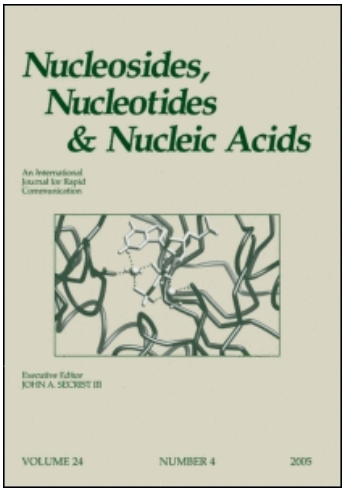

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Selective Photo-Cross-Linking of 2'-*O*-Psoralen-Conjugated Oligonucleotide with Rnas Having Point Mutations

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SELECTIVE PHOTO-CROSS-LINKING OF 2′-*O*-PSORALEN-CONJUGATED OLIGONUCLEOTIDE WITH RNAS HAVING POINT MUTATIONS

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□ *It has been reported that point mutations in genes are responsible for various cancers and the selective regulation of the gene expression is an important issue to develop a new type of anticancer drugs. In this report, we present a new type of antisense molecule that photo-cross-links to an oligoribonucleotide having a point mutation site in a sequence specific manner. 2′-O-psoralen-conjugated adenosine was synthesized in four steps from adenosine and introduced in the middle of an oligodeoxyribonucleotide (2′-Ps-oligo). Compared with 5′-O-psoralen-conjugated oligodeoxyribonucleotide (5′-Ps-oligo), which has a psoralen at the 5′-end, 2′-Ps-oligo more selectively photo-cross-linked to a pyrimidine base of the site of alteration from purine to pyrimidine in the oligoribonucleotide.*

Keywords Antisense oligonucleotide; psoralen; photo-cross-linking; ras; Point mutation

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INTRODUCTION

The first stage of the human genome project has reached its completion and has reported that the number of genes could be ca. 30,000.[1] Recently, the FANTOM consortium has reported drastic facts.[2,3] Non-coding RNAs (ncRNAs) have been transcribed and shown to possibly participate in the regulation of gene expression. Reports suggest that more than 70% regions of the genome transcribe RNAs which play important roles in the cell survival, and moreover, more than 50% of transcribed RNAs are ncRNAs. Clarification of the function of ncRNAs becomes an urgent theme of life science and plays an important part of second stage of the human genome project, which is focused on clarifying the functions of genes. Among several strategies for studying the functions, an antisense strategy[4] is a certainly suitable choice.[5–9]

The antisense strategy has acquired the reliance on its potential in regulating gene expression and has been deployed into clinical trials of various diseases such as cancers and viral infections[10] Vitravene of ISIS Pharmaceuticals Inc. obtained the approval of the United States Food and Drug Administration for cytomegalovirus retinitis in AIDS patients is the first antisense drug.[11]

In our previous study concerning the antisense strategy, we designed a photo-cross-linking reagent conjugated at 5′-end of oligo(nucleoside phosphorothioate)s (psoralen-conjugated S-oligo).[12] Psoralen derivatives have an ability to cross-link covalently to pyrimidine bases, favorably with thymine and uracil, upon UV irradiation (320∼400 nm).[13] As S-oligos (oligo(nucleoside phosphorothioate)s) are so far the most effective antisense molecule[14,15] and have fairly long half-lives in cell culture conditions,[16] we used them as the mother antisense oligonucleotide. We demonstrated the regulatory effects of psoralen-conjugated S-oligo on cervical carcinoma cells. Psoralen-conjugated S-oligo drastically inhibited the cellular proliferation of the cancer cells in a sequence specific manner only upon UVA irradiation ($IC_{50} = 16$ nM) without any drug delivery systems.[17]

Among the target genes of the antisense molecules studied to date, it is still difficult to discriminate the sites of point mutations. The point mutation of K-*ras* gene is the typical example, which is reportedly responsible for various cancers including pancreas cancer and colorectal cancer.[18–20] The point mutation in the *ras* gene occurs at codon 12, where G is changed to T. The transversion causes the alternation of an amino acid from Gly-12 to Val-12; this mutation eliminates GTPase activity from K-Ras protein. Some groups reported the suppression of the functions of *ras*-mRNA with a point mutation using an antisense S-oligo in a sequence-specific manner.[21–25] However, the report has not been well verified to date. This might be due to the difficulty in discriminating the site of the point mutation by antisense oligonucleotides under physiological conditions. It is necessary to regulate such a site in a strictly sequence-specific manner. In this report, we present

a new type of antisense molecule that photo-cross-links to mRNAs having point mutation sites in a sequence-specific manner with the maximum strictness. The molecule is 2′-*O*-psoralen-conjugated oligonucleotide (2′-Ps-oligo). The psoralen of 5′-*O*-psoralen-conjugated oligonucleotide (5′-Ps-oligo) in our previous study could interact with a pyrimidine base in the vicinity of the psoralen even if the pyrimidine base is not the target base. This might be due to the flexible tether. 2′-Ps-oligo exhibited even more strict sequence specificity than 5′-Ps-oligo.

