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Novel photoresponsive cross-linking oligodeoxyribonucleotides having a caged α-chloroaldehyde

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ABSTRACT

We have developed photoresponsive cross-linking oligodeoxyribonucleotides (ODNs) for sequence-selective interstrand covalent bond formation toward target nucleotides. A phosphoramidite derivative of α-chloroaldehyde whose carbonyl group was converted to a bis(2-nitrobenzyl)acetal group was prepared for the synthesis of photoresponsive α-chloroaldehyde (PCA)-conjugated ODN. The bis(2-nitrobenzyl)acetal group of a PCA–thymidine conjugate was completely removed by UV irradiation at 365 nm (400 mW/cm^2) for 1 min. Photo-cross-linking studies revealed that PCA–ODN selectively reacted with the target nucleotides having an adenine or a cytosine moiety at the frontal position of the α-chloroaldehyde group.

1. Introduction

Oligonucleotide analogues forming covalent bonds with complementary nucleotides in a sequence-specific manner under physiological conditions are of potential clinical and biological interest.[1,2] In particular, photoresponsive oligonucleotide analogues which cross-link with complementary nucleotides using photo-irradiation as a trigger of the reaction have been developed to investigate and control gene functions without damaging living systems.[3,4] These oligonucleotide analogues have photoresponsive moieties introduced on a site which does not affect duplex or triplex formation. The introduced photoresponsive moieties, which are non-reactive or less reactive toward bio-molecules in their original form, generate highly reactive species after photo-irradiation. Arylazido[5] and ziazirine[6] derivatives that generate highly reactive electron-deficient species after UV irradiation have been introduced to nucleotide analogues and used for site-specific cross-linking to complementary nucleotides in a sequence specific manner. Thionucleobase[7] derivatives, which mainly give 2+2 photo-adducts of the excited C–S bond onto the 5,6 double bond of pyrimidines under UV irradiation, have been incorporated into functionalized nucleotides, such as ribozymes[8] and protein-recognized RNA,[9] to elucidate active-structures and protein-RNA interactions. 5-Cyanovinyldeoxyuridine[10] incorporated to the 5′-end of oligoODN reacted to an adenine residue in a complementary ODN to yield end-capping DNA duplexes. 4,5′,8-Trimethylpsoralen (Ps) derivatives have been introduced at the 5′-end,[11,12] and the internal strand[13,14] and used for photo-cross-linking with pyrimidine moieties in complementary nucleotides. We have reported that oligonucleotides having a Ps at the 2′-*O* hydroxy group of adenosine (2′-Ps-eom[15]) recognize one base difference in the target sequences under clinically relevant conditions. Using 2′-Ps-eom, we successfully achieved the inhibition of K-*ras*-immortalized cell proliferation (K12V) but not of Vco cells that contain the wild-type K-*ras* gene.[16] Considering the potential benefits of photoresponsive cross-linking reagent-nucleotide conjugates, it is important to develop new photoresponsive groups that could give high yields and exhibit specific reactive characteristics to target nucleobases located in specific positions in the target sequence.

α-Chloroacetaldehyde (CAA), which is generated by the metabolism of vinylchloride,[17,18] reacts with deoxycytidine (dC), deoxyadenosine (dA) under slightly acidic and neutral conditions, yielding 1,N^6-ethenoadenine (εA), and 3,N^4-ethenocytosine (εC), respectively.[19–23] Under neutral conditions, CAA subtly reacts with guanine, yielding N^2,3-ethenoguanine (εG), with decomposition of CAA. Quantitative analysis of the formation of etheno adducts in DNA duplexes by the CAA reaction reveals that the relationship can be represented as follows: εC > εA > εG.[24] Grant and Dervan reported[25] a sequence-selective cross-linking reaction that formed a triple helix by using an α-haloacetamide-attached ODN and the target DNA duplex. Detailed product analysis of the target strand using alkaline treatment for reactive species-selective cleavage revealed that an α-haloacetamide attached to the 5′-end of ODN selectively reacted with guanine-N7 in the target DNA duplex. Summerton and Barlet also reported that an α-haloketone-conjugated ODN[26] forms a guanine-N7 adduct in the DNA duplexes. Although cross-linking studies on α-haloacetamides and α-haloketones having chemical structures similar to α-chloroaldehyde have

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previously been reported, we believe that α-haloaldehyde has not previously been incorporated into nucleotides and has not been used for the sequence-selective modification of target nucleotides.[27]

In this study, we newly synthesized photo-cross-linking ODNs having a photoresponsive α-chloroaldehyde (PCA) group at the 5′-end of the ODN. The PCA group was comprised of an α-chloro bis(2-nitrobenzyl)acetal group, which was converted to an α-chloroaldehyde group after 1 min of UV irradiation. Photo-cross-linking studies revealed that the oligonucleotide conjugates underwent sequence-selective cross-linking to target nucleotides in a time-dependent manner under physiological conditions.

