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Synthesis of antisense oligonucleotides containing 2′-*O*-psoralenylmethoxyalkyl adenosine for photodynamic regulation of point mutations in RNA

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ABSTRACT

2′-*O*-Psoralen-conjugated antisense oligonucleotide was able to recognize a point mutation of mRNA. It had outstanding ability to photo-cross-link only to oligoribonucleotides (ORN) having a point mutation. This type of antisense molecule is the only one of its kind so far. To give high photo-cross-linking efficiency and sequence selectivity to antisense molecules, we synthesized novel photo-reactive oligonucleotides (2′-Ps-***x***om) containing psoralen at the *2′-O-position* adenosine via an ethoxymethylene (2′-Ps-eom), propoxymethylene (2′-Ps-pom) and butoxymethylene (2′-Ps-bom) linker, respectively. We evaluated the photo-cross-linking efficiency and sequence selectivity in photo-cross-linking of 2′-Ps-***x***om to the complementary ORN and to an ORN having a mismatch base. Among them, 2′-Ps-eom exhibited superior photo-cross-linking efficiency with high sequence selectivity.

1. Introduction

In recent years, it has been reported that point mutations in genes are responsible for various cancers.[1–4] The mutation affects cellular proliferation and elicits tumorigenic properties. It has been desired to inhibit the gene expression of disease-causing mutations selectively without affecting the functions of normal genes. The strict regulation of the expression of genes having point mutations in their sequence could lead to the development of novel anti-cancer therapy. The point mutation of the *ras* gene is a typical example which is reportedly responsible for various cancers.[5–8] A considerable number of studies have been carried out using chemically modified antisense oligonucleotides[9–11] and siRNA.[12–14] Some groups have reported the successful suppression of mutant *ras* mRNA using antisense oligonucleotides or siRNA, though these methods inhibited the expression of not only mutant *ras* mRNA but also wild-type *ras* mRNA.[15–20] This undesired suppression could lead to serious side effects in curing cancers. Thus, novel conceptual attempts are needed.

We have attempted to regulate the expression of mRNA having a point mutation using psoralen derivatives. They have an ability to photo-cross-link covalently to pyrimidine bases, especially thymine and uracil, upon UV irradiation (320–400 nm).[21] When we localize psoralen in the close vicinity of a mutationally-altered pyrimidine base using psoralen-conjugated antisense oligonucleotides, the sequence specific suppression of the functions of designated mRNA could be achieved.

Previously, a photo-reactive antisense oligonucleotide whose *5′-O-position* was modified with a 4,5′,8-trimethylpsoralen (psoralen) via an aminoethylaminomethyl linker (5′-Ps-oligo) was reported.[22,23] 5′-Ps-oligo complementary to the E6 region of human papillomavirus type 18 (HPV-18) mRNA significantly elicited the apoptotic death of HPV18-positive cervical carcinoma cells upon UVA irradiation (365 nm) for 2 min in vitro and in vivo.[24,25] With these results, photodynamic antisense therapy (PDA-therapy) is proceeding to the clinical stage. For future developments of PDA-therapy, we have to minimize the side reactions of psoralen derivatives with off-target mRNAs and the cytotoxicity of UVA used for photo-cross-linking. The psoralen of 5′-Ps-oligo in our previous study could photo-cross-link to a pyrimidine base in the vicinity of the psoralen even though the pyrimidine base is not the target base. This might be due to the flexibility of the psoralen conjugated at the 5′-end. Instead of 5′-end modification, we chose the *2′-O-position* of adenosine as the modification site of psoralen and synthesized a photo-reactive antisense oligonucleotide having a 2′-*O*-psoralen-conjugated adenosine (2′-Ps-oligo). Adenosine was *conjugated* with a psoralen via a methylene linker at the *2′-O-position* and the conjugated adenosine was incorporated into the designated position of the antisense oligonucleotides (2′-Ps-met).[26] The 2′-Ps-met photo-cross-linked to the complementary oligoribonucleotides (ORN) upon UVA irradiation (365 nm) with appreciable efficiency, but hardly at all to ORN having a mismatch base at the opposite site of the 2′-*O*-psoralen-conjugated adenosine. The introduction of psoralen to the *2′-O-position* of adenosine greatly enhanced the sequence specificity of the photo-cross-linking reaction compared with 5′-Ps-oligo. However the efficiency was largely decreased. The photo-cross-linking efficiency of 2′-Ps-met with

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complementary ORN upon UVA irradiation was ca. 50% after 150 min irradiation, while the photo-cross-linking efficiency of 5′-Ps-oligo was ca. 50% after 18 min irradiation. As UVA (320–400 nm) is mutagenic and carcinogenic for cells,[27] 2′-Ps-met is not substantially applicable for clinical uses and therefore, photo-irradiation time should be minimized by appropriate molecular design of antisense molecules.

In this report, we designed advanced 2′-Ps-oligo to enhance the photo-cross-linking efficiency and sequence selectivity by focusing on the linker molecules. We synthesized novel photo-reactive oligonucleotides (2′-Ps-***x***om) containing psoralen at the *2′-O-position* of adenosine via an ethoxymethylene (2′-Ps-eom), propoxymethylene (2′-Ps-pom) and butoxymethylene (2′-Ps-bom) linker, and examined their photo-cross-linking properties to ORNs. It was found that the 2′-Ps-eom and 2′-Ps-pom exhibited an outstanding ability to photo-cross-link only to complementary ORN. These results suggest that 2′-Ps-eom and 2′-Ps-pom were able to recognize a point mutation of mRNA and selectively regulate the expression. This type of antisense molecule is the only one of its kind so far.

2. Results and discussion

2.1. Molecular design

In a previous study, we reported that 2′-Ps-met photo-cross-linked to complementary ORN upon UVA irradiation but hardly at all to ORN having a mismatch base.[26] It was noteworthy that 2′-Ps-met discriminated a single base mutation in RNA. However, the long UVA irradiation time (150 min for 50% cross-linking yield) must be avoided, because of the mutagenic and carcinogenic properties of UVA.[27] We have also observed that the cell viability was drastically decreased upon UVA irradiation.[24] Accordingly, 2′-Ps-met is not practical for clinical purposes. To pursue PDA-therapy, it is essential to enhance the photo-cross-linking efficiency of 2′-Ps-oligo without sacrificing its sequence specificity. We designed other 2′-Ps-oligos focusing on the length and flexibility of the linkage that connects psoralen to the *2′-O-position* of adenosine. We adopted alkoxymethylene as the linker molecule as shown in Figure 1. 2′-Ps-oligos are denoted as 2′-Ps-***x***om, where ***x*** represents the first letter of the name of the linker molecules. As the target of 2′-Ps-***x***om, we chose a codon12 region of K-*ras* mRNA. A single nucleotide mutation at codon 12 ([12]Gly→Val; GGU→GUU) is common among K-*ras* mRNA mutation. When K-*ras* underwent a point mutation, the mutated K-*ras* transformed normal cells into cancer cells. This type of event is frequently observed in various cancers such as colorectal cancer and pancreas cancer.[3] Selective suppression of mutated K-*ras* expression could lead to the development of a novel methodology in cancer therapy. The sequences of 2′-Ps-***x***om are shown in Table 1. As the target, mutant-type K-*ras* ORN (ORN-**U**) and wild-type K-*ras* ORN (ORN-**G**) were prepared. Prior to the molecular design of 2′-Ps-***x***om, the location of psoralen in a duplex of 2′-Ps-***x***om and ORN-**U** was evaluated by conformational calculations using the AMBER* force field[28] in MacroModel (ver.8.0, Schrödinger).[29] The initial position of psoralen was as follows: 5′–UG*UU-3′/3′-AC^{PS}AA-5′ (*, designated intercalation site; U, target uracil; ^{PS}A, psoralen-conjugated adenosine). In the case of 2′-Ps-eom and 2′-Ps-pom, it was demonstrated that the psoralen was located in the close vicinity of the target uracil (U). In the case of 2′-Ps-bom, there is a possibility that the psoralen is located in close range of both the target uracil (U) and the neighboring uracil (U). To evaluate whether psoralen of 2′-Ps-***x***om can photo-cross-link to the target uracil (U), the average distances between the 5,6-carbon of uracil and 3,4-carbon of 4,5′,8-trimethylpsoralen were estimated based on 3D-structures presented by MacroModel. The distances were 4.5 Å in 2′-Ps-eom, 3.6 Å in 2′-Ps-pom, 4.3 Å in 2′-Ps-bom and 5.9 Å in 2′-Ps-pnom. The results suggest that psoralen of 2′-Ps-eom, 2′-Ps-pom, and 2′-Ps-bom can locate in the close range of the target uracil compared with 2′-Ps-met (5.6 Å); the psoralen of 2′-Ps-eom and 2′-Ps-pom can cross-link only to the target uracil (U) of the ORN-**U**; and the psoralen of 2′-Ps-bom can cross-link not only to the designated uracil (U) but also to the neighboring uracil (U). On the other hand, 2′-Ps-pnom was supposed not to intercalate between base pairs. According, we synthesized 2′-Ps-eom, 2′-Ps-pom, and 2′-Ps-bom, and examined their characteristics.

