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Cross-Linking Antisense Oligodeoxyribonucleotides with a Photoresponsive α-Chloroaldehyde Moiety for RNA Point Mutations

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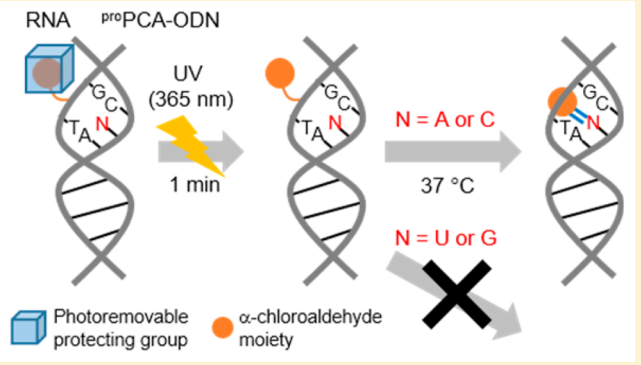
Supporting Information

ABSTRACT: Because point mutations in GTPase-coding genes have been reported to be responsible for the transformation of cells, anticancer reagents that react effectively and sequence selectively with target RNAs having a point mutation are highly desired. In this study, we developed novel photo-cross-linking oligodeoxyribonucleotides ([pro]PCA-ODNs) that had a caged α-chloroaldehyde group conjugated to a 2-methylpropanediyl backbone ([pro]PCA) in the middle of the strand. A kinetic study of the deprotection reaction of [pro]PCA-ODN revealed that the bis(2-nitrobenzyl)acetal group was completely deprotected within 1 min. Photo-cross-linking studies of [pro]PCA-ODNs with complementary oligoribonucleotides (ORNs) revealed that [pro]PCA-ODNs reacts efficiently and selectively with the target ORNs that have an adenosine or cytidine residue at a frontal position of the [pro]PCA residue without adverse effects of bases adjacent to the mutation site.



INTRODUCTION

Particular point mutations in the RAS-RAF-MEK-ERK-MAP kinase pathway, which mediated cellular responses to growth signal, have been reported to be related to cellular transformation.[1] Three isoforms of RAS genes (K, H, and NRAS) are the most common oncogenes in human tumors; RAS gene mutations were found in about 16% of human tumors and 63% of pancreas tumors.[2] Molecular biological and genome screening studies revealed that point mutations occurring mainly at codons 12, 13, and 61 in KRAS gene are potential triggers of cellular transformation.[3] In 2002, Davies et al. reported that point mutations, especially T1796A transversion mutation, in BRAF genes were found in 66% of malignant melanomas.[4] These point mutations in humans elevate human RAF1 kinase activity and induce cellular transformation. Ras-related C3 botulinum toxin substrate (RAC) 1 and RAC2 genes, which belong to the RHO family of small GTPase,[5] are also known as oncogenes. Point mutations of the active site-related sequence in RAC1 and RAC2 genes rendered Rac proteins constitutively active and highly oncogenic.[6] Although these findings have provided new therapeutic opportunities in human cancers, the development of anticancer drugs that regulate the translation of point-mutated mRNAs encoded by oncogenes without affecting those of normal mRNAs is still in the preliminary stages.

Photo-cross-linking oligonucleotides that bond covalently with complementary oligonucleotides with photoirradiation as a reaction trigger have been developed to control the activities of biomolecules without affecting biological activities. The cross-linking oligonucleotides have photoactivatable moieties, which become reactive by photoirradiation and capable of forming a new covalent bond with neighboring chemical species, at sites that do not hamper the formation of highly ordered structures. Oligonucleotides with arylazido[7] or diazirine[8] derivatives, which can be activated by UV-light of particular wavelength, have been developed and used for photo-cross-linking reaction with complementary nucleotides. 4-Thiopyrimidine analogues,[9] which preferentially form photo-adducts with neighboring pyrimidine nucleobases under photoirradiation conditions, have been used for elucidation of the three-dimensional conformations and active structures of the ribozymes[10] and protein-recognized RNAs.[11] For the purpose of regulating the translation of point-mutated mRNAs, photo-cross-linking oligonucleotides with photoactive

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groups that selectively react with pyrimidine nucleotides under photoirradiation conditions were reported by us[12] and others.[13] In 2010, we reported the sequence-selective inhibition of KRAS mRNAs with a point mutation from GGU to GUU using a psoralen-conjugated photo-cross-linking oligoribonucleotide (ORN).[12] The photo-cross-linking ORN suppressed the proliferation of NIH3T3 cells containing mutant KRAS genes under UV irradiation, whereas the ORN hardly affected their proliferation either without UV irradiation or containing wild-type KRAS genes. In 2012, Fujimoto et al. reported the selective and effective inhibition of the reverse transcription and translation activities of mutant KRAS mRNA extracted from a pancreatic cancer cell line using ^{CNV}K-conjugated antisense oligodeoxyribonucleotides (ODNs).[13] Considering the potential benefits of photoresponsive cross-linking reagent-nucleotide conjugates, it is important to develop photoresponsive nucleosides that could give high yields and exhibit specific reactive characteristics to target nucleobases located at specific positions in the target sequences.

