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Synthesis and Properties of Oligonucleotides Containing Novel Fluorescent Biaryl Units

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We describe the synthesis and properties of oligonucleotides (ONs) containing biaryl units, which are composed of a bis(hydroxymethyl)benzene residue and a naphthalene or pyrene moiety. We found that by introducing the biaryl units into the ONs, the aromatic chromophores were suitably arrayed in the DNAs. Further, we succeeded in the detection of a single-base mismatch in RNA by using the ON containing the biaryl unit as a molecular beacon.

Introduction

One major goal of research in biotechnology and nanotechnology is to develop assemblies of novel biomaterials that can be used in analytical, industrial, and therapeutic applications. To date, a wide variety of oligonucleotide analogs (ONAs), which contain fluorescent chromophores, have been synthesized, and their properties have been extensively investigated to evaluate their use in the above-mentioned applications.[1–36]

Recently, we reported the synthesis and properties of an oligonucleotide (ON) that consists of a benzene–phosphate backbone.[37] The building blocks of this ON are composed of bis(hydroxymethyl)benzene residues that are connected to nucleobases through a biaryl-like axis (Figure 1). The thermal denaturation of the duplexes composed of such ONs revealed that the ONA having the benzene–phosphate backbone forms a thermally and thermodynamically stable duplex in itself.[37] Furthermore, we have designed and synthesized a molecular beacon (MB) that has the benzene–phosphate backbone at the stem moiety; this MB successfully detected a target RNA with excellent efficiency.[38] The results of this study prompted us to examine the synthesis and properties of ONs that contain biaryl units composed of aromatic chromophores (Figure 1). We speculated that since the structures of biaryl units **3** and **4** are such that interplanar angles are formed between the aromatic chromophores and the benzene moieties, the chromophores can efficiently stack on top of each other in DNA duplexes; as a result of this stacking, the duplexes will be thermally stabilized. The stacked aromatic chromophores would form exciplexes within the duplexes, and hence, the duplexes will be functionalized.

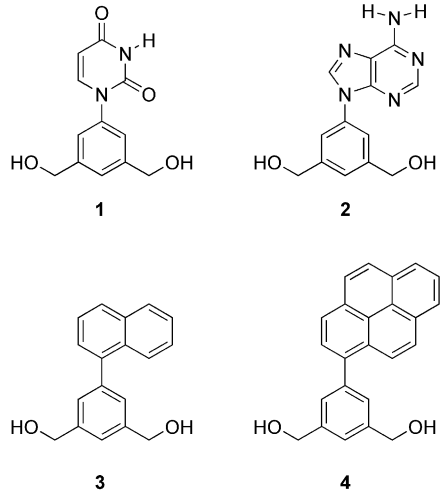

Figure 1. Chemical structures of monomers.

In this paper, we report the synthesis and properties of ONs containing biaryl units that are composed of aromatic chromophores. We also investigated RNA detection by using a MB containing the same biaryl unit at the stem moiety.

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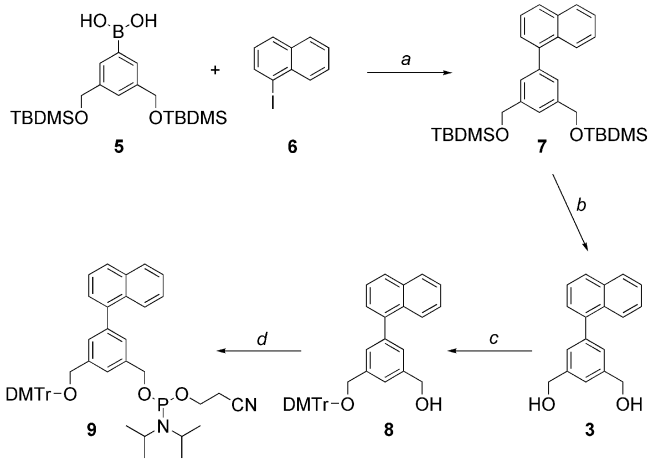
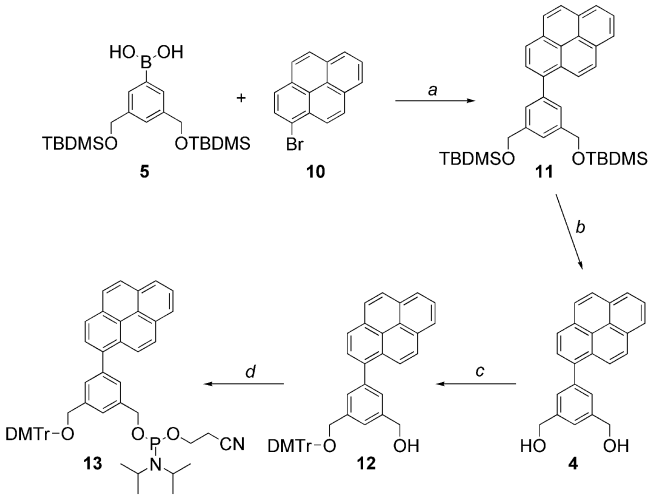
Results and Discussion

