexitropsin **5** and 5'-ATTG-3' (complex A) of the DNA is given in Figure 6A.

Since the above results for complex A are without precedent, additional experiments were performed to verify the above findings. Accordingly, ^{1}H NMR studies of the 1:1 complex of lexitropsin **8**, which has a second methylene group at the C-terminus, and the decadeoxyribonucleotide d-[CGCAATTGCG]$_2$ were performed.

Binding of Lexitropsin 8 to d-[CGCAATTGCG]$_2$. The nonexchangeable (Figure 1F) and imino proton NMR signals of complex B were assigned with use of the strategy discussed earlier. Individual assignments of the lexitropsin and DNA proton NMR signals are given in Tables I and II, respectively.

The imino ^{1}H NMR spectrum of complex B obtained at 21 °C reveals four imino proton signals. The two lower field signals are assigned to the exchanging peaks for imino protons V and IV at 14.52 and 13.25 ppm, respectively. The remaining two signals at 13.03 and 12.76 ppm are therefore assigned to protons II and III, respectively. The chemical shifts of protons IV and V of the DNA are most affected by the binding of **8**, when compared to the free DNA,[7] and this suggests that the ligand is, as expected, located in the minor groove at the central AATT core of the decadeoxyribonucleotide. The peak for imino proton I is detected at 13.14 ppm at 4 °C as shown in Figure 2B. The signals of protons IV, V, II, and III at 4 °C are found at 14.56, 13.52, 12.98, and 12.72 ppm, respectively.

A plot of the lexitropsin-induced chemical shift changes ($\Delta\delta$) of selected protons versus the DNA sequence, as shown in Figure 7, establishes the location of **8** on the AATT core of the DNA (also see Table II). The location and orientation of the N to C-termini of lexitropsin **8** on the 5'-AATT-3' sequence are confirmed by NOE difference studies (data not shown). Saturation of the AH2(5) signal at 8.05 ppm produced NOEs at 6.68 and 6.77 ppm for H3 and H9 of **8** (also see Table I). When the AH2(4) signal at 7.35 ppm was irradiated, NOEs were seen at 8.18 and 3.39 ppm for H1 and H15 on **8**. A model for the 1:1 complex of lexitropsin **8** to the AATT sequence on the DNA is shown in Figure 6B. Saturation of the signal at 7.35 for CH6(3,9) produced NOEs for their sugar protons, with the pattern of intensities H2' $\gg$ H1' $\geq$ H3', which is in accord with B-DNA.

These results clearly demonstrate that the second methylene moiety, as in **8**, is in close proximity to AH2(4) and is responsible for reading the terminal AT base pair. Therefore, binding of **8** to ATTG is prevented by steric interactions between the second methylene group and guanine-2-NH$_2$.

Molecular Modeling. The computer-generated diagrams of complexes A and B are shown in Figure 8, A and B, respectively. Figure 8A shows that lexitropsin **5** is bound snugly to the ATTG sequence in the minor groove. The amide protons are on the concave face, and they are hydrogen bonded to the purine-N3 or pyrimidine-O2 as illustrated by the dotted lines. Generation of the model of complex A (Figure 8A) is constrained by NOE measurements to establish the intermolecular contacts between the lexitropsin **5** and DNA. This requires that H1 and NH1 be adjacent to imino proton V for dA(5).dT(6) base pair and the H14 protons of the C-terminus be juxtaposed to GH8(8).

For complex B (Figure 8B), the diagram was similarly generated according to the NOE measurements. Lexitropsin **8** fits snugly in the minor groove. The AATT sequence is hydrogen bonded to **8**, as indicated by the dotted lines. The intermolecular contacts for complex B are in good agreement with those of the 1:1 complex of G$^+$A-Py-Im-CH$_2$CH$_2$-Am$^+$ and d-[CGCAATTGCG]$_2$.[7]

Exchange Processes for Complexes A and B. The ^{1}H NMR spectra for complexes A and B are depicted in Figure 1, E and F, respectively. The ^{1}H NMR signals for the DNA in complex A are broader than those for complex B under comparable conditions. The broadening of signals in complex A could be due to the relatively slow exchange of lexitropsin **5** between the two equivalent binding sites (Figure 8A) with respect to the NMR time scale. These results suggest that the exchange of lexitropsin **8** between the two equivalent sites in complex B is faster than that in complex A.

