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Methylcytosine-selective fluorescence quenching by osmium complexation

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Abstract—We report on the control of the emission from a fluorophore fixed on DNA using the methylcytosine-selective addition of an osmium-bipyridine complex. We have synthesized DNA modified by a microenvironment-sensitive fluorophore, 2-dimethylamino-6-acyl-naphthalene. The emission from the fluorophore tethered to a probe DNA was effectively quenched by a methylcytosine glycol-osmium-bipyridine triad, which was located in the immediate neighborhood of the fluorophore. The discrimination of the cytosine methylation status at a methylation hot spot in the p53 gene was also executed using a well-designed fluorescent DNA probe.

1. Introduction

Cytosine methylation plays a crucial role in the regulation of chromatin stability, gene regulation, parental imprinting, and X chromosome inactivation in females.[1–4] In addition, erroneous DNA methylation may contribute to the etiopathogenesis of tumorigenesis and aging.[5,6] Therefore, a simple diagnosis of cytosine methylation status is highly desirable for further understanding of the role of 5-methylcytosine (M) in human genes.

There have been very few reports on cytosine methylation analysis based on chemical concepts; for example, cytosine hydrolysis with sodium bisulfite.[7–9] To construct an effective method for a high-throughput detection of methylation in the future, the establishment of optical analysis systems, particularly one utilizing fluorescence emission, is essential, but there remains the difficulty in developing these, because there is only a slight difference in the photophysical properties of cytosine and methylcytosine.

Here, we report on the control of the fluorescence emission from a fluorophore fixed on DNA using the methylcytosine-selective addition of an osmium–bipyridine complex. The DNA modified with a microenvironment-sensitive fluorophore was synthesized, and the fluorescence was effectively quenched by the addition of the osmium–bipyridine complex to methylcytosine, which is located in the immediate neighborhood of the fluorophore.

2. Results and discussion

2.1. Synthesis of a fluorescence-labeled nucleoside, D

We designed a DNA probe with a microenvironment-sensitive fluorophore, 6-dimethylamino-2-acylnaphthalene (DAN). This has a unique fluorescence property: the emission intensity and wavelength vary sharply with the degree of protonation and solvent polarity, and with any microstructural changes.[10–12] We incorporated this fluorophore into DNA. The purine base modified with DAN (D) forms a wobble base pair with pyrimidines, and in addition, the acyl group of the DAN is expected to form an extra hydrogen bond with the amino group of the base-pairing cytosine (Fig. 1).[13,14] The synthesis of D-containing DNA is outlined in Scheme 1. The DAN derivative **2**, which was prepared from 6-dimethylamino-2-naphthaldehyde (**1**) in four steps, was cou-

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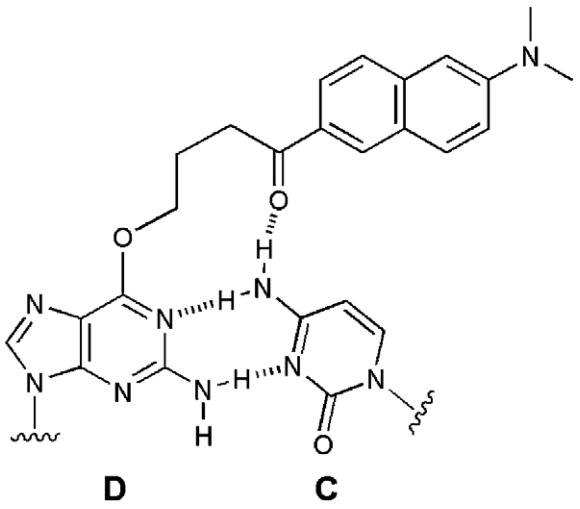
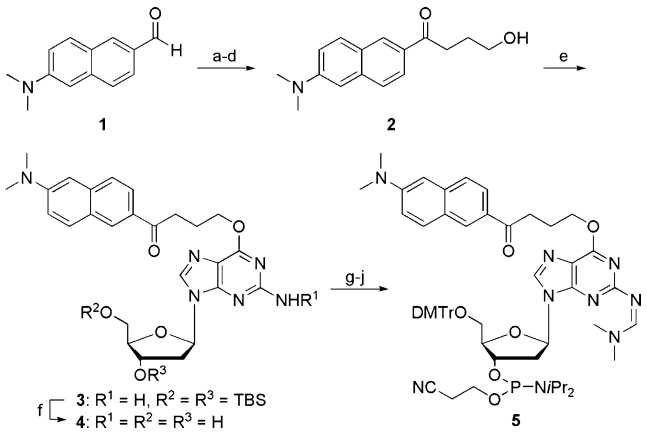


Figure 1. Our proposed base-pairing model of a fluorescence-labeled purine base, D, and cytosine.



Scheme 1. Synthesis of a fluorescent nucleoside, D. Reagents and conditions: (a) *O*-(*tert*-butyldimethylsilyl)propargyl alcohol, *n*-butyl lithium, THF, −78 °C, rt, 30 min, 96%; (b) H_2, Pd–C, methanol, rt, 9 h, 65%; (c) tetrapropylammonium perruthenate, 4-methylmorpholine *N*-oxide, dichloromethane, rt, 2 h, 87%; (d) tetrabutylammonium fluoride, THF, rt, 6 h, 95%; (e) 3′*O*,5′*O*-bis(*t*-butyldimethylsilyl)-2′-deoxyguanosine, triphenylphosphine, diethyl azodicarboxylate, dioxane, 70 °C, 6 h, 73%; (f) tetrabutylammonium fluoride, THF, rt, 2 h, 73%; (g) dimethylformamide dimethylacetal, DMF, rt, 6 h, 94%; (h) 4,4′-dimethoxytrityl chloride, pyridine, 50 °C, 2 h, 87%; and (i) $(i\mathrm{Pr}_2\mathrm{N})_2\mathrm{PO}(\mathrm{CH}_2)_2\mathrm{CN}$, 1*H*-tetrazole, acetonitrile, rt, 2 h.

pled with silyl-protected 2′-deoxyguanosine[15] to give **3**. The deprotection of **3** provided a nucleoside **4**, which was subsequently converted into the phosphoramidite **5** for use in a DNA autosynthesizer. The synthesized oligodeoxyribonucleotides (ODN) are summerized in Table 1.

