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PAPER

Facile synthesis of hydroxymethylcytosine-containing oligonucleotides and their reactivity upon osmium oxidation†

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DNA strands containing a 5-hydroxymethylcytosine (^{hm}C), which have recently been found in neuron cells and embryonic stem cells, were synthesized through a facile synthetic technique. The ^{hm}C-containing strands were efficiently oxidized at ^{hm}C using an osmium oxidation assay. The ^{hm}C was oxidized as easily as 5-methylcytosine, which can be distinguished from unmethylated cytosine.

Introduction

5-Hydroxymethylcytosine (^{hm}C) is a newly discovered natural nucleobase, which is abundant in Purkinje neuron cells[1] and mouse embryonic stem cells.[2] ^{hm}C is induced by modification of 5-methylcytosine (^{m}C) with TET proteins, which have potential roles in epigenetic regulation. Given the critical role of ^{m}C in epigenetic regulation, ^{hm}C may have an important biological role *in vivo*, such as acting as an intermediate in a pathway for active DNA demethylation.

To analyze and understand ^{hm}C in DNA, effective detection methods for ^{hm}C are required. The bisulfite technique, which is a conventional method for distinguishing cytosine (C) from ^{m}C,[3] has already been reported to show that neither ^{hm}C nor ^{m}C in DNA undergo deamination, although it is likely that ^{hm}C was converted into cytosine 5-methylenesulfonate by bisulfite treatment.[4,5] Osmium oxidation is an effective method for ^{m}C detection, which makes it possible to analyze ^{m}C in DNA sequence-selectively and ^{m}C-positively in a short time without any DNA scission.[6,7] ^{m}C is oxidized efficiently by exposure to a reaction mixture of potassium osmate, potassium hexacyanoferrate(III), and a bipyridine ligand, a stable methylcytosine glycol–osmate–bipyridine complex being formed, making possible a clear distinction from inefficient oxidation of unmethylated cytosine. Osmium oxidation may be effective not only for detection of ^{m}C but also for detection of ^{hm}C. Extending the range of ^{hm}C detection methods would be very useful for the next generation of epigenetic studies.

Herein, we report a facile preparation protocol for ^{hm}C-containing oligodeoxynucleotides (ODNs) and their reactivity upon osmium oxidation. The ^{hm}C in ODNs showed high reactivity upon osmium oxidation. The osmium oxidation method gave a stable ternary complex at ^{hm}C in ODNs.

Results and discussion

Several routes for the synthesis of ^{hm}C-containing ODNs have been reported.[8,9] They include troublesome synthetic steps and/or many purification processes. We initially developed a facile synthetic route for ^{hm}C-containing ODNs to obtain an efficient supply of ODNs for the chemical analysis of ^{hm}C (Scheme 1). The synthesis started from thymidine (**1**). The two hydroxy groups of **1** were initially protected with acetyl groups. After a simple extraction process, bromination of the C5 methyl group was carried out using 2,2′-azobisisobutyronitrile (AIBN) and *N*-bromosuccinimide. The crude product was converted into the modified thymidine **2** by mixing with 3-hydroxypropionitrile and subsequent purification with silica gel column chromatography. The C4 of **2** was activated using phosphorus oxychloride and 1,2,4-triazole, and then aminated with ammonia in dioxane. After the acetyl groups were completely removed using ammonia in methanol, the C4 amino group was protected with a di(n-butyl)formamidine group[10,11] to give a 5-hydroxymethyl-2′-deoxycytidine derivative **3**. The nucleoside **3** was converted into the phosphoramidite form **4** for the DNA autosynthesizer. The synthesis of ^{hm}C-containing ODN was achieved through the conventional phosphoramidite method in a DNA autosynthesizer and subsequent deprotection in 28% ammonia at 70 °C for 16 h (Fig. 1).

