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A 2-AMINO-6-METHYLPYRIDIN-5-YL NUCLEOBASE FOR GC BASE PAIR RECOGNITION IN THE PARALLEL TRIPLEX DNA

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Dedicated to Professor Dr. Eiichi Negishi on the occasion of his 77th birthday

Abstract – DNA triple helices containing consecutive C^+H•GC triplets are unstable at neutral pH because protonations of cytosines into the third strand are necessary. Previously, a 2-aminopyridin-5-yl nucleobase (**P**) was developed and has been widely used as a substitute for the cytosine nucleobase. In this work, we designed a 2-amino-6-methylpyridin-5-yl nucleobase ($\mathbf{P_{Me}}$), which could preferentially adopt an anti-orientation by the 6-methyl group. This might lead to formation of a stable base triplet with a GC base pair compared to **P**. We also synthesized 15-mer triplex-forming oligonucleotides (TFOs) containing $\mathbf{P_{Me}}$ by the standard solid-phase method and evaluated the triplex stability of the TFOs at neutral pH by UV melting experiments.

INTRODUCTION

Sequence specific binding of triplex-forming oligonucleotides (TFOs) to double-stranded DNA (dsDNA) can selectively regulate gene expression and can be applied to genetic diagnostics.[1] Pyrimidine-rich TFOs bind to the major groove of dsDNA with homopurine tracts by Hoogsteen hydrogen bonding. A thymine (T) and a protonated cytosine (C^+H) in the third strand recognize an adenine (A) of an AT base pair (T•AT) and a guanine (G) of a GC base pair (C^+H•GC), respectively. However, the C residues in TFOs prevent stable triple helix formation at neutral pH. In particular, when TFOs containing contiguous cytosine residues are used, formation of triplex with target DNA is quite difficult. The development of non-natural nucleobases which form a stable triplet with a GC base pair in triplex DNA has been attempted to overcome this problem.[2,3] For example, replacement of cytosine by 5-methylcytosine in TFOs leads to an increase in the stability of triple helix formation.[2] However, in the case of target dsDNA

containing contiguous GC base pairs, even TFO using 5-methylcytosine can show inadequate stability for triple helix formation with the dsDNA.[4] In 1996, Leumann's group and Niedle's group independently reported that 2-aminopyridin-5-yl nucleobases (**P**[4,5] and ^{Me}**P**[4]) as cytosine analogues were more basic than cytosine, and these nucleobases were useful for GC recognition of target dsDNA[6] and 15-mer TFO bearing four contiguous ^{Me}**P** residues could form a stable triple helix at neutral conditions (Figure 1).

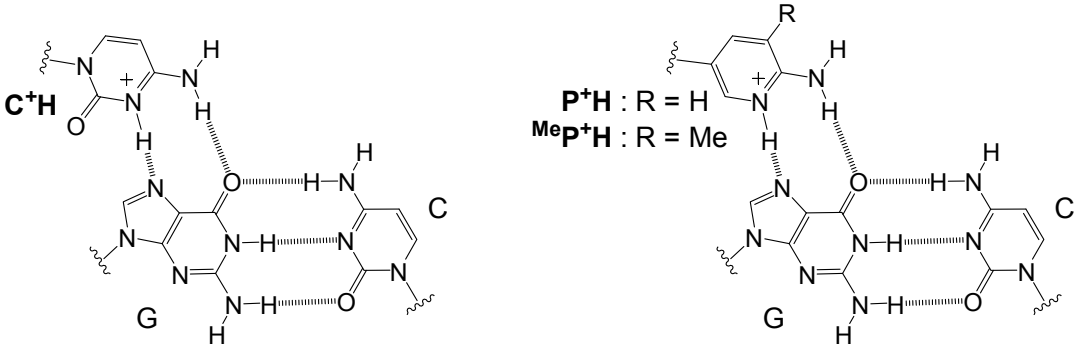


Figure 1. C$^+$H•GC, **P**$^+$**H**•GC and ^{Me}**P**$^+$**H**•GC triplets.

The glycosidic bonds of **P** and ^{Me}**P** can freely rotate. If the rotation was restricted to the anti-orientation, appropriate for recognition of a GC base pair, an increase of the affinity to a GC base pair was considered (Figure 2).[7] Thus, we designed a 2-amino-6-methylpyridin-5-yl nucleobase (**P**$_{\mathbf{Me}}$) to restrict the glycosidic bond in the anti-orientation by steric repulsion between the 6-methyl group and the furanose ring. In this paper, we describe the synthesis of oligonucleotides containing **P**$_{\mathbf{Me}}$ and **P**, as well as evaluation of their binding affinity to dsDNA at neutral pH.

