Recognition of All Four Base Pairs of Double-Helical DNA by Triple-Helix Formation: Design of Nonnatural Deoxyribonucleosides for Pyrimidine•Purine Base Pair Binding

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Abstract: The sequence-specific recognition of double-helical DNA by oligonucleotide-directed triple-helix formation is limited mostly to purine tracts. Design leads that could expand the recognition code to all four Watson–Crick base pairs would provide one step toward a general solution targeting single sites in megabase size DNA. The nonnatural deoxyribonucleoside 1-(2-deoxy-β-D-ribofuranosyl)-4-(3-benzamidophenyl)imidazole (D_3) was synthesized in four steps and incorporated by automated methods into pyrimidine oligodeoxyribonucleotides. Within a pyrimidine oligonucleotide, D_3 binds pyrimidine•purine base pairs with higher affinity than it binds purine•pyrimidine base pairs. From affinity-cleaving analysis, the stabilities of base triplets decrease in the order D_3•TA ~ D_3•CG > D_3•AT > D_3•GC. Such specificity allows binding by triple-helix formation at an 18 base pair site in SV40 DNA containing all four base pairs at physiologically relevant pH and temperature. The stabilities of these novel triplets may be an example of shape-selective recognition of CG and TA Watson–Crick base pairs in the major groove.

Introduction

Pyrimidine oligodeoxyribonucleotides bind specifically to purine sequences in double-stranded DNA to form local triple-helical structures.[1-3] Pyrimidine oligodeoxyribonucleotides bind in the major groove of DNA parallel to the purine Watson–Crick strand through the formation of specific Hoogsteen hydrogen bonds to the purine Watson–Crick base.[1-5] Specificity is derived from thymine (T) recognition of adenine•thymine (AT) base pairs (T•AT triplet) and protonated cytosine (C^+) recognition of guanine•cytosine (GC) base pairs (C^+GC triplet) (Figure 1).[1-5] The discovery of other natural base triplets such as G•TA[2a,b] and the development of linked pyrimidine oligonucleotides for alternate-strand triple-helix formation[2c-f] have extended the number of sites capable of being recognized by this motif. More recently, a second triple-helix motif has been characterized on the basis of a purine third strand.[6] Purine-rich oligodeoxyribonucleotides bind in the major groove of DNA antiparallel to the purine Watson–Crick strand through the formation of specific hydrogen bonds to the purine Watson–Crick base.[6b,c] Specificity is derived from G recognition of GC base pairs (G•GC triplet) and A or T recognition of AT base pairs (A•AT or T•AT triplets).[6] Although oligonucleotide-directed triple-helix formation offers a powerful chemical approach for the sequence-specific recognition of double-helical DNA, both pyrimidine and purine triple-helical motifs provide limited recognition codes and specify for mostly purine tracts of double-helical DNA. General methods for recognition of mixed sequences containing all four base pairs of duplex DNA are lacking (Figure 1).

We describe here initial efforts to design heterocycles that can bind CG or TA Watson–Crick base pairs selectively within the pyrimidine•purine•pyrimidine triple-helix motif. The rationale was to engineer a base compoment that will (i) sterically match the edges of pyrimidine•purine Watson–Crick base pairs in the major groove, (ii) position hydrogen bond donors and acceptors to form hydrogen bonds in the major groove with CG or TA base pairs, (iii) maintain a backbone geometry compatible with the pyrimidine•purine•pyrimidine triple-helix motif, and (iv) allow energetically favorable stacking of the bases in the third strand. From model building, we chose two nonnatural base components, 4-phenylimidazole (D_2) and 4-(3-benzamidophenyl)imidazole (D_3) (Figure 2). A single bond was employed to connect the two aromatic rings. This provides a rotational degree of freedom to allow the nonnatural base to adopt a favorable geometry for interaction with pyrimidine•purine base pairs. D_2 was prepared with the anticipation of forming one hydrogen bond with a CG base pair between imidazole N3 and the NH_2 of C. The D_3 heterocycle was designed to span both strands of the Watson–Crick CG base pair and form two hydrogen bonds. This differs from

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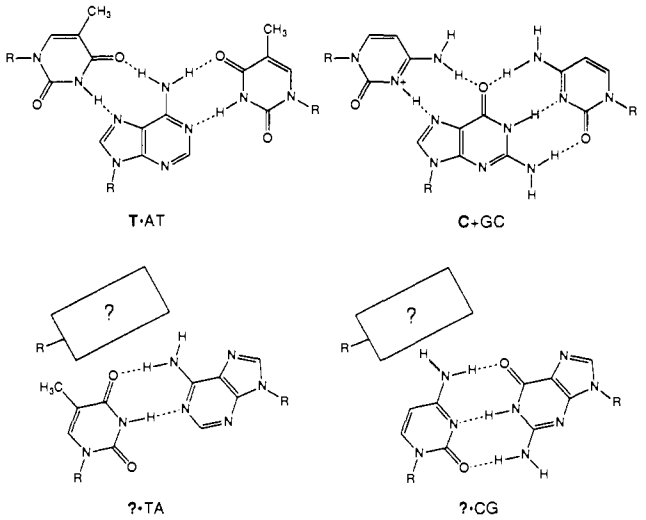

Figure 1. Top: Natural base triplets T·AT and C⁺GC. The third-strand base is bound in the major groove by Hoogsteen hydrogen bonds to the purine in the Watson–Crick duplex. Bottom: Novel heterocycles must be designed for recognition of TA and CG Watson–Crick base pairs compatible with the position of the phosphate–deoxyribose backbone in the pyrimidine·purine·pyrimidine triple-helix motif.

hydrogen-bonding patterns of the pyrimidine·purine·pyrimidine and purine·pyrimidine·purine motifs, wherein the third-strand base forms hydrogen bonds only to the Watson–Crick purine base.[1,4,6]

We find that the base 4-(3-benzamidophenyl)imidazole (D_3) affords novel binding affinity and sequence specificity for duplex DNA, *different* however from those anticipated by the original design rationale. Rather than preferential CG base pair binding, D_3 recognizes *both* pyrimidine·purine base pairs (TA and CG) in preference to purine·pyrimidine base pairs (AT and GC) within the pyrimidine triple-helix motif. Utilizing this expanded recognition code containing two natural triplets (T·AT, C⁺GC) and two nonnatural triplets (D_3·TA, D_3·CG), we report here the site-specific binding of an 18 base pair sequence in SV40 plasmid DNA *containing all four base pairs* by a pyrimidine oligonucleotide containing the synthetic base D_3.

Results and Discussion

Syntheses of Deoxyribonucleosides D_2 and D_3. A scheme for the synthesis of the phosphoramidites **7** and **8** is depicted in Figure 3. For the synthesis of **7** containing the base component D_3, the sodium salt of 4-(3-nitrophenyl)imidazole[7] was condensed with 1-chloro-2-deoxy-3,5-di-O-*p*-toluoyl-α-D-ribofuranose[8] to give the imidazole N-1 β-anomer **2** as the major product in 65% yield.[9] A crystal structure of reaction product **2** confirmed the regio- and stereochemical course of this reaction.[10] The *p*-toluoyl protecting groups were removed,[11] and the nitro group was subjected to hydrogenation to generate the aminobenzene derivative **4**. The aromatic amino group was selectively acylated by the transient protection method to provide benzamide **5**.[12] Treatment of the resulting amide with 4,4′-dimethoxytrityl chloride furnished the protected nucleoside **6**,[13] which was then activated with 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite to afford phosphoramidite **7**.[13,14] The phosphoramidite **8** containing the novel base component D_2 was synthesized in a similar manner.