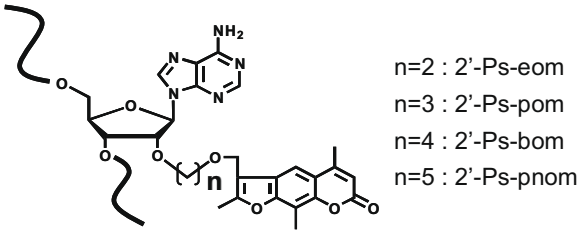


Figure 1. Structure of photo-reactive oligonucleotides containing 2′-*O*-psoralenylmethoxyalkyl adenosine.

2.2. Synthesis of 2′-*O*-psoralenylmethoxyalkyl adenosine

2′-Ps-***x***om was synthesized by the introduction of 2′-*O*-psoralen-conjugated adenosine into the designated site of the oligodeoxyribonucleotides. As the key adenosine derivative, 4′-chloroalkoxymethyl-4,5′,8-trimethylpsoralens were synthesized as shown in Scheme 1. 4′-Chloromethyl-4,5′,8-trimethylpsoralen (**1**) and 4′-hydroxyethoxymethyl-4,5′,8-trimethylpsoralen (**2a**) were synthesized according to the reported procedure.[30,31] Compound **1** was treated by a glycol as a linker molecule. Compound **1** was reacted with ethylene glycol, 1,3-propanediol, or 1,4-butanediol without solvent to give **2a**, **2b** or **2c**, respectively. Chlorination of **2a**, **2b** and **2c** was then carried out in carbon tetrachloride with triphenylphosphine to give **3a**, **3b**, and **3c**. The overall yields of **3a**, **3b**, and **3c** from **1** were 64%, 78%, and 62%, respectively.

2′-*O*-Psoralen-conjugated adenosines were synthesized as shown in Scheme 2. Unprotected adenosine was activated by sodium hydride in dry DMF followed by treatment with **3a**, **3b** or **3c** at 80 °C for 32 h giving both *2′-O-position* and *3′-O-position* modified adenosine. In this case, it was found that more than 90% of the product was a 2′-*O*-psoralen-modified product. 2′-*O*-Psoralen-conjugated adenosines (**4a**, **4b** and **4c**) were isolated by silica gel chromatography in 18%, 16% and 28% yields, respectively. These relatively low yields were attributed to the β-elimination reaction of 4′-chloroalkoxymethyl-4,5′,8-trimethylpsoralens; further, the suppression of the side reaction was unsuccessful. **4a**, **4b** and **4c** were treated with benzoyl chloride according to the general procedures of Ti et al.[32] to give **5a**, **5b**, and **5c**. The 5′-hydroxyl moieties of **5a**, **5b** and **5c** were protected by dimethoxytrityl chloride in pyridine followed by the treatment of 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite in the presence of 1*H*-tetrazole as a catalyst in dry acetonitrile. Without purification, these reaction mixtures were added to 5′-detritylated d(ACCGCAT)-CPG, and the mixtures were allowed to react at room temperature for 2 h. The CPG support was further processed to give 2′-Ps-***x***om with the designated sequence. The sequences of 2′-Ps-***x***om complementary to the codon12 region of K-*ras* mRNA are shown in Table 1.

2.3. Interaction between 2′-Ps-*x*om and ORNs

The behaviors of oligonucleotides to which psoralen had been introduced were examined. The thermal stability of the duplexes of 2′-Ps-***x***om and ORNs were evaluated by UV melting profiles (Table

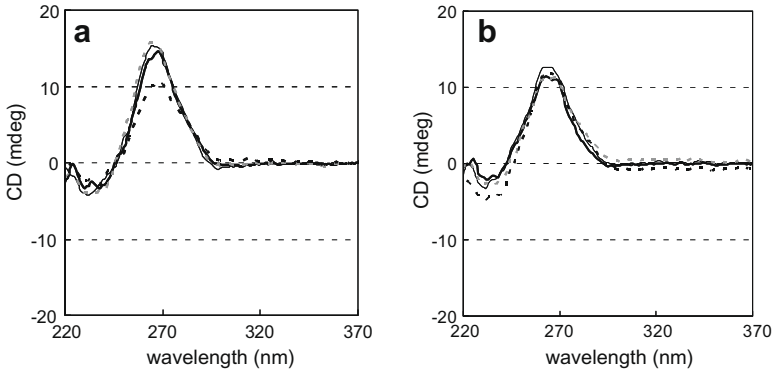


Figure 2. CD spectra of the duplexes of 2′-Ps-***x***om and (a) ORN-**U** or (b) ORN-**G**. Key: Kr-ODN and ORN (thin line), 2′-Ps-eom and ORN (thick line), 2′-Ps-pom and ORN (gray broken line) 2′-Ps-bom and ORN (black broken line), conditions : [2′-Ps-***x***om] = [Kr-ODN] = [ORN] = 2 mM in 0.1 M phosphate buffer containing 0.1 M NaCl (pH 7.0). Measurements were carried out at 10 °C.

Table 1
Sequences of 2′-Ps-oligos and ORNs

Name	Sequence	ESI-MS (*m*/*z*)	
		Calcd	Found
2′-Ps-eom	5′ dTACGCCAA^{Ps}CAGCTCC 3′[a]	([M−6H]$^{-6}$) 795.642	795.623
2′-Ps-pom	5′ dTACGCCAA^{Ps}CAGCTCC 3′[a]	([M−6H]$^{-6}$) 797.977	798.069
2′-Ps-bom	5′ dTACGCCAA^{Ps}CAGCTCC 3′[a]	([M−7H]$^{-7}$) 685.839	685.903
Kr-ODN	5′ dTACGCCAACAGCTCC 3′	([M−7H]$^{-7}$) 638.963	638.907
ORN-**U**	5′ rGGAGCUGUUGGCGUA 3′	([M−6H]$^{-6}$) 813.107	813.131
ORN-**G**	5′ rGGAGCUGGUGGCGUA 3′	([M−5H]$^{-6}$) 968.125	968.155

[a] Aps = 2′-O-psoralen-conjugated adenosine.

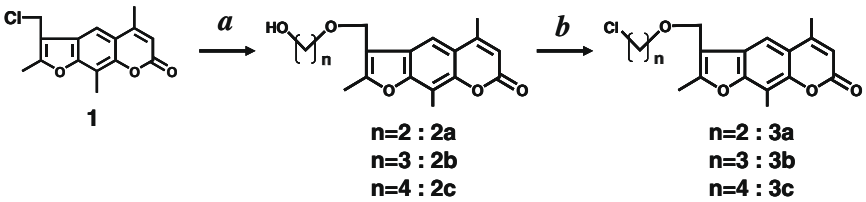
2). The melting temperatures (Tms) of the duplexes of 2′-Ps-***x***om and ORN-**U** suggested that the introduction of psoralen to adenosine via alkoxymethylene linkers destabilized the duplex to some extent. However, the Tms was high enough for 2′-Ps-***x***om to function as an antisense oligonucleotide under physiological conditions. It was also demonstrated that the duplex of 2′-Ps-***x***om and ORN-**G** was also stably formed under physiological conditions.

UV spectra of the duplexes showed no appreciable spectrum change, which might be due to the broad absorption band attributed to psoralen around 300–360 nm. The location of the psoralen in the duplex was examined by circular dichroism (CD) spectroscopy (Fig. 2). CD spectra of the duplexes of 2′-Ps-***x***om and ORN-**U** except for 2′-Ps-bom and ORN-**U** showed substantially the same spectra as the duplex of psoralen-unmodified ODN, suggesting that the duplex was A-form-like. The duplex of 2′-Ps-bom and ORN-**U** also showed an A-form-like spectrum while the cotton effect was significantly diminished. Induced circular dichroism (ICD) around 300–350 nm was not observed.