2.2. Quenching of D fluorescence by osmium complexation

The ODN containing a D base, 5′-d(TTTTTTCDC TTTTTT)-3′ (**cODN(D)**), formed a stable duplex with ODNs, 5′-d(AAAAAAGXGAAAAAA)-3′ (**ODN(X)**), containing cytosine (X = C) or 5-methylcytosine (X = M) opposite a D base (T_m = 33.2 and 31.9 °C, respectively, Table 2), although the addition of a hydrophobic fluorophore decreased the duplex stability, compared with the stability of the duplex with a C/A mismatched base pair. **ODN(C)/cODN(D)** was more stable than **ODN(M)/cODN(D)**, implying the formation of an extra hydrogen bond between cytosine and D as shown in Figure 1 and/or the M/D base pair destabilization caused by steric hindrance between a 5-methyl group of methylcytosine and the DAN chromophore of D. These fluorescent duplexes showed similar fluorescence intensities at 515 nm (Φ_F = 0.075 and 0.061, respectively, excited at 390 nm, Table 3). At this stage, the fluorescence of D was not much affected by the presence/absence of the C5 methyl group of a cytosine ring.

To more effectively distinguish 5-methylcytosine from cytosine, we modified **ODN(X)** by the addition of an osmium–bipyridine complex. The addition of osmium tetroxide and bipyridine to pyrimidine bases is known to provide a pyrimidine glycol-osmium-bipyridine triad.[16–18] We have previously established the sequence-selective complexation of 5-methylcytosine in DNA using potassium osmate and bipyridine.[19] This treatment drastically changed the fluorescence intensity of the D-containing duplex with a high methylcytosine selectivity (Fig. 2a). **ODN(X)** (5 μM) was added to a solution of 5 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM bipyridine, and 1 mM EDTA in 100 mM Tris–HCl buffer (pH 7.7) and 10% acetonitrile. This mixture was then incubated at 37 °C for 3 h. The fluorescence spectra of the resulting ODNs were measured after desalting and hybridization with **cODN(D)**. The fluorescence of the duplex **ODN($\mathbf{M_{Os}}$)/cODN(D)**, which was produced via osmium addition to **ODN(M)**, was significantly quenched (Φ_F = 0.018, Table 3) compared to the fluorescence emission of **ODN(C)/cODN(D)** and **ODN(M)/cODN(D)** (Figs. 2b and c). The decay in fluorescence was strongly influenced by the osmium complex formation. A new rapid component (τ_1 = 43 ps) was observed only for **ODN ($\mathbf{M_{Os}}$)/cODN(D)** (Table 4), suggesting that photo-induced electron transfer from the excited D base to the osmium complex occurred, and that this contributed to the quenching process.

Accordingly, the distance between the D base and the osmium complex is very important for efficient fluorescence quenching. We measured the fluorescence intensity of several duplexes that had different distances between D and the osmium complex attached to 5-methylcytosine (Fig. 3) or thymine (Fig. 4). Little significant fluorescence quenching was observed for all of the duplexes that contained osmium complexes on one or two of the bases next to the opposite D base. The results clearly show that fluorescence quenching occurred only on osmium complexation at the methylcytosine located opposite to the D base. Although many 5-methylcytosines and thymines that can form complexes with osmium may be included in a DNA strand, the fluorescence of D was very sensitive only to complexation of 5-methylcytosine opposite D. The selective quenching suggests that the methylation status of cytosine at the site of