Syntheses of Amidite Units

The syntheses of the phosphoramidite units of biaryl units **3** and **4** are shown in Schemes 1 and 2, respectively. Arylboronic acid derivative **5**, which was synthesized by a previously reported method,[38] was coupled with 1-iodonaphthalene (**6**) in the presence of $PdCl_2$(dppf) [dppf = 1,1′-bis(diphenylphosphanyl)ferrocene] at 65 °C; this coupling reaction afforded biaryl derivative **7**; subsequently, **7** was desilylated upon treatment with tetra-*n*-butylammonium fluoride (TBAF) to give biaryl unit **3** in a yield of 84%. One out of the two hydroxy groups of **3** was protected by a 4,4′-dimethoxytrityl (DMTr) group to give mono-DMTr derivative **8** in 37% yield. Derivative **8** was phosphitylated by the standard procedure to afford the corresponding phosphoramidite **9** in 88% yield. In a similar manner, phosphoramidite **13** was synthesized from 1-bromopyrene (**10**); the total yield of **13** was 29%.



Scheme 1. Reagents and conditions: (a) $PdCl_2$(dppf)·CH_2Cl_2, NaOH, THF/H_2O (5:1), 65 °C, 24 h; (b) TBAF, THF, room temp., 1 h, 84% (2 steps); (c) DMTrCl, pyridine, room temp., 4 h, 37%; (d) chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphane, *i*Pr_2NEt, THF, room temp., 1 h, 88%.



Scheme 2. Reagents and conditions: (a) $PdCl_2$(dppf)·CH_2Cl_2, NaOH, THF/H_2O (5:1), 65 °C, 48 h; (b) TBAF, THF/CH_2Cl_2 (1:1), room temp., 1 h, 54% (2 steps); (c) DMTrCl, pyridine, room temp., 4 h, 58%; (d) chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphane, *i*Pr_2NEt, THF, room temp., 1 h, 93%.

Before introducing biaryl units **3** and **4** into the ONs, their fluorescence properties were studied. Biaryl unit **4** showed emission maxima at 380 and 400 nm under 341-nm excitation in methanol; biaryl unit **3** exhibited an emission band at 348 nm under 287-nm excitation in methanol. The quantum yield (Φ_{em}) of biaryl unit **4** in methanol was found to be 0.07.

Oligonucleotide Syntheses

The various sequences of ONs used in this study are summarized in Table 1. All ONs were synthesized with a

Table 1. Sequences of oligonucleotides used in this study.[a]

Duplex	ON	Sequence
Duplex**1**	ON **1**	5′-AGCTCGGTCATCGAGAGTGCA-3′
	ON **2**	3′-TCGAGCCAGTAGCTCTCACGT-5′
Duplex**2**	ON **3**	5′-AGCTCGGTCA**3**CGAGAGTGCA-3′
	ON **4**	3′-TCGAGCCAGT**3**GCTCTCACGT-5′
Duplex**3**	ON **5**	5′-AGCTCGGTC**33**CGAGAGTGCA-3′
	ON **6**	3′-TCGAGCCAG**33**GCTCTCACGT-5′
Duplex**4**	ON **7**	5′-AGCTCGGT**333**CGAGAGTGCA-3′
	ON **8**	3′-TCGAGCCA**333**GCTCTCACGT-5′
Duplex**5**	ON **9**	5′-AGCTCGGTCA**4**CGAGAGTGCA-3′
	ON **10**	3′-TCGAGCCAGT**4**GCTCTCACGT-5′
Duplex**6**	ON **11**	5′-AGCTCGGTC**44**CGAGAGTGCA-3′
	ON **12**	3′-TCGAGCCAG**44**GCTCTCACGT-5′
Duplex**7**	ON **13**	5′-AGCTCGGT**444**CGAGAGTGCA-3′
	ON **14**	3′-TCGAGCCA**444**GCTCTCACGT-5′
–	ON **15**	5′-GCA**4**AGC-GAAGGTCAAGGTATCTCT-GCT**4**TGC-3′
–	RNA **16**	3′-r(ACCCCU*CUUCCAGUUCCAUAGAGA*CCUGGAG)-5′
–	RNA **17**	3′-r(ACCCCU*CUUCCAGUUA**C**AUAGAGA*CCUGGAG)-5′

[a] The italicized letters indicate complementary sequences to the loop regions of the MB. The bolded, italicized letter represents the mismatched base.