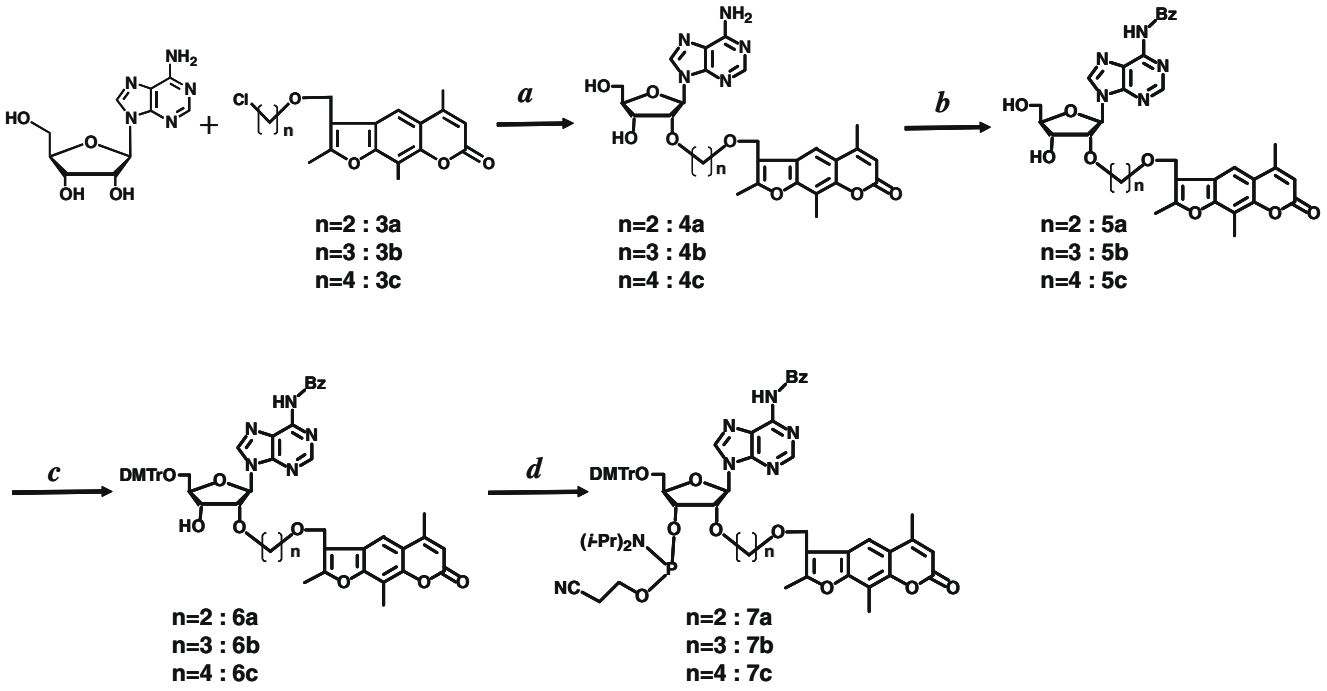
Fluorescence spectroscopy gave considerable information concerning psoralen localization. Figure 3 shows the fluorescence spectra of duplexes of 2′-Ps-***x***om and ORNs. It was demonstrated that the fluorescence intensity of the psoralen at 465 nm was significantly quenched when psoralen was incorporated into ODN. When 2′-Ps-***x***om formed a duplex with ORN-**U**, the fluorescence intensities around 465 nm were further quenched compared with 2′-Ps-***x***om alone. The degree of fluorescence quenching of 2′-Ps-eom was most significant among the three molecules. Moreover, the fluorescence spectra of 2′-Ps-***x***om showed a slight blue shift. It was reported that the fluorescence spectrum due to psoralen was quenched and blue-shifted when psoralen was intercalated between base pairs of the hybrid.[33] These spectroscopic characterizations suggest that 2′-Ps-***x***om formed a stable A-form-like duplex with ORNs and that the psoralen of 2′-Ps-***x***om was intercalated between base pairs of the duplex.

2.4. Photo-cross-linking properties of 2′-Ps-*x*om

UVA irradiation of equimolar solutions of 2′-Ps-***x***om and ORNs was carried out at 35 °C on a transilluminator (365 nm, 1.6 mW/cm^2). To evaluate the photo-cross-linking efficiency and selectivity in sequence recognition, these photo-reactions were monitored by reversed-phase HPLC. Major photoproduct peaks (*) appeared in a time-dependent manner (Fig. 4). As it was reported that psoralen–pyrimidine adducts were regenerated to psoralen and pyrimidine by irradiation at 240–290 nm,[34] we used the photo-reaction to identify the new peaks. Peaks with an asterisk were isolated and then UV-irradiated at 254 nm for 30 min. The photo-regeneration reactions of the photoproducts were analyzed by reversed-phase HPLC (Fig. 5). It was observed that the peak with an asterisk in the mixture of 2′-Ps-eom and ORN-**U** regenerated both starting ORN-**U** and 2′-Ps-eom. Same reaction was observed in the mixture of 2′-Ps-pom and ORN-**U**. On the other hand, the photoproduct in the mixture of 2′-Ps-bom and ORN-**U** regenerated only starting 2′-Ps-bom but not ORN-**U**, suggesting that the photoproduct in the 2′-Ps-bom mixture was an intramolecularly photo-cross-linked products of 2′-Ps-bom. The butoxymethylene linker in 2′-Ps-bom might be too long to photo-cross-link to the designated uracil of ORN. Furthermore, the photoproduct in the mixture of 2′-Ps-***x***om and ORN-**G** regenerated only starting 2′-Ps-***x***om. These results suggest that 2′-Ps-eom and 2′-Ps-pom photo-cross-linked to ORN-**U** in a sequence specific manner and that they were applicable for the treatment of cancer cells having crucial point mutations in gene.



Scheme 1. Reagents and conditions: (a) excess diol, 110 °C; (b) triphenylphosphine, carbon tetrachloride, pyridine, r.t.



Scheme 2. Reagents and conditions: (a) 4′-chloroalkoxymethyl-4,5′,8-trimethylpsoralen, NaH, DMF, 75 °C, (b) 1—TMSCl, pyridine, r.t; 2—BzCl, pyridine, r.t; 3—NH_3, r.t, (c) DMTrCl, pyridine, r.t; (d) 2-Cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite, 1*H*-tetrazole, acetonitrile, r.t.

Table 2
Thermal stabilities of duplexes of 2′-Ps-***x***om with ORNs

	Tm (°C)	
	ORN-**U**	ORN-**G**
2′-Ps-eom	63.1	59.1
2′-Ps-pom	62.7	58.8
2′-Ps-bom	64.0	60.2
Kr-ODN	67.3	58.9

The photo-cross-linking efficiencies were evaluated based on remaining intact ORN and the time courses are shown in Figure 6. The photo-cross-linking efficiencies of 2′-Ps-eom and 2′-Ps-pom with ORN-**U** were ca. 75% and ca. 50% upon UVA irradiation for 1 min at 35 °C, respectively. In the case of ORN-**G**, the efficiencies of 2′-Ps-eom and 2′-Ps-pom were ca. 7% and ca. 9%, respectively. In our previous report, the photo-cross-linking efficiency of 2′-Ps-met and ORN-**U** was ca. 3% upon UVA irradiation for 1 min and ca. 35% for 120 min.[26] Compared with the results of 2′-Ps-met, it was found that the adoption of an ethoxymethylene or a propoxymethylene as a linker molecule of psoralen conjugation dramatically enhanced the photo-cross-linking efficiency and the sequence selectivity. The photo-cross-linking efficiency of 2′-Ps-eom was the highest among all types of psoralen-conjugated oligonucleotides ever reported,[22–24] and the irradiation time for photo-cross-linking could be allowed for clinical use.

3. Conclusion

In conclusion, we developed new types of photo-cross-linking antisense oligonucleotides containing adenosine in which the *2′-O-position* was modified with psoralen via alkoxymethylene linkers, and 2′-Ps-eom and 2′-Ps-pom showed the high photo-cross-linking efficiency and the sequence selectivity. This is the first time that we have come across such an antisense molecule, and it could show the outstanding ability to control certain types of mutations. Assuming that this photo-reaction proceeds in actual cancer cells having point mutations in specified genes (e.g., G→U or A→U), 2′-Ps-eom and 2′-Ps-pom could regulate the proliferation of the cancer cells. The attempt to apply these antisense molecules to cancer cells having a point mutation in gene is now underway.