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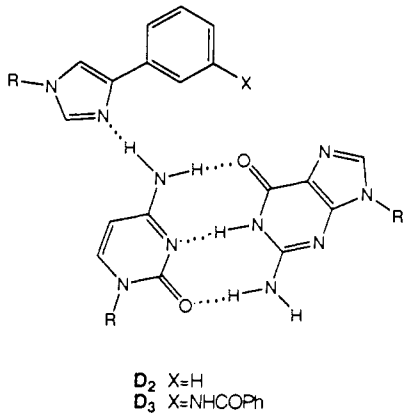

Figure 2. Design rationale for recognition of Watson–Crick CG base pairs by nonnatural bases D_2 and D_3 within a pyrimidine·purine·pyrimidine triple-helix motif.

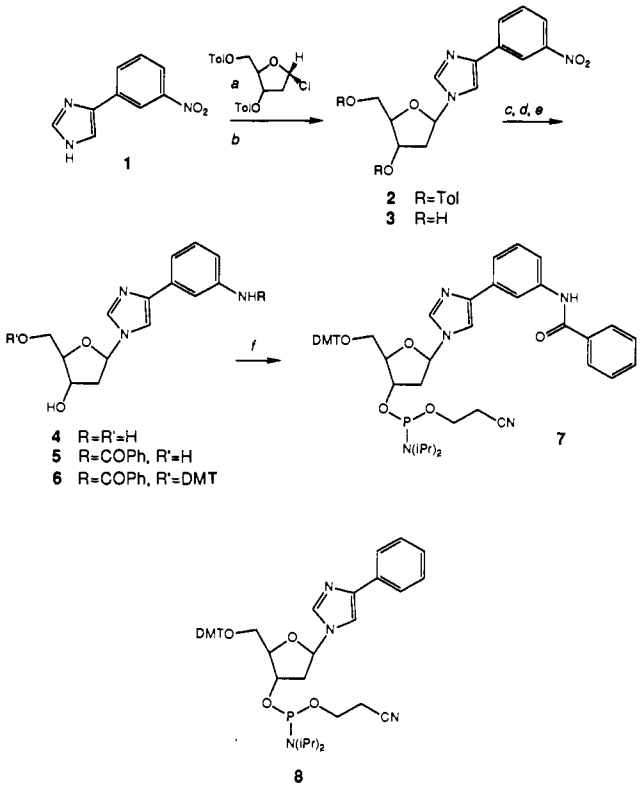

Figure 3. Scheme for the synthesis of the 2-cyanoethyl phosphoramidites of D_3. Reaction conditions: (a) 1.1 equiv of NaH, CH_3CN, room temperature, 65%; (b) 1% NaOH, MeOH, room temperature, 99%; (c) H_2, Pd/C, MeOH, room temperature, 99%; (d) 5 equiv of TMSCl, pyridine, 0 °C, followed by 5 equiv of PhCOCl, 0 °C to room temperature, then H_2O, 0 °C to room temperature,92%; (e) 1.4 equiv of DMTCl, pyridine, 4 °C, 88%; (f) 1.5 equiv of 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite, 3 equiv of diisopropylethylamine, CH_2Cl_2, room temperature 98%.

Synthesis of Oligodeoxyribonucleotides Containing D_2 and D_3 Heterocycles. Oligodeoxyribonucleotides **9–16** were synthesized by automated methods using 2-cyanoethyl phosphoramidite chemistry.[13,14] The phosphoramidites containing the novel bases D_2 and D_3 coupled with efficiencies equal to those observed for A, G, C, and T phosphoramidites (>97%). Because oligodeoxyribonucleotides **9–16** contained T* (thymidine–EDTA), 0.1 N sodium hydroxide was utilized in the deprotection step.[15] To examine the stabilities of the novel bases under the conditions for automated synthesis and subsequent deprotection, tetramers 5′-

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T-T-D_2-T-3′ and 5′-T-T-D_3-T-3′ were synthesized and characterized by ^{1}H NMR. Neither glycosidic bond anomerization nor base modification of D_2 and D_3 was observed under these conditions.[16] The benzamido group of D_3 was found to be stable to the deprotection conditions.

The integrity of the nonnatural nucleosides D_2 and D_3 after automated synthesis and deprotection was analyzed by HPLC. Purified oligodeoxyribonucleotides containing D_2 and D_3 were treated with snake venom phosphodiesterase and calf intestine alkaline phosphatase to afford the corresponding nucleoside monomers. Correlation of HPLC retention times and UV spectra with those of authentic samples provided a means of product identification. These results suggest that D_2 and D_3 can be utilized in automated oligodeoxyribonucleotide synthesis without complications.

Analysis of Binding Specificity by Affinity Cleaving. The relative affinities of the bases D_2 and D_3 for all four Watson–Crick base pairs within a pyrimidine·purine·pyrimidine triple-helix motif were examined by affinity cleaving.[1a,2a] Oligodeoxyribonucleotides equipped with the DNA-cleaving moiety thymidine–EDTA·Fe(II) (T*)[15] allowed the relative stabilities of triple-helix formation at a 15 base pair purine tract within 30 base pair (bp) oligonucleotide duplexes containing one variable base pair site d(AAAAAAAX-AAAAAAA)·d(TTTTTTTYTTTTTTT) (XY = AT, GC, CG, or TA) and a series of 15 nucleotide oligomers differing at one base position 5′-TTTTTTTNTTTTTTT-3′ (N = T, C, D_2, D_3) to be determined (Figure 4A). The 30-bp duplexes were labeled with ^{32}P at the 5′ end of the Watson–Crick strand containing the target sequence, 5′-TTTTTTTYTTTTTTT-3′. The DNA-affinity-cleaving reactions were performed under conditions sensitive to a single-base mismatch in the triple-helical complex (pH 7.4, 35 °C, 40% ethanol). The most efficient cleavage was observed for the combinations N·XY = C·GC, T·AT, D_3·TA, and D_3·CG (Figure 4B,C). The cleavages observed in the reactions of two of these oligonucleotides (Figure 4B, lanes 3 and 8) indicate the known stabilities of T·AT and C^+GC base triplets, respectively. With oligonucleotides possessing modified bases, efficient cleavage was observed when D_3 was opposite a TA or CG base pair (D_3·TA and D_3·CG base triplets) (Figure 4B, lanes 17 and 18). Within this motif, the D_3 base shows an affinity for pyrimidine·purine base pairs over purine·pyrimidine base pairs (D_3·TA ~ D_3·CG ≥ D_3·AT > D_3·GC). The base D_2 exhibits weak binding affinity for all four base pairs which is comparable to that observed for the natural base triplet mismatches[2a,17] (Figure 4C).

From examination of the two-dimensional representation of base triplets containing D_3, it is difficult to assign the observed selectivity to specific hydrogen-bonding interactions (Figure 4D). The differences between D_2 and D_3 suggest that the benzamido group is responsible for a specific interaction stabilizing triple-helix formation. Affinity-cleaving analyses of three amide analogs reveal that the benzamide component is important. When the benzamide is replaced by acetamide, cyclohexanecarboxamide, or 1-naphthamide, diminished affinity and specificity are observed.[18] Modeling studies suggest D_3 may lie out of the plane of the opposite Watson–Crick base pair to avoid steric clashes between the benzamide group and the purine base in a D_3·TA or D_3CG triplet.[18,19] The observation that D_3 prefers binding both TA and CG base pairs but not AT or GC base pairs suggests that the specificity could be due to *shape-selective* discrimination. Perhaps interactions between the walls of the major groove and the benzamido group are important.