^{hm}C-containing ODN formed a stable duplex with the complementary DNA. For example, the melting temperature (T_m) of the duplex formed by **ODN1(^{hm}C)** 5′-CAATG^{hm}CGCTAGT-3′ and the complementary ODN **ODN1′(^{hm}C)** 5′-ACTAG^{hm}CGCATTG-3′ (1 μM) was 50 °C in 50 mM sodium phosphate (pH = 7.0) and 100 mM sodium chloride, which was almost the same value as that of a methylated duplex **ODN1(^{m}C)/ODN1′(^{m}C)** (49 °C). The T_m values of hemimethylated duplexes **ODN1(^{hm}C)/ODN1′(^{m}C)** and **ODN1(^{m}C)/ODN1′(^{hm}C)** were 50 °C and 49 °C, respectively,

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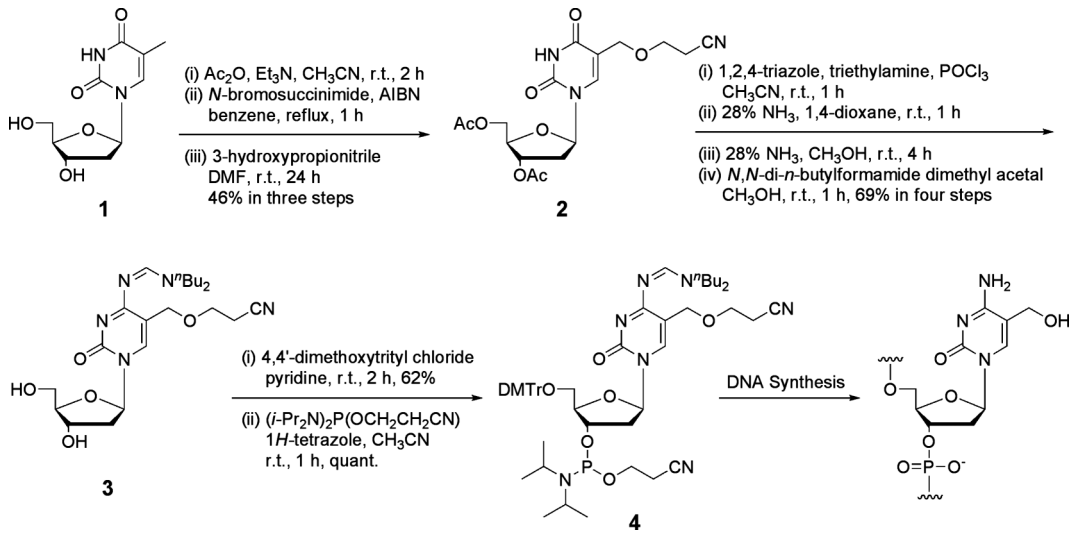


Scheme 1 Facile synthesis of ^{hm}C-containing ODN.

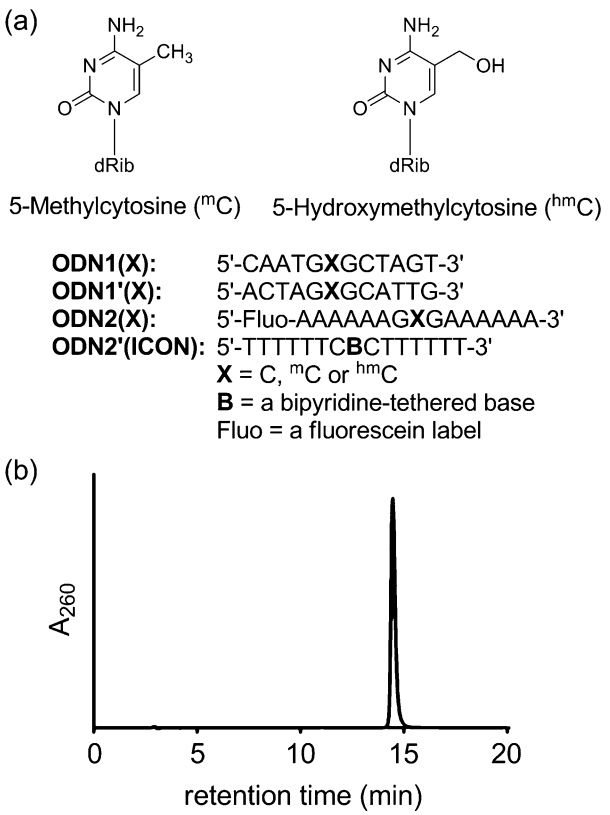


Fig. 1 ODNs containing 5-hydroxymethylcytosine. (a) ^{hm}C- or ^{m}C-containing ODN sequences used in this study. (b) Reverse phase HPLC profile of synthesized **ODN1(^{hm}C)**. The sample was eluted with a solvent mixture of 0.1 M triethylammonium acetate (pH = 7.0), linear gradient over 20 min from 5% to 20% acetonitrile.

suggesting that the addition of one hydroxy group has little influence on the duplex stability in this case. The CD spectrum of the duplex **ODN1(^{hm}C)/ODN1′(^{hm}C)** exhibits a positive Cotton effect at 270 nm and a negative Cotton effect at 250 nm, suggesting that the duplex exhibits a typical B-type DNA structure.