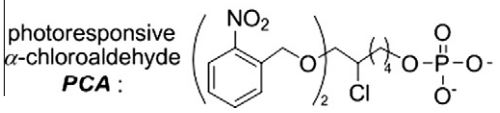
2. Results and discussion

2.1. Synthesis of PCA-conjugated ODNs

The synthetic route of α-chloroaldehyde derivatives is shown as Scheme 1. Photo-labile protective groups known as caging group have been widely introduced to append the photo-responsivity to the bio-active reagents for controlling living systems in a spatial and temporal fashion. Therefore, the bis(2-nitrobenzyl)acetal group, which was first reported in 1963[28] as a photo-removal protective group of carbonyl groups, was chosen for the protection of the α-chloroaldehyde group, which is usually unstable and extremely reactive with nucleophiles.[29,30] *N*-Chlorosuccinimide was used for the chlorination of 6-oxohexanoyl benzoate,[31] which was prepared using the reported procedures. Because **2** was inherently unstable and purification of **2** from a mixture of the starting material and the bis-chlorinated derivative did not succeed, crude products were used for introduction of the bis(2-nitrobenzyl)acetal group. After the protection of the carbonyl group using 2-nitrobenzyl alcohol and naphthalene sulfonic acid, the benzoyl group was deprotected by methanolic ammonia. Under such basic conditions, the α-chloro bis(2-nitrobenzyl)acetal group was stable. A phosphoramidite derivative was prepared using the standard procedures and was used for the synthesis of the α-chloroaldehyde-conjugated ODNs, **PCA-T**, **PCA–ODN1**, and **PCA–ODN2** (Fig. 1). **PCA–ODN1** and **PCA–ODN2** contain the antisense sequences of bcl2 mRNA[32] at positions 401–421 and 402–422, respectively.

PCA-ODN1:
PCA-GTT CTC CCA GCG TGC G 3'
PCA-ODN2:
PCA-TTC TCC CAG CGT GCG C 3'

photoresponsive α-chloroaldehyde ***PCA*** :



N-ODN1 (N=C, A, G, or T):
5' CGC ACG CTG GGA GAA CNG GGT 3'
N-ODN2 (N=C, A, G, or T):
5' GCG CAC GCT GGG AGA ANA GGG 3'
A-ORN:
5' CGC ACG CUG GGA GAA CAG GGU 3'

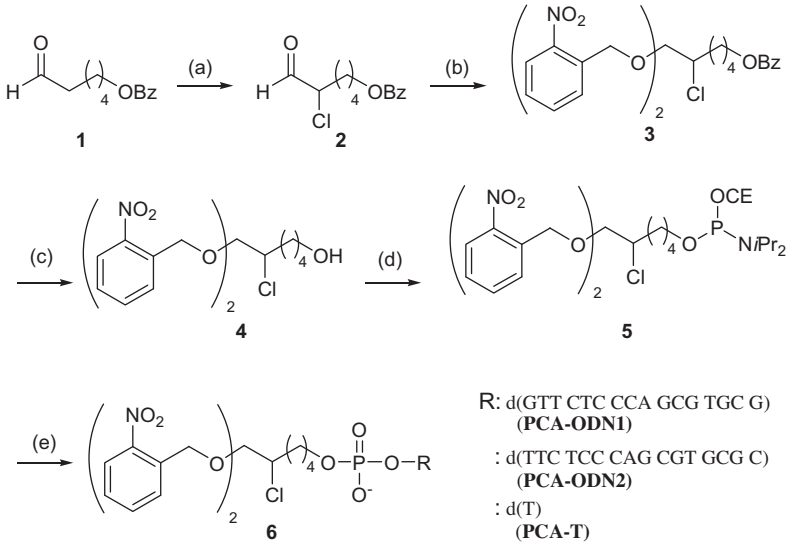
Figure 1. Oligonucleotides used in this study.

