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Design of a Nonnatural Deoxyribonucleoside for Recognition of GC Base Pairs by Oligonucleotide-Directed Triple Helix Formation

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Abstract: The deoxyribonucleoside 1-(2-deoxy-β-D-ribofuranosyl)-3-methyl-5-amino-1*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (**P1**) was designed such that two specific hydrogen bonds would form with guanine (G) of a Watson–Crick guanine–cytosine (GC) base pair in the major groove of double-helical DNA. One edge of the **P1** heterocycle mimics N3-protonated cytosine, which would circumvent the pH dependence observed for the formation of triple helices containing C + GC base triplets. **P1** was synthesized in five steps and incorporated by automated methods in pyrimidine oligodeoxyribonucleotides. From affinity cleaving analyses, the stabilities of base triplets decrease in the order P1·GC ≫ P1·CG ≫ P1·AT ~ P1·TA (pH 7.4, 35 °C). **P1** binds GC base pairs within a pyrimidine triple-helix motif as selectively and strongly as C but over an extended pH range. Oligodeoxyribonucleotides containing **P1** residues were shown to bind within plasmid DNA a single 15 base pair site containing five GC base pairs at pH 7.8 and a single 16 base pair site containing six *contiguous* GC base pairs at pH 7.4.

Introduction

Oligodeoxyribonucleotide-directed triple-helix formation offers a powerful chemical approach for the sequence-specific recognition of double-helical DNA.[1,2] Pyrimidine oligodeoxyribonucleotides bind specifically to purine sequences in double-helical DNA to form a local triple-helical structure.[1,2] These oligonucleotides bind in the major groove of DNA parallel to the purine Watson–Crick (W–C) strand through the formation of specific (Hoogsteen) hydrogen bonds.[1-4] Specificity is derived from thymine (T) recognition of adenine–thymine (AT) base pairs (T·AT triplets) and protonated cytosine (C+) recognition of guanine–cytosine (GC) base pairs (C + GC triplets). Complexes of triple-helical nucleic acids containing cytosine (C) and thymine (T) on the Hoogsteen strand are stable in acidic to neutral solutions but dissociate with increasing pH.[1-4] An important factor is the required protonation of the cytosine N3 in the third strand to enable the formation of two hydrogen bonds.[3] Moreover, the stability of triple-helical complexes depends on the number and positions of C + GC triplets. In particular, we have observed that the stability of complexes containing *contiguous* C + GC triplets decreases markedly with increasing pH.[5] Oligodeoxyribonucleotides which contain 5-methylcytosine (mC) bind duplex DNA with greater affinities and over an extended pH range.[1c,2e,6] However, protonation of N3 is still required (mC + GC triplets). For the binding of *contiguous* tracts of GC base pairs on double-helical DNA by the pyrimidine motif[1,2] over a wide range of pH, even oligodeoxyribonucleotides containing 5-methylcytosine may be inadequate.

We report the design of nonnatural bases that bind GC base pairs without protonation in a pyrimidine-motif triple-helical complex.[7,8] Our rationale was to use a molecular frame that would place hydrogen bond donors and acceptors in the appropriate positions to form two specific hydrogen bonds to GC base pairs while maintaining a phosphate–deoxyribose backbone geometry compatible with the pyrimidine triple-helix motif. From mod-

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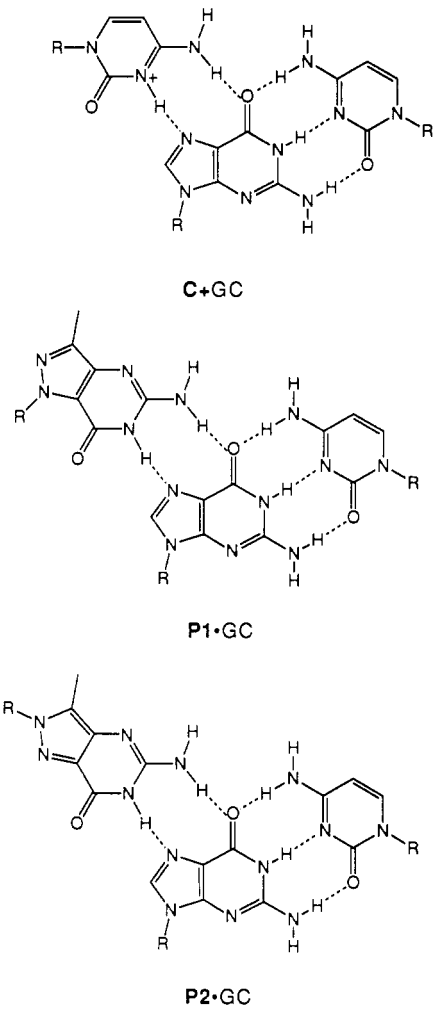

Figure 1. Base triplets C + GC, P1·GC and P2·GC. For the pyrimidine motif, the third strand is in the major groove of the Watson–Crick duplex parallel to the purine W–C strand and antiparallel to the pyrimidine W–C strand.[1-3]

el-building studies, we chose to synthesize the nucleosides **P1** and **P2** (Figure 1). The pyrazole analogues **P1** and **P2** possess a donor–acceptor hydrogen-bonding pattern on one edge that would mimic an N3-protonated cytosine and thus could form two hydrogen bonds to G in the purine W–C strand with no requirement for protonation (Figure 1).

Proton NMR, mass spectral analysis, and enzyme digestion studies showed that **P1** and **P2** nucleosides are stable during the oligonucleotide automated synthesis and base deprotection.[9] The sequence specificity of **P1** for GC base pairs is documented by characterizing the relative stabilities of P1·GC, P1·AT, P1·TA and P1·CG triplets by the affinity cleaving method. When incorporated in a pyrimidine oligonucleotide, **P1** was found to recognize GC base pairs as selectively and strongly as C but over an extended pH range. The utility of **P1** is demonstrated in the site-specific binding of a 15 base pair site containing five GC base pairs (pH 7.8) and a single 16 base pair site containing six *contiguous* GC base pairs (pH 7.4) in plasmid DNA.

Results and Discussion

Synthesis of Nucleosides P1 and P2. A scheme for the five-step syntheses of nucleosides **P1** and **P2** is shown in Figure 2. Ethyl 3-methyl-4-nitropyrazole-5-carboxylate (**1**)[10] was condensed with 1-chloro-2-deoxy-3,5-di-O-*p*-toluoyl-α-D-ribofuranose (**2**)[11] to give the N1 and N2 β anomers, **3a** and **3b**, which could be readily separated by column chromatography. The structures of isomers **3a** and **3b** were assigned by ^{1}H NMR (NOE) and X-ray crystallographic analysis.[9] Irradiation at the C–H1′ proton enhanced the methylene proton peaks of ethyl 5-carboxylate of one isomer by 8.9% and the 5-methyl protons of the other isomer by 13.3%. These were assigned to **3a** and **3b**, respectively. These assignments were confirmed by X-ray crystallographic analysis of crystalline **3b**. Ammonolysis of **3a** in methanol afforded carboxamide **4a**, which in turn was reduced (H_2, 10% Pd/C) to amine **5a**. Treatment of **5a** with phenyl isothiocyanate provided thiol **6a**,[12] which was oxidized to its corresponding sulfonate. Displacement with saturated aqueous ammonium hydroxide[13] affords the amine, **P1**. Nucleoside **P1** was selectively acylated on N5 by the transient protection method[14] to afford **7a**, and the 5′-hydroxy group was treated with dimethoxytrityl chloride to give the DMT-protected nucleoside **8a**. Reaction of **8a** with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite afforded **9a**, the 5′-DMT-protected phosphoramidite of **P1**.[14] Nucleoside **P2** and the corresponding DMT-protected phosphoramidite **9b** were prepared by the same methods.

Synthesis of Oligodeoxyribonucleotides Containing Nucleosides P1 and P2. Oligodeoxyribonucleotides **10–20** were synthesized using standard solid-phase β-cyanoethyl phosphoramidite chemistry.[14] **P1**, **P2**, and the abasic phosphoramidites[15,16] coupled with efficiencies equal to those of A, G, C, and T phosphoramidites. Oligonucleotides **10–20**, which contain T* (thymine-EDTA), were deprotected with 0.1 N NaOH.[17] To examine the stability of the novel bases through several machine-assisted coupling cycles and base deprotection, two short oligodeoxyribonucleotides, 5′-T-T-P1-T-3′ and 5′-T-T-P2-T-3′, were synthesized on a 10-μmol scale for ^{1}H NMR and mass spectral studies. From the ^{1}H NMR and mass spectral analysis of the purified tetramer oligodeoxyribonucleotides, it was found that the *N*-isobutyroyl protecting group of **P1** or **P2** was completely removed to afford a free amine and the novel pyrazole base.[9]

Base Composition Analysis of Oligodeoxyribonucleotides Containing P1 and P2. The integrity of the nonnatural nucleosides **P1** and **P2** after automated synthesis and deprotection steps was analyzed by enzymatic degradation. The base composition of oligodeoxyribonucleotides containing nucleosides **P1** and **P2** was analyzed by HPLC to ensure that the pyrazole heterocycle was not chemically altered during automated synthesis. Purified oligodeoxyribonucleotides containing **P1** or **P2** were treated with snake venom phosphodiesterase and calf intestine alkaline phosphatase to give the corresponding nucleoside monomers. Analysis by HPLC of the enzymatic digestion products of oligonucleotide 5′-T*(T)$_4$(P$_1$T)$_5$-3′ revealed T*, **P1** and T nucleosides. Similarly, oligonucleotide 5′-T*(T)$_3$C(T)$_4$(P$_1$)$_6$T-3′ afforded T*, T, C, and **P1** nucleosides. Oligonucleotide 5′-TP$_2$TT-3′ afforded T and **P2** nucleosides. These analyses suggest that the **P1** and **P2** nucleosides can be used in automated oligodeoxyribonucleotide synthesis without complications.