MATERIALS AND METHODS

1. Materials

,5′,8-Trimethylpsoralen was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Reagents for the oligonucleotide synthesis were purchased from Glen Research, Inc. (Sterling, VA, USA) Reagents for the gel electrophoresis were purchased from Nacalai Tesque Co. (Kyoto, Japan). 5′-Ps-oligo was synthesized according to the reported procedure.[26] Oligodeoxyribonucleotides (ODNs) and oligoribonucleotides (ORNs) were synthesized via the conventional procedure using a 391 DNA synthesizer (Applied Biosystems, Foster City, CA, USA). The other reagents were purchased from Wako Pure Chemicals Inc. (Osaka, Japan) and used without further purification. Silica gel column chromatography and thin-layer chromatography (TLC) were carried out on Wako gel C-200 and Merck 60 F_{254}.

2. Synthesis of 2′-*O*-Psoralen-Conjugated Oligonucleotide

2.1. Synthesis of 2′-O-[(4,5′,8-trimethyl)psoralen-4′-ylmethyl]adenosine (II)

4′-Chloromethyl-4,5′,8-trimethylpsoralen (**I**) was synthesized according to the reported procedure.[27] Adenosine (1.4 g, 5.4 mmol) was stirred with NaH (259 mg, 6.5 mmol) in dry DMF (10 ml) at room temperature for 1 hour prior to the addition of **I** (500 mg, 1.8 mmol) in dry DMF (25 ml). The mixture was stirred at 0°C for 12 hours. Phosphate buffer (1.0 ml, 100 mM sodium phosphate, pH 7.0) was added to the reaction mixture and the solution was evaporated to dryness; then the residue was resolved in CH_2Cl_2. The resulting solution was washed with aqueous 5% $NaHCO_3$ (20 ml × 3). The organic solution was dried over Na_2SO_4 and the solution was concentrated to dryness. The residue was dissolved in MeOH followed by silica gel column chromatography. Elution with CH_2Cl_2-MeOH (25:1, v/v) gave fractions of 2′-*O*-[(4,5′,8-trimethyl)psoralen-4′-ylmethyl]adenosine (**II**) (208 mg, 23% yield). TLC (CH_2Cl_2-MeOH 4:1, v/v) R_f 0.60; ^{1}H-NMR (DMSO-d_6) δ2.2–2.5(m, 9H, 4,5′8-trimethyls of psoralen), 3.5–3.7(m, 2H, 5′-H), 4.0(s, 1H, 4′-H), 4.4(t, 1H, 3′-H), 4.0(s, 1H, 4′-H), 4.6–4.8(m, 2H, 4′-CH_2 of psoralen), 4.7(m, 1H, 2′-H), 5.4(d, 1H, 3′-OH), 5.6(m, 1H, 5′-OH), 5.9(d, 1H, 1′-H), 6.3(s, 1H, 3-H of psoralen), 7.0(bs, 2H, NH_2), 7.3(s, 1H, 5′-H of psoralen), 7.8(s, 1H, 2-H), 8.0(s, 1H, 8-H).

2.2. Synthesis of 6-N-Bz-2′-O-[(4,5′,8-trimethyl)psoralen-4′-ylmethyl]adenosine (V)

The title compound was synthesized according to the general procedure of Ti et al.[28] Compound **II** (100 mg, 0.2 mmol) in dry pyridine (2.5 ml) was allowed to react with trimethylchlorosilane (130 μl, 1.0 mmol) and was stirred at room temperature for 2 hours to give 2′-*O*-[(4,5′,8-trimethyl)psoralen-4′-ylmethyl]-3′,5′-*O*-bis(trimethylsilyl)adenosine (**III**). To the reaction mixture of **III**, benzoyl chloride (100 μl, 0.8 mmol) was added and the resulting solution was stirred at room temperature for 4 hours to give 6-*N*,*N*-dibenzoyl-2′-*O*-[(4,5′,8-trimethyl)psoralen-4′-ylmethyl]-3′,5′-*O*-bis(trimethylsilyl)adenosine (**IV**). Concentrated ammonium hydroxide (1.0 ml) was added to the reaction mixture of **IV**, and the solution was stirred at room temperature for 1 hour, followed by evaporation to dryness. The residue was resolved in CH_2Cl_2, and the resulting solution was washed with aqueous 5% $NaHCO_3$ (20 ml × 3). The organic solution was dried over Na_2SO_4, and the solution was concentrated to dryness. The residue was dissolved in CH_2Cl_2, followed by a silica gel column chromatography. The eluting with CH_2Cl_2-MeOH (50:1, v/v) gave 6-*N*-benzoyl-2′-*O*-[(4,5′,8-trimethyl)psoralen-4′-ylmethyl]adenosine (**V**) (79.4 mg, 66% yield). TLC (CH_2Cl_2-MeOH 9:1, v/v) R_f 0.6 (**III**), 0.8 (**IV**), 0.4 (**V**); ^{1}H-NMR ($CDCl_3$) δ2.4–2.5(m, 9H, 4,5′8-trimethyls of psoralen), 2.8(bs, 1H, 3′-OH), 3.7–4.0(m, 2H, 5′-H), 4.3(s, 1H, 4′-H), 4.6(t, 1H, 3′-H), 4.5–4.9(m, 2H, 4′-CH_2 of psoralen), 5.1(m, 1H, 2′-H), 5.6(m, 1H, 5′-OH), 5.8(d, 1H, 1′-H), 6.2(s, 1H, 3-H of psoralen), 7.1(s, 1H, 5′-H of psoralen), 7.5–8.1(m, 5H, Ar), 7.6(s, 1H, 2-H), 8.5(s, 1H, 8-H), 8.7(bs, 1H NH).