Previously, we developed photo-cross-linking ODNs with a photoresponsive α-chloroaldehyde (PCA-ODN)[14] and photoresponsive α-bromoaldehyde (PBA-ODN)[15] residue at the 5′ end of the strand as shown in Figure 1a. These ODNs were

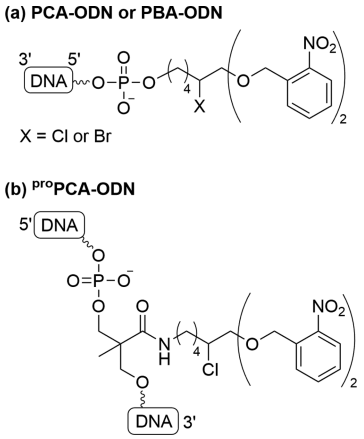


Figure 1. (a) Chemical structure of PCA-ODN (X = Cl) and PBA-ODN (X = Br). (b) Chemical structure of ^{pro}PCA-ODN.

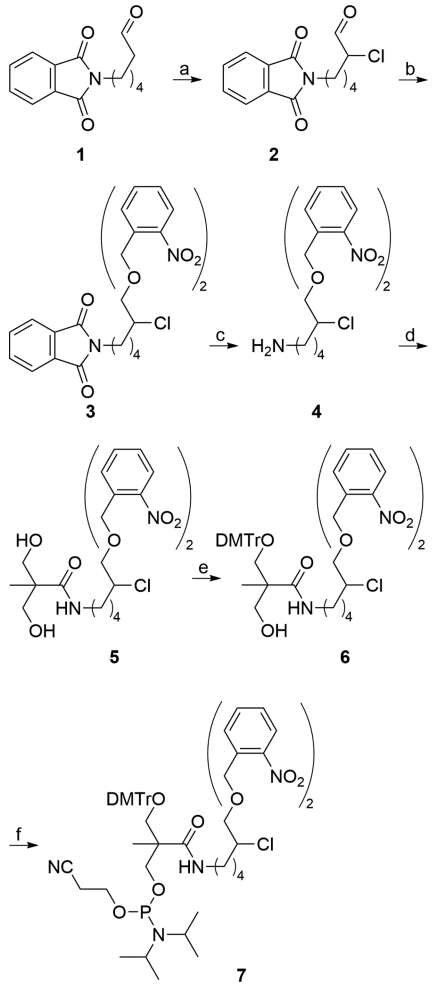
activated by UV irradiation and formed covalent bonds with target DNAs or RNAs with an adenine or a cytosine nucleobase at the frontal position of the photoreactive moieties.

Here, we developed novel cross-linking ODNs, ^{pro}PCA-ODNs, with a photoresponsive α-chloroaldehyde (PCA) moiety in the middle of the ODNs. The ^{pro}PCA moiety consists of the PCA moiety connected to a 2-methylpropane-diyl backbone via a flexible linker (Figure 1b). A ^{pro}PCA phosphoramidite unit was newly synthesized and used for the preparation of ^{pro}PCA-ODNs. Photo-cross-linking studies of ^{pro}PCA-ODNs with ORNs revealed that these ^{pro}PCA-ODNs effectively and sequence selectively reacted with ORNs having single base mutations.

RESULTS AND DISCUSSION

The ^{pro}PCA phosphoramidite unit was synthesized as shown in Scheme 1. According to our previous studies,[14,15] the bis(2-nitrobenzyl)acetal group was applied to the caging group of the α-chloroaldehyde moiety, which is extremely reactive with adenine and cytosine bases.[16−18] *N*-Chlorosuccinimide was

Scheme 1. Synthetic Scheme of a ^{pro}PCA Phosphoramidite Unit[a]



[a]Reagents and conditions: (a) NCS, L-proline, CH_2Cl_2, 0 °C then rt, 82%; (b) 2-nitrobenzyl alcohol, 2-naphthalenesulfonic acid, molecular sieves 4 Å, toluene, 80 °C, 28%; (c) H_2NNH_2, EtOH, reflux, 91%; (d) $CH_3C(CH_2OH)_2COOH$, EDC, HOBt, DMF, rt, 58%; (e) DMTrCl, pyridine, rt, 53%; (f) iPr$_2$NP(Cl)OCH$_2$CH$_2$CN, *N*,*N*-diisopropylethylamine, CH_2Cl_2, rt, 34%.

used for the chlorination of 6-phthalimidohexanal (**1**), which was prepared from *N*-(6-hydroxyhex-1-yl)phthalimide via Swern oxidation.[19] After the protection of the aldehyde group of **2** using 2-nitrobenzyl alcohol and 2-naphthalenesulfonic acid, the phthalimide group of **3** was converted to an amino group by hydrazine monohydrate. The caged α-chloroaldehyde derivative **4** was conjugated to the backbone segment by condensation with 2,2-bis(hydroxymethyl)propanoic acid. The phosphoramidite unit **7** was obtained by dimethoxytritylation and phosphitylation of **5** and used for the synthesis of ^{pro}PCA-conjugated ODNs, **^{pro}PCA-ODNn** (n = 1−5; Table 1). **^{Pro}PCA-ODN2** contains an antisense sequence of pEGFP-N2[20] mRNA at positions 684−700. All ^{pro}PCA-ODNs were purified by reversed-phase HPLC and identified by electrospray ionization mass spectrometry (ESI-MS). The reversed-phase HPLC profile of **^{pro}PCA-ODN2** showed twin peaks around 27 min (Figure S1, Supporting Information). The faster and slower peaks gave the same mass as the calculated mass [m/z: $[M - 5H]^{5-}$ calcd for the faster peak, 1069.18, found 1069.17; calcd for the slower peak, 1069.18; found 1069.17]. Asanuma et