P1·GC and P2·GC Triplet Stabilities Characterized by Affinity Cleaving. The relative affinities of the novel bases **P1** and **P2** for all four Watson–Crick base pairs within a pyrimidine triple-helix motif were examined (Figure 3A). As a control, these were compared with an abasic residue φ, which contains no base at that same position.[15,16] Oligodeoxyribonucleotides equipped with the DNA-cleaving moiety, thymidine-EDTA·Fe^{II} (T*), allowed the relative stabilities of triple-helix formation between 30 base pair

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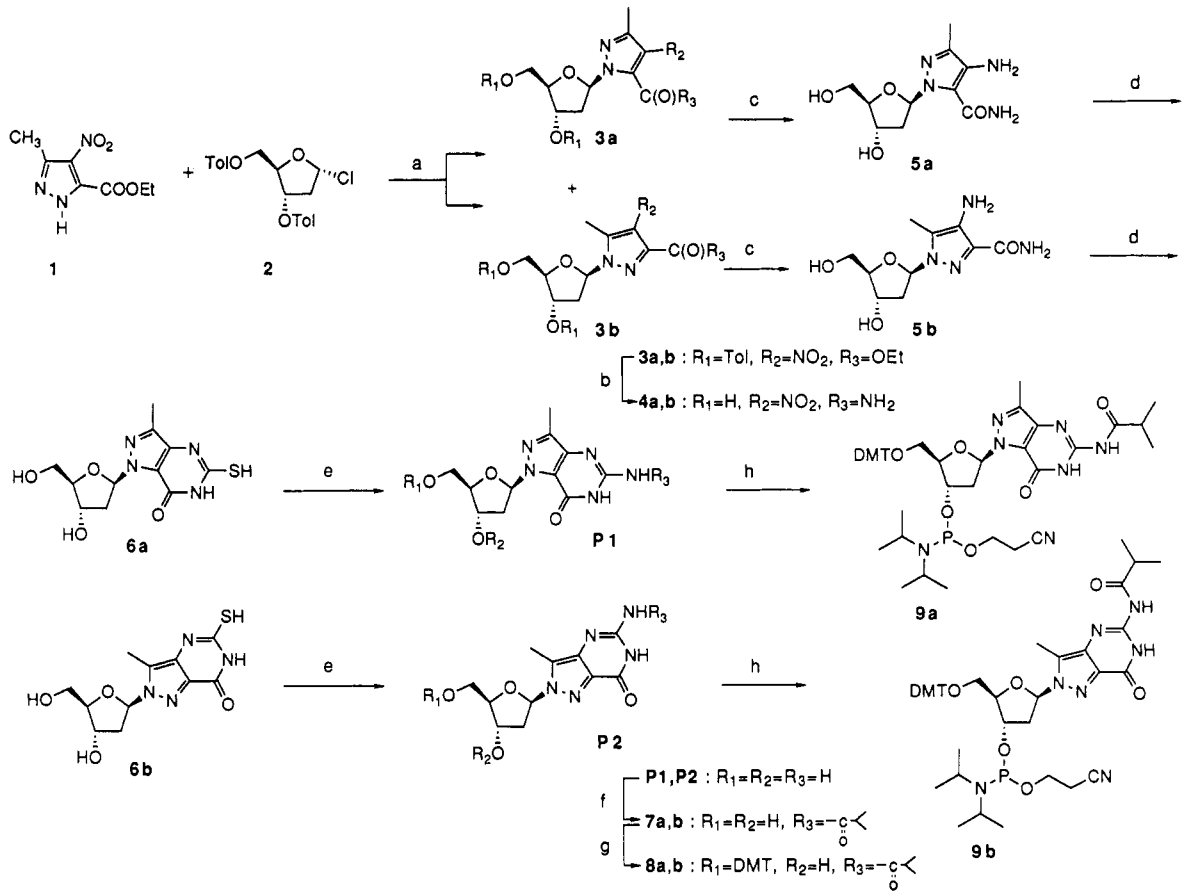

Figure 2. Syntheses of the DMT-protected phosphoramidites of **P1** and **P2**: (a) NaH, CH_3CN; (b) NH_3, MeOH, 120 °C; (c) 10% Pd/C, H_2, $EtOH/H_2O$; (d) PhNCS, pyridine, reflux; (e) NH_4OH; H_2O_2; NH_3, 120 °C; (f) TMS-Cl; isobutyric anhydride, pyridine; (g) DMTCl, pyridine; (h) *N,N*-diisopropylethylamine, β-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, CH_2Cl_2.

(bp) DNA duplexes containing one variable site $d(A_7XA_7)\cdot d(T_7YA_7)$ (XY = AT, GC, TA, or CG) and a series of 15-mer oligonucleotides **10–13** differing at one base position $d(T_7NT_7)$ (N = C, **P1**, **P2**, ϕ) to be determined by the affinity cleaving method.[1] The 30-bp duplexes were labeled with ^{32}P at the 5′-end of the Watson–Crick target strand $d(T_7YT_7)$. The DNA-cleaving reactions with oligodeoxyribonucleotides **10–13** were performed under conditions that were sensitive to the stability of the variable base triplet in the middle of the triple-helical complex (pH 7.4, 35 °C, 40% ethanol). The most intense cleavage was observed when N = C, XY = GC and N = **P1**, XY = GC (Figures 3B and C). The binding for one of these combinations demonstrates the known ability of C to form C + GC base triplets.[1-4] Efficient cleavage was observed when **P1** was opposite a GC base pair (P1·GC triplet), while moderate cleavage was observed with the isomer **P2** (P2·GC triplet). The strong cleavage observed for the P1·GC triplet would be consistent with two hydrogen bonds formed. The weaker cleavage pattern observed for the P2·GC triplet indicates that there may be an energetically unfavorable distortion of the third-strand backbone or that the methyl group disfavors the anti conformation of **P2** in the triple helix. For comparison, oligodeoxyribonucleotides containing the abasic residue ϕ at the variable position show weak binding with a modest preference for TA and CG base pairs.[16]

Specific Binding of Sites Containing a $(GA)_6$ Sequence. The ability of oligonucleotides containing several **P1** residues to target single sites containing the 12 base pair purine tract $(GA)_6$ was characterized by affinity cleaving. A 4.06-kbp plasmid containing the 15 base pair purine target site, 5′-AAAAAGAGAGAGA-GA-3′, 1.02 kbp from the end was labeled with ^{32}P. This DNA was allowed to react with four oligonucleotides-EDTA·Fe, **14–17**, which differ at five variable positions and contain C, mC, **P1**, and **P2** residues, respectively, (25 °C, pH 6.2–7.8). Analysis of the products by gel electrophoresis revealed one major cleavage product 1.02 kbp in size. The site-specific cleavage efficiency of oligonucleotide **14** containing residues C/T decreased sharply above pH 7.0. Replacement of C with mC (oligonucleotide **15**) extended the binding/cleavage reaction to pH 7.4. However, oligonucleotide **15** (mC/T) did not bind strongly at pH 7.8. Substitution of **P1** for mC (oligonucleotide **16**) afforded the same cleavage efficiency as C and mC at pH 7.0, but extended the pH range for binding of the target to pH 7.8 (Figure 4B and C). Oligodeoxyribonucleotide **17** containing five isomeric **P2** residues showed no cleavage even at lower pH (Figure 4B).

Specific Binding of Sites Containing a $(G)_6$ Sequence. The ability of oligodeoxyribonucleotides **18–20** to bind single sites on double-helical DNA containing six *contiguous* GC base pairs was examined by affinity cleaving. Plasmid DNA, which contained the naturally occurring site from the HIV-LTR region, 5′-AAA-AGAAAAGGGGGGA-3′, located 1.7 and 3.2 kbp from the ends, was allowed to react with oligodeoxyribonucleotides-EDTA·Fe **18–20** (30 °C, pH 6.2–7.4) which differ at six contiguous positions by substitution of C, mC, or **P1**, respectively. Separation of cleavage products by gel electrophoresis revealed a major cleavage site producing two DNA fragments, 1.7 and 3.2 kb in size (Figure 5B and C). Sequence-specific cleavage by oligodeoxyribonucleotides **18** and **19** containing six contiguous C and mC residues was observed at pH 6.2, but this decreased above pH 6.6 (Figures 5B and C). Perhaps, charge repulsion disfavors the protonation at N3 of all cytosines and 5-methylcytosines in contiguous C + GC and mC + GC triplets. These results suggest that the pK_a's of C + GC and mC + GC triplets may be sequence dependent, which may limit the utility of 5-methylcytosine for oligonucleotide-directed triple-helix formation for certain G-rich sequences. However, oligodeoxyribonucleotide **20** containing six contiguous **P1** residues cleaved the single site in plasmid DNA up to pH 7.4 (30 °C). Moreover, oligodeoxyribonucleotide **20** at neutral pH can bind the HIV-LTR G-rich sequence at physiological tem-

