Nitrogen-15-Labeled Oligodeoxynucleotides. 6. Use of ^{15}N NMR To Probe Binding of Netropsin and Distamycin to {d[CGCGAATTCGCG]}$_2$

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Abstract: The binding of netropsin and distamycin to the duplex {d[CGCGAATTCGCG]}$_2$, in which adenine residues A5 or A6 were [3-^{15}N] labeled, was monitored by ^{15}N NMR to probe the sensitivity of these ^{15}N labels to drug binding. The ^{15}N chemical shifts were obtained from a ^1H–^{15}N heteronuclear 2D experiment. The ^{15}N atoms in the asymmetric complexes were assigned with use of, in addition, a ^1H–^1H 2D NOESY experiment. The results for both complexes show that water is excluded from the center of the binding site, and that there is hydrogen bonding to the N3 at the common propylamidinium termini of the drugs. Further, in the distamycin complex, there is a second drug·H bond at the formamide terminus of the drug. These results demonstrate that these ^{15}N labels are useful monitors of drug binding.

Introduction

NMR of ^{15}N-labeled DNA and RNA fragments is finding increasing use for determination of nucleic acid structure.[1–19] Studies in this laboratory using specifically ^{15}N-labeled DNA fragments have focused on nitrogen chemical shift changes, where the ^{15}N label functions as a local probe of hydrogen bonding or protonation, and also can be used for unambiguous assignment of a particular ^1H resonance.[13–19] Other researchers, using fully or partially ^{15}N- and ^{13}C-labeled RNA fragments, have concentrated on the ability of multidimensional NMR to resolve overlapping ^1H resonances, thereby greatly enhancing and extending the ability of NMR to provide three-dimensional structural information.[9–12]

In recent work probing tetraplex structures of small guanine-rich DNA fragments, comparison of the ^{15}N chemical shifts of the tetraplex, duplex, and single-strand species present suggested that the ^{15}N might also be a monitor of hydration.[17] Hydration may be regarded as the simplest, and most ubiquitous, ligand interaction of nucleic acids. The work presented below uses two well-studied drug·DNA complexes to probe the ability of [3-^{15}N]-labeled adenine residues in the complexes to define features of the drug binding. The results demonstrate that these ^{15}N labels are sensitive both to drug·DNA hydrogen bonding and to the exclusion of water in the center of the binding site.

The binding of netropsin and distamycin to {d[CGCGAATTCGCG]}$_2$, and to related molecules, has been studied extensively by both X-ray[20–23] analysis and NMR.[24–30] These studies have shown that binding of each of these drugs occurs at A·T rich sites, and that it involves replacement of the B-DNA spine of hydration with the drug. In the work reported below we have used netropsin and distamycin complexes of d[CGCG($^{15}N^3$)AATTCGCG] (**1**) and d[CGCGA($^{15}N^3$)ATTCGCG] (**2**). The [3-^{15}N]-2′-deoxyadenosine used in this work was prepared as reported previously.[31]

Results

Assignment of ^{15}N Resonances in the Drug·DNA Complexes. A diagrammatic representation showing the numbering of the netropsin and distamycin complexes with {d[CGCGAATTCGCG]}$_2$ is presented in Figure 1. Upon binding of either netropsin or distamycin, the symmetry of the DNA duplex is removed, so that there are two adenine N3 resonances for the single [3-^{15}N] label in d[CGCG($^{15}N^3$)AATTCGCG] (**1**) and d[CGCGA($^{15}N^3$)ATTCGCG] (**2**) in each complex. The adenine [3-^{15}N] resonances were identified (and detected) by their coupling

