A Universal, Photocleavable DNA Base: Nitropiperonyl 2′-Deoxyriboside

Michael C. Pirrung,* Xiaodong Zhao, and Shannon V. Harris

Department of Chemistry, Levine Science Research Center, Duke University, Durham, North Carolina 27708-0317

pirrung@chem.duke.edu

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A universal, photochemically cleavable DNA base analogue would add desirable versatility to a number of methods in molecular biology. A novel C-nucleoside, nitropiperonyl deoxyriboside (NPdR, P*), has been investigated for this purpose. NPdR can be converted to its 5′-DMTr-3′-CE-phosphoramidite and was incorporated into pentacosanucleotides by conventional synthesis techniques. The destabilizing effect on hybrid formation with a complementary strand when this P* base opposes A, T, and G was found to be 3−5 kcal/mol, but 9 kcal/mol when it opposes C. Brief irradiation (λ > 360 nm, 20 min) of DNA containing the P* base and piperidine treatment causes strand cleavage giving the 3′- and 5′-phosphates. Two significant recent interests, universal/non-hydrogen-bonding base analogues and photochemical backbone cleavage, have thus been combined in a single molecule that serves as a light-based DNA scissors.

Introduction

Most techniques for DNA manipulation in molecular biology involve polymerase chain reaction (PCR) or processing by restriction enzymes and ligases. Chemical methods to manipulate DNA can offer advantages in terms of substrate generality and enzyme-free reaction products. For example, chemical ligation of oligonucleotides through an S_N2 reaction of 5′-phosphorothioate and phosphoroselenoate DNAs has been recently reported by Kool.[1] Photochemical processes to manipulate nucleic acids offer a further advantage of being fully reagent-free. For example, photochemical generation of single-strand breaks[2] and "sticky ends" has been reported by Taylor.[3]

A DNA base that recognizes the four native bases in DNA equally and is photochemically cleavable could have a number of uses in nucleic acid fragmentation. A variety of new techniques for nucleic acid analysis/sequencing, such as mass spectrometry[4] and DNA "chips",[5] best utilize relatively short, single-stranded analytes. Existing fragmentation methods[6] have drawbacks in their sequence bias and processing steps needed to remove reagents/byproducts. A nucleoside analogue incorporated stochastically into a nucleic acid chain and activated for backbone cleavage by long-wavelength UV light would create single-strand nicks. The average length of uninterrupted double-stranded nucleic acid would thereby be shortened, facilitating conversion to single strands. Alternatively, the ability to selectively destroy a short oligonucleotide in the spatially and temporally defined manner uniquely permitted by light could enable powerful studies of chemical, biochemical, or biological processes. For example, with no added reagents, catalysts, or treatments, oligonucleotide fragmentation could be selectively promoted inside cells or during PCR reactions.

Many workers have developed functional analogues of the heterocyclic bases in nucleic acids. For example, Bergstrom[7] and Brown[8] have advanced nitropyrrole and nitroindole as heterocycles that will be accepted opposite any of the natural bases in double stranded nucleic acid. Such "universal" bases[9] have uses including simplified syntheses of short oligonucleotides that are used as probes for hybridization and primers for DNA sequencing and nucleic acid amplification. Hydrophobic, self-pairing bases[10] developed by Schultz and Romesberg serve as universal terminators.[11] On the other hand, Kool has shown that high-fidelity complementation of an unnatural base that cannot participate in hydrogen bonding can occur during DNA polymerase reactions. Difluorotoluene (F) deoxyriboside[12] specifically substitutes for thymidine (T) opposite a natural adenine (A) either in the template strand[13] or as an incoming triphosphate.[14]

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This work was aimed at combining the concepts of universal bases with nucleotide substitutes that can be cleaved by irradiation into two portions (and thereby cleave the backbone of the nucleic acid). Because the known photocleavable compounds do not resemble natural nucleotides, they cannot form duplex structures and could not be DNA polymerase substrates. We designed the C-nucleoside called P* (nitropiperonyl) based primarily on the substitution pattern known to provide optimal nitrobenzyl photochemistry and without regard to base pairing. Given its monocyclic structure with an oxygen at C4, P* resembles T. The C4 carbonyl of thymine participates in a hydrogen bond with the amino group of adenosine in the natural T−A pair, but the C2 carbonyl is not involved in any hydrogen bonding. The nitro group of P* is at the position corresponding to C2, is expected to play no role in pairing, and can occupy free space. Because of the resemblance of the nitroaromatic to the nitroheterocyclic universal bases, P* offers the potential to be accepted by nucleic acid modifying enzymes. Preliminary reports on some aspects of this work have appeared.[15]