2.3. Synthesis of 6-N-Bz-5′-O-DMTr-2′-O-[(4,5′,8-trimethyl)psoralen-4′-ylmethyl] adenosine (VI)

Compound **V** (79.6 mg, 0.13 mmol) in dry pyridine and THF(1:1, v/v, 4.0 ml) was allowed to react with 4,4′-Dimethoxytrityl chloride (220 mg, 0.65 mmol) in the presence of $AgNO_3$ (110 mg, 0.65 mmol), and the mixture was stirred at room temperature for 1.5 hours. The reaction was quenched by adding ice-water, and the solution was evaporated to dryness. The residue was dissolved in CH_2Cl_2 (50 ml), and the resulting solution was washed with aqueous 5% $NaHCO_3$ (20 ml × 3). The organic solution was dried over Na_2SO_4, and the solution was concentrated to dryness. The residue was dissolved in CH_2Cl_2, followed by silica gel column chromatography. Elution with CH_2Cl_2-MeOH (500:1, v/v) gave 6-*N*-Bz-5′-*O*-DMTr-2′-*O*-[(4,5′,8-trimethyl)psoralen-4′-ylmethyl]adenosine (**VI**) (91.1 mg, 77% yield). TLC (CH_2Cl_2-MeOH 9:1, v/v) R_f 0.5 (VI); ^{1}H-NMR ($CDCl_3$) δ2.4–2.6(m, 9H, 4,5′8-methyls of psoralen), 2.7(bs, 1H, 3′-OH), 3.4–3.5(m, 2H, 5′-H), 3.8(s, 6H, methoxy), 4.3(s, 1H, 4′-H), 4.5(t, 1H, 3′-H), 4.8–4.9(m, 2H, 4′-CH_2 of psoralen), 4.8(m, 1H, 2′-H), 6.1(d, 1H, 1′-H), 6.2(s, 1H, 3-H of psoralen),

TABLE 1 Sequence of Ps-oligos and oligo-RNAs

Name	Sequence
2′-Ps-oligo	5′d(TACGCCAA^{Ps}CAGCTCC)3′*
ODN1	5′d(TACGCCAACAGCTCC)3′
cORN1	5′ r(GGAGCUGUUGGCGUA)3′
Mismatch-ORN1	5′ r(GGAGCUGGUGGCGUA)3′
5′-Ps-oligo	5′ d(Ps-ACAGCTCCAACTACC)3′
cORN2	5′ r(GGUAGUUGGAGCUGUUGGCG)3′
Mismatch-ORN 2	5′ r(GGUAGUUGGAGCUGGUGGCG)3′

*APs = 2′-*O*-psoralen-conjugated adenosine.

6.8–7.4(m, 13H, Ar of DMTr), 7.3(s, 1H, 5′-H of psoralen), 7.5–8.1(m, 5H, Ar), 7.9(s, 1H, 2-H), 8.6(s, 1H, 8-H), 8.9(bs, 1H NH).

2.4. Synthesis of 6-N-Bz-5′-O-DMTr-2′-O-[(4,5′,8-trimethyl)psoralen-4′-ylmethyl] adenosine 3′-(2-cyanoethyl-N, N′-diisopropylphosphoramidite) (VII) and introduction it to oligonucleotide

The sequence of 2′-Ps-oligo was complementary to that of K-*ras* mRNA containing codon12 region (Table 1).

The fully protected 5′-*O*-DMTr-ACCGCAT-CPG (1.0 μmol) was synthesized using a DNA synthesizer. Compound **VI** (9.1 mg, 10 μmol) in dry acetonitrile (72 μl) was allowed to react with 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite (3.5 μl, 11 μmol) in the presence of 0.5 M 1*H*-tetrazole (24 μl, 11 μmol), and the mixture was stirred at room temperature for 1 hour. Without purification, the reaction mixture was added to 5′-detritylated ACCGCAT-CPG in the presence of 1 equiv. of 1*H*-tetrazole in dry acetonitrile and the mixture was allowed to react at room temperature for 4 hours.