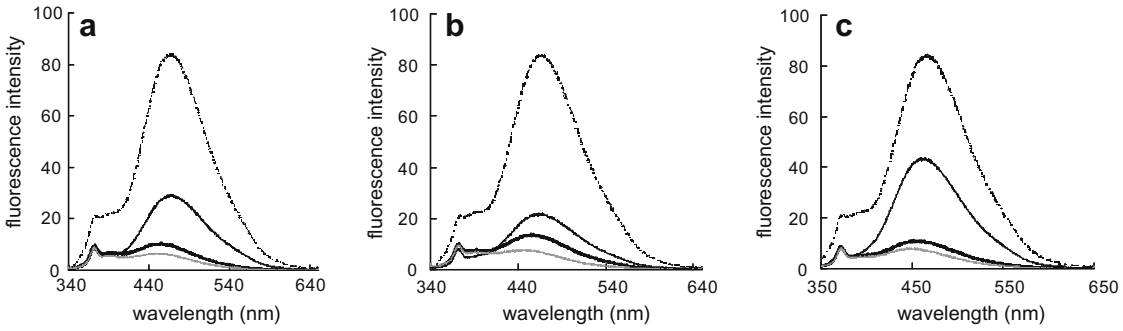


Figure 3. Fluorescence spectra of the duplexes of ORNs and (a) 2′-Ps-eom, (b) 2′-Ps-pom, (c) 2′-Ps-bom. Key: the single-strand 2′-Ps-***x***om (thin line), the duplex of 2′-Ps-***x***om and ORN-**U** (thick line), the duplex of 2′-Ps-***x***om and ORN-**G** (gray line), 4′-chloroalkoxymethyl-4,5′,8-trimethylpsoralen (broken line). Conditions: [2′-Ps-***x***om] = [ORNs] = 2 μM in 0.1 M phosphate buffer containing 0.1 M NaCl (pH 7.0). Measurements were carried out at 10 °C. (λ_{ex} = 330 nm).

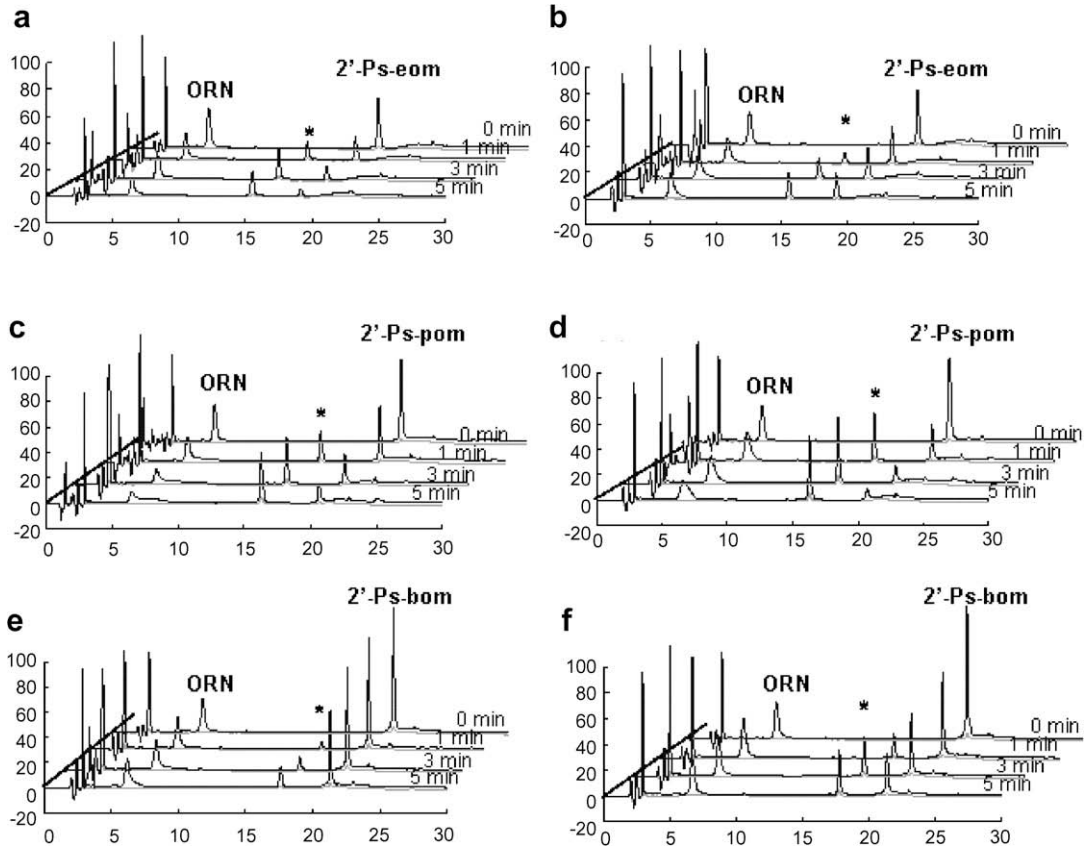


Figure 4. Time course of photo-cross-linking reactions of equimolar mixture of 2′-Ps-*x*om and ORNs analyzed by reversed-phase HPLC. (a) 2′-Ps-eom and ORN-**U**, (b) 2′-Ps-eom and ORN-**G**, (c) 2′-Ps-pom and ORN-**U**, (d) 2′-Ps-pom and ORN-**G**, (e) 2′-Ps-bom and ORN-**U**, (f) 2′-Ps-bom and ORN-**G**. Key: absorbance at 260 nm (black line), absorbance at 330 nm (gray line), Conditions: [2′-Ps-*x*om] = [ORNs] = 2 μM in 0.1 M phosphate buffer containing 0.1 M NaCl (pH 7.0), HPLC conditions: the acetonitrile gradient in TEAA (pH 7.0) was as follows; 0–10 min, a linear gradient from 7.5% to 15%; 10–20 min, a linear gradient from 15% to 50%; 20–30 min maintained at 50%, column oven; 40 °C.

4. Experimental

4.1. General

All reagents and solvents were purchased from commercial sources and used without further purification. Silica gel column chromatography was carried out on Wako gel C-200. NMR spectra were measured with Bruker Biospin DRX500 spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C with a deuterated solvent and TMS as an internal standard. ESI-MS spectra were recorded on Bruker DALTONICS® microTOF. Oligodeoxyribonucleotides and oligoribonucleotides were synthesized via the conventional procedure using a Beckmen Oligo1000 DNA synthesizer. Abbreviation of some reagents: Bz, benzoyl; DMTr, 4,4′-dimethoxytriphenylmethyl.

4.2. Synthesis

4.2.1. 4′-(3″-Hydroxypropoxymethyl)-4,5′,8-trimethylpsoralen (2b)

Compound **1** (1.59 g, 5.76 mmol) was suspended in 1,3-propanediol (40 ml, 553 mmol), and heated up to 110 °C to dissolve **1**. The mixture was stirred at 110 °C for 10 min and after cooling to room temperature, water (60 ml) was added. The solution was extracted four times with CH_2Cl_2 (80 ml). The organic phase was dried over Na_2SO_4, filtrated, and concentrated to dryness. The crude residue was dissolved in acetonitrile (70 ml). The solution was kept at 0 °C overnight, and a white precipitate was collected. The crystal was isolated by filtration to give **2b** (1.52 g, 83%). ^{1}H NMR ($CDCl_3$) δ 1.85–1.89 (m, 2H), 2.50 (s, 6H), 2.54 (s, 3H), 3.67–3.69 (t, J = 5.86, 2H), 3.75–3.78 (t, J = 5.71, 2H), 4.64 (s, 2H), 6.21 (d, J = 1.05, 1H), 7.58 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 8.4, 12.3, 19.3, 32.2, 61.5, 63.3, 68.8, 109.1, 111.4, 111.8, 112.7, 116.1, 125.0, 149.1, 153.4, 154.6, 154.8, 161.5. HRMS m/z: calcd for $C_{18}H_{20}NaO_5$ ($[M+Na]^+$) 339.121, found 339.119.

4.2.2. 4′-(4″Hydroxybutoxymethyl)-4,5′,8-trimethylpsoralen (2c)

Compound **1** (767 mg, 2.77 mmol) was suspended in 1,4-butanediol (25 ml, 282 mmol), and heated up to 110 °C to dissolve **1**. The mixture was stirred at 110 °C for 10 min and after cooling to room temperature, water (40 ml) was added. The solution was extracted four times with CH_2Cl_2 (60 ml). The organic phase was dried over Na_2SO_4, filtrated, and concentrated to dryness. The crude residue was dissolved in acetonitrile (50 ml). The solution was kept at 0 °C overnight, and a white precipitate was collected. The crystal was isolated by filtration to give 2c (652 mg, 71%). ^{1}H NMR ($CDCl_3$) δ 1.65–1.74 (m, 4H), 2.49 (s, 6H), 2.54 (s, 3H), 3.52–3.55 (t, J = 5.93, 2H), 3.62–3.64 (t, J = 6.03, 2H), 4.63 (s, 2H), 6.21 (d, J = 1.10, 1H), 7.59 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 8.4, 12.3, 19.3, 26.6, 30.0, 62.5, 63.0, 70.0, 109.0, 111.6, 111.9, 112.7, 116.1, 125.0, 149.1, 153.4, 154.6, 154.8, 161.5. HRMS m/z: calcd for $C_{19}H_{22}NaO_5$ ($[M+Na]^+$) 353.137, found 353.113.