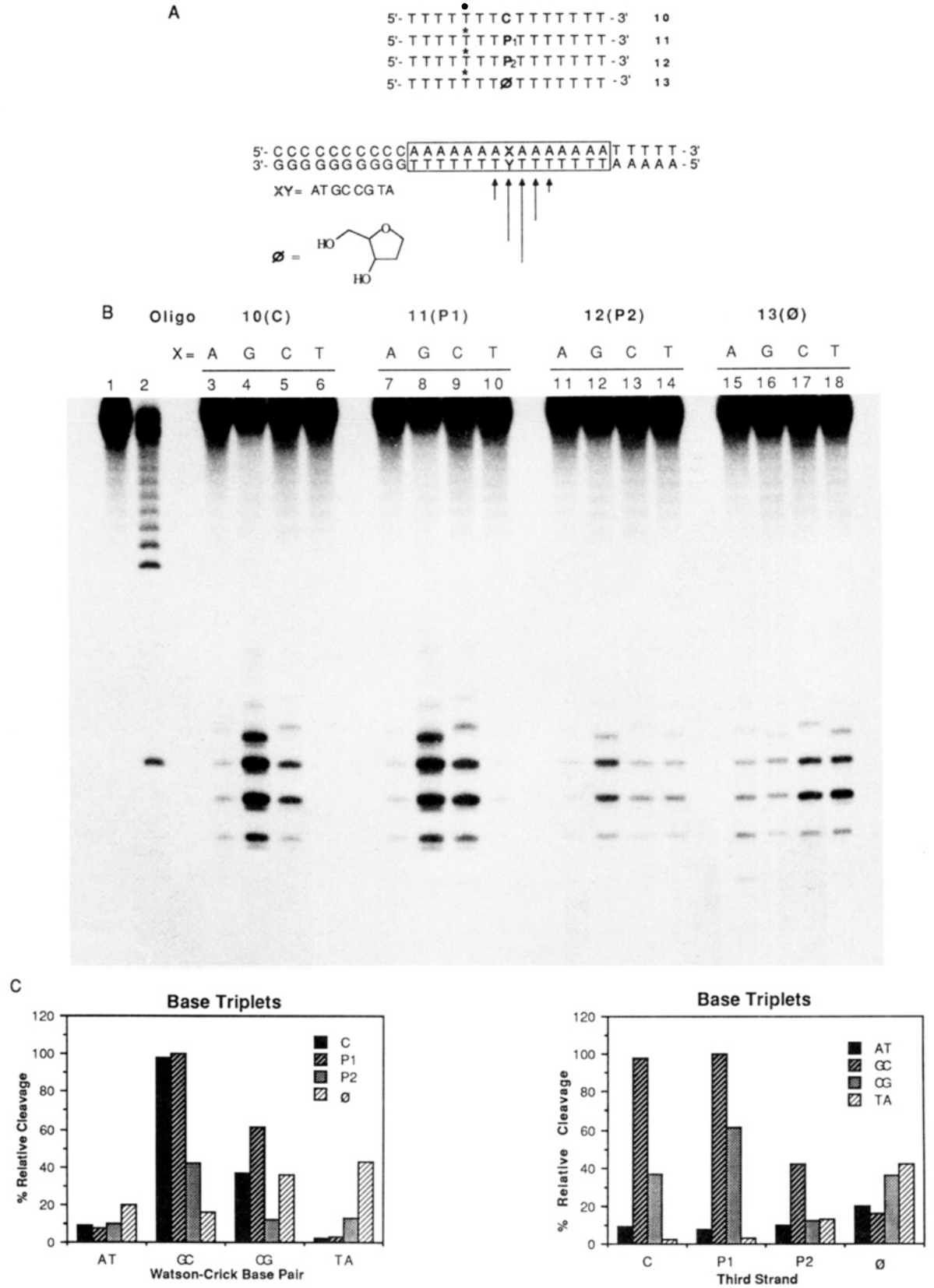

Figure 3. (A, top) Sequence of oligodeoxyribonucleotides-EDTA (**10**–**13**) where T* is the position of thymidine-EDTA. The oligonucleotides differ at one base position, indicated in bold type. (Bottom) The box indicates the double-stranded sequence bound by oligonucleotides-EDTA·Fe (**10**–**13**). The Watson–Crick base pair (AT, GC, TA or CG) 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 oligodeoxyribonucleotide-EDTA (2 μM), spermine (1 mM), and Fe(II) (25 μM) with the ^{32}P-end-labeled 30-mer duplex in a solution of Tris-acetate (50 mM, pH 7.4), NaCl (100 mM), calf thymus DNA (100 μM bp), and 40% ethanol and incubating at for 1 h. Cleaving reactions were initiated by the addition of dithiothreitol (DTT; 3.3 mM) and allowed to proceed for 6 h at 37 °C. The reactions were stopped by freezing and lyophilization. The cleavage products were analyzed by gel electrophoresis. Key: (Lanes 1–18) 5′-end-labeled 30-mer duplex ($A_5T_7YT_7G_{10}$) (Lane 1) intact 5′-labeled 30-bp DNA standard obtained after treatment according to the cleavage reaction in the absence of oligonucleotide-EDTA. (Lane 2) products of Maxam–Gilbert G + A sequence reaction. (Lanes 3–18) DNA-cleavage products produced by oligonucleotides-EDTA·Fe (**10**, **11**); **10** (lanes 3–6), **11** (lanes 7–10), **12** (lanes 11–14), **13** (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 representing the relative cleavage efficiencies (±10%) from densitometric analysis of (B). Sixteen base triplets were examined for binding specificity compatible with the pyrimidine triple-helix motif by the experiment described in (B).

