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ORIGINAL PAPER

Synthesis of a new intercalating nucleic acid analogue with pyrenol insertions and the thermal stability of the resulting oligonucleotides towards DNA over RNA

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Abstract A new intercalating nucleic acid monomer **Y** was obtained via alkylation of pyren-1-ol with (*S*)-(+)-2-(2,2-dimethyl-1,3-dioxolan-4-yl)ethanol under Mitsunobu conditions followed by hydrolysis with 80% aqueous acetic acid to give a diol which was tritylated with 4,4′-dimethoxytrityl chloride followed by treatment with 2-cyanoethyltetraisopropylphosphordiamidite in the presence of *N*,*N*′-diisopropylammonium tetrazolide. In this way the monomer **Y** was obtained as its dimethoxytrityl-protected phosphoramidite building block for standard DNA synthesis. The corresponding oligonucleotides from **Y** have nearly identical hybridization properties with those of intercalating nucleic acid (INA) where neighboring oxygen and carbon atoms are interchanged in the linker. The synthesis of monomer **Y** avoids the use of allergic intermediates which are a problem in the synthesis of INA.

Keywords Bioorganic chemistry · Intercalating nucleic acid · DNA · Fluorescence · Oligonucleotides · Pyren-1-ol

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Introduction

In recent years, our group has reported the synthesis of intercalating nucleic acid INA (**Z**, Fig. 1) with bulge insertions of 1-*O*-(pyrenylmethyl)glycerol. These oligos have higher duplex stabilities and have high hybridization specificity towards DNA over RNA [1–3]. Quadruplex-forming INA has been designed and studied for its capacity to inhibit the expression of the KRAS oncogene in pancreatic adenocarcinoma cells [4]. Nucleic acid duplexes are stabilized both by hydrogen bonding interactions and by base stacking [5–11]. Therefore polyaromatic units with hydrophobicity in conjunction with a large surface area can be used in place of the natural nucleic bases for mimicking base stacking in Watson–Crick/Hoogsteen base pairing [12–14]. The design and the synthesis of various oligonucleotides containing planar polycyclic chromophores such as pyrene, anthracene, dansyl, and others, which have a long fluorescence lifetime and possibilities to form π-stacking interactions in aqueous solutions, have been the subject of active research during recent years [15, 16]. Pyrene has previously been tested as an intercalator covalently attached to the O2′ in a uracil nucleotide through a methylene linker. The investigation showed a promising stabilization when hybridized to DNA, whereas hybridization to RNA caused destabilization. Insertion in RNA caused destabilization when hybridized to both RNA and DNA [16–18]. Interestingly, it was discovered that a deoxyoligonucleotide with two such neighboring modified nucleotides is an excellent capture and detection probe [19]. Furthermore, there are numerous studies of covalent attachment of pyrene to glycerol for insertions in nucleic acids [20–25]. In our ongoing study we are looking for a new and safe method for the preparation of intercalating nucleic acids. The problem in the reported synthesis of INA

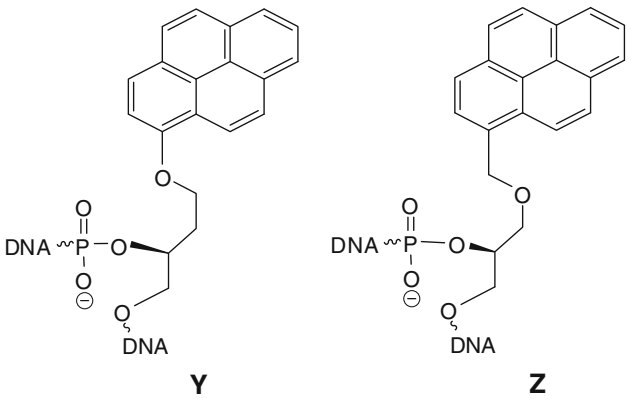

Fig. 1 The synthesized intercalating nucleic acid **Y** and **Z** (INA)

is the alkylation step of 2,2-dimethyl-1,3-dioxolan-4-ylmethanol with 1-(chloromethyl)pyrene [1], which is one of the strongest skin sensitizers ever reported [26–28] and we have several times had severe problems in our laboratory due to improper handling of this compound. Therefore, the major objective of this work was to explore the intercalating effectiveness when interchanging the oxygen atom and methylene group in the linker of INA so that the oxygen atom is attached directly to the intercalating pyrene moiety instead of the methylene group. The linker properties are not expected to be different, but the stacking properties of the pyrene moiety cannot be predicted. Besides studying thermal stabilization properties of corresponding oligonucleotides, the fluorescence properties are considered interesting because the fluorescence of INA shows a decrease in fluorescence emission upon excitation at 340 nm upon hybridization to DNA [1–3] indicating that the pyrene moiety intercalates into the DNA double helix [29, 30].