The enzymatic digestion of ^{hm}C-containing ODNs showed almost the same result as that of ^{m}C-containing ODNs. In treatment with phosphodiesterase and alkaline phosphatase for 2 h at 37 °C, the ^{hm}C-containing ODN **ODN1(^{hm}C)** was digested completely to nucleosides. Treatment of **ODN1(^{hm}C)/ODN1′(^{hm}C)** with the methylation-sensitive restriction enzyme *Hha*I at 37 °C for 1 h showed no cleavage for the methylated duplex **ODN1(^{m}C)/ODN1′(^{m}C)**, while the unmethylated duplex **ODN1(C)/ODN1′(C)** was completely digested.

Osmium oxidation is an excellent method for detection of 5-methylated pyrimidines.[7,12,13] Based on this reaction, we have reported the sequence-selective oxidation of ^{m}C.[14] The ^{hm}C nucleotide may also be reactive on osmium oxidation, although the effect of addition of a hydroxy group on the reactivity is unknown. If ^{hm}C shows oxidation reactivity as high as that of ^{m}C, ^{hm}C detection using osmium oxidation will be possible as with the detection of ^{m}C. The osmium oxidation of ^{hm}C-containing ODNs was tested under the reaction conditions as follows: 5 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), and 100 mM bipyridine in 100 mM Tris-HCl buffer (pH = 7.7), 1 mM EDTA, and 10% acetonitrile. The fluorescein-labeled ODN, 5′-Fluo-AAAAAAGXGAAAAAA-3′ (**ODN2(X)**, X = ^{hm}C, ^{m}C, or C) was added to the reaction mixture and incubated at 0 °C for a period of 5 min. Then, an aliquot of the reaction sample was treated with hot piperidine to cleave the strands at the reaction sites. After the reaction, we observed new retarded bands for **ODN2(^{hm}C)** and **ODN2(^{m}C)** in the PAGE analysis (Fig. 2a), whereas the intensity of the new band was weak for **ODN2(C)**. After hot piperidine treatment of an aliquot of the reaction product, these retarded bands disappeared, and instead new bands appeared in the lanes of **ODN2(^{hm}C)** and **ODN2(^{m}C)**. The band mobility, agreeing with that of the short fragment 5′-Fluo-AAAAAAGp-3′ (p = 3′-phosphate end), indicated that ^{hm}C and ^{m}C were selectively oxidized and subsequently cleaved (Fig. 2b). The reactivity of **ODN2(^{hm}C)** (61%) was almost the same as that observed for **ODN2(^{m}C)** (72%). The reactivity of **ODN2(C)** was much lower (28%). The MALDI-TOF MS data of **ODN2(^{hm}C)** after oxidation suggested that a stable hydroxymethylcytosine

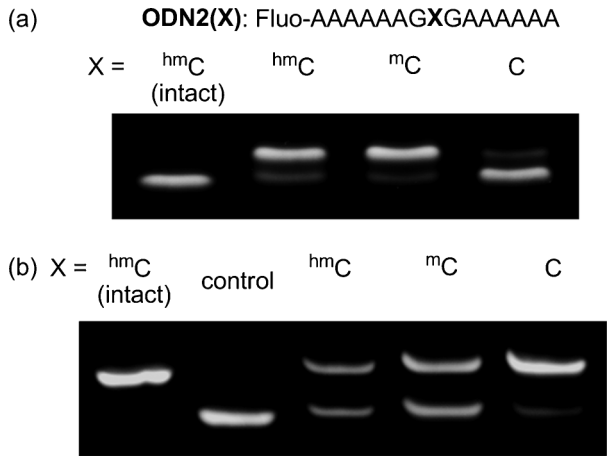
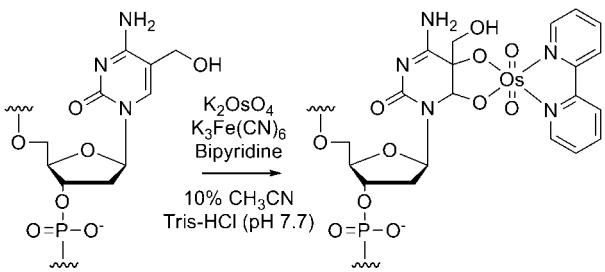