Results

The target for synthesis was (nitropiperonyl)deoxyriboside. It is prepared by treatment of ditoluoylribosyl chloride[16] with the diarylcadmium[17] reagent derived from benzodioxol-4-yl Grignard. This method for preparation of the C-nucleoside was chosen because of its superiority (for simple aryl nucleosides) to some other known methods.[18] The coupling reaction was examined at three different temperatures, 4 °C, room temperature, and refluxing THF. Higher reaction temperature may cause side reactions, as colored products result. Reaction at room temperature results in the highest yield (87%), providing a mixture of epimers ($\alpha/\beta = 10/1$). Reaction at 4 °C does not show a notable improvement of yield, and the ratio of α/β epimers is independent of reaction temperature. Kool originally performed similar couplings at room temperature and saw anomer ratios favoring α by 5:1 to 10:1. Using Kool's *p*-TsOH/toluene equilibration procedure,[19] our initial mixture is converted to a 1:1.55 α/β ratio that is rectified by chromatography. The α anomer can be recycled to give good overall throughput to the β anomer (76%). Nitration ($Cu(NO_3)_2/Ac_2O$, 90%) yields **1** (λ_{max} 360 nm, H_2O) (Chart 1). There is no detectable α/β isomerization under these nitration conditions, which were the first examined.

Before examining the incorporation of P* into DNA, we address the issue of whether the synthesized artificial base has the desired structural features. The structure was confirmed through X-ray crystallography of the deprotected C-deoxyriboside and NOESY studies of both α and β anomers of **1**. They show very distinct patterns of NOE cross-peaks (Table 1). In the α anomer, one of the H2′ protons (α) has coupling with H1′ but not H3′. The other H2′ proton (β) has coupling with both H3′ and H1′. In the β anomer, the H2′α proton has strong coupling with H1′ and weak coupling with H3′. The other H2′ proton (β) has strong coupling with H3′ and weak coupling with H1′. Unlike the observation in the α anomer, the longer distance couplings of H2′α-H3′ and H2′β-H1′ appear as weak NOESY peaks rather than being completely absent. This may be due to the flexibility of the 2′-deoxyribofuranose conformation of the β anomer, since AM1 calculations (Spartan) of the β anomer suggest that there may be a steric interaction between the 5′-CH_2 and the aryl group. It is also worth noting that H2′β ($\delta = 1.92$ ppm) in the β anomer and H2′α ($\delta = 1.85$ ppm) in the α anomer resonate at higher field due to diamagnetic shielding by the aromatic ring. The nitropiperonyl-2′-deoxyriboside was crystallized from methanol solution and subjected to X-ray crystallography.[20] The structure demonstrates most of the properties of natural nucleosides, with the base in an anti conformation (based on analogy to T; vide infra) and a 3′-exo sugar pucker in the 2′-deoxyribose. The AM1 calculation also suggests the most stable conformation of the base is anti.

Using conventional methods,[21] **1** can be converted to the 5′-dimethoxytrityl-3′-cyanoethyl(diisopropyl)phosphoramidite **2** (52%) and used in automated DNA synthesis (Chart 1). It was used to prepare the pentacosanucleotide **4**, which substitutes P* for T in parent sequence **3**. Hybridization properties of **4** were examined against the four variants of sequence **5**, giving all four pairings with P*. Using known methods,[22] the thermodynamics of melting were determined with the base pairs in the boxed region. The results, summarized in Table 2, support the idea that P* resembles T as well as exhibiting universal