Results and discussion

Chemistry

The synthetic route for obtaining the acyclic amidite **5** is shown in Scheme 1. The synthesis of (*S*)-4-(pyren-1-yloxy)butane-1,2-diol (**3**) was achieved under Mitsunobu conditions. In this way (*S*)-2-(2,2-dimethyl-1,3-dioxolan-4-yl)ethanol (**1**), diethyl azodicarboxylate (DEAD), triphenylphosphine, and 1-pyrenol (**2**) in dry ice-cooled tetrahydrofuran (THF) were reacted overnight [31, 32]. Subsequent treatment with 80% aqueous acetic acid gave the diol **3** in 87% yield after purification by silica column chromatography. The primary hydroxy group was protected with 4,4′-dimethoxytrityl chloride (DMT-Cl) and NEt_3 in CH_2Cl_2 under nitrogen to give the *O*-DMT-protected compound **4** in 60% yield. The secondary hydroxy group was phosphitylated with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite in anhydrous CH_2Cl_2 under argon in the presence of *N*,*N*′-diisopropylammonium tetrazolide as an activator to give the target amidite **5** in 88% yield.

The DMT-protected phosphoramidite **5** was incorporated into DNA oligonucleotides by using normal procedures. The coupling efficiency was over 96% as determined by detritylation measurement. All modified oligonucleotides were confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis (see "Experimental"). The purity of the final oligos was confirmed by ion-exchange HPLC.

Thermal stability studies

The project was designed to study the effect of interchanging a carbon and an oxygen atom in the linker of INA with the oxygen atom being attached directly to the intercalating pyrene moiety. When bulge insertion of the monomer **Y** (Table 1, entry 2) is compared with the wild-type oligo, an increase in melting temperature of the DNA/(INA analogue) duplex ($\Delta T_m = 4.6$ °C) was observed. This is a 1.6 °C increase in thermal melting temperature when compared with the single insertion of the original pyrene monomer **Z**. To investigate further the stabilization phenomena, two monomers were inserted with varying distances of one to four nucleobases between the two insertions. The trend for insertions of pyrene monomer **Y** is to give slightly higher thermal melting temperatures than the pyrene monomer **Z**. The melting temperatures increased steadily on increasing the number of bases between the two inserted bases. When **Y** was inserted twice with six nucleobases in between a stabilization of 15.7 °C (7.9 °C per modification) was observed. When compared with a single insertion with $\Delta T_m = 4.6$ °C (Table 1), the results could also indicate a dependence on the insertion sites. Destabilization of the pyrene monomer **Y** was observed towards RNA when compared with the wild-type DNA/RNA duplex. The difference in melting temperature between the DNA/(INA analogue) duplex and the corresponding RNA/(INA analogue) duplex is 14 °C when pyrene monomer **Y** inserted as a bulge (Table 1, entry 2). This difference is 8.8 °C larger than in the unmodified oligo. This means that intercalating nucleic acids containing insertions of pyrene **Y** moiety are selective and discriminate between DNA over RNA, as the DNA/DNA duplexes were stabilized while DNA/RNA duplexes were destabilized. For both stabilization and discrimination, the difference in melting temperature between the DNA/(INA analogue) duplex and the RNA/(INA analogue) is up to 27.7 °C when two pyrene **Y** moieties are inserted.