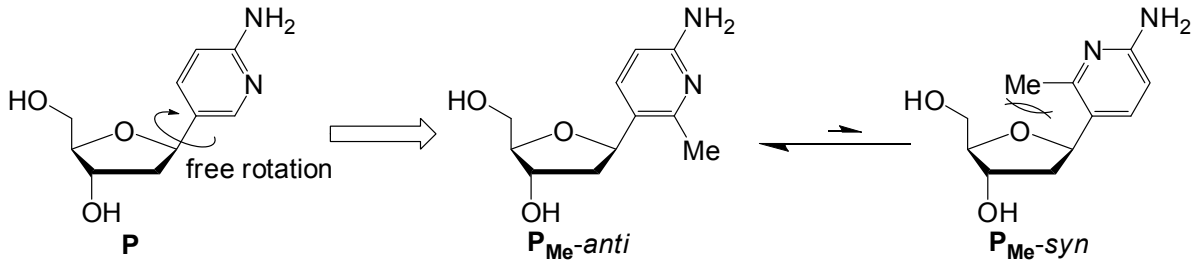
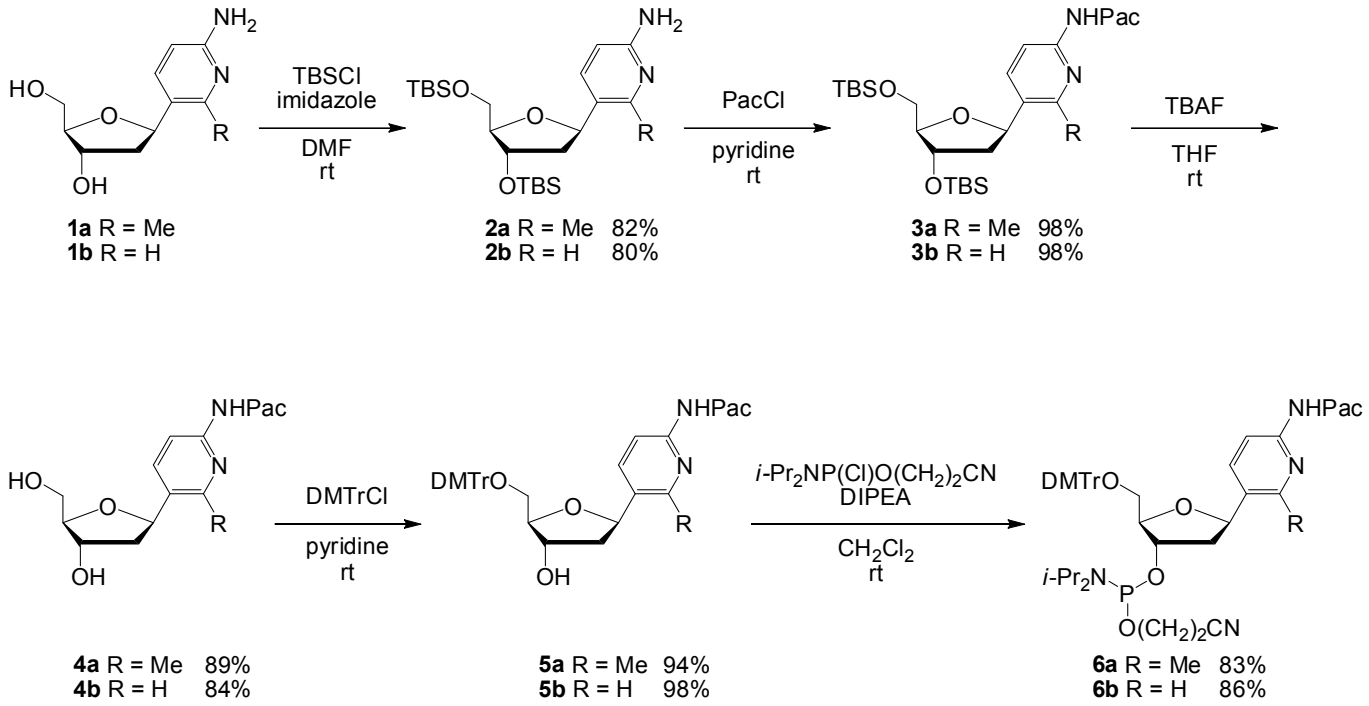


Figure 2. Design concept of **P**$_{\mathbf{Me}}$.

RESULTS AND DISCUSSION

The syntheses of *C*-nucleosides with **P**$_{\mathbf{Me}}$ and **P** are shown in Scheme 1. According to a previous report by Leumann's group, relatively hard conditions, *i.e.*, 40% aqueous methylamine (70 °C, 26 h) was required to efficiently deprotect oligonucleotides containing the benzoyl-protected **P**.[4] Thus, we decided to protect the exocyclic amino function in **P**$_{\mathbf{Me}}$ and **P** using the phenoxyacetyl (Pac) group which could be removed

under mild and/or standard conditions. *C*-Nucleosides **1a** and **1b** were synthesized as previously reported by McLaughlin's group.[8,9] After protection of the 3'- and 5'-hydroxyl groups of **1a** using TBDMS groups (**2a**; 82%), the protection of amino group was performed with PacCl (**3a**; 98%) and desilylation of **3a** with tetra-*n*-butylammonium fluoride (TBAF) gave the desired *C*-nucleoside **4a** in 89% yield. As we expected, it was clarified that the nucleobase moiety of **4a** preferentially exists in anti-orientation by the NOE correlation between H5' in the sugar moiety and H4, not 6-methyl group, in the nucleobase (Figure 3). The phosphoramidite building block **6a** was obtained by dimethoxytritylation of **4a** with 4,4'-dimethoxytrityl chloride (**5a**; 94%) followed by phosphitylation of **5a** with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (**6a**; 83%). The phosphoramidite **6b** was synthesized in the same way. Using **6a** and **6b**, the 15-mer **TFO-1**–**TFO-8** oligonucleotides listed in Figure 4 were synthesized under standard conditions on an automated DNA synthesizer.



Scheme 1. Syntheses of nucleosides $\mathbf{P_{Me}}$ and **P**.

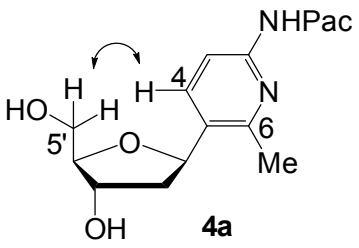


Figure 3. NOE correlation of **4a**.