DNA synthesizer. The fully protected ONs (1.0 μmol each) linked to solid supports were deprotected upon treatment with concentrated NH_4OH at 55 °C for 12 h. The ONs that were released from the support were purified by using 20% denaturing polyacrylamide gel electrophoresis (PAGE) to afford ONs **3**–**15**. These ONs were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS); the observed molecular weights were in good agreement with their structures. All ONs are soluble in water.

Thermal Stabilities of Duplexes

The thermal stabilities of the DNA duplexes containing the biaryl units were studied by carrying out thermal denaturation in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl (Table 2). Their UV absorbances were measured at 260 nm. It was found that biaryl units **3** and **4** thermally stabilize the DNA duplexes. The stabilities of the duplexes depended on the number and type of the biaryl units. Biaryl unit **4**, which has a pyrene moiety, stabilized the duplex more efficiently than **3**, which has a naphthalene moiety. The thermal stability of the duplexes increased as the number of units **3** or **4** increased, till four residues of **3** or **4** were incorporated into the duplexes. In contrast, the stability of the duplex (T_m = 66.0 °C) containing six residues of **3** was slightly lower than that (T_m = 67.0 °C) of the duplex containing two residues of **3**; moreover, the stability of the duplex (T_m = 74.0 °C) containing six residues of **4** was comparable to that (T_m = 74.5 °C) of the duplex containing four residues of **4**. Although the exact reason for the low thermal stability of the duplexes (duplexes **4** and **7**) containing six residues of the biaryl units as compared to that of the duplexes (duplexes **3** and **6**) containing four residues of the units is not elucidated, we speculate that it may be attributed to the distance between the aromatic chromophores in the duplex. The number of carbon atoms between the hydroxy groups in the biaryl units (five carbons) is greater than that in a natural nucleoside (three carbons); that is, the distances between the aromatic chromophores in the duplexes are probably slightly greater than those in the case of natural nucleosides. As a result, the stacking interaction between the aromatic chromophores may become weak with increasing number of the biaryl units.

Table 2. Melting temperatures.[a]

Duplex	T_m [°C]	ΔT_m [°C]	Duplex	T_m [°C]	ΔT_m [°C]
Duplex**1**	62.9	–	Duplex**1**	62.9	–
Duplex**2**	67.0	+4.1	Duplex**5**	71.4	+8.5
Duplex**3**	68.7	+5.8	Duplex**6**	74.5	+11.6
Duplex**4**	66.0	+3.1	Duplex**7**	74.0	+11.1

[a] T_m values at 3.0 μM duplex concentrations.

To determine which factor is responsible for the stabilization of the duplexes, thermodynamic parameters of the duplexes (duplexes **1** and **6**) on duplex formation were studied; these parameters were determined by calculations using the slope of a $1/T_m$ vs. $\ln(C_T/4)$ plot, where C_T is the total concentration of single-stranded DNAs. The calculated parameters are summarized in Table 3. The $\Delta G°_{310}$ values of duplexes **1** and **6** were –21.0 and –23.8 kcal mol^{-1}, respectively. Thus, it was found that incorporation of biaryl unit **4** thermodynamically stabilized the DNA duplex. The $\Delta\Delta H°$ and $\Delta\Delta S°$ values between duplexes **1** and **6** were calculated to be +22 kcal mol^{-1} and +78 cal mol^{-1} K^{-1}, respectively. Thus, duplex formation between ONs **11** and **12**, which contain biaryl unit **4**, is less favorable in terms of enthalpy but more favorable in terms of entropy than duplex formation between ONs **1** and **2**, which consist of the natural nucleosides. These results suggest that the enhanced thermodynamic stability of duplex **6** is contributed by the entropy term, which is associated with the hydrophobic interactions between the pyrene residues of duplex **6**.

Table 3. Thermodynamic parameters.

