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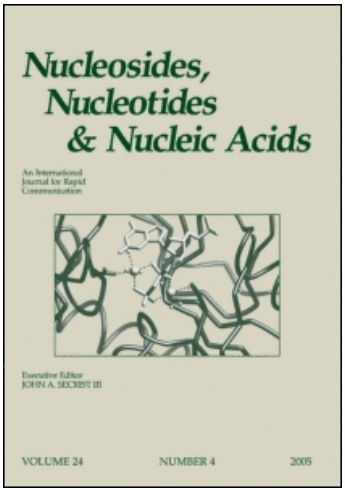

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SYNTHESIS OF DUPLEX DNA CONTAINING A SPIN LABELED ANALOG OF 2'-DEOXYCYTIDINE

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Abstract. We report the chemical synthesis of phosphoramidite **8**, containing a spin labeled analog of deoxycytidine, C*, and its incorporation into synthetic DNA. The EPR characteristics of the resulting DNAs indicated that the motion of the spin label was well-correlated with the uniform modes of the macromolecule, but that correlation of the spin label with internal motion was less effective than that achieved using a spin labeled quinolone, Q.

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INTRODUCTION

Duplex DNA is a conformationally dynamic molecule. The finding that DNA in a variety of DNA-protein complexes is substantially reorganized relative to the canonical double helix suggests that these dynamics may be important in the biological function of DNA.[1] It is possible that the sequence-dependent dynamical properties of DNA will play a role in determining the sequence-dependent recognition properties of DNA. For this reason, we have for some years pursued the goal of determining the dynamics of nucleic acids as a function of nucleotide sequence. We selected electron paramagnetic resonance (EPR) spectroscopy as the experimental approach, because the motions of molecules which occur anywhere from the millisecond to nanosecond time scale can be monitored by this technique. EPR possesses the additional advantage that very small quantities of sample are required compared to many other spectroscopies. An obstacle in applying EPR to the study of nucleic acids is the absence in these substances of an unpaired electron. Not only must this unpaired electron be incorporated to make EPR spectroscopy useful in this regard, but the motion of this electron must be correlated with the DNA motion.

Our earliest work reported the spin labeling of DNA with two analogs of thymidine.[2-5] The first of these was the thymidine analog T*, which was incorporated into duplex DNA as in base pair **1**[2,4]. While the availability of DNA containing this base pair was a considerable advance, there remained some ambiguity as to how well the motion of

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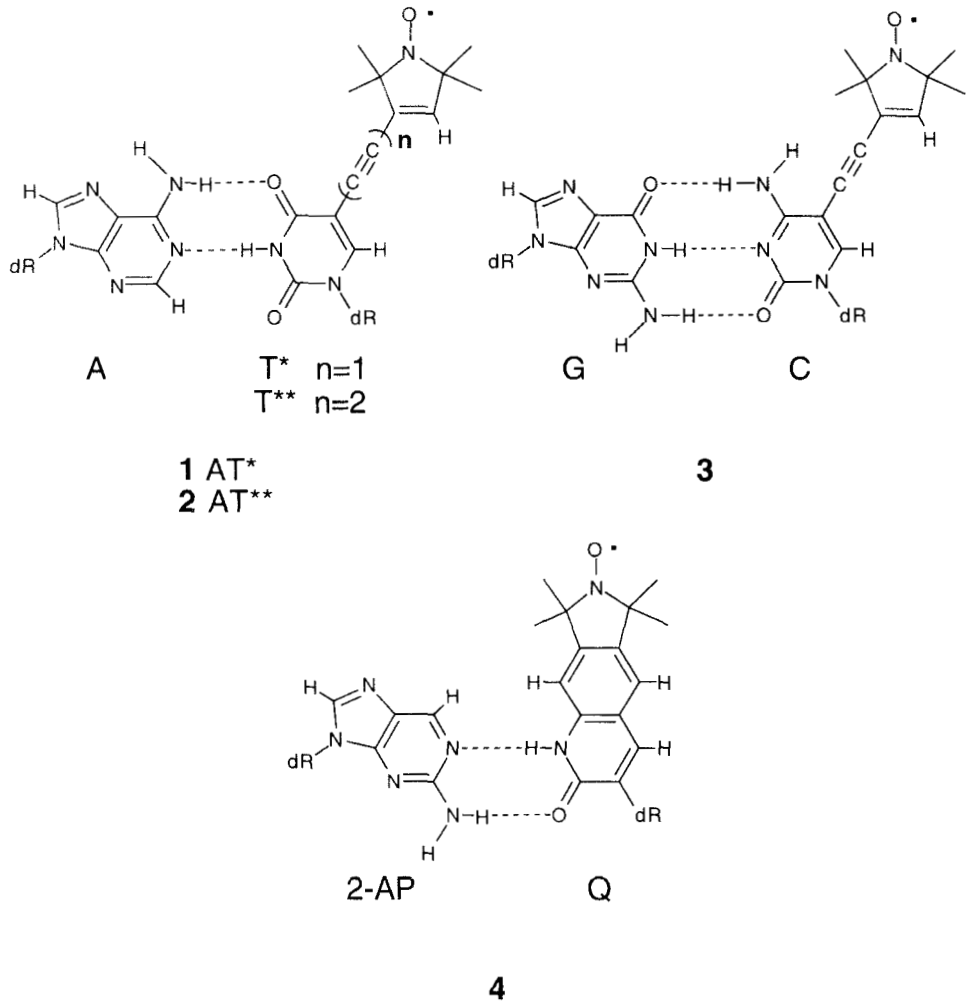