Scheme 1

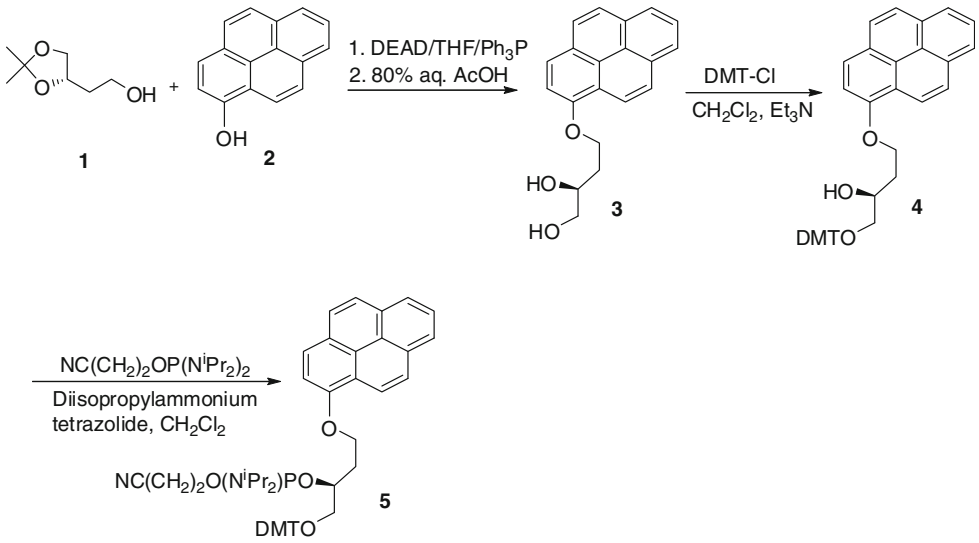

Table 1 Melting temperatures of DNA/DNA and DNA/RNA duplexes with **X = Y** inserted as a bulge

Entry	Oligo	Target DNA 5′-AGCTTGCTTGAG-3′ T_m (°C)		Target RNA 5′-AGCUUGCUUGAG-3′ T_m (°C)		Discrimination T_m(DNA)−T_m(RNA) ΔT_m (°C)					
1	3′-TCGAACG AACTC-5′	47.4		42.2		5.2					
Entry	Oligo	**X = Y**		**X = Z**		**X = Y**		**X = Z**		**X = Y**	**X = Z**
		T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)	ΔT_m (°C)	ΔT_m (°C)
2	3′-TCGAAC**X**GAACTC-5′	52.0	4.6	50.4	3.0	38.0	−4.2	37.8	−4.4	14.0	12.6
3	3′-TCGAAC**X**G**X**AACTC-5′	51.7	4.3	51.4	4.0	35.1	−7.1	34.2	−8.0	16.6	17.2
4	3′-TCGAAC**X**GA**X**ACTC-5′	58.1	10.7	55.6	8.2	36.1	−6.1	32.6	−9.6	22.0	23.0
5	3′-TCGA**X**ACGA**X**ACTC-5′	61.0	13.6	60.8	13.4	33.3	−8.9	35.0	−7.2	27.7	25.8
6	3′-TCG**X**AACGAA**X**CTC-5′	63.1	15.7	–[a]	–[a]	41.7	−0.5	–[a]	–[a]	21.4	–[a]

Data of T_m values **X** = **Z** were taken from a previous study for comparison [1]

T_m (°C) was determined as the first derivative of melting curves by measuring the absorbance at 260 nm against increasing temperature (1.0 °C/min) on equimolar mixtures (1.5 μM of each strand) in 140 mM NaCl, 10 mM sodium phosphate buffer, 1 mM EDTA, pH 7.0

[a] Not determined

Fluorescence properties

Upon single insertion of **Y** the fluorescence spectrum bands with maxima at 390 and 410 nm upon excitation at 340 nm (Fig. 2) were quenched when hybridized to the complementary single-stranded (ss) DNA, indicating that the bulging pyrene moiety **Y** interacts strongly with the double helix by intercalation. The quenching effect upon hybridization with complementary ssDNA is more pronounced than for ssRNA (Fig. 2), which was expected due to lower stability of RNA/INA-type duplexes. Double pyrene insertions as next nearest neighbors in the oligonucleotide gave the same results as for the single insertion (data not shown). Surprisingly, no excimer formation was observed for ssDNA with two insertions of **Y** as next nearest neighbors. This is in contrast to INA in a previous study which showed an excimer band at 480 nm [1] and this is also in contrast with other examples of excimer bands where perylene and pyrene moieties were inserted as next nearest neighbors in peptide nucleic acid (PNA) analogues of oligonucleotides [33, 34]. The missing excimer bands may be an advantage because of reduced risk of interference with fluorescence markers that are routinely conjugated to oligos for detection purposes.