The DMTr cations released from the CPG were quantified by UV spectroscopy and the coupling yield was 23%. 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 ammonium hydroxide at 55°C for 11 hours, and the ammonium hydroxide solution was evaporated in vacuo. Purification of 2′-Ps-oligo was performed with a reverse-phase HPLC with an acetonitrile gradient in TEAA (pH 7.0). Column: CAPCELL PAK C18, 5 μm, 4.6ϕ × 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 (gradient: 7.5% to 15% for 15 minutes, 15–40% for 10 minutes, 40% for 5 minutes). The fractions containing the oligonucleotides with DMTr groups were concentrated; then the DMTr groups were removed with treatment by 80% acetic acid for 30 minutes.

3. Measurements of UV-Melting Profiles of 2′-Ps-Oligo and ORN Hybrid Duplexes

UV melting profiles of the hybrid between 2′-Ps-oligo and ORN were obtained by a UV spectrophotometer (U2000 A, Hitachi Co., Tokyo, Japan) equipped with a programmed thermo-controller with the increase rate of 1.0°C/minute. 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 5.0 μM.

4. Fluorescence Spectra of 2′-Ps-Oligo and ORN Duplexes

Fluorescence spectra were obtained by a spectrophotometer (RF-5300 PC, Shimadzu Co., Kyoto, Japan) equipped with a thermal controller. An equimolar solution of 2′-Ps-oligo and ORN (5.0 μM each) in 100 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl was denatured at 85°C for 5 minutes and slowly cooled to the designated temperature prior to measurement. No attempt was made to eliminate dissolved oxygen in the buffer.

5. CD Spectra of 2′-Ps-Oligo and ORN Hybrid Duplexes

Circular dichroism (CD) spectra were obtained on a CD spectrophotometer (J-720, JASCO, Tokyo, Japan) equipped with a thermo controller (RET-100, Neslab, Portsmouth, NH, USA). An equimolar solution of 2′-Ps-oligo and ORN (5.0 μM each) was prepared in a buffer containing 100 mM sodium phosphate (pH 7.0) and 0.1M NaCl.

6. Photo-Cross-Linking Reaction of Psoralen-Conjugated Oligonucleotide (Ps-Oligo) and ORN

The equimolar solution of Ps-oligo and ORN (5.0 μM each) was denatured at 85°C for 5 minutes and slowly cooled to 37°C in a buffer containing 100 mM sodium phosphate (pH 7.0) and 0.1 M NaCl for 20 minutes at 37°C. After incubation, the reaction mixture was irradiated by UVA on a transilluminator (FTI-LW, 365 nm, 1.4 mW/cm^2, Funakoshi Co., Ltd., Tokyo, Japan), and then analyzed by denatured PAGE (18% polyacrylamide, 7M urea/TBE, 300 V, 2 h). The bands in the denatured PAGE were visualized by UV shadowing procedure. The photo-cross-linking yields were evaluated from the amount of the intact ORN by reverse-phase HPLC.

RESULTS AND DISCUSSION

Design of Photo-Cross-Linking Antisense Oligonucleotide

The fidelity of sequence recognition of target mRNAs by ODN is a crucial issue of the antisense strategy. Other than ODN with phosphodiester linkages, various types of antisense DNAs such as PNA,[29] morpholino oligonucleotide,[30] and BNA (or LNA)[31,32] have been designed to increase the fidelity. The fidelity was usually evaluated by a comparison of UV-melting temperatures of fully matched hybrids with those of mismatched hybrids. Significant difference in melting temperature (Tm) suggests that those antisense DNAs possess enough potential to discriminate mismatched sequences from fully matched ones. However, it is unclear whether those DNAs can discriminate single mismatched sequences in cellular systems. It is necessary to design an antisense DNA that strictly inactivates designated mRNAs with high sequence fidelity.