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Table 1. Sequences of Oligonucleotides Used in This Study[a]

	sequences		
^{pro}PCA-ODNl	5′-t ^{Pro}PCA	t-3′	
^{pro}PCA-ODN2	5′-ctcgccct	^{Pro}PCA	gctcacca-3′
^{pro}PCA-ODN3	5′-ctcgcccg	^{Pro}PCA	tctcacca-3′
^{pro}PCA-ODN4	5′-ctcgcccg	^{Pro}PCA	gctcacca-3′
^{pro}PCA-ODN5	5′-ctcgccct	^{Pro}PCA	tctcacca-3′
ORN(CN_1A)[b]	5′-UGGUGAGC	N_1	AGGGCGAG-3′
ODN(tN_2g)[c]	5′-ctcgccct	N_2	gctcacca-3′
ODN(cN_2a)[c]	5′-tggtgagc	N_2	agggcgag-3′
ORN(N_3UN_4)[d]	5′-UGGUGAGN$_3$	U	N_4GGGCGAG-3′

[a]A, U, G, C: RNA; a, t, g, c: DNA. [b]N_1 = A, U, G, C. [c]N_2 = a, t, g, c. [d]N_3 = A, C; N_4 = A, C.

al. reported that the reversed-phase HPLC analyses of ODNs containing azobenzene moieties conjugated with 2-methylpropanediyl backbones gave twin peaks attributed to the chirality of the 2-methylpropanediyl residues.[21] In our case, the ODN containing the *R* isomer of the caged α-haloaldehyde derivative and that containing the *S* isomer at the 5′ end of the strand were not obtained as two distinct peaks in the reversed-phase HPLC analyses.[14,15] Although a more detailed analysis of the obtained diastereomeric ODNs is required, we used both products, named **^{pro}PCA-ODN2f** and **^{pro}PCA-ODN2s**, for cross-linking studies.

First, the UV-deprotection conditions of the bis(2-nitrobenzyl)acetal group in the ^{pro}PCA moiety were evaluated using **^{pro}PCA-ODN1**. The reversed-phase HPLC profiles of the uncaging reaction of **^{pro}PCA-ODN1** are shown in Figure 2.

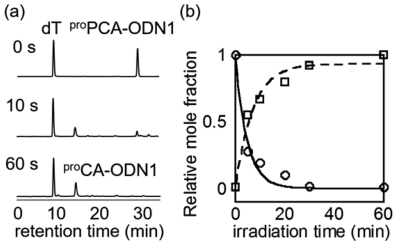

Figure 2. (a) HPLC analyses of the photodeprotection reaction of the bis(2-nitrobenzyl)acetal group of the ^{pro}PCA moiety. **^{Pro}CA-ODN1** represents deprotected **^{pro}PCA-ODN1**. (b) Time course of the photodeprotection reaction. Solid line and circles: **^{pro}PCA-ODN1**, broken line and squares: **^{pro}CA-ODN1**.

After UV irradiation at 365 nm (400 mW/cm^2) for 10 s, the peak of **^{pro}PCA-ODN1** (Figure 2a) was decreased, and a peak appeared simultaneously at around 14 min. As UV irradiation time was increased, the intensity of the peak of **^{pro}PCA-ODN1** decreased, whereas that of the new peak increased. The new product corresponding to the peak at around 14 min was analyzed by ESI-TOF-MS and found to correspond precisely to uncaged **^{pro}PCA-ODN1** (ESI-TOF-MS *m*/*z*: [M − 2H]$^{2-}$ calcd for **^{pro}CA-ODN1** 435.59, found 435.59). A kinetic study of the deprotection reaction shown in Figure 2b revealed that the deprotection of the bis(2-nitrobenzyl)acetal group was completed within 1 min at a kinetic rate of $t_{1/2}$ = 3.3 s (Figure 2b).

Next we analyzed the photo-cross-linking reactions of ^{pro}PCA-ODNs with complementary ORNs, which contained one of four ribonucleotides at the frontal position of the ^{pro}PCA moiety (Table 1). Before cross-linking studies, we examined the duplex stabilities of **^{pro}PCA-ODN2f** and **^{pro}PCA-ODN2s** hybridized with complementary **ORN(CN_1A)**. Although the melting temperatures of **^{pro}PCA-ODN2f** or **^{pro}PCA-ODN2s**/**ORN(CN_1A)** were 5.5−10.2 °C lower than that observed for the full match **ODN(tN_2g)**/**ORN(CN_1A)**, the duplexes were formed sufficiently under cross-linking reaction conditions (Figure S2, Supporting Information). ^{32}P-labeled ORNs were used as tracers for denaturing PAGE analyses. Reaction mixtures of 0.1 μM **^{pro}PCA-ODN2f** or **^{pro}PCA-ODN2s**, 0.1 μM ORNs, and 0.02 nM tracers in 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl were irradiated at 365 nm (400 mW/cm^2) for 1 min and incubated at 37 °C up to 48 h. The reaction mixtures of **^{pro}PCA-ODN2f** with ORNs were analyzed by denaturing PAGE followed by autoradiography (Figure 3a−d). In the case of **ORN(CAA)**, a