Figure 1. Spin labeled base pairs described herein.

the spin label was correlated with the motion of the macromolecule. Specifically, there was concern that hindered rotation about the acetylene axis of T* might influence the resulting spectra of DNA spin labeled as in **1**. We demonstrated that such motion was in fact hindered, by synthesizing DNA containing the spin labeled base T** shown in base pair **2**, in which the spacer between the spin-label-containing heterocycle and the DNA base was extended from a monoyne to a diyne.[3,5] EPR spectra of macromolecules labeled with this base pair were satisfactorily simulated by assuming a rapid (unhindered) motion about the

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diyne axis. The extent to which a related, but hindered motion influences the spectra of **1** remains at this time under investigation.

To eliminate the possibility of rotation about an alkyne linkage, we have more recently focused attention on the synthesis of DNA duplexes containing a spin labeled quinolone derivative Q present as in base pair, **4**[6]. The great effort to prepare this label has been repaid by the information content of the resulting EPR spectra, a point briefly noted below and to be described in detail in future publications. The analysis of these spectra has prompted us to return, for purposes of comparison, to the more conveniently available alkyne-tethered spin labels. We report herein the synthesis and incorporation into duplex DNA of a spin labeled analog of deoxycytidine, C*, as shown in base pair **3**. With this new substance, it its now possible to spin label either AT or GC base pairs with the alkyne-tethered spin labels. The characterization by EPR of DNA sequences site-specifically spin labeled with C* is reported herein. We comment on the relative advantages and disadvantages of T*, C*, and Q.

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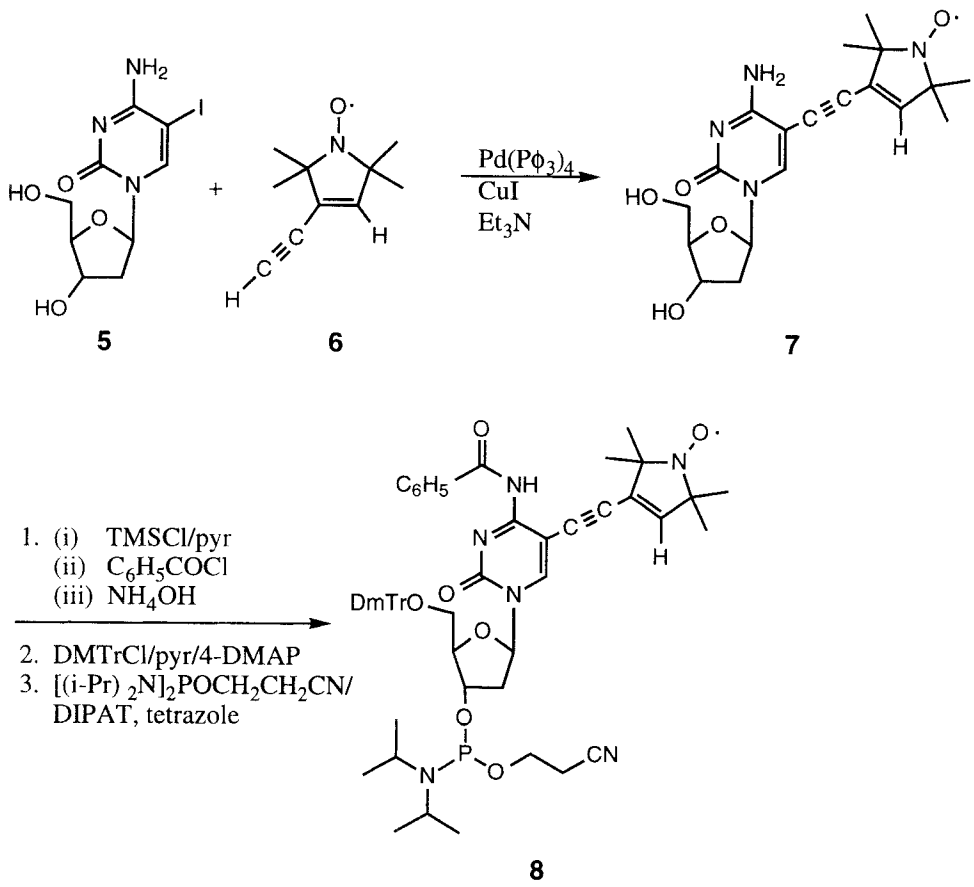
RESULTS AND DISCUSSION