TFO-1 : 5'-TTTTTC̲T**P$_{Me}$**TC̲TC̲TC̲T-3'
TFO-2 : 5'-TTTTTC̲T**P**TC̲TC̲TC̲T-3'
TFO-3 : 5'-TTTTTC̲TCTC̲TC̲TC̲T-3'
TFO-4 : 5'-TTTTTC̲TC̲TC̲TC̲TC̲T-3'
TFO-5 : 5'-TTTTT**P$_{Me}$P$_{Me}$P$_{Me}$**TC̲TC̲TC̲T-3'
TFO-6 : 5'-TTTTT**PPP**TC̲TC̲TC̲T-3'
TFO-7 : 5'-TTTTTCCCTC̲TC̲TC̲T-3'
TFO-8 : 5'-TTTTTC̲C̲C̲TC̲TC̲TC̲T-3'

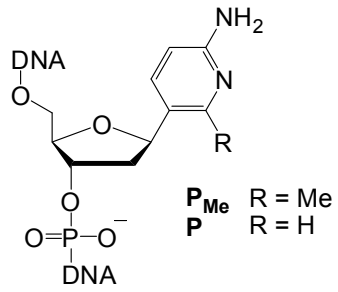


Figure 4. Sequences of TFOs used in this study. C̲ : 2'-Deoxy-5-methylcytidine.

Table 1. T_m Values (°C) of triplexes between TFOs and hairpin-loop dsDNA.

Triplexes	TFO	T_m value (°C)
5'-TTTTTC̲T**X**TC̲TC̲TC̲T-3' 5'-GGCAAAAAGA**G**AGAGAGACGC—C18spacer 3'-CCGTTTTTCT**C**TCTCTCTGCG—	**TFO-1** (X = **P$_{Me}$**)	41
	TFO-2 (X = **P**)	42
	TFO-3 (X = C)	39
	TFO-4 (X = C̲)	42
5'-TTTTT**XXX**TC̲TC̲TC̲T-3' 5'-GGCAAAAA**GGG**AGAGAGACGC—C18spacer 3'-CCGTTTTT**CCC**TCTCTCTGCG—	**TFO-5** (X = **P$_{Me}$**)	37
	TFO-6 (X = **P**)	42
	TFO-7 (X = C)	32
	TFO-8 (X = C̲)	36

Conditions : 1.89 μM Each strand, 10 mM sodium cacodylate buffer (pH 6.8), 100 mM KCl, 50 mM $MgCl_2$, 5 °C to 90 °C (0.5 °C/min). C̲ : 2'-Deoxy-5-methylcytidine.

The dsDNA binding properties of the synthesized TFOs were examined under neutral conditions by means of thermal denaturation experiments. We used hairpin-loop dsDNA linked to a hexa(ethyleneglycol) unit (C18-spacer) as target dsDNA. Because the hairpin-loop structure increased the thermal stability of the duplex, it could prevent the transition of dsDNA to ssDNA from overlapping with that of triplex to duplex. The results obtained by UV melting experiments are summarized in Table 1. The T_m value of the triplex formed between the complementary dsDNA and **TFO-1** containing single **P$_{Me}$** modification was 41 °C, comparable to that of **TFO-2** (X = **P**) and **4** (X = C̲) and slightly higher than that of **TFO-3** (X = C). On the other hand, in the case of **TFO-5** bearing three contiguous **P$_{Me}$** modifications, the stability (T_m = 37 °C) was significantly decreased compared with that (T_m = 42 °C) of the **P**-modified **TFO-6**, though **TFO-5** showed a similar T_m value to the **C̲**-modified **TFO-8**. The UV-melting curves using TFOs containing **P$_{Me}$** or **P** are shown in Figure 5. The hyperchromicities of triplex by singly

modified **TFO-1** and **TFO-2** were almost same. However, in the case of three contiguous modifications (**TFO-5** and **TFO-6**), the hyperchromicities significantly decreased and **TFO-5** clearly showed smaller hyperchromicity than **TFO-6**. This suggests that $\mathbf{P_{Me}}^{+}$**H•**GC triplet may not have enough stacking interaction with adjacent triplets due to the steric bulkiness of the methyl group of $\mathbf{P_{Me}}$. Eventually, this may cause a decrease in stability of triplex with contiguous $\mathbf{P_{Me}}$ modifications compared to that with **P**.

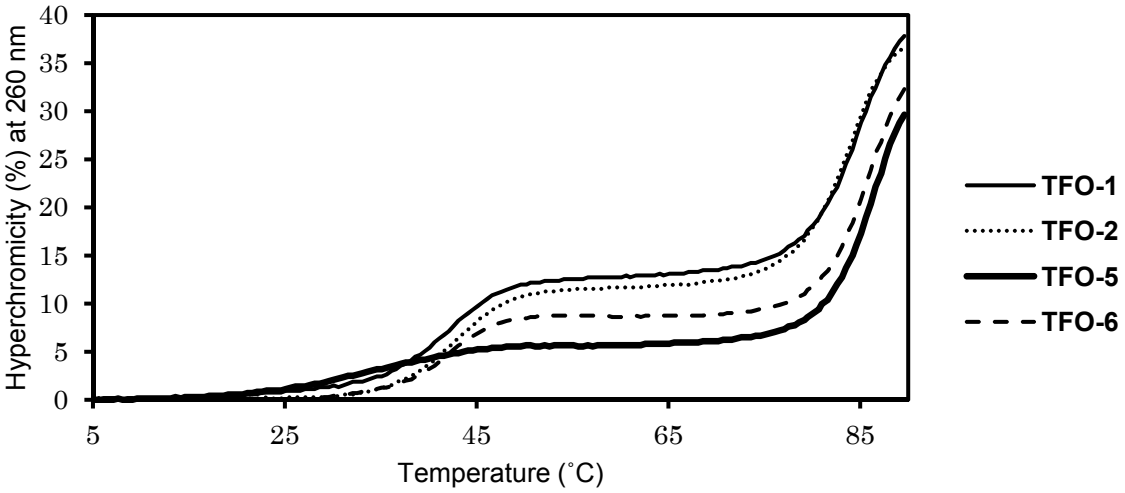


Figure 5. UV-Melting profiles of triplexes between TFOs and dsDNA.