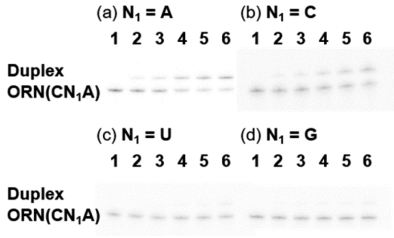

Figure 3. (a−d) Denaturing PAGE analyses of photo-cross-linking reactions of **^{pro}PCA-ODN2f** with complementary ORNs. Lane 1: incubation for 1 week at 37 °C without UV-irradiation, lanes 2−6: UV-irradiated and incubated at 37 °C for following times, lane 2: 3 h, lane 3: 6 h, lane 4: 12 h, lane 5: 24 h, lane 6: 48 h. (a) **ORN(CAA)**, (b) **ORN(CCA)**, (c) **ORN(CUA)**, (d) **ORN(CGA)**.

new single band in the higher molecular weight region was observed in the first 3 h of incubation (Figure 3a, lane 2), whereas no new bands were observed in the control reaction mixture (Figure 3a, lane 1). The intensity of the new band increased in a time-dependent manner with a concomitant decrease in the intensity of the **ORN(CAA)**. In Figure 3b, a new single band in the higher molecular weight region was also observed in the case of **ORN(CCA)** in a time-dependent manner. In contrast to **ORN(CAA)** and **ORN(CCA)**, only a faint band of the cross-linked duplexes were observed in the reaction with **ORN(CUA)** or **ORN(CGA)**. To quantify the cross-linking reactions between **^{pro}PCA-ODN2f** and **ORN(CN_1A)** (N_1 = A, U, G, or C), the cross-linking efficiencies were estimated using the band density of the cross-linking products, and the kinetic rates of the reactions were determined by fitting the cross-linking efficiencies as pseudo-first-order reactions (Figure 4a). The cross-linking efficiency of **^{pro}PCA-ODN2f** with **ORN(CAA)** reached 65% after 48 h of incubation at 37 °C at a kinetic rate of $t_{1/2}$ = 3.4 h. The cross-linking efficiency of **^{pro}PCA-ODN2f** with **ORN(CCA)** reached around 59% after 48 h of incubation at 37 °C with a modest kinetic rate ($t_{1/2}$ = 20 h). In the cases of **ORN(CUA)** and **ORN(CGA)**, the cross-linking efficiencies were less than 10%. As for **^{pro}PCA-ODN2f**, **^{pro}PCA-ODN2s** selectively formed cross-linked duplexes with **ORN(CAA)** or **ORN(CCA)** with similar kinetic rates (Figure 4b). To estimate the steric effect of the chiral backbone, energy-optimized duplex structures between **ORN(CAA)** or **ORN(CCA)** and uncaged ^{pro}PCA-ODNs containing the *R* or *S* isomer of the propanediyl backbone were obtained using molecular mechanics calculations with the AMBER* force field (Figure S7, Supporting Information). The distances between the carbonyl carbon atom of the α-chloroaldehyde

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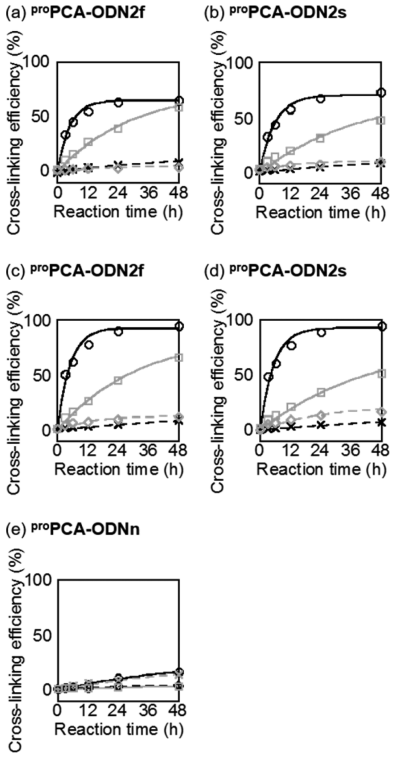

Figure 4. Time course of the photo-cross-linking reactions between ORNs and (a) 1 equiv of **^{pro}PCA-ODN2f**, (b) 1 equiv of **^{pro}PCA-ODN2s**, (c) 3 equiv of **^{pro}PCA-ODN2f**, and (d) 3 equiv of **^{pro}PCA-ODN2s**. The cross-linking efficiencies were obtained as follows: (band density of duplex)/(band density of duplex + band density of ORNs). Black solid line and circles: N_1 = A, gray solid line and squares: N_1 = C, gray broken line and diamonds: N_1 = G, black broken line and crosses: N_1 = U. (e) Time course of the photo-cross-linking reactions between ORNs and 1 equiv of **^{pro}PCA-ODNn** (n = 2−5). Gray solid line and squares: **ORN(CUA)**/**^{pro}PCA-ODN2**, black solid line and circles: **ORN(AUC)**/**^{pro}PCA-ODN3**, black broken line and diamonds: **ORN(CUC)**/**^{pro}PCA-ODN4**, gray broken line and crosses: **ORN(AUA)**/**^{pro}PCA-ODN5**.

group and the amino group of the adenosine or cytidine residue at the frontal position of the α-chloroaldehyde group are summarized in Table 2; the distances for the *S* isomer-