Synthesis of Spin-labeled DNA. We have previously prepared DNA spin labeled with analogs of deoxythymidine as in **1** and **2** by the phosphoramidite method.[2,3] The phosphoramidite **8** was selected for use in the synthesis of DNA spin labeled with an analog of deoxycytidine, as in **3**, and was prepared by analogy to the synthesis of the thymidine analog (Scheme 1).[2] The commercially available nucleoside 5-iodo-2'-deoxycytidine (**5**) was coupled using Hobbs' modification[7] of the method of Robins to the previously reported 3-ethynyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy (**6**),[2] affording the substituted deoxycytidine **7** in 93% yield. The benzoyl group was then installed by the transient protection method of Jones[8] in ca. 50% yield. A by-product of this step was a substance identified by its mass and ^{1}H NMR spectra as a dibenzoyl analog, which was isolated in 20% yield. Installation of the dimethoxytriphenylmethyl protecting group (47%) and the phosphitylation reaction (87%) were then accomplished by standard methods to complete the synthesis of phosphoramidite **8**.

DNA polymers containing this new spin label were synthesized predominantly by the automated phosphoramidite method, except for the coupling of the spin labeled phosphoramidite which was conducted manually to minimize consumption of the synthetic phosphoramidite. Colorimetric quantitation of the released trityl cation from the manual coupling indicated that this step had proceeded in ca. 50% yield, much lower than is typical in DNA synthesis, presumably due to competing hydrolysis from water contamination. Because this yield was more than adequate for our purposes, it was not further optimized.



Scheme 1. Synthesis of phosphoramidite used for the incorporation of C* into DNA.

All DNAs were deprotected with aqueous ammonia (55°, ca. 16 h), and purified by denaturing polyacrylamide gel electrophoresis.

The incorporation of the C* residue into DNA was demonstrated by quantitation of released deoxynucleosides following exhaustive enzymatic hydrolysis of the phosphodiester backbone. The DNA, 5'-d(CGCGGATC*CGCG) was enzymatically hydrolyzed using a mixture of calf intestinal alkaline phosphatase, crotalus adamantus venom phosphodiesterase I, DNase I, DNase II and nuclease S1. The hydrolysate was analyzed by HPLC, showing that it contained only the four natural deoxynucleosides in the expected ratios plus a strongly retained substance which in a separate HPLC run coeluted with authentic admixed synthetic nucleoside **7** (See Figure 2). The UV spectrum of **7**

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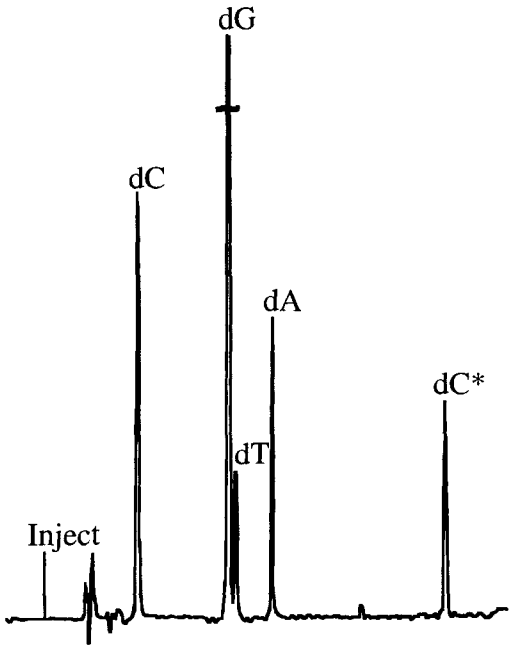

Figure 2. HPLC chromatogram of the enzymatic hydrolysate of 5'd(CGCGCGATC*CGCG).

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recovered from DNA subjected to enzymatic hydrolysis was identical to synthetic **7** (λ_{max} = 266, 280, 302 nm).

The stoichiometry of incorporation of the spin label into DNA oligomers was independently verified by double integration of EPR spectra. A solution of a singly spin labeled oligomer of concentration 0.60 ± 0.06 mM determined by UV absorbance at 260 nm was found by double integration to be 0.50 ± 0.05 mM in unpaired electrons. This finding is consistent with the free radical quantitatively surviving the synthesis and purification protocols.