Fig. 2 Osmium oxidation of ^{m}C- and ^{hm}C-containing ODN. (a) Formation of adduct. The fluorescein-labeled **ODN2(X)** (5 μM) to be examined was incubated in a solution of 5 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM bipyridine, 0.5 mM EDTA, and 1 M sodium chloride in 50 mM Tris-HCl buffer (pH 7.7) and 10% acetonitrile at 0 °C for 5 min. (b) Cleavage at the oxidation site by hot piperidine treatment. The reaction sample was heated in 50 μL of 10% piperidine at 90 °C for 20 min. The control DNA was 5′-AAAAAAGp-3′.

glycol–dioxidoosmium–bipyridine ternary complex was formed in the ODN ([M – OH]$^-$, calcd. 5604.99, found 5604.45; [M – 2O – H]$^-$, calcd. 5588.99, found 5590.14; [M – 3O – H]$^-$, calcd. 5572.99, found 5575.40) (Scheme 2). The complex formation was supported by the generation of a retarded band in the PAGE analysis after osmium oxidation. Another fluorescein-labeled ODN, 5′-Fluo-AAA^{hm}CGAG^{hm}CGAAAAAA-3′ (**ODN3(^{hm}C)**) also exhibited osmium oxidation at each ^{hm}C (Fig. S1). Osmium oxidation was observed at ^{hm}Cs regardless of the number of ^{hm}Cs in the sequences.



Scheme 2 Osmium oxidation of an ^{hm}C-containing ODN.

The structure of the complex was determined by X-ray crystallography of the Os-oxidized product of hydroxythymidine, which was prepared by the deblocking of **2**. The complex obtained from the osmium oxidation of hydroxythymidine was a hydroxythymidine glycol–dioxidoosmium–bipyridine ternary complex, which had a slightly distorted octahedral geometry with coplanar glycol oxygens and bipyridine nitrogens (Fig. 3). The two Os–O double bonds were *trans*. The structure of glycol in the complex was the 5*R*,6*S*-configuration. In addition, the hydroxymethyl group extended from the nucleobase did not coordinate to an osmium complex core. The *N*-glycosyl bond was in an *anti*-conformation, and the puckering of the ribose ring remained in the C2′-*endo* conformation. The ternary complex structure is close to that reported for the osmium oxidation of thymidine.[15]

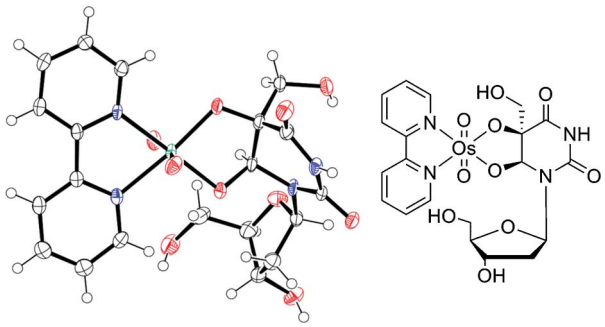


Fig. 3 The crystal structure of the (5*R*,6*S*)-hydroxythymidine glycol—dioxidoosmium(VI)–bipyridine ternary complex.