Experimental

NMR spectra were recorded on a Varian Gemini 2000 NMR spectrometer at 300 MHz for ^{1}H, 75 MHz for ^{13}C,

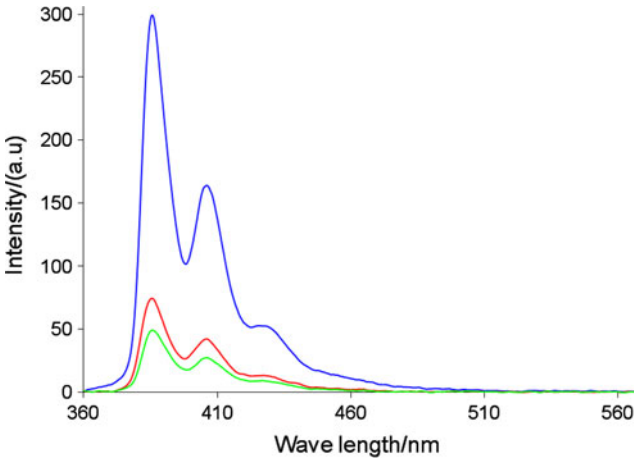

Fig. 2 Fluorescence measurements (**X** = **Y**) of a 13mer, single-stranded oligo (INA analogue) with pyrene insertion (*top*), its duplex with complementary 12mer RNA (*middle*), and its duplex with complementary 12mer DNA (*bottom*). The sequences are as shown in Table 1, entry 2

and 121.5 MHz for ^{31}P with TMS as an internal standard for ^{1}H, deuterated solvents $CDCl_3$ ($\delta = 77.00$ ppm), DMSO-d_6 ($\delta = 39.44$ ppm) for ^{13}C, and 85% H_3PO_4 as an external standard for ^{31}P. Chemical shifts are reported in parts per million (δ). Electrospray ionization mass spectra (ESI–MS) were recorded on a 4.7 T HiResESI Uitima (FT) mass spectrometer. This spectrometer is controlled by the OMEGA Data System. All modified oligonucleotides were confirmed by MALDI-TOF MS analysis on a Voyager Elite Biospectrometry research station from PerSeptive Biosystems. Analytical silica gel TLC plates 60 F_{254} were purchased from Merck and were visualized in UV light (254 nm). The silica gel (0.040–0.063 mm) used for column chromatography was purchased from Merck. Solvents used for column chromatography were distilled prior to use. THF was distilled from Na/benzophenone. Triethylamine and CH_2Cl_2 were dried over 4 Å molecular sieves.

(S)-4-(Pyren-1-yloxy)butane-1,2-diol (**3**, $C_{20}H_{18}O_3$)
An ice-cooled solution of 0.197 cm^3 diethyl azodicarboxylate (1.268 mmol) in 28 cm^3 dry THF was treated with 0.150 cm^3 (*S*)-2-(2,2-dimethyl-1,3-dioxolan-4-yl)ethanol (1.056 mmol) for 25 min. Then 0.3 g 1-pyrenol (1.375 mmol) and 0.332 g triphenylphosphine (1.268 mmol) were added to the mixture. The resultant mixture was stirred in an ice bath for 30 min and then allowed to warm to room temperature overnight. The mixture was quenched with 18.5 cm^3 saturated NH_4OH and extracted with ethyl acetate. The organic layer was washed with water, dried ($MgSO_4$), and concentrated under reduced pressure. Without further purification the residue was added to 25 cm^3 aqueous solution of acetic acid (80%) and stirred at room temperature for 24 h. The solvent was removed in vacuo and the residue was coevaporated with 2 × 30 cm^3 toluene/EtOH (5:1, v/v). The residue was dried under reduced pressure. The residue was purified by silica gel column chromatography (5% MeOH/CH_2Cl_2, v/v) to afford 0.261 g **3** (87%) as a clear colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 1.88$–2.19 (m, 2H, C*H*$_2$ CH_2O), 3.45 (m, 2H, C*H*$_2$OH, C*H*OH), 3.87 (m, 1H, C*H*$_2$ OH), 4.48 (m, 2H, CH_2O), 4.64, 4.74 (2 × s, 2H, 2 × OH), 7.77 (d, 1H, $J = 8.7$ Hz, Ar), 7.94–8.26 (m, 7H, Ar), 8.39 (d, 1H, $J = 9.3$ Hz, Ar) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): $\delta = 33.28$ (*C*$H_2$$CH_2O$), 65.55 ($CH_2O$), 66.05 ($CH_2$ OH), 68.12 (CHOH), 109.71, 119.31, 120.85, 124.02, 124.24, 124.55, 125.98, 126.14, 126.31, 127.24, 128.60, 128.76, 131.04, 131.19, 131.47, 152.70 (C_{arom}) ppm; HRMS (ESI): $m/z = 329.1145$ ([M + Na]$^+$, calcd.: 329.1150).