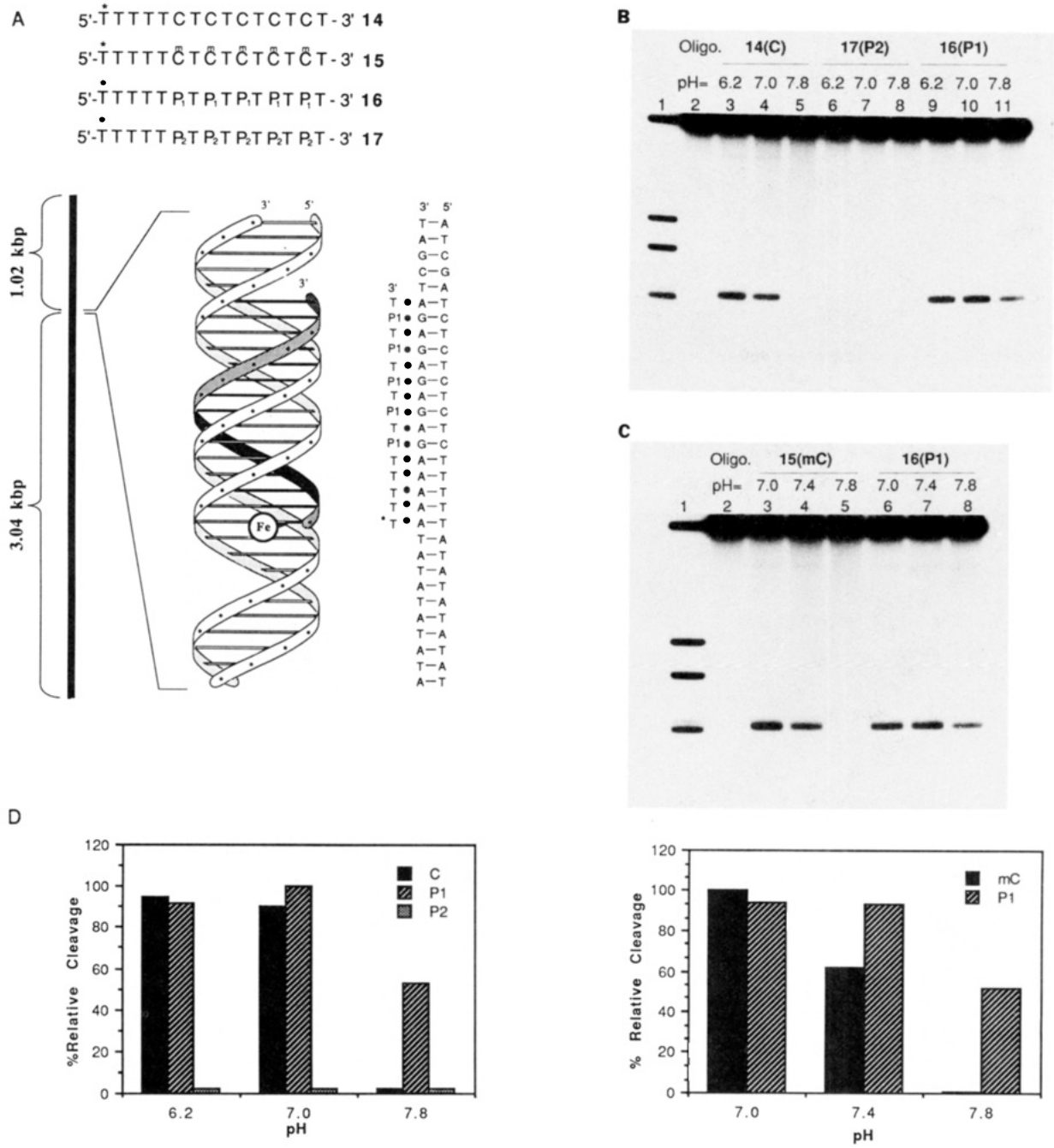

Figure 4. (A) (Top) Oligodeoxyribonucleotides **14–17** containing cytosine (C), 5-methylcytosine (mC), thymine (T), **P1**, **P2**, and thymidine-EDTA (T*). (Bottom) Ribbon model of triple helical complex between oligonucleotide-EDTA **16** and a 15 base pair purine site in plasmid DNA (4.06 kbp). (B, C) Double-strand cleavage of plasmid DNA analyzed on a 0.9% agarose gel. Plasmid pDMAG10 was linearized with *Sty*I and labeled with (α-^{32}P)TTP, producing a 4.06-kbp restriction fragment. The ^{32}P-end-labeled DNA was dissolved in buffer containing NaCl, Tris-acetate, and spermine and was mixed with oligonucleotides **15–18** previously equilibrated for 30 min with 1.5 equiv of Fe(II). After incubation at 25 °C for 30 min, the reactions were initiated by the addition of DTT (final concentration. 25 mM Tris-acetate, 1 mM spermine, 100 mM NaCl, calf thymus DNA (100 μM base pairs), 2 μM oligonucleotide-EDTA·Fe^{II}, and 2.5 mM DTT). The cleavage reactions were allowed to proceed for 6 h at 25 °C. The reactions were stopped by precipitation with ethanol and the cleavage products were analyzed by gel electrophoresis. Key: (B) (Lane 1) DNA size markers (bp) obtained by digestion of *Sty*I linear pDMAG10 with *Eco*RI (1060), *Bam*HI (998), *Asp*700 (1460), and *Pst*I (1814). (Lane 2) Control containing no oligonucleotide-EDTA·Fe^{II}. (Lanes 3–5) **14**, (lanes 6–8) **17**, (lanes 9–11) **16**. Lanes 3, 6, and 9 are at pH 6.2; lanes 4, 7, and 10 are at pH 7.0; lanes 5, 8 and 11 are at pH 7.8. (C) (Lane 1) DNA size markers as above. (Lane 2) Control containing no oligonucleotide-EDTA·Fe^{II}. (Lanes 3–5) **15**, (lanes 6–8) **16**. Lanes 3 and 6 are at pH 7.0; lanes 4 and 7 are at pH 7.4; lanes 5 and 8 are at pH 7.8. (D) Bar graph representing the relative cleavage efficiencies (±10%) from the densitometric analysis of (B) and (C).

peratures (37 °C) (Figure 5D).

Conclusion

Triple-helical complexes containing contiguous C + GC or mC + GC triplets are sensitive to pH. Although 5-methylcytosine extended the pH range of triple-helix stability for the site $A_5(GA)_5$, a similar pH extension was not observed for the site, $dA_4GA_4G_6A$. Oligodeoxyribonucleotides containing **P1** show less pH sensitivity in triple-helix formation and bind G-rich purine tracts in double-helical DNA at pH 7.8. **P1** enables the sequence-specific binding of double-helical DNA sites containing six contiguous GC base pairs at physiological pH[18] by oligonucleotide-directed tri-

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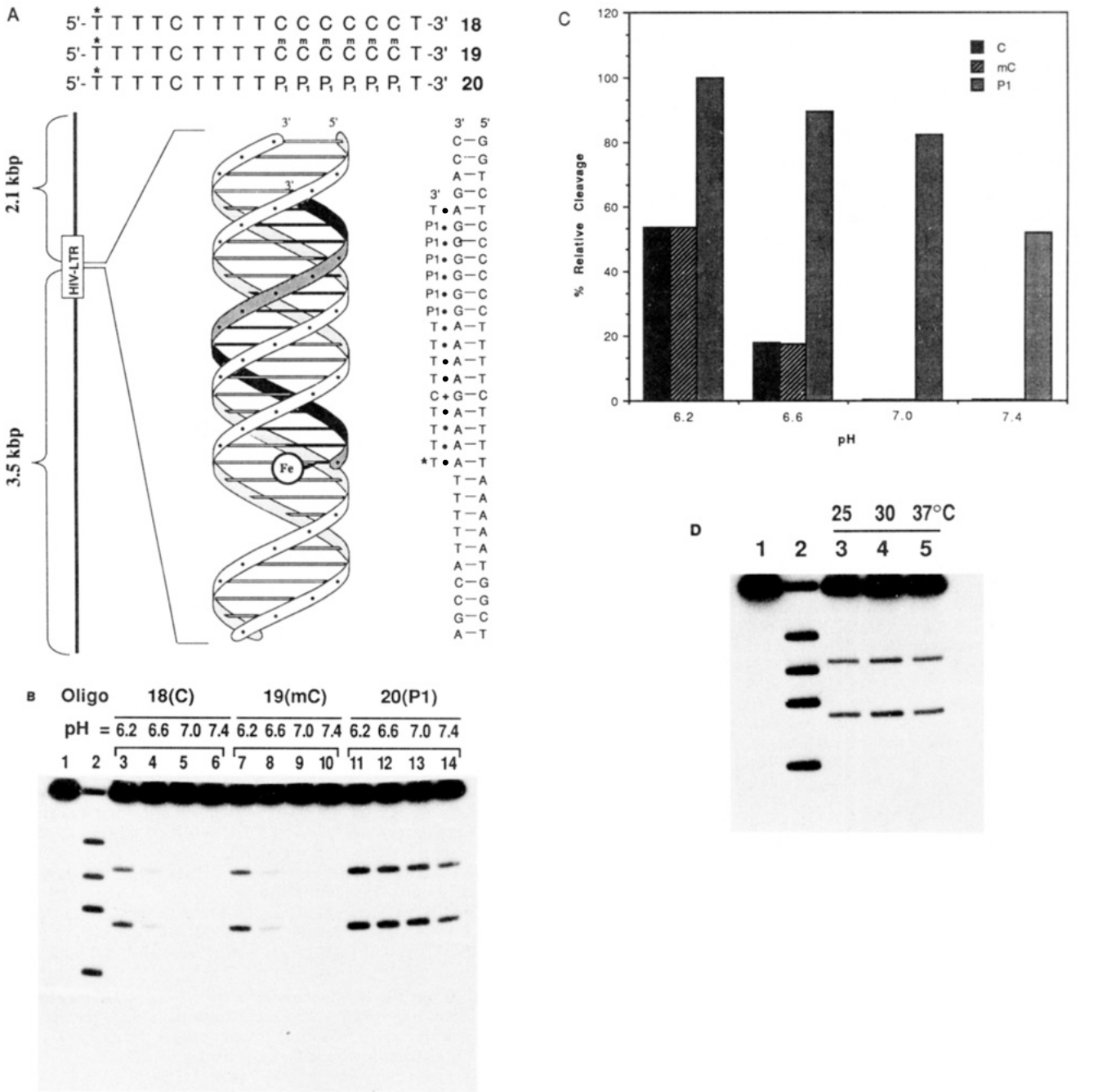

Figure 5. (A) (Top) Oligodeoxyribonucleotides **18**–**20** containing cytosine (C), 5-methylcytosine (mC), thymine (T), **P1**, and thymidine-EDTA (T*). (Bottom) Ribbon model of triple-helical complex between oligonucleotides-EDTA **20** and a 16 base pair purine site in plasmid DNA (4.95 kbp). (B) Autoradiogram of double-strand cleavage of pHIV-CAT DNA (4.95 kbp) analyzed on a 0.9% agarose gel. The reactions were carried out by combining a mixture of oligonucleotide-EDTA **18**–**20** (2 μM), spermine (1 mM), and Fe(II) (2 μM) with the ^{32}P-labeled linearized plasmid in a solution of Tris-acetate (25 mM), NaCl (100 mM), and calf thymus DNA [100 μM (bp)] and incubating for 1 h at the reaction temperature. Cleavage reactions were initiated by the addition of ascorbate (1 mM) and allowed to proceed for 10 h at 30 °C. The reactions were stopped by precipitation with ethanol and the cleavage products were analyzed by agarose gel electrophoresis. Key: (Lanes 1–8) pHIV-CAT linearized with *Bam*HI and 3′-end labeled at both ends. (Lane 1) Control containing no oligonucleotide-EDTA·Fe^{II}. (Lane 2) DNA size markers obtained by digestion of *Bam*HI linearized pHIV-CAT with *Hind*III and *Xho*I 5.6 (undigested), 4.6, 3.3, 2.3, and 0.96 kbp. (Lanes 3–6) DNA cleavage products produced by **18**; (lanes 7–10) DNA cleavage products produced by **19**; (lanes 11–14) DNA cleavage products produced by **20**. Lanes 3, 7, and 11 are at pH 6.2; lanes 4, 8, and 12 are at pH 6.6; lanes 5, 9, and 13 are at pH 7.0; lanes 6, 10, and 14 are at pH 7.4. (C) Bar graph representing the relative cleavage efficiencies (±10%) from the densitometric analysis of (B). (D) Autoradiogram of double-strand cleavage of pHIV-CAT DNA (5.6 kbp) analyzed on a 0.9% agarose gel. The reactions were carried out by combining a mixture of oligonucleotide-EDTA **20** (2 μM), spermine (1 mM), and Fe(II) (2 μM) with the ^{32}P-labeled linearized plasmid in a solution of Tris-acetate (25 mM pH 7.0), NaCl (100 mM), calf thymus DNA [100 μM (bp)] and incubating for 1 h at the reaction temperature. Cleavage reactions were initiated by the addition of ascorbate (1 mM) and allowed to proceed for 10 h at the reaction temperature. The reactions were stopped by precipitation with ethanol and the cleavage products were analyzed by agarose gel electrophoresis. Key: (Lanes 1–5) pHIV-CAT linearized with *Bam*HI and 3′-end labeled at both ends. (Lane 1) Control containing no oligonucleotide-EDTA·Fe^{II}. (Lane 2) DNA size markers obtained by digestion of *Bam*HI-linearized pHIV-CAT with *Hind*III and *Xho*I 5.6 (undigested), 4.6, 3.3, 2.3, and 0.96 bp. (Lane 3) DNA cleavage products produced by **20** at 25 °C; (lane 4) DNA cleavage products produced by **20** at 30 °C; (lane 5) DNA cleavage products produced by **20** at 37 °C.