It is clear from the variable-temperature NMR studies of complex A (Figure 9) that the rate of exchange of **5** on the equivalent binding sites (ATTG) is reduced by lowering the temperature. As a result, the exchanging signals, such as those for T-CH$_3$(6), became apparent at temperatures below 15 °C (Figure 9C). Coalescence of the T-CH$_3$(6), GH8(8), and AH2(5) signals occurs at about 15 °C (Figure 9C). At 4 °C, the exchange signals for T-CH$_3$(6), GH8(8), and AH2(5) are well resolved. The rate and free energy of transition for the exchange of **5** on the equivalent sites of the DNA at temperatures above, below, and at coalescence can be estimated by using the equations reported by Dalling and co-workers.[29] The exchange signal for

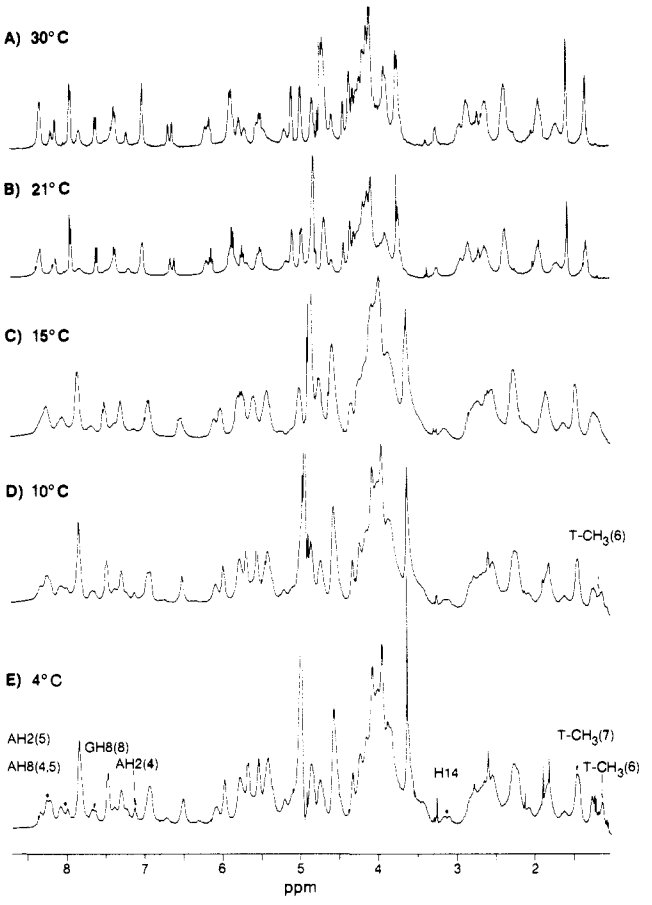

Figure 9. Variable-temperature ^1H NMR spectra of complex A.

T-CH_3(6) at 1.29 ppm was used to calculate the rate of exchange at several temperatures. A plot of the rate of exchange of **5** in complex A against temperature is shown in Figure 10. The rate of exchange of **5** between the two ATTG sites is about 185 s^{-1} at 294 K, and the free energy of transition for the exchange process is 58 ± 5 kJ mol^{-1}. However, in the NMR spectrum for complex B at 4 °C, the line width at half-height for the T-CH_3(6) peak is 11.5 Hz which is similar to that for complex A at 294 K. Therefore the rate of exchange of lexitropsin **8** between the two AATT sites is greater than 180 s^{-1} at 4 °C.

Only four imino ^1H NMR signals of complex A can be detected at room temperature (Figure 2A). This indicates that the terminal base pairs are fraying as is observed for the free DNA.[7] In the ^1H NMR spectrum of complex A, the pyrrole protons of **5** did not undergo doubling of resonances even at 4 °C (Figure 9E). Thus the exchange process does not result in a change in chemical environments for the lexitropsin molecules. Thus, the intramolecular "slide-swing" mechanism (Figure 11Aa,d,c) is unlikely since this would result in distinct changes in the chemical environment for the lexitropsin during the exchange process. However, this is insufficient evidence for excluding the intramolecular exchange mechanism, and thus we consider that the exchange mechanism is largely but probably not exclusively an intermolecular process. Since the intermolecular exchange mechanism is without precedent for the systems we have examined to date, additional experiments were performed to confirm this exchange mechanism. Intermolecular exchange processes are dependent on the concentration of the two components in complex A. Therefore the effect of the concentration of complex A on the rate of exchange was studied. At the concentrations of complex A of 0.74, 0.37, and 0.074 mM, the temperature of coalescence occurs at about 15, 10, and 4 °C, respectively. These results indicate that the rate of exchange of