Because the focus of the present study was the dynamic properties of *duplex* DNA, we verified that DNA oligomers containing C* were in fact in the duplex form using native gel electrophoresis. A variety of DNA strands spin labeled with C* were electrophoresed in the presence and absence of their complementary strands (data not shown). Admixed complementary strands afforded a gel mobility at 12 °C which was significantly retarded relative to either single strand alone. This clearly indicated that the melting temperatures of these DNAs were well above the 2-4 °C temperatures at which the EPR spectra were recorded. The thermodynamic parameters of these DNAs were not further studied.

EPR Characterization. We have previously shown that the EPR spectra of DNA structures spin labeled with the thymidine analog T* are useful for distinguishing various DNA structures, as a consequence of their differing dynamical properties.[9] For example, a monodeoxynucleoside, single strand oligodeoxynucleotide, and duplex oligodeoxynucleotides were readily distinguished from one another. The same situation is now revealed with C* (Figure 3). EPR spectra of the monodeoxynucleoside **7**, the single strand 5'-d(GCC TAC ATG C*GA CG) (hereafter 14-mer) and the corresponding 14-mer duplex are shown in Figure 3. The relatively sharp lines in the monomer (Figure 3a) are broadened upon incorporation into a single strand (Figure 3b), and in turn further broadened by addition of the complementary opposite strand (Figure 3c). The spectrum of this duplex is consistent with anisotropic correlation times of 8 ns ($\tau_{/\!/}$) and 19 ns ($\tau_{\perp}$), the approximate correlation times expected[2] for a duplex 14-mer tumbling in aqueous solution at 0 °C.

To study the extent to which the motion of the spin label was correlated with that of the attached DNA, as well as to compare this with two better characterized spin labels, we acquired spectra of DNA in 50% aqueous sucrose at 0°C. In this viscous medium the global tumbling (uniform modes) of 14-mer duplexes have relatively long correlation times, on the microsecond time scale. The EPR spectrum of the 14-mer duplex labeled with C* (Figure 3d) closely resembled the spectrum of the analogous thymidine spin-labeled DNA duplex in this same medium (Figure 3e). In contrast, this sequence labeled with the quinolone shown in **4** (Figure 3f) shows substantially sharper lines, closer to the expectation for a spin label rigidly tethered to the DNA.

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Comparison of T*, C* and Q. The complexity and breadth of the spectral features for the T* and C* labeled DNAs in sucrose suggest the existence in these samples of multiple structural and dynamical components. Space filling molecular models of duplex DNA provide an interpretation for the structural heterogeneity, indicating that the pyrrolinyloxy ring in C* or T* may preferentially align itself with the sugar-phosphate backbone in the major groove of DNA, resulting in two distinct orientations for the spin label relative to the helix axis.[3] The two dynamical components have been interpreted as a fast vibrational component (hindered rotation about the acetylenic linkage) and the slow motion of the DNA.[4] This particular attribution cannot be experimentally confirmed and must be viewed as speculative. In contrast, spectra of DNA sequences spin labeled with Q, where the spin label is rigidly attached and has no opportunity to rotate, lack this apparent structural and dynamical heterogeneity. The narrow lines suggest that just one kind of motion is present, namely that of the DNA. This probe appears best suited to providing relevant dynamical information about DNA.