We have developed a functional nucleoside in which an adenine base and a bipyridine ligand were connected with an alkyl chain. The ODN containing this nucleotide was called the ICON probe, which is useful for sequence-selective detection of ^{m}C in DNA mediated by osmium complexation. We next prepared a fluorescein-labeled DNA containing C, ^{m}C, or ^{hm}C, **ODN2(X)**, and the ICON probe was designed so that a bipyridine-labeled adenine base (B) was positioned opposite the target X base, 5′-TTTTTTCBCTTTTTT-3′ (**ODN2′(ICON)**) (Fig. 4a). The reaction mixture was incubated at 25 °C for a period of 10 min. Reaction of **ODN2(C)** was not observed in the mixture containing oxidants and **ODN2′(ICON)**. Conversely, the reaction of **ODN2(^{hm}C)** in the presence of both potassium osmate and

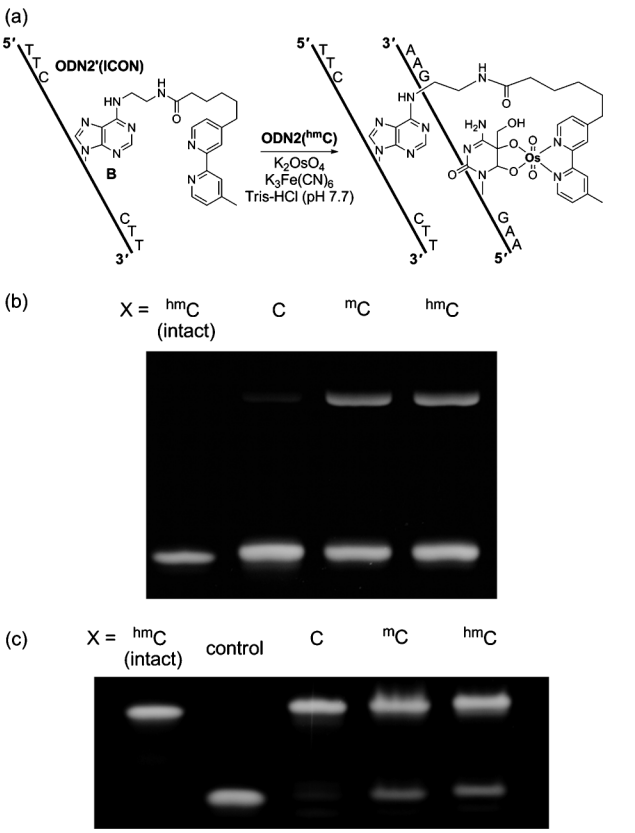


Fig. 4 Osmium oxidation mediated by the ICON probe. (a) ICON probe and the interstrand cross-link with ^{hm}C-containing ODN. (b) Interstrand cross-link formation. (c) PAGE analysis after hot piperidine treatment of the samples shown in (b).

ODN2′(ICON) produced a product with a higher molecular weight, which appeared as a band of low mobility on PAGE analysis (Fig. 4b). It was similar to the reaction product from **ODN2(^{m}C)** and **ODN2′(ICON)**. Treatment of the reaction product with hot piperidine eliminated the high-molecular-weight band and produced a new band that indicated cleavage of the strand at ^{hm}C (Fig. 4c). The appearance of this new band suggests that an interstrand cross-link, mediated by osmium complexation, occurred between **ODN2(^{hm}C)** and **ODN2′(ICON)**. The mass spectrometric data for the product generated after osmium complexation with **ODN2(^{hm}C)/ODN2′(ICON)** directly indicate the production of a cross-linked adduct ([M – OH]$^-$, calcd 10238.15, found 10236.40). The reactivity of **ODN2(^{hm}C)** in the presence of **ODN2′(ICON)** was 23%, close to that of **ODN2(^{m}C)** (24%), clearly distinguishable from that of **ODN2(C)** (4%). For effective oxidation of ^{hm}C, hybridization of the ICON probe with the target DNA sequence is essential. For example, a noncomplementary ODN, 5′-Fluo-AGCAA^{hm}CGAAGCAAAA-3′ (**ODN4(^{hm}C)**), exhibited osmium oxidation at ^{hm}C when the combination of potassium osmate, potassium hexacyanoferrate(III) and bipyridine was used, whereas oxidation was not observed at ^{hm}C in **ODN4(^{hm}C)** at all when **ODN2′(ICON)** was used in the presence of potassium osmate and potassium hexacyanoferrate(III), because **ODN2′(ICON)** does not hybridize with **ODN4(^{hm}C)** (Fig. S2).