Table 1. ODN sequences used in this study and their MS values

	Sequences	Calcd	Found[a]
ODN(C)	5′-AAA AAA GCG AAA AAA-3′	—	—
ODN(M)	5′-AAA AAA GMG AAA AAA-3′	4657.13[b]	4657.14
ODN(M_{Os})	5′-AAA AAA GM_{Os}G AAA AAA-3′	5051.54[c]	5051.36
ODN(T)	5′-AAA AAA GTG AAA AAA-3′	—	—
ODN(T_{Os})	5′-AAA AAA GT_{Os}G AAA AAA-3′	5052.53[c]	5052.72
cODN(D)	5′-TTT TTT CDC TTT TTT-3′	4734.24[b]	4734.51
cODN(G)	5′-TTT TTT CGC TTT TTT-3′	—	—
ODN(M5′-1)	5′-AAA AAM GCG AAA AAA-3′	4633.11[b]	4633.76
ODN(M5′-2)	5′-AAA AMA GCG AAA AAA-3′	4633.11[b]	4633.81
ODN(M3′-1)	5′-AAA AAA GCG MAA AAA-3′	4633.11[b]	4633.13
ODN(M3′-2)	5′-AAA AAA GCG AMA AAA-3′	4633.11[b]	4633.86
ODN(M_{Os}5′-1)	5′-AAA AAM_{Os} GCG AAA AAA-3′	5027.52[c]	5028.01
ODN(M_{Os}5′-2)	5′-AAA AM_{Os}A GCG AAA AAA-3′	5027.52[c]	5028.51
ODN(M_{Os}3′-1)	5′-AAA AAA GCG M_{Os}AA AAA-3′	5027.52[c]	5027.66
ODN(M_{Os}3′-2)	5′-AAA AAA GCG AM_{Os}A AAA-3′	5027.52[c]	5027.71
ODN(T5′-1)	5′-AAA AAT GCG AAA AAA-3′	—	—
ODN(T5′-2)	5′-AAA ATA GCG AAA AAA-3′	—	—
ODN(T3′-1)	5′-AAA AAA GCG TAA AAA-3′	—	—
ODN(T3′-2)	5′-AAA AAA GCG ATA AAA-3′	—	—
ODN(T_{Os}5′-1)	5′-AAA AAT_{Os} GCG AAA AAA-3′	5028.89[c]	5028.87
ODN(T_{Os}5′-2)	5′-AAA AT_{Os}A GCG AAA AAA-3′	5028.89[c]	5029.77
ODN(T_{Os}3′-1)	5′-AAA AAA GCG T_{Os}AA AAA-3′	5028.89[c]	5028.65
ODN(T_{Os}3′-2)	5′-AAA AAA GCG AT_{Os}A AAA-3′	5028.89[c]	5027.31
cODN(D5′-1)	5′-TTT TTT CDC ATT TTT-3′	4743.25[b]	4743.99
cODN(D5′-2)	5′-TTT TTT CDC TAT TTT-3′	4743.25[b]	4743.15
cODN(D3′-1)	5′-TTT TTA CDC TTT TTT-3′	4743.25[b]	4743.01
cODN(D3′-2)	5′-TTT TAT CDC TTT TTT-3′	4743.25[b]	4744.31
p53(C)	5′-GCTATCTGAGCAGCGCTCATGGTGGGGG CAGCGCCTCACAACCTCCGTCATGTGCTGTGA-3′	—	—
p53(M)	5′-GCTATCTGAGCAGCGCTCATGGTGGGGGCAGM GCCTCACAACCTCCGTCATGTGCTGTGA-3′	—	—
cODN(p53)	5′-GTG AGG CDC TGC CCC-3′	4808.27[b]	4809.62

[a] Mass spectra were taken by MALDI-TOF MS with 2′,3′,4′-trihydroxyacetophenone as a matrix.
[b] These values were calculated by $[M-H]^-$.
[c] These values were calculated by $[M-OH]^-$.

Table 2. Melting temperatures of D-containing ODNs

Duplexes	T_m/°C[a]
ODN(C)/cODN(G)	48.6
ODN(C)/cODN(A)	35.1
ODN(C)/cODN(D)	33.2
ODN(M)/cODN(D)	31.9
ODN(M_{Os})/cODN(D)	35.5

[a] 2.5 μM duplexes in 50 mM sodium phosphate and 0.1 M sodium chloride (pH 7.0). Detection wavelength was at 260 nm.

Table 3. Fluorescence quantum yields of DAN in ODNs

	Φ_F[a]
cODN(D)	0.068
ODN(T)/cODN(D)	0.068
ODN(C)/cODN(D)	0.075
ODN(M)/cODN(D)	0.061
ODN(T_{Os})/cODN(D)	0.035
ODN(M_{Os})/cODN(D)	0.018

[a] **cODN(D)** hybridized with ODNs was measured (2.5 μM in 50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, 25 °C). λ_{ex} = 390 nm, λ_{em} = 515 nm.

interest could be assessed fluorometrically without any sequence dependence using osmium complexation. Therefore, this phenomenon would be applicable to a fluorescent epigenotyping method.

2.3. Detection of methylcytosine in p53 sequence

We prepared a 60-mer DNA strand, 5′-d(…GGCAGXGCCTC…)-3′, which contained a methylation hotspot (X = M or C) at codon 175 in exon 5 of the p53 gene (**p53(X)**).[20] We treated this DNA with the osmium oxidation reagents described above. After desalting through a gel filter, the recovered DNA was hybridized with a D-containing DNA probe, **cODN(p53)**, in a 96-well microliter plate, and its fluorescence intensity at 510 nm was evaluated. Compared to the control sample, a small difference in the fluorescence intensity was observed for the nonmethylated sample, whereas the fluorescence of the methylated sample was markedly decreased (Fig. 5). Using this fluorescent system, cytosine methylation at the sequence of interest can be effectively analyzed.

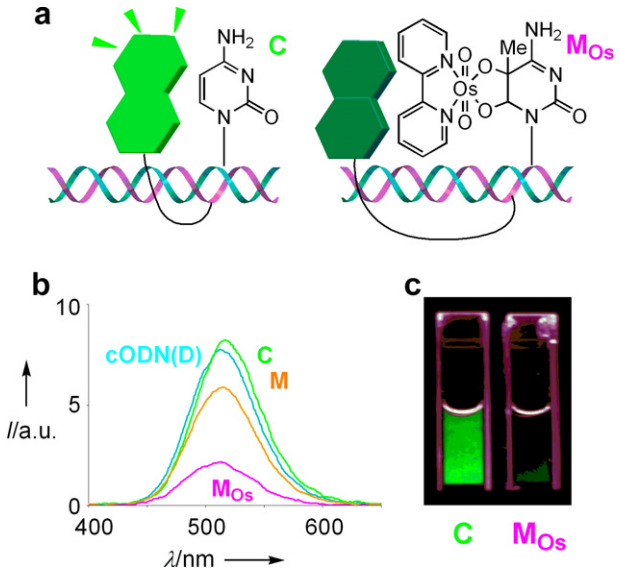


Figure 2. Fluorescence quenching by 5-methylcytosine-selective osmium complexation: (a) a new way to distinguish methylcytosine from cytosine using a fluorophore-tethered modified base; (b) fluorescence spectra of 2.5 μM **cODN(D)** (light blue line) hybridized with 2.5 μM **ODN(C)** (green line), **ODN(M)** (orange line), and **ODN(M_{Os})** (magenta line) in 50 mM sodium phosphate (pH 7.0) and 0.1 M sodium chloride at 25 °C. Excitation was at 390 nm; and (c) fluorescence image of **ODN(C)/cODN(D)** (left) and **ODN(M_{Os})/cODN(D)** (right). The image was taken using a transilluminator (366 nm).