Additional studies have shown that neighboring base triplets influence the stabilities of D_3·TA and D_3·CG triplets. They are most stable when flanked by T·AT triplets and less stable when C^+GC triplet is on the 3′ side.[20] Undoubtedly, two-dimensional models are inadequate, and further understanding must await direct structural studies.

Site-Specific Double-Strand Cleavage of Plasmid DNA. In a formal sense, the D_3·TA and D_3·CG triplets constitute a degenerate code for recognition of CG or TA base pairs by triple-helix formation. The specificity of oligonucleotides containing D_3 targeted toward single sites in large DNA was examined. Cleavage of plasmid DNA under conditions sensitive to a single-mismatch triplet allows for a demonstration of the stabilizing effect of the D_3·TA and D_3·CG triplets within a local triple-helical complex. The ability of pyrimidine oligonucleotide–EDTA·Fe(II) complexes containing D_3 to cause site-specific double-strand breaks at a naturally occurring mixed sequence containing all four base pairs in SV40 DNA (5.2 kbp in size) was examined under physiologically relevant pH and temperature. (Figure 5A). SV40 was digested with *Bcl*I and radiolabeled at both 3′ ends to produce a ^{32}P-labeled 5.2-kbp fragment, which contained the 18 base pair target sequence d(AAATAAAAGACAAAAAGA) located 2.2 and 3.0 kbp from the ends. This purine-rich target site contains fourteen AT base pairs, two GC base pairs, one TA base pair, and one CG base pair. The oligonucleotide–EDTA·Fe(II) complexes of sequence composition 5′-T*TTXTTTTCTYTTTTTCT-3′ (where X, Y = T, T; T, D_3; D_3, T; and D_3, D_3; for oligonucleotides **13–16**, respectively) were synthesized, and relative binding affinities were compared by the affinity-cleaving method (pH = 7.0, 37 °C). The double-mismatch controls at the CG and TA base pair positions (oligonucleotide **13**, where X, Y = T, T) reveal no site-specific cleavage (Figure 5A). The single-mismatch controls at CG and TA base pair positions (oligonucleotides **14** and **15**, where X, Y = T, D_3 and D_3, T, respectively) reveal no site-specific cleavage (Figure 5A). However, oligonucleotide **16** (where X, Y = D_3, D_3) binds and double-strand cleaves at the target site, producing DNA products 3.0 and 2.2 kbp in size. This result indicates that the D_3·TA and D_3·CG triplets stabilize the triple-helical complex.

Concluding Remarks. The novel base 4-(3-benzamidophenyl)imidazole (D_3) enables the selective binding of TA and CG Watson–Crick base pairs within a pyrimidine·purine·pyrimidine triple helix. When used in combination with the natural triplets T·AT and C^+GC, this allows the oligonucleotide-directed sequence-specific recognition of double-helical DNA sequences containing *all four base pairs*, without the need for alternate strand crossover junctions.[2c–f,21] Although the D_3·TA and D_3·CG triplets formally extend triple-helix specificity to all four possible base pairs of double-helical DNA, limitations do exist. The first is that D_3 does not distinguish between TA and CG base pairs. A second limitation is that there are nearest neighbor interactions that influence the stabilities of D_3·TA and D_3·CG triplets, indicating that consideration of the sequence composition of target sites will be important.[20] The 4-(3-benzamidophenyl)imidazole binding of pyrimidine·purine base pairs is likely an example of recognition of Watson–Crick base pairs by triple-helix formation dominated by an ensemble of van der Waals interactions rather than specific hydrogen bonds. These results may provide structural insight into the design of deoxyribonucleosides with nonnatural heterocycles with specific recognition properties directed toward a more general solution to sequence-specific recognition of double-helical DNA.

Experimental Section

^{1}H and ^{13}C NMR spectra were recorded at 400 MHz on a JEOL GX400 NMR spectrometer. IR spectra were recorded on a Perkin-Elmer 1600 Series FTIR spectrophotometer. High-resolution mass spectra (HRMS) were recorded using electron ionization (EI) or fast atom bombardment (FAB) techniques at the Midwest Center for Mass Spectrometry at the University of Nebraska, Lincoln, or at the Mass Spectrometry Facility at the University of California, Riverside. Melting points are uncorrected. Elemental analyses were performed by the analytical laboratory at the California Institute of Technology. Acetonitrile, dichloromethane, chlorotrimethylsilane, and *N,N*-diisopropylethylamine were distilled from calcium hydride. All other solvents and reagents were commercially available and used without further purifi-

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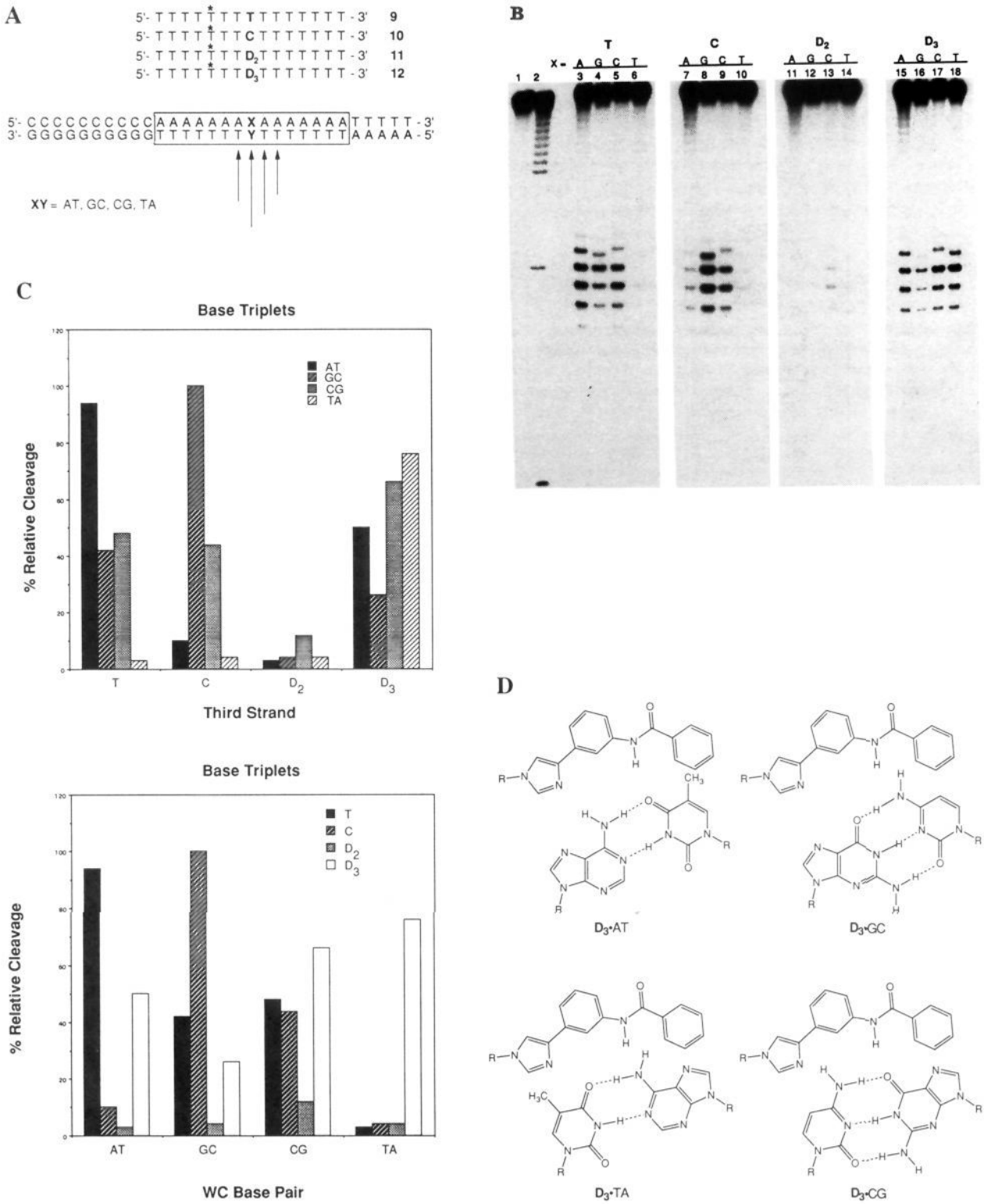
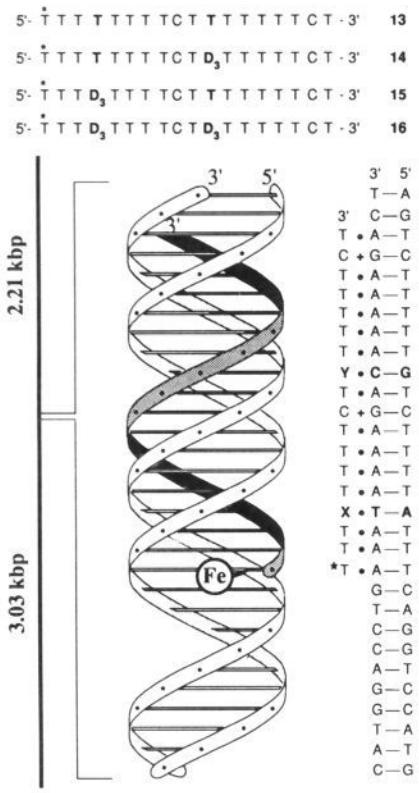