2.2. Deprotection conditions of the bis(2-nitrobenzyl)acetal group

Deprotection conditions of the bis(2-nitrobenzyl)acetal group attached to the 5′-end of thymidine (**PCA-T**) were examined by reversed-phase HPLC. After UV irradiation at 365 nm (400 mW/cm^2) for 30 s, the peak of **PCA-T** (Fig. 2a) almost disappeared, concomitant with an increase in the new product that peaked at approximately 18 min. The high-resolution mass of the purified product precisely corresponded to deprotected **PCA-T**, α-chloroaldehyde-conjugated thymidine (**CA-T**); calcd 453.08, found 453.08. Kinetic study of the deprotection reaction revealed that deprotection of the bis(2-nitrobenzyl)acetal group was completed within 1 min at a kinetic rate $t_{1/2}$ = 1.9 s (Fig. 2b). Therefore, 1 min UV irradiation at 365 nm (400 mW/cm^2) was used for the cross-linking studies described below.

2.3. Cross-linking studies of PCA-conjugated ODNs

The specificity and efficiency of the cross-linking reaction of α-chloroaldehyde-conjugated ODNs were examined by gel



Scheme 1. (a) *N*-chlorosuccinimide, L-proline, 10-camphorsulfonic acid, CH_2Cl_2, −5 °C, 20 h, 99%; (b) 2-nitrobenzyl alcohol(HONB), 2,2-dimethoxypropane, 2-naphthalenesulfonic acid, 95 °C, 24 h, 57%; (c) 1% NaOH/MeOH, rt, 12 h, 89%; (d) *O*-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, 1*H*-tetrazole, rt, acetonitrile, 1 h, 59%; (e) standard phosphoramidite method, d(GTT CTC CCA GCG TGC G)-CPG and d(TTC TCC CAG CGT GCG C)-CPG for **PCA–ODN1** and **PCA–ODN2**, respectively.

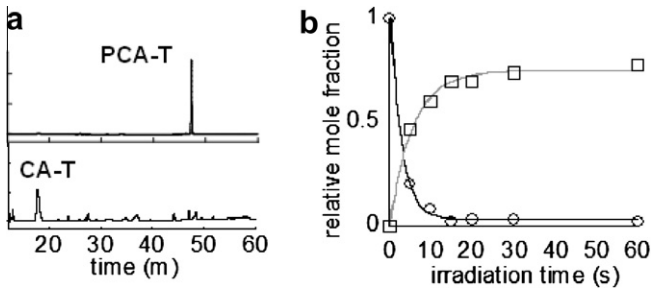


Figure 2. Deprotection of **PCA-T** under UV irradiation. (a) Reversed-phase HPLC spectra of **PCA-T** (upper: without UV irradiation. lower: after 30 s UV irradiation at 365 nm). (b) Variation of relative mole fractions of **PCA-T** and **CA-T** over time were determined by analyzing the reversed-phase HPLC profiles of the reaction mixtures. Black line: relative mole fraction of **PCA-T**. Gray line: relative mole fraction of **CA-T**.