Duplex	$\Delta H°$ [kcal mol^{-1}]	$\Delta S°$ [cal mol^{-1} K^{-1}]	$\Delta G°_{310}$ [kcal mol^{-1}]
Duplex**1**	–166	–466	–21.0
Duplex**6**	–144	–388	–23.8

Absorption Properties

Next, we performed a series of temperature-dependent UV/Vis absorption experiments on the double-stranded DNAs. Figure 2 shows the UV absorbance profiles of duplexes **5**–**7** in the range 300–400 nm, which corresponds to the absorbance range of pyrene moieties. The intensity of

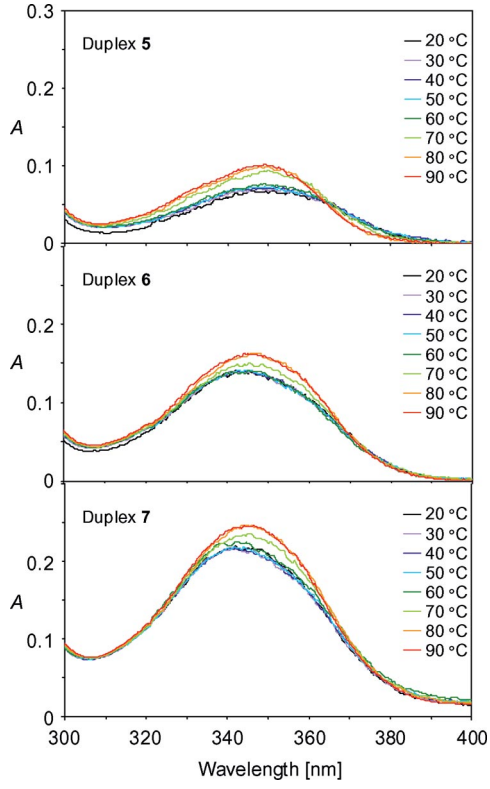


Figure 2. UV absorbance measurement.

the UV absorbance was dependent on the temperatures at which they were measured. In the case of all duplexes, the UV absorbance drastically decreased around T_m (measured at 260 nm) of the duplexes. This finding strongly suggests that the pyrene moieties participate in interstrand stacking interactions, because stacking interactions of chromophores are generally accompanied with a decrease in the absorption intensity (hypochromic effect). In the profile of duplex **5**, two isosbestic points were observed at 356 and 364 nm.[29] This indicates that there exists two different types of interactions between the pyrene moieties in duplex **5**. The first isosbestic point observed at 364 nm, which was formed by the curves measured below T_m (71.4 °C), is attributable to the dissociation of the duplex; the second isosbestic point observed at 356 nm, which was formed by the curves measured above T_m, is attributable to a conformational change in the single-stranded DNAs. In contrast, no isosbestic point was observed in the profiles of duplexes **6** and **7**.

Fluorescence Properties

To elucidate the fluorescence properties of the ONs containing biaryl unit **4**, their emission spectra were measured from 370 to 670 nm. The single-stranded DNAs, ONs **9** and **10**, which contain a single residue of **4**, showed monomer fluorescence around 395 nm under 342-nm excitation (Figure 3c). In contrast, duplex **5**, which contains ONs **9** and **10**, exhibited excimer fluorescence at 490 nm and no monomer fluorescence.[39] This result indicates that the pyrene moieties in the ONs interact with each other in the duplex. The intensities of the emissions of the duplexes increased with increasing number of biaryl unit **4** and were clearly higher than the sum of emission intensities of the corresponding single-stranded DNAs (Figure 3a,b). These observations indicate that the presence of a benzene–phosphate backbone ensures that the aromatic chromophores are suitably arrayed in the duplexes.

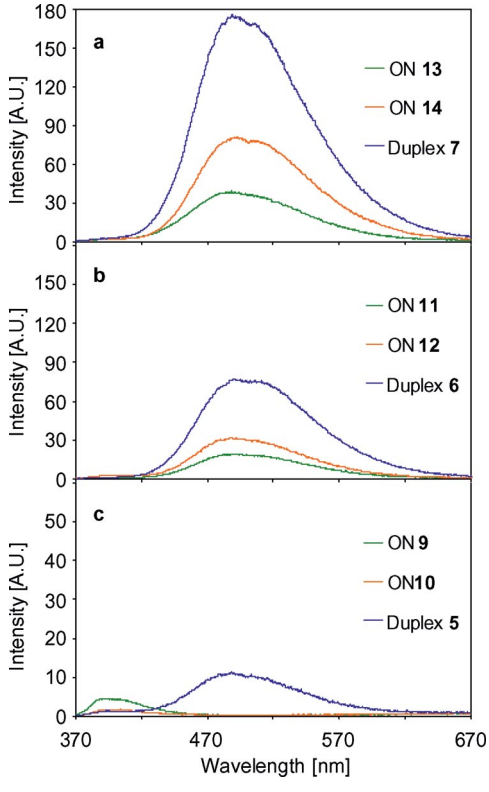


Figure 3. Fluorescence intensity measurement of duplexes.