Figure 4. (A) Above: Sequences of oligonucleotide–EDTA **9–12**, where **T*** is the position of the thymidine–EDTA.[15] The oligonucleotides differ at one base position indicated in bold type. Below: Box indicating the double-stranded sequence bound by oligonucleotide–EDTA·Fe(II) **9–12**. The Watson–Crick base pair (AT, GC, CG, or TA) opposite the variant base in the oligonucleotide is also in bold type. (B) Autoradiogram of the 20% denaturing polyacrylamide gel. The cleavage reactions were carried out by combining a mixture of oligonucleotide–EDTA (2 μM), spermine (1 mM), and Fe(II) (25 μM) with the ^{32}P-labeled 30-mer duplex [~0.5 mM (bp)] in a solution of Tris–acetate, pH 7.4 (25 mM), NaCl (50 mM), calf thymus DNA [100 μM (bp)], and 40% ethanol and incubating at 35 °C for 1 h. Cleavage reactions were initiated by addition of DTT (3 mM) and allowed to proceed for 6 h at 35 °C. The reactions were stopped by freezing and lyophilization, and the cleavage products were analyzed by gel electrophoresis. (Lanes 1–18) duplexes containing 5′-end-labeled d($A_5T_7YT_7G_{10}$): (lane 1) control showing intact 5′-labeled 30-bp DNA standard obtained after treatment according to the cleavage reactions in the absence of oligonucleotide–EDTA; (lane 2) products of G + A chemical sequencing reaction; (lanes 3–18) DNA cleavage products produced by oligonucleotide–EDTA·Fe(II) [**9** (lanes 3–6), **10** (lanes 7–10), **11** (lanes 11–14), **12** (lanes 15–18)]. XY = AT (lanes 3, 7, 11, 15); XY = GC (lanes 4, 8, 12, 16); XY = CG (lanes 5, 9, 13, 17); XY = TA (lanes 6, 10, 14, 18). (C) Bar graph presenting the relative cleavage data from densitometric analysis of part B. Sixteen base triplets were examined for binding specificity compatible with the pyrimidine triple-helix motif. The data are reproducible within ±10% of reported values. (D) Two-dimensional models of D_3·AT, D_3·GC, D_3·TA, and D_3·CG triplets.



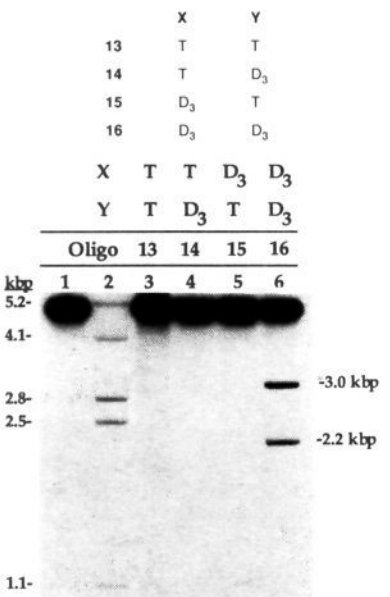


Figure 5. (A, Top) Above: Sequences of oligonucleotide–EDTA complexes **13–16**. Below: Ribbon model of the triple-helix complex between the bound oligonucleotide–EDTA·Fe(II) **16** and a single site containing all four base pairs within the 5.24-kbp plasmid DNA. (B, Bottom) Autoradiogram of double-strand cleavage of SV40 length analyzed on a 1% agarose gel. The cleavage reactions were carried out by combining a mixture of oligonucleotide–EDTA **13** (4 μM), spermine (1 mM), and Fe(II) (5 μM) with the ^{32}P-labeled linearized plasmid [17 μM (bp)] in a solution of Tris–acetate, pH 7.0 (50 mM), NaCl (50 mM), and calf thymus DNA [100 μM (bp)] and incubating 1 h for at 37 °C. Cleavage reactions were initiated by addition of DTT (4 mM) and allowed to proceed for 10 h at 37 °C. The reactions were stopped by precipitation with ethanol, and the cleavage products were analyzed by gel electrophoresis. (Lanes 1–6) SV40 linearized with *Bcl*I and labeled at both 3′ ends with ^{32}P: (lane 1) control containing no oligonucleotide–EDTA·Fe(II); (lane 2) DNA size markers obtained by digestion of *Bcl*I linearized SV40 with *Acc*I and *Bgl*I 5243 (undigested DNA), 4101, 2778, 2465, 1142; (lanes 3–6) DNA cleavage products produced by oligonucleotide–EDTA·Fe(II) complexes [**13** (lane 3), **14** (lane 4), **15** (lane 5), **16** (lane 6)].

cation. Reactions were performed in oven-dried flasks under positive argon or nitrogen pressure unless otherwise noted. Reactions were monitored by analytical thin-layer chromatography (TLC) with E. Merck silica-gel 60F plates (0.25 mm). Flash chromatography was executed with E. Merck silica gel (230–400 mesh).[22]