In conclusion, we synthesized TFOs containing $\mathbf{P_{Me}}$ and **P** and evaluated their triplex-forming abilities. We found that the affinity of $\mathbf{P_{Me}}$ to a GC base pair was the same degree as that of 5-methylcytosine, but lower than that of **P**. Moreover, it was suggested that the 6-methyl group of $\mathbf{P_{Me}}$ might disturb enough stacking interaction in triplex DNA.

EXPERIMENTAL

All moisture-sensitive reactions were carried out in well-dried glassware under N_2 atmosphere. ^{1}H, ^{13}C, and ^{31}P spectra were recorded on JEOL JNM-EX300 or JEOL JNM-EX400 spectrometers. Chemical shifts are reported in parts per million referenced to tetramethylsilane (δ = 0.00 ppm) for ^{1}H NMR spectra, $CDCl_3$ (δ = 77.0 ppm) and CD_3OD (δ = 49.0 ppm) for ^{13}C NMR spectra, and phosphoric acid (δ = 0.00 ppm) for ^{31}P NMR spectra. IR spectra were recorded on a JASCO FT/IR-4200 spectrometer. Optical rotations were recorded on a JASCO P-2200 polarimeter. FAB mass spectra were measured on JEOL JMS-600 or JEOL JMS-700 mass spectrometers. MALDI-TOF mass spectra were recorded on a Bruker Daltonics Autoflex II TOF/TOF mass spectrometer. Fuji Silysia silica gel PSQ-60B (0.060 mm) and FL-60D (0.060 mm) were used for flash column chromatography. For HPLC, SHIMADZU LC-10AT$_{VP}$, SHIMADZU SPD-10A$_{VP}$ and SHIMADZU CTO-10$_{VP}$ instruments were used.

2-Amino-5-[3',5'-di-*O-tert*-butyldimethylsilyl-(2'-deoxy-D-ribofuranosyl)]-6-methylpyridine (2a)

Under an N_2 atmosphere, imidazole (62 mg, 0.914 mmol) and TBDMSCl (66 mg, 0.439 mmol) were added to a solution of **1a** (41 mg, 0.183 mmol) in anhydrous DMF (3 mL) at 0 °C and the mixture was stirred at room temperature for 11 h. After the addition of water at 0 °C, the reaction mixture was diluted with Et_2O, washed with water and brine, dried over Na_2SO_4, and concentrated. The crude product was purified by flash column chromatography (2:1 Hexane-EtOAc) to give **2a** (68 mg, 82%); Yellow oil; $[\alpha]_D^{28}$ 34.6 (*c* 1.03, $CHCl_3$); IR ν_{max} (KBr) 1610, 1545, 1485, 1389, 1275 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.08 (6H, s), 0.09 (6H, s), 0.91 (9H, s), 0.91 (9H, s), 1.75 (1H, ddd, *J* = 5.5, 10.5, 12.5 Hz), 2.08 (1H, ddd, *J* = 2.0, 5.5, 12.5 Hz), 2.37 (3H, s), 3.65 (1H, dd, *J* = 5.5, 11.0 Hz), 3.76 (1H, dd, *J* = 4.0, 11.0 Hz), 3.91 (1H, ddd, *J* = 2.0, 4.0, 5.5 Hz), 4.33 (2H, brs), 4.41 (1H, ddd, *J* = 2.0, 2.0, 5.5 Hz), 5.24 (1H, dd, *J* = 5.5, 10.5 Hz), 6.34 (1H, d, *J* = 8.5 Hz), 7.59 (1H, d, *J* = 8.5 Hz); ^{13}C-NMR ($CDCl_3$, 101 MHz) δ: -5.5, -5.4, -4.7, -4.6, 18.0, 18.3, 21.5, 25.8, 25.9, 42.9, 63.6, 74.2, 76.1, 87.5, 106.1, 125.4, 135.8, 153.1, 156.7; MS (FAB) *m/z* 453 $[M+H]^+$; HRMS (FAB) *m/z* Calcd for $C_{23}H_{45}N_2O_3Si_2$ $[M+H]^+$: 453.2963. Found 453.2967.

2-Amino-5-[3',5'-di-*O-tert*-butyldimethylsilyl-(2'-deoxy-D-ribofuranosyl)]pyridine (2b)