Table 2. Distances between the Carbonyl Carbon Atom of the ^{pro}PCA Moiety and the Amino Nitrogen Atom of the Adenosine or Cytidine

	ORN(CAA)	ORN(CCA)
S isomer	3.5 Å	3.6 Å
R isomer	3.6 Å	4.0 Å

containing ODN with **ORN(CAA)** or **ORN(CCA)** were 3.5 and 3.6 Å, respectively, whereas those for the *R* isomer were 3.6 and 4.0 Å, respectively. These results suggest that the length and flexibility of the linkage between the α-chloroaldehyde group and the propanediyl backbone were sufficient for the cross-linking reactions. We further investigated the effect of the amount of the ^{pro}PCA-ODNs on the cross-linking efficiencies. Figure 4c,d shows the results of the cross-linking reactions of ORNs with 3 equiv of **^{pro}PCA-ODN2f** or **^{pro}PCA-ODN2s**, respectively. The cross-linking efficiencies were improved by increasing the amount of ^{pro}PCA-ODNs and reached over 80% for **ORN(CAA)**.

The cross-linking reactivities of ^{pro}PCA-ODNs with ODNs containing one of the four deoxyribonucleotides at the frontal position of the ^{pro}PCA moiety were also investigated (Figure S5, Supporting Information). These results revealed that α-chloroaldehyde-conjugated ODNs preferentially formed cross-linking products with the target nucleotides with dA or dC at the frontal position of ^{pro}PCA after UV irradiation; the quantitative relationship is represented as dA > dC ≫ dG = dT.

We previously found that PCA-ODNs with an α-chloroaldehyde group at the 5′ end of the strand modestly reacted with ODNs with 3′ dangling ends composed of a 5′-AGGG-3′ sequence.[14] These results suggested that the α-haloaldehyde group introduced at the 5′ end of the strand reacted with the neighboring bases of the mutation sites. Therefore, we examined the effects of the neighboring bases of the mutation site in the target RNAs on the cross-linking reactions of ^{pro}PCA-ODNs. **^{Pro}PCA-ODNn** (n = 2−5) had 5′-M_1^{pro}PCAM_2-3′ (M_1, M_2 = T or G) sequences and **ORN-(N_3UN_4)**, which had 5′-N_3UN_4-3′ (N_3, N_4 = C or A) sequences, were complementary ORNs to **^{pro}PCA-ODNn** (n = 2−5). Because **^{pro}PCA-ODN2f** has cross-linking efficiencies similar to those of **^{pro}PCA-ODN2s**, **^{pro}PCA-ODNn** (n = 2−5) which were obtained from the polar main peak in reversed-phase HPLC were used for evaluation of adverse effect of bases adjacent to the mutation site, with the exception of **^{pro}PCA-ODN5**, which showed a single peak in reversed-phase HPLC analysis. As shown in Figure 4e, a series of **^{pro}PCA-ODNn** (n = 2−5) hardly cross-linked with **ORN(N_3UN_4)** (N_3, N_4 = C or A); the yields reached only 20%. These results suggest that the α-chloroaldehyde group hardly reacted with base-pair-forming adenosines or cytosines, even if these bases were close to the reactive aldehyde group. Previously, discrimination of a point mutation from a purine nucleobase to a pyrimidine nucleobase in ORNs was achieved using a 2 + 2 cyclization type reaction under photoirradiation conditions;[12,13] however, that from guanine or uracil to adenine or cytosine bases had not been established. In our cases, ^{pro}PCA-ODN realized the discrimination of the adenine or cytosine bases from other nucleobases using photoirradiation as a trigger of the cross-linking reaction for the first time.

■ CONCLUSION

In this study, we synthesized ^{pro}PCA-ODNs with ^{pro}PCA moieties in the middle of the strands for sequence-selective cross-linking reactions with RNAs having a point mutation. Photo-cross-linking studies by denaturing PAGE revealed that ^{pro}PCA-ODNs selectively cross-linked with adenine or cytosine bases at the frontal position of the α-chloroaldehyde moiety without any significant side reactions. These results suggest that ^{pro}PCA-ODNs have great potential to selectively inhibit point-mutated mRNAs. Now the studies on the inhibition of the cellular RNA activities using ^{pro}PCA-conjugated oligonucleotides are in progress.

■ EXPERIMENTAL SECTION

Materials. Reagents and solvents were purchased from commercial sources. Flash column chromatography was performed using silica gel (200 mesh). NMR spectra were recorded on a 300 MHz NMR spectrometer. Gel permeation chromatography was performed using a recycling preparative HPLC system. ^{Pro}PCA-ODNs were purified using reversed-phase HPLC system with a C-18 reversed-phase column (HPLC conditions: 0.8 mL/min; solvent A = 0.1 M triethylammonium acetate (TEAA); solvent B = 50% or 80%

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CH_3CN/0.1 M TEAA linear gradient, monitored at 260 nm). ORNs and ODNs were purchased from commercial sources and purified by 20% denaturing polyacrylamide gel electrophoresis. The 5′-radiolabeled ORNs and ODNs were prepared by reactions with [γ-^{32}P] ATP and T4 polynucleotide kinase. 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.