4-(3-Nitrophenyl)imidazole (1). This compound was prepared by a modificadtion of the procedure employed by Bredereck and Theilig.[7] A solution of 1-nitro-3-(bromoacetyl)benzene[23] (4.67 g, 19.1 mmol) in formamide (100 mL) was heated in an oil bath to 190 °C. The mixture changed from colorless to a deep orange. After the reaction was allowed to stir at this temperature for 1 h, the solution was cooled to ambient temperature and diluted with water (30 mL) to produce a brown slurry. The mixture was poured onto a short column of ion-exchange resin (Biorad AG-50W-X8, hydrogen form). The column was first washed with water, neutralized with 10% ammonium hydroxide (100 mL), and finally washed with methanol (3 × 200 mL). The methanol fractions were concentrated under reduced pressure, and the product was purified by flash chromatography (triethylamine/methanol/ethyl acetate/hexane, 1:1:9:9) to yield 2.57 g (71%) of a yellow powder. Crystallization from water afforded yellow crystals: mp 223–224 °C; IR (KBr) (cm^{-1}) 3448, 3103, 1524, 1347, 866; ^{1}H NMR (400 MHz, CD_3OD) δ 8.59 (t, J = 1.8 Hz, 1 H), 8.11–8.07 (m, 2 H), 7.80 (d, J = 1.1 Hz, 1 H), 7.66 (d, J = 1.1 Hz, 1 H), 7.59 (t, J = 8.1 Hz, 1 H). HRMS (EI) for $C_9H_7N_3O_2$ (M^+): calcd 189.0538, found 189.0532.

1-[2-Deoxy-3,5-bis-*O*-(4-methylbenzoyl)-β-D-ribofuranosyl]-4-(3-nitrophenyl)imidazole (2). 4-(3-Nitrophenyl)imidazole (**1**) (10.0 g, 52.9 mmol) was suspended in acetonitrile (800 mL), and this mixture was cooled to 0 °C. Sodium hydride (1.90 g, 80% dispersion in mineral oil, 63.3 mmol) was added, and the solution was allowed to stir under Ar for 40 min. 1-Chloro-2-deoxy-3,5-bis-O-(4-methylbenzoyl)-α-D-ribofuranose[8] (42.4 g, 109 mmol) was added, the cooling bath was removed, and the solution was allowed to stir for 3 h. After filtration through celite, the solvent was evaporated. The residue was dissolved in CH_2Cl_2 and passed through a short column of silica gel, eluting with EtOAc/hexane (3:1). Concentration of the resulting solution and recrystallization from EtOAc/hexane (3:1) gave 7.39 g of the product as yellow crystals. A further 11.16 g was obtained by evaporating the mother liquor and chromatographing the residue using EtOAc/hexane (3:1): Overall yield 18.55 g (65%); IR (KBr) (cm^{-1}) 3061, 2953, 1730, 1714, 1520, 1349, 1268, 1104, 868; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.28 (t, J = 1.1 Hz, 1 H), 7.88 (d, J = 8.1 Hz, 1 H), 7.80–7.76 (m, 4 H), 7.65 (d, J = 7.7 Hz, 1 H), 7.57 (d, J = 1.1 Hz, 1 H), 7.28–7.24 (m, 2 H), 7.12–7.06 (m, 4 H), 6.01 (t, J = 6.2 Hz, 1 H), 5.57–5.54 (m, 1 H), 4.59 (dd, J = 13.2, 4.4 Hz, 1 H), 4.47–4.44 (m, 2 H), 2.62–2.57 (m, 2 H), 2.27 (s, 3 H), 2.22 (s, 3 H); ^{13}C NMR (62.9 MHz, $CDCl_3$) δ 166.0, 165.8, 148.7, 144.5, 144.3, 141.0, 136.5, 135.7, 130.5, 129.7, 129.5, 129.3, 129.2, 126.7, 126.4, 121.3, 119.5, 113.1, 86.5, 82.9, 74.9, 63.9, 39.5, 21.6, 21.5. HRMS (FAB) for $C_{30}H_{28}N_3O_7$ (MH^+): calcd 542.1927, found 542.1930. Anal. Calcd for $C_{30}H_{27}N_3O_7$: C, 66.54; H, 5.03; N, 7.76. Found: C, 66.74; H, 5.05; N, 7.44.

1-(2-Deoxy-β-D-ribofuranosyl)-4-(3-nitrophenyl)imidazole (3). The protected nucleoside **2** (2.32 g, 4.30 mmol) was dissolved in 1% sodium hydroxide in methanol (50 mL). The reaction was stirred for 1 h at room temperature. The solution was concentrated under reduced pressure until ca. 10 mL remained. Silica gel was added (ca. 10 g), and the remaining solvent was evaporated. The resulting residue was added as a slurry to the top of a flash-chromatography column (ethyl acetate/hexane/methanol, 72:18:1) for purification of the reaction product. The deprotected nucleoside was obtained as a yellow solid (1.30 g, 99%): IR (KBr) (cm^{-1}) 3300, 2917, 1528, 1342, 871; ^{1}H NMR (400 MHz, CD_3OD) δ 8.57–8.56 (m, 1 H), 8.09–8.05 (m, 2 H), 7.99 (br s, 1 H), 7.91 (br s, 1 H), 7.57 (t, J = 8.1 Hz, 1 H), 6.14–6.11 (m, 1 H), 4.50–4.47 (m, 1 H), 3.99–3.96 (m, 1 H), 3.78–3.68 (m, 2 H), 2.56–2.49 (m, 1 H), 2.45–2.39 (m, 1 H); ^{13}C NMR (62.9 MHz, CD_3OD) δ 150.2, 142.3, 138.5, 137.1, 131.7, 130.9, 122.3, 120.3, 116.1, 89.2, 88.0, 72.4, 63.2, 42.7. HRMS (EI) for $C_{14}H_{15}N_3O_5$ (M^+): calcd 305.1012, found 305.1025.

1-(2-Deoxy-β-D-ribofuranosyl)-4-(3-aminophenyl)imidazole (4). To the nucleoside derivative **3** (0.724 g, 2.37 mmol) dissolved in methanol (5 mL) at room temperature was added 5% palladium on carbon (ca. 10 mg). The reaction flask was evacuated under reduced pressure and then repressurized with hydrogen gas. After the mixture was stirred for 2 h, the catalysts was removed by filtration through Celite, and the resulting colorless solution was concentrated under reduced pressure. Filtration through silica gel (methylene chloride/methanol, 9:1) yielded 0.650 g (99%) of product as a white foam: IR (KBr) (cm^{-1}) 3352, 2909, 1376, 1305, 1185, 1095, 1047; UV (CH_3OH) [λ, nm (ϵ, mol^{-1} cm^{-1})] 228 (34 100, λ_{max}), 260 (15 200), 280 (4610); ^{1}H NMR (400 MHz, CD_3OD)

(22) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.
(23) Evans, W. L.; Brooks, B. T. *J. Am. Chem. Soc.* **1908**, *30*, 404–412.

δ 7.90 (d, J = 1.1 Hz, 1 H), 7.60 (d, J = 1.1 Hz, 1 H), 7.11–7.04 (m, 3 H), 6.63–6.60 (m, 1 H), 6.09 (t, J = 6.6 Hz, 1 H), 4.48–4.44 (m, 1 H), 3.97–3.94 (m, 1 H), 3.73 (dd, J = 12.1, 4.0 Hz, 1 H), 3.68 (dd, J = 12.1, 4.6 Hz, 1 H), 2.54–2.47 (m, 1 H), 2.42–2.36 (m, 1 H); ^{13}C NMR (62.9 MHz, CD_3OD) δ 148.9, 143.5, 137.6, 135.8, 103.3, 116.2, 115.5, 114.2, 113.1, 89.0, 87.8, 72.4, 63.2, 42.5. HRMS (FAB) for $C_{14}H_{18}N_3O_3$ (MH^+): calcd 276.1348, found 276.1351.