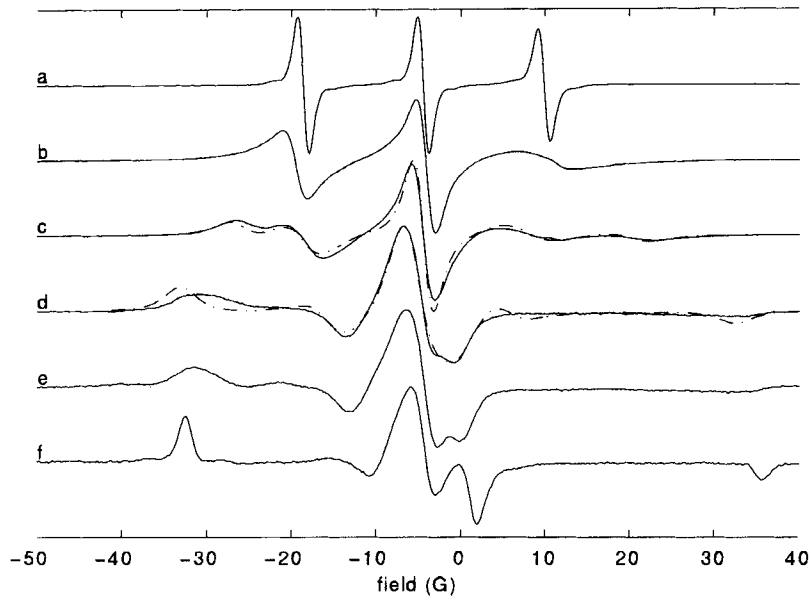

Figure 3. CW-EPR spectra of (a) **7**; (b) [5'-d(GCC TAC ATG C*GA CG)] at 0 °C in aqueous solution; (c) [5'-d(GCC TAC ATG C*GA CG)·5'-d(CG TCG CAT GTA GGC)] at 0 °C in aqueous solution (solid line) overlaid with a simulated spectrum (see below); (d) [5'-d(GCC TAC ATG C*GA CG)·5'-d(CG TCG CAT GTA GGC)] at 0 °C in 50% aqueous sucrose (solid line) overlaid with a simulated spectrum; (e) [5'-d(GCC TAC ATG T*GA CG)-5'·d(CG TCA CAT GTA GGC)] at 0 °C in 50% aqueous sucrose; (f) [5'-d(GCC TAC ATG QGA CG)-5'-d(CG TC(2-AP) CAT GTA GGC)] at 0 °C in 50% aqueous sucrose. Samples contained 10 mM phosphate buffer (pH 7.0), 115 mM Na^+, and 0.1 mM EDTA. A least-squares fit to spectrum d yielded the following tensor values: A_{xx} = 5.251 G, A_{yy} = 10.831 G, A_{zz} = 31.06 G, g_{xx} = 2.0050, g_{yy} = 2.0041, g_{zz} = 2.0016 (R_{sq} = 0.9746). Spectrum c was fit to a model of anisotropic rigid body motion and the correlation times $\tau_{\parallel}$= 8.44 ns and $\tau_{\perp}$ = 18.82 ns which are appropriate for duplex DNA of this length.[4,10] We find the internal rms oscillation amplitude β = 23°, which corresponds to an order parameter $S = \langle D^2_{00}(\beta)\rangle = 0.77$.

In the present context it is important to distinguish the *thermodynamic* and *dynamic* consequences of incorporation of these unnatural residues into DNA. Froehler et al.[11] have reported that 5-propynyl substitution (similar to the alkynyl nitroxides discussed herein) on deoxyuridine and deoxycytidine raise the melting temperature of certain duplex and triplex structures; we have reported that DNA duplexes containing Q have a lower melting temperature than appropriate controls.[5] These changes in melting temperature indicate that incorporation of these unnatural residues alters the *thermodynamic* properties of one or both of the helix and/or coil states. This fact tells us nothing about possible alterations in

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dynamic properties caused by the unnatural residues. It seems plausible to speculate that the dynamic properties near the site of incorporation will be influenced more greatly than motions at a distance from the label. For this reason, in work to be described elsewhere, we have used these labels to study the impact on dynamics experienced by the spin label of nucleotide sequence changes distant from the spin label itself.

The relative benefits of the spin label Q are mitigated somewhat by its labor-intensive synthesis. The preparation of the phosphoramidite precursor to Q involves some 20 synthetic operations beginning from commercial starting materials.[6] Phosphoramidite **8** and its thymidine analog (the precursor to T*) are prepared by much shorter syntheses, with fewer than ten steps from commercial substances. Because the phosphoramidite leading to T* lacks any base protection, it is the more easily prepared of the two.

The spin labels also differ in thermal stability. After 36 hours of exposure to 80°C, the EPR spectra of both T*- and C*-containing duplexes show a fast component which resembles the EPR spectrum of the nucleoside monomer that has not been incorporated into DNA. This fast component can be explained by depyrimidination of the spin labeled residue, in which the unnatural base with its nitroxide moiety comes off the large DNA molecule, thus becoming a fast-tumbling, small molecule.[3] The C-nucleoside, Q, is inherently more stable to thermal degradation, showing no such tendency to undergo decomposition on routine handling including gentle warming.

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The spin labels comprising the family described in this paper, T*, C*, and Q, have differing uses. Because of the high cost in synthesizing Q, the other spin labels, T* and C*, are better for low resolution screening of DNA motion. Although these two spin labels yield EPR spectra compromised by the spin label motion, they are very sensitive to *changes* in structure.[9] The independent motion of spin label can actually be used to advantage, amplifying the changes in motion resulting from changes in structure. However, the spin labels T* and C* give less useful information about the structures or the motions themselves, due to the fundamental limitation that the motion of the spin label independent of the DNA cannot be distinguished from the motion of the DNA itself.

CONCLUSIONS

We report the chemical synthesis of a spin labeled analog of deoxycytidine which has been incorporated site-specifically into both single stranded and duplex DNAs. Enzymatic hydrolysis of the C* spin labeled DNA showed that the unnatural nucleoside was incorporated unaltered. This spin labeled analog of deoxycytidine is the third in a family of spin labels developed in this laboratory over the last 10 years. The behavior of this spin label appears similar to that of a previously observed spin labeled analog of

thymidine. Both the spin labeled deoxycytidine and thymidine analogs are relatively conveniently available, but have deficiencies in that they are thermally labile and provide EPR spectra characteristic of heterogeneous mixtures. EPR spectra of a less conveniently synthesized spin labeled quinolone, Q, possess narrower lines which appear more characteristic of a structurally and dynamically homogeneous sample. The latter thus appears capable of providing the most rigorous analysis of both internal and global dynamics of DNA.