*Synthesis of 2-Chloro-6-phthalimidohexanal (**2**).* Compound **1**[19] (795.5 mg, 3.2 mmol) was dissolved in dry CH_2Cl_2 (6.5 mL) and cooled to 0 °C. L-Proline (38.3 mg, 0.33 mmol) and *N*-chlorosuccinimide (439.1 mg, 3.3 mmol) were added to the solution, which was then stirred for 1 h at 0 °C and then for 2.5 h at rt. The solution was washed with saturated sodium thiosulfate, saturated sodium hydrogen carbonate and saturated sodium chloride, dehydrated with sodium sulfate, and evaporated under reduced pressure. The residue was purified by flash column chromatography with hexane/ethyl acetate at a 4:1 ratio, plus 1% triethylamine, yielding compound **2** (748.1 mg, 82%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.49 (d, J = 2.1, 1H), 7.85 (dd, J = 5.4, 3.0, 2H), 7.72 (dd, J = 5.4, 3.0, 2H), 4.16 (ddd, J = 8.4, 5.3, 2.1, 1H), 3.71 (t, J = 6.8, 2H), 2.12−2.00 (m, 1H), 1.94−1.83 (m, 1H), 1.81−1.69 (m, 2H), 1.67−1.59 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 195.0, 168.4, 134.0, 132.1, 123.2, 63.6, 37.4, 31.4, 27.9, 22.9; HRMS (ESI-TOF) m/z: [M + Na]$^+$ calcd for $C_{14}H_{14}ClNO_3Na$ 302.0554; found 302.0563.

*Synthesis of N-(5-Chloro-6,6-bis(2-nitrobenzyloxy)hex-1-yl)-phthalimide (**3**).* Compound **2** (748.1 mg, 2.7 mmol), 2-nitrobenzyl alcohol (1.10 g, 7.2 mmol), 2-naphthalenesulfonic acid (125.6 mg, 0.60 mmol), and a few pieces of 4 Å molecular sieves were added to dry toluene (9.0 mL) and stirred for 2 days at 80 °C. The solution was washed with saturated sodium hydrogen carbonate and saturated sodium chloride, dehydrated with sodium sulfate, and evaporated under reduced pressure. The residue was purified by gel permeation chromatography with ethyl acetate as a eluent, yielding compound **3** (428.7 mg, 28%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.07 (d, J = 8.4 2H), 7.88−7.81 (m, 4H), 7.73−7.63 (m, 4H), 7.45 (t, J = 7.6, 2H), 5.10 (dd, J = 14.7, 19.5, 4H), 4.91 (d, 5.4, 1H), 4.10 (ddd, J = 9.6, 5.1, 2.7, 1H), 3.71 (t, J = 6.8, 2H), 2.14−2.05 (m, 1H), 1.89−1.63 (m, 4H), 1.54−1.49 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.4, 147.0, 146.9, 133.9, 132.1, 128.8, 128.3, 128.2, 124.7, 123.2, 104.8, 67.0, 66.3, 61.4, 37.6, 31.8, 28.0, 23.4; HRMS (ESI-TOF) m/z: [M + Na]$^+$ calcd for $C_{28}H_{26}ClN_3O_8Na$ 590.1301; found 590.1306.

*Synthesis of 6-Amino-2-chloro-1,1-bis(2-nitrobenzyloxy)hexane (**4**).* Compound **3** (542.0 mg, 0.95 mmol) and hydrazine monohydrate (280 μL, 5.7 mmol) were dissolved in ethanol (5.0 mL) and refluxed for 3 h. The solution was evaporated under reduced pressure. The residue was extracted with diethyl ether cooled at 0 °C. The extract was evaporated under reduced pressure and purified by flash column chromatography with CH_2Cl_2/MeOH/triethylamine at a 96:3:1 ratio, yielding compound **4** (380.7 mg, 91%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.08 (d, J = 8.1, 2H), 7.84 (dd, J = 18.3, 7.8, 2H), 7.66 (t, J = 7.4, 2H), 7.46 (t, J = 7.8, 2H), 5.10 (s, 4H), 4.92 (d, J = 5.1, 1H), 4.11 (m, 1H), 2.73 (t, J = 6.4, 2H), 2.07−1.97 (m, 2H), 1.84−1.78 (m, 2H), 1.73−1.62 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 147.1, 147.0, 133.9, 133.8, 128.9, 128.4, 128.3, 124.8, 124.7, 104.8, 67.0, 66.3, 61.6, 41.5, 32.2, 30.9, 23.5; HRMS (ESI-TOF) m/z: [M + H]$^+$ calcd for $C_{20}H_{25}ClN_3O_6$ 438.1426; found 438.1422.

*Synthesis of N-(5-Chloro-6,6-bis(2-nitrobenzyloxy)hex-1-yl)-2,2-bis(hydroxylmethyl)propanamide (**5**).* 2,2-Bis(hydroxymethyl)-propanoic acid (53.8 mg, 0.40 mmol), EDC hydrochloride (77.6 mg, 0.40 mmol), and HOBt (53.6 mg, 0.35 mmol) were dissolved in dry DMF (0.5 mL) following the addition of compound **4** (139.6 mg, 0.32 mmol). After being stirred for 3 h at rt, the solution was washed with saturated sodium hydrogen carbonate and saturated sodium chloride, dehydrated with sodium sulfate, and evaporated under reduced pressure. The residue was purified by flash column chromatography with hexane/ethyl acetate at a 4:1 ratio, plus 1% triethylamine, yielding compound **5** (127.7 mg, 58%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.07 (d, J = 8.1, 2H), 7.82 (dd, J = 16.6, 7.6, 2H), 7.67 (t, J = 7.6, 2H), 7.46 (t, J = 7.4, 2H), 6.99 (m, 1H), 5.09 (s, 4H), 4.91 (d, J = 5.4, 1H), 4.13−4.07 (m, 1H), 3.74 (dd, J = 15.0, 11.1, 4H), 3.30 (dd, J = 5.7, 4.4, 2H), 1.98−1.76 (m, 1H), 1.73−1.47 (m, 4H), 1.01 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.6, 147.1, 147.0, 133.9, 133.8, 129.0, 128.5, 128.4, 124.9, 124.8, 104.8, 68.2, 67.1, 66.4, 61.5, 47.6, 39.0, 31.9, 28.8, 23.4, 18.0; HRMS (ESI-TOF) m/z: [M + Na]$^+$ calcd for $C_{25}H_{32}ClN_3O_9Na$ 576.1719; found 576.1718.