1-(2-Deoxy-β-D-ribofuranosyl)-4-(3-benzamidophenyl)imidazole (5). A solution of aniline **4** (0.373 g, 1.35 mmol) in pyridine (5 mL) was cooled to 0 °C, and neat chlorotrimethylsilane (0.88 mL, 6.93 mmol) was added dropwise over 5 min. After the resulting mixture was stirred for 0.5 h at 0 °C, benzoyl chloride was introduced (0.80 mL, 6.89 mmol) and the reaction was warmed to room temperature. The solution was stirred for 2.5 h at ambient temperature, recooled to 0 °C, and quenched with water (3 mL). After 10 min, a saturated solution of ammonium hydroxide (3 mL) was added, and the reaction was stirred for 1 h. The mixture was then concentrated under reduced pressure. To the residue was added a saturated solution of aqueous sodium bicarbonate (ca. 5 mL). The resulting solution was extracted with ethyl acetate (3 × 5 mL), and the combined organic layers were washed with brine and dried ($MgSO_4$). Concentration of the organic extracts followed by purification by flash chromatography (methylene chloride/methanol, 19:1 → 7:3) provided 0.474 g (92%) of the product as a white solid: IR(KBr) (cm^{-1}) 3244, 2922, 1614, 1504, 1255, 1094, 1050; UV (CH_3OH) [λ, nm (ε, mol^{-1} cm^{-1})] 206 (20 600, λ_{max}), 260 (18 300, λ_{max}), 280 (9530); ^{1}H NMR (400 MHz, CD_3OD) δ 7.99–7.94 (m, 3 H), 7.73 (d, J = 1.1 Hz, 1 H), 7.65 (dt, J = 8.1, 1.1 Hz, 1 H), 7.61–7.40 (m, 3 H), 7.37 (t, J = 8.1 Hz, 1 H), 7.23–7.08 (m, 2 H), 6.13 (t, J = 6.6 Hz, 1 H), 4.50–4.47 (m, 1 H), 3.99–3.96 (m, 1 H), 3.78–3.68 (m, 2 H), 2.57–2.50 (m, 1 H), 2.42 (ddd, J = 13.6, 6.2, 3.7 Hz, 1 H). HRMS (EI) for $C_{21}H_{21}N_3O_4$ (M^+): calcd 379.1532, found 379.1521.

1-[5-O-[Bis(4-methoxyphenyl)phenylmethyl]-2-deoxy-β-D-ribofuranosyl]-4-(3-benzamidophenyl)imidazole (6). To a stirred solution of nucleoside **5** (0.442 g, 1.16 mmol) in pyridine (3 mL) at 0 °C were added triethylamine (0.65 mL, 4.6 mmol), 4-(N,N-dimethylamino)pyridine (21 mg), and 4,4′-dimethoxytrityl chloride (0.601 g, 1.77 mmol). The mixture was stirred for 4 h and then quenched with an aqueous solution of saturated sodium bicarbonate (ca. 4 mL). The resulting solution was extracted with ethyl acetate (2 × 5 mL), and the combined organic layers were washed with brine and dried ($MgSO_4$). Concentration of the extracts followed by flash chromatography (ethyl acetate/hexane/methanol, 6:3:1) furnished 0.697 g (88%) of the protected nucleoside as a white solid: IR (KBr) (cm^{-1}) 3345, 3059, 2932, 1654, 1610, 1509, 1252, 1032; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87–7.81 (m, 4 H), 7.67 (s, 1 H), 7.58–7.18 (m, 15 H), 6.81 (d, J = 8.8 Hz, 2 H), 6.79 (d, J = 8.8 Hz, 2 H), 6.06 (t, J = 6.6 Hz, 1 H), 4.61–4.58 (m, 1 H), 4.11–4.09 (m, 1 H), 3.72 (s, 6 H), 3.41–3.33 (m, 2 H), 2.60–2.55 (m, 1 H), 2.47–2.44 (m, 1 H). HRMS (FAB) for $C_{42}H_{40}N_3O_6$ (MH^+): calcd 682.2917, found 682.2883.

Phosphoramidite of DMT-Protected D_3 (7). A solution of the protected nucleoside **6** (0.213 g, 312 μmol) in methylene chloride (0.5 mL) was cooled to 0 °C. After the addition of N,N-diisopropylethylamine (163 μL, 936 μmol), 2-cyanoethyl N,N-diisopropylphosphoramidochloridite(104 μL, 468 μmol) was introduced, and the reaction was stirred for 30 min. The reaction was quenched with an aqueous solution of saturated sodium bicarbonate (1 mL), and the resulting solution was partitioned with ethyl acetate (3 × 1 mL). The combined organic layers were washed with brine and dried ($MgSO_4$). Concentration of the extracts and purification by flash chromatography (methylene chloride/hexane/triethylamine/methanol, 48:48:1:3) afforded 0.270 g (98%) of the diastereomeric phosphoramidites as a brittle white foam: IR (KBr) (cm^{-1}) 3427, 2967, 2253, 1670, 1508, 1252, 1032, 829; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.23 (s, 1 H), 8.00–7.97 (m, 2 H), 7.94 (s, 1 H), 7.73 (s, 1 H), 7.66–7.51 (m, 4 H), 7.39–7.36 (m, 2 H), 7.30–7.17 (m, 10 H), 6.86–6.81 (m, 4 H), 6.18–6.14 (m, 1 H), 4.63–4.59 (m, 1 H), 4.12–3.98 (m, 1 H), 3.79–3.30 (m, 10 H), 3.22–3.18 (m, 2 H), 2.93–2.88 (m, 1 H), 2.82–2.79 (m, 1 H), 2.71–2.49 (m, 2 H), 1.29–1.04 (m, 12 H). HRMS (FAB) for $C_{51}H_{57}N_5O_7P$ (MH^+): calcd 882.3996, found 882.3966.

1-[2-Deoxy-3,5-bis-O-(4-methylbenzoyl)-β-D-ribofuranosyl]-4-phenylimidazole. A dispersion of 4-phenylimidazole (2.88 g, 20.0 mmol) in acetonitrile (180 mL) was deprotonated with sodium hydride (0.88, 60% dispersion in mineral oil, 22 mmol) in the same manner as **2**. 1-chloro-2-deoxy-3,5-di-O-p-toluoyl-α-D-ribofuranose[9] (8.5 g, 22 mmol) was added, and the reaction was quenched according to the procedure described for **2**. Purification by flash chromatography (hexane/methylene chloride/acetonitrile, 3:2:1) followed by recrystallization from ethanol/methylene chloride afforded 5.26 g of the product (53%) as a white crystalline solid: mp 160–161 °C; IR (KBr) (cm^{-1}) 3023, 2921, 1708, 1610, 1492, 1270, 1104; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (d, J = 8.1 Hz, 2 H), 7.95 (d, J = 8.1 Hz, 2 H), 7.70 (d, J = 1.1 Hz, 1 H), 7.55–7.52 (m, 2 H), 7.31–7.19 (m, 8 H), 6.16 (t, J = 7.0, 1 H), 5.74–5.72 (m, 1 H), 4.78–4.73 (m, 1 H), 4.63–4.57 (m, 2 H), 2.76–2.72 (m, 2 H), 2.44 (s, 3 H), 2.42 (s, 3 H). HRMS (FAB) for $C_{30}H_{29}N_2O_5$ (MH^+): calcd 497.2076, found 497.2091. Anal. Calcd for $C_{30}H_{28}N_2O_5$: C, 72.56; H, 5.68; N, 5.64. Found: C, 72.42; H, 5.67; N, 5.64.

