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Changes in ^{13}C NMR chemical shifts of DNA as a tool for monitoring drug interactions

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Abstract

The antibiotic drug, netropsin, was complexed with the DNA oligonucleotide duplex $[d(GGTATACC)]_2$ to explore the effects of ligand binding on the ^{13}C NMR chemical shifts of the DNA base and sugar carbons. The binding mode of netrospin to TA-rich tracts of DNA has been well documented and served as an attractive model system. For the base carbons, four large changes in resonance chemical shifts were observed upon complex formation: -0.64 ppm for carbon 4 of either Ado4 or Ado6, 1.36 ppm for carbon 2 of Thd5, 1.33 ppm for carbon 5 of Thd5 and 0.94 for carbon 6 of Thd5. AdoC4 is covalently bonded to a heteroatom that is hydrogen bonded to netropsin; this relatively large deshielding is consistent with the known hydrogen bond formed at AdoN3. The three large shielding increases are consistent with hydrogen bonds to water in the minor groove being disrupted upon netropsin binding. For the DNA sugar resonances, large changes in chemical shifts were observed upon netropsin complexation. The 2′, 3′ and 5′ ^{13}C resonances of Thd3 and Thd5 were shielded whereas those of Ado4 and Ado6 were deshielded; the ^{13}C resonances of 1′ and 4′ could not be assigned. These changes are consistent with alteration of the dynamic pseudorotational states occupied by the DNA sugars. A significant alteration in the pseudorotational states of Ado4 or Ado6 must occur as suggested by the large change in chemical shift of -1.65 ppm of the C3′ carbon. In conclusion, ^{13}C NMR may serve as a practical tool for analyzing structural changes in DNA-ligand complexes.

Keywords: Netropsin; Ligand; NMR; DNA; Drug; Chemical shift

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1. Introduction

^{13}C NMR[1] chemical shifts are very sensitive to changes in the environment of nuclei, which should make it a practical, atomic-level parameter for monitoring drugs interacting with target nucleic acids. This would require, however, a better understanding of the relative sensitivity of DNA ^{13}C chemical shifts to various binding events such as forming hydrogen bonds, dehydration, changes in base stacking, and helix distortions. Previous investigations have begun to shed light into this by monitoring changes in chemical shifts upon duplex formation in model oligonucleotides. Early ^{1}H NMR studies on single- and double-stranded oligonucleotides indicate that almost all the base protons are shielded as temperature is decreased through the transition from the random coils to base-stacked structures [1–4]. Likewise, ^{13}C NMR studies of *r*(AAA) and *r*(AAG), both of which lack a hydrogen-bonded duplex structure, indicate that all the base carbons become shielded upon decreasing the temperature [5]. Such changes have been attributed to the gain of ring current shielding [6], and additionally for ^{13}C, an increase in steric contact between the stacked bases [5]. For several DNA oligonucleotide duplexes, we observed that most of the base carbons exhibit similar (shielding) chemical shifts upon lowering the temperature from the random coils. Surprisingly, approximately 25% of the base carbons exhibited large (approx. 1 ppm) deshielding changes upon duplex formation; this was attributed to making Watson–Crick H-bonds [7,8].

^{13}C NMR studies of RNA di- and trinucleotides [5,9] and DNA oligonucleotides [7,10] have monitored chemical shifts as a function of changes in sugar pucker. The major factor affecting chemical shifts was attributed to sugar pucker changes while other factors apparently contribute to a smaller extent.

The well-characterized system of netropsin binding to the minor groove of DNA oligonucleotides [11–21] offers a possibility for further exploring the effects of H-bonding and sugar puckering on the chemical shifts of DNA carbons. It is known that netropsin binds sequence specifically to double stranded DNA, and at least four contiguous A-T base pairs are required for netropsin to bind with maximum strength. Several sources including H-bonding are responsible for the specificity and binding of netropsin. Dickerson and colleagues report from crystallographic data that netropsin H-bonds to the TO2 and AN3 sites in its complex with the dodecamer duplex $[d(CGCGAATTCGCG)]_2$. They propose that water binds in the minor groove of A-T regions of B-DNA in a 'spine of hydration' [22,23]. As many as 12 of these water molecules are displaced from the dodecamer duplex upon binding to netropsin.

Sugar pucker changes were also reported for the interaction of netropsin to DNA oligonucleotides. Dickerson and colleagues [11,16] showed crystallographic data on the sugar pucker changes of $[d(CGCGAATTCGCG)]_2$ upon interaction with netropsin. Changes in the ^{1}H NMR [13] and ^{13}C [24] and ^{15}N NMR [24,25] chemical shifts were also monitored for DNA oligonucleotide binding to netropsin. Changes in ^{15}N chemical shifts upon netropsin binding were attributed to H-bonding.

In this work, we investigate the relation between changes in ^{13}C NMR shifts of the DNA duplex $[d(GGTATACC)]_2$ upon binding to netropsin as a model system. Netropsin (Fig. 1a) is known to bind to the minor groove of the 'TATA' central portion of the DNA duplex (Fig. 1b) and form H-bonds to AN3 and TO2 (Fig. 1c).

2. Materials and methods

$[d(GGTATACC)]_2$ was synthesized from phosphoramidites using the syringe method [26] and purified from shorter chain length impurities by C_8 reverse-phase HPLC using tetrabutylammonium acetate as an ion-pairing buffer in

[1] *Abbreviations:* A, adenosine; T, thymidine; G, guanosine; C, cytidine; NMR, nuclear magnetic resonance; H-bond, hydrogen bond; δ, chemical shift; Δδ, change in chemical shift; Tm, melting temperature; Net, netropsin; ppm, parts per million; the oligonucleotide discussed in this paper has no terminal phosphates unless specifically denoted.