ple-helix formation. The marked difference between isomeric nucleosides **P1** and **P2** for recognition of GC base pairs suggests that, for the design of novel base triplets, positioning the phosphate–deoxyribose backbone and controlling glycosidic conformation (syn–anti) will be as important as matching hydrogen bond donors–acceptors of heterocycles for base-specific triple-helix

formation. We would anticipate that, for binding single sites in megabase DNA, **P1** increases the number of purine sequences available by oligonucleotide-directed triple-helix formation.[1h,k]

Experimental Section

^{1}H NMR spectra were recorded at 400 MHz on a JEOL-GX 400 NMR spectrometer. The NOE spectra were measured in degassed $CDCl_3$. Chemical shifts are reported in parts-per-million downfield from tetramethylsilane. IR spectra were recorded on a Shimadzu IR-435 infrared spectrometer. 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 or the Mass Spectrometry Laboratory at the University of California, Riverside. Elemental analyses were performed by the analytical laboratory at the California Institute of Technology. Reagent grade chemicals were used as received unless otherwise noted. Diisopropylethylamine was distilled from CaH_2. Acetonitrile, pyridine, and methylene chloride were purchased as anhydrous solvents from Aldrich. Flash chromatography was carried out using EM science Kieselgel 60 (230–400 mesh). Thin-layer chromatography (TLC) was performed on EM Reagents silica gel plates (0.25 μm).

Ethyl 1-[2-Deoxy-3,5-bis-*O*-(4-methylbenzoyl)-β-D-ribofuranosyl]-3-methyl-4-nitro-1*H*-pyrazole-5-carboxylate (3a) and Ethyl 1-[2-Deoxy-3,5-bis-*O*-(4-methylbenzoyl)-β-D-ribofuranosyl]-5-methyl-4-nitro-1*H*-pyrazole-3-carboxylate (3b). To a stirred suspension of sodium hydride (60% dispersion in oil, 4.4 g, 0.11 mol) in dry CH_3CN (1 L) was added ethyl (3-methyl-4-nitropyrazole-5-carboxylate (**1**)[10] (19.9 g, 0.1 mol) portionwise at 23 °C under argon. After 40 min, 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-ribofuranose (**2**)[11] (42.8 g, 0.11 mol) was added, and the solution was allowed to stir for an additional 1 h. The reaction mixture was poured into 500 mL of CH_2Cl_2 and washed with H_2O (100 mL) and brine, and dried ($MgSO_4$). Concentration followed by flash chromatography (hexane/ether, 2:1, v/v, TLC R_f = 0.3, 0.45) afforded 25.3 g (46%) of **3a** (R_f = 0.45) and 24.3 g (44%) of **3b** (R_f = 0.3). Isomer **3a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (d, J = 8.03 Hz, 2 H), 7.95 (d, J = 8.03 Hz, 2 H), 7.24 (d, J = 8.03 Hz, 2 H), 7.19 (d, J = 8.03 Hz, 2 H), 6.38 (t, J = 6.72 Hz, 1 H), 5.81 (m, 1 H), 4.62 (m, 2 H), 4.20 (m, 3 H), 3.32 (m, 1 H), 2.63 (m, 1 H), 2.41 (s, 6 H), 2.38 (s, 3 H), 1.37 (t, J = 6.72 Hz, 3 H); IR 3000, 1730, 1720, 1610, 1503, 1380, 1250 cm^{-1}; HRMS (EI) for $C_{28}H_{29}N_3O_9$ calcd 551.1903, found 551.1898. Anal. Calcd for $C_{28}H_{29}N_3O_9$: C, 60.97; H, 5.30; N, 7.61. Found: C, 60.98; H, 5.35; N, 7.63. Isomer **3b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (d, J = 8.06 Hz, 2 H), 7.90 (d, J = 8.06 Hz, 2 H), 7.22 (d, J = 8.06 Hz, 2 H), 7.18 (d, J = 8.06 Hz, 2 H), 6.21 (t, J = 6.74 Hz, 1 H), 5.26 (m, 1 H), 4.23–4.62 (m, 5 H), 3.61 (m, 1 H), 2.71 (s, 3 H), 2.65 (m, 1 H), 2.37 (s, 3 H), 2.42 (s, 3 H), 1.32 (t, J = 6.76 Hz, 3 H); IR (KBr) 3000, 1735, 1730, 1710, 1618, 1380, 1270, 1130 cm^{-1}; HRMS (EI) for $C_{28}H_{29}N_3O_9$ calcd 551.1903, found 551.1897. Anal. Calcd for $C_{28}H_{29}N_3O_9$: C, 60.97; H, 5.30; N, 7.61. Found: C, 60.98; H, 5.32; N, 7.63.

1-(2-Deoxy-β-D-ribofuranosyl)-3-methyl-4-nitro-1*H*-pyrazole-5-carboxamide (4a). Ethyl ester **3a** (2.04 g, 4.0 mmol) was heated in saturated NH_3/MeOH (50 mL) in a sealed tube at 120 °C for 24 h. Cooling, concentration, and flash chromatography (MeOH/ethyl acetate, 1:19, v/v; TLC R_f = 0.42) yielded 1.0 g (88%) of a clear colorless oil: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.49 (s, 1 H), 8.28 (s, 1 H), 6.00 (t, J = 6.13 Hz, 1 H), 5.28 (d, J = 4.25 Hz, 1 H, 3′ OH), 4.71 (t, J = 5.12 Hz, 1 H, 5′ OH), 4.32 (m, 1 H), 3.83 (m, 1 H), 3.45 (m, 1 H), 3.35 (m, 1 H), 2.68 (m, 1 H), 2.43 (s, 3 H), 2.23 (m, 1 H); IR (KBR) 3330, 3170, 2950, 1690, 1650, 1435, 1320, 1080, 1020 cm^{-1}; HRMS (FAB) for $C_{10}H_{15}N_4O_6$ (M + H) calcd 287.0991, found 287.0974.

1-(2-Deoxy-β-D-ribofuranosyl)-5-methyl-4-nitro-1*H*-pyrazole-3-carboxamide (4b). Ethyl ester **3b** (2.04 g, 4.0 mmol) was treated similarly as described for the preparation of amide **4a** to afford **4b**. Purification by flash chromatography (MeOH/ethyl acetate, 1:18, v/v; TLC R_f = 0.20) yielded 0.98 g (85%) of a clear colorless oi: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.95 (s, 1 H), 7.70 (s, 1 H), 6.24 (t, J = 6.87 Hz, 1 H), 5.30 (d, J = 4.26, 1 H, 3′ OH), 4.71 (t, J = 5.14 Hz, 1 H, 5′ OH), 4.34 (m, 1 H), 3.80 (m, 1 H), 3.20–3.50 (m, 3 H), 2.82 (m, 1 H), 2.64 (s, 3 H), 2.22 (m, 1 H); IR (KBr) 3350, 2950, 1670, 1500, 1370, 1380, 1080, 1060 cm^{-1}; HRMS (FAB) for $C_{10}H_{15}N_4O_6$ (M + H) calcd 287.0991, found 287.0974.

1-(2-Deoxy-β-D-ribofuranosyl)-3-methyl-4-amino-1*H*-pyrazole-5-carboxamide (5a). A solution of nitro compound **4a** (8.6 g, 30 mmol) in EtOH/H_2O, (8:2, v/v; 300 mL) was shaken with 10% Pd/C (800 mg) under H_2 (50 psi) for 5 h at room temperature. Filtration and concentration afforded 7.0 g (90%) of a clear colorless oil, which solidified upon standing: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.49 (s, 2 H), 6.56 (t, J = 6.13 Hz, 1 H), 5.12 (d, J = 4.25 Hz, 1 H, 3′ OH), 4.72 (t, J = 5.12 Hz, 1 H, 5′ OH), 4.32 (m, 3 H), 3.71 (m, 1 H), 3.42 (m, 1 H), 3.23 (m, 1 H), 2.76 (m, 1 H), 2.04 (m, 4 H); IR (KBr) 3400, 2950, 1700, 1590, 1215 cm^{-1}; HRMS (EI) for $C_{10}H_{16}N_4O_4$ calcd 256.1172, found 256.1167.

1-(2-Deoxy-β-D-ribofuranosyl)-5-methyl-4-amino-1*H*-pyrazole-3-carboxamide (5b). Amine **5b** was prepared analogously from the nitro compound **4b** (8.6 g, 30 mmol) as described for the synthesis of **5a**. Filtration and concentration gave a colorless oil, which solidified upon standing: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.08 (s, 1 H), 7.04 (s, 1 H), 6.04 (t, J = 6.14 Hz, 1 H), 5.13 (d, J = 4.24 Hz, 1 H, 3′ OH), 4.66 (t, J = 5.12 Hz, 1 H, 5′ OH), 4.31–4.50 (m, 3 H), 3.75 (m, 1 H), 3.20–3.50 (m, 2 H), 2.93 (m, 1 H), 2.15 (m, 4 H); IR (KBr) 3400, 2950, 1672, 1600, 1320, 1300 cm^{-1}; HRMS (EI) for $C_{10}H_{16}N_4O_4$ calcd 256.1172, found 256.1167.