(S)-1-(Bis(4-methoxyphenyl)phenylmethoxy)-4-(pyren-1-yloxy)butan-2-ol (**4**, $C_{41}H_{36}O_5$)
Compound **3** (0.200 g, 0.653 mmol) and 0.5 cm^3 anhydrous NEt_3 were dissolved in 10 cm^3 anhydrous CH_2Cl_2 and 0.265 g 4,4′-dimethoxytrityl chloride (0.784 mmol) was added under nitrogen at room temperature overnight. When TLC showed no more starting material, the reaction mixture was quenched with 2 cm^3 MeOH. Ethyl acetate (100 cm^3) was added, and the mixture was extracted with saturated aqueous $NaHCO_3$ (2 × 40 cm^3). The water phase was extracted with ethyl acetate (2 × 20 cm^3). The combined organic phases were dried (Na_2SO_4), filtered, and evaporated under reduced pressure. The residue was coevaporated with toluene/EtOH (1:1, v/v, 2 × 25 cm^3). The residue was purified by silica gel column chromatography (ethyl acetate/cyclohexane/NEt_3 30:70:1, v/v/v), which afforded 0.239 g **4** (60%) as a clear oil. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.10$–2.15 (m, 2H, C*H*$_2$$CH_2O$), 3.24–3.27 (m, 1H, C*H*HODMT), 3.36–3.40 (m, 1H, C*H*OH), 3.71 (s, 6H, 2 × OCH_3), 3.79 (s, 1H, OH), 4.19–4.23 (m, 1H, CH*H*ODMT), 4.38–4.44 (m, 2H, CH_2O), 6.77 (d, 4H, $J = 8.7$ Hz, DMT), 7.20–7.33 (m, 7H, arom), 7.43–7.51 (m, 3H, arom), 7.86–7.99 (m, 4H, arom), 8.05–8.11 (m, 3H, arom), 8.34 (d, 1H, $J = 9$ Hz, arom) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 33.31$ (*C*$H_2$$CH_2O$), 55.12 (2 × OCH_3), 65.65 (CH_2O), 67.52 (CHOH), 68.67 (*C*H_2ODMT), 86.26 (O*C*Ph$_3$), 109.19, 113.12, 121.10, 124.14–131.71, 135.91, 144.83, 152.81, 158.49 (arom, DMT) ppm; HRMS (ESI): $m/z = 631.2474$ ([M + Na]$^+$, calcd.: 631.2455).

(S)-1-(Bis(4-methoxyphenyl)phenylmethoxy)-4-(pyren-1-yloxy)butan-2-yl 2-cyanoethyl diisopropylphosphoramidite (**5**, $C_{50}H_{53}N_2O_6P$)
Compound **4** (0.170 g, 0.279 mmol) was dissolved under argon in 20 cm^3 anhydrous CH_2Cl_2 and 72 mg *N*,*N*′-diisopropylammonium tetrazolide (0.42 mmol) was added