4.2.3. 4′-(2″-Chloroethoxymethyl)-4,5′,8-trimethylpsoralen (3a)

Compound **2a** (1.49 mg, 4.95 mmol) was dissolved in dry pyridine (50 ml). To this solution, triphenylphosphine (3.25 g, 12.4 mmol) and carbon tetrachloride (l.60 ml, 12.4 mmol) were added and stirred at room temperature for 7 h followed by evaporation to dryness. The residue was resolved in CH_2Cl_2 (120 ml) and

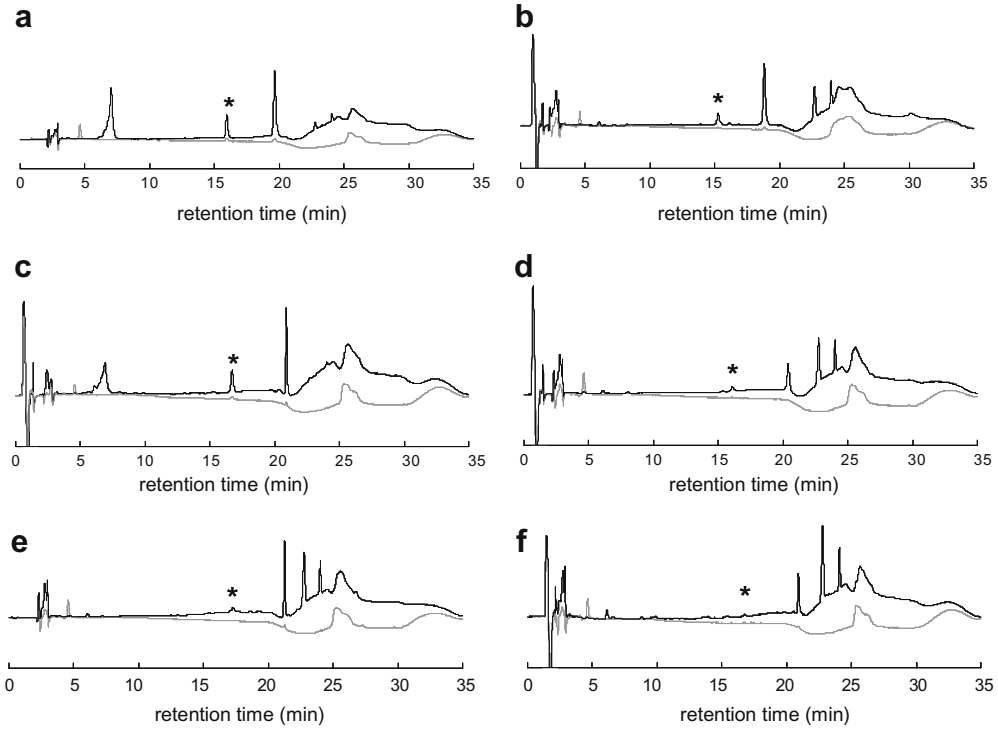


Figure 5. Photo-regeneration reactions were analyzed by reversed-phase HPLC. Isolated products obtained from the reaction mixture of (a) 2′-Ps-eom and ORN-**U**, (b) 2′-Ps-eom and ORN-**G**, (c) 2′-Ps-pom and ORN-**U**, (d) 2′-Ps-pom and ORN-**G**, (e) 2′-Ps-bom and ORN-**U**, (f) 2′-Ps-bom and ORN-**G**, were irradiated at 254 nm for 30 min. Key: absorbance at 260 nm (black line), absorbance at 330 nm (gray line). HPLC conditions: the acetonitrile gradient in TEAA (pH 7.0) was as follows; 0–10 min, a linear gradient from 7.5% to 15%; 10–20 min, a linear gradient from 15% to 50%; 20–30 min maintained at 50%, column oven; 40 °C. HPLC conditions: the acetonitrile gradient in TEAA (pH 7.0) was as follows; 0–10 min, a linear gradient from 7.5% to 15%; 10–20 min, a linear gradient from 15% to 50%; 20–35 min maintained at 50%, column oven; 40 °C.

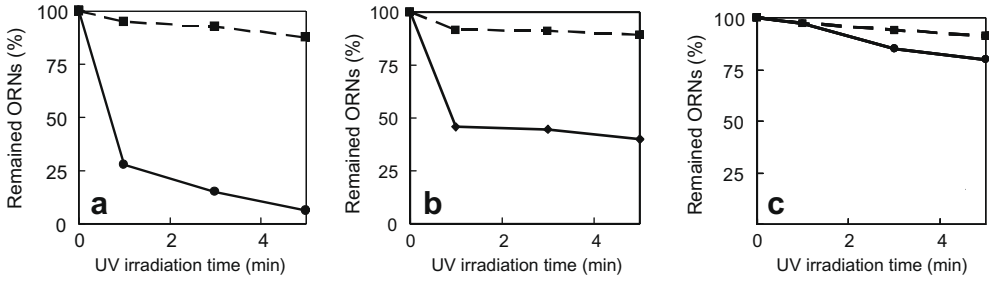


Figure 6. Time course of photo-cross-linking efficiencies of (a) 2′-Ps-eom, (b) 2′-Ps-pom, (c) 2′-Ps-bom, and ORN-**U** (thin line) or ORN-**G** (broken line).

washed with aqueous 5% $NaHCO_3$ (120 ml). The organic layer was dried over Na_2SO_4, filtrated, and concentrated to dryness. The residue was purified by flash chromatography to give **3a** (1.37 g, 87%) as white powder. ^{1}H NMR ($CDCl_3$) δ 2.49 (d, J = 1.35, 6H), 2.56 (s, 3H), 3.67–3.69 (m, 2H), 3.76–3.78 (m, 2H), 4.71 (s, 2H), 6.23 (d, J = 1.10, 1H), 7.68 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 8.4, 12.3, 19.4, 43.2, 63.6, 69.9, 109.0, 111.6, 111.9, 112.8, 116.2, 124.9, 149.2, 153.4, 154.6, 154.7, 161.5. HRMS m/z: calcd for $C_{17}H_{17}NaO_4$ ($[M+Na]^+$) 343.071, found 343.074.

4.2.4. 4′-(3″-Chloropropoxymethyl)-4,5′,8-trimethylpsoralen (3b)

Compound **2b** (900 mg, 2.84 mmol) was dissolved in dry pyridine (20 ml). To this solution, triphenylphosphine (1.28 g, 4.87 mmol) and carbon tetrachloride (530 μl, 5.49 mmol) were added and stirred at room temperature for 6 h followed by evaporation to dryness. The residue was resolved in CH_2Cl_2 (70 ml) and washed with aqueous 5% $NaHCO_3$ (70 ml). The organic layer was dried over, filtrated, and concentrated to dryness. The residue was purified by flash chromatography to give **3b** (896 mg, 94%) as white powder. ^{1}H NMR ($CDCl_3$) δ 2.02–2.07 (m, 2H), 2.49 (s, 6H), 2.55 (s, 3H), 3.64–3.67 (m, 4H), 4.62 (s, 2H), 6.22 (d, J = 1.00, 1H), 7.57 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 8.4, 12.3, 19.4, 32.5, 41.8, 63.3, 66.2, 109.1, 111.5, 111.9, 112.8, 116.2, 125.1, 149.2, 153.3, 154.7, 154.8, 161.5. HRMS m/z: calcd for $C_{18}H_{19}NaO_4$ ($[M+Na]^+$) 357.087, found 357.092.