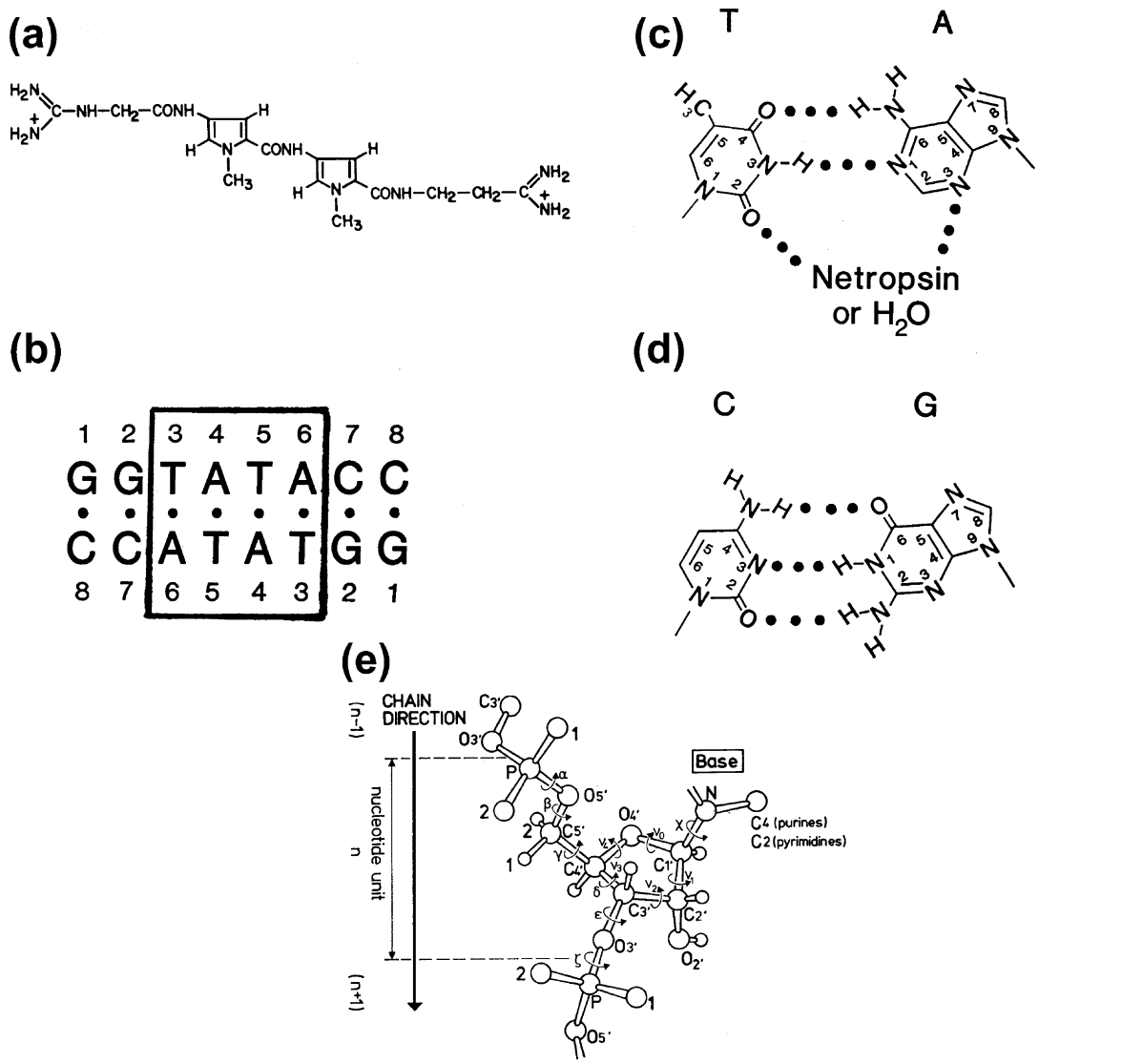

Fig. 1. (a) The molecular formula for netropsin is shown. (b) Numbering scheme for the self-complementary duplex [*d*(GGTATACC)]$_2$. The boxed in portion of the duplex is the binding site of netropsin. (c) Water/Na^+ and netropsin H-bond sites on the A–T base pairs are shown. (d) A representation of the C–G base pair. (e) The nomenclature of a deoxyribose sugar is shown (adapted from Saenger [38]).

CH_3CN/H_2O gradients. The 8-mer was then purified from salts, i.e. tetrabutylammonium acetate, using a Sephadex A-25 column. The column was pre-equilibrated with 0.01 M cacodylate solution, the sample was loaded, washed with five column volumes of 0.1 M NaCl solution, and then eluted with 1 M NaCl solution. The excess NaCl was removed from the 8-mer using a Sephadex G-10 column with distilled water as the solvent. Netropsin was purchased from Serva Fine Biochemicals. The pure [*d*(GGTATACC)]$_2$ sample was put into a 5-mm NMR tube (7 mM in duplex, 14 mM in single strands) containing 0.1 M NaCl, 10^{-4} M EDTA, 10 mM cacodylate pH 7.2,

20% D_2O in a total volume of 0.5 ml. Netropsin was added incrementally to the duplex and spectra recorded at drug: duplex ratios of 0.1, 0.2, 0.33, 0.5, 0.67, 1.0 and 1.5. Spectra at several temperatures were measured at ratios of 0.2, 0.5 and 1.0 (three different samples were used to do all these experiments). Natural abundance spectra were taken at 125.8 MHz for ^{13}C and 500 MHz for ^{1}H in a 5-mm dual probe on a General Electric GN-500. Unless otherwise indicated, the spectra were taken at 23°C. For the ^{13}C spectra, a one pulse experiment was used with a 90°C pulse, repetition rates of 3 sec and 'MLEV' decoupling. 10 000–12 000 transients were averaged with 16k data tables (8k real + 8k imaginary) and sweep width of 30 kHz. The ^{1}H spectra were recorded in aqueous solution using the Jump-Return experiment to suppress the H_2O peak [27]. ^{13}C spectra were referenced to the cacodylate peak at 20.11 ppm. ^{1}H spectra taken at 23°C were referenced to the H_2O peak at 4.75 ppm. Molecular Graphics analysis was performed on a Silicon Graphics Iris 2400 Turbo using the UCSF Midas program and crystal coordinates of $[d(CGCGAATTCGCG)]_2$ and its complex with netropsin obtained from the PDB (Protein Data Bank).

The ^{13}C base resonances of $[d(GGTATACC)]_2$ have been assigned by a combination of the ^{1}H-$\{^{13}C\}$-HMQC experiment (two-dimensional ^{1}H-detected ^{1}H-^{13}C correlation) and the comparative method as reported elsewhere [8,28,29]. The ^{13}C sugar resonances were assigned by the ^{1}H-$\{^{13}C\}$-HMQC experiment [10,28].

