Supporting Information

Quencher Free, End Stacking Oligonucleotides for Probing Single-Base Mismatches in DNA

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Experimental Procedures for the Synthesis of Fluorene and pyrene-Labeled Deoxyadenosine Derivatives.

Synthesis of Fluorene -Labeled Deoxyadenosine Derivatives.

Reagents and conditions: (i) 2-Ethynylfluorene, (PPh₃)₂PdCl₂, CuI, Et₃N/THF (1:3), 45–50 °C, 2 h, 75%; (ii) 2-cyanoethyl diisopropylchlorophosphoramidite, 4-methylmorpholine, CH₂Cl₂, rt, 30 min, 95%.

N₆-Benzoyl-5'-O-[bis(4-methoxyphenyl)phenylmethyl]-8-(2-ethynylfluorenyl)-2'-deoxyadenosine(**2**).

(PPh₃)₂PdCl₂ (20 mg, 0.028 mmol) and CuI (6 mg, 0.028 mmol) were added to a solution of **1** (203 mg, 0.276 mmol) and 2-ethynylfluorene (58 mg, 0.030 mmol) in Et₃N (2 mL) and THF (6 mL). Argon was bubbled through the mixture for 2 min before the mixture was subjected ten times to a pump/purge cycle and then stirred at 45–50 °C for 2 h. After evaporation of the solvent in vacuo, the residue was subjected to chromatography (SiO₂; hexane/EtOAc, 1:2) to yield **2** (174 mg, 75%). M.p. 145–147 °C. IR (film): ν 3426, 3064, 2932, 2835, 1714, 1610, 1537, 1508, 1454, 1389, 1353, 1301, 1250, 1177, 1095, 1032, 912, 829, 790, 767, 542 cm⁻¹. ¹H NMR (300 MHz, CDCl₃): δ 9.06 (s, 1H; NH), 8.60 (s, 1H; H-2), 8.02–7.15 (m, 21H; fluorene-H, DMT-H, Bz-H), 6.75–6.70 (m, 5H; DMT-H, H-1'), 4.99 (br s, 1H; H-3'), 4.19–4.18 (br s, 1H; H-4'), 3.90 (s, 2H; ArCH₂), 3.72 and 3.70 (2s, 6H; OCH₃), 3.53–3.42 (m, 3H; H-5', H-2'), 2.50 (br s, 2H; H-2', OH). ¹³C NMR (75 MHz, CDCl₃): δ 164.6, 158.5, 145.6, 133.1, 132.2, 130.2, 129.7, 128.7, 128.5, 128.3, 127.9, 127.0, 113.2, 86.4, 62.9, 55.4, 46.1, 36.6. HRMS–FAB (*m/z*): [M + Na]⁺ calcd for C₅₃H₄₅N₅O₆Na, 868.3111; found, 868.3116.

N₆-Benzoyl-5'-O-[bis(4-methoxyphenyl)phenylmethyl]-8-(2-ethynylfluorenyl)-3'-bis[1-phosphoramidyl]cyanoethyl-2'-deoxyadenosine(**3**).

2-Cyanoethyl diisopropyl chlorophosphoramidite (140 μ L, 0.617 mmol) was added dropwise to a solution of **2** (174 mg, 0.206 mmol) and 4-formylmorpholine (70 μ L, 0.617 mmol) in CH₂Cl₂ (6 mL) at room temperature. After the reaction reached completion (30 min), the mixture was concentrated in vacuo and purified by chromatography through a short column of SiO₂ (hexane/EtOAc, 1:1) to yield **3** (205 mg, 96%). M.p. 84–85 °C. [α]_D²⁵ = –21° (*c* = 0.90, CHCl₃). IR (film): ν 2963, 2926, 1699, 1607, 1508, 1489, 1457, 1250, 1178, 1033, 6671 cm⁻¹. ¹H NMR (300