Under an N_2 atmosphere, imidazole (210 mg, 3.08 mmol) and TBDMSCl (215 mg, 1.43 mmol) were added to a solution of **1b** (125 mg, 0.595 mmol) in anhydrous DMF (6 mL) at 0 °C and the mixture was stirred at room temperature for 13 h. After the addition of water at 0 °C, the reaction mixture was diluted with Et_2O, washed with water and brine, dried over Na_2SO_4, and concentrated. The crude product was purified by flash column chromatography (1:1 Hexane-EtOAc) to give **2b** (208 mg, 80%); Yellow oil; $[\alpha]_D^{26}$ 15.4 (*c* 1.02, $CHCl_3$); IR ν_{max} (KBr) 2954, 2929, 2886, 2857, 1622, 1503, 1471, 1360, 1255, 1089, 1031 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.07 (6H, s), 0.09 (6H, s), 0.91 (9H, s), 0.91 (9H, s), 1.90 (1H, ddd, *J* = 5.5, 10.5, 12.5 Hz), 2.03 (1H, ddd, *J* = 1.5, 5.0, 12.5 Hz), 3.59 (1H, dd, *J* = 6.0, 10.5 Hz), 3.74 (1H, dd, *J* = 4.0, 10.5 Hz), 3.92 (1H, ddd, *J* = 2.0, 4.0, 6.0 Hz), 4.39 (2H, brs), 4.42 (1H, ddd, *J* = 1.5, 2.0, 5.5 Hz), 5.03 (1H, dd, *J* = 5.0, 10.5 Hz), 6.48 (1H, d, *J* = 9.0 Hz), 7.49 (1H, dd, *J* = 2.0, 9.0 Hz), 8.03 (1H, d, *J* = 2.0 Hz); ^{13}C-NMR ($CDCl_3$, 101 MHz) δ: -5.5, -5.4, -4.7, -4.7, 18.0, 18.4, 25.8, 25.9, 43.7, 63.9, 74.5, 77.9, 87.9, 108.4, 127.3, 136.3, 146.4, 158.0; MS (EI) *m/z* 438 (M^+, 100); HRMS (EI) *m/z* Calcd for $C_{22}H_{42}N_2O_3Si_2$: 438.2734. Found 438.2732; Anal. Calcd for $C_{22}H_{42}N_2O_3Si_2$: C, 60.22; H, 9.65; N, 6.38. Found: C, 60.13; H, 9.82; N, 6.21.

5-[3',5'-Di-*O-tert*-butyldimethylsilyl-(2'-deoxy-D-ribofuranosyl)]-6-methyl-2-phenoxyacetylamino-pyridine (3a)

Under an N_2 atmosphere, PacCl (46 μL, 0.331 mmol) was added to a solution of **2a** (125 mg, 0.276 mmol) in anhydrous pyridine (4 mL) at 0 °C and the mixture was stirred at room temperature for 10 h.

After the addition of water at 0 °C, the reaction mixture was diluted with EtOAc, washed with water, saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated. The crude product was purified by flash column chromatography (15:1 Hexane-EtOAc) to give **3a** (159 mg, 98%); Colorless oil; $[\alpha]_D^{27}$ 14.9 (*c* 1.02, $CHCl_3$); IR ν_{max} (KBr) 2952, 2930, 1702, 1591, 1523, 1496, 1461, 1393, 1361, 1252, 1084, 1034 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.09 (6H, s), 0.10 (6H, s), 0.91 (9H, s), 0.92 (9H, s), 1.76 (1H, ddd, *J* = 6.0, 10.0, 12.5 Hz), 2.15 (1H, ddd, *J* = 2.0, 5.5, 12.5 Hz), 2.45 (3H, s), 3.68 (1H, dd, *J* = 5.5, 10.5 Hz), 3.79 (1H, dd, *J* = 3.5, 10.5 Hz), 3.95 (1H, ddd, *J* = 2.0, 3.5, 5.5 Hz), 4.43 (1H, ddd, *J* = 2.0, 2.0, 6.0 Hz), 4.61(2H, s), 5.28 (1H, dd, *J* = 5.5, 10.0 Hz), 7.01 (2H, d, *J* = 8.5 Hz), 7.05 (1H, t, *J* = 8.5 Hz), 7.34 (2H, t, *J* = 8.5 Hz), 7.89 (1H, d, *J* = 8.5 Hz), 8.06 (1H, d, *J* = 8.5 Hz), 8.81 (1H, brs); ^{13}C-NMR ($CDCl_3$, 101 MHz) δ: -5.5, -5.4, -4.7, -4.7, 18.0, 18.3, 21.5, 25.8, 25.9, 42.9, 63.5, 67.5, 74.1, 76.0, 87.8, 111.6, 114.9, 122.3, 129.8, 132.6, 135.9, 148.4, 153.3, 157.0, 166.7; MS (EI) *m/z* 586 (M^+, 40); HRMS (EI) *m/z* Calcd for $C_{31}H_{50}N_2O_5Si_2$: 586.3258. Found 586.3265; Anal. Calcd for $C_{31}H_{50}N_2O_5Si_2$: C, 63.44; H, 8.59; N, 4.77. Found: C, 63.04; H, 8.74; N, 4.58.

5-[3',5'-Di-*O*-*tert*-butyldimethylsilyl-(2'-deoxy-D-ribofuranosyl)]-2-phenoxyacetylaminopyridine (3b)