EXPERIMENTAL SECTION

General. Water sensitive reactions were conducted in flame dried glassware under positive argon pressure. Commercial reagents were used as received except for the following: dichloromethane, pyridine and triethylamine were distilled under argon from calcium hydride; copper(I) iodide was recrystallized from 40% methyl sulfide in diethyl ether; tetrahydrofuran was distilled under argon from sodium benzophenone ketyl. Column chromatography was performed under slight positive pressure on Merck silica gel 60 (230-400 mesh). TLC was performed on precoated silica gel 60 plates (0.25 mm). Proton nuclear magnetic resonance spectra (^{1}H NMR) were determined on a Bruker AF-300 MHz spectrometer and are reported in parts per million downfield from internal tetramethylsilane (0.00 ppm). Low resolution mass spectra (LRMS) and high resolution mass spectra (HRMS) were measured on a VG70SEQ mass spectrometer. Ultraviolet (UV) spectra were measured on a Hewlett-Packard model 8452A UV/vis spectrometer, and are reported as wavelengths in nanometers (nm). ^{1}H NMR spectra of spin labeled compounds were recorded in $CDCl_3$ or d_6-acetone following treatment with phenylhydrazine or sodium dithionite/D_2O respectively, prior to acquisition of the ^{1}H NMR spectra. EPR spectra were recorded on an X-band (9 GHz) EPR spectrometer. EPR acquisition parameters were as follows: 128 Gauss sweep width, 0.1 milliwatts power, 1.0 Gauss modulation amplitude, 10 kHz modulation frequency, 1024 points, 0.125 s per point, 1 or 4 scans, 0 °C.

DNA synthesis was executed on an Applied Biosystems Model 392 Synthesizer using ABI reagents, on a 1 μmol scale, under full automation, except for the coupling with phosphoramidite **8** as described below. HPLC was conducted on a Beckman 421A HPLC with Beckman 110B Pumps, equipped with a Biorad Model 1706 UV/Vis Monitor (260 nm) and an HP 3390A Integrator.

Preparation of 5-[2-(3-(2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy))ethynyl]-2'-deoxycytidine, 7. To a deoxygenated solution of 320 mg (0.9 mmol) of 5-iodo-2'-

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deoxycytidine, **5**, and 100 mg (0.61 mmol) of 3-ethynyl-2,2,5,5-tetramethyl-pyrrolin-1-yloxy, **6**, in 4.5 mL of DMF at 25 °C was added 230 mg (1.2 mmol) of copper(I) iodide and 104 mg (0.09 mmol) of tetrakistriphenylphosphine palladium(0). The head space above the resulting slurry was argon flushed. Triethylamine, 0.58 g (5.7 mmol) was added, and the mixture was stirred for 2 h. The reaction mixture was concentrated in vacuo, and the resulting gum was stirred with 3 mL of 20% methanol in dichloromethane. The resulting slurry was filtered, and the filtrate concentrated in vacuo. The residue was purified on silica gel, with 12% methanol in dichloromethane, to afford 220 mg (93%) of the spin labeled nucleoside **7** as a yellow solid: ^{1}H NMR (300 MHz, d_6-acetone, D_2O, $Na_2S_2O_4$) δ 1.45 (6H, s, 2xCH_3), 1.55 (6H, s, 2xCH_3), 2.35 (1H, m, H2' or H2"), 2.55 (1H, m, H2' or H2"), 3.9 (2H, m, H5', H5"), 4.15 (1H, m, H3'), 4.55 (1H, m, H4'), 6.25 (1H, m, H1'), 6.3 (1H, s, HC=C), 8.5 (1H, s, H6); LRMS (ES): 390 ($M+H^+$), 274; EPR (acetone): 3 lines; UV (12 μM in H_2O prepared by dissolving 1.16 mg in 500 μL of EtOH followed by serial dilution with H_2O): λ_{max} = 266 (ε 10,593 $M^{-1}cm^{-1}$), 280 (ε 10,424 $M^{-1}cm^{-1}$), 302 nm (ε 9,322 $M^{-1}cm^{-1}$).