Synthesis of MB

Nucleic acid probes modified with fluorescent dyes are widely used to detect specific DNA or RNA molecules.[40–48] MBs are self-reporting nucleic acid probes that have complementary arm sequences and a loop that is complementary to a target sequence (Figure 4). MBs possess a fluorophore and a quencher at the termini and form a closed structure to bring these fluorophore labels into juxtaposition under unhybridized conditions; the juxtaposition of the labels leads to fluorescence quenching. When the MBs bind to a complementary target, they adopt an open conformation, wherein the labels are spatially separated. As a result, the MBs brightly fluoresce in the presence of a nucleic acid target sequence.

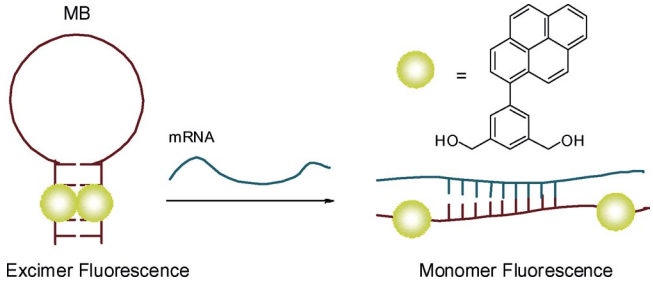


Figure 4. Structure of molecular beacon.

Next, we attempted to introduce biaryl unit **4** into the stem moiety of the MB (Figure 4). We predicted that by introducing **4** into the middle of the stem moiety, we can realize a new type of MB that does not possess a quencher and has two free ends (5′- and 3′-ends). We believed that a target RNA could be detected from the monomer and excimer fluorescence signal of **4** observed in its emission spectra.

The sequences of the MBs and the corresponding target RNA are shown in Table 1. We selected an mRNA of a human CYP2C9 (position 1060–1090) as the target sequence. The functionality of ON **15** as a MB was studied by carrying out fluorescence titration experiments in the presence of fully complementary target RNA **16** (Figure 5a). The addition of RNA **16** (0.1–1.0 equiv.) to a solution containing ON **15** caused a decrease in excimer fluorescence intensity (around 480 nm) and an increase in the monomer fluorescence intensity (around 390 nm). This result implies that ON **15** effectively functions as a MB.

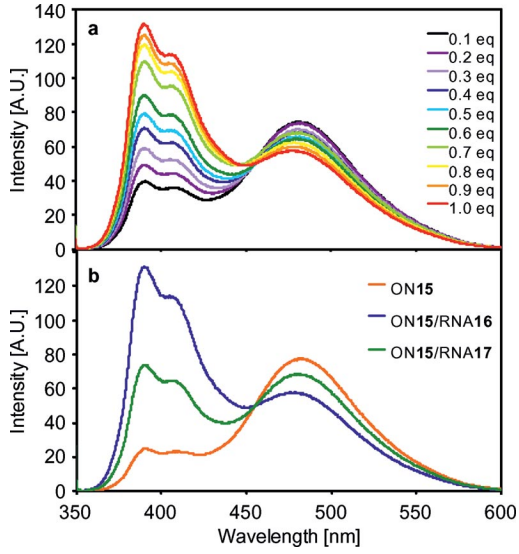


Figure 5. Fluorescence intensity measurement of MB.

We next examined the ability of the MB to discriminate a single-base mismatch in the target RNA. RNA **17** contains one base mismatch in the middle of the strand. Figure 5b shows the emission spectra of the solution containing hybrids of ON **15** and RNA **16** or ON **15** and RNA **17**. Ratios of fluorescence intensities of the excimer to monomer emission (I_{390nm}/I_{480nm}) of ON **15**/RNA **16** and ON **15**/RNA **17** hybrids were 2.3 and 1.1, respectively. Thus, it can be concluded that ON **15** as a MB can effectively discriminate a single-base mismatch in a 31mer RNA.

Conclusions

In the present study, ONs containing biaryl units **3** and **4** composed of naphthalene and pyrene moieties were synthesized, and their properties were studied. The thermal denaturation of the synthesized duplexes revealed that incorporation of biaryl unit **3** or **4** into the duplex structures enhanced their thermal and thermodynamic stabilities. Biaryl unit **4** formed an excimer in the duplexes. The intensities of the emissions of the duplexes increased with increasing number of units **4**. These results indicate that the presence of the benzene–phosphate backbone ensures that the aromatic chromophores are suitably arrayed in the duplexes. Further, we succeeded in the detection of a single-base mismatch in RNA by using the ON containing **4** as a MB. Thus, biaryl compounds **3** and **4** effectively functionalize the ONs. Further studies to develop applications of biaryl compounds to nanodevices are currently being conducted in our laboratory.