4.2.5. 4′-(4″-Chlorobutoxymethyl)-4,5′,8-trimethylpsoralen (3c)

Compound **2c** (586 mg, 1.77 mmol) was dissolved in dry pyridine (10 ml). To this mixture, triphenylphosphine (1.40 g, 5.31 mmol) and carbon tetrachloride (510 μl, 5.32 mmol) were added and stirred at room temperature for 9 h followed by evaporation to dryness. The residue was dissolved in CH_2Cl_2 (70 ml) washed with aqueous 5% $NaHCO_3$ (70 ml). The organic phase was dried over Na_2SO_4, filtrated, and concentrated to dryness. The residue was purified by flash chromatography to give **3c** (546 mg, 88%) as white powder. ^{1}H NMR ($CDCl_3$) δ 1.75–1.79 (m, 2H), 1.85–1.91 (m, 2H), 2.50 (m, 6H), 2.56 (s, 3H), 3.51–3.55 (t,

J = 5.93, 4H), 4.62 (s, 2H), 6.23 (d, J = 0.82, 1H), 7.58 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 8.4, 12.3, 19.3, 27.0, 29.6, 44.8, 63.1, 69.1, 109.1, 111.5, 112.0, 112.8, 116.1, 125.1, 149.2, 153.3, 154.7, 161.5. HRMS m/z: calcd for $C_{19}H_{21}NaO_4$ ([M+Na]$^+$) 371.103, found 371.120.

4.2.6. 2′-*O*-[[(4,5′,8-trimethyl) psoralen-4′-ylmethoxy] ethyl] adenosine (4a)

Adenosine (7.23 g, 26.8 mmol) was stirred with 60% NaH (1.23 g, 32.2 mmol) in dry DMF (50 ml) at room temperature for 1 h, then **3a** (980 mg, 2.8 mmol) in dry DMF (15 ml) was added. The mixture was stirred at 75 °C for 27 h. Phosphate buffer (1.0 ml, 100 mM sodium phosphate, pH 7.0) was added to the reaction mixture, the solution was evaporated to near dryness, and the residue was dissolved in CH_2Cl_2. The resulting solution was washed with aqueous 5% $NaHCO_3$ (150 ml × 3). The organic layer was dried over Na_2SO_4, filtrated, and concentrated to dryness. The residue was purified by silica gel column chromatography. Elution with CH_2Cl_2–MeOH (25:1, v/v) gave fractions of **4a** (270 mg, 18% yield). ^{1}H NMR ($CDCl_3$) δ 2.50 (s, 3H), 2.53 (s, 3H), 2.58 (s, 3H), 3.49 (m, 1H), 3.55–3.59 (m, 2H), 3.72 (m, 2H), 3.86 (s, 1H), 3.93 (d, J = 12.8, 1H), 4.32 (s, 1H), 4.51 (d, J = 4.35, 1H), 4.67 (t, J = 3.35, 2H), 4.79 (m, 1H), 5.76 (s, 2H), 5.80 (d, J = 7.6, 1H), 6.26 (s, 1H), 6.70 (d, J = 11.9, 1H), 7.60 (s, 1H), 7.77 (s, 1H), 8.30 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 8.5, 12.3, 19.3, 60.4, 63.2, 63.4, 68.4, 69.6, 70.8, 81.6, 88.1, 89.6, 109.3, 111.0, 111.4, 113.0, 116.3, 124.7, 141.0, 149.3, 152.4, 153.3, 154.6, 155.5, 156.0, 161.4. HRMS m/z: calcd for $C_{27}H_{29}NaO_8$ ([M+Na]$^+$) 574.191, found 574.198.

4.2.7. 2′-*O*-[[(4,5′,8-Trimethyl) psoralen-4′-ylmethoxy] propyl] adenosine (4b)

Adenosine (8.86 g, 33.1 mmol) was stirred with 60% NaH (1.60 g, 39.8 mmol) in dry DMF (50 ml) at room temperature for 1 h, then **3b** (740 mg, 2.20 mmol) and potassium iodide (512 mg 3.10 mmol) in dry DMF (20 ml) were added. The mixture was stirred at 75 °C for 27 h. Phosphate buffer (1.0 ml, 100 mM sodium phosphate, pH 7.0) was added to the reaction mixture, the solution was evaporated to near dryness, and the residue was dissolved in CH_2Cl_2. The resulting solution was washed with aqueous 5% $NaHCO_3$ (100 ml × 3). The organic layer was dried over Na_2SO_4, filtrated, and concentrated to dryness. The residue was purified by silica gel column chromatography. Elution with CH_2Cl_2–MeOH (25:1, v/v) gave fractions of **4b** (203 mg, 16% yield). ^{1}H NMR ($CDCl_3$) δ 2.02 (m, 2H), 2.47 (s, 3H), 2.48 (s, 3H), 2.49 (s, 3H), 3.36–3.52 (m, 6H), 4.01 (m, 1H), 4.13 (s, 1H), 4.26 (s, 1H), 4.46 (s, 2H), 4.79 (m, 1H), 5.42 (s, 1H), 5.70 (s, 2H), 5.93 (m, 1H), 6.30 (d, J = 11.9, 1H), 7.67 (s, 1H), 8.08 (s, 1H), 8.34 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 8.7, 12.4, 19.2, 30.2, 61.8, 62.3, 63.8, 66.5, 67.3, 67.8, 72.9, 79.7, 86.7, 87.9, 108.3, 112.6, 112.8, 116.2, 129.1, 132.0, 140.0, 140.3, 148.9, 152.9, 153.2, 154.1, 155.3, 156.6, 160.5. HRMS m/z: calcd for $C_{28}H_{31}NaO_8$ ([M+Na]$^+$) 588.207, found 588.211.

4.2.8. 2′-*O*-[[(4,5′,8-Trimethyl) psoralen-4′-ylmethoxy] butyl] adenosine (4c)

Adenosine (5.70 g, 21.6 mmol) was stirred with 60% NaH (1.02 g, 25.7 mmol) in dry DMF (30 ml) at room temperature for 1 h, then **3c** (500 mg, 1.43 mmol) and potassium iodide (332 mg 2.00 mmol) in dry DMF (20 ml) were added. The mixture was stirred at 75 °C for 31 h. Phosphate buffer (1.0 ml, 100 mM sodium phosphate, pH 7.0) was added to the reaction mixture, the solution was evaporated to near dryness, and the residue was dissolved in CH_2Cl_2. The resulting solution was washed with aqueous 5% $NaHCO_3$ (100 ml × 3). The organic layer was dried over Na_2SO_4, filtrated, and concentrated to dryness. The residue was purified by silica gel column chromatography. Elution with CH_2Cl_2–MeOH (25:1, v/v) gave fractions of **4c** (232 mg, 28% yield). ^{1}H NMR ($CDCl_3$) δ 2.02–2.10 (s, 2H), 2.44 (s, 3H), 2.47 (s, 3H), 2.49 (s, 3H), 3.36–3.64 (m, 6H), 3.95 (d, J = 3.1, 1H), 4.26 (s, 1H), 4.42–4.44 (t, J = 5.6, 1H), 4.50 (s, 2H), 4.60 (s, 1H), 5.39 (s, 1H), 5.74 (s, 2H), 5.94 (d, J = 6.2, 1H), 6.30 (s, 1H), 7.65 (s, 1H), 8.09 (s, 1H), 8.35 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 8.7, 12.5, 19.1, 26.1, 26.4, 26.8, 61.9, 62.2, 69.4, 69.5, 70.0, 81.3, 86.5, 86.8, 108.2, 112.6, 112.7, 112.8, 116.2, 119.7, 125.3, 140.1, 148.9,149.4, 152.9, 154.1, 154.3, 155.1, 156.6, 160.5. HRMS m/z: calcd for $C_{29}H_{33}NaO_8$ ([M+Na]$^+$) 602.223, found 602.207.

4.2.9. 6-*N*-Bz-2′-*O*-[[(4,5′,8-Trimethyl) psoralen-4′-ylmethoxy] ethyl] adenosine (5a)

Compound **4a** (260 mg, 0.5 mmol) was dried by repeated coevaporation with pyridine and was dissolved in dry pyridine (5 ml). To the solution was added trimethylchlorosilane (300 μl, 2.4 mmol). After the mixture was stirred for 15 min, benzoyl chloride (275 μl, 2.4 mmol) was added and maintained at room temperature for 2 h. The mixture was then cooled in ice bath and water (1 ml) was added. After 5 min, 28% aqueous ammonia (2.5 ml) was added and stirred at room temperature for 0.5 h. The reaction mixture was then evaporated to near dryness and the residue was dissolved in $CHCl_3$ (60 ml). The solution was washed three times with water (60 ml). The organic layer was dried over Na_2SO_4, filtered, and concentrated to dryness. The residue was purified by flash chromatography to give **5a** (284 mg, 93%) as yellow powder. ^{1}H NMR (DMSO-d_6) δ 2.48 (m, 9H), 3.55 (s, 3H), 3.65 (m, 2H), 3.67 (m, 1H), 3.97 (d, J = 3.7, 1H), 4.57 (d, J = 7.05, 3H), 5.15 (s, 2H), 6.14 (d, J = 5.4, 1H), 6.30 (s, 1H), 7.54 (m, 3H), 8.68 (s, 1H), 8.69 (s, 1H), 11.17 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 8.7, 12.5, 19.1, 61.4, 61.7, 66.1, 66.9, 67.4, 69.1, 73.9, 81.6, 86.2, 86.3, 93.4, 107.6, 108.4, 112.6, 112.9, 116.2, 125.2, 128.9, 129.0, 132.9, 133.7, 143.2, 148.9, 150.9, 154.3, 154.3, 155.3, 160.5. HRMS m/z: calcd for $C_{34}H_{33}NaO_9$ ([M+Na]$^+$) 678.218, found 678.209.