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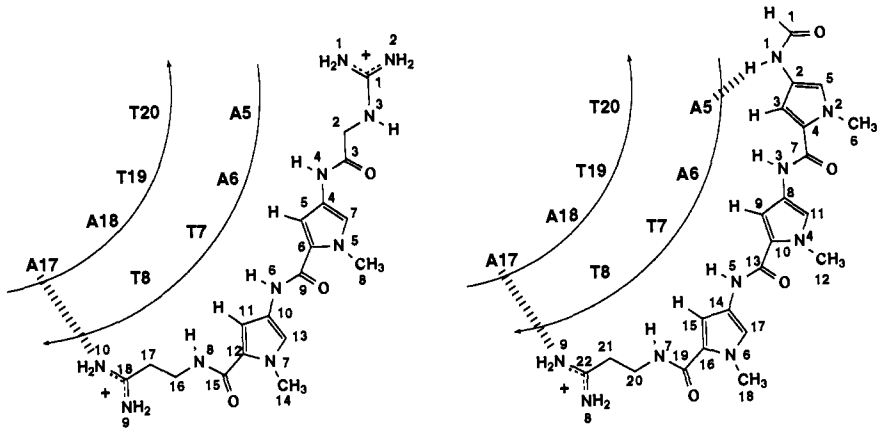

Figure 1. Diagrammatic representation of (A, left) the netropsin·{d[CGCGAATTCGCG]}$_2$ complex and (B, right) the distamycin·{d[CGCGAATTCGCG]}$_2$ complex, showing the drug numbering and the numbering of the AATT binding sites. The dashed lines indicate hydrogen-bonding interactions between the indicated adenine N3 atoms and the drug.