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Preparation of N^4-Benzoyl-5-[2-(3-(2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy))ethynyl]-2'-deoxycytidine. The nucleoside was acylated by the transient protection method of Jones et al.[8] Spin labeled nucleoside **7**, 70 mg (0.18 mmol) was dried by concentration twice from anhydrous pyridine, then dissolved in 1.5 mL of pyridine, and cooled to 0 °C. To this solution was added 150 μL (128.4 mg, 1.2 mmol) of chlorotrimethylsilane. After 1 h, an additional 100 μL of chlorotrimethylsilane was added, and to this suspension was added 70 μL (85 mg, 0.6 mmol) of benzoyl chloride dropwise over a period of 5 min at 0 °C. The cooling bath was removed, and the resulting solution was stirred for a total of 2.5 h at 25 °C. The mixture was cooled to 0 °C, treated with 300 μL of water, stirred for 15 min, then treated with 80 μL of concentrated aqueous ammonia. The mixture was stirred for 1.5 h, cooled to 0 °C, and concentrated in vacuo. The resulting slurry was triturated with methanol and the insoluble solid was removed by filtration. Following concentration of the filtrate in vacuo, the residue was chromatographically purified on silica (10% methanol in dichloromethane) to afford 90 mg (70%) of the title compound as a yellow solid: ^{1}H NMR (300 MHz, d_6-acetone, D_2O, $Na_2S_2O_4$) δ 1.45 (6H, s, 2xCH_3), 1.50 (6H, s, 2xCH_3), 2.45 (1H, m, H2' or H2"), 2.65 (1H, m, H2', or H2"), 3.95 (2H, m, H5', H5"), 4.20 (1H, m, H3'), 4.6 (1H, m, H4'), 6.20 (1H, s, HC=C), 6.3 (1H, m, H1'), 7.63 (2H, t, Ar), 7.67 (1H, t, Ar), 8.1 (2H, d, Ar), 8.9 (1H, s, H6); LRMS (ES): 494 ($M+H^+$), 378, 325; HRMS (FAB, 2HEDS): Calc'd for $C_{26}H_{30}N_4O_6Na$, 516.1978; found, 516.1985 ; EPR (acetone): 3 lines.

Preparation of N^4-Benzoyl-$O^{5'}$-(4'',4'''-dimethoxytriphenylmethyl-5-[2-(3-(2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy))ethynyl]-2'-deoxycytidine. The benzoylated diol, 60 mg (0.12 mmol), was dried by concentration in vacuo from anhydrous acetonitrile for 2 h. To this reaction flask was added 60 mg (0.30 mmol) of 4,4'-dimethoxytriphenylmethyl chloride, and a crystal of 4-dimethylaminopyridine. The reaction mixture was held in vacuo for an additional 2 h. At that time, 600 μL of pyridine and 10 μL of triethylamine (0.07 mmol) were added and the reaction mixture was stirred at 25 °C for 2.5 h. Methanol, 1 mL, was added, and the mixture was stirred for 10 min. The resulting solution was concentrated in vacuo, and purified chromatographically on silica gel to afford 45 mg (47%) of the dimethoxytriphenylmethyl derivative as a yellow solid: ^1H NMR (300 MHz, $CDCl_3$, phenyl hydrazine) δ 1.35 (6H, s, 2xCH_3), 1.45 (6H, s, 2xCH_3), 2.35 (1H, m, H2' or H2''), 2.65 (1H, m, H2' or H2''), 3.24 (2H, m, H5' or H5''), 3.72 (6H, s, 2xOCH_3), 4.13 (1H, m, H3'), 4.52 (1H, m, H4'), 5.00 (1H, s, HC=), 6.32 (1H, m, H1'), 6.0-7.5 (18 H, m, Ar), 8.45 (1H, s, H6); FAB-LRMS: 797 ($M+H^+$), 781, 765, 679, 645, 378, 325, 303; FAB-HRMS (2HEDS): Calc'd for $C_{47}H_{48}N_4O_8$ ($M+H^+$): 796.3472; found, 796.3438 ; EPR (acetone): 3 lines.

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Preparation of Phosphoramidite, 8. A flame-dried flask was charged with 35 mg (0.04 mmol) of the product of the previous reaction and the resulting flask was dried twice by concentration from anhydrous acetonitrile. Diisopropylammonium tetrazolide, 5 mg (0.03 mmol) was added and the resulting mixture was held at 0.05 mm Hg for 3 h. Following addition of argon, 400 μL of dichloromethane and 40 mg (0.13 mmol) of 2-cyanoethyl bis(diisopropylamino)-2-cyanoethylphosphorodiamidite, the mixture was stirred for 3 h. The mixture was then concentrated in vacuo. A basic silica gel column was prepared by washing with a solution of 5 drops of triethylamine in 100 mL of 50% ethyl acetate/hexane. The reaction mixture was loaded onto this basic silica gel column and was chromatographed with 50% ethyl acetate/hexane to afford 38 mg (87%) of the two diastereoisomeric forms of phosphoramidite **8** as a yellow oil: EPR: 3 lines.