4.2.10. 6-*N*-Bz-2′-*O*-[[(4,5′,8-Trimethyl) psoralen-4′-ylmethoxy] propyl] adenosine (5b)

Compound **4b** (200 mg, 0.35 mmol) was dried by repeated coevaporation with pyridine and was dissolved in dry pyridine (4 ml). To the solution was added trimethylchlorosilane (230 μl, 1.82 mmol). After the mixture was stirred for 15 min, benzoyl chloride (205 μl, 1.80 mmol) was added and maintained at room temperature for 2 h. The mixture was then cooled in ice bath and water (1 ml) was added. After 5 min, 28% aqueous ammonia (2 ml) was added and stirred at room temperature for 0.5 h. The reaction mixture was then evaporated to near dryness and the residue was dissolved in $CHCl_3$ (60 ml). The solution was washed three times with water (25 ml). The organic layer was dried over Na_2SO_4, filtered, and concentrated to dryness. The residue was purified by flash chromatography to give **5b** (122 mg, 52%) as yellow powder. ^{1}H NMR (DMSO-d_6) δ 1.97–2.00 (m, 2H), 2.42 (d, 6H), 2.48 (s, 3H), 3.39–3.40 (m, 2H), 3.55–3.68 (m, 4H), 3.96 (d, J = 3.9, 1H), 4.30 (m, 1H), 4.44 (m, 1H), 4.48 (s, 2H), 5.13–5.18 (m, 2H), 6.07 (d, J = 5.5, 1H), 6.28 (s, 1H), 7.50–7.54 (m, 2H), 7.62 (m, 1H), 7.67 (s, 1H), 7.99–8.02 (m, 2H), 8.70 (m, 2H),11.2 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 8.7, 12.5, 19.1, 29.9, 61.4, 62.3, 66.5, 66.8, 67.4, 69.1, 73.9, 81.6, 86.2, 86.5, 93.5, 107.6, 108.3, 112.6, 112.8, 116.2, 125.2, 128.9, 129.0, 132.9, 133.7, 143.2, 148.9, 150.9, 154.2, 154.3, 155.3, 160.5. HRMS m/z: calcd for $C_{35}H_{35}NaO_9$ ([M+Na]$^+$) 692.233, found 692.222.

4.2.11. 6-*N*-Bz-2′-*O*-[[(4,5′,8-Trimethyl) psoralen-4′-ylmethoxy] butyl] adenosine (5c)

Compound **4b** (208 mg, 0.36 mmol) was dried by repeated coevaporation with pyridine and was dissolved in 4 ml of dry pyridine. To the solution was added trimethylchlorosilane (228 μl, 1.8 mmol). After the mixture was stirred for 15 min, benzoyl chloride (210 μl, 1.8 mmol) was added and maintained at room temperature for 2 h.

The mixture was then cooled in ice bath and water (0.8 ml) was added. After 5 min, 28% aqueous ammonia (1.5 ml) was added and stirred at room temperature for 0.5 h. The reaction mixture was then evaporated to near dryness and the residue was dissolved in $CHCl_3$ (50 ml). The solution was washed three times with water (30 ml). The organic layer was dried over Na_2SO_4, filtered, and concentrated to dryness. The residue was purified by flash chromatography to give **5b** (248 mg, 94%) as yellow powder. ^{1}H NMR (DMSO-d_6) δ 2.20 (m, 4H), 2.42–2.48 (m, 9H), 3.39–3.40 (m, 2H), 3.46–3.78 (m, 6H), 3.97 (d, J = 3.4, 1H), 4.30 (s, 1H), 4.48 (m, 1H), 4.50 (s, 2H), 5.16 (m, 1H), 5.23 (d, J = 5.5, 1H), 6.09 (d, J = 5.8, 1H), 6.29 (s, 1H), 7.48–7.53 (m, 3H), 7.69 (s, 1H), 7.98–8.02 (m, 2H), 8.69 (s, 1H),8.72 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 8.7, 12.5, 19.0, 26.1, 26.3, 26.8, 61.9, 62.2, 69.4, 69.7, 70.0, 81.3, 86.5, 86.8, 108.2, 112.6, 112.7, 112.8, 116.2, 119.7, 125.3,128.8, 130.0, 132.7, 133.7, 140.1, 148.7,149.5, 152.9, 154.1, 154.3, 155.0, 156.6, 160.5. HRMS m/z: calcd for $C_{36}H_{37}NaO_9$ ([M+Na]$^+$) 706.249, found 706.252.

4.2.12. 6-*N*-Bz-5′-*O*-DMTr-2′-*O*-[[(4,5′,8-Trimethyl) psoralen-4′-ylmethoxy] ethyl] adenosine (6a)

Compound **5a** (82 mg, 0.12 mmol) was dried by repeated coevaporation with pyridine and was dissolved in dry pyridine (1.5 ml). To the solution was added 4,4′-dimethoxytrityl chloride (104 mg, 0.30 mmol), and the mixture was stirred at room temperature for 8 h, then water (20 ml) was added. The solution was extracted three times with CH_2Cl_2 (20 ml). The organic layer was dried over Na_2SO_4, filtered, and concentrated to dryness. The residue was purified by flash chromatography to give **6a** (63 mg, 55%) as white foam. ^{1}H NMR (DMSO-d_6) δ 2.48 (m, 9 H), 3.58–3.68 (m, 2H), 3.69 (s, 6H), 3.81 (m, 1H), 4.01 (m, 1H), 4.42 (d, J = 7.05, 1H), 4.59 (s, 1H), 4.67 (t, J = 7.05, 1H), 5.15 (d, 1H), 6.18 (d, J = 5.4, 1H), 6.27 (s, 1H), 6.78–6.81(m, 4H), 7.17–7.23 (m, 6H), 7.30–7.32 (m, 3H), 7.53–7.55 (m, 2H), 7.62 (m, 1H), 7.70 (s, 1H), 8.03 (d, J = 3.7, 2H) 8.52 (s, 1H), 8.60 (s, 1H), 11.18 (s, 1H); ^{13}C NMR ($CDCl_3$) 8.4, 12.3, 19.3, 55.2, 62.9, 63.0, 69.8, 70.9, 81.7, 84.2, 86.6, 87.1, 109.2, 11.5, 111.9, 112.6, 113.2, 116.1, 127.0, 127.4, 127.9, 128.0, 128.2, 128.5, 128.8, 130.0, 130.2, 131.9, 132.8, 134.4, 135.5, 141.4, 144.4, 149.1, 153.3, 154.6, 154.9, 158.6, 161.5. HRMS m/z: calcd for $C_{55}H_{51}NaO_{11}$ ([M+Na]$^+$) 980.348, found 980.366.