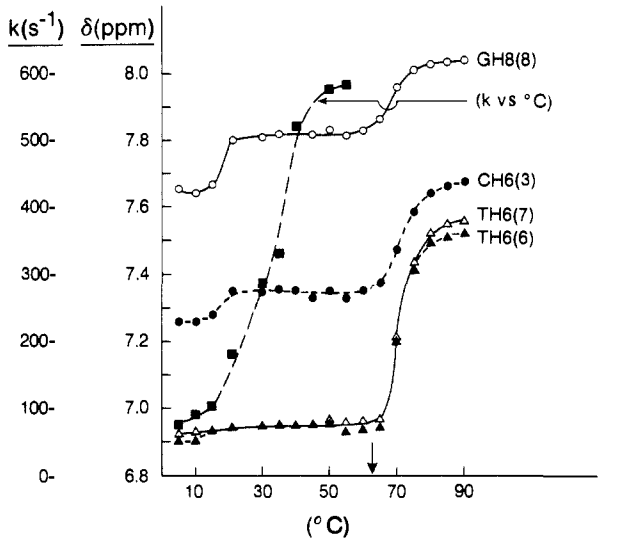

Figure 10. A plot of the chemical shifts (δ) and rates of exchange of lexitropsin **5** between the two equivalent sites on the DNA (k, s^{-1}) versus temperature (°C) for complex A.

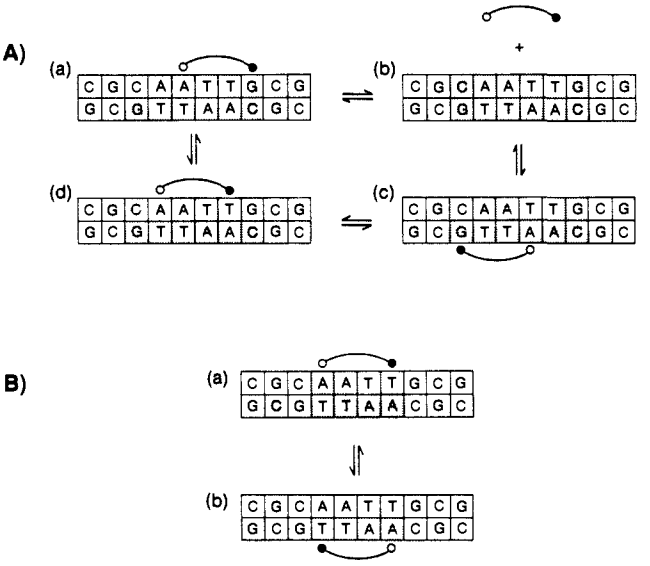

Figure 11. Representation of the dynamic processes in the complexes formed between lexitropsin **5** and **8** and d-[CGCAATTGCG]$_2$. The disymmetric ligands are denoted by two linked circles: A, complex A; B, complex B.

5 on the two equivalent sites is dependent on the concentration of A, and thereby they provide additional evidence for the intermolecular mechanism. Therefore, in the 1:1 complex of **5** and DNA (complex A), lexitropsin **5** must dissociate from the complex (Figure 11Ab) during the exchange process. Subsequently the free ligand **5** recombines with another ATTG site (Figure 11Ac). This is compatible with an intermolecular exchange mechanism (Figure 11Aa,b,c).

From the variable-temperature NMR studies of complex A, the chemical shifts of individual protons versus temperature are plotted as shown in Figure 10. The curves show two distinct cooperative melting transitions, with T_m values at 18 and 70 °C. Under these low salt conditions the helix-coil transition of the self-complementary octadeoxyribonucleotide d-[GGAATTCC]$_2$ occurs between 12 to 21 °C depending on the concentration of the DNA.[30] Therefore, the lower T_m (18 °C) is probably due to the melting of drug-free decadeoxyribonucleotide, while the

(29) (a) Dalling, D. K.; Grant, D. M.; Johnson, L. F. *J. Am. Chem. Soc.* **1971**, *93*, 3678. (b) Shaw, D. *Fourier Transform NMR Spectroscopy*: Elsevier Scientific Publishing Co.: New York, 1976; pp 231–233.