Experimental Section

General Remarks: NMR spectra were recorded at 400 MHz (^{1}H) and 100 MHz (^{13}C) with a Jeol JNM-AL400 instrument and are reported in ppm downfield from tetramethylsilane. The coupling constants (*J*) are expressed in Hertz. Mass spectra were obtained by the electron ionization (EI) method with a JEOL JMS-700/GI instrument. Thin-layer chromatography was carried out on Merck coated plates 60F_{254}. Silica gel column chromatography was carried out on Wakogel C-300.

1-[3,5-Bis(hydroxymethyl)phenyl]naphthalene (3): A solution of 3,5-bis(*tert*-butyldimethylsilyloxymethyl)phenylboronic acid (1.00 g, 2.44 mmol)[38] in THF/H_2O (5:1, 12 mL) was added to a solution of 1-iodonaphthalene (0.62 g, 2.44 mmol) and $PdCl_2$(dppf)·CH_2Cl_2 (0.122 g, 0.149 mmol) in THF/H_2O (5:1, 12 mL). NaOH (2 M, 3.66 mL) was added to the mixture, and this mixture was stirred at 65 °C for 24 h. The mixture was partitioned between EtOAc and H_2O. The organic layer was washed with aqueous $NaHCO_3$ (saturated) and brine, dried (Na_2SO_4), and concentrated. The residue was dissolved in THF (7.3 mL). TBAF (1 M in THF, 7.3 mL) was added to the solution, and the mixture was stirred at room temperature for 1 h. The solvent was evaporated in vacuo, and the resulting residue was purified by column chromatography (SiO_2, 2% MeOH in $CHCl_3$) to give **3** (0.545 g, 84%). UV (MeOH): λ (ε, L mol^{-1} cm^{-1}) = 222 (53000), 290 (10000) nm. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.00–7.27 (m, 10 H, H_{Ar}), 5.25 (t, *J* = 5.8 Hz, 2 H, OH), 4.58 (d, *J* = 5.8 Hz, 4 H, CH_2) ppm. ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 143.2, 140.4, 140.1, 134.0, 131.4, 128.8, 128.0, 127.3, 126.7, 126.4, 126.0, 125.9, 124.2, 63.5 ppm. HRMS (EI): calcd. for $C_{18}H_{16}O_2$ 264.1150; found 264.1146. $C_{18}H_{16}O_2$·1/10H_2O (266.12): calcd. C 81.24, H 6.14; found C 80.96, H 6.13.

1-[3-(4,4′-Dimethoxytrityloxymethyl)-5-(hydroxymethyl)phenyl]-naphthalene (8): A mixture of **3** (0.545 g, 2.05 mmol) and DMTrCl (0.909 g, 2.68 mmol) in pyridine (10 mL) was stirred at room temperature for 4 h. The mixture was partitioned between $CHCl_3$ and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 6% MeOH in $CHCl_3$) to give **8** (0.429 g, 37%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.86–6.75 (m, 23 H, H_{Ar}), 4.74 (s, 2 H), 4.23 (s, 2 H), 3.74 (s, 6 H, OCH_3) ppm. ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 158.4, 145.2, 143.3, 140.0, 139.8, 139.0, 135.9, 133.7, 131.0, 129.9, 128.7, 128.2, 127.9, 127.1, 127.0, 126.9, 126.8, 126.6, 126.2, 125.9, 125.5, 124.3, 113.5, 86.2, 65.3, 63.1, 55.3 ppm. $C_{39}H_{34}O_4$·6/5H_2O (588.30): calcd. C 79.62, H 6.24; found C 79.57, H 6.31.

1-[3-{[(2-Cyanoethoxy)(*N*,*N*-diisopropylamino)phosphanyl]oxymethyl}-5-(4,4′-dimethoxytrityloxymethyl)phenyl]naphthalene (9): A mixture of **8** (0.43 g, 0.759 mmol), *N*,*N*-diisopropylethylamine (0.48 mL, 2.75 mmol), and chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphane (0.35 mL, 1.57 mmol) in THF (7 mL) was stirred at room temperature for 1 h. The mixture was partitioned between $CHCl_3$ and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (a neutralized SiO_2, EtOAc) to give **9** (0.513 g, 88%). ^{31}P NMR (160 MHz, $CDCl_3$): δ = 149.0 ppm.