Under an N_2 atmosphere, PacCl (15 μL, 0.109 mmol) was added to a solution of **2b** (32 mg, 0.0729 mmol) in anhydrous pyridine (2 mL) at 0 °C and the mixture was stirred at room temperature for 10 h. After the addition of water at 0 °C, the reaction mixture was diluted with EtOAc, washed with water, saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated. The crude product was purified by flash column chromatography (8:1 Hexane-EtOAc) to give **3b** (41 mg, 98%); Colorless oil; $[\alpha]_D^{28}$ 9.52 (*c* 1.05, $CHCl_3$); IR ν_{max} (KBr) 2953, 2929, 2857, 1704, 1521, 1496, 1253, 1093, 1032 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ : 0.08 (6H, s), 0.10 (6H, s), 0.91 (9H, s), 0.92 (9H, s), 1.89 (1H, ddd, *J* = 5.5, 10.5, 12.5 Hz), 2.12 (1H, ddd, *J* = 1.5, 5.0 12.5 Hz), 3.63 (1H, dd, *J* = 5.5, 11.0 Hz), 3.76 (1H, dd, *J* = 4.0, 11.0 Hz), 3.97 (1H, ddd, *J* = 2.0, 4.0, 5.5 Hz), 4.44 (1H, ddd, *J* = 1.5, 2.0, 5.5 Hz), 4.63(2H, s), 5.14 (1H, dd, *J* = 5.0, 10.5 Hz), 7.01 (2H, d, *J* = 7.5 Hz), 7.05 (1H, t, *J* = 7.5 Hz), 7.34 (2H, t, *J* = 7.5 Hz), 7.77 (1H, dd, *J* = 2.0, 9.0 Hz), 8.24 (1H, d, *J* = 9.0 Hz), 8.29 (1H, d, *J* = 2.0 Hz), 8.93 (1H, brs); ^{13}C-NMR ($CDCl_3$, 101 MHz) δ : -5.5, -5.3, -4.7, -4.6, 18.0, 18.4, 25.8, 25.9, 44.2, 63.8, 67.4, 74.4, 77.6, 88.3, 113.8, 114.9, 122.4, 129.9, 134.4, 136.5, 146.0, 149.8, 156.9, 166.8; MS (FAB) *m/z* 573 $[M+H]^+$; HRMS (FAB) *m/z* Calcd for $C_{30}H_{49}N_2O_5Si_2$ $[M+H]^+$: 573.3175. Found 573.3184; Anal. Calcd for $C_{30}H_{48}N_2O_5Si_2$: C, 62.90; H, 8.45; N, 4.89. Found: C, 62.52; H, 8.42; N, 4.66.

5-(2'-Deoxy-D-ribofuranosyl)-6-methyl-2-phenoxyacetylaminopyridine (4a)

Under an N_2 atmosphere, 1 M tetra-*n*-butylammonium fluoride in THF (1.23 mL, 1.23 mmol) was added to a solution of **3a** (235 mg, 0.410 mmol) in anhydrous THF (5 mL) at 0 ºC and the mixture was stirred at

room temperature for 12 h. The reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (1:10 Hexane-EtOAc) to give **4a** (131 mg, 89%); Colorless crystals; mp 149-152 °C; $[\alpha]_D^{28}$ 48.5 (*c* 1.00, CH_3OH); IR ν_{max} (KBr): 3381, 2935, 1694, 1595, 1532, 1495, 1461, 1394, 1295, 1241, 1083, 1049 cm^{-1}; ^{1}H-NMR (CD_3OD, 400 MHz) δ: 1.84 (1H, ddd *J* = 6.5, 10.5, 13.0 Hz), 2.27 (1H, ddd, *J* = 1.5, 5.0, 13.0 Hz), 2.46 (3H, s), 3.69 (2H, d, *J* = 5.0 Hz), 3.94 (1H, dt, *J* = 2.5, 5.0 Hz), 4.33 (1H, ddd, *J* = 1.5, 2.5, 6.5 Hz), 4.68(2H, s), 5.29 (1H, dd, *J* = 5.0, 10.5 Hz), 7.01 (1H, t, *J* = 7.5 Hz), 7.05 (2H, d, *J* = 9.0 Hz), 7.33 (2H, dd, *J* = 7.5, 9.0 Hz), 7.98 (1H, d, *J* = 8.5 Hz), 8.00 (1H, d, *J* = 8.5 Hz); ^{13}C-NMR (CD_3OD, 101 MHz) δ: 21.6, 43.3, 63.8, 68.3, 74.3, 77.3, 89.0, 112.9, 115.9, 123.0, 130.7, 133.7, 137.4, 150.2, 154.9, 158.9, 169.3; MS (EI) *m/z* 358 (M^+, 100); HRMS (EI) *m/z* Calcd for $C_{19}H_{22}N_2O_5$: 358.1529. Found 358.1524; Anal. Calcd for $C_{19}H_{22}N_2O_5$: C, 63.67 H, 6.19; N, 7.82. Found: C, 63.26; H, 6.13; N, 7.79.

5-(2'-Deoxy-D-ribofuranosyl)-2-phenoxyacetylaminopyridine (4b)

Under an N_2 atmosphere, 1 M tetra-*n*-butylammonium fluoride in THF (0.630 mL, 0.630 mmol) was added to a solution of **3b** (145 mg, 0.253 mmol) in anhydrous THF (2 mL) at 0 °C and the mixture was stirred at room temperature for 8 h. The reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (1:10 Hexane-EtOAc) to give **4b** (73 mg, 84%); Colorless crystals; mp 121-124 °C; $[\alpha]_D^{25}$ 32.3 (*c* 1.04, CH_3OH); IR ν_{max} (KBr) 3374, 1702, 1600, 1523, 1495, 1411, 1343, 1306, 1242, 1087,1052 cm^{-1}; ^{1}H-NMR (CD_3OD, 400 MHz) δ: 1.96 (1H, ddd, *J* = 6.0, 10.5, 13.0 Hz), 2.22 (1H, ddd, *J* = 1.5, 5.5, 13.0 Hz), 3.67 (1H, d, *J* = 5.0 Hz), 3.94 (1H, m), 4.34 (1H, m), 4.69 (2H, s), 5.13 (1H, dd, *J* = 5.5, 10.5 Hz), 7.00 (1H, t, *J* = 8.0 Hz), 7.04 (2H, d, *J* = 8.0 Hz), 7.31 (2H, t, *J* = 8.0 Hz), 7.86 (1H, dd, *J* = 1.5, 9.0 Hz), 8.14 (1H, d, *J* = 9.0 Hz), 8.32 (1H, d, *J* = 1.5 Hz); ^{13}C-NMR (CD_3OD, 101 MHz) δ: 44.7, 63.9, 68.3, 74.4, 79.0, 89.4, 115.2, 115.9, 123.0, 130.7, 135.6, 137.9, 147.1, 151.6, 159.0, 169.4; MS (EI) *m/z* 344 (M^+, 100); HRMS (EI) *m/z* Calcd for $C_{18}H_{20}N_2O_5$: 344.1372. Found 344.1386; Anal. Calcd for $C_{18}H_{20}N_2O_5$: C, 62.78; H, 5.85; N, 8.13. Found: C, 62.49; H, 5.81; N, 8.04.