(30) Patel, D. J.; Canuel, L. L. *Eur. J. Biochem.* **1979**, *96*, 267.

high-temperature cooperative melting (T_m = 70 °C) is due to the 1:1 complex of **5** and DNA.[31]

The appearance of the H14 signal at 3.23 ppm is characteristic of complex formation.[7,11] As the temperature was raised from 60 to 65 °C, the signal at 3.23 ppm disappeared. This indicates that at 63 ± 2 °C lexitropsin **5** is no longer bound to the DNA, and by raising the temperature beyond this point (indicated by a thick arrow in Figure 10) the DNA starts to melt.

Only four imino proton NMR signals of complex B can be detected at room temperature; therefore, the duplex DNA is weakly stabilized and the exchange of the imino proton I to the medium is fast. The exchange process in complex B is independent of the concentration of the 1:1 complex. Furthermore there is no doubling of ^{1}H NMR signals of **8** in complex B which indicates that there is no change in the chemical environment of the lexitropsin in the exchange process. Therefore the exchange of **8** is likely to proceed without dissociation of the ligand from the DNA, i.e., via an intramolecular flip-flop (Figure 1B) process. On the basis of the significantly different rates of exchange for complexes A and B, the intramolecular component of the exchange process in complex A is minimal. The difference in the exchange mechanism of complexes A and B could be due to the different electrostatic potential of the ATTG versus AATT sequences.[10]

Conclusions

The application of NOE difference measurements and COSY techniques permit the assignment of the nonexchangeable and imino proton resonances for complexes A and B. The ^{1}H NMR results indicate that the "truncated" lexitropsin **5**, which has a methylene group at the C-terminus, binds preferentially to the sequence 5′-ATTG-3′ on the decadeoxyribonucleotide d-[CGCAATTGCG]$_2$. Ligand **5** resides on the floor of the minor groove and forms hydrogen bonds to the purine-N3 and pyrimidine-O2 positions thus displacing the shell of hydration on the DNA.[4] However, lexitropsin **8**, which has a second methylene group at the C-terminus, as expected, binds to the 5′-AATT-3′ sequence. These results demonstrate that in such cases the van der Waals interactions between the methylene protons at the C-terminus and adenine-H2 or guanine-2-NH$_2$ are responsible for reading the base pair at the 3′-end. Thus, owing to steric interactions between the second methylene group and G-2-NH$_2$, lexitropsins such as **8**, netropsin, and distamycin are prevented from reading a 3′-terminal GC base pair, and, by default, thereby read an AT base pair.

NOE measurements of the two complexes show that both DNA duplexes exist as right-handed helices of the B family, which are similar to the conformation of the free decadeoxyribonucleotide. Therefore binding of lexitropsins **5** and **8** to the DNA results in minimal conformational distortion of the DNA.

Exchange ^{1}H NMR effects allow an estimate of ≈185 s^{-1} with $\Delta G^{\ddagger}$ of 58 ± 5 kJ mol^{-1} for the exchange of **5** on the two equivalent ATTG sites on the DNA at 294 K. However, the exchange of **8** on the AATT sites is fast (>180 s^{-1}) even at 277 K. There is evidence to suggest that the exchange of **5** on the DNA proceeds predominantly via an intermolecular process. The intramolecular flip-flop mechanism most simply explains the data for the exchange of lexitropsin **8** between the two AATT sites.

These results provide structural information on the effects of the methylene groups at the C-terminus of lexitropsins in molecular recognition to DNA. In combination with other factors that contribute to the molecular recognition process, such as electrostatic attraction and introduction of hydrogen bond accepting heterocycles, we are now at the stage of the examination of rationally designed lexitropsins that exhibit predictable sequence-specific binding properties for development as gene probes. These results will be reported in due course.

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(31) The melting behavior observed by UV and NMR experiments is due to the different experimental conditions employed. Thus relatively high salt concentrations were used to study the helix-to-coil transition by UV, whereas relatively low ionic strength conditions obtained in the ^{1}H NMR study. The biphasic melting behavior found in the latter experiments (see Figure 10) is fairly typical of such systems. For example, in the paper by Patel and Canuel (Patel, D. J.; Canuel, L. L. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5207), "Figure 2 shows...the experimental biphasic melting curves were assigned to the opening of the drug-free dA-dT base pair regions (lower temperature cooperative transition) and the opening of the dA-dT base pair regions centered on the intercalated drug (higher temperature cooperative transition)." Thus it is consistent to attribute the biphasic transitions shown in our Figure 4 as follows. The lower temperature transition corresponds to opening of the central dA-dT region of the free DNA under the low salt conditions and the higher ~70 °C melting corresponds to melting of the stabilized drug–DNA complex.