Table 4. Fluorescence analysis for D-containing ODNs[a]

	τ_1/ns (α_1)	τ_2/ns (α_2)	τ_3/ns (α_3)	χ^2
ODN(M)/cODN(D)	—	1.156 (60)	3.352 (40)	0.96
ODN(M_{Os})/cODN(D)	0.043 (50)	1.448 (29)	3.814 (21)	1.06

[a] **cODN(D)** hybridized with **ODN(M)** or **ODN(M_{Os})** was measured (2.5 μM in 50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, 25 °C). λ_{ex} = 337 nm, λ_{em} = 510 nm.

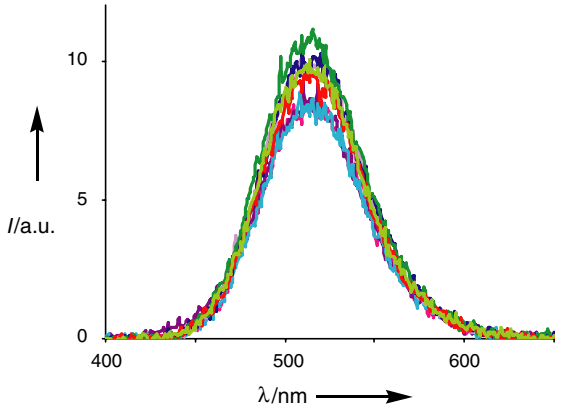


Figure 3. Fluorescence spectrum of the duplexes with different distances between M and D, **ODN(M5′-1)/cODN(D5′-1)** (blue line), **ODN(M5′-2)/cODN(D5′-2)** (red line), **ODN(M3′-1)/cODN(D3′-1)** (pink line), **ODN(M3′-2)/cODN(D3′-2)** (light blue line), **ODN(M_{Os}5′-1)/cODN(D5′-1)** (green line), **ODN(M_{Os}5′-2)/cODN(D5′-2)** (light green line),**ODN(M_{Os}3′-1)/cODN(D3′-1)** (purple line),**ODN(M_{Os}3′-2)/cODN(D3′-2)** (light purple line). Reaction solutions containing 2.5 μM duplexes and 0.1 M sodium chloride in 50 mM sodium phosphate (pH 7.0) were excited at 390 nm at 25 °C.

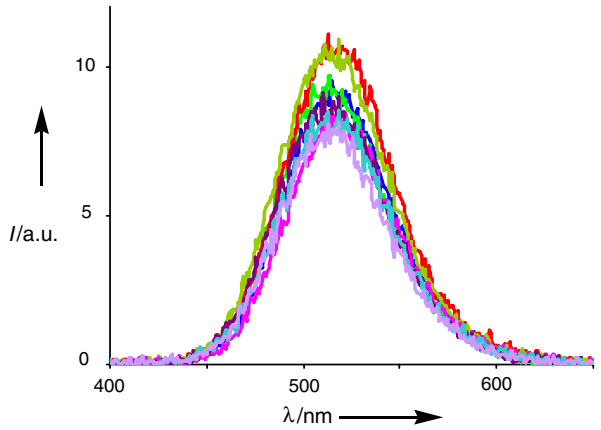


Figure 4. Fluorescence spectrum of the duplexes with different distances between T and D, **ODN(T5′-1)/cODN(D5′-1)** (blue line), **ODN(T5′-2)/cODN(D5′-2)** (red line), **ODN(T3′-1)/cODN(D3′-1)** (pink line), **ODN(T3′-2)/cODN(D3′-2)** (light blue line), **ODN(T_{Os}5′-1)/cODN(D5′-1)** (green line), **ODN(T_{Os}5′-2)/cODN(D5′-2)** (light green line),**ODN(T_{Os}3′-1)/cODN(D3′-1)** (purple line),**ODN(T_{Os}3′-2)/cODN(D3′-2)** (light purple line). Reaction solutions containing 2.5 μM duplexes and 0.1 M sodium chloride in 50 mM sodium phosphate (pH 7.0) were excited at 390 nm at 25 °C.

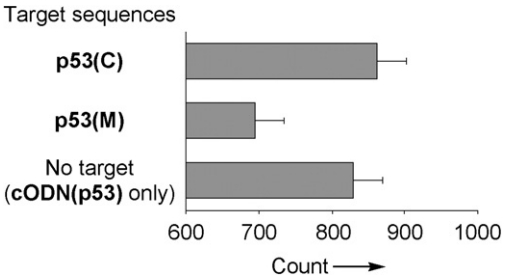


Figure 5. Distinction between cytosine and methylcytosine at a p53 methylation hotspot using the fluorescence quenching of **cODN(p53)** (50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, 25 °C). Excitation was at 380 ± 10 nm, and the fluorescence intensity was measured through 510 ± 5 nm band-pass filter. The data points represent the averages of five experimental results, and the 10% error bars are retained from the individual sets.

3. Conclusion

In conclusion, we have synthesized a DAN-labeled nucleoside, D, whose fluorescence is very sensitive to osmium complexation at the 5-methylcytosine site opposite D. This fluorescence quenching enables us to distinguish 5-methylcytosines from cytosines. Thus, the combination of a DAN-labeled DNA probe and osmium complexation would be useful as a fluorescent epigenotyping system.