electrophoresis and autoradiography. As target sequences of **PCA–ODN1**, we used 21-mer ODNs (**N-ODN1**), which consist of sequences complementary to **PCA–ODN1** and a 5-nucleotide dangling end containing one of the four nucleotides (N = A, G, C, and T) at the frontal position of the PCA. Reaction mixtures of **PCA–ODN1** and ^{32}P-labeled **N-ODN1** in 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl were irradiated for 1 min at 365 nm (400 mW/cm^2) and incubated at 37 °C for 192 h. Analyses of these reactions by gel electrophoresis followed by autoradiography are shown in Figure 3a–e. In the case of **A-ODN1**(N = A), a new single band in the high-molecular-weight region was observed in the first 6 h of incubation (Fig. 3a, lane 2), whereas the control reaction mixture (Fig. 3a, lane 1), which was not irradiated, showed no new bands even after 7 days. The intensity of the new band was increased in a time-dependent manner with a concomitant decrease in that of **A-ODN1**. **PCA–ODN1** also reacted with **C-ODN1** (Fig. 3b) and gave two new bands, **Band 1** and **Band 2**, in the high-molecular-weight region under the same reaction conditions. The intensity variation of obtained the new bands was further analyzed by changing the reaction temperature in accordance with previously reported methods[33,34] (Fig. 3c). The intensity of the higher-molecular-weight band (**Band 1**) was decreased and that of the lower-molecular-weight band (**Band 2**) was increased after incubation initially at 37 °C for 24 h, and later at 80 °C for 4 h. Wiewiorowski and co-workers[35] reported that the reaction of cytosine and adenine derivatives with CAA consisted of two steps (Fig. 4): [1]. Alkylation of the endocyclic nitrogen and cyclization by addition of an exoamine group to the carbonyl group, resulting in the formation of cyclic intermediates (**7**), and (**8**); and [2] double bond formation by dehydration, resulting in the formation of etheno derivatives. They also reported that the cyclic intermediate (**8**) showed higher stability in comparison with that of (**7**) and that isolation of the intermediate (**7**) was difficult. In consideration of these results, a cross-linking product containing an intermediate (**8**) and that containing 3,N^4-ethenocytosine might be distinctly observed during the 3,N^4-ethenocytosine-forming reaction in Figure 3b and c. Although an N-7 adduct, 1,N^2-etheno adduct, and N^2,3-etheno adduct of chloroacetaldehyde with guanine were reported by Bartsch and co-workers,[36] cross-linking products in the reaction between **PCA–ODN1** and **G-ODN1** were scarcely observed under the reaction conditions used (Fig. 3e). To quantify the cross-linking reaction between **PCA–ODN1** and **N-ODN1**, the cross-linking efficiency was estimated and is summarized in Figure 5a. The cross-linking yield of **PCA–ODN1** to **A-ODN1** was increased with time and reached 60% after 48 h of incubation at 37 °C at a kinetic rate of $t_{1/2}$ = 22 h. To determine the cross-linking efficiency of **PCA–ODN1** and **C-ODN1**, the combined intensity of both generated bands was used. The results showed that **C-ODN1** had modest reactivity with **PCA–ODN1**. In the case of N = T or G (Fig. 3d and e), the cross-linking efficiency was less than 5% at 48 h in both cases. These results indicate that α-chloroaldehyde-conjugated ODNs preferentially formed the cross-linking products with the target nucleotides having A or C at the frontal position of the PCA after UV irradiation and the quantitative relationship is represented as follows: A > C >> G = T. In addition, the cross-linking reactivity of **PCA–ODN1** to the complementary RNA (**A-ORN**) was analyzed (Fig. 3f). **PCA–ODN1** reacted with **A-ORN** just as did with **A-ODN1**, and the cross-linking efficiencies at 48 h and 168 h were 68% and 76%, respectively. These results suggest that the cross-linking reactions of **PCA–ODN1** were applicable to not only the complementary DNA strand but also the complementary RNA strand.

The reactivity of the adjacent nucleoside to the frontal base of the α-chloroaldehyde was examined using **PCA–ODN2** and **N-ODN2**, which contain one of the four nucleotides (N = A, G, C, and T) at the frontal position of the PCA and deoxyadenosine next to the frontal position of PCA. The cross-linking reactions of **PCA–ODN2** with **A-ODN2** and **C-ODN2** proceeded in the same manner as those of **PCA–ODN1** with **A-ODN1** and **C-ODN1**. The cross-linking efficiencies of **T-ODN2** and **G-ODN2** at 48 h were approximately 20% (Fig. 5b), whereas those of **T-ODN1** and **G-ODN1** were less than 5% (Fig. 5a). Although further detailed conformational studies will be needed, these results might suggest that the α-chloroaldehyde group can react with a neighboring 2′-deoxyadenosine at the frontal base. Finally, the fluorescence spectra of the reaction mixtures between **PCA–ODN1** and **N-ODN1** (N = A or T) after 48 h incubation at 37 °C were measured using an excitation wavelength