MHz, CDCl₃): δ 9.10 (s, 1H; NH), 8.62 and 8.61 (2s, 1H; H-2), 8.05–7.07 (m, 21H; fluorene-H, DMT-H, Bz-H), 6.76–6.72 (m, 5H; DMT-H, H-1'), 5.00 (br s, 1H; H-3'), 4.16–4.05 (br, 1H; H-4'), 3.95 (s, 2H; ArCH₂), 3.89–3.42 (m, 13H; OCH₃, NCH, OCH₂, H-5', H-2'), 2.67–2.48 (m, 3H; CH₂CN, H-2'), 1.31–1.14 (m, 12H; NCHCH₃). ¹³C NMR (75 MHz, CDCl₃): δ 164.4, 158.5, 153.1, 149.2, 145.0, 144.0, 143.6, 140.7, 138.0, 136.2, 131.7, 129.1, 128.4, 128.0, 127.9, 127.4, 125.4, 120.8, 120.3, 113.1, 86.3, 85.7, 63.8, 60.6, 58.2, 55.4, 47.7, 43.6, 40.0, 29.9, 24.8, 22.9, 20.2. ³¹P NMR (121 MHz, CDCl₃): δ 149.9, 149.5. HRMS–FAB (*m/z*): [M + Na]⁺ calcd for C₆₂H₆₀N₇O₇PNa, 1068.4190; found, 1068.4192.

Synthesis of pyrene-labeled deoxyadenosine derivatives.

8-(1-Ethynylpyrenyl)-2'-deoxyadenosine (2'). (PPh₃)₄Pd (63 mg, 0.09 mmol) and CuI (34 mg, 0.18 mmol) were added to a solution of **1'** (300 mg, 0.90 mmol) in DMF (5 mL) under nitrogen and then 1-ethynylpyrene (260 mg, 11.8 mmol) and *N,N*-diisopropylethylamine (235 μ L, 13.6 mmol) were added to the solution. After degassing, the reaction mixture was stirred at 45–50 °C for 8 h and monitored by TLC. Water was added to this solution and then the product was filtered off and purified by column chromatography (SiO₂; EtOAc/MeOH, gradient elution). After evaporating the solvents, the product was dried in vacuo (190 mg, 88%). M.p. 217–218 °C. [α]_D²³ = +184° (*c* = 1.12, DMF). IR (KBr): ν 3346, 3188, 2926, 2202, 1599, 1554, 1512, 1332, 1097, 841 cm⁻¹. ¹H NMR (300 MHz, DMSO-*d*₆): δ 8.49 (s, 1H; H-2), 8.41–8.11 (m, 9H; ArH), 7.69 (br s, 2H; NH₂), 6.75 (t, *J* = 6.9 Hz, 1H; H-1'), 5.41 (br s, 1H; OH), 5.36 (br s, 1H; OH), 4.55–4.49 (m, 1H; H-3'), 3.98–3.92 (m, 1H; H-4'), 3.71–3.63 (m, 1H; H-5'), 3.59–3.51 (m, 1H; H-5'), 2.39–2.33 (m, 2H; H-2') ppm. ¹³C NMR (75 MHz, DMSO-*d*₆): δ 156.2, 153.5, 148.7, 133.3, 132.0, 131.8, 130.7, 130.3, 130.0, 129.7, 129.3, 127.2, 127.0, 126.6, 126.5, 125.0, 124.1, 123.5, 123.2, 119.9, 113.9, 93.7, 88.4, 85.5, 84.2, 71.3, 62.2, 37.7 ppm. HRMS–FAB (*m/z*): [M + H]⁺ calcd for C₂₈H₂₂N₅O₃, 476.1723; found, 476.1724.