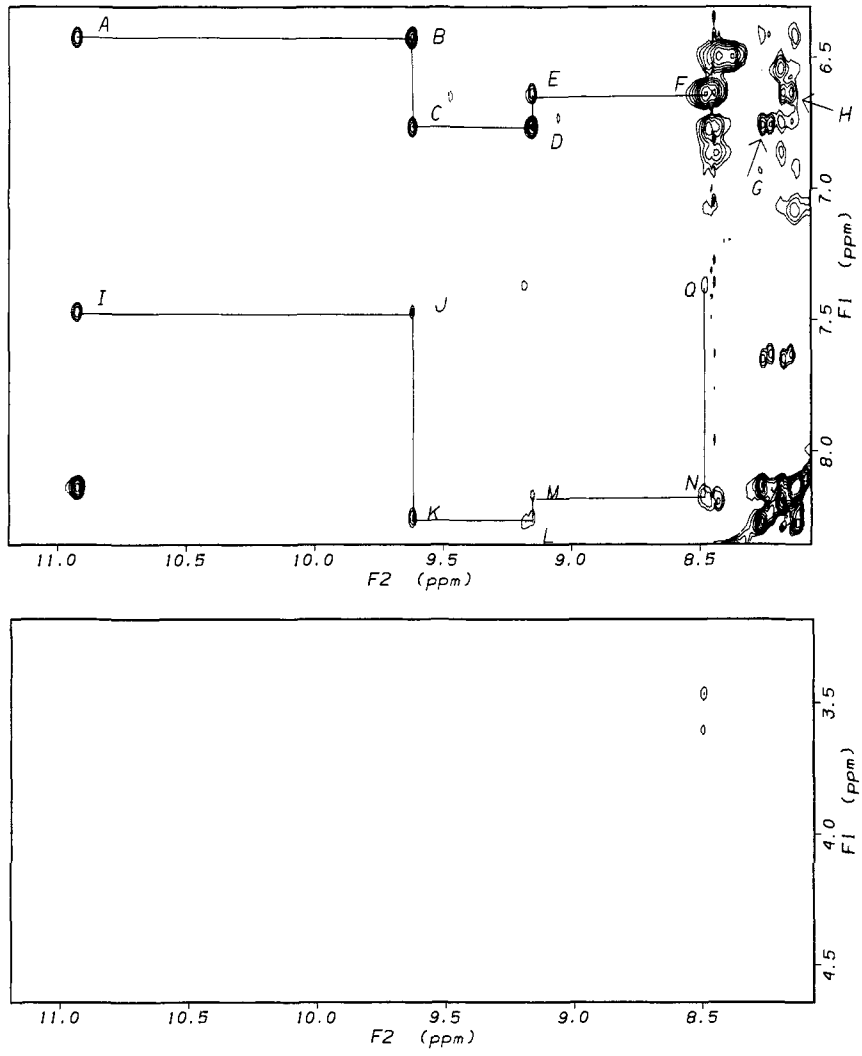

Figure 2. 2D NOESY spectra of the distamycin·**2** complex showing (A, top) cross peaks and connectivities between the distamycin pyrrole protons and amide protons, cross peaks between the amide protons and the adenine C2 protons, and (B, bottom) cross peaks between the distamycin N7H (~ 8.5 ppm) and the distamycin propylamidinium methylene protons (~3.5 ppm). The labeled cross peaks in part A are the following: A, N1H–C3H; B, N3H–C3H; C, N3H–C9H; D, N5H–C9H; E, N5H–C15H, F, N7H–C15H; G, A6 C2H–C9H; H, A18 C2H–C15H; I, A5 C2H–N1H; J, A5 C2H–N3H; K, A6 C2H–N3H; L, A6 C2H–N5H; M, A18 C2H–N5H; N, A18 C2H–N7H; Q; A17 C2H–N7H. The spectrum was recorded at 500 MHz and 15.0 °C, with a mixing time of 150 ms, a t_1 acquistion time of 0.03 s, a t_2 acquisition time of 0.2048 s, and a total acquisition time of 26 h. The NOESY experiment was performed with use of a jump-and-return sequence for the last pulse,[41] with the maximum excitation 7 ppm from the water resonance. A 0.02-s homospoil pulse was applied at the beginning of the mixing period to improve spectrum quality.

to the adenine C2 proton. The adenine C2 protons of the drug·DNA complexes were assigned from their NOEs to the drug amide protons. The ^{1}H–^{1}H 2D NOESY spectra used for these assignments are shown in Figures 2 and 3. For both complexes, the resonance at ~8.5 ppm was assigned to the amide proton closest to the propylamidinium end of the drug because