*Synthesis of N-(5-Chloro-6,6-bis(2-nitrobenzyloxy)hex-1-yl)-2-(4,4′-dimethoxytrityloxymethyl)-2-hydroxymethylpropanamide (**6**).* Compound **5** (407.7 mg, 0.74 mmol) and 2,2-dimethoxytrityl chloride (315.5 mg, 0.93 mmol) were dissolved in dry pyridine (1.0 mL) and stirred for 30 min at rt. The solution was washed with saturated sodium hydrogen carbonate and saturated sodium chloride, dehydrated with sodium sulfate, and evaporated under reduced pressure. The residue was purified by flash column chromatography with hexane/ethyl acetate at a 1:1 ratio plus 1% triethylamine, yielding compound **6** (331 mg, 53%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.07 (dt, J = 8.1, 1.4, 2H), 7.81 (dd, J = 16.5, 7.5, 2H), 7.64 (m, 2H), 7.44 (t, J = 8.6, 2H), 7.40 (d, J = 7.2 2H), 7.31 (m, 4H), 7.27 (d, J = 4.2, 2H), 7.20 (t, J = 7.2, 1H), 7.04 (t, J = 5.1, 1H), 6.84 (d, J = 8.7, 4H), 5.07 (t, J = 15.9, 4H), 4.88 (dd, J = 5.3, 1.4, 1H), 4.01 (m, 1H), 3.77 (s, 6H), 3.60 (d, J = 5.7, 2H), 3.30−3.21 (m, 4H), 2.02−1.94 (m, 1H), 1.81−1.77 (m, 1H), 1.69−1.42 (m, 4H), 1.20 (d, J = 1.2, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.3, 158.6, 147.1, 147.0, 144.3, 135.3, 135.1, 133.9, 129.9, 128.9, 128.4, 128.3, 128.1, 127.9, 127.1, 124.8, 124.7, 113.3, 104.8, 86.8, 68.1, 67.3, 67.1, 66.4, 61.5, 55.2, 47.2, 39.0, 31.9, 29.0, 23.6, 19.0; HRMS (ESI-TOF) m/z: [M + Na]$^+$ calcd for $C_{46}H_{50}ClN_3O_{11}Na$ 878.3026; found 878.3029.

*Synthesis of ^{pro}PCA Phosphoramidite Unit (**7**).* Compound **6** (72.0 mg, 0.084 mmol) and *N*,*N*-diisopropylethylamine (75 μL, 0.42 mmol) were dissolved in dry CH_2Cl_2 (1.0 mL). 2-Cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (38 μL, 0.17 mmol) was added to the solution, followed by stirring for 15 min at rt. The solution was washed with saturated sodium hydrogen carbonate and saturated sodium chloride, dehydrated with sodium sulfate, and evaporated under reduced pressure. The residue was purified by flash column chromatography with hexane/ethyl acetate at a 4:1 ratio plus 1% triethylamine, yielding compound **7** (30.5 mg, 34%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.07 (dt, J = 6.9, 1.5, 2H), 7.82 (dd, J = 17.2, 7.6, 2H), 7.64 (td, J = 7.5, 3.7, 2H), 7.47−7.41 (m, 4H), 7.31 (d, J = 9.0, 4H), 7.28 (d, J = 3.9, 2H), 7.19 (t, J = 7.2, 1H), 6.83 (d, J = 9.0, 4H), 6.79 (t, J = 5.7, 1H), 5.08 (t, J = 15.9, 4H), 4.88 (d, J = 5.1, 1H), 4.01−3.99 (m, 1H), 3.77 (s, 6H), 3.74−3.67 (m, 2H), 3.55−3.50 (m, 2H), 3.29 (s, 2H), 3.21 (q, J = 7.2, 2H), 2.53 (q, J = 6.6, 2H), 2.02−1.90 (m, 1H), 1.81−1.52 (m, 3H), 1.52−1.38 (m, 4H), 1.22 (m, 3H), 1.15 (d, J = 6.9, 6H), 1.09 (dd, J = 6.6, 2.7, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.5, 147.0, 144.6, 135.6, 133.9, 133.8, 130.1, 128.9, 128.4, 128.0, 127.9, 126.9, 124.8, 124.7, 113.2, 104.8, 86.3, 67.1, 66.3, 61.6, 55.2, 43.2, 43.1, 32.0, 29.1, 24.6, 24.5, 23.7, 18.8; ^{31}P NMR (121 MHz, $CDCl_3$) δ 148.3; HRMS (ESI-TOF) m/z: [M + Na]$^+$ calcd for $C_{55}H_{67}ClN_5O_{12}PNa$ 1078.4105; found 1078.4116.