5-(5'-*O*-Dimethoxytrityl-2'-deoxy-D-ribofuranosyl)-6-methyl-2-phenoxyacetylaminopyridine (5a)

Under an N_2 atmosphere, DMTrCl (33 mg, 0.0971 mmol) was added to a solution of **4a** (29 mg, 0.0809 mmol) in anhydrous pyridine (1.5 mL) at 0 °C and the mixture was stirred at room temperature for 6 h. After the addition of water at 0 °C, the reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (100:1 CH_2Cl_2-CH_3OH) to give **5a** (50 mg, 94%); White powder; mp 62-63 °C; $[\alpha]_D^{28}$ 12.9 (*c* 1.02, $CHCl_3$); IR ν_{max} (KBr): 1699, 1600, 1516, 1461, 1394, 1297, 1245, 1178, 1081, 1035 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.89 (1H, ddd *J* = 6.5, 10.0, 13.0 Hz), 2.27 (1H, ddd, *J* = 2.5, 5.5, 13.0 Hz), 2.43 (3H, s), 3.28 (1H, dd, *J* = 5.5, 10.0 Hz), 3.40 (1H, dd, *J* = 5.0,

10.0 Hz), 3.80 (6H, s), 3.93 (1H, ddd, *J* = 3.0, 5.0, 5.5 Hz), 4.42 (1H, ddd, *J* = 2.5, 3.0, 6.5 Hz), 4.69 (2H, s), 5.29 (1H, dd, *J* = 5.5, 10.0 Hz), 6.80-6.85 (4H, m), 7.22-7.46 (14H, m), 7.79 (1H, d, *J* = 9.0 Hz), 7.86 (1H, brs), 7.96 (1H, d, *J* = 9.0 Hz); ^{13}C-NMR ($CDCl_3$, 101 MHz) δ: 21.1, 43.6, 55.2, 64.3, 67.4, 74.6, 77.6, 86.3, 86.5, 113.2, 113.8, 114.9, 122.4, 126.9, 127.8, 128.1, 129.8, 130.1, 133.5, 135.9, 136.4, 144.7, 146.0, 149.9, 156.9, 158.4, 166.8; MS (FAB) *m/z* 661 $[M+H]^+$; HRMS (FAB) *m/z* Calcd for $C_{40}H_{41}N_2O_7$ $[M+H]^+$: 661.2908. Found 661.2917.

5-(5'-*O*-Dimethoxytrityl-2'-deoxy-D-ribofuranosyl)-2-phenoxyacetylaminopyridine (5b)

Under an N_2 atmosphere, DMTrCl (64 mg, 0.290 mmol) was added to a solution of **4b** (50 mg, 0.145 mmol) in anhydrous pyridine (2 mL) at 0 °C and the mixture was stirred at room temperature for 10 h. After the addition of water at 0 ºC, the reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (100:1 CH_2Cl_2-CH_3OH) to give **5b** (93 mg, 98%); White powder; mp 60-61 °C; $[\alpha]_D^{24}$ 10.0 (*c* 1.01, $CHCl_3$); IR ν_{max} (KBr): 1606, 1510, 1496, 1303, 1250, 1176, 1083, 1035 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 2.04 (1H, ddd *J* = 6.5, 10.0, 13.0 Hz), 2.26 (1H, ddd, *J* = 2.5, 6.0, 13.0 Hz), 3.26 (1H, dd, *J* = 6.0, 10.0 Hz), 3.36 (1H, dd, *J* = 4.5, 10.0 Hz), 3.79 (6H, s), 4.07 (1H, ddd, *J* = 3.0, 4.5, 6.0 Hz), 4.46 (1H, ddd, *J* = 2.5, 3.0, 6.5 Hz), 4.69 (2H, s), 5.18 (1H, dd, *J* = 6.0, 10.0 Hz), 6.81-6.85 (4H, m), 6.99 (2H, d, *J* = 7.5 Hz), 7.05 (1H, t, *J* = 7.5 Hz), 7.20-7.37 (9H, m), 7.44 (2H, d, *J* = 7.5 Hz), 7.75 (1H, dd, *J* = 2.0, 8.5 Hz), 8.24 (1H, d, *J* = 8.5 Hz), 8.30 (1H, d, *J* = 2.0 Hz), 8.93 (1H, brs); ^{13}C-NMR ($CDCl_3$, 101 MHz) δ: 43.6, 55.1, 64.3, 67.3, 74.4, 77.5, 86.2, 86.5, 113.1, 113.8, 114.7, 122.3, 126.8, 127.8, 128.1, 129.8, 130.0, 133.9, 135.8, 136.4, 144.7, 145.8, 149.8, 156.9, 158.4, 166.8; MS (FAB) *m/z* 647 $[M+H]^+$; HRMS (FAB) *m/z* Calcd for $C_{39}H_{39}N_2O_7$ $[M+H]^+$: 647.2752. Found 647.2761; Anal. Calcd for $C_{39}H_{38}N_2O_7$: C, 72.43 H, 5.92; N, 4.33. Found: C, 72.23; H, 6.22; N, 4.13.