4.2.13. 6-*N*-Bz-5′-*O*-DMTr-2′-*O*-[[(4,5′,8-Trimethyl) psoralen-4′-ylmethoxy] propyl] adenosine (6b)

Compound **5b** (56 mg, 0.08 mmol) was dried by repeated coevaporation with pyridine and was dissolved in dry pyridine (1 ml). To the solution was added 4,4′-dimethoxytrityl chloride (45 mg, 0.13 mmol), and the mixture was stirred at room temperature for 5 h, then water (20 ml) was added. The solution was extracted three times with CH_2Cl_2 (20 ml). The organic layer was dried over Na_2SO_4, filtered, and concentrated to dryness. The residue was purified by flash chromatography to give **6b** (49 mg, 58%) as white foam. ^{1}H NMR ($CDCl_3$) δ 1.90 (m, 2H), 2.40 (s, 3H), 2.49 (s, 3H), 2.51 (s, 3H), 3.24 (s, 1H), 3.38 (m, 1H), 3.47 (s, 1H), 3.55–3.60 (m, 2H), 3.78 (s, 8H), 3.92 (m, 1H), 4.15 (s, 1H), 4.41 (s, 1H), 4.44 (s, 1H), 4.59–4.63 (m, 2H), 5.96 (s, 1H), 6.15 (s, 1H), 6.80–6.82 (m, 4H), 7.21–7.31(m, 6H), 7.41 (s, 1H), 7.43 (s, 1H), 7.52–7.57 (m, 5H), 8.03 (d, J = 6.8, 1H), 8.17 (s, 1H), 8.69 (s, 1H), 9.12 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 8.5, 12.3, 19.3, 29.6, 55.2, 62.8, 62.9, 66.5, 68.3, 69.6, 81.7, 84.1, 86.6, 87.0, 109.2, 111.4, 11.5, 112.8, 113.2, 116.1, 124.9, 127.0, 127.8, 127.9, 128.1, 128.9, 130.0, 130.1, 132.8, 135.5, 135.6, 141.4, 144.4, 152.6, 153.1, 155.2, 158.6, 161.4, 164.6. HRMS m/z: calcd for $C_{56}H_{53}NaO_{11}$ ([M+Na]$^+$) 994.364, found 994.380.

4.2.14. 6-*N*-Bz-5′-*O*-DMTr-2′-*O*-[[(4,5′,8-Trimethyl) psoralen-4′-ylmethoxy] butyl] adenosine (6c)

Compound **5c** (235 mg, 0.34 mmol) was dried by repeated coevaporation with pyridine and was dissolved in dry pyridine (3 ml). To the solution was added 4,4′-dimethoxytrityl chloride (230 mg, 0.69 mmol), and the mixture was stirred at room temperature for 6 h, then water (20 ml) was added. The solution was extracted three times with CH_2Cl_2 (20 ml). The organic layer was dried over Na_2SO_4, filtered, and concentrated to dryness. The residue was purified by flash chromatography to give **6c** (105 mg, 31%) as white foam. ^{1}H NMR ($CDCl_3$) δ 2.15 (m, 4H), 2.47–2.48 (t, 9H), 3.16 (s, 1H), 3.48–3.52 (m, 5H), 3.75–3.76 (m, 8H), 4.22 (d, J = 3.0, 2H), 4.52 (m, 2H), 4.59–4.62 (m, 2H), 6.14–6.15 (d, J = 3.5, 2H), 6.79–6.82 (m, 4H), 7.25–7.33 (m, 4H), 7.41–7.43 (m, 4H), 7.48–7.51 (m, 2H), 8.05 (d, J = 6.8, 1H), 8.16 (s, 1H), 8.69 (s, 1H), 9.12 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 8.4, 12.3, 19.3, 26.1, 26.3, 26.8, 30.9, 55.2, 62.9, 63.0, 69.7, 69.8, 70.9, 81.7, 84.1, 86.6, 87.0, 109.2, 11.5, 111.9, 112.8, 113.2, 116.1, 127.0, 127.4, 127.9, 128.0, 128.1, 128.5,128.8, 130.0, 130.1, 131.9, 132.8, 133.4, 135.5, 141.4, 144.4, 149.1, 153.3, 154.6, 154.9, 158.6, 161.5; HRMS m/z: calcd for $C_{57}H_{55}NaO_{11}$ ([M+Na]$^+$) 1008.380, found 1008.375.

4.2.15. Synthesis of 6-*N*-Bz-5′-*O*-DMTr-2′-*O*-{[(4,5′,8-trimethyl) psoralen-4′-ylmethoxy] alkyl} adenosine 3′-(2-cyanoethyl-*N,N*′-diisopropylphosphoramidite) (7a,7b,7c) and introduction to oligonucleotide

The fully protected 5′-*O*-DMTr-ACCGCAT (1.0 μmol) was synthesized on CPG using a DNA synthesizer. Each compound, **7a**, **7b**, and **7c** (10 μmol) in dry acetonitrile (72 μl) was allowed to react with 2-cyanoethyl *N,N,N*′,*N*′-tetraisopropylphosphorodiamidite (4 μl, 11 μmol) in the presence of 0.5 M 1*H*-tetrazole (24 μl, 11 μmol), and these mixtures were stirred at room temperature for 1 h. Without purification, the reaction mixtures were added to 5′-detritylated ACCGCAT-CPG in the presence of 1 equiv. of 1*H*-tetrazole in dry acetonitrile, and the mixtures were allowed to react at room temperature for 2 h. The DMTr cations released from the CPG were quantified by UV spectroscopy and the coupling yield was ca. 50%. After capping, the CPG support was then set to the DNA synthesizer and the oligonucleotide synthesis was continued. The CPG support was treated with concentrated 28% aqueous ammonia at 55 °C for 7 h, and the solution was concentrated to dryness. Purification of 2′-Ps-***x***om was performed with a reverse-phase HPLC with an acetonitrile gradient in TEAA (pH 7.0). Column, CAPCELL PAK C18, 5 μm, 4.6 $\Phi \times$ 150 mm, (Shiseido, Co. Ltd., Tokyo, Japan); mobile phase, (A) 0.1 M triethylammonium acetate (TEAA, pH 7.0) and (B) 50% CH_3CN in 0.1 M TEAA at the flow rate of 0.8 ml/min (acetonitrile gradient: 7.5–15% for 15 min, 15–50% for 10 min, 50% for 10 min). The fractions containing the oligonucleotides with DMTr groups were concentrated, and then the DMTr groups were removed by treatment with 80% acetic acid for 30 min.

4.3. Measurements of UV-melting profiles of duplexes of 2′-Ps-oligo and ORN

UV melting profiles of the duplex 2′-Ps-***x***om and ORN were obtained by a UV spectrophotometer equipped with a programmed thermal controller at an increase rate of 1.0 °C/min. The sample solutions were prepared in 100 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl, and the concentration of the oligonucleotides was fixed at 2.0 μM.

4.4. CD spectra of duplexes of 2′-Ps-oligo and ORN

Circular dichroism (CD) spectra were obtained on a CD spectrophotometer (J-720, JASCO, Tokyo, Japan) equipped with a thermal controller (RET-100, Neslab, Portsmouth, NH, USA). An equimolar solution of 2′-Ps-***x***om (or Kr-ODN) and ORNs (2.0 μM each) was prepared in a buffer containing 100 mM sodium phosphate (pH 7.0) and 0.1 M NaCl.

4.5. Fluorescence spectra of duplexes of 2′-Ps-oligo and ORN

Fluorescence spectra were obtained by a Fluorescence spectrophotometer (RF-5300PC, Shimadzu Co. Kyoto, Japan) equipped with a thermal controller at 15 °C. An equimolar solution of 2′-Ps-oligo and ORN (2.0 μM each) in 100 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl was heated at 85 °C for 5 min and slowly cooled to the designated temperature prior to measurements. No attempt was made to eliminate dissolved oxygen in the buffer. The excitation bandwidth was 5 nm. The emission bandwidth was 5 nm.

4.6. Photo-cross-linking reaction of psoralen-conjugated oligonucleotide (Ps-oligo) and ORN

An equimolar solution of 2′-Ps-***x***om and ORNs (2.0 μM each) was denatured at 85 °C for 5 min and slowly cooled to 37 °C in a buffer containing 100 mM sodium phosphate (pH 7.0) and 0.1 M NaCl. The reaction mixture was irradiated by UVA on a transilluminator (FTI-LW, 365 nm, 1.6 mW/cm^2, Funakoshi Co., Ltd., Tokyo, Japan), and analyzed by reversed-phase HPLC with an acetonitrile gradient in TEAA (pH 7.0). Column, CAPCELL PAK C18, 5 μm, 4.6 $\Phi \times 150$ mm, (Shiseido, Co. Ltd., Tokyo, Japan); column oven, 40 °C; mobile phase, (A) 0.1 M triethylammonium acetate (TEAA, pH 7.0) and (B) 50% CH_3CN in 0.1 M TEAA at a flow rate of 0.8 ml/min (acetonitrile gradient: 7.5–15% for 15 min, 15–50% for 10 min, 50% for 10 min). The photo-cross-linking yields were evaluated from the amount of the intact ORNs.

Photo-regeneration reaction was carried out as follows: isolated samples by HPLC were dried up and then irradiated by a short wavelength illuminator (MINERALIGHT® LAMP, UVGL-58, 254 nm, Funakoshi Co., Ltd., Tokyo, Japan). The photo-reversal samples were dissolved in 0.1 M TEAA and analyzed by reversed-phase HPLC.

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