followed by dropwise addition of 0.266 cm^3 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (0.837 mmol) under external cooling with an ice–water bath. After 24 h, analytical TLC showed no more starting material and the reaction was quenched with 15 cm^3 water. Layers were separated and the organic phase was washed with 15 cm^3 water. The combined water layers were washed with 25 cm^3 CH_2Cl_2. The combined organic phases were dried (Na_2SO_4) and filtered, and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography (ethyl acetate/cyclohexane/NEt_3 30:70:1, v/v/v), which afforded 0.20 g **5** (88%) as clear colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.08$–1.17 (m, 12H, 2 × CH(CH_3)$_2$), 2.20–2.30 (m, 2H, CH_2CH_2O), 2.40 (t, 2H, $J = 6.6$ Hz, CH_2CN), 3.15–3.20 (m, 1H, C*H*HODMT), 3.33–3.40 (m, 1H, C*H*OP), 3.54–3.80 (m, 10H, 2 × OCH_3, 2 × C*H*(CH_3)$_2$, CH_2CH_2OP), 4.30–4.50 (m, 3H, CH*H*ODMT, CH_2O), 6.73–6.85 (m, 4H, DMT), 7.15–7.40 (m, 7H, arom), 7.45–7.57 (m, 3H, arom), 7.86–8.15 (m, 7H, arom), 8.37–8.45 (m, 1H, arom) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 20.10$ (CH_2CN), 24.49, 24.61, 24.70 (2 × CH(CH_3)$_2$), 33.51 (CH_2CH_2O), 43.07, 43.23 (2 × $C(CH_3)_2$), 55.12 (2 × OCH_3), 58.04 (OCH_2CH_2CN), 64.98 (CH_2O), 66.50 (CHOP), 71.15 (CH_2ODMT), 86.02 ($OCPh_3$), 109.04, 113.00, 121.27, 124.10–130.13, 136.08, 144.96, 158.37 (arom, DMT) ppm; ^{31}P NMR (121.5 MHz, $CDCl_3$): $\delta = 149.71$, 150.18 ppm in a ratio of 5:4; HRMS (ESI): $m/z = 831.3542$ ($[M + Na]^+$, calcd.: 831.3533).

Oligonucleotide synthesis, purification, and melting temperature determination

DMT-on oligodeoxynucleotides (ODNs) were prepared at 0.2 μmol scales on 500 Å CPG supports with an Expedite™ Nucleic Acid Synthesis System Model 8909 from Applied Biosystems, using 1*H*-tetrazole as an activator for the coupling reaction. A 0.05 mM solution of the phosphoramidite **5** in anhydrous CH_2Cl_2 was used and inserted into the growing oligonucleotides by using an extended coupling time of 10 min. DMT-on oligonucleotides bound to CPG supports were treated with 1 cm^3 aqueous ammonia (32%) at room temperature and then at 55 °C overnight. Purification of 5′-*O*-DMT-on ONs was accomplished by reversed-phase semipreparative HPLC on a Waters Xterra MS C_{18} column with a Waters Delta Prep 4000 Preparative Chromatography System; buffer A = 0.05 M triethylammonium acetate in water (pH 7.4), buffer B = 75% CH_3CN in water, flow = 2.5 cm^3 min^{-1}, using the following gradient: 2 min 100% A, linear gradient to 70% B in 38 min, linear gradient to 100% B in 3 min, and then 100% A in 10 min. ODNs were DMT-deprotected in 100 mm^3 80% acetic acid for 20 min. Afterwards, 100 mm^3 water and 50 mm^3 aqueous AcONa (3 M) were added, and 15 mm^3 sodium perchlorate and the ONs were precipitated from 500 mm^3 acetone. All modified ODNs were confirmed by MALDI-TOF analysis on a Voyager Elite Biospectroscopy research station from PerSeptive Biosystems. ODN found *m*/*z* (calculated *m*/*z*) as follows: oligo entry 2: 3,981.6 (3,981.8); oligo entry 3: 4,351.3 (4,349.1); oligo entry 4: 4,351.8 (4,349.1); oligo entry 5: 4,351.6 (4,349.1); oligo entry 6: 4,351.0 (4,349.1). The purity of the final ODNs was found to be over 90%, checked by ion-exchange chromatography using a LaChrom system from Merck Hitachi on Genpak-Fax column (Waters). Melting temperature measurements were performed on a Perkin-Elmer UV–Vis spectrometer Lambda 35 fitted with a PTP-6 temperature programmer. All ODNs were measured in a buffer consisting of 140 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.0, with a concentration of 1.5 mm^3 each strand. The solutions were heated to 80 °C for 5 min and cooled to 5 °C and were then kept at this temperature for 10 min. The melting temperature (T_m, °C) was determined as the maximum of the first-derivative plots of the melting curves obtained by absorbance at 260 nm against increasing temperature (1.0 °C/min). All melting temperatures are within the uncertainly ±1.0 °C as determined by repetitive experiments.

Fluorescence measurements

The fluorescence measurements were measured on a Perkin-Elmer LS-55 luminescence spectrometer fitted with a Julabo F25 temperature controller set at 10 °C in a buffer consisting of 140 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.0, with a concentration of 1.5 mm^3 each strand. Excitation wavelength was set to 340 nm and detection at 360–600 nm. The excitation and emission slits were set to 4 and 2.5 nm, respectively.

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