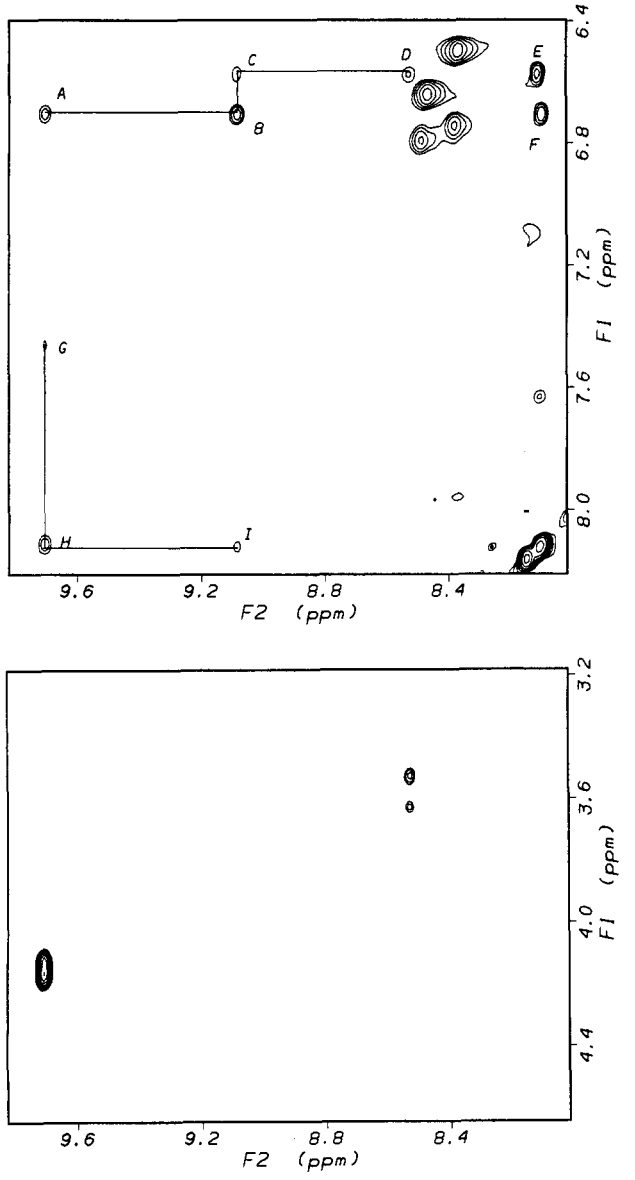

Figure 3. 2D NOESY spectra of the netropsin·**1** complex showing (A, top) cross peaks and connectivities between the netropsin pyrrole protons and amide protons, cross peaks between the amide protons and the adenine C2 protons, and (B, bottom) cross peaks between the netropsin N8H (~8.5 ppm) and the netropsin propylamidinium methylene protons (~3.5 ppm) as well as a cross peak between the netropsin N4H (~9.7 ppm) an the glycine methylene protons at the guanidinium terminus. The labeled cross peaks in part A are the following: A, N4H–C5H; B, N6H–C5H; C, N6H–C11H; D, N8H–C11H; E, A18 C2H–C11H; F, A6 C2H–C5H; G, N4H–A5 C2H; H, N4H–A6 C2H; I, N6H–A6 C2H. The spectrum was obtained as described for Figure 2.

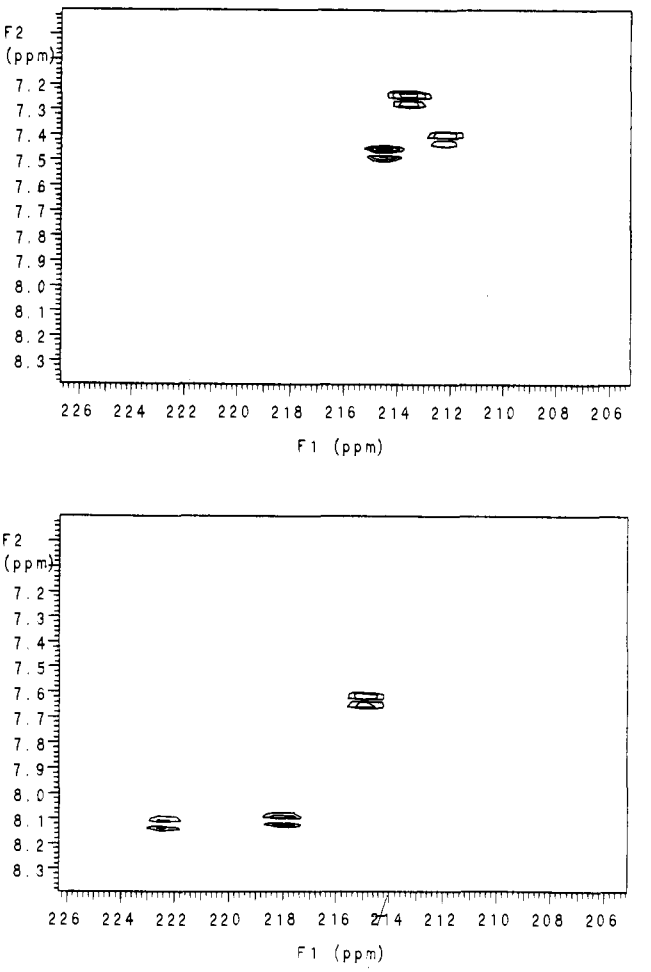

Figure 4. Representative 2D ^1H–^{15}N HSQC spectra of (A, top) the 0.4:1 netropsin·**1** sample and (B, bottom) the 0.4:1 netropsin·**2** sample, at 30 °C. The data were obtained by using an anti-phase version of the HSQC experiment: $90°(H_x)$–$1/(4 * {}^2J_{NH})$–$180°(H)$, $180°(N)$–$1/(4 * {}^2J_{NH})$–$90°(H_y)$, $90°(N_{\pm x})$–$t_1/2$–$180°(H)$–$t_1/2$–$90°(H_x)$, $90°(N_x)$–Acq.(±). The spectra were recorded at a ^1H frequency of 500 MHz with a resolution of 0.6 ppm/point for the ^{15}N dimension. Two to four transients for each FID were acquired at a total acquisition time of 2–15 min.