We developed an antisense molecule so as to cross-link to the pyrimidine base at the site of the point mutation in the target RNA, but not to randomly cross-link to off-target pyrimidine bases. For this purpose, we designed an adenosine derivative anchoring a psoralen derivative at the 2′-position of D-ribose via a methylene linker (Aps) as shown in Figure 1a, and Aps was introduced into ODNs by a phosphoramidite protocol giving a photo-reactive antisense ODN (2′-Ps-oligo). The conformational calculation of the hybrid of 2′-Ps-oligo with its complementary ORN (cORN1), whose sequence was originated from c-K-*ras*-mRNA, was carried out by Macro Model (ver.5.5; CA, USA) using the Amber* algorithm.[33,34] The psoralen moiety of the single-stranded 2′-Ps-oligo was localized between adjacent bases as shown in Figure 1b. When 2′-Ps-oligo formed a hybrid with the cORN1, the psoralen was localized between the designated base pairs as shown in Figure 1c, site **a**. The distance between the double bond of the pyrimidine base, 5–6, and that of the psoralen, 4′-3′, was calculated to be 5.6 Å. The distance is close enough for two moieties to interact with each other in the ground state and to react to form a cyclobutane adduct upon UVA irradiation. Other several semi-stable structures also were postulated by the calculation, and they were substantially similar to the model shown in Figure 1c. As a comparison, a similar calculation was carried out for the 5′-*O*-psoralen-conjugated oligonucleotide (5′-Ps-oligo). Figures 1d and 1e suggest that the psoralen of 5′-Ps-oligo could intercalate in two ways (site **b** and site **c**). That is, the sequence selective inactivation of mRNAs having a point mutation by 5′-Ps-oligo is not realistic, and 2′-Ps-oligo could be suitable for the purpose.

Synthesis of 2′-*O*-Psoralen-Conjugated Oligonucleotide

2′-*O*-[(4,5′,8-trimethyl)psoralen-4′-ylmethyl]adenosine (**II**) was synthesized from unprotected adenosine and 4′-chloromethyl-4,5′,8-trimethylp-

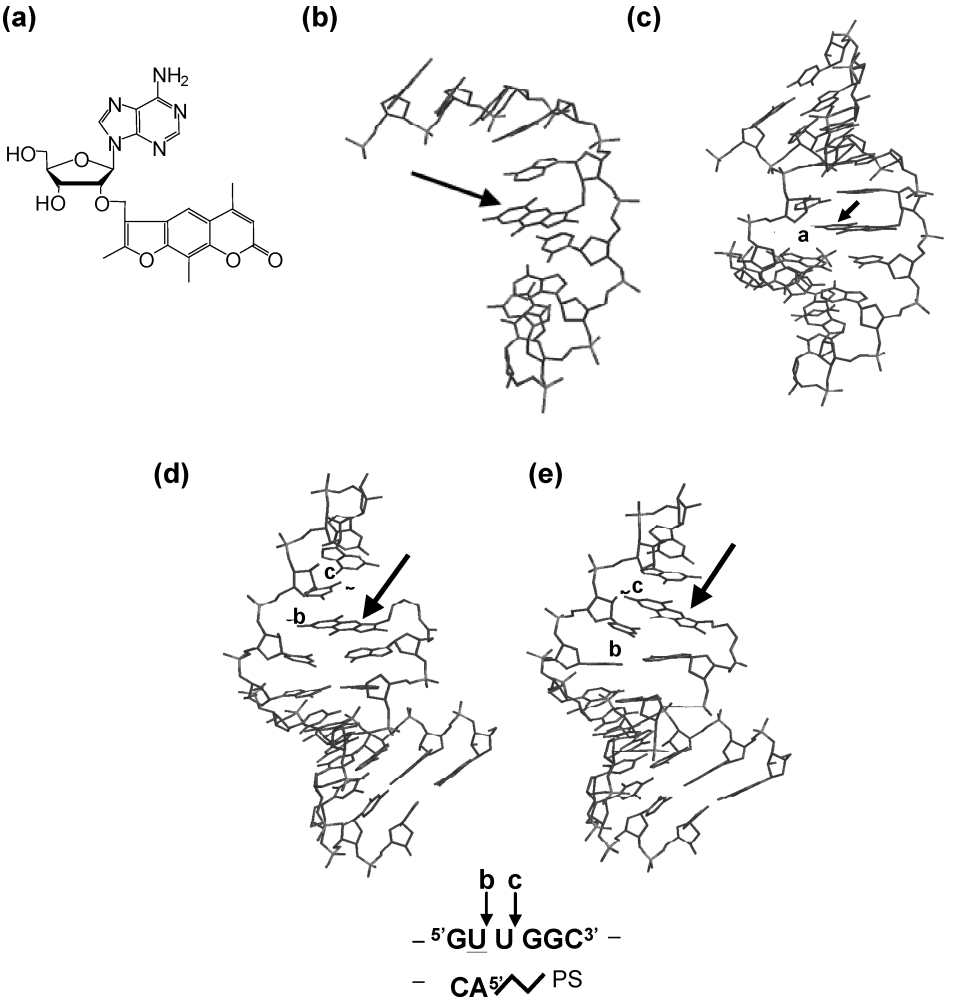
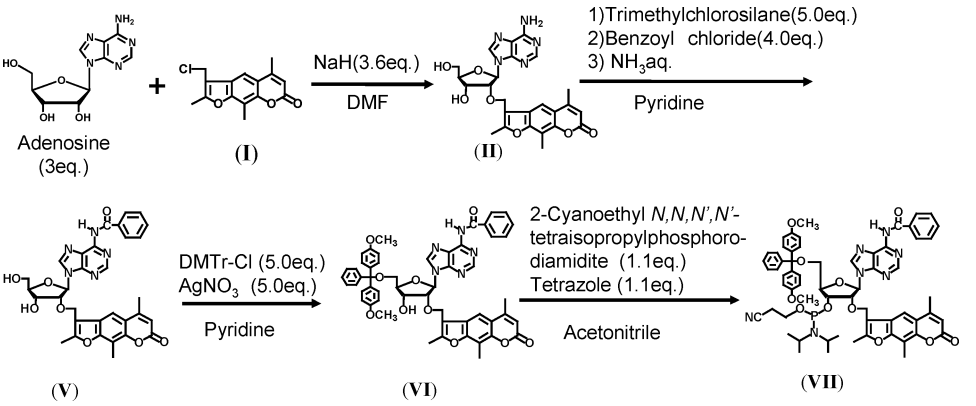