1-[3,5-Bis(hydroxymethyl)phenyl]pyrene (4): A solution of 3,5-bis(*tert*-butyldimethylsilyloxymethyl)phenylboronic acid (1.00 g, 2.44 mmol) in THF/H_2O (5:1, 24 mL) was added to a solution of 1-bromopyrene (0.686 g, 2.44 mmol) and $PdCl_2$(dppf)·CH_2Cl_2 (0.122 g, 0.149 mmol) in THF/H_2O (5:1, 12 mL). NaOH (2 M, 3.66 mL) was added to the mixture, and this mixture was stirred at 65 °C for 48 h. The mixture was partitioned between EtOAc and H_2O. The organic layer was washed with aqueous $NaHCO_3$ (saturated) and brine, dried (Na_2SO_4), and concentrated. The residue was dissolved in THF/CH_2Cl_2 (1:1, 45 mL). TBAF (1 M in THF, 1.14 mL) was added to the solution, and the mixture was stirred at room temperature for 1 h. The solvent was evaporated in vacuo,

and the resulting residue was purified by column chromatography (SiO_2, 6–9% MeOH in $CHCl_3$) to give **4** (0.416 g, 54%). UV (MeOH): λ (ε, $L\,mol^{-1}\,cm^{-1}$) = 202 (51100), 243 (43400), 277 (36400), 342 (27600) nm. ^1H NMR (400 MHz, [D$_6$]DMSO): δ = 8.36–7.43 (m, 12 H, H_{Ar}), 5.31 (t, J = 5.9 Hz, 2 H, OH), 4.64 (d, J = 5.9 Hz, 4 H, CH_2) ppm. ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 142.9, 140.0, 137.7, 131.1, 130.6, 130.2, 127.8, 127.7, 127.6, 127.5, 126.8, 126.6, 125.5, 125.1, 125.0, 124.9, 124.3, 124.2, 123.9, 63.1 ppm. HRMS (EI) calcd. for $C_{24}H_{18}O_2$ 338.1307; found 338.1312. $C_{24}H_{18}O_2 \cdot 1/5H_2O$ (342.00): calcd. C 84.29, H 5.42; found C 84.21, H 5.37.

1-[3-(4,4′-Dimethoxytrityloxymethyl)-5-(hydroxymethyl)phenyl]pyrene (12): A mixture of **4** (0.41 g, 1.21 mmol) and DMTrCl (0.46 g, 1.36 mmol) in pyridine (10 mL) was stirred at room temperature for 4 h. The mixture was partitioned between $CHCl_3$ and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 2–9% MeOH in $CHCl_3$) to give **4** (0.113 g, 28%) and **12** (0.448 g, 58%). ^1H NMR (400 MHz, $CDCl_3$): δ = 8.62–6.82 (m, 25 H, H_{Ar}), 4.84 (s, 2 H, CH_2), 4.33 (s, 2 H, CH_2), 3.77 (s, 6 H, OCH_3) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 158.5, 145.0, 141.4, 141.1, 139.9, 137.5, 136.2, 131.5, 131.0, 130.6, 130.1, 129.1, 128.7, 128.5, 128.2, 127.9, 127.6, 127.5, 127.4, 127.4, 126.8, 126.0, 125.3, 125.1, 125.0, 124.9, 124.8, 124.6, 124.5, 113.1, 86.5, 65.6, 65.5, 55.2 ppm. $C_{45}H_{36}O_4 \cdot 4/5H_2O$ (655.18): calcd. C 82.49, H 5.78; found C 82.49, H 5.92.

1-[3-{[(2-Cyanoethoxy)(*N*,*N*-diisopropylamino)phosphanyl]oxymethyl}-5-(4,4′-dimethoxytrityloxymethyl)phenyl]pyrene (13): A mixture of **12** (0.44 g, 0.687 mmol), *N*,*N*-diisopropylethylamine (0.565 mL, 3.24 mmol), and chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino) phosphane (0.307 mL, 1.38 mmol) in THF (4 mL) was stirred at room temperature for 1 h. The mixture was partitioned between $CHCl_3$ and aqueous $NaHCO_3$ (saturated). The organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (neutralized SiO_2, 30% acetone in EtOAc) to give **13** (0.539 g, 93%). ^{31}P NMR (160 MHz, $CDCl_3$): δ = 149.3 ppm.