***N*₆-Benzoyl-5'-*O*-[bis(4-methoxyphenyl)phenylmethyl]-8-(1-ethynylpyrenyl)-2'-deoxyadenosine (4').** (PPh₃)₄Pd (18 mg, 0.026 mmol) and CuI (10 mg, 0.053 mmol) were added to a solution of **3'** (190 mg, 0.26 mmol) in DMF (3 mL) under Ar and then the mixture was stirred for 5 min at room temperature. 1-Ethynylpyrene (60 mg, 0.26 mmol) and *N,N*-diisopropylethylamine (68 μL, 0.39 mmol) were added to this solution. After degassing, the reaction mixture was stirred at 45–50 °C for 5 h and monitored by TLC. Water was added to the solution and the product was extracted with excess EtOAc. The organic phase was washed twice with water. After evaporation of solvent in vacuo, the residue was subjected to chromatography (SiO₂; hexane/EtOAc, 1:1) to yield **4'** (170 mg, 77%). M.p. 140–141 °C. $[\alpha]_D^{23} = +33^\circ$ (*c* = 0.525, CH₂Cl₂). IR (film): ν 3366, 2923, 2203, 1684, 1582, 1508, 1464, 1339, 1174, 846, 702 cm⁻¹. ¹H NMR (300 MHz, CDCl₃): δ 9.15 (s, 1H; NH), 8.64 (s, 1H; H-2), 8.25–7.15 (m, 23H; ArH), 6.89 (t, *J* = 6.9 Hz, 1H; H-1'), 6.68–6.73 (m, 4H; ArH), 5.02–5.08 (m, 1H; H-3'), 4.22 (dd, *J* = 10.1, 6.0 Hz, 1H; H-4'), 3.70 (s, 6H; OCH₃), 3.71–3.65 (m, 1H; H-2'), 3.47–3.44 (m, 2H; H-5'), 2.45–2.34 (m, 1H; H-2'), 2.34 (br s, 1H; OH) ppm. ¹³C NMR (75 MHz, CDCl₃): δ 163.9, 158.0, 157.9, 152.5, 148.7, 144.2, 137.4, 135.5, 135.3, 133.3, 132.5, 132.3, 130.5, 130.3, 129.6, 129.5, 129.4, 129.0, 128.4, 127.7, 127.4, 127.3, 127.1, 126.7, 126.6, 126.3, 126.2, 125.9, 124.4, 124.0, 123.7, 123.4, 113.5, 112.6, 96.8, 85.9, 85.0, 82.5, 72.7, 63.6, 54.7, 36.9 ppm. HRMS–FAB (*m/z*): [M + H]⁺ calcd for C₅₆H₄₄N₅O₆, 882.3294; found, 882.3288.

***N*₆-Benzoyl-5'-*O*-[bis(4-methoxyphenyl)phenylmethyl]-8-(1-ethynylpyrenyl)-3'-{bis[1-(methylethyl)phosphoramidyl]cyanoethyl}-2'-deoxyadenosine (5').**

4-Methylmorpholine (55 μL, 0.501 mmol) was added to a solution of **4'** (150 mg, 0.16 mmol) in CH₂Cl₂ (2 mL) under nitrogen and then the mixture was stirred at room temperature for 5 min. 2-Cyanoethyldiisopropylaminochlorophosphoramidite (48 μL, 0.217 mmol) was added and the mixture was stirred for 1 h and monitored by TLC. The solvent was evaporated in vacuo and the residue was purified by chromatography on a short column (SiO₂; hexane/EtOAc/Et₃N, 97:97:6) to yield **5'** (160 mg, 87%). M.p. 110–112 °C. $[\alpha]_D^{23} = +81^\circ$ (*c* = 1.06, CHCl₃). IR (film): ν 3447, 2968, 2932, 1702, 1608, 1580, 1508, 1463, 1250, 1178, 1117, 1087, 979, 849, 829, 706 cm⁻¹. ¹H NMR (300 MHz, CDCl₃): δ 9.10 (s, 1H; NH), 8.65–8.05 (m, 9H; Ar_{py}H), 7.56–7.14 (m, 14H; ArH), 6.68 (dd, *J* = 11.9, 6.3 Hz, 1H; H-1'), 6.71–6.65 (m, 4H; ArH), 5.27–4.98 (m, 1H; H-3'), 4.43–4.40 (m, 1H; H-4'), 3.74–3.41 (m, 13H; OCH₃, H-2', NCH, OCH₂, H-5'), 2.67–2.54 (m, 1H; H-2'), 2.52–2.36 (m, 2H; CH₂CN), 1.15–1.06 (m, 12H; NCHCH₃) ppm. ¹³C NMR (75 MHz, CDCl₃): δ 163.9, 157.9, 152.5, 150.4, 148.7, 144.3, 137.7, 135.5, 135.4, 133.4, 132.6, 132.4, 132.3, 130.7, 130.4, 129.9, 129.6, 129.5, 129.0, 128.4, 127.7, 127.4, 127.2, 126.7, 126.2, 125.9, 124.5, 124.1, 123.9, 123.5, 122.8, 117.0, 113.6, 112.5, 96.8, 85.7, 85.4, 85.3, 82.7, 73.5, 63.4, 63.2, 59.9, 58.2, 57.9, 54.7, 42.9, 24.0, 19.7 ppm. ³¹P NMR (121 MHz, CDCl₃): δ 151.6, 151.3 ppm. MS (FAB) *m/z*: 1082.3 [M + H]⁺.