4. Experimental

^{1}H NMR spectra were measured with Varian Mercury 400 (400 MHz) spectrometer. ^{13}C NMR spectra were measured with JEOL JNM α-500 (125 MHz) spectrometer. Coupling constants (*J* value) are reported in Hertz.

The chemical shifts are expressed in ppm downfield from tetramethylsilane, using residual chloroform (δ = 7.24 in ^{1}H NMR, δ = 77.0 in ^{13}C NMR) as an internal standard. FAB mass spectra were recorded on JEOL JMS DX-300 spectrometer or JEOL JMS SX-102A spectrometer. Masses of oligodeoxyribonucleotides were determined with a MALDI-TOF mass spectroscopy (PerSeptive Biosystems Voyager Elite, acceleration voltage 21 kV, negative mode) with 2′,3′,4′-trihydroxyacetophenone as matrix, using dT_8 ([M−H]$^-$ 2370.61) and dT_{17} ([M−H]$^-$ 5108.37) as an internal standard. HPLC was performed on a Cosmosil 5C-18AR or CHEMCOBOND 5-ODS-H column (4.6× 150 mm) with a Gilson Chromatography Model 305 using a UV detector Model 118 at 254 nm.

4.1. Synthesis

4.1.1. Compound 2. To a solution of *O*-(*tert*-butyldimethylsilyl)propargyl alcohol (2.31 g, 13.5 mmol) in THF (40 mL) was added 1.6 M *n*-BuLi (8.48 ml, 13.5 mmol) at −78 °C, and stirred at 0 °C for 30 min. 6-Dimethylamino-2-naphthaldehyde (1.50 g, 7.54 mmol) in THF (15 mL) was added to the mixture at −78 °C and then stirred at room temperature for 30 min. After the reaction was quenched with NH_4Cl aq at 0 °C, the mixture was evaporated under reduced pressure. The reaction mixture was extracted with NH_4Cl aq and ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The crude product was purified by silica gel column chromatography (hexane/ethyl acetate = 6:1) to yield the adduct (2.88 g, 7.49 mmol, 96%) as a yellow solid: ^{1}H NMR ($CDCl_3$) δ 7.82 (s, 1H), 7.70 (d, 1H, *J* = 9.2 Hz), 7.66 (d, 1H, *J* = 8.6 Hz), 7.52 (dd, 1H, *J* = 1.8, 8.6 Hz), 7.17 (dd, 1H, *J* = 2.6, 9.2 Hz), 6.92 (s, 1H), 5.59 (s, 1H), 4.44 (d, 2H, *J* = 1.8 Hz), 3.05 (s, 6H), 2.36 (s, 1H), 0.92 (s, 9H), 0.13 (s, 6H): ^{13}C NMR ($CDCl_3$) δ 148.8, 134.7, 134.0, 128.9, 126.7, 126.3, 125.2, 125.0, 116.6, 106.4, 85.1, 84.8, 64.7, 51.8, 40.8, 25.8, 18.2, −5.2: MS (FAB), *m*/*z* 369[M$^+$]; HRMS (FAB) calculated for $C_{22}H_{31}O_2NSi$ [M$^+$] 369.2131; found: 369.2124.

A mixture of the adduct (2.88 g, 7.49 mmol) and 10% Pd/C (2.0 g) in methanol (100 mL) was stirred under hydrogen atmosphere for 9 h at room temperature. The mixture was filtered through Celite and then washed with methanol. The filtrate and washings were combined and evaporated under reduced pressure. The crude product was purified by silica gel chromatography (hexane/ethyl acetate = 5–3:1) to give the alcohol (1.74 g, 4.66 mmol, 65%) as a yellow solid: ^{1}H NMR ($CDCl_3$) δ 7.71–7.64 (m, 3H), 7.39 (d, 1H, *J* = 9.5 Hz), 7.19 (d, 1H, *J* = 7.7 Hz), 6.98 (br s, 1H), 4.81 (t, 1H, *J* = 6.0 Hz), 3.68 (dt, 2H, *J* = 5.9, 1.6 Hz), 3.05 (s, 6H), 1.95–1.89 (m, 2H), 1.70–1.58 (m, 4H), 0.91 (m, 9H), 0.07 (m, 6H): ^{13}C NMR ($CDCl_3$) δ 148.6, 138.5, 134.4, 128.7, 126.6, 126.5, 124.5, 124.2, 116.6, 106.6, 74.4, 63.4, 40.9, 36.2, 29.2, 25.9, 18.3, −5.4: MS (FAB) *m*/*z* 373 [M$^+$]; HRMS (FAB) calculated for $C_{22}H_{35}O_2NSi$ [M$^+$] 373.2437; found: 373.2439.