1-(2-Deoxy-β-D-ribofuranosyl)-3-methyl-5-mercapto-1*H*-pyrazolo-[4,3-*d*]pyrimidin-7-one (6a). Nucleoside **5a** (5.12 g, 20 mmol) and phenyl isothiocyanate (2.7 g, 20 mmol) were dissolved in dry pyridine (50 mL) and heated at reflux temperature for 45 min.[12] Concentration followed by flash chromatography (5% MeOH in ethyl acetate) gave 5.1 g (85%) of **6a**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.02 (s, 1 H), 12.02 (s, 1 H), 6.68 (t, J = 6.15 Hz, 1 H), 5.27 (d, J = 4.16 Hz, 1 H, 3′ OH), 4.68 (t, J = 5.25 Hz, 1 H, 5′ OH), 4.35 (m, 1 H), 3.78 (m, 1 H), 3.45 (m, 1 H), 3.31 (m, 1 H), 2.71 (m, 1 H), 2.31 (s, 3 H), 2.20 (m, 1 H); IR (KBr) 3400, 3100, 1700, 1600, 1290, 1200, 1160 cm^{-1}; HRMS (EI) for $C_{11}H_{14}N_4O_4S$ calcd 298.0735, found 298.0737.

2-(2-Deoxy-β-D-ribofuranosyl)-3-methyl-5-mercapto-1*H*-pyrazolo-[4,3-*d*]pyrimidin-7-one (6b). Nucleoside **6b** was analogously prepared from nucleoside **5b** (5.12 g, 20 mmol) as described for the preparation of **6a**. Evaporation and trituration of the resulting oil with ethyl acetate gave 4.7 g (80%) of **6b**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.61 (s, 1 H), 12.08 (s, 1 H), 6.25 (t, J = 6.15 Hz, 1 H), 5.29 (d, J = 4.24 Hz, 1 H, 3′ OH), 4.73 (t, J = 5.15 Hz, 1 H, 5′ OH), 4.37 (m, 1 H), 3.81 (m, 1 H), 3.21–3.50 (m, 2 H), 2.90 (m, 1 H), 2.47 (s, 3 H), 2.25 (m, 1 H); IR (KBr) 3300, 3100, 1700, 1620, 1600, 1280 cm^{-1}; HRMS (EI) for $C_{11}H_{14}N_4O_4S$ calcd 298.1735, found 298.0727.

1-(2-Deoxy-β-D-ribofuranosyl)-3-methyl-5-amino-1*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (P1). To a solution of nucleoside **6a** (2.98 g, 10 mmol) in 10 mL of concentrated NH_4OH at 0 °C was added 30% hydrogen peroxide (3.0 mL) dropwise.[13] After being stirred for 40 min at 0 °C, the reaction mixture was saturated with ammonia (at 0 °C), transferred to a sealed tube, and heated for 120 °C for 12 h.[11] Concentration afforded a brown solid, which was recrystallized from water to give 2.01 g of colorless crystals (71%) of **P1**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.96 (s, 1 H), 6.73 (t, J = 6.92 Hz, 1 H), 6.13 (s, 2 H), 5.18 (d, J = 4.32 Hz, 1 H, 3′ OH), 4.69 (t, J = 5.23 Hz, 1 H, 5′ OH), 4.44 (m, 1 H), 3.76 (m, 1 H), 3.30–3.65 (m, 2 H), 2.72 (m, 1 H), 2.22 (s, 3 H), 2.13 (m, 1 H); IR (KBr) 3400, 3330, 2950, 1690, 1650, 1570, 1435, 1320 cm^{-1}; UV (H_2O) λ_{max} nm (ε) 228 (20 900), 260 (3390), 310 (6020); HRMS (FAB) for $C_{11}H_{16}N_5O_4$ (M + H) calcd 282.1202, found 282.1212.

2-(2-Deoxy-β-D-ribofuranosyl)-3-methyl-5-amino-1*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (P2). Nucleoside **P2** was analogously prepared from nucleoside **6b** (2.98 g, 10 mmol) as described for the transformation of **6a** to **P1**. Concentration and flash chromatography (MeOH) yielded 1.9 g (67%) of a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.55 (s, 1 H), 6.33 (t, J = 6.67 Hz, 1 H), 6.00 (s, 2 H), 5.32 (d, J = 4.34 Hz, 1 H, 3′ OH), 4.75 (t, J = 5.23 Hz, 1 H, 5′ OH), 4.40 (m, 1 H), 3.79 (m, 1 H), 3.39 (m, 1 H), 3.26 (m, 1 H), 2.92 (m, 1 H), 2.31 (s, 3 H), 2.24 (m, 1 H); IR (KBr) 3400, 3320, 2960, 1695, 1630, 1600, 1320 cm^{-1}; UV (H_2O) λ_{max} nm (ε) 224 (23 000), 260 (4142), and 310 (3857); HRMS (FAB) for $C_{11}H_{16}N_5O_4$ (M + H) calcd 282.1202, found 282.1210.

***N*-[1-(2-Deoxy-β-D-ribofuranosyl)-3-methyl-7-oxo-1*H*-pyrazolo[4,3-*d*]pyridin-5-yl]-2-methylpropanamide (7a).** To a stirred suspension of nucleoside **P1** (1.41 g, 5 mmol) in dry pyridine (25 mL) at 0 °C under argon was added chlorotrimethylsilane (2.16 mL, 25 mmol) dropwise.[14] After 30 min, isobutyric anhydride (3.95 mL, 25 mmol) was slowly added. The reaction mixture was allowed to warm to room temperature and allowed to stir for 3 h. Neutralization by addition of saturated aqueous $NaHCO_3$, followed by coevaporation several times with toluene and EtOH, afforded a viscous residue. The residue was dissolved in ethyl acetate, washed with H_2O, and dried (Na_2SO_4). Chromatography (ethyl acetate R_f = 0.5) yielded 1.31 g (75%) of isobutyroyl-protected nucleoside **7a**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.15 (s, 1 H), 11.55 (s, 1 H), 6.77 (dd, J = 6.41, 6.74 Hz, 1 H), 5.23 (d, J = 4.27 Hz, 1 H, 3′ OH), 4.68 (t, J = 5.65 Hz, 1 H, 5′ OH), 4.49 (m, 1 H), 3.79 (m, 1 H), 3.48 (m, 1 H), 3.31 (m, 1 H), 2.75 (m, 2 H), 2.31 (s, 3 H), 2.21 (m, 1 H), 1.05 (d, J = 6.84 Hz, 6 H); IR (KBr) 3250, 2980, 1680, 1610, 1490, 1300 cm^{-1}; HRMS (FAB) for $C_{15}H_{22}N_5O_5$ (M + H) calcd 352.1620, found 352.1608.

***N*-[2-(2-Deoxy-β-D-ribofuranosyl)-3-methyl-7-oxo-1*H*-pyrazolo[4,3-*d*]pyrimidin-5-yl]-2-methylpropanamide (7b).** Nucleoside **7b** was analogously prepared from nucleoside **P2** (1.41 g, 5 mmol) as described for the preparation of **8a**. Chromatography (ethyl acetate, R_f = 0.35) yielded 1.30 g (75%) of a light yellow solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.89 (s, 1 H), 11.37 (s, 1 H), 6.32 (dd, J = 5.86, 6.35 Hz, 1 H), 5.28 (d, J = 4.18 Hz, 1 H, 3′ OH), 4.69 (t, J = 5.24 Hz, 1 H, 5′ OH), 4.39 (m, 1 H), 3.81 (m, 1 H), 3.37 (m, 1 H), 3.22 (m, 1 H), 2.94 (m, 1 H), 2.69 (m, 1 H), 2.43 (s, 3 H), 2.22 (m, 1 H), 1.07 (d, J = 6.83, 6 H); IR (KBr) 3250, 2980, 1680, 1610, 1490, 1300 cm^{-1}; HRMS (FAB) for $C_{15}H_{22}N_5O_5$ (M + H) calcd 352.1620, found 352.1610.

***N*-[1-[5-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-2-deoxy-β-D-ribofuranosyl]-3-methyl-7-oxo-1*H*-pyrazolo[4,3-*d*]pyrimidin-5-yl]-2-methylpropanamide (8a).** To a solution of nucleoside **7a** (1.06 g, 3 mmol) in dry pyridine (6 mL) was added 4,4′-dimethoxytrityl chloride (DMT-Cl; 1.14 g, 3.3 mmol). The reaction mixture was allowed to stir at 4 °C under argon for 16 h. An additional portion of DMT-Cl (35 mg, 0.1 mmol) was added until most of the starting material was converted to product, as determined by TLC analyses. The reaction was quenched by the addition of MeOH (2 mL) and diluted with CH_2Cl_2 (50 mL). The organic layer was washed three times with H_2O, dried (Na_2SO_4), filtered, and concentrated to a yellow foam. Chromatography (CH_2Cl_2/ethyl acetate, 2:1, v/v; R_f = 0.31) yielded 1.46 g (75%) of **8a** as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.28 (s, 1 H), 11.59 (s, 1 H), 7.31 (d, J = 7.84 Hz, 2 H), 7.16 (m, 7 H), 6.76 (m, 5 H), 5.27 (d, J = 4.70 Hz, 1 H, 3′ OH), 4.45 (m, 1 H), 3.92 (m, 1 H), 3.70 (s, 3 H), 3.69 (s, 3 H), 3.00 (m, 2 H), 2.73 (m, 2 H), 2.26 (m, 1 H), 2.18 (s, 3 H), 1.10 (d, J = 6.84 Hz, 6 H); IR (KBr) 3400, 3200, 2980, 1670, 1610, 1500, 1250 cm^{-1}; HRMS (FAB) for $C_{36}H_{40}N_5O_7$ (M + H) calcd 654.2927, found 654.2938.