Assignment of the DNA resonances in the netropsin: $[d(GGTATACC)]_2$ complex were secured by comparison of spectra (at several temperatures) of incremented drug concentrations with the spectrum of pure duplex. This method of direct comparison was possible since the DNA signals moved as average peaks as the netropsin concentration was increased. Partial assignment of the ^{13}C resonances of $[d(GGTATACC)]_2$ in complex with netropsin have been previously reported [24] (i.e. those of the protonated base carbons and the C1′, C3′ and C4′ sugar carbons). Here, we additionally report the assignment of ^{13}C resonances of the quaternary base carbons and the C2′ and C5′ sugar carbons.

3. Results

3.1. Nomenclature

In this paper, specific carbons are referred to with the base type designated first by letter, the chain position second, and the carbon class last. For example, G2,8 represents carbon number eight in the guanine base of the second residue from the 5′-end of $[d(GGTATACC)]_2$, whereas TC6 designates the class: thymine carbon 6. If another type of atom is being discussed, the atom type will be designated, for example G2,H8.

3.2. ^{13}C chemical shift changes of the bases

The lowest-field portion of the 125.8 MHz ^{13}C NMR spectra of the $[d(GGTATACC)]_2$ duplex and its complex with netropsin are shown in Fig. 2a and Fig. 2b, respectively. This part of the spectrum contains nearly all of the quaternary base carbons, including the carbonyls and amino-bonded carbons, which are involved in the hydrogen bonds between bases. Most of the resonances are well resolved, e.g. in Fig. 2a, 18 lines can be distinguished for the 20 carbons in this region. At 23°C, the DNA signals move as average peaks (netropsin complexed and uncomplexed) as netropsin concentration is increased (e.g. see Fig. 3 showing the C3′ resonances) indicating that netropsin is in intermediate to rapid exchange on the NMR time scale. A plot of $\Delta\delta$ for these base carbons vs. the ratio of netropsin: $[d(GGTATACC)]_2$ is shown in Fig. 4. T5,2 and one of the AC4 carbons exhibit the largest $\Delta\delta$ values. The $\Delta\delta$ values of all of the T and A base carbons are collected in Table 1; large chemical shift changes are indicated in boldface.

3.3. ^{13}C chemical shift changes of the sugars

The sugar resonances of $[d(GGTATACC)]_2$ fall in the aliphatic region between 35 and 95 ppm. The C2′, C3′ and C5′ groups of resonances fall in separate isolated regions of the spectrum, which made assignments and analysis straightforward. The C1′ and C4′ resonance groups overlap with each other; assignments of the uncomplexed DNA

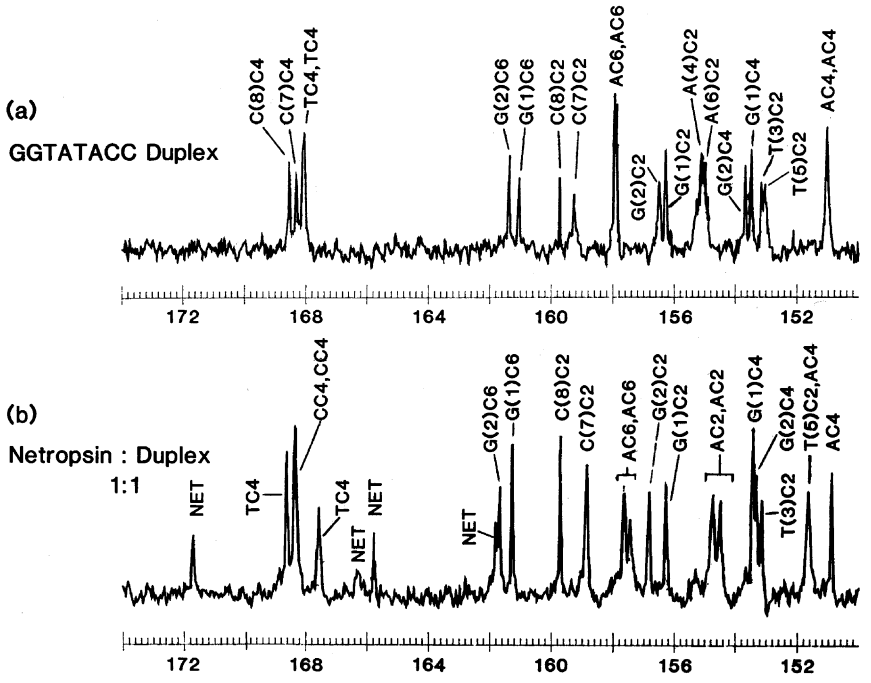

Fig. 2. 125.8 MHz ^{13}C NMR spectra (10 000–12 000 transients) of the lowest field base carbons of pure [*d*(GGTATACC)]$_2$ and the 1:1 complex with netropsin. The resonances are assigned to specific duplex carbons. The netropsin resonances are designated by 'NET' above the resonance.

resonances were obtained but assignments were not possible when netropsin complexed. The C3′ carbon class exhibited larger changes in chemical shifts than C2′ and C5′ (see Table 2). Fig. 3 shows the C3′ region as netropsin was incrementally added up to a 1:1 duplex to drug ratio. Seven of the eight expected resonances are well resolved. The resonances of C3′, C5′ and C2′ carbons belonging to the central 'TATA' nucleotides of the DNA duplex exhibited larger Δδ values than the G1, G2, C7 and C8 nucleotides (see Table 2). Note that resonances of T3 and T5 exhibit positive Δδ values (except T3,3′) whereas those of nucleotides A4 and A6 are negative.

4. Discussion

4.1. Hydrogen bonding effects on ^{13}C chemical shifts

Previous studies showed that some classes of base carbon resonances (e.g. GC2, GC6, and TC4) exhibited large deshielding changes upon duplex association from single strands; these were largely carbons that are bonded to heteroatoms involved in Watson–Crick H-bonds (see Fig. 1c,d). The large deshielding changes were attributed to H-bond formation upon strand association [7]. The remaining majority of base carbons exhibited large shielding changes (e.g. GC8 and GC5) or small shielding increases (e.g. GC4). The shielding was attributed to an increase in ring current and steric compression effects (present for all base carbons) upon forming base stacked structures. These effects are apparently small compared to the deshielding due to H-bond formation at some sites. At a site such as GC4, shielding and deshielding effects must nearly balance [5,7,8]. The observed magnitude of Δδ from H-bonding is certainly attenuated by stacking effects and breaking of H-bonds with solvent water. Hence, H-bonding effects on chemical shifts are attenu-