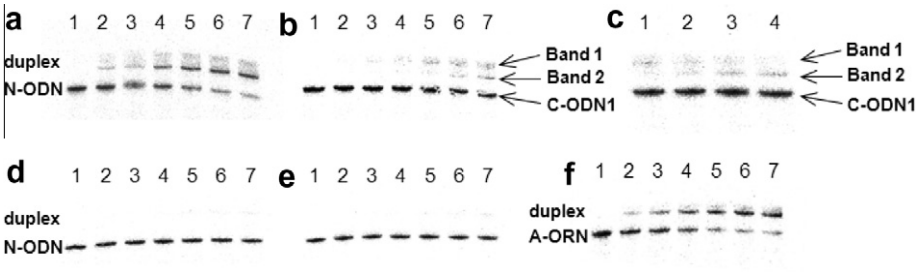


Figure 3. Photo-induced cross-linking study of **PCA-ODN1** with target nucleotides. (a–f) **N-ODN1** and **A-ORN** were labeled at the 5′-end with ^{32}P and hybridized with **PCA–ODN1** in 100 mM of sodium phosphate buffer (pH 7.0) containing 150 mM of NaCl. The reaction mixture was irradiated at 365 nm for 1 min and then incubated for up to 7 days at 37 °C. Autoradiograms of 20% polyacrylamide/7 M urea gel were obtained to analyze the cross-linking reaction of **PCA–ODN1** in the presence of 100 nM of **N-ODN1**: (a) N = A, (b) N = C, (d) N = T, and (e) N = G. Lane 1, without UV irradiation; Lane 2, 6 h; Lane 3, 12 h; Lane 4, 24 h; Lane 5, 48 h; Lane 6, 96 h; Lane 7, 168 h. (c) Photo-induced cross-linking study of the duplex between **PCA–ODN1** and **C-ODN1**. The reaction mixtures were irradiated at 365 nm for 1 min, then incubated initially at 37 °C for 24 h, and later at 80 °C for several hours. Lane 1, incubation at 37 °C for 24 h; Lane 2, incubation at 37 °C for 24 h, and at 80 °C for 1 h ; lane 3, incubation at 37 °C for 24 h, and at 80 °C for 2 h ; lane 4, incubation at 37 °C for 24 h, and at 80 °C for 4 h. (f) Autoradiogram of the cross-linking reaction mixture in the presence of **A-ORN**.

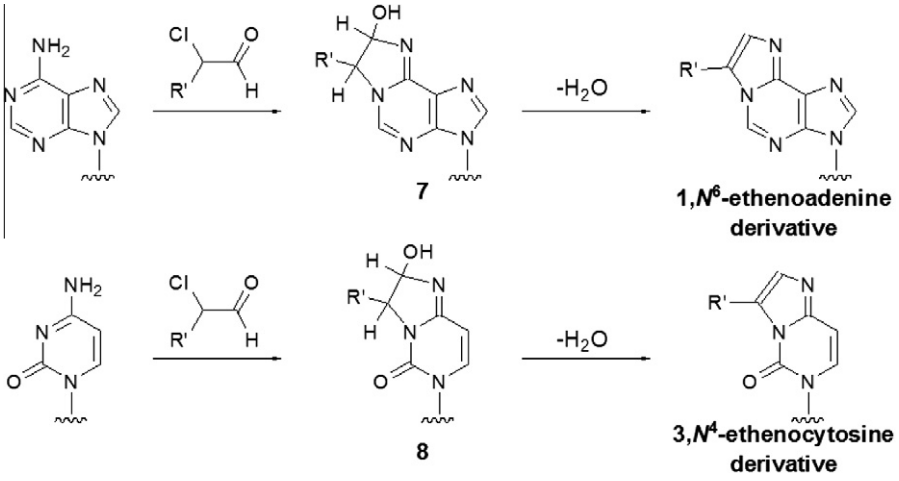


Figure 4. The crosslinking reaction of adenine and cytosine with a chloroaldehyde derivative.

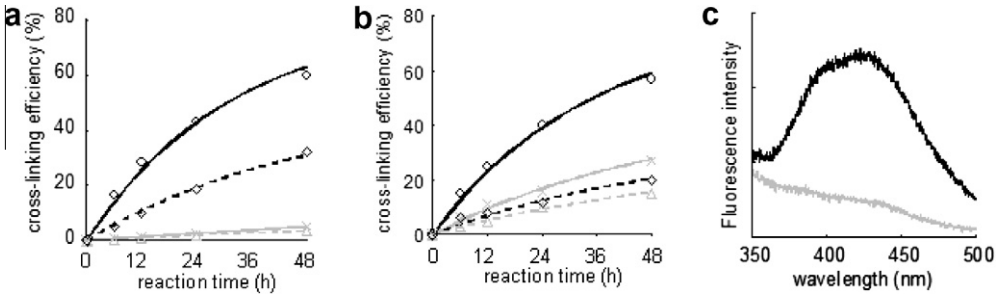


Figure 5. (a) Time course of the cross-linking efficiency of **PCA–ODN1**. The cross-linking efficiencies were obtained as follows: (band density of duplex)/(band density of duplex + band density of **N-ODN1**). Black solid line: N = A; black broken line: N = C; gray solid line: N = G; gray broken line: N = T. (b) Time course of the cross-linking efficiency of **PCA–ODN2**. (c) Fluorescence spectra of **PCA–ODN1** in the presence of **N-ODN1** (N = A, black line; N = T, gray line) after 48 h of incubation at 37 °C. The excitation wavelength was 282 nm.

at 282 nm (Fig. 5c). A typical fluorescent signal derived from C10-substituted 1,N^6-ethenoadenine[34] with a peak (maximum) at a wavelength of approximately 420 nm was observed only in the case of N = A, whereas no fluorescence was observed in the case of N = T. These results strongly suggested that 1,N^6-ethenoadenine was formed in the reaction between **PCA–ODN1** and **A-ODN1**.