Conclusions

In conclusion, we synthesized ODN strands containing ^{hm}C and investigated their reactivity on osmium oxidation. The osmium oxidation of ^{hm}C-containing ODN provided a 5-hydroxymethyl-2′-deoxycytidine glycol–dioxidoosmium–bipyridine ternary complex in the ODN. Characterization of the structure of the osmium-centered complex in the ODN would support new drug design for efficient DNA hydroxymethylation analysis. Although there still remain further aspects to be examined toward an easier-to-use hydroxymethylation analysis, such as distinguishing between ^{hm}C and ^{m}C, we anticipate that this new concept of effective hydroxymethylation analysis supported by the chemical basis will be the starting point for an epoch-making epigenotyping assay, which will supersede conventional methods.

Experimental

General

^{1}H, ^{13}C, and ^{31}P NMR spectra were measured with a Varian NMR system 500. Coupling constants (J value) are reported in hertz. The chemical shifts are shown in ppm downfield from tetramethylsilane, using residual chloroform ($\delta = 7.27$ in ^{1}H NMR, $\delta = 77.0$ in ^{13}C NMR) and methanol ($\delta = 3.30$ in ^{1}H NMR, $\delta = 49.0$ in ^{13}C NMR) as an internal standard. An external H_3PO_4 standard (δ = 0.00 ppm) was used for ^{31}P NMR measurements. ESI mass spectra were recorded on a Bruker BioApex II. MALDI-TOF mass spectra were measured with a Bruker Daltonics Reflex.

3′*O*,5′*O*′-Diacetyl-5-(2-cyanoethoxy)methyl-2′-deoxyuridine (2)

Thymidine (2.42 g, 10 mmol) was suspended in acetonitrile (20 mL). Triethylamine (8.4 mL, 60 mmol) and acetic anhydride (3.8 mL, 40 mmol) were added to the suspension. The mixture was stirred at room temperature for 2 h. After concentrating, ethyl acetate (100 mL) and brine (100 mL) were added to the residue. The product was extracted to the organic layer. The organic layer was washed with saturated sodium chloride (100 mL) twice, dried over magnesium sulfate, and filtered. The solvent was removed by evaporation, and the residue was dried under reduced pressure. *N*-Bromosuccinimide (1.96 g, 11 mmol) and benzene (20 mL) were added to the residue. The mixture was stirred and repeatedly degassed by reduction of the pressure and injection of nitrogen gas. After addition of AIBN (164 mg, 1 mmol), the mixture was refluxed for 1 h. The reaction mixture was cooled and the precipitate was removed by filtration. The solvent was removed by evaporation and the residue was dried under reduced pressure. *N*,*N*-Dimethylformamide (10 mL) and 3-hydroxypropionitrile (4.1 mL, 60 mmol) were added to the residue. The mixture was stirred at room temperature for 24 h. Ethyl acetate (100 mL) and brine (100 mL) were added to the reaction mixture. The product was extracted to the organic layer. The organic layer was washed with brine (100 mL) twice, dried over magnesium sulfate, and filtered. The solvent in the organic phase was removed by evaporation. The product was purified by silica gel column chromatography (2 : 1–1 : 0 ethyl acetate/hexane). The product **2** was obtained as a white foam (1.80 g, 46%): R_f 0.48 (ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 9.33 (br, 1H), 7.63 (t, J = 1.0, 1H), 6.31 (dd, J = 8.4, 5.5, 1H), 5.24–5.22 (m, 1H), 4.41–4.27 (m, 5H), 3.80–3.76 (m, 2H), 2.52 (ddd, J = 14.2, 5.6, 1.7, 1H), 2.24 (ddd, J = 14.2, 8.3, 6.4, 1H), 2.13 (s, 3H), 2.12 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 170.24, 170.23, 162.7, 150.1, 137.4, 117.8, 111.4, 85.3, 82.3, 74.2, 65.3, 64.9, 63.7, 37.4, 20.72, 20.65, 18.59; HRMS (ESI) calcd for $C_{17}H_{21}N_3O_8Na$ ([M + Na]$^+$) 418.1221, found 418.1221; UV (chloroform) λ_{max} 264 nm (ε 34260). Thymidine diacetate (719 mg, 22%) was also collected: R_f 0.63 (ethyl acetate).