Oligonucleotide Synthesis and Purification. Phosphoramidite **8** was manually incorporated into DNA as follows. After detritylation of the residue 3' to the intended position for C*, the automated cycle was interrupted. The controlled pore glass (CPG) column was removed from the machine and attached to two syringes, one filled with 100 μL of 0.1 M tetrazole in anhydrous CH3CN and the other with 100 μL of a 130 mM solution of **8** (12 mg, 13 mmol) also in anhydrous CH3CN. The solutions were passed back and forth through the column approximately 100 times over a 2.5 min period. The liquid was discarded and the column returned to the synthesizer to finish DNA synthesis

under full automation. DNA was cleaved from the resin and deprotected in concentrated aqueous ammonia at 55°C for 16 h and then dried in a Savant Speed Vac concentrator (4 h).

DNA was purified by DPAGE as follows. About 30 OD of dried, crude DNA was dissolved in 50% (v/v) formamide in water containing 5 mM Na_2EDTA and loaded onto a 20% denaturing polyacrylamide gel [19:1 acrylimide:bisacrylamide, 8 M Urea, 1 X in TBE buffer (90 mM Tris-Borate, 1.0 mM Na_2EDTA), 1.5 mm thick, 14 X 16 cm, 5-toothed comb, 10 OD/lane, 450V, 4 h]. The gel was run until xylene cyanol loaded into an adjacent lane had traveled about 9 cm from the origin. DNA was visualized by UV shadowing and isolated from the gel by cutting it out with a razor blade. The DNA was recovered from the gel matrix by the following crush and soak procedure. The gel slices were macerated with a glass rod and incubated with nutation (12-16 h, 25 °C) with about 4 volumes of elution buffer (0.5 M NH_4OAc, 10 mM Na_2EDTA, pH 7.8). The supernatant was removed and the gel material was incubated with an additional 4 volumes of elution buffer (15 min). A Waters SepPak® C_{18} Cartridge was prepared by sequential washes with 10 mL of CH_3CN and 10 mL of H_2O. The supernatants containing DNA were then pooled and passed through the column. After the DNA sample was added, the column was washed sequentially with 10 mL of 10 mM aqueous NH_4OAc and 4 mL of H_2O and the DNA eluted with 4 mL of 25% aqueous CH3CN. The DNA eluent was concentrated in a Savant Speed Vac Concentrator until it was approximately 0.5 OD / 5 mL.

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Enzymatic Hydrolysis of d(CGCGGATC*CGCG). The self-complementary DNA (0.4 OD) was hydrolyzed enzymatically in a total volume of 30 μL of hydrolysis buffer (5 mM Tris/Tris-HCl, 10 mM $MgCl_2$, pH 7.5) including 4 μL calf intestinal alkaline phosphatase (BMB, 10 units), 2 μL crotalus adamantus venom phosphodiesterase I, (USB/Amersham, 2 units), 2 μL Dnase I (Sigma, 10 units), 2 μL Dnase II (Sigma, 4 units), and 2 μL nuclease S1 (Gibco, 1800 units). Reactions proceeded 3 h at 37°C. HPLC analysis used the following gradient profile (Solvent A = 100 mM NH_4OAc, pH 7.0, Solvent B = 100% CH3CN, flow rate of 1 mL/min, about 1500 psi): 7 min isocratic at 8% B, 7 min linear gradient from 8% to 30% B, 13 min linear gradient from 30%-40% B, 10 min linear gradient from 40% - 8% B, 5 min isocratic at 8% B. Response factors of 0.48(dC): 0.81(dG): 0.57(dT): 1.00(dA) were obtained from the hydrolysate of a control DNA contained the four residues in equimolar amounts, 5'-d(TATCCCGGGATA). The relative ratios of the returned nucleosides in the spin labeled DNA 5'-d(CGCGGATC*CGCG) were: 3.8(dC): 5.3(dG): 1.0(dT): 1.0(dA) (calc'd 4.0: 5.0: 1.0: 1.0). The HPLC chromatogram also showed a strongly retained substance which coeluted with an admixed an authentic sample of the nucleoside C* on coinjection.

EPR spectra. Samples were prepared for EPR by diluting 0.5 OD of the spin labeled DNA in a total of 10 μL of buffer, (about 0.25 mM in spin-labeled strands of DNA, 10 mM sodium phosphate, 100 mM NaCl, 0.1 mM Na_2EDTA, pH 7.0). For spectra in 50% sucrose, the sample was DNA was dissolved in a solution 1:1 (w/v) solution of sucrose and this same buffer. For non-self complementary DNA duplexes, the unlabeled complementary strand was admixed in roughly 1.5-fold molar excess over the spin-labeled strand.

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