3. Conclusions

We have prepared synthetic ODNs containing photoresponsive α-chloroaldehyde at the 5′-end. Photo-irradiation is the key to the cross-linking reaction between the caged ODN and the target nucleotides. From the quantitative analysis of the cross-linking products, α-chloroaldehyde-conjugated ODNs can be seen to react with the target nucleotides having an adenine and a cytosine at the frontal position of the PCA. Preliminary product studies using fluorescence analysis indicated that 1,N^6-ethenoadenine derivatives were generated in the reaction. Although the efficiency and kinetic rate of cross-linking reactions need to be improved, the caged α-chloroaldehyde analogues are still applied to the antisense therapy and nucleotide manipulation in vivo, and biological assays using this principle are now in progress.

4. Experimental section

4.1. General methods and materials

Reagents and solvents were purchased from Wako Pure Chemical Industries, TCI, or Sigma. Reactions were monitored with TLC plates precoated with Merck silica gel 60 F_{254}. Spots were visualized with UV light or staining with molybdic acid (a solution of 10 g $(NH_4)_6Mo_7O_{24}$ and 4 g $Ce(NH_4)_4(SO_4)_4$ in 360 mL of 10% aqueous H_2SO_4 in 330 mL ethanol). Wakogel C-200 was used for silica gel flash chromatography. ^1H and ^{13}C NMR spectra were measured on a BRUKER ADVANCE II 300 (^1H spectra at 300 MHz; ^{13}C spectra at 75 MHz; ^{31}P spectra at 121 MHz) spectrometer. FAB mass spectra were recorded on a JEOL JMS-700 MStation. Gel permiation chromatography (JAI) was performed using an LC 9201 recycling preparative HPLC system with JAIGEL-GS310 GPC column. DNA and RNA oligonucleotides were purchased from GeneDesign, Inc. and FASMAC Co., Ltd, respectively. DNA oligonucleotides containing PCA were prepared and purified as described below. **N-ODN1**, **N-ODN2**, and **A-ORN** were purified by preparative 20% denaturing polyacrylamide gel electrophoresis. The 5′ radiolabeling was done with [γ-32P] ATP (PerkinElmer) and T4 polynucleotide kinase, which was obtained from TaKaRa Bio Inc. A Milli-Q PF system was used to purify the distilled deionized water that was used to make all the buffers. Autoradiography was performed on a BAS-1800II (Fuji Film. Data analysis was performed with Image Gauge (version 3.4) and rate constants and dissociation constants were determined with Kaleidagraph (version 3.6.1) software.

4.2. 5-Chloro-6-oxohexanoyl benzoate(2)

(+)-10-Camphorsulfonic acid (2.4 mg, 0.01 mmol) and L-proline (20.2 mg, 0.04 mmol) in CH_2Cl_2 (2 mL) were added to the solution of 6-oxohexanoyl benzoate (**1**) (42.2 mg, 0.2 mmol) in CH_2Cl_2 (1 mL) at −10 °C under nitrogen atmosphere and stirred for

5 min, and then *N*-chlorosuccinimide in CH_2Cl_2 (2 mL) was added. After stirring for 20 h at −10 °C, triethylamine (200 μL, 1.4 mmol) was added and the reaction mixture was washed with saturated sodium thiosulfate three times, dried over sodium sulfate, and evaporated under reduced pressure. 5-Chloro-6-oxohexanoyl benzoate (**2**) (50.5 mg, 99%) was obtained without further purification. ^{1}H NMR (300 MHz $CDCl_3$) δ 9.51 (1H, d, J = 2.1 Hz), 8.03 (2H, d, J = 7.8 Hz), 7.56 (1H, t, J = 6.3 Hz), 7.44 (2H, t, J = 5.9 Hz), 4.34 (2H, t, J = 6.3 Hz), 4.20 (1H, m), 2.12–2.03 (1H, m), 1.98–1.58 (7H, m); ^{13}C NMR (300 MHz, $CDCl_3$) δ 195.1, 166.6, 132.9, 130.2, 129.5, 128.4, 64.4, 63.7, 31.6, 28.2, 22.3. (ESI-TOF Mass) Found $[M+Na]^+$ = 277.043, Calcd $[M+Na]^+$ = 277.061.