Oligonucleotide Synthesis: The synthesis was carried out with a DNA/RNA synthesizer (Applied Biosystems Model 3400) by the phosphoramidite method. In the case of the coupling of amidites **9** and **13**, a 0.12 M solution of amidite **9** or **13** in CH_3CN and a coupling time of 15 min were used. Deprotection of the bases and phosphates was performed in concentrated NH_4OH at 55 °C for 16 h. The oligonucleotides were purified by 20% PAGE containing 7 M urea to give the highly purified oligonucleotides, ON **3** (28), ON **4** (14), ON **5** (13), ON **6** (20), ON **7** (15), ON **8** (16), ON **9** (16), ON **10** (15), ON **11** (16), ON **12** (22), ON **13** (11), ON **14** (13), and ON **15** (14). The yields are indicated in parentheses as OD units at 260 nm starting from 1.0 μmol scale. The extinction coefficients of the oligonucleotides were calculated from those of mononucleotides and dinucleotides according to the nearest-neighbor approximation method.[49]

MALDI-TOF/MS Analyses of Oligonucleotides: Spectra were obtained with a time-of-flight mass spectrometer. ON **3**: calculated mass, 6492.1; observed mass, 6495.0. ON **4**: calculated mass, 6394.1; observed mass, 6393.8. ON **5**: calculated mass, 6507.2; observed mass, 6500.1. ON **6**: calculated mass, 6418.1; observed mass, 6423.6. ON **7**: calculated mass, 6546.2; observed mass, 6548.2. ON **8**: calculated mass, 6417.1; observed mass, 6420.4. ON **9**: calculated mass, 6564.2; observed mass, 6563.8. ON **10**: calculated mass, 6466.1; observed mass, 6467.4. ON **11**: calculated mass, 6651.2; observed mass, 6653.2. ON **12**: calculated mass, 6562.2; observed mass, 6566.7. ON **13**: calculated mass, 6762.2; observed mass, 6764.1. ON **14**: calculated mass, 6633.2; observed mass, 6634.2. ON **15**: calculated mass, 10042.7; observed mass, 10041.5.

Thermal Denaturation Study: The solution containing the duplex in a buffer comprising 10 mM sodium phosphate (pH 7.0) and 0.1 M NaCl was heated at 95 °C for 3 min, cooled gradually to an appropriate temperature, and then used for the thermal denaturation study. The thermal-induced transition of each mixture was monitored at 260 nm with a spectrophotometer. The sample temperature was increased by 0.5 °C min^{-1}. The thermodynamic parameters of the duplexes on duplex formation were determined by calculations based on the slope of a $1/T_m$ vs. $\ln(C_T/4)$ plot, where C_T (2, 4, 6, 10, 16, 26, 44, 70, 120, and 200 μM) is the total concentration of single strands.

Absorption Experiments: Absorption spectra (200–500 nm) were obtained with a Beckman Coulter DU640 spectrophotometer fitted with a temperature controller in quartz cuvettes with a path length of 1.0 cm and a 3.0 μM duplex concentration in a buffer of 10 mM Tris-HCl (pH 7.2) containing 100 mM NaCl.

Fluorescence Experiments: Steady-state fluorescence emission spectra (370–670 nm) were obtained with a Shimadzu RF-5300PC spectrophotometer fitted with a temperature controller in quartz cuvettes with a path length of 1.0 cm and a concentration of strands in T_m buffer of 3.0 μM. Spectra were recorded with use of excitation slit of 3.0 nm and emission slit of 3.0 nm. The fluorescence quantum yield (Φ_{em}) was determined by use of quinine as a reference with the known Φ_{em} value of 0.58 (22 °C) in 0.1 M H_2SO_4. The quantum yield was calculated according to the following equation: $\Phi_{em(S)}/\Phi_{em(R)} = [I_{(S)}/I_{(R)}] \times [A_{(S)}/A_{(R)}] \times [n_{(S)}^2/n_{(R)}^2]$. Here, $\Phi_{em(S)}$ and $\Phi_{em(R)}$ are the fluorescence quantum yields of the sample and the reference, respectively, $I_{(S)}$ and $I_{(R)}$ are the integrated fluorescence intensities of the sample and the reference, respectively, $A_{(S)}$ and $A_{(R)}$ are the respective optical density of the sample and the reference solutions at the wavelength of excitation, and $n_{(S)}^2$ and $n_{(R)}^2$ are the values of the refractive index for the respective solvents.

Supporting Information (see footnote on the first page of this article): Graphical data of a $1/T_m$ vs. $\ln(C_T/4)$ plot.

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