Chart 1

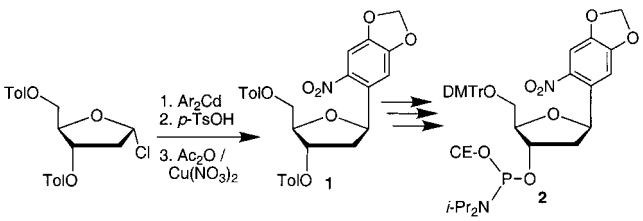

Table 1. Observed NOE Cross Peaks in NOESY of 2′-Deoxy-1′-(2-nitropiperonyl)-D-ribofuranose

	protons	H1′[a]	H2′α[a]	H2′β[a]	H3′[a]
	H1′		++	+	−
β-anomer	H2′α (2.63 ppm)	++		++	−
	H2′β (1.92 ppm)	+	++		++
	H3′	−	+	++	
	H1′		−	++	−
α-anomer	H2′α (1.85 ppm)	-		++	−
	H2′β (3.00 ppm)	++	++		++
	H3′	−	−	++	

[a] ++, strong; +, detectable; −, not detectable.

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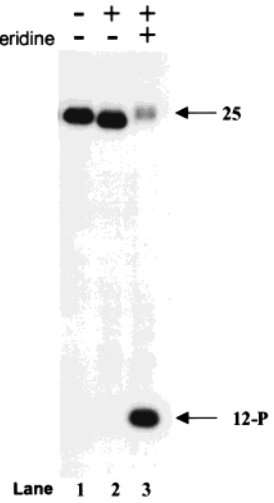


Figure 1. Photochemical backbone cleavage. Oligonucleotide **4** was ^{32}P-end labeled with polynucleotide kinase. A solution of 0.1 pmol of **4** in 10 μL of TE was irradiated in a Rayonet reactor at 400 μW/cm^2 for 20 min. Aqueous piperidine (90 μL of 1 M solution) was added, and the reaction was heated to 90 °C for 30 min. The solvent was removed under vacuum, and the products were suspended in formamide/loading dye and then subjected to 12% denaturing polyacrylamide gel electrophoresis. Lane 1 is starting **4**. Lane 2 is the irradiated product before backbone cleavage. Lane 3 is the cleaved product, which has the mobility of a 13-mer, indicating it is a 12-mer bearing a 3′-phosphate.

5'-GTA GAA TTC TTT T CC TTC TAG ATC G-3' **3**
5'-GTA GAA TTC TTT P*CC TTC TAG ATC G-3' **4**
3'-CAT CTT AAG AAA X GG AAG ATC TAG C-5' **5**
5'-GTA GAA P*TC TTT TCC TTC TAG ATC G-3' **6**

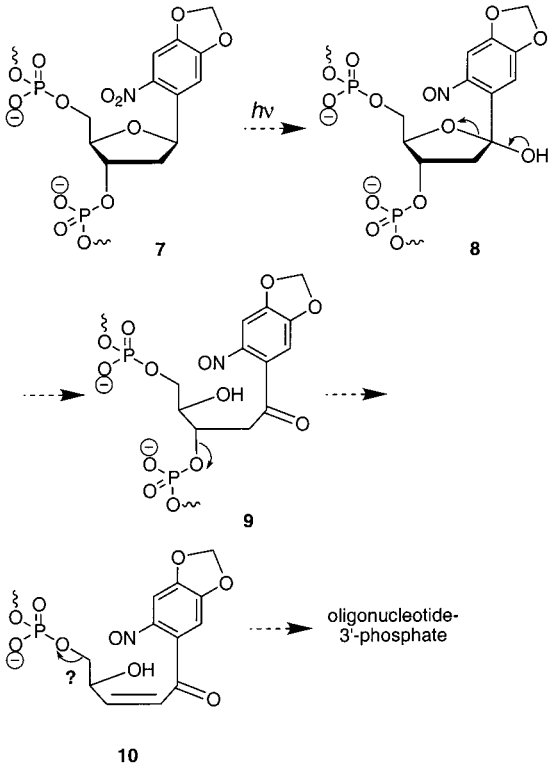
Table 2. Thermodynamics of Hybridization of Oligonucleotides 3 and 4 (Containing the P* Base) to Oligonucleotide 5

base pair (**3** or **4**−**5**)	T_m (°C)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (kcal/mol·K)	$\Delta G°$ (kcal/mol)
T−A	62.0	−195.9	−0.555	−30.5
P*−A	57.0	−187.7	−0.538	−27.2
P*−T	55.0	−163.9	−0.469	−24.1
P*−G	55.0	−154.4	−0.440	−23.2
P*−C	53.5	−143.1	−0.408	−21.5
T−T	56.0	−145.6	−0.412	−22.7