4.3. 5-Chloro-6,6-bis(2-nitrobenzyl)hexanoyl benzoate (3)

2-Nitrobenzyl alcohol (HONB) (118.3 mg, 0.7 mmol), 2,2-dimethoxypropane (28 μL, 0.31 mmol), and 2-naphthalenesulfonic acid (8.0 mg, cat) were added to the solution of **2** (70.5 mg, 0.28 mmol) in anhydrous toluene (3 mL) under a nitrogen atmosphere and stirred for 48 h at 80 °C. After addition of triethylamine (300 μL, 2.1 mmol), $CHCl_3$ (3 mL) was added and the organic solution was washed with saturated sodium bicarbonate three times, dried over sodium sulfate, and evaporated under reduced pressure. The residue was purified by silica column chromatography (hexane/chloroform 4:1) to yield **3**. (92.0 mg, 57% from **1**) ^{1}H NMR (300 MHz $CDCl_3$) δ 8.03 (4H, m), 7.85 (1H, d, J = 7.5 Hz), 7.80 (1H, d, J = 7.8 Hz), 7.64 (2H, m), 7.53 (1H, m), 7.42 (4H, m), 5.10 (4H, s), 4.93 (1H, d, J = 5.1 Hz), 4.35 (2H, t, J = 5.7 Hz), 4.13 (1H, m), 2.04–1.64 (6H, m); ^{13}C NMR (300 MHz, $CDCl_3$) δ 166.6, 147.1, 133.9, 132.9, 130.3, 129.5, 129.1, 128.9, 128.3, 124.8, 104.9, 67.1, 66.3, 64.6, 61.5, 32.0, 28.2, 22.8, 21.5; (ESI-TOF Mass) Found $[M+Na]^+$ = 565.134, Calcd $[M+Na]^+$ = 565.135.

4.4. 5-Chloro-6,6-bis(2-nitrobenzyl)hexan-1-ol (4)

Compound **3** (92 mg, 0.16 mmol) and sodium hydroxide (30 mg, 0.75 mmol) in MeOH (3 mL) were stirred at room temperature for 12 h. $CHCl_3$ (10 mL) was added and the organic solution was washed with brine (3 × 50 mL), dried over sodium sulfate, and evaporated under reduced pressure. The residue was purified by silica column chromatography (hexane/chloroform 4:1) to yield **4**. (60.0 mg, 88%) ^{1}H NMR (300 MHz $CDCl_3$) δ 8.06 (2H, d, J = 6.6 Hz), 7.86 (1H, d, J = 7.8 Hz), 7.80 (1H, d, J = 7.8 Hz), 7.65 (2H, m), 7.45 (2H, t, J = 7.8 Hz), 5.10 (4H, s), 4.92 (1H, d, J = 5.4 Hz), 4.12 (1H, m), 3.68 (2H, t, J = 6.0 Hz), 1.89–1.50 (6H, m); ^{13}C NMR (300 MHz, $CDCl_3$) δ 147.1, 133.8, 129.0, 128.4, 124.8, 104.8, 67.0, 66.3, 62.6, 61.6, 32.1, 22.5.; (ESI-TOF Mass) Found $[M+Na]^+$ = 461.084, Calcd $[M+Na]^+$ = 461.109.

4.5. *O*-(5-Chloro-6,6-bis(2-nitrobenzyl)hexan-1-yl)-*O*′-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (5)

To a solution of **4** (60 mg, 0.14 mmol) in anhydrous CH_3CN was added *O*-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (27 μL, 0.15 nmol) and 1*H*-tetrazole (6.1 mg, 0.15 mmol). After stirring for 1 h at room temperature, the reaction mixture was quenched with 5% sodium bicarbonate solution and extracted with diisopropyl ether. The organic layer was washed with 0.1 N NaOH aq, dried over sodium sulfate, and concentrated in vacuo. The crude product (**4**) (70.9 mg, 79%) was purified by gel permiation chromatography (GPC). ^{1}H NMR (300 MHz $CDCl_3$) δ 8.06 (2H, d, J = 8.1 Hz), 7.87 (1H, d, J = 7.8 Hz), 7.81 (1H, d, J = 7.5 Hz), 7.66 (2H, t, J = 7.5 Hz), 7.45 (2H, t, J = 7.8 Hz), 5.10 (4H, s), 4.91 (1H, d, J = 5.4 Hz), 4.11 (1H, m), 3.83 (2H, m), 3.61 (2H, m), 2.63 (2H, t, J = 6.3 Hz), 1.89–1.50 (6H, m), 1.19–1.12 (12H, m); ^{13}C NMR (300 MHz, $CDCl_3$) δ 147.1, 147.0, 133.9, 133.9, 133.8, 128.9, 128.5, 128.4, 128.3, 124.8, 124.7, 117.7, 104.9, 67.0, 66.3, 63.4, 63.2, 61.7, 58.4, 58.2, 43.1, 42.9, 32.1, 30.7, 30.6, 29.7, 24.7, 24.6, 24.5, 22.7, 20.4, 20.3, 19.9; ^{31}P NMR (300 MHz $CDCl_3$) δ 147.35.; (ESI-TOF Mass) Found $[M+Na]^+$ = 661.217, Calcd $[M+Na]^+$ = 661.217.