To a solution of the alcohol (189 mg, 0.51 mmol) in dichloromethane (4 mL) was added flame-dried molecular sieves (4A, 1/16, 100 mg), 4-methylmorpholine *N*-oxide (90 mg, 0.77 mmol), and tetrapropylammonium perruthenate (18 mg, 0.05 mmol) at 0 °C, and the mixture was stirred at room temperature for 2 h. After the reaction was completed, the mixture was diluted with diethyl ether (80 mL) and Florisil (150–250 μm, 60–100 mesh, 100 mg) was added to the solution and stirred at room temperature for 15 min. The mixture was filtered through Celite and washed with diethyl ether. The filtrate and washings were combined and evaporated under reduced pressure. The residue was extracted with Na_2SO_3 aq and ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The crude product was purified by silica gel column chromatography (hexane/ethyl acetate = 5:1) to yield the ketone (162 mg, 0.44 mmol, 87%) as a yellow solid: ^{1}H NMR ($CDCl_3$) δ 8.35 (d, 1H, *J* = 1.5 Hz), 7.95 (dd, 1H, *J* = 1.8, 8.8 Hz), 7.79 (d, 1H, *J* = 9.2 Hz), 7.63 (d, 1H, *J* = 8.8 Hz), 7.15 (dd, 1H, *J* = 2.7, 9.2 Hz), 6.87 (d, 1H, *J* = 2.4 Hz), 3.75 (t, 2H, *J* = 6.0 Hz), 3.14 (t, 2H, *J* = 7.1 Hz), 3.09 (s, 6H), 2.05–1.98 (m, 2H), 0.93 (s, 9H), 0.08 (s, 6H): ^{13}C NMR ($CDCl_3$) δ 99.6, 150.1, 137.5, 130.6, 129.7, 126.1, 125.0, 124.5, 116.2, 105.2, 62.4, 40.3, 34.5, 27.8, 25.9, 18.3, −5.3: MS (FAB) *m*/*z* 371 [M$^+$]; HRMS (FAB) calculated for $C_{22}H_{33}O_2NSi$ [M$^+$] 371.2281; found: 371.2283.

To a solution of the ketone (2.87 g, 7.72 mmol) in tetrahydrofuran (20 mL) was added a 1.0 M solution of tetra-*n*-butylammonium fluoride in tetrahydrofuran (9.24 mL, 9.24 mmol), and the mixture was stirred for 6 h at room temperature. The reaction mixture was concentrated and purified by column chromatography on silica gel, eluting with hexane/ethyl acetate (1:1) to give **2** (1.90 g, 95 %) as a yellow solid:^{1}H NMR ($CDCl_3$) δ 8.35 (d, 1H, *J* = 1.6 Hz), 7.93 (dd, 1H, *J* = 1.8, 8.8 Hz), 7.78 (d, 1H, *J* = 9.0 Hz), 7.62 (d, 1H, *J* = 9.0 Hz), 7.16 (dd, 1H, *J* = 2.6, 9.2 Hz), 6.86 (d, 1H, *J* = 2.6 Hz), 3.77 (t, 2H, *J* = 5.9 Hz), 3.21 (t, 2H, *J* = 7.0 Hz), 3.10 (s, 6H), 2.15 (br, 1H), 2.09–2.02 (m, 2H): ^{13}C NMR ($CDCl_3$) δ 200.1, 150.1, 137.6, 130.6, 130.2, 129.9, 126.1, 124.9, 124.4, 116.1, 105.1, 62.3, 40.2, 35.0, 27.3: MS (FAB) *m*/*z* 257 [M$^+$]; HRMS (FAB) calculated for $C_{16}H_{19}O_2N$ [M$^+$] 257.1416; found: 257.1417.

4.1.2. Compound 3. Triphenylphosphine (262 mg, 1 mmol), 3′*O*,5′*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxyguanosine (496 mg, 1 mmol), and **2** (257 mg, 1 mmol) were placed in a dried flask under vacuum for 15 min. After the flask was refilled with argon, dry dioxane (5 mL) was added via syringe and stirred at 70 °C. Diethylazodicarboxylate (460 μL, 1 mmol) was added to the resulting suspension slowly via syringe. After the solution became homogeneous, the reaction mixture was stirred for an additional 6 h. The reaction mixture was concentrated and purified by column chromatography on silica gel, eluting with 1 : 2 hexane–ethyl acetate to give **3** (537 mg, 73%) as a yellow solid: ^{1}H NMR ($CDCl_3$) δ 8.35 (d, 1H, *J* = 1.6 Hz), 7.93 (dd, 1H,

J = 1.8, 8.8 Hz), 7.90 (s, 1H), 7.78 (d, 1H, J = 9.2 Hz), 7.62 (d, 1H, J = 8.8 Hz), 7.16 (dd, 1H, J = 2.6, 9.2 Hz), 6.87 (d, 1H, J = 2.4 Hz), 6.31 (t, 1H, J = 6.6 Hz), 4.80 (br, 2H), 4.64 (t, 2H, J = 6.0 Hz), 4.58 (dt, 1H, J = 3.5, 5.7 Hz), 3.99–3.96 (m, 1H), 3.81 (dd, 1H, J = 4.2, 11.2 Hz), 3.75 (dd, 1H, J = 3.3, 11.0 Hz), 3.31 (t, 2H, J = 7.0 Hz), 3.10 (s, 6H), 2.56 (ddd, 1H, J = 5.9, 7.14, 13.0 Hz), 2.38–2.31 (m, 3H), 0.91 (d, 18H, J = 1.8 Hz), 0.10 (s, 6H), 0.08 (d, 6H, J = 1.1 Hz): ^{13}C NMR($CDCl_3$) δ 198.9, 161.2, 159.3, 153.5, 150.1, 137.5, 137.3, 130.6, 130.4, 129.8, 126.0, 125.0, 124.4, 116.1, 115.7, 105.2, 87.5, 83.5, 71.9, 66.2, 62.7, 62.0, 40.8, 40.3, 34.5, 25.9, 25.7, 23.7, 18.3, 17.9, 14.3, −4.8, −4.9, −5.5, −5.6: MS (FAB) m/z 734 [M^+]; HRMS (FAB) calculated for $C_{38}H_{58}O_5N_6Si_2$ [M^+] 734.4007; found: 734.4017.