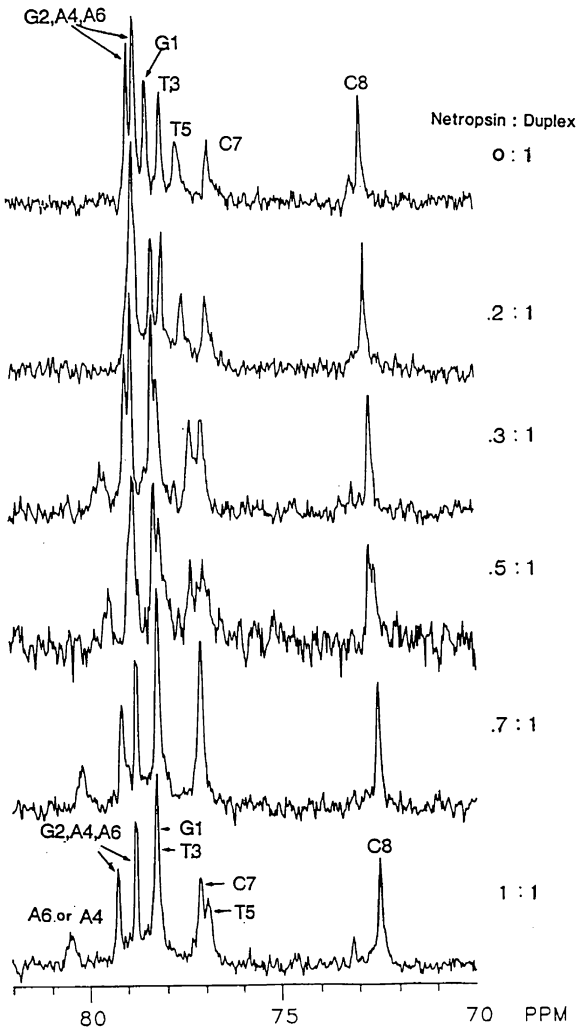

Fig. 3. The incremental addition of netropsin to the DNA duplex is shown for the C3′ region of the ^{13}C spectrum of $[d(GGTATACC)]_2$. The nucleotide assignment is given beside the resonance.

ated, but the observation of a large deshielding of a ^{13}C resonance may indicate that a nearby heteroatom is involved in H-bond formation.

4.2. H-bonding and netropsin: $[d(GGTATACC)]_2$

Upon complex formation there are no large changes in chemical shifts for the G and C bases, and notably large changes for the T and A bases (the largest are indicated in boldface in Table 1). These observations suggest that netropsin binds to the A–T rich center of $[d(GGTATACC)]_2$. The only large negative Δδ deshielding occurs for one of the two AdoC4 (−0.64 ppm). Unfortunately it cannot be specifically assigned since both AC4 ^{13}C signals overlap at all temperatures in the absence of netropsin. The large negative Δδ of the single AC4 may indicate that the adjacent heteroatom AN3 is involved in H-bonding to the netropsin ligand. Since each resonance represents a carbon from each strand, two H-bonds are identified. Hydrogen bonding at AN3

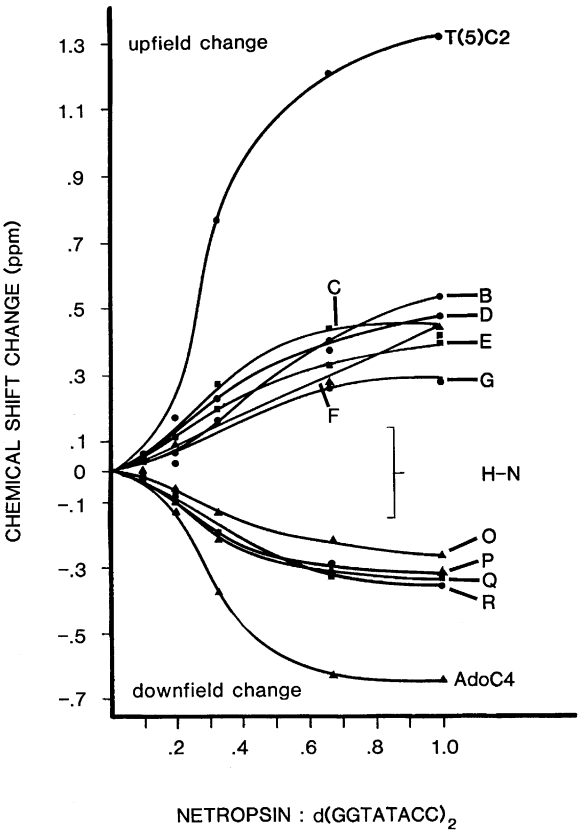

Fig. 4. Δδ vs. netropsin: $[d(GGTATACC)]_2$ for the lowest field base carbons. A positive Δδ upon netropsin additional indicates an increase in shielding or an upfield change. The T5,2 and AC4 resonances are specifically labeled and the other resonances are: B,[A6,2]; C,[AC6]; D,[TC4]; E,[C7,2]; F,[A4,2]; G,[AC6]; H-N,[G1,2, G1,4, C8,2, T3,2, C8,4, AC4]; O,[G1,6]; P,[G2,6]; Q,[G2,4]; R,[G2,2].

Table 1
^{13}C base resonance assignments of $[d(GGTATACC)]_2$ and chemical shift changes upon complexation with netropsin[a]

δ (ppm)[b]	Assignments[c]	Δδ[d]
168.58	C8,4	0.18
168.34	C7,4	0.10
168.08	T(3 or 5),4	−0.32
168.08	T(3 or 5),4	0.47
161.37	G2,6	−0.32
161.05	G1,6	−0.26
159.76	C8,2	0.01
159.26	C7,2	0.39
157.96	A(4 or 6),6	0.27
157.90	A(4 or 6),6	0.41
156.52	G2,2	−0.35
156.29	G1,2	−0.02
155.14	A4,2[e]	0.39
155.05	A6,2[e]	0.53
153.70	G(1 or 2),4	−0.33
153.49	G(1 or 2),4	0.03
153.17	T3,2[e]	−0.02
153.05	**T5,2**[e]	**1.36**
151.05	A(4 or 6),4	0.12
151.05	**A(4 or 6),4**	**−0.64**
143.99	C8,6	0.15
143.26	C7,6	0.45
142.14	A4,8	0.06
141.99	A6,8	−0.09
139.81	G1,8	0.44
138.99	T3,6	0.67
138.99	**T5,6**	**0.94**
138.81	G2,8	0.26
120.81	A(4 and 6),5	0.68
118.98	G1,5	0.55
118.28	G2,5	0.50
113.64	T3,5[e]	0.30
113.55	**T5,5**[e]	**1.33**
98.84	C(7 or 8),5	0.21
98.64	C(7 or 8),5	0.45
14.19	T3,CH3	0.03
14.19	T5,CH3	0.03

[a]The individual duplex base carbon resonances (in the absence of netropsin) are listed by decreasing ppm. The assignments and also the Δδ values upon netropsin addition are listed for these pure duplex resonances.