4-*N*,*N*-Di-n-butylformamidine-5-(2-cyanoethoxy)methyl-2′-deoxycytidine (3)

Nucleoside **2** (1.80 g, 4.55 mmol) was dissolved in acetonitrile (80 mL). Ethyl acetate (6.98 mL, 50.1 mmol) and phosphorus oxychloride (0.85 mL, 9.1 mmol) were added to the solution with stirring. The reaction mixture was stirred at room temperature for 1 h. The solvent was evaporated, and ethyl acetate (100 mL) and brine (100 mL) were added to the residue. The product was extracted to the organic layer. The organic layer was washed with brine twice and dried over magnesium sulfate. The solvent was removed by evaporation, and the residue was dried under reduced pressure. 1,4-Dioxane (100 mL) and 28% ammonia (15 mL) were added to the residue. The mixture was stirred at room temperature for 1 h. The solvent was removed by evaporation. Methanol (15 mL) and 28% ammonia (15 mL) were added to the residue. The mixture was stirred at room temperature for 4 h. The solvent was removed by evaporation, and then the residue was azeotropically dried with methanol under reduced pressure. Methanol (20 mL) and *N*,*N*-di-n-butylformamide dimethyl acetal (4.64 g, 22.8 mmol) were added to the residue. The mixture was stirred at room temperature for 1 h. The solvent was removed by evaporation. The product was purified by silica gel column chromatography (0–10% methanol/ethyl acetate). The product **3** was obtained as a white solid (1.41 g, 69%): mp 67 °C; ^{1}H NMR (CD_3OD) δ 8.64 (s, 1H), 8.21 (s, 1H), 6.27 (t, J = 6.4, 1H), 4.50–4.44 (m, 2H), 4.37

(ddd, J = 6.4, 3.9, 3.9, 1H), 3.96 (m, 1H), 3.82 (dd, J = 12.1, 3.3 Hz, 1H), 3.75 (dd, J = 12.0, 3.9, 1H), 3.71 (t, J = 6.1, 2H), 3.60 (t, J = 7.6, 2H), 3.45 (t, J = 7.3, 2H), 2.71 (t, J = 6.0, 2H), 2.41 (ddd, J = 13.6, 6.2, 4.0, 1H), 2.17 (ddd, J = 13.4, 6.7, 6.7, 1H), 1.69–1.62 (m, 4H), 1.42–1.32 (m, 4H), 0.99–0.95 (m, 6H); ^{13}C NMR (CD_3OD) δ 171.5, 159.1, 158.3, 142.4, 119.7, 112.7, 89.0, 88.0, 71.9, 67.6, 66.3, 62.7, 53.4, 47.1, 42.3, 32.0, 30.2, 21.2, 20.7, 19.3, 14.2, 14.0; HRMS (ESI) calcd for $C_{22}H_{35}N_5O_5Na$ ([M + Na]$^+$) 472.2530, found 472.2530; UV (methanol) λ_{max} 325 nm (ε 45690), 233 nm (ε 42980).

Phosphoramidite (4)