of its NOE to two of the propylamidinium methylene protons which resonate at ~3.5 ppm, shown in Figures 2B and 3B. Further, in the netropsin complex, the amide proton at 9.71 ppm is identified as the N4H because of its NOE to the C2 methylene protons at the guanidinium end (Figure 3B). The connectivities between the amide protons, the pyrrole protons, and the adenine C2 protons are shown in Figures 2A and 3A. These ^1H assignments are consistent with those reported previously by other researchers for similar or identical complexes.[24-27] Assignment of the A5 and A17 N3 resonances of the drug·**1** complexes, and the A6 and A18 N3 resonances of drug·**2** complexes, was then made by using the corresponding ^1H–^{15}N heteronuclear 2D HSQC spectra. An example of the latter is shown in Figure 4.

Hydrogen Bonding. The chemical shifts of the N3 label in **1**, both with and without drug,[32] are listed in Tables I–III (Supplementary Material) and plotted in Figure 5, over a temperature range of 0–50 °C. It was not possible to follow the chemical shift through the melting transition because of signal broadening. In both the netropsin and distamycin complexes, the N3 resonance of A17 is shifted upfield, relative to its position in the free duplex, by ~2 ppm. Hydrogen bonding to an sp^2 nitrogen is expected to bring about an upfield shift of the ^{15}N resonance.[33] The upfield shift observed thus supports hydrogen bonding between this adenine N3 and the terminal propylamidinium group. In an X-ray structure of a netropsin·{d[CGCGAATT(5-Br)CGCG]}$_2$ complex the only drug·DNA H-bonding interaction that was within 3 Å (2.65 Å) was that between the A17 N3 and one of the drug propylamidinium amino groups.[20]

The A5 resonances, in contrast to the A17 resonances, are shifted in opposite directions upon drug binding. In the netropsin complex the A5 N3 is shifted slightly downfield, while in the distamycin complex there is an upfield shift analogous to that of the A17 N3. These data support H bonding to the A5 N3 in the distamycin complex, but not in the netropsin complex. In fact, the only potential H-bonding interaction under 3 Å that was found in the ^1H NMR structure of the distamycin complex was that between the A5 N3 and the distamycin formamide N1 H.[27]

(32) A drug·DNA ratio of 0.4 to 1 was used for this experiment, so that each sample contained free DNA.

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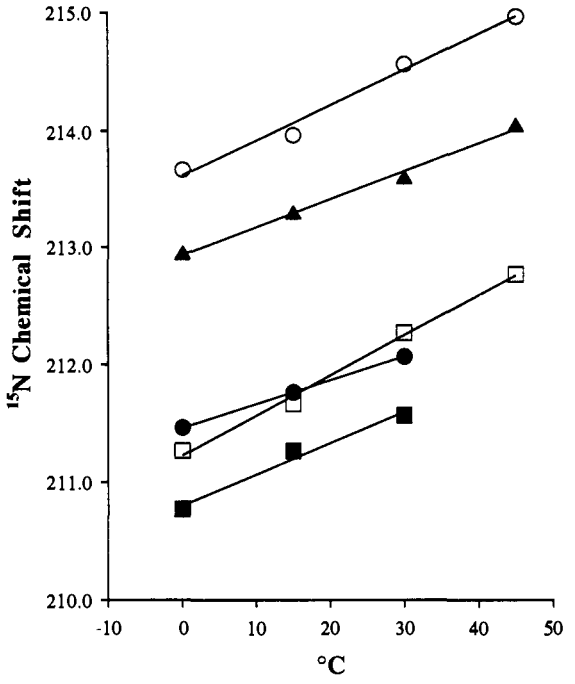

Figure 5. Plot of the ^{15}N chemical shift vs temperature data in Tables I–III (see supplementary material) for **1** in each drug complex and without drug: (■) A17 N3 in the **1**·distamycin complex; (□) A17 N3 in the **1**·netropsin complex; (●) A5 N3 in the **1**·distamycin complex; (○) A5 N3 in the **1**·netropsin complex; (▲) average of the chemical shifts of the N3 in the absence of drug.