4.1.3. Compound 4. To a solution of **3** (1.66 g, 2.26 mmol) in tetrahydrofuran (20 mL) was added a 1.0 M solution of tetra-*n*-butylammonium fluoride in tetrahydrofuran (6.77 mL), and the mixture was stirred for 2 h at room temperature. The reaction mixture was concentrated and purified by column chromatography on silica gel, eluting with 30:1 chloroform/methanol to give **4** (830 mg, 73%) as a yellow solid: ^{1}H NMR ($CDCl_3$) δ 8.34 (d, 1H, J = 1.5 Hz), 7.92 (dd, 1H, J = 1.8, 8.6 Hz), 7.77 (d, 1H, J = 9.2 Hz), 7.62–7.60 (m, 2H), 7.14 (dd, 1H, J = 2.6, 9.2 Hz), 6.85 (d, 1H, J = 2.6 Hz), 6.21 (dd, 1H, J = 5.7, 9.5 Hz), 4.92 (br, 2H), 4.74 (d, 1H, J = 5.1 Hz), 4.62 (t, 2H, J = 6.0 Hz), 4.20–4.19 (m, 1H), 3.95 (dd, 1H, J = 1.6, 12.6 Hz), 3.75 (dd, 1H, J = 1.8, 12.8 Hz), 3.29 (t, 2H, J = 7.1 Hz), 3.09 (s, 6H), 2.99–2.96 (m, 1H), 2.33 (quintet, 2H, J = 6.4 Hz), 2.27–2.22 (m, 1H): ^{13}C NMR ($CDCl_3$) δ 199.0,161.7, 158.7,152.2,150.2,138.8,137.6, 130.7,130.3, 130.0,126.1, 125.0,124.4,116.8,116.2,105.2,89.2,87.3,73.1,66.4,63.4,40.3, 34.5,25.3,23.7: MS(FAB) m/z 506 [M^+]; HRMS(FAB) calculated for $C_{26}H_{30}O_5N_6$ [M^+] 506.2278; found: 506.2285.

4.1.4. Compound 5. A solution of **4** (253 mg, 0.5 mmol) and *N*,*N*-dimethylformamide dimethylacetal (1 mL) in *N*,*N*-dimethylformamide (3 mL) was stirred for 6 h at room temperature. The reaction mixture was concentrated to a yellow oil and purified by column chromatography on silica gel, eluting with 10:1 ethyl acetate–methanol to give the 2-NH_2-protected nucleoside (266 mg, 94%) as a yellow solid: ^{1}H NMR (DMSO-d_6) δ 8.60 (s, 1H), 8.44 (d, 1H, J = 1.1 Hz), 8.27 (s, 1H), 7.86 (d, 1H, J = 9.2 Hz), 7.82 (dd, 1H, J = 1.6, 8.6 Hz), 7.65 (d, 1H, J = 8.8 Hz), 7.24 (dd, 1H, J = 2.7, 9.2 Hz), 6.92 (d, 1H, J = 2.4 Hz), 6.34 (dd, 1H, J = 6.2, 7.7 Hz), 5.29 (br, 1H), 5.09 (br, 1H), 4.59 (t, 2H, J = 6.6 Hz), 4.41–4.39 (m, 1H), 3.88–3.86 (m, 1H), 3.61 (dt, 1H, J = 4.6, 11.7 Hz), 3.52 (ddd, 1H, J = 4.6, 5.5, 10.8 Hz), 3.25 (t, 2H, J = 7.1 Hz), 3.11 (s, 3H), 3.04 (s, 6H), 2.99 (s, 3H), 2.66 (ddd, 1H, J = 5.9, 7.7, 13.4 Hz), 2.25 (ddd, 1H, J = 2.9, 6.2, 13.2 Hz), 2.17 (quintet, 2H, J = 6.8 Hz): ^{13}C NMR (DMSO-d_6) δ 198.5, 161.9, 160.0, 158.3, 153.5, 150.3, 140.0, 137.4, 130.7, 130.0, 129.9, 126.0, 124.7, 124.0, 117.1, 116.5, 104.9, 88.0, 83.6, 79.3, 71.0, 65.4, 62.0, 40.5, 40.0, 34.6, 34.0, 23.8: MS (FAB) m/z 561 [M^+]; HRMS (FAB) calculated for $C_{29}H_{35}O_5N_7$ [M^+] 561.2700; found: 561.2692.

To a solution of the 2-NH_2-protected nucleoside (84 mg, 0.15 mmol) in pyridine (3 mL) was added 4,4′-dimethoxytrityl chloride (56 mg, 0.17 mmol), and the mixture was stirred for 2 h at 50 °C. The reaction mixture was concentrated and purified by column chromatography on silica gel, eluting with chloroform–methanol (30:1) to give the tritylated nucleoside (112 mg, 87%) as a yellow solid: ^{1}H NMR (DMSO-d_6) δ 8.57 (s, 1H), 8.44 (s, 1H), 8.16 (s, 1H), 7.87–7.81 (m, 2H), 7.65 (d, 1H, J = 8.8 Hz), 7.32–7.15 (m, 10H), 6.92 (d, 1H, J = 2.2 Hz), 6.79–6.74 (m, 4H), 6.37 (t, 1H, J = 6.4 Hz), 5.37 (d, 1H, J = 4.4 Hz), 4.59 (t, 2H, J = 7.0 Hz), 4.44–4.42 (m, 1H), 3.90–3.95 (m, 1H), 3.69 (s, 6H), 3.26 (t, 2H, J = 7.1 Hz), 3.15 (dd, 1H, J = 4.8, 10.6 Hz), 3.12–3.11 (m, 1H), 3.09 (s, 3H), 3.04 (s, 6H), 3.00 (s, 3H), 2.87–2.73 (m, 1H), 2.37–2.30 (m, 1H), 2.18 (quintet, 2H, J = 6.6 Hz): ^{13}C NMR (DMSO-d_6) δ 198.5, 160.0, 158.14, 158.10, 158.06, 153.5, 150.3, 145.0, 139.8, 137.4, 135.71, 135.67, 130.7, 130.0, 129.9, 129.8, 129.7, 127.8, 126.7, 126.0, 124.7, 124.0, 117.2, 116.5, 113.2, 104.9, 86.0, 85.6, 83.0, 79.3, 70.8, 65.5, 64.4, 55.08, 55.07, 40.3, 40.0, 34.7, 34.1, 23.8: MS (FAB) m/z 864 [$(M+H)^+$]; HRMS (FAB) calculated for $C_{50}H_{54}O_7N_7$ [$(M+H)^+$] 864.4085; found: 864.4083.