[b]Chemical shifts of $[d(GGTATACC)]_2$ resonances uncomplexed to netropsin.

[c]^{13}C resonance assignments of $[d(GGTATACC)]_2$ uncomplexed to netropsin.

[d]Changes in chemical shift upon complexation with netropsin ($\Delta\delta = \delta$ uncomplexed $- \delta$ complexed, units are in ppm). A negative Δδ indicates a downfield shift. A positive Δδ indicates an upfield shift.

[e]Assignments were obtained from base stacking arguments.

sites requires that netropsin is physically binding in the duplex minor groove. The lack of change in chemical shift of the methyl resonances further suggests that netropsin is not bound in the major groove.

The clear majority of the large Δδ values in Table 1 occur at T5 in the central base pairs of the complex, illustrated in Fig. 5. Caution should be observed for the unique assignments of T5,5 and T5,2 since their distinction from T3,5 and T3,2 was made by ring current assessments [8]. The T5,6 assignment was made directly from the ^{1}H-{^{13}C}-HMQC experiment ([8], and see Section 2). The fact that the magnitude and direction of Δδ for both T5,5 and T5,2 agree with T5,6 (Table 1) reinforces these assignments. The large and moderately large positive Δδ values observed at T5,2, T5,5 and T5,6 cannot be explained by formation of H-bonds to nearby covalent-bonded heteroatoms (large deshielding would have been expected). Besides, T5,6 and T5,5 exist at the floor of the major groove whereas netropsin binds in the minor groove. It is possible that the positive Δδ of T5,2 may be a result of breaking a H-bond with solvent water upon netropsin displacement of the 'spine of hydration'. The Δδ of T5,5 and T5,6 may be a consequence of electronic rearrangement resulting from breaking an H-bond to water at TO2. Another tentative explanation is that a bound water molecule (Fig. 5a) tends to constrain the A–T pairs in their naturally propeller twisted state, or may even accentuate the twist. This would have the effect of moving these atoms away from their A4 neighbor. When netropsin binds and displaces water, T5,5 and T5,6 may then come into stronger steric contact with the A4 base, thus shielding these nuclei more [5]. The insignificant Δδ for T3,2 and the other AC4 resonances (Table 1) could be a result of replacing a hydrogen bond to water with one to netropsin.

Upon addition of netropsin, most ^{13}C resonances of $[d(GGTATACC)]_2$ experience at least small changes in chemical shift. Most of the values in Table 1 are positive for those carbons which had positive slopes in their δ vs. tempera-

Table 2
^{13}C sugar resonance assignments of $[d(GGTATACC)]_2$ and chemical shift changes upon complexation with netropsin[a]

δ (ppm)[b]	Assignments[c]	Δδ[d]
78.87[e]	G2,3′	0.10
78.72[e]	A4,3′ or A6,3′	−1.65
78.72[e]	A4,3′ or A6,3′	−0.53
78.43	G1,3′	0.18
78.05	T3,3′	−0.20
77.66	T5,3′	0.70
76.84	C7,3′	−0.32
72.93	C8,3′	0.44
68.00[f]	A4,5′ and A6,5′	−0.70 and −0.35
67.90[f]	G2,5′	0.05
67.81	C7,5′	−0.10
67.32[g]	T3,5′ and T5,5′	0.60 and 0.60
67.20[g]	C8,5′	0.08
64.40	G1,5′	0.00
42.05	C8,2′	0.30
40.84	G1,2′ or G2,2′	0.33
40.58	A4,2′ or A6,2′	−0.41
40.58	G1,2′ or G2,2′	−0.23
40.10	A4,2′or A6,2′	−0.41
39.87	C7,2′	−0.23
39.69	T3,2′	0.29
39.69	T5,2′	0.29

[a]The individual duplex carbon resonances (in the absence of netropsin) are listed by decreasing ppm.
[b]Chemical shifts of resonances of free $[d(GGTATACC)]_2$.
[c]Assingments of resonances of free $[d(GGTATACC)]_2$.
[d]Changes in duplex chemical shift upon complexation with netropsin ($\Delta\delta = \delta$ uncomplexed−δ complexed, units are in ppm). A negative Δδ indicates a downfield shift. A positive Δδ indicates an upfield shift.
[e]The A4,3′, A6,3′ and G2,3′ carbons resonante at 78.87 and 78.72 ppm; it was logically assumed that the smaller change in chemical shift belonged to the terminal G2 residue.
[f]The A4,5′, A6,5′ and G2,5′ carbons resonante at 68.00 and 67.90 ppm; it was logically assumed that the smaller change in chemical shift belonged to the terminal G2 residue.
[g]The T3,5′, T5,5′ and C8,5′ carbons resonante at 67.32 and 67.20 ppm; it was logically assumed that the smaller change in chemical shift belonged to the terminal C8 residue.

ture profiles (in the absence of netropsin) [8]. Likewise, most of the negative values for all four bases are associated with carbons having negatively sloping temperature profiles. This is probably a consequence of netropsin raising the T_m for dissociating the strands approximately 15°C [14,15,30].

Large effects on ^{13}C chemical shifts due to ring current of the netropsin pyrroles are unlikely.