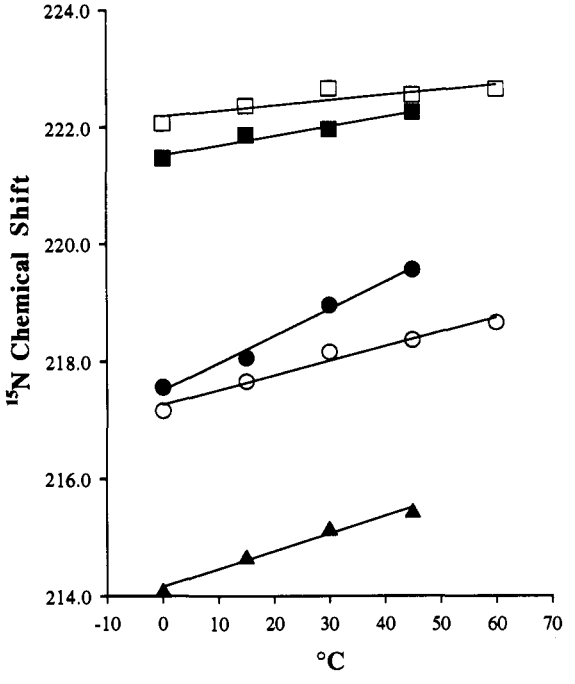

Figure 6. Plot of the ^{15}N chemical shift vs temperature data in Tables IV–VI (see supplementary material) for **2** in each drug complex and without drug: (■) A18 N3 in the **2**·distamycin complex; (□) A18 N3 in the **2**·netropsin complex; (●) A6 N3 in the **2**·distamycin complex; (○) A6 N3 in the **2**·netropsin complex; (▲) average of the chemical shifts of the N3 in the absence of drug.

No drug·DNA H bond was found by X-ray analysis between the A5 N3 and netropsin.[20,23]

Exclusion of Water. The chemical shifts of the N3 label in **2**, both with and without each drug,[32] are listed in Tables IV–VI (Supplementary Material) and plotted in Figure 6, over a temperature range of 0–60 °C. In this case, the N3 resonances are shifted nearly identically for both drug·DNA complexes. Thus, both A6 resonances are shifted downfield by ~3 ppm, while both A18 resonances are shifted downfield by ~7 ppm. These large downfield shifts are consistent with tight binding of each drug, with resulting exclusion of water from the center of the drug binding site. This exclusion of water was also noted in previous X-ray and ^{1}H NMR studies.[20–23,25,27]

Discussion

Tight ligand binding at a particular site in a nucleic acid may give rise to replacement of hydrogen bonds to water molecules with hydrogen bonds to the ligand. Alternatively, the water·nucleic acid hydrogen bonds may be displaced in favor of ligand·nucleic acid van der Waals contacts. Both types of ligand·nucleic acid interaction are present in the drug·DNA complexes examined here. Where the hydration of the adenine N3 atom is replaced with a drug H-bonding interaction, an upfield shift of the order of 2 ppm is observed. In contrast, where the water spine is replaced not by a drug H bond but by van der Waals interactions, the shift is both downfield and larger (3–7 ppm). The direction of the ^{15}N chemical shift changes with increasing temperature that we and others have observed in both monomers and short DNA fragments is uniformly downfield for an H bond acceptor nitrogen and upfield for an H bond donor nitrogen.[2,13,17,19,34–36] Further, in the present study, the direction of chemical shift change observed is consistent with the known features of these well-studied complexes.