1-(2-Deoxy-β-D-ribofuranosyl)-4-phenylimidazole. A solution of 1-[2-deoxy-3,5-bis-O-(4-methylbenzoyl)-β-D-ribofuranosyl]-4-phenylimidazole (1.99 g, 4.00 mmol) was stirred in a 1% sodium hydroxide/methanol solution (80 mL) at room temperature in analogy to the method employed for the synthesis of **3**. Purification by flash chromatography (methylene chloride/toluene/methanol, 8:1:1) furnished the diol as a white solid in 96% yield: IR (KBr) (cm^{-1}) 3442, 3122, 2920, 1609, 1495, 1228, 1103; UV (CH_3OH) [λ, nm (ε, mol^{-1} cm^{-1})] 208 (19 700, λ_{max}), 260 (11 500, λ_{max}), 280 (2810); ^{1}H NMR (400 MHz, CD_3OD) δ 7.93 (d, J = 1.1 Hz, 1 H), 7.73–7.70 (m, 2 H), 7.69 (d, J = 1.1 Hz, 1 H), 7.37–7.32 (m, 2 H), 7.25–7.21 (m, 1 H), 6.11 (t, J = 6.6 Hz, 1 H), 4.50–4.46 (m, 1 H), 4.00–3.96 (m, 1 H), 3.75 (dd, J = 11.7, 4.0 Hz, 1 H), 3.69 (dd, J = 11.7, 4.4 Hz, 1 H), 2.52–2.49 (m, 1 H), 2.43–2.38 (m, 1 H). HRMS (EI) for $C_{14}H_{16}N_2O_3$ (M^+): calcd 260.1161, found 260.1160.

1-[5-O-[Bis(4-methoxyphenyl)phenylmethyl]-2-deoxy-β-D-ribofuranosyl]-4-phenylimidazole. A solution of 1-(2-deoxy-β-D-ribofuranosyl)-4-phenylimidazole (0.94 g, 3.6 mmol) was dissolved in pyridine (6 mL) and cooled to 4 °C, and 4,4′-dimethoxytrityl chloride (1.22 g, 3.60 mmol) was added. The reaction was allowed to stir at this temperature for 6 h. During the course of the reaction, additional portions of 4,4′-dimethyoxytrityl chloride (2 × 0.60 g, 3.5 mmol) were added until no starting material remained. The reaction was quenched by the addition of methanol (3 mL), and the resulting solution was diluted with methylene chloride (50 mL). This mixture was extracted with water (2 × 50 mL), and the organic layer was dried (Na_2SO_4). Concentration of the extracts followed by purification by flash chromatography (ethyl acetate/hexane/methanol, 20:10:0 then 20:10:1) provided 1.72 g (85%) of the product as a white foam: IR (KBr) (cm^{-1}) 3385, 2933, 1608, 1508, 1252, 1177, 1033; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57 (d, J = 0.7 Hz, 1 H), 7.49–7.47 (m, 2 H), 7.39–7.12 (m, 13 H), 6.75 (d, J = 9.2 Hz, 4 H), 5.96–5.93 (m, 1 H), 4.60–4.57 (m, 1 H), 4.14–4.11 (m, 1 H), 3.64 (s, 6 H), 3.33–3.29 (m, 2 H), 2.48–2.41 (m, 1 H), 2.34 (ddd, J = 13.6, 5.9, 2.9 Hz, 1 H). HRMS (FAB) for $C_{35}H_{35}N_2O_5$ (MH^+): calcd 563.2546, found 563.2544.

Phosphoramidite of DMT-Protected D_2 (8). To a solution of 1-β-(4-phenylimidazolyl)-2-deoxy-5-O-(4,4′-dimethoxytrityl)ribose (0.169 g, 300 μmol) in methylene chloride (1.2 mL) was added diisopropylethylamine (156 μL, 900 μmol) followed by 2-cyanoethyl N,N-diisopropylphosphoramidochoridite (100 μL, 450 μmol). The resulting mixture was stirred for 1 h at room temperature quenched with ethanol (0.5 mL), and diluted with ethyl acetate (30 mL). This solution was washed with an aqueous solution of saturated sodium bicarbonate (2 × 30 mL) and brine (2 × 30 mL). The organic layer was dried (Na_2SO_4). Concentration of the organic layer followed by purification by flash chromatography (ethyl acetate/hexane/methanol/triethylamine, 32:16:1:1) afforded 0.215 g (94%) of the diastereomeric phosphoramidites as a brittle white foam: IR (KBr) (cm^{-1}) 2967, 2238, 1609, 1508, 1252, 1179, 1033, 829; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.67 (s, 1 H), 7.54–7.51 (m, 2 H), 7.42–7.40 (m, 3 H), 7.31–7.15 (m, 10 H), 6.80–6.76 (m, 4 H), 6.06–6.02 (m, 1 H), 4.72–4.62 (m, 1 H), 4.26–4.19 (m, 1 H), 3.72–3.54 (m, 8 H), 3.39–3.28 (m, 2 H), 2.62–2.43 (m, 6 H), 1.18–1.08 (m, 12 H). HRMS (FAB) for $C_{44}H_{52}N_4O_6P$ (MH^+): calcd 763.3625, found 763.3593.

Synthesis of Oligonucleotides. Oligonucleotides were synthesized on a 1-μmol scale on a Beckman System 1 Plus or ABI 380B DNA synthesizer using 2-cyanoethyl phosphoramidite chemistry.[13,14] The phosphoramidite derivative of the nucleoside analog T* was prepared according to published procedures.[15] Phosphoramidite derivatives **7** and **8** were prepared as described and utilized in automated synthesis as 0.1 M solutions in acetonitrile. Coupling yields for modified bases were comparable to those obtained for the standard phosphoramidite monomers (97–99%). Unmodified oligonucleotides were produced by deprotection under standard conditions using ammonium hydroxide. Oligonucleotides containing modified bases were treated with 0.1 N NaOH (1.2 mL) at 55 °C for 20 h, and the resulting solutions were neutralized with glacial acetic acid (ca. 6 μL).[15] The resulting mixture was applied to a column of Sephadex G10-120 and eluted with water. The crude oligonucleotides were lyophilized and then purified by electrophoresis on 20% denaturing polyacrylamide gels (19:1 monomer/bis). The major UV-absorbing bands were excised, crushed, and eluted (0.2 N NaCl, 1 mM EDTA, 37 °C, 24 h). After filtration through a Quik-Sep filter (Isolab), the resulting solution was then exhaustively dialyzed against water. After dialysis, the oligonucleotide solutions were passed through a 0.45-μm Centrex filter (Schleicher and Schuell). The concentrations of the oligonucleotides were determined by UV measurements (A_{260}) with-

a Perkin-Elmer Lambda 4C UV-VIS or Hewlett Packard 8452A diode array spectrophotometer.