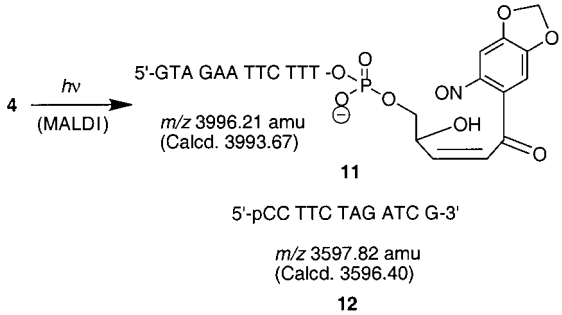
base properties. It pairs best with A, but also fairly well with G and T. Qualitatively, a P*−A pair leads to a ∼5 °C depression in T_m compared to a T−A pair.

The photochemical behavior of P* in DNA strands was next examined. The 5′-^{32}P-labeled **4** was subjected to irradiation at 350 nm, followed by base treatment (piperidine, 90 °C), resulting in a 12-mer oligonucleotide fragment bearing a 3′-phosphate (Figure 1) (and presumably an unlabeled 12-mer). A second 25-mer **6** was synthesized that places P* asymmetrically to ensure reliable identification of the fate of the strands 5′ of P* and 3′ of it. Irradiation of its 5′-end-labeled derivative and base treatment leads to a 6-mer product as the 3′-phosphate (5′-GTA GAAp-3′) as established by PAGE (data not shown). A similar experiment with unlabeled oligonucleotide gives two products as established by HPLC (C18, 100 mM Et_3NHOAc/MeCN 0−50% gradient), 5′-GTA GAAp-3′ (14.8 min), and 5′-TC TTT TCC TTC TAG ATC G-3′ (28.0 min).

A mechanism can be proposed for photochemical cleavage of oligonucleotides containing P* (**7**) to give the observed products. Very well precedented nitrobenzyl photochemistry[23] should lead to **8**, which is also similar to known pathways for creation of abasic sites through formation of a C-1′ radical.[24] Ring-chain tautomerism produces ketone **9**, which can undergo β-elimination of the 3′-phosphate to **10**. To generate the product derived



from the segment of oligonucleotide to the 5′ of P* (its 3′-phosphate), a second elimination reaction likely occurs. Extensive efforts to establish this mechanism through isolation of intermediates and products in the irradiation of **1** were unsuccessful. Support for the mechanism comes from direct MALDI-TOF mass spectrometry of **4**, wherein laser excitation cleaves the backbone. The observed ions correspond to the 5′-fragment (**11**; before phosphate elimination) and the 3′-fragment (**12**).



Discussion

The relatively good stability of DNA duplexes containing the P* base is likely a consequence of its nitroaryl group, which is known from the nitropyrrole and nitroin-

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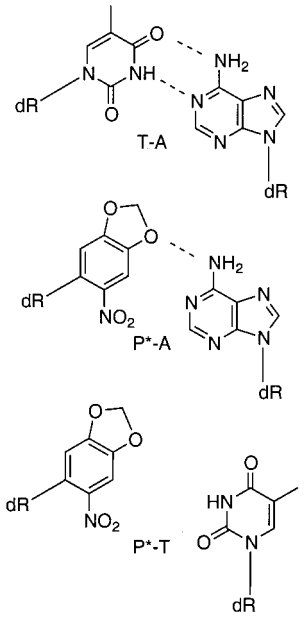
Chart 2



dole precedents to facilitate stacking interactions even when hydrogen bonding across the helix is not possible. The nitro group thus serves a dual purpose, as it also imparts the photochemical reactivity required to cleave the backbone. The hybridization of P*-containing oligonucleotides in several sequence contexts shows some selectivity for P*−A pairs. Progression from the T−A perfect match to the P*−A pairing to the T−P* pairing (Chart 2) results in a ∼3 kcal/mol difference in duplex stability at each step, perhaps reflecting the loss of a hydrogen bond. Generally, P* is accepted in duplexes through base stacking, fulfilling the intent if not the definition of a universal base. However, given that earlier workers have *not* found a correlation between thermodynamic duplex stability and the acceptance of unnatural bases by enzymes such as DNA polymerases, further experimentation will be required to define the true nature of P* as a universal base.