FIGURE 1 Structure of psoralen-conjugated oligonucleotide. The arrows indicated trimethylpsoralen. (a) 2′-*O*-psoralen-conjugated adenosine (Aps), (b) single strand of 2′-Ps-oligo, (c) duplex of 2′-Ps-oligo and cORN1, (d), (e) two intercalation patterns in the duplex of 5′-Ps-oligo and cORN2. a,b,c: psoralen intercalation sites.

soralen (**I**) in the presence of NaH. **I** was introduced to either the 2′-*O*- or 3′-*O*-position. The ratio of **II** to 3′-*O*-modified adenosine was ca.17:1 estimated by ^{1}H-NMR. **II** was then separated from 3′-*O*-conjugated adduct by silica gel chromatography, and was used for consecutive reactions to obtain **VI** (Scheme 1). The overall yield of **VI** from **I** was 12%. Aps was phosphytilated at 3′-OH to form **VII** and it was introduced to the designated position of an ODN by a syringe protocol followed by an elongation protocol using a DNA synthesizer. The sequences of 2′-Ps-oligo and related oligonucleotides are shown in Table 1.



SCHEME 1 Synthesis of 2′-*O*-psoralen-conjugated adenosine derivative.

Interaction between 2′-Ps-Oligo and Its Complementary RNA

In order to evaluate the effect of the introduced psoralen moiety on the stability of the hybrid with ORN, the UV-thermal stability was examined (Table 2). The Tm of the hybrid of 2′-Ps-oligo with the complementary ORN (cORN1) was lower than that of the hybrid of ODN1 with cORN1. In the case that the mismatched base pair located in the close vicinity of the psoralen, Tm of the hybrid of 2′-Ps-oligo with mismatch-ORN1 also was decreased. These results suggest that the modification of a psoralen to the 2′-*O*-position of oligonucleotide destabilized the hybrid to some extent.

The location of the psoralen in the hybrid was examined by electronic spectroscopy. The UV spectra of the hybrid did not show any appreciable spectrum change, which might due to the broad absorption band attributed to psoralen around 300–360 nm.

The CD spectrum of the hybrid of 2′-Ps-oligo and cORN1 clearly showed a similar spectrum of the hybrid of ODN1 and cORN1, suggesting that the hybrid was A-form-like (Figure 2). The induced circular dichroism (ICD) around 300–350 nm due to the psoralen of 2′-Ps-oligo was not observed.

TABLE 2 The melting temperatures of the hybrid of 2′-Ps-oligo with ORNs

	Tm (°C)	
	cORN1	Mismatch-ORN1
2′-Ps-olig	61.6	59.0
ODN1	64.6	60.0

Measurements were carried out at 260 nm for the equimolar mixture of 2′-Ps-oligo (ODN1) and ORN in 0.1 M phosphate buffer (pH 7.0) with 0.1 M NaCl.

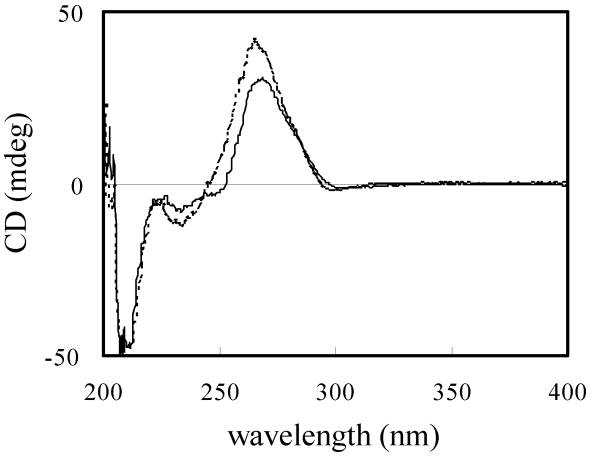


FIGURE 2 CD spectra of the duplexes of psoralen-conjugated oligonucleotide with its complementary oligoribonucleotide.—: 2′-Ps-oligo and cORN1, ----: ODN1 and cORN1. [2′-Ps-oligo] = [ODN1] = [cORN1] = 5 μM in 0.1M phosphate buffer/0.1 M NaCl (pH 7.0). Measurements were carried out at 37°C.