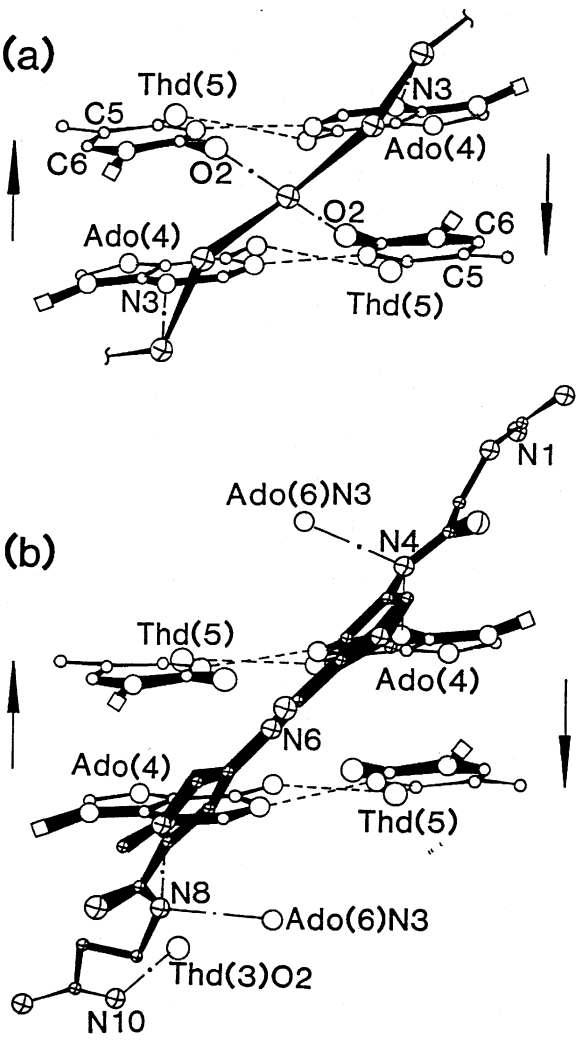

Fig. 5. A view into the minor groove of the central 5′ApT3′ step in $[d(GGTATACC)]_2$, showing (a) a portion of the spine of hydration which binds specifically to ThdO2 and AdoN3 (oxygen atoms of the water molecules (or Na^+) are illustrated as cross-hatched circles), and (b) a bound netropsin molecule (cross-hatched circles). The two ends of netropsin are slightly different, producing an asymmetric complex. Netrpsin N6 is too distant from TO2 to form classical hydrogen bonds. Netropsin N4 and N8 should form bifurcated H-bonds to A4 and A6 N3 atoms, and netropsin N10 should H-bond to T3,2O based on consideration of the X-ray model for the netropsin: $[d(CGCGAATTCGCG)]_2$ complex (Fig. 2 of [11] was used to produce this drawing). Both duplexes have the central ApT step in common, although the other residues are different; it is assumed here that the TO2/AN3 H-bond sites in the minor groove are nearly isomorphous. The C1′ atoms are indicated as squares; otherwise the diameter of the circles indicates the type of atom, increasing in the order: C, N, O. Arrows show the 5′-3′ direction of the chains.

Distances from the center of the netropsin pyrrole ring to A and T base carbons were determined by molecular graphics to be greater than 4.5 Å with most in the range of 5–6 Å. At these distances and considering the angular dependence of the ring current effect, none of the carbons should be affected by more than 0.1 ppm [31,32].

4.3. Sugar pucker effects on ^{13}C chemical shifts

The ^{13}C deoxyribose and base signals of DNA are largely influenced by different factors. The ^{13}C deoxyribose signals are particularly sensitive to sugar pucker conformation, backbone torsion angles and the 'χ' torsion angle (see Fig. 1e). Lankhorst et al. [9] determined sugar pucker conformation of small RNA di- and trinucleotides using ^{1}H NMR methods and concurrently measured ^{13}C chemical shifts. They report a direct correlation of sugar pucker and chemical shift change. The other factors affecting ^{13}C $\Delta\delta$ are apparently small compared to the effects of sugar pucker.

Another ^{13}C study, including our molecule $[d(GGTATACC)]_2$, showed that there were large changes in chemical shifts of deoxyribose signals upon duplex association from single strands [10]. Negative slopes characterized most of the A and G chemical shift vs. temperature profiles, whereas positive slopes were usually observed for the C and T profiles. At high temperature the distribution of pseudorotational states for the eight nucleotides are similar. As temperature is decreased the distribution changes. These trends were consistently observed for all sugar carbons (except C1′) of $[d(GGTATACC)]_2$ and other DNA oligomers. In agreement with ^{1}H NMR and X-ray work, at least part of the differences in sugar pucker may be attributed to interstrand purine base clashes in the minor groove. A recent theoretical and experimental study also confirms the sensitivity of ^{13}C chemical shifts to sugar pucker [33].

4.4. Sugar pucker and netropsin: $[d(GGTATACC)]_2$

Large changes in chemical shifts were observed for deoxyribose resonances of the central 'TATA' nucleotides as indicated in Table 2. Unfortunately, the C1′ and C4′ resonances could not be assigned for the complex therefore, only $\Delta\delta$ values for C2′, C3′ and C5′ are listed. The $\Delta\delta$ values for the deoxyribose resonances of G and C nucleotides were smaller, suggesting that netropsin binds to the A–T rich center of $[d(GGTATACC)]_2$. It is interesting to note in Table 2 that the pyrimidines (residues T3 and T5) have positive $\Delta\delta$ values (except T3,3′) whereas the purines (residues A4 and A6) have negative $\Delta\delta$ values. What could be the source of the changes in chemical shifts and why do the purines and pyrimidines consistently have opposite signs for $\Delta\delta$?

The changes in chemical shifts of the deoxyribose resonances are most likely due to changes in the distribution of pseudorotational states that the sugars occupy. Upon binding netropsin, most sugar resonances of all nucleotides change to some degree. This is probably a consequence of netropsin raising the T_m for dissociating the strands by 15°C [14,15,30]. Hence, the expected changes in chemical shifts would give rise to opposite signs for purines and pyrimidines.

The large $\Delta\delta$ values in Table 2 may be from modifications of the occupied pseudorotational states beyond that of stabilization of the helix. A possible explanation for the changes may be due to alterations of the glycosyl torsion angles. Replacing the H-bonds between the bases to the spine of hydration with those to the netropsin molecule may force the bases to occupy different gylcosyl torsion angles (even if slightly). The large $\Delta\delta$ values of Table 2 suggests that this may be occurring for the four central 'TATA' nucleotides; however one nucleotide is affected to a larger degree as indicated by the $\Delta\delta$ value of $C3' = -1.65$ for either A4 or A6. In considering the ^{13}C $\Delta\delta$ values of the base carbons T5,5 and T5,6, the large change may be the A4 nucleotide. It is possible that a change in the glycosyl torsion angle of the A4 residue could force the A4 and T5 bases into strong steric contact (this thus takes into account the large shielding $\Delta\delta$ of T5,5 and T5,6 base carbons).