Nucleoside **3** (1.41 g, 3.14 mmol) and 4,4′-dimethoxytrityl chloride (1.38 g, 4.08 mmol) were dissolved in pyridine, and the solution was stirred at room temperature for 2 h. Methanol (1 mL) was added to the reaction mixture, and the solvent was evaporated. Pyridine in the residue was removed by coevaporation with dichloromethane and hexane. The product was purified by silica gel column chromatography (1% triethylamine, 0–2% methanol/ethyl acetate). The product (1.46 g, 62%) was obtained as a white foam: mp 70 °C; ^{1}H NMR ($CDCl_3$) δ 8.79 (s, 1H), 8.07 (s, 1H), 7.46–7.19 (m, 9H), 6.84–6.82 (m, 4H), 6.47 (t, J = 6.5, 1H), 4.58 (br, 1H), 4.52 (br, 1H), 4.19–4.15 (m, 2H), 3.96 (d, J = 11.5, 1H), 3.77 (s, 6H), 3.55–3.47 (m, 3H), 3.35–3.29 (m, 5H), 2.69 (ddd, J = 13.5, 5.6, 3.2, 1H), 2.22 (ddd, J = 13.6, 6.7, 6.7, 1H), 2.09 (t, J = 6.8, 2H), 1.64–1.56 (m, 4H), 1.38–1.24 (m, 4H), 0.96–0.92 (m, 6H); ^{13}C NMR ($CDCl_3$) δ 169.6, 158.2, 158.2, 157.5, 156.1, 144.4, 140.6, 135.5, 135.4, 129.9, 129.8, 127.9, 127.6, 126.6, 117.4, 112.9, 110.5, 86.1, 86.0, 85.8, 71.4, 66.1, 64.5, 63.2, 54.9, 52.1, 45.5, 42.0, 30.7, 28.7, 19.9, 19.5, 17.7, 13.5, 13.4; HRMS (ESI) calcd for $C_{43}H_{54}N_5O_7$ ([M + H]$^+$) 752.4018, found 752.4019; UV (chloroform) λ_{max} 324 nm (ε 32010), 242 nm (ε 23590). The tritylated nucleoside (270 mg, 0.36 mmol) and 1*H*-tetrazole (50 mg, 0.72 mmol) were dissolved in acetonitrile (5 mL), and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropyl phosphorodiamidite (343 μL, 1.08 mmol) was added to the solution. The reaction mixture was stirred at room temperature for 1 h. A mixture of ethyl acetate (25 mL) and saturated sodium bicarbonate (25 mL) was added to the reaction mixture. The product was extracted to the organic layer. The organic layer was washed with brine twice and dried over magnesium sulfate. The solvent was removed by evaporation. The product was dried by coevaporation with acetonitrile and further dried under reduced pressure. ^{31}P NMR ($CDCl_3$): δ 149.660, 149.028; HRMS (ESI) calcd for $C_{52}H_{71}N_7O_8P$ ([M + H]$^+$) 952.5096, found 952.5099. Acetonitrile (2.4 mL) was added to the residue, and the solution was passed through a 0.45 μm filter. The resulting solution was used for automated DNA synthesis without further purification.

DNA synthesis and characterization

Artificial DNA was synthesized by the conventional phosphoramidite method using an NTS H-6 DNA/RNA synthesizer. Synthesized DNA was 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 (pH = 7.0), linear gradient over 20 min from 5% to 20% acetonitrile at a flow rate of 3.0 mL min^{-1}). Each DNA was characterized by MALDI-TOF MS. **ODN1(**^{hm}**C)**, [M + H]$^+$, calcd 3676.4, found 3675.6; **ODN1′(**^{hm}**C)**, [M + H]$^+$, calcd 3676.4, found 3676.3; **ODN2(**^{hm}**C)**, [M + H]$^+$, calcd 5212.6, found 5211.5; **ODN2′(ICON)**, [M + H]$^+$, calcd 4790.3, found 4790.9.

Osmium oxidation (bipyridine use)

The fluorescein-labeled **ODN2(X)** (5 μM) to be examined was incubated in a solution of 5 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM bipyridine, 0.5 mM EDTA, and 1 M sodium chloride in 50 mM Tris-HCl buffer (pH 7.7) and 10% acetonitrile at 0 °C for 5 min. The reaction solution was filtered to deionize it, and then the sample was precipitated in ethanol. After drying *in vacuo*, the precipitated DNA was redissolved in 50 μL of 10% piperidine (v/v), heated at 90 °C for 20 min, and then evaporated to dryness by vacuum rotary evaporation.

Osmium oxidation (ICON DNA use)

The fluorescein-labeled **ODN2(X)** (8 μM) to be examined was incubated in a solution of 5 μM **ODN2′(ICON)**, 5 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 0.5 mM EDTA, and 1 M sodium chloride in 50 mM Tris-HCl buffer (pH 7.7) at 25 °C for 10 min. The reaction solution was filtered to deionize it, and then the sample was precipitated in ethanol. After drying *in vacuo*, the precipitated DNA was redissolved in 50 μL of 10% piperidine (v/v), heated at 90 °C for 20 min, and then evaporated to dryness by vacuum rotary evaporation.

Melting temperature (T_m) measurements

All T_m measurements of the DNA duplexes (1 μM, final duplex concentration) were made in 50 mM sodium phosphate buffer (pH 7.0) containing 100 mM sodium chloride. Absorbance *vs.* temperature profiles were measured at 260 nm with a Shimadzu UV-2550 spectrophotometer equipped with a Peltier temperature controller using a cell with a 1 cm path length. The absorbance of the samples was monitored at 260 nm from 10 °C to 90 °C, at a heating rate of 1 °C min^{-1}.

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