ICD often is used to identify the locations of intercalators. In this case, ICD might be too weak and broad to be observed. Therefore, CD was not suitable to specify the location of the psoralen in the hybrid.

In contrast, the fluorescence spectra showed a drastic change in fluorescence due to the psoralen. The fluorescence of 2′-Ps-oligo alone showed diminished fluorescence around 450 nm with slight shifts compared with 4′-hydroxymethyl-4,5′,8-trimethylpsoralen. This phenomenon might be due to the interaction of the psoralen with adjacent bases (Figure 3). When 2′-Ps-oligo formed a hybrid with cORN1, the fluorescence was further quenched to about 30% with a slight blue shift, suggesting that the psoralen relocated so as to alter its location to a stable one. In general, when psoralen was intercalated between base pairs, the fluorescence was appreciably quenched with a slight shift.[35] These characteristic changes of the fluorescence suggest that the psoralen moiety intercalates between base pairs and that the location allowed the psoralen to photo-cross-link to the adjacent pyrimidine base. These spectroscopic characteristics showed good accordance with the results from the calculation by the Amber* algorithm as shown in Figures 1b and 1 c. In addition, when 2′-Ps-oligo formed a hybrid with mismatch-ORN1, the fluorescence was further quenched to about 55% with a slight blue shift. The greater decrease observed in the mismatch hybrid than the full match hybrid suggests that the intercalation of psoralen in the mismatch region was more favored. We think that psoralen in the mismatch hybrid located in the certain pocket produced by mismatch hybrid formation. The decrease in Tm in the mismatch hybrid can be explained

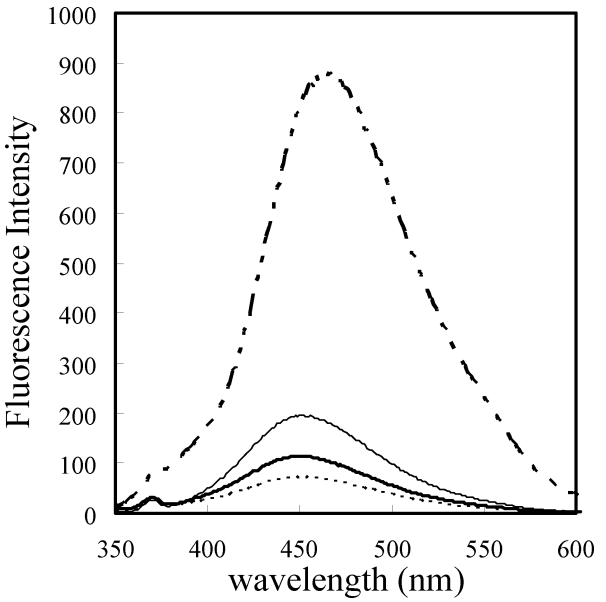


FIGURE 3 Fluorescence spectra of the duplexes of psoralen-conjugated oligonucleotide with its complementary oligoribonucleotide.—: the single-strand 2′-Ps-oligo, — : 2′-Ps-oligo and cORN1, ----: 2′-Ps-oligo and mismatch-ORN1, – ·· – : 4′-Hydroxymethyl-4,5′,8-trimethyl-psoralen monomer (HMT) [2′-Ps-oligo] = [ORN (cORN1 or mismatch-ORN1)] = [HMT] = 2 μM in 0.1 M phosphate buffer/0.1 M NaCl (pH 7.0). Measurements were carried out at 11°C. (λex = 330 nm).

that the stabilization due to the intercalation could not compensate the destabilization due to the mismatch hybridization.

Comparison of the Cross-Linking Efficacy between 5′-Ps-Oligo and 2′-Ps-Oligo

When 2′-Ps-oligo alone was irradiated by UVA (365 nm) in the physiological media, a fast-moving band appeared along with the parent band in PAGE. The intensity of the fast-moving band increased as the irradiation time increased. As 2′-Ps-oligo had a cytidine adjacent to the psoralen of 2′-Ps-oligo, the newly appeared band presumably corresponded to a self-cross-linked adduct.