To characterize the stability of the modified bases under the conditions employed for oligonucleotide synthesis, oligonucleotide 4-mers 5'-TTD_2T-3' and 5'-TTD_3T-3' were synthesized on a 10-μmol scale and deprotected using 0.1 N NaOH. After deprotection, the oligonucleotides were desalted on Sephadex G-10-120 columns, passed through 0.45-μm filters, and then lyophilized. 5'-TTD_2T-3': ^{1}H NMR (400 MHz, DMSO-d_6, partial) δ 7.96 (s, 1 H), 7.91 (s, 1 H), 7.82 (s, 1 H), 7.79–7.78 (m, 3 H), 7.74 (s, 1 H), 7.34–7.30 (m, 2 H), 7.18–7.14 (m, 1 H), 6.21–6.12 (m, 3 H). 5'-TTD_3T-3': ^{1}H NMR (400 MHz, DMSO-d_6, partial) δ 10.56 (s, 1 H), 8.18 (s, 1 H), 8.06 (d, J = 1.5 Hz, 1 H), 8.04 (s, 1 H), 7.92 (s, 1 H), 7.84–7.81 (m, 2 H), 7.77 (s, 1 H), 7.55–7.48 (m, 4 H), 7.29–7.26 (m, 1 H).

HPLC Analyses. Analytical HPLC was performed with a Hewlett-Packard 1090 liquid chromatographer using a reverse-phase Brownlee Labs Aquapore OD-300 4.6 mm × 22 cm, 7-μm C18 column. The purified oligodeoxyribonucleotide (3 nmol) was digested simultaneously with snake venom phosphodiesterase (6 μg) and calf intestine alkaline phosphatase (3 units) in 50 mM Tris-HCl (pH 8.1), 100 mM $MgCl_2$. The reaction mixture was incubated at 37 °C for 2 h and lyophilized. The sample was dissolved with water (10 μL), and an aliquot of the solution (5 μL) was injected onto a C18 reverse-phase column. The products were eluted with 10 mM ammonium phosphate (pH 5.1) with a gradient of 8–50% methanol and detected by UV absorption at 260 nm. Comparison and co-injection with a standard solution containing C, T, and the modified base (D_2 or D_3) established the composition of the oligonucleotide.

DNA Manipulations. Distilled deionized water was employed in all aqueous reactions and dilutions. Enzymes were purchased from Boehringer-Mannheim or New England Biolabs. Deoxynucleoside triphosphates were purchased from Pharmacia as 100 mM solutions. The radiolabeled triphosphates 5'-(α-^{32}P)dNTPs (>3000 Ci/mM) and 5'-(γ-^{32}P)ATP were obtained from Amersham. The SV40 DNA was purchased from Bethesda Research Laboratories. Calf thymus DNA, obtained from Sigma, was sonicated and subjected to a series of extractions with water saturated phenol (3 × 0.2 volumes) and then 24:1 chloroform/isoamyl alcohol (1 × 0.2 volumes), followed by chloroform (1 × 0.2 volumes). The resulting solution was extensively dialyzed against water and passed through a 0.45-μM Centrex filter (Schleicher & Schuell), and the DNA concentration was determined by UV assuming ϵ_{260} = 11 800 L (mol bp cm). Agarose gel electrophoresis and polyacrylamide gel electrophoresis was executed in TBE buffer.[23] 3'-End-labeling of DNA was accomplished with the Klenow fragment of DNA polymerase I via standard procedures.[24] Radioactivity (Cerenkov) was measured with a Beckman LS 2801 scintillation counter. Autoradiography was carried out using Kodak X-AR film. Optical densitometry was performed utilizing an LKB Broma Ultrascan XL Laser Densitometer operating at 633 nm. Relative peak area for each cleavage band or locus was equated to the relative cleavage efficiency at that site.

Affinity-Cleaving Reactions of 30-mer Duplex Targets. For the preparation of the duplex targets, each single-stranded oligonucleotide (50 pmol) 5'-AAAAATTTTTTTYTTTTTTTGGGGGGGGGG-3' (Y = T, C, G, A) was labeled at the 5' end with T4 polynucleotide kinase and (γ-^{32}P)ATP according to standard procedures.[24] The radiolabeled oligonucleotides were then hybridized to their complement (100 mM NaCl, 50 mM Tris-acetate, pH 7.4), and the resulting duplexes were then purified on nondenaturing 15% polyacrylamide gels. The end-labeled duplexes were visualized by autoradiography, and desired bands were excised from the gel, crushed, and eluted (1 mL 0.2 M NaCl, 37 °C, 24 h). The eluents were passed through 0.45-μm Centrex filters and exhaustively dialyzed against water. Maxam-Gilbert (A^+G chemical sequencing reactions[25] were carried out on the purified 5'-end-labeled 30-mer duplexes according to standard procedures.

The cleaving reactions were executed in a total volume of 20 μL with final concentrations of each species as indicated.[26] A mixture of the oligonucleotide-EDTA (2 μM) and ferrous ammonium sulfate (25 μM) was added to the ^{32}P-end-labeled duplex (ca. 0.5 mM (bp)) in a solution of Tris-acetate (25 mM, pH 7.4), NaCl (100 mM), sonicated calf thymus DNA (100 μM (bp)), spermine tetrahydrochloride (Aldrich, 1 mM, pH 7.4), and 40% ethanol. The oligonucleotide-EDTA·Fe was allowed to equilibrate with the DNA duplex target at 35 °C for 1 h. The reactions were then initiated by the addition of dithiothreitol (3 mM) and allowed to proceed at 35 °C for 6 h. Termination of the reaction was accomplished by freezing followed by lyophilization. The residue was resuspended in 5 μL in 100 mM Tris-borate-EDTA and 80% formamide solution. The ^{32}P-labeled products were separated by 20% denaturing polyacrylamide gel electrophoresis. Cleavage products were visualized by autoradiography, and their relative amounts were analyzed by densitometry.

Double-Strand Cleavage of SV40. SV40 was linearized with *Bcl*I and end-labeled with 5'-(α-^{32}P)dATP and the Klenow fragment of DNA polymerase I according to standard procedures.[24] After the labeling reaction, the DNA was applied to a G50-80 sephadex column, eluted with water, and precipitated with ethanol. Molecular weight markers were produced by digestion of the radiolabeled DNA with *Acc*I and *Bgl*I.

The affinity-cleaving reactions were executed in a total volume of 40 μL with final concentrations of each species as indicated. A mixture of oligonucleotide-EDTA (4 μM) and ferrous ammonium sulfate (5 μM) was added to the ^{32}P-3'-end-labeled restriction fragment (17 μM (bp)) in a solution of Tris-acetate (50 mM, pH 7.0), NaCl (50 mM), sonicated calf thymus DNA (100 μM (bp)), and spermine tetrahydrochloride (1 mM). The oligonucleotide was allowed to equilibrate with the DNA duplex target at 37 °C for 1 h. The reactions were then initiated by the addition of dithiothreitol (4 mM) and allowed to proceed at 37 °C for 10 h. Termination of the reaction was accomplished by precipitation with ethanol, and the resulting pellet was rinsed with cold 70% ethanol (100 μL) and briefly dried in vacuo. The residue was resuspended in TE buffer.[23] The reaction products were separated by electrophoresis on a 1% agarose gel.

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(26) The pH values are not corrected for temperature or the presence of ethanol and are given for the 10-fold concentrated buffer solutions at 25 °C.