The biophysical evidence suggests that our synthetic compound has many desirable features for an artificial nucleoside. The photochemistry of P* leads to DNA strand cleavage exclusively at the modified position, demonstrating that the behavior of P* is not due to a general DNA cleaving property of nitro compounds under irradiation.[25] The photochemistry of this P* base parallels chemistry recently reported for the photochemical introduction of deoxyribonolactone lesions into oligodeoxyribonucleotides through irradiation of nitroindole deoxyribosides, though this work was not extended to backbone cleavage.[26]

Experimental Section

2′-Deoxy-1′-piperonyl-3′,5′-di-*O*-toluoyl-D-ribofuranose. To 5 mL of dry tetrahydrofuran (THF) and magnesium turnings (120 mg, 5 mmol) was added 4-bromo-1,2-methylenedioxybenzene (1.03 g, 5 mmol).[27] The reaction was initiated by adding a few I_2 crystals and kept at ∼40 °C for 1 h. Cadmium chloride powder (450 mg, 2.5 mmol) was added. After being refluxed for 1.5 h, the mixture was cooled to room temperature, 1′α-chloro-3′,5′-di-*O*-toluoyl-2′-deoxyribose (1.50 g, 3.8 mmol), prepared as reported by Hoffer, was added, and the resulting mixture was allowed to stir for 6 h. The reaction was quenched with aqueous NH_4Cl and extracted with ethyl acetate. The extracts were washed with saturated aqueous $NaHCO_3$ and saturated NaCl solution and dried over anhydrous Na_2SO_4. The mixture was filtered, and the solvent was removed. The products were purified by flash column chromatography on silica gel with 12% ethyl acetate/hexane as the eluant. The products (1.60 g, 3.3 mmol) were obtained as clear oil (epimer α/*β*, 10:1) in 87% yield.

Equilibration to 2′-Deoxy-1′*β*-piperonyl-3′,5′-di-*O*-toluoyl-D-ribofuranose. To 75 mL of toluene were added the α/*β* epimer mixture (1.00 g), a catalytic amount of *p*-toluenesulfonic acid (100 mg), one drop of concentrated sulfuric acid, and five drops of water. After the mixture was refluxed for 2 h, the toluene was removed and the residue was neutralized with saturated aqueous $NaHCO_3$ and extracted with ethyl acetate. The extracts were washed with saturated NaCl solution and dried over anhydrous Na_2SO_4. The α and *β* epimers were isolated by flash chromatography on silica gel with 12.5% ethyl acetate/hexane as the eluent. α Epimer: 0.30 g (30%); ^{1}H NMR (300 MHz, $CDCl_3$) *δ* 7.95 (2H, d, *J* = 8.2 Hz), 7.78 (2H, d, *J* = 8.2 Hz), 7.23 (2H, d, *J* = 8.3 Hz), 7.20 (2H, d, *J* = 8.3 Hz), 6.97−6.76 (3H, m), 5.94 (2H, s, CH_2), 5.58 (1H, m, H1′), 5.26 (1H, m, H3′), 4.66 (1H, m, H4′), 4.56 (2H, m, H5′,5″), 2.89 (1H, m, H2′*β*), 2.40 (6H, s, 2 × CH_3), 2.11 (1H, m, H2′α); IR (thin film, cm^{-1}) 2922, 2852, 1720, 1271, 1104. *β* Epimer, 0.46 g (46%): ^{1}H NMR (300 MHz, $CDCl_3$) *δ* 7.97 (2H, d, *J* = 8.3 Hz), 7.96 (2H, d, *J* = 8.3 Hz), 7.27 (2H, d, *J* = 8.0 Hz), 7.23 (2H, d, *J* = 8.0 Hz), 6.90−6.74 (3H, m), 5.94 (2H, s, CH_2), 5.58 (1H, dd, *J* = 1.3, 4.8 Hz, H1′), 5.16 (1H, dd, *J* = 5.0, 10.9 Hz, H3′), 4.63 (2H, m, H5′5″), 4.50 (1H, m, H4′), 2.49 (1H, m, H2′α), 2.44 (3H, s, CH_3), 2.41 (3H, s, CH_3), 2.22 (1H, m, H2′*β*); IR (thin film, cm^{-1}) 2921, 1719, 1611, 1271, 1105; HRMS (FAB) *m*/*z* calcd for $C_{28}H_{26}O_7$ 474.1678, found 474.1682.