When a hybrid of 2′-Ps-oligo (or 5′-Ps-oligo) with cORN1 (or cORN2) was irradiated by UVA (365 nm), the psoralen cross-linked to the adjacent pyrimidine, giving photo-adducts as shown in Figures 4a and 4b. In the case of 5′-Ps-oligo, two main photo-adducts were observed. The slow-moving band, indicated by an arrow, was isolated and the extracted compound was irradiated by UVB (254 nm). Two main bands were observed in PAGE, both of which corresponded to the starting materials. This result suggests that the slow-moving band was the photo-cross-linked adduct between 5′-Ps-oligo and cORN2. The fast-moving materials were probably the self-cross-linked

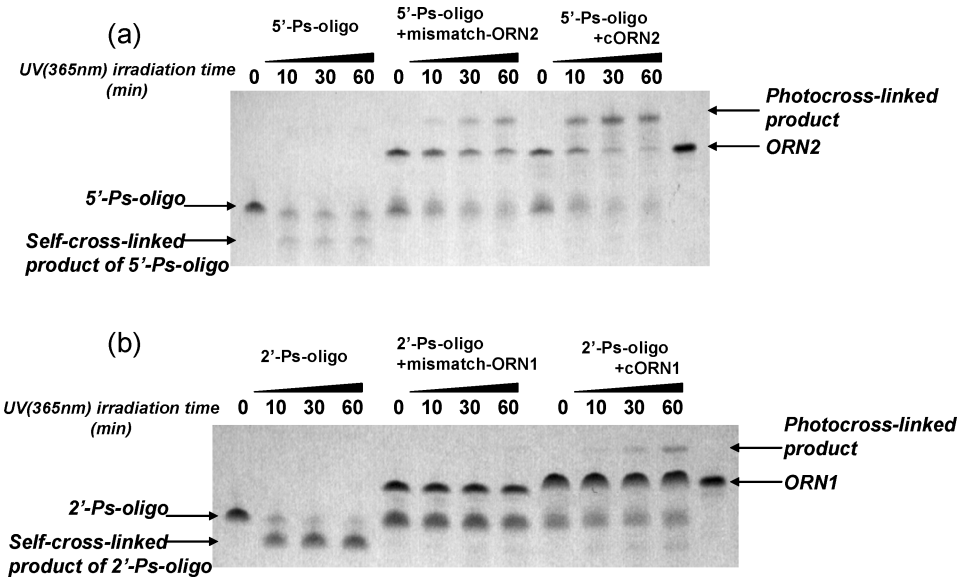


FIGURE 4 Time course of photo-cross-linking reactions of psoralen-conjugated oligonucleotide with the target oligoribonucleotide. (a) 5′-Ps-oligo and ORN (cORN2 or mismatch-ORN2), and (b) 2′-Ps-oligo and ORN (cORN1 or mismatch-ORN1). 18% denatured polyacrylamide gel. [2′-Ps-oligo or 5′-Ps-oligo] = [ORN] = 5 μM in 0.1M phosphate buffer/0.1 M NaCl (pH 7.0).

photo-adduct. When mismatch-ORN2, which contained a mismatched base around the region of the 5′-end of 5′-Ps-oligo, was used as the target RNA, the photo-cross-linked hybrid also was observed. The photo-cross-linking yield of the hybrid of 5′-Ps-oligo and cORN2 was ca.70% upon UVA-irradiation for 60 minutes. The result suggests that 5′-Ps-oligo cross-linked to an undesignated base to some extent. This was expected from the calculated structure shown in Figures 1d and 1e. The tether was long enough to alter the location of the psoralen of 5′-Ps-oligo. The change of the tether could increase the fidelity of photo-cross-linking. That is, in conclusion, 5′-Ps-oligo might not be suitable for the selective inhibition of mRNAs with point mutation sites as it is. When 2′-Ps-oligo was used, it also formed a stable hybrid with both the cORN1 and mismatch-ORN1. However, after UVA irradiation, 2′-Ps-oligo photo-cross-linked predominantly to the cORN1 and scarcely photo-cross-linked to mismatch-ORN1. The photo-cross-linking yield of the hybrid of 2′-Ps-oligo and cORN1 was ca. 25% and that of the hybrid of 2′-Ps-oligo and mismatch-ORN1 was not more than 3% upon UVA-irradiation for 60 minutes. These results suggest that 2′-Ps-oligo fairly discriminated the sites of single point mutation. Namely, the introduction of a psoralen at the 2′-*O*-position of a nucleoside enhanced the selectivity of the photo-cross-linking.

This kind of functionalized antisense oligonucleotide will be needed to regulate and consequently to clarify the functions of genes with point

mutations. Recently, RNA-interference has attracted the enthusiastic interest of researchers in molecular biology and medicinal science. The concept might dominate the scientific field of gene silencing in the near future. Despite the widespread use of RNAi to "knock down" gene functions, the RNAi pathway itself remains poorly understood. Also, short-interference RNA (siRNA), is not always applicable to all purposes. Many reports suggest that the off-target gene regulation by siRNA could be serious issues.[36–38] The ability to discriminate single-base difference in RNAs could be another high hurdle for the siRNA strategy. Although this weak points for sequence specific inhibition are shared by the antisense strategy, our 2′-psoralen-conjugated antisense oligonucleotide could overcome the weak point.

So far, the selectivity in cross-linking has drastically increased; however, the efficiency in cross-linking has not been sufficient for extensive uses. By designing the tether between a ribose and a psoralen, and by choosing the structure of the tether, a more effective photo-cross-linkable antisense DNA could be designed.

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