Synthesis of ^{pro}PCA-Conjugated Oligonucleotides. ODNs containing a ^{pro}PCA moiety (**^{pro}PCA-ODNn** (n = 1−5)) were synthesized by the conventional solid-phase phosphoramidite method. The coupling time for the ^{pro}PCA phosphoramidite unit (**7**) was 1 h. The ODNs were deprotected in concentrated ammonium hydroxide (55 °C, 12 h), and Tr-ON ODNs were purified by reversed-phase HPLC (HPLC conditions for **^{pro}PCA-ODN1**; solvent A = 0.1 M TEAA; solvent B = 50% CH_3CN/0.1 M TEAA linear gradient from 0% to 100% over 50 min; for **^{pro}PCA-ODNn** (n = 2−5); solvent A = 0.1 M TEAA; solvent B = 80% CH_3CN/0.1 M TEAA linear gradient from 0% to 100% over 50 min). In the case of **^{pro}PCA-ODN4**, the diastereomers observed at around 27 min in the HPLC profile were isolated at this stage. The dimethoxytrityl group was removed by 80% CH_3COOH aq and resulted ODNs were purified by reversed-phase HPLC (HPLC conditions: **^{pro}PCA-ODN1**; solvent A = 0.1 M TEAA; solvent B = 50% CH_3CN/0.1 M TEAA linear gradient from 0% to 100% over 50 min; **^{pro}PCA-ODNn** (n = 2−5); solvent A = 0.1 M TEAA; solvent B = 80% CH_3CN/0.1 M TEAA two linear gradient from 0% to 10% over 1 min and from 10% to 50% over 40 min). **^{Pro}PCA-ODNn** (n = 2, 3) gave twin peaks at around 27 min, respectively, while **^{pro}PCA-ODN5** gave a single peak. All oligonucleo-

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tides were characterized by ESI-TOF-MS spectrometry. ESI-TOF-MS m/z: $[M - 2H]^{2-}$ calcd for **^{pro}PCA-ODN1** 579.63, found 579.70; $[M - 5H]^{5-}$ calcd for **^{pro}PCA-ODNn** (n = 2, 3) 1069.18, **^{pro}PCA-ODN2f**, found 1069.17; **^{pro}PCA-ODN2s** found 1069.17; **^{pro}PCA-ODN3** found 1069.18; $[M - 5H]^{5-}$ calcd for **^{pro}PCA-ODN4** 1074.19, found 1074.13; $[M - 5H]^{5-}$ calcd for **^{pro}PCA-ODN5** 1064.18, found 1064.13.

Photodeprotection Reaction. **^{pro}PCA-ODN1** (0.5 OD) and dT (0.3 OD) as an internal standard were dissolved in 10 mM phosphate buffer (pH 7.0) with 100 mM NaCl. The reaction mixture was heated to 90 °C, incubated for 5 min, and cooled to 37 °C at −1 °C/min. Then 50 μL of the solutions was transferred to a 96-well black plate and irradiated at 365 nm (400 mW/cm^2) for up to 1 min. The UV-irradiated samples were analyzed by reversed-phase HPLC (solvent A = 0.1 M TEAA; solvent B = 50% CH_3CN/0.1 M TEAA linear gradient from 0% to 100% over 50 min).

Radiolabeling of ORNs and ODNs with γ-^{32}P ATP. One picomole of ORNs or ODNs was reacted with 5 pmol of [γ-^{32}P]-ATP and 20 U of T4 polynucleotide kinase in 50 μL of kinase buffer at 37 °C for 30 min. According to the manufacturer's protocol, the reaction mixtures were purified by a C-18 reversed-phase column.

Photo-Cross-Linking Reactions. **^{pro}PCA-ODNn** (n = 2−5) (0.1 or 0.3 μM), ORN or ODN (0.1 μM), and ^{32}P-labeled ORN or ODN (0.02 nM) were dissolved in 50 μL of 10 mM phosphate buffer (pH 7.0) with 100 mM NaCl. The reaction mixtures were heated to 90 °C, incubated for 5 min, and cooled to 37 °C at −1 °C/min. Then 5 μL of the samples was transferred as non-UV-irradiated samples, and the other samples were irradiated at 365 nm (400 mW/cm^2) for 1 min and incubated at 37 °C up to 48 h. All samples were quenched by addition of the same amount of formamide. All samples were analyzed by 20% denaturing polyacrylamide gel electrophoresis at 600 V for 120 min (gels containing 7 M urea, 25% formamide). The bands were visualized by autoradiography with 1-h exposure to an Image Plate. The resulting autoradiograms were quantified using Image Gauge software to generate plots of product formed as a function of time. The resulting data were fitted to a single-exponential plot to determine $t_{1/2}$.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.5b02573.

^{1}H, ^{13}C, and ^{31}P NMR spectra of new compounds, denaturing PAGE analyses, reversed-phase HPLC profiles of oligonucleotides, thermal denaturation curves, T_m values, energy-optimized duplex structures of ^{pro}PCA-ODNs with ORNs, and mass spectra of **^{pro}PCA-ODN2f** and **^{pro}PCA-ODN2s** (DOC)

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Notes

The authors declare no competing financial interest.

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