Figure S1. Absorption and emission spectra of ODN **S4** (NB A). Spectra were recorded using an excitation wavelength of 420 nm at 20 °C in a buffer of 1 mM MgCl_2 and 100 mM Tris-HCl (pH 8).

Figure S2. Energy-minimized (MM+) structures of the hairpin states of ODNs **S1** and **S2**. (a, c) Side views; (b, d) views from below. The violet regions indicate the units involved in the π -stacking of the pyrene unit at the terminus of the stem. Energy minimization was followed by molecular dynamics for 50 ps at a simulation temperature of 300 K and then further energy minimization using Hyperchem (v. 7.0) software and the MM+ force field. In the energy-minimized conformations of ODNs **S1** and **S2**, we observe that the pyrene moiety of ODN **S1** is located at the 5' end (anti form) and stacks with the NB in the hairpin; the distance between their planes is 3.40 Å (i.e., the pyrene units form stacks). In contrast, the pyrene-stacking position of ODN **S2** is different (syn form): it is located at the 3' end. The higher value of T_m (56 °C) of ODN **S2** probably arises from this structure.

Table S1. Oligonucleotide Sequences.

Entry		Sequence								
1	5'-d-	A ^{FL} GC	GAG	AAG	TTA	GAA	CCT	ATG	CTC	GC
2	5'-d-	U ^{FL} GC	GAG	AAG	TTA	GAA	CCT	ATG	CTC	GC
3	5'-d-	GC A ^{PY}	GAG	AAG	TTA	GAA	CCT	ATG	CTC	TGC
4	5'-d-	GC U ^{PY}	GAG	AAG	TTA	GAA	CCT	ATG	CTC	AGC

Table S2. MALDI-TOF Mass Spectral Data and Melting Temperatures.

Sequence	MALDI-TOF signal [M ⁺]		<i>T_m</i> (°C)
	Calcd. <i>m/z</i>	Found <i>m/z</i>	
S1	8229	8228	53
S2	8206	8201	56
S3	7691	7693	47
S4	8228	8234	47
S5	8228	8227	46
S6	8229	8231	55
S7	8213	8211	47
S8	8204	8207	46
S9	8189	8193	52
S10	8253	8251	45
S11	8244	8240	46
S12	8269	8267	46
S13	4542	4549	N.D.
S14	4551	4552	N.D.
S15	4567	4567	N.D.
S16	4527	4526	N.D.
1	8195	8198	43
2	8171	8163	60
3	8533	8534	46
4	8510	8514	55

“None” refers to natural oligonucleotides. N.D.: Not determined.

Details of discrimination factor and Figure 3 experiment.

Details of discrimination factor

The relative fluorescence emission intensities between the hairpin state and fully matched duplex state were measured by using MD-5020 PTI model microscope photometer. We integrated each emission intensity by using 2003 MS excel program and then we divided the duplex emission integration value by hairpin emission integration value. The resulting value is the discrimination factor.

Details of Figure 3 experiment

Steady-state fluorescence (FL) spectra were recorded on a MD-5020 PTI model microscope photometer using a bandwidth of 15 nm and 0.5×2 cm quartz cuvettes with a light pass of 1 cm. The cell holder was thermostated with circulating water controlled by a PolyScience digital temperature controller 9110. An excitation wavelength of 420 nm was used, and all excitation and emission slits were set to 1 nm. All hybridizations and fluorescence experiments were carried out in 1 mM MgCl_2 , 100 mM Tris-HCl buffer (pH 7.2) containing a one-to-one ratio of complementary ODNs at 1.5 μM concentration. The concentration of all oligonucleotides was determined by using UV measurement.