4.6. Synthesis and characterization of PCA-T, PCA–ODN1, and PCA–ODN2

PCA phosphoramidite (**5**) was introduced to 0.1 μmole of thymidine, 5′-d(GTT CTC CCA GCG TGC G)-3′ and 5′-d(TTC TCC CAG CGT GCG C)-3′ attached on CPG, which were purchased from GeneDesign, Inc., using standard β-cyanoethyl phosphoramidite chemistry. The coupling times for the PCA phosphoramidites (**5**) was 600 s. All oligonucleotides were deprotected in concentrated ammonium hydroxide (55 °C, 16 h) and purified by reversed-phase HPLC on a SHIMADZU 10A system with a SHISEIDO CAPCELL PAK C-18 reversed-phase column (HPLC conditions: 0.8 ml/min; solvent A = 0.1 M TEAA; solvent B = 50% CH_3CN/0.1 M TEAA linear gradient from 0% to 60% over 30 min, monitored at 260 nm). All oligonucleotides were characterized by ESI-TOF mass spectrometry. **PCA-T**, Calcd $[M-H]^{1-}$ = 741.158, Found = 741.181.; **PCA–ODN1**, Calcd $[M-6H]^{6-}$ = 890.143, Found =890.105.; **PCA–ODN2**, Calcd $[M-6H]^{6-}$ = 887.311, Found = 887.261.

4.7. Molar absorption coefficient of PCA-T

0.2 OD of **PCA-T** was fully digested with calf intestine alkaline phosphatase (25 units, 1 μL) and snake venom phophodiesterase (1 unit, 30 μL) in alkaline phosphatase buffer at 37 °C for 40 h. The digested solution was analyzed by reversed-phase HPLC. The molar absorption coefficient of **PCA-T** was determined as 16,700 by comparing peak areas with a standard solution containing 0.1 OD of 2′-deoxyadenosine.

4.8. Deprotection conditions of PCA-T

0.50 OD of **PCA-T** was dissolved in 50 μL of 10 mM phosphate buffer containing 100 mM NaCl. The solution was irradiated at 365 nm on an LED Spot Light (HLV2, 365 nm, 400 mW/cm^2, CCS Inc.) for up to 1 min and analyzed by reversed-phase HPLC. The concentration of **PCA-T** was determined by comparing peak areas with a standard solution containing 0.30 OD of T.

4.9. Photo-cross-linking reactions of PCA–ODNs

1.0 pmol of the N-ODNs and A-ORN were radiolabeled on the 5′-end using [γ-32P]-ATP with T4 polynucleotide kinase (TaKaRa Bio Inc.) at 37 °C. Excess [γ-32P]-ATP was removed using a C-18 reversed-phase column according to the manufacturer's protocol. For crosslinking studies, additional nonradioactive N-ODNs or A-ORN was added to the labeled strand to yield a concentration of 2% labeled nucleic acids. An equimolar solution of N-ODNs (N = A, C, G, T) or A-ORN and PCA–ODNs (100 nM each) was denatured at 95 °C for 5 min and slowly cooled at 37 °C in 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl. The reaction mixtures were irradiated by UVA on an LED Spot Light (HLV2, 365 nm, 400 mW/cm^2, CCS Inc.) for 1 min, then incubated at 37 °C up to 7 days. The reaction mixtures were analyzed by denatured PAGE (20% polyacrylamide, 7 M Urea, 25% formamide/TBE, 500 V, 45 °C, 90 min). The separation of high-molecular-weight nucleic acid fragments arising from the product and the 21-nucleotide fragment originating from the N-ODNs or A-ORN was visualized using autoradiography by exposure to an Image Plate (Fuji Film) for 1 h. The resulting autoradiograms were quantitated using Image Gauge (version 3.4) software to generate plots of prod-

uct formed as a function of time. The resulting data were fitted to a single-exponential to determine $k_{1/2}$.

4.10. Fluorescence measurements

Fluorescence spectra of the samples in 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl were recorded at 20 °C on an RF-5300 spectrofluorometer (Shimadzu) equipped with a thermal controller. Emission scans were measured from 250 to 500 nm, using slits of 5 nm band-pass (ex, em) with an excitation wavelength at 282 nm.

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Supplementary data

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