Overall, the ^{15}N data reported here show differences in the complexes only at the guanidinium/formamide termini, where the drugs differ in structure. The behavior of the A17, A18, and A6 N3 resonances is nearly identical for each complex, while the A5 chemical shift change is opposite for the two complexes. The A5 chemical shift change shows drug H bonding to this N3 atom in the distamycin complex, but not in the netropsin complex, in accord with expectations. The drug·DNA hydrogen bonding to the A17 N3 seen here for both drugs was found also in X-ray structures of netropsin complexed with {d[CGCGAATT(5-Br)-CGCG]}$_2$[20] and with {d[CGCGATATCGCG]}$_2$,[22] although it was not observed in the ^{1}H NMR structure of a distamycin·{d[CGCGAATTCGCG]}$_2$[27] complex, or in the X-ray structure of a netropsin·{d[CGCGAATTCGCG]}$_2$[23] complex. The downfield shift of the A6 and A18 resonances is in accord with disruption of hydration at these two sites, as expected, although the origin of the difference in magnitude of the shifts is not apparent. Additional experiments with other complexes will be necessary to probe further the limits of the ability of the ^{15}N chemical shift to define drug·DNA interactions.

Summary

The results presented above demonstrate that the ^{15}N chemical shifts of two ^{15}N-labeled drug·DNA complexes can be used to identify sites of drug·DNA hydrogen bonding and sites of exclusion of water. This type of information should be generally useful for defining drug or other ligand binding sites, particularly for larger molecules that may not be amenable to detailed X-ray or ^{1}H NMR structure determination.

Experimental Section

General Methods. The ^{1}H and ^{15}N NMR spectra were recorded on Varian XL-400 or VXR-500 spectrometers. Oligonucleotide syntheses were carried out on a Milligen/Biosearch 8750 synthesizer. Reversed-phase HPLC was performed on a system consisting of a Waters 6000A pump and U6K injector, with an Autochrom CIM to allow a single pump gradient, and either a Waters 440 detector for analytical work or a Beckman 153B detector for preparative separations. The purification

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and analyses of **1** and **2** were carried out with use of the columns and conditions listed below.

Synthesis and Purification of d[CGCG($^{15}N^3$)AATTCGCG] (1) and d[CGCGA($^{15}N^3$)ATTCGCG] (2). The syntheses were carried out by an H-phosphonate method with a tentagel polystyrene/polyethylene glycol support.[37,38] The crude products were purified by HPLC both before and after detritylation on a Beckman Ultrapore C-3 reversed-phase column (10 mm × 25 cm). The first purification used a gradient of 2–50% acetonitrile:0.1 M triethylammonium acetate in 45 min at a flow rate of 2 mL/min. Detritylation was effected with use of 0.1 M acetic acid for 20–40 min. The second purification used a gradient of 2–20% acetonitrile: 0.1 M triethylammonium acetate in 45 min at a flow rate of 2 mL/min. A third purification using a gradient of 2–20% acetonitrile:0.1 M ammonium acetate in 45 min at a flow rate of 2 mL/min was necessary for these molecules. Impure fractions were combined and repurified. Purification of these molecules is complicated by the persistence of the duplex form during chromatography, which causes **1** and **2** to elute as two peaks on analytical HPLC. Analytical HPLC was carried out on a Beckman C-3 Ultrapore column (4.6 mm × 7.5 cm), using a gradient of 2–20% acetonitrile:0.1 M triethylammonium acetate in 5 min at a flow rate of 2 mL/min. The pure products were converted to the sodium form by ion exchange with sodium form Bio-Rad AG50W-X4 resin, after hplc using the C-3 column with acetonitrile and 0.1 M ammonium bicarbonate (2–25% in 15 min at a flow rate of 2 mL/min) to remove all triethylammonium acetate. The ratio of monomers produced upon enzymatic degradation[39] of the purified molecules was determined by integration of the peaks obtained by HPLC on a Waters C-18 Novapak column using Baseline chromatography software. The results of the syntheses are summarized in Table VII (Supplementary Material). Chromatograms of **1** and **2** after purification, and after enzymatic degradation, also are included in the Supplemental Material.

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Supplementary Material Available: Tables of ^{15}N chemical shifts of **1** and **2**, both with and without each drug, a summary of the syntheses of **1** and **2**, and HPLC profiles of **1** and **2**, after purification and after enzymatic degradation (4 pages). Ordering information is given on any current masthead page.

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