Given the system being studied here (a DNA

oligonucleotide interacting with a minor groove binding drug), ring current effects from netropsin should be apparently small due to the inherent distance and angular dependence. The distances would have to be very short and the carbon atom directly above or below the pyrrole ring or carbonyl group to give such large Δδ values [31]. Neither ring current nor steric contact effects would be expected to consistently result in opposite signs for purines and pyrimidines as observed for all three carbon classes listed in Table 2; however, it may explain the exception of the unusual Δδ sign of T3,3′.

4.5. Comparison with X-ray and ^{1}H NMR data of the bases

Dickerson's laboratory has proposed that water binds in the minor groove of A–T regions of B-DNA in a 'spine of hydration' [22,23]. This model has been recently revised based upon a very high resolution X-ray structure that shows an inner spine composed of Na^+ ions and water, and an outer spine of water [34]. From X-ray data, Dickerson's work indicates that netropsin displaces as many as 12 of the water/Na^+ in the inner spine; the spine components bind to each other, TO2, and AN3 [11,16,23]. Fig. 5a shows the central two base pairs in the duplex $[d(CGCGAATTCGCG)]_2$ and the oxygens (or Na^+) of five of the waters in the spine. TO2 and AN3 are nearly isomorphous in a regular B-DNA geometry, so any sequence of A–T pairs allows the spine to form; at least four contiguous A–T pairs are required for netropsin to bind with maximum strength. Fig. 5b shows that netropsin displaces the specifically bound waters/Na^+ and that its amide nitrogens are located in similar positions to the water oxygens (or Na^+) in Fig. 5a. The pyrrole rings conflict sterically with the adenine bases, forcing netropsin N6 to be approximately 3.7 Å from the H-bond acceptors at T5,O2; this is too distant to form H-bonds. Patel and Shapiro [14,15] also report from ^{1}H-NOESY data that the netropsin pyrrole protons are in close contact with the AH2 protons of $[d(GGTATACC)]_2$. They and others [25] further suggest that these close contacts rule out the presence of water/Na^+ molecules being sandwiched between the netropsin and $[d(GGTATACC)]_2$ at the binding site. In our study, a large positive $\Delta\delta = 1.36$ ppm at T5,2 of $[d(GGTATACC)]_2$ is consistent with loss of a H-bond with water/Na^+ and lack of formation of a H-bond with netropsin at the T5,O2 sites.

Table 3
Approximate average distances between nearby hydrogen bond donor and acceptor sites in the netropsin: $[d(GGTATACC)]_2$ complex[a]

Netropsin donor	DNA acceptor	Average N–N or N–O distance (Å)
N6H	T5,O2	3.7
N1H or N10H	T3,O2	3.2
N4H or N8H	A4,N3	3.4
N4H or N8H	A6,N3	2.6

[a]Adapted from the netropsin: $[d(CGCGAATTCGCG)]_2$ complex [11,16].

Patel and Shapiro [14] state that in rapid exchange netropsin would unbind and jump rapidly between two states, the one shown in Fig. 5b and one where N1 is at the lower left. The H-bond distances from the Dickerson data in Table 3 should closely describe the averaged complex. The Dickerson model shows that the outer netropsin amides should form short H-bonds to A6,N3 which may be the source of the deshielding effect for one AC4 resonance in our data. For the other TC2 and AC4 resonances, replacing a H-bond to water/Na^+ with one to netropsin at T3 of TO2 and AN3 apparently has little effect on T3,2 and the AC4 resonance.

In the same studies, Dickerson and coworkers have also shown that the binding of netropsin to $[d(CGCGAATTCGCG)]_2$ has little effect on the DNA structure. The structural changes observed were a base displacement at the T8-C9 step and an induced 8° bending back of the helix axis away from the binding sites at the point of attachment. In the netropsin: $[d(GGTATACC)]_2$ complex, the T5 base may be involved in base displacement and helix bending. ^{1}H-NOESY cross-peaks [14] could not be used to identify this in the netropsin: $[d(GGTATACC)]_2$ complex since line broadening

in the 'TATA' portion complicated structural conclusions.

Patel and Shapiro [14] also measured Δδ for the ^{1}H resonances of $[d(GGTATACC)]_2$ upon binding netropsin. There were three resonances which had larger Δδ values than 0.2 ppm. The A4,H2 and A6,H2 resonances became deshielded by 0.49 and 0.40 ppm. Since the AH2 protons are juxtaposed with the netropsin pyrrole rings, the source of Δδ is most likely from a steric factor and ring-current deshielding by the netropsin pyrrole rings (Fig. 5). The T5,H6 resonance was shielded by 0.30 ppm. This is consistent with the shielding observed for T5,C6 and T5,C5 and provides additional support that the assignments of T5,5 and T5,2 are correct.

4.6. Comparisons with X-ray and ^{1}H NMR data of the sugars

Dickerson's laboratory reported that netropsin binding has little effect on $[d(CGCGAATTCGCG)]_2$. They indicated that the overall changes in sugar pucker (given here as the average change in the sugar torsion angle approximately the C4′–C3′ bond-see Fig. 1e) was 7.6° for the purines and 2.5° for the pyrimidines. This is consistent with the hypothesis that ^{13}C Δδ of the deoxyribose ring is sensitive to sugar pucker changes [10] given that the observed ^{13}C Δδ values of the 'TATA' nucleotides are 0.67 for the purines and 0.45 for the pyrimidines. Dickerson's laboratory also reports a base displacement at the T8-C9 step of $[d(CGCGAATTCGCG)]_2$ and an induced 8° bending back of the helix away from the binding site at the point of attachment. Given the base and sugar data reported here, it is possible that a significant local change occurs for $[d(GGTATACC)]_2$ at the A4-T5 step. Differences between the X-ray and ^{13}C NMR data may arise from the fact that two different DNA sequences were studied and that the X-ray structure shows the netropsin molecule in one orientation whereas the NMR data is from the netropsin molecule in equilibrium binding between two orientations.