5-{5'-*O*-Dimethoxytrityl-3'-*O*-2-cyanoethoxy(diisopropylamino)phosphino-2'-deoxy-D-ribofuranosyl}-6-methyl-2-phenoxyacetylaminopyridine (6a)

Under an N_2 atmosphere, DIPEA (53 μL, 0.303 mmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (20 μL, 0.0908 mmol) were added to a solution of **5a** (40 mg, 0.0605 mmol) in anhydrous CH_2Cl_2 (1.5 mL) at 0 °C and the mixture was stirred at room temperature for 12h. After the addition of saturated aqueous $NaHCO_3$ at 0 °C, the reaction mixture was stirred at room temperature for 1h and diluted with EtOAc, washed with saturated aqueous $NaHCO_3$, water and brine, dried over Na_2SO_4, and concentrated. The crude product was purified by flash column chromatography (4:1 Hexane-EtOAc) to give **6a** (43 mg, 83%); White amorphous; mp 41-43 °C; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.08-1.29 (12H, m), 1.86-1.89 (1H, m), 2.25-2.40 (1H, m), 2.44 (3H, s), 2.41-2.63 (2H, m), 3.15-3.90 (12H, m), 4.18-4.22 (1H, m), 4.42-4.48 (1H, m), 4.60-4.61 (2H, m), 5.25-5.31 (1H, m), 6.78-6.85 (4H, m), 6.98-7.08 (3H, m), 7.17-7.38 (11H, m), 7.85-7.92 (1H, m), 8.03-8.08 (1H, m), 8.84

(1H, brs); ^{31}P-NMR ($CDCl_3$, 162 MHz) δ: 148.6, 149.0; MS (FAB) *m/z* 861 $[M+H]^+$; HRMS (FAB) *m/z* Calcd for $C_{49}H_{58}N_4O_8P$ $[M+H]^+$: 861.3987. Found 861.3967.

5-{5'-*O*-Dimethoxytrityl-3'-*O*-2-cyanoethoxy(diisopropylamino)phosphino-2'-deoxy-D-ribofuranosyl}-2-phenoxyacetylaminopyridine (6b)

Under an N_2 atmosphere, DIPEA (36 μL, 0.209 mmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (14 μL, 0.0626 mmol) were added to a solution of **5b** (27 mg, 0.0417 mmol) in anhydrous CH_2Cl_2 (2 mL) at 0 °C and the mixture was stirred at room temperature for 3.5 h. After the addition of saturated aqueous $NaHCO_3$ at 0 °C, the reaction mixture was stirred at room temperature for 1 h and diluted with EtOAc, washed with saturated aqueous $NaHCO_3$, water and brine, dried over Na_2SO_4, and concentrated. The crude product was purified by flash column chromatography (4:1 Hexane-EtOAc) to give **6b** (30 mg, 86%); White amorphous; mp 40-42 °C; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.09-1.20 (12H, m), 1.98-2.07 (1H, m), 2.31-2.48 (2H, m), 2.62 (1H, t, *J* = 6.5 Hz), 3.23-3.34 (2H, m), 3.54-3.87 (10H, m), 4.20-4.24 (1H, m), 4.51-4.57 (1H, m), 4.63 (2H, s), 5.14-5.18 (1H, m), 6.80-6.83 (4H, m), 6.99-7.07 (3H, m), 7.20-7.46 (11H, m), 7.76-7.79 (1H, m), 8.23-8.25 (1H, m), 8.32-8.34 (1H, m), 8.93 (1H, brs); ^{31}P-NMR ($CDCl_3$, 162 MHz) δ: 148.0, 148.1; MS (FAB) *m/z* 847 $[M+H]^+$; HRMS (FAB) *m/z* Calcd for $C_{48}H_{56}N_4O_8P$ $[M+H]^+$: 847.3830. Found 847.3836.

Synthesis of oligonucleotides

TFO-1–**TFO-8** were synthesized on an automated DNA synthesizer (Applied Biosystems Expedite™ 8909) using a standard phosphoramidite protocol (DMTr-ON mode).[10] Syntheses were performed on 0.2 or 1.0 μmol scale. TFOs were cleaved from the CPG supports and deprotected by treatment with ca. 1 mL 28% ammonium aqueous solution (room temperature, 2 h to 55 °C, 24-48 h). After removal of ammonia *in vacuo*, the obtained crude TFOs were purified with Sep-Pak® Plus C18 cartridges (Waters) followed by reversed-phase HPLC (Waters XBridge® MS C_{18} 2.5 μm, 10 x 50 mm). The composition of the TFOs was confirmed by MALDI-TOF mass analysis. MALDI-TOF mass data ($[M-H]^-$) for **TFO-1**–**TFO-8**; **TFO-1**, found 4478.62 (calcd 4478.01); **TFO-2**, found 4463.73 (calcd 4463.99); **TFO-3**, found 4479.54 (calcd 4480.98); **TFO-4**, found 4494.27 (calcd 4495.00); **TFO-5**, found 4442.21 (calcd 4443.05); **TFO-6**, found 4400.50 (calcd 4400.97); **TFO-7**, found 4451.99 (calcd 4451.94); **TFO-8**, found 4494.35 (calcd 4494.02).

UV-Melting experiments

UV-Melting experiments were carried out on SHIMADZU UV-1650 and SHIMADZU UV-1800 spectrometers equipped with T_m analysis accessory. Equimolecular amounts of the target hairpin-loop dsDNA and TFOs were dissolved in 10 mM sodium cacodylate buffer (pH 6.8) containing 100 mM KCl and 50 mM $MgCl_2$ to give a final each strand concentration of 1.89 μM. The samples were annealed by

heating at 100 °C followed by slow cooling to 5 °C. The melting profiles were recorded at 260 nm from 5 ºC to 90 °C at a scan rate of 0.5 °C/min.

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