2′-Deoxy-1′*β*-(2-nitropiperonyl)-3′,5′-di-*O*-toluoyl-D-ribofuranose. To a solution of 2′-deoxy-1′*β*-piperonyl-3′,5′-di-*O*-toluoyl-D-ribofuranose (1.40 g, 2.95 mmol) in 175 mL of acetic anhydride immersed in an ice−water bath was added $Cu(NO_3)_2 \cdot 3H_2O$ (2.57 g, 9.38 mmol).[28] The mixture was stirred for 25 min and then poured into 200 mL of saturated aqueous $NaHCO_3$ and extracted with ethyl acetate. The extracts were washed with saturated NaCl solution and dried over anhydrous Na_2SO_4. The product was purified by flash chromatography on silica gel with 18% ethyl acetate/hexane as the eluant. The product (1.38 g, 2.65 mmol) was obtained as a yellow oil in 90% yield: ^{1}H NMR (300 MHz, $CDCl_3$) *δ* 7.98 (2H, d, *J* = 8.2 Hz, Tol-H), 7.93 (2H, d, *J* = 8.2 Hz, Tol-H), 7.54 (1H, s, Ar-H), 7.37 (1H, s, Ar-H), 7.27 (2H, d, *J* = 8.0 Hz, Tol-H), 7.22 (2H, d, *J* = 8.0 Hz, Tol-H), 6.09 (1H, d, *J* = 6.6 Hz, CH_2), 6.08 (1H, d, *J* = 6.6 Hz, CH_2), 5.77 (1H, dd, *J* = 5.1, 10.2 Hz, H1′), 5.58 (1H, dd, *J* = 1.8, 4.8 Hz, H3′), 4.72 (2H, m, H5′,5″), 4.51 (1H, m, H4′), 2.94 (1H, ddd, *J* = 1.2, 5.1, 6.4 Hz, H2′α), 2.43 (3H, s, CH_3), 2.40 (3H, s, CH_3), 2.05 (1H, m, H2′*β*); IR (thin film, cm^{-1}) 2922, 2853, 1712, 1611, 1503, 1482, 1268, 1177, 1106; HRMS (FAB) *m*/*z* calcd for $C_{28}H_{25}NO_9$ 519.1529, found 519.1539.

2′-Deoxy-1′*β*-(2-nitropiperonyl)-D-ribofuranose. To a solution of 2′-deoxy-1′*β*-(2-nitropiperonyl)-3′,5′-di-*O*-toluoyl-D-ribofuranose (0.22 g, 0.42 mmol) in 30 mL of methanol was added 0.5 M sodium methoxide in methanol (7.5 mL). The mixture was stirred for 45 min and then quenched by the addition of NH_4Cl powder (1.5 g). The mixture was filtered, concentrated, and flash chromatographed on silica gel with 80% ethyl acetate/hexane. The product was obtained as yellow solid (0.12 g, 0.42 mmol) in a quantitative yield: UV (H_2O) λ_{max} 360.5 nm, ϵ_{max} 1.3×10^4, UV (CH_3OH) λ_{max} 346.0 nm, ϵ

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$_{max}$ 1.4 ×10^4; mp 133−134 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.54 (1H, s, Ar−H), 7.31 (1H, s, Ar−H), 6.12 (1H, d, J = 3.0 Hz, CH_2), 6.11 (1H, d, J = 3.0 Hz, CH_2), 5.68 (1H, dd, J = 6.0, 9.0 Hz, H1′), 4.40 (1H, m, H3′), 4.02 (1H, m, H4′), 3.93 (2H, m, H5′,5”), 2.63 (1H, ddd, J = 3.0, 6.0, 8.9 Hz, H2′α), 1.92 (1H, m, H2′β); IR (thin film, cm^{-1}) 3396 (br), 2931, 2857, 1520, 1504, 1482, 1329, 1255, 1033; HRMS (FAB) *m/z* calcd for $C_{12}H_{13}NO_7$ 283.0692, found 283.0686.