***N*-[2-[5-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-2-deoxy-β-D-ribofuranosyl]-3-methyl-7-oxo-2*H*-pyrazolo[4,3-*d*]pyrimidin-5-yl]-2-methylpropanamide (8b).** DMT-protected nucleoside **8b** was analogously prepared from nucleoside **7b** (1.06 g, 3 mmol) as described in the preparation of **8a**. Chromatography (CH_2Cl_2/ethyl acetate, 2:1, v/v; R_f = 0.23) yielded 1.43 g (75%) of **8b**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.94 (s, 1 H), 11.39 (s, 1 H), 7.00–7.30 (m, 9 H), 6.75 (d, J = 8.98 Hz, 2 H), 6.73 (d, J = 8.55 Hz, 2 H), 6.41 (m, 1 H), 5.35 (d, J = 5.13 Hz, 1 H, 3′ OH), 4.57 (m, 1 H), 3.96 (m, 1 H), 3.68 (m, 3 H), 3.66 (s, 3 H), 3.00 (m, 2 H), 2.81 (m, 1 H), 2.72 (m, 1 H), 2.55 (s, 3 H), 2.27 (m, 1 H), 1.09 (d, J = 6.86 Hz, 6 H); IR (KBr) 3400, 3200, 2980, 1670, 1620, 1500, 1470, 1290, 1250 cm^{-1}; HRMS (FAB) for $C_{36}H_{40}N_5O_7$ (M + H) calcd 654.2927, found 654.2950.

Phosphoramidite of DMT-Protected P1 (9a). 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (200 μL, 0.90 mmol) was added dropwise to a solution of **9a** (392 mg, 0.60 mmol) and *N*,*N*-diisopropylethylamine (312 μL, 1.80 mmol) in dry CH_2Cl_2 (4 mL) under argon. After 1 h, the reaction was quenched with ethanol (1.0 mL), diluted with ethyl acetate (60 mL), and washed with saturated aqueous $NaHCO_3$ (2 × 60 mL) and brine (2 × 60 mL). The organic layer was dried (Na_2SO_4) and concentrated. Flash chromatography (EtOAc/CH_2Cl_2/triethylamine/isopropyl alcohol, 5:90:2:2, v/v/v/v; R_f = 0.70) gave 430 mg (83%) of a white solid **9a** consisting of a mixture of diastereomers: ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.29 (s, 1 H), 11.61 (s, 1 H), 7.37 (m, 2 H), 7.16 (m, 7 H), 6.76 (m, 5 H), 4.75 (m, 1 H), 4.06 (m, 1 H), 3.40–3.85 (m, 10 H), 3.09 (m, 1 H), 2.80 (m, 2 H), 2.72 (m, 2 H), 2.65 (m, 1 H), 2.45 (m, 1 H), 2.18 (s, 3 H), 0.90–1.40 (m, 18 H); IR (KBr) 3200, 2980, 2250, 1680, 1610, 1500, 1305, 1250 cm^{-1}; HRMS (FAB) for $C_{45}H_{57}N_7O_8P$ (M + H) calcd 854.4006, found 854.4036.

Phosphoramidite of DMT-Protected P2 (9b). Phosphoramidite **9b** was analogously prepared from protected nucleoside **8b** (392 mg, 0.60 mmol) as described for the preparation of **9a**. Chromatography (EtOAc/CH_2Cl_2/triethylamine/isopropyl alcohol, 5:90:2:2, v/v/v/v; R_f = 0.44) gave (418 mg; 82% yield) **9b** as a white solid consisting of a mixture of diastereomers: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.94 (s, 1 H), 11.40 (s, 1 H), 7.40–6.90 (m, 9 H), 6.75 (m, 4 H), 6.50 (t, J = 4.27 Hz, 1 H), 4.89 (m, 1 H), 4.10 (m, 1 H), 3.40–3.80 (m, 10 H), 3.21 (m, 1 H), 3.05 (m, 1 H), 2.30–2.80 (m, 8 H), 0.8–1.50 (m, 18 H); IR (KBr) 3250, 2990, 1680, 1610, 1245 cm^{-1}; HRMS (FAB) for $C_{45}H_{57}N_7O_8P$ (M + H) calcd 854.4006, found 854.4026.

Oligonucleotides. Oligonucleotides **10–20** were synthesized by automated methods on a Beckman System 1 Plus DNA synthesizer using β-cyanoethyl phosphoramidite chemistry.[14] Synthetic nucleosides coupled as 0.1 M solutions in CH_3CN with >97% efficiency. The oligonucleotides were removed from the support and deprotected by treatment with 0.1 N NaOH (1.5 mL/μmol of oligonucleotide, 55 °C, 24 h), neutralized with glacial acetic acid (6–7 μL/1.5 mL 0.1 N NaOH), applied to a column of Sephadex G-10–120, and eluted with water. The crude oligonucleotides were lyophilized and purified by electrophoresis on 20% denaturing polyacrylamide gel (19:1 monomer/bis). The major UV-absorbing bands were cut out and eluted (0.2 N NaCl, 1 mM EDTA, 37 °C, 24 h), then passed through Sephadex G-10-120, and exhaustively dialyzed against water. Concentrations of oligonucleotides were determined by UV measurements (A_{260}) using a Perkin-Elmer Lambda 4C UV–vis spectrophorometer. Oligodeoxyribonucleotides 5′-T-T-P1-T-3′ and 5′-T-T-P2-T-3′ were synthesized on a 10-μmol scale in a similar manner for ^{1}H NMR and HRMS studies. Chromatography over a Sephadex G-10–120 column gave 6.12 mg of a white solid. T-T-P1-T: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.30 (m, 4 H), 7.90 (s, 1 H), 7.87 (s, 1 H), 7.81 (s, 1 H), 6.71 (t, J = 5.01 Hz, 1 H), 6.31 (s, 2 H), 6.18 (m, 3 H), 5.89 (m, 1 H), 4.82 (m, 2 H), 4.75 (m, 1 H), 4.45 (m, 1 H), 4.20–3.50 (m, 13 H), 2.98 (m, 1 H), 2.25 (s, 3 H), 2.50–2.00 (m, 7 H), 1.85 (s, 6 H), 1.29 (s, 3 H); HRMS (FAB) for $C_{41}H_{52}N_{11}O_{25}P_3Na_3$ (M + H) calcd 1260.2041, found 1260.2009. T-T-P2-T: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.20 (m, 4 H), 7.81 (s, 1 H), 7.79 (s, 1 H), 7.74 (s, 1 H), 6.22 (m, 4 H), 6.01 (m, 1 H), 5.78 (m, 1 H), 4.81 (m, 1 H), 4.72 (m, 1 H), 4.65 (m, 1 H), 4.38 (m, 1 H), 4.31 (m, 1 H), 3.22–4.00 (m, 16 H), 3.00 (m, 1 H), 2.31 (s, 3 H), 2.60–1.90 (m, 7 H), 1.78 (s, 6 H), 1.73 (s, 3 H); HRMS (FAB) for $C_{41}H_{52}N_{11}O_{25}P_3Na_3$ (M + H) calcd 1260.2041, found 1260.2075.

HPLC Analysis. Analytical HPLC was performed with a Hewlett-Packard 1090 liquid chromatograph using a reversed-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 (3 μL, 2 μg/μL) and calf intestine alkaline phosphatase (3 μL, 1 unit/μL) in 50 mM Tris (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 10 μL of water, and 5 μL of the solution was injected onto a C18 reversed-phase column, utilizing 10 mM ammonium phosphate (pH 5.1)/8.0% MeOH as the eluent and detecting at A_{260}. Comparison and coinjection of 5 μL of digested oligonucleotide solution with 5 μL of a standard solution containing C (4 nmol), T (4 nmol), **P1** (12 nmol), and **P2** (12 nmol) established the composition of the oligonucleotide.

DNA Manipulations. Distilled, deionized water was used for all aqueous reactions and dilutions. Enzymes were purchased from either Boehrinnger-Manheim or New England Biolabs. Deoxynucleoside triphosphates were purchased from Pharmacia as 100 mM solutions. 5′-(α-^{32}P)dNTPs (>3000 Ci/mmol) and 5′-(γ-^{32}P)ATP (>5000 Ci/mmol) were obtained from Amersham. The plasmid pDMAG10 (4.06 kbp) contains the site d(AAAAAGAGAGAGAGA). Calf thymus DNA was purchased from Sigma, sonicated, and extracted with 3 × 0.2 volumes of water-saturated phenol, 0.2 volumes of 24:1 $CHCl_3$/isoamyl alcohol, and 0.2 volumes of $CHCl_3$. After extensive dialysis against water, the DNA was passed through a 4.5-mL Centrex filter (Schleicher-Schuell) and the oligonucleotide concentration was measured by UV assuming ϵ_{260} = 11 800 L/mol·bp·cm. Agarose gel electrophoresis was performed in TAE buffer and polyacrylamide gel electrophoresis was performed in TBE buffer.[19] 3′-End-labeling of DNA was accomplished with the Klenow fragment of DNA polymerase I using standard procedures.[19] Radioactivity (Cerenkov) was measured with a Beckman LS 2801 scintillation counter. Autoradiography was carried out using Kodak X-Omat film. Optical densitometry was performed using an LKB Broma Ultrascan XL laser densitometer operating at 630 nm. The relative peak area for each cleavage band or locus was equated to the relative cleavage efficiency at that site.