To a solution of the tritylated nucleoside (70 mg, 81.0 μmol) in acetonitrile (900 μL) were added 2-cyanoethyl tetraisopropyldiphosphoramidite (31 μL, 98.9 μmol) and tetrazole (7 mg, 98.9 μmol). The reaction mixture was stirred at room temperature for 2 h. The mixture of **5** was filtered and used with no further purification.

4.1.5. Oligodeoxyribonucleotide synthesis and characterization. Oligodeoxyribonucleotides (ODNs) were synthesized by the conventional phosphoramidite method by using an Applied Biosystems 392 DNA/RNA synthesizer. Synthesized ODNs were purified by reverse phase HPLC on a 5-ODS-H column (10× 150 mm, elution with a solvent mixture of 0.1 M triethylammonium acetate (TEAA), pH 7.0, linear gradient over 30 min from 5% to 20% acetonitrile at a flow rate of 3.0 mL/min). An aliquot of purified ODN solution was fully digested with calf intestine alkaline phosphatase (50 U/mL), snake venom phosphodiesterase (0.15 U/mL), and P1 nuclease (50 U/mL) at 37 °C for 3 h. Digested solution was analyzed by HPLC on Cosmosil 5C-18AR or CHEMCOBOND 5-ODS-H column (4.6× 150 mm), elution with a solvent mixture of 0.1 M TEAA, pH 7.0, linear gradient over 20 min from 0% to 20% acetonitrile at a flow rate of 1.0 mL/min. Concentration of each ODN was determined by comparing a given peak area with those of 0.1 mM standard solution containing dA, dC, dG, and dT. Each ODN was characterized by MALDI-TOF MS.

4.2. Photophysical and photochemical measurements

4.2.1. Melting temperature (T_m) measurements. All T_ms of the ODNs (2.5 μM, final duplex concentration) were taken in 50 mM sodium phosphate buffer (pH 7.0) containing 100 mM sodium chloride. Absorbance vs temperature profiles were measured at 260 nm using a

Shimadzu UV-2550 spectrophotometer equipped with a Peltier temperature controller using 1 cm path length cell. The absorbance of the samples was monitored at 260 nm from 5 to 90 °C with a heating rate of 1 °C/min. From these profiles, first derivatives were analyzed to determine T_m values.

4.2.2. Osmium oxidation of oligodeoxyribonucleotides. Osmium oxidation and fluorescence measurement of ODNs were carried out as follows: The ODN (5 μM) to be examined was incubated in a solution of 5 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM bipyridine, and 1 mM EDTA in 100 mM Tris–HCl buffer (pH 7.7) and 10% acetonitrile at 37 °C for 3 h. ODN samples were purified by reverse phase HPLC or through gel filters. Recovered ODN samples were hybridized with D-containing DNA probes to obtain 2.5 μM duplex solutions in 50 mM sodium phosphate buffer (pH 7.0) containing 100 mM sodium chloride. Fluorescence spectra of the duplexes were taken at 25 °C using a Shimadzu RF-5300PC spectrofluorophotometer and 1 cm path length cell. The excitation bandwidth was 1.5 nm. The emission bandwidth was 1.5 nm.

4.2.3. Fluorescence quantum yields. The emission bandwidth was 1.5 nm. The fluorescence quantum yields (Φ_F) were determined using 9,10-diphenylanthracene as a reference with a known Φ_F of 0.95 in ethanol according to the reference, *J. Phys. Chem.* **1976**, *80*, 969–974. The area of the emission spectrum was integrated using the software available in the instrument, and the quantum yield is calculated according to the following equation,

$$\Phi_{F(S)}/\Phi_{F(R)} = [A_{(S)}/A_{(R)}] \times [(\mathrm{Abs})_{(R)}/(\mathrm{Abs})_{(S)}] \times [n^2_{(S)}/n^2_{(R)}] \quad (1)$$

Where, $\Phi_{F(S)}$ and $\Phi_{F(R)}$ are the fluorescence quantum yield of the sample and the reference, respectively. $A_{(S)}$ and $A_{(R)}$ are the area under the fluorescence spectra of the sample and the reference, respectively, $(\mathrm{Abs})_{(S)}$ and $(\mathrm{Abs})_{(R)}$ are the respective optical densities of the sample and the reference solution at the wavelength of excitation, and $n_{(S)}$ and $n_{(R)}$ are the values of refractive index for the respective solvents used for the sample (1.333) and the reference (1.383).

4.2.4. Fluorescence decay measurements. ODN solutions were prepared as described in the T_m measurement experiment. Fluorescence decay was measured by a two-dimensional photon-counting method with the picosecond fluorescence lifetime measurement system (C4780, Hamamatsu). A N_2 laser (337 nm) was used as the excitation light source. The fluorescence emission was collected at 510 nm.

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