2′-deoxy-1′α-(2-nitropiperonyl)-D-ribofuranose was prepared similarly from 2′-deoxy-1′α-piperonyl-3′,5′-di-O-toluoyl-D-ribofuranose: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.56 (1H, s, Ar-H), 7.35 (1H, s, Ar-H), 6.12 (1H, d, J = 4.6 Hz, CH_2), 6.11 (1H, d, J = 4.6 Hz, CH_2), 5.68 (1H, t, J = 7.1 Hz, H1′), 4.44 (1H, m, H3′), 4.22 (1H, m, H4′), 3.76 (2H, m, H5′,5″), 3.00 (1H, m, H2′β), 1.85 (1H, m, H2′α).

2′-Deoxy-1′β-(2-nitropiperonyl)-5′-*O*-dimethoxytrityl-D-ribofuranose. To a solution of 2′-deoxy-1′β-(2-nitropiperonyl)-D-ribofuranose (0.11 g, 0.39 mmol), triethylamine (0.15 mL), and *N*,*N*-dimethylaminopyridine (5 mg, 0.041 mmol) in 10 mL of pyridine was added dimethoxytrityl chloride (0.194 mg, 0.50 mmol). After being stirred for 6 h, the reaction was poured into 50 mL of water and extracted with ethyl acetate. The extracts were washed with saturated aqueous $NaHCO_3$ and saturated NaCl solution and dried over anhydrous Na_2SO_4. The product was obtained as a yellow foam (0.16 g, 0.27 mmol) in 69% yield after flash chromatography on silica gel with 27.5% ethyl acetate/hexane as the eluant: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.55−7.19 (11H, m, Ar-H), 6.84 (4H, d, J = 8.8 Hz), 6.09 (2H, 2 d, J = 8.4 Hz, CH_2), 5.69 (1H, dd, J = 6.2, 8.8 Hz, H1′), 4.38 (1H, m, H3′), 4.06 (1H, m, H4′), 3.79 (6H, s, 2×OCH_3), 3.38 (2H, m, H5′,5″), 2.62 (1H, ddd, J = 3.1, 6.1, 9.3 Hz, H2′α), 1.96 (1H, m, H2′β); IR (thin film, cm^{-1}) 2927, 1511, 1480, 1257, 1034; HRMS (FAB) *m/z* calcd for $C_{33}H_{31}NO_9$ 585.1998, found 585.1983.

2′-Deoxy-1′β-(2-nitropiperonyl)-3′-*O*-phosphoramidite-5′-*O*-dimethoxytrityl-D-ribofuranose. 2′-Deoxy-1′β-(2-nitropiperonyl)-5′-*O*-dimethoxytrityl-D-ribofuranose (60 mg, 0.103 mmol) was dissolved in 4 mL of CH_2Cl_2 and 1 mL of triethylamine, and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.10 mL, 0.44 mmol) was added. After the mixture was stirred for 3 h, the solvent was removed under vacuum and the products were purified by column chromatography on silica gel eluted with 30% ethyl acetate/hexane. A mixture of two isomeric products was obtained as a pale yellow oil (60 mg, 0.076 mmol) in 76% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.55−7.20 (11H, m, Ar−H), 6.84 (4H, m), 6.10 (2H, m, CH_2), 5.67 (1H, m, H1′), 4.46 (1H, m), 4.21 (1H, m), 3.86 (1H, m), 3.80 (3H, s, OCH_3), 3.79 (3H, s, OCH_3), 3.62 (2H, m), 3.32 (1H, m), 2.78 (1H, m), 2.65 (2H, m, CH_2), 2.43 (2H, m, CH_2), 1.92 (1H, m), 1.17 (12H, d, J = 6.8 Hz, 4 × CH_3); ^{31}P NMR (162 MHz, $CDCl_3$, referenced to TMP at 0.00) δ 149.24, 148.33; IR (thin film, cm^{-1}) 2958, 2923, 2853, 1605, 1511, 1480, 1462, 1256, 1033; HRMS (FAB) *m/z* calcd for $C_{42}H_{48}N_3O_{10}P$ 785.3077, found 785.3020.