Cleavage of 30-Mer Duplex. Each single-stranded oligonucleotide (50 pmol) $A_5T_7YT_7G_{10}$ (Y = T, C, G, A) was labeled at the 5′-end with T4 polynucleotide kinase according to standard procedures.[19] The 30-mer oligonucleotides were hybridized (100 mM NaCl, 50 mM Tris-acetate, pH 7.4) with the complementary 30 mer and the resulting duplexes were purified by 15% nondenaturing polyacrylamide gel electrophoresis. The four bands (Y = T, C, G, A) of end-labeled DNA were visualized by autoradiography, excised from the gel, crushed, and eluted into 0.2 N NaCl at 37 °C for 24 h. The end-labeled 30-mer duplexes were recovered by passing the eluents through a 0.45-μm Centrax filter and dialyzed against water. The ^{32}P-end-labeled DNA was used for all cleavage reactions. In a typical cleavage experiment, the DNA (20 000 cpm/reaction) was mixed with salts, buffer, spermine, and the oligonucleotide-EDTA·Fe^{II}. This solution was incubated for 30 min at 37 °C before the cleavage reaction was initiated by the addition of DTT, giving a final volume of 40 μL. The reaction was stopped by freezing followed by lyophilization. Electrophoretic separation of the cleavage products was achieved on a 20% denaturing polyacrylamide gel. Cleavage products were visualized by autoradiography and the relative amounts of cleavage products were analyzed by densitometry.

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Double-Strand Cleavage of pDMAG10. The plasmid pDMAG10[20] was linearized with *Sty*I and labeled at one end with ^{32}P using the Klenow fragment of DNA polymerase I according to standard procedures.[19] In a typical cleavage experiment, the end-labeled DNA (20 000 cpm/reaction) was mixed with salts, buffer, spermine, and oligonucleotide-EDTA·Fe. This solution was incubated for 30 min at 25 °C before the cleavage reaction was initiated by the addition of DTT, in a final volume of 40 μL. The reaction was stopped by ethanol precipitation. The pellets were rinsed once with 70% cold ethanol (50 μL), briefly dried in vacuo, and redissolved in TE buffer. Electrophoretic separation of the cleavage products was achieved on a 0.9% agarose gel/TAE buffer. The gel was dried and the cleavage products were visualized by autoradiography.

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Double-Strand Cleavage of HIV-CAT DNA. pHIV-CAT[21] was digested with *Bam*HI and the linearized DNA was labeled with α-(^{32}P)-dGTP using the Klenow fragment of DNA polymerase I according to standard procedures.[19] Purification of the labeled DNA and performance of the cleavage experiments were carried out as described for the double-stranded cleavage of pDMAG10 except with sodium ascorbate instead of DTT as the reducing agent.

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Communications to the Editor

Structures of the Beticolins, the Yellow Toxins Produced by *Cercospora beticola*†

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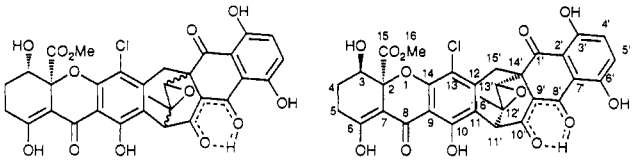
The genus *Cercospora* includes several species responsible for leaf spot diseases in many plants. Among them, *Cercospora beticola* Sacc. is the casual agent of cercosporiose, the most important leaf disease of sugar beet.[1] Two major toxins are produced by several *C. beticola* strains including a red pigment, cercosporin,[2,3] which can initiate the peroxidation of membrane lipids,[4] and a yellow compound successively named "gelben fraktion" (GF)[5] and then "*Cercospora beticola* toxin" (CBT).[1]

In this work, we report the structure of this yellow toxin, which we call beticolin.[6] In fact, there are two isomeric beticolins: beticolin 1 (**1**)[7] and beticolin 2 (**2**).[7] Both compounds exhibit the same biological activities as those already reported for CBT.[8]



1 **2**

On the basis of HREIMS measurments[9] as well as examination

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(6) *C. beticola* strain CM was grown on diluted V8 medium in Roux flasks. After 10 days, the mycelium was removed from the medium and extracted with ethyl acetate until the filtrate was colorless. The filtrate was washed with water and then evaporated in a vacuum rotary evaporator. The beticolins were separated by flash chromatography using silica gel pretreated with $Ca(H_2PO_4)_2 \cdot H_2O$ (Balis, C.; Payne, M. G. *Phytopathology* **1971**, *61*, 1477) and eluted with $CHCl_3$. On TLC (plates pretreated with H_3PO_4 and $Ca(H_2PO_4)_2 \cdot H_2O$ and reactivated) with $CHCl_3/CH_3OH/CH_3CO_2H$, 100/2/1, as solvent, the R_f values were 0.54 and 0.33 for beticolin 2 and beticolin 1, respectively. Both were crystallized from hexane/ethyl acetate.

(7) Beticolin 1 (**1**): mp = 250 °C dec; $[\alpha]_D$ = +950.4° (c = 0.043, CH_2Cl_2); ^1H NMR (CD_3COCD_3; 400 MHz δ (ppm) 15.25 (s, 1 H), 13.62 (s, 1 H), 12.39 (s, 1 H), 11.87 (s, 1 H), 11.38 (s, 1 H), 7.31, 7.39 (2 H, J = 13.4 Hz, H-4′, H-5′), 5.72 (d, 1 H (exchangeable with D_2O), J = 4.5 Hz), 4.57 (dt, 1 H, J = 11.4, 4.5 Hz, H-3), 2.91 (ddd, 1 H, J = 18, 11.4, 5.2 Hz, H-5β), 2.64 (ddd, 1 H, J = 18, 5.7, 0.8 Hz, H-5α), 2.33 (qd, 1 H, J = 11.4, 5.2 Hz, H-4α), 2.11 (tdd, 1 H J = 11.4, 4.5, 0.8 Hz, H-4β), 3.74 (s, 3 H, H-16), 4.95 (d, 1 H, H-11′, J = 1.37 Hz), 4.04 (d, 1 H, H-13′, J = 1.3 Hz), 3.31 (s, 2 H, H-15′), 1.7 (s, 3 H, H16′); ^{13}C NMR (100.57 MHz) δ (ppm) 86.9 (C-2), 71.1 (C-3), 25.4 (C-4), 28.1 (C-5), 181.05 (C-6), 101.05 (C-7), 187.1 (C-8), 106.3 (C-9), 157.5 (C-10), 116.4 (C-11), 114.7 (C-12), 144.5 (C-13), 155.6 (C-14), 169.94 (C-15), 53.1 (C-16), 183.4 (C-1′), 114.2 (C-2′), 155.0 (C-3′), 130.9 (C-4′), 127.7 (C-5′), 158.1 (C-6′), 112.4 (C-7′), 186.6 (C-8′), 103.2 (C-9′), 202.0 (C-10′), 44.2 (C-11′), 58.85 (C-12′), 60.04 (C-13′), 49.1 (C-14′), 40.05 (C-15′), 19.4 (C-16′). Beticolin 2 (**2**): mp = 225 °C; $[\alpha]_D$ = +443.3° (c = 0.042, CH_2Cl_2); ^1H NMR (CD_3COCD_3; 400 MHz) δ (ppm) 15.25 (s, 1 H), 14.0 (s, 1 H), 12.5 (s, 1 H), 12.15 (s, 1 H), 11.5 (s, 1 H), 7.45, 7.4 (2 H, J = 9.8 Hz, H-4′, H-5′), 5.67 (s, 1 H (exchangeable with D_2O)), 4.15 (t, 1 H, J = 4 Hz, H-3), 2.92 (ddd, 1 H, J = 13, 10, 5 Hz, H-5β), 2.51 (dd, 1 H, J = 13, 4 Hz, H-5α), 2.1 (m, 2 H, H-4α, H-4β), 3.67 (s, 3 H, H-16), 4.68 (s, 1 H, H-11′), 4.05 (s, 1 H, H-13′), 3.5 (AB system, 2 H, H-15′, J = 12 Hz), 1.55 (s, 3 H, H16′); ^{13}C NMR (100.57 MHz) δ (ppm) 84.5 (C-2), 65.0 (C-3), 23.4 (C-4), 23.2 (C-5), 180.7 (C-6), 99.2 (C-7), 185.6 (C-8), 104.3 (C-9), 155.7 (C-10), 114.6 (C-11), 113.05 (C-12), 143.25 (C-13), 153.2 (C-14), 169.2 (C-15), 52.5 (C-16), 182.2 (C-1′), 111.4 (C-2′), 151.3 (C-3′), 129.2 (C-4′), 125.7 (C-5′), 156.7 (C-6′), 100.5 (C-7′), 182.3 (C-8′), 101.5 (C-9′), 200.1 (C-10′), 42.6 (C-11′), 53.0 (C-12′), 59.8 (C-13′), 47.7 (C-14′), 38.1 (C-15′), 16.2 (C-16′). Assignment of the nonprotonated carbons was made on the basis of the long-range ^1H–^{13}C coupling; one-bond CH correlations were used to assign protonated carbons.

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