Patel and Shapiro [13] have measured Δδ for the sugar ^{1}H resonances of $[d(GGTATACC)]_2$ upon binding netropsin. There were six relatively large Δδ values −0.21 for A6,H3′, 0.30 for T3,H2′, 0.34 for A6,H2′, −0.30 for T3,H1′, −0.36 for T5,H1′ and −0.57 for A6,H1′. These changes occur for the central 'TATA' nucleotides and the largest changes are at the T3 and A6 residues. Unlike ^{13}C Δδ, qualitative assessment of changes in sugar pucker are impossible by ^{1}H Δδ alone given the uninterpretable melange of factors influencing ^{1}H shifts (e.g. sugar pucker and steric contact effects, and anisotropic effects from the netropsin pyrrole ring and carbonyl groups). The consistent purine/pyrimidine pattern observed for the ^{13}C Δδ values for the 'TATA' nucleotides in Table 2 is not found for the ^{1}H Δδ values. Some ^{1}H Δδ values have opposite sign as the ^{13}C Δδ values of the carbon to which the proton is bonded. In addition, the signs of ^{1}H Δδ are the same for the purines and pyrimidines of a proton class. In conclusion, it may be safest to claim that the ^{1}H Δδ values indicate that netropsin binds to the 'TATA' site.

Patel and Shapiro [13] have studied the two-dimensional NOESY spectra of $[d(GGTATACC)]_2$ uncomplexed and when bound to netropsin. They state that interpretation of changes in NOE cross-peak intensities is complicated by resonance broadening due to equilibrium binding of netropsin between two orientations. The largest number of changes of NOE's occurred between the bases of T5 and A6 nucleotides with the sugar protons of the same nucleotide. This may be a result of changes in sugar pucker, although the observed differences in NOE cross-peaks may be due to changes in ^{1}H–^{1}H distances and/or to resonance broadening. It is possible that the observation of a weaker NOE between the base A6,H8 and sugar ^{1}H of T3,H1′ is consistent with the changes between the A4 and T5 nucleotides as suggested by this ^{13}C work.

4.7. Binding mode and titration curves of netropsin with DNA

Equilibrium constants for netropsin binding to DNA molecules with A–T tracts of 4–5 base-pairs have been reported to be in the range of 10^{7}–10^{8} in 20 mM Na^{+} (summarized in [19]). The complexes exhibited 1:1 netropsin/duplex stoi-

chiometry, and had apparently simple binding isotherms.

The titration profiles in Fig. 4 are consistent with a 1:1 stoichiometry at saturation. However, the sigmoidal shapes are not consistent with a simple binding equilibrium. How can a 1:1 system give the appearance of cooperativity in binding? The system cannot be a simple one-step complex formation. Presumably, the water–sodium ion spine [34–36] is reasonably stable, and a threshold concentration of netropsin is required to disrupt it. Further analysis and experimentation are planned to explore and parameterize the multiple equilibria.

We must also consider why this sigmoidal behavior was not apparent in other studies using calorimetry, van't Hoff analysis of UV melting, and circular dichroism [19] or proton NMR [12,14,15,24,30]. As there are probably issues due to Na^+ binding and concentration-dependent equilibria, part of the discrepancy may be due to differences in solution conditions. The non-NMR studies were routinely performed on samples at approximately 1 μM concentration in 0.02 M Na^+, whereas the NMR work was done at ~10 mM DNA and netropsin in 0.1 or 0.2 M Na^+. It is also possible that multiple equilibria in thermodynamic analyses can be masked if transitions with similar enthalpies and heat capacities are involved. This might be the case for the DNA–spine and DNA–netropsin complexes, where the number of hydrogen-bonds is nearly conserved. The circular dichroism induced in the netropsin chromophore upon binding DNA was monitored at 310 nm [19], a wavelength where the response to binding is small. Sigmoidally curved profiles could be drawn within the apparent error limits on the CD binding isotherms (see Fig. 4 of [19]). The published analyses monitoring netropsin binding by ^{1}H-NMR [12,14,15,24,30] did not routinely plot chemical shift profiles in the range of low netropsin concentration where the unusual effects on ^{13}C chemical shifts are most pronounced. Finally, hydrogen bonding has only indirect effects on non-exchangeable ^{1}H-NMR, CD, and thermodynamic measurements; perhaps it should not be surprising that the existence of multiple equilibria would be revealed in ^{13}C chemical shifts known to be exquisitely sensitive to H-bonding effects [7].

5. Conclusion

The experiments described here were designed to explore the effects of H-bonding and sugar puckering on the chemical shifts of the DNA carbon resonances. When the ^{13}C Δδ were analyzed without considering other studies, the results were consistent with formation of H-bonds from netropsin to AN3 in the minor groove of the duplex. The observation is a shielding decrease at two of the AC4 atoms. The observation of large Δδ of the sugar resonances is consistent with changes in sugar pucker and changes of glycosyl torsion angles. The sugar and base Δδ values considered together suggest local changes at the A4–T5 step. In light of other studies, our results are consistent with: (1) specific hydration at TO2, supporting the idea that a 'spine of hydration' is disrupted upon netropsin binding in the minor groove of A–T-rich DNA segments; (2) replacement of H-bonds (e.g. to water/Na^+) with new ones in a complex may produce no large changes in chemical shift; and (3) changes in sugar pucker and glycosyl torsion angles.

This work reinforces the idea that changes in ^{13}C chemical shifts are related to sugar pucker and to the formation or loss of specific hydrogen bonds between nucleic acids and ligands. For systems in which there is no conclusive X-ray or ^{1}H NOE data, our experiments suggest that ^{13}C NMR spectroscopy may provide a technique for distinguishing the positions on nucleic acid bases involved in H-bonds and changes in sugar pucker.

It may be possible to use characteristic changes in ^{13}C chemical shift to locate the sites involved in hydrogen bonding interactions in nucleic acid self-complexes, and in complexes with proteins, drugs, and various other ligands. ^{13}C NMR may also provide a technique for monitoring changes in the state of hydration and dynamics [37] of nucleic acids. Experiments are planned to test the effects of H-bonding at other sites in the major and minor grooves, as well as in RNA duplexes, non-Watson–Crick base pairs and DNA–interca-

lator interactions. Given sufficient local mobility, it should be possible to extend the method to complexes in the molecular weight range of 100 000 Da. For such large complexes, use of ^{13}C-enriched nucleotides would increase the sensitivity of chemical shift observation by a factor of 100, greatly simplify the spectra, and allow unequivocal assignments.

Finally, it is worth mentioning that monitoring the ^{13}C shifts by direct detection, one-dimensional ^{13}C spectra requires a relatively high concentration of DNA ($\sim$ 10 mM used here). Lower concentrations may be used by applying the more sensitive, single-bond (HSQC) and multiple-bond (HMBC) ^{1}H–^{13}C correlation experiments.

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