Oligonucleotide Synthesis. Oligonucleotides were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer with standard phosphoramidite chemistry. An additional 10 min was added to the coupling time for the P* nucleoside to ensure complete incorporation, though it was not established that this long a coupling was necessary. The oligomers were purified by either HPLC or 12% polyacrylamide gel electrophoresis. MALDI analysis of **3** gave an observed mass of 7617.85 amu, comparable (±0.1%) to the calculated value of 7610.0 amu. MALDI analysis of **4** gave no parent ion at the calculated 7669.04, only the two fragment peaks at 3996.21 (calcd 3993.67) and 3597.82 amu (calcd 3596.40).

Melting Temperature. Double-stranded oligonucleotide 25-mers (1−20 μM) were dissolved in a buffer containing 100 mM NaCl, 10 mM sodium phosphate, pH 7.0. Absorbance was measured in quartz cuvettes with optical path lengths of 1 cm or 1 mm using an Aviv 62DS spectrophotometer. The absorbance of the oligonucleotides at 260 nm was in the range of 0.4−1.2. The samples were heated in the spectrophotometer from 40 to 75 °C at increments of 0.5. Each incremental data point was equilibrated for 30 s with a maximum fluctuation of 0.1 °C. The melting temperature (T_m) of each duplex oligonucleotide was determined at three to four different concentrations by taking the first derivative of the melting curve (absorbance vs temperature). Curve fitting was performed with SigmaPlot using a simplified nonlinear least-squares model, and the data were plotted in MS Excel. Thermodynamic data were calculated from a two-state model using the method of Marky & Breslauer. ΔG was determined for 25 °C (298 K), and T_m was calculated at 1 μM.

MALDI-TOF MS. Oligonucleotides were precipitated with ammonium acetate/ethanol. After resuspension in 1:1 acetonitrile/water at ∼20 pmol/μL, 0.8 μL was placed on a MALDI plate and allowed to air-dry. An 0.4 μL aliquot of AG-50W X ion-exchange resin that had been previously treated with 5M ammonium acetate was placed on top of the DNA spot and allowed to dry. Matrix (0.8 μL of 6-aza-2-thiothymine solution, 0.1 g/mL with ammonium citrate) was added and allowed to dry. MALDI mass spectra were acquired in the linear, negative-ion mode using a nitrogen laser (337 nm). All spectra were collected using an acceleration voltage of 25 kV, a grid voltage of 23 kV, a guide wire voltage of 75 V, and a delay time of 225 ns. Each spectrum was the sum of 156 to 256 laser shots. The raw intensity versus time data in each mass spectrum were smoothed using a Savitsky−Golay routine prior to mass calibration using internal standards.

Irradiation Experiments. Oligonucleotides **4** or **6** were treated with T4 polynucleotide kinase (NEB) and ATP at 37 °C for 2 h in kinase buffer. The products were size-separated on 19% PAGE, and the desired product was excised from the gel, eluted into 1 × TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH 7.5), and precipitated with ethanol. The 5′-labeled oligonucleotide 25-mer (0.1 pmol) was suspended in 10 μL of TE and irradiated at 350 nm in a Rayonet photochemical reactor maintained close to room temperature by a fan. A Pyrex filter was used to block light <300 nm. Photolysis was complete at a fluence of 400 μW/cm^2 for 20 min, after which 90 μL of 1 M piperidine in water was added and the reaction was heated to 90 °C for 30 min. The solvent was removed under vacuum, and the products were resuspended in 10 μL of formamide/dye and resolved on a 12% denaturing polyacrylamide gel.

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Supporting Information Available: Melting curves for six duplexes and ORTEP plot of P* nucleoside. This material is available free of charge via the Internet at http://pubs.acs.org.

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