

Induced cross-linking reactions to target genes using modified oligonucleotides

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Synthetic oligonucleotides (ONs) are valuable tools that interfere with gene expression by specifically binding to target genes in a sequence-specific manner. Reactive ONs containing cross-linking agents are expected to induce efficient inhibition because they bind covalently to target genes. In recent years, researchers have reported several cross-linking reactions that target DNA induced by external stimuli. This short review highlights recently developed novel cross-linking reactions, focusing particularly on nucleoside derivatives developed by our group.

Introduction

Even a small change in DNA may cause disorders in gene expression, leading to diseases such as cancer. Thus, a strategy for regulating gene expression is of major importance from a therapeutic viewpoint.

Synthetic oligonucleotides (ONs) artificially inhibit gene expression by binding specifically to target genes in a sequence-specific manner. Much effort has been spent on improving ON properties.¹ Major applications of single stranded ONs include an mRNA-targeted antisense method,^{2,3} an antigene method based on triplex formation⁴⁻⁶ and a method involving small

interfering RNAs (siRNAs), a new class of RNA-based ONs.⁷⁻⁹ Recent genomic studies have shown that micro RNAs (miRNAs) regulate gene expression by means of translational repression or mRNA degradation,¹⁰ providing novel targets for the application of ONs.¹¹⁻¹⁴ In these ON strategies, the functions of natural ONs rely on non-covalent hybridization and their inhibitory effects may be transient. In contrast, ONs modified with reactive appendages induce irreversible chemical modifications of the targeted sequences.^{15,16}

Cross-linking reactions between complementary duplexes or triplexes are an important means of improving the stability of the complex by covalent bond formation. These reactions are expected to increase the inhibition efficiency for gene expression. In addition, cross-linking reactions have the potential to lead site-directed mutagenesis.¹⁷⁻²⁰ This short review focuses on the cross-linking reactions using modified ONs to react natural bases in duplexes, and describes our recent work on the development of selective cross-linking reactions.

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Inducible cross-linking reactions activated by UV irradiation or chemical reactions

An ideal cross-linking agent for use in cells would induce a reaction when triggered by a signal such as UV irradiation or a chemical reaction. Psoralen is a natural product that reacts with pyrimidine bases, predominantly thymine, to form cyclobutane linkages at the 5'-TA-3' duplex sites under UV irradiation (Fig. 1).

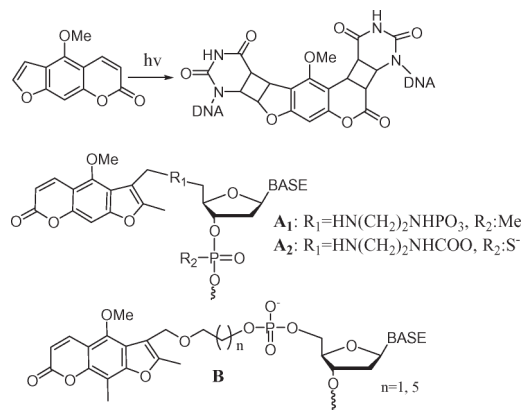


Fig. 1 Psoralen-conjugated ODN.

The functional group of psoralen is relatively stable until activated by UV irradiation. It has been reported that psoralen-conjugated ONs (**A**₁) at the 5' end cross-link specifically with mRNA targets and inhibit translation of the mRNA target *in vitro*.²¹ In addition, psoralen-conjugated oligonucleoside phosphorothioates (**A**₂) drastically inhibit cell growth with irradiation in a sequence-dependent manner.²²

Phosphoroamidite derivatives are commercially available for preparing psoralen conjugated ON (**B**) and psoralen conjugated ONs are applicable in cells. A psoralen (**B**; *n* = 5) covalently attached to a triple helix-forming oligonucleotide (TFO) can generate interstrand cross-links between two DNA strands at specific ON binding sequences. Furthermore, psoralen-ON conjugates have been reported to block transcription upon irradiation.^{23–25} Psoralen-modified TFOs have also been used for photoinduced directed mutagenesis of specific sites in duplex DNA and targeted gene knock out in cells.^{26–28} Thus, psoralen-conjugated ONs have been widely applied to several *in vitro* and *in vivo* studies related to antisense and antigene methods. Recently, psoralen has been conjugated at the adenosine 2'-*O*-position through alkoxy methylene linkers²⁹ and directly at the sugar 1'-position³⁰ to improve its flexibility when conjugated at the ON 5' end (Fig. 2).

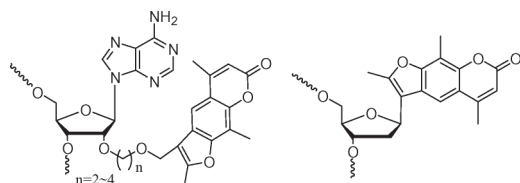


Fig. 2 Novel psoralen-conjugated ONs.

Although psoralen is a very useful cross-linking agent, it has some serious drawbacks. Its reactivity to pyrimidines is sharply restricted, and it has a strong bias towards thymines, with cross-

link formation limited to 5'-TA or AT sites. In addition, the photo-cross-linked DNAs, *via* a [2 + 2] cycloaddition between psoralen and a thymine base, regenerate to the parent DNA upon irradiation at 254 nm, resulting in fatal damage to normal DNA. To overcome these problems, many researchers have been seeking alternative types of photo-cross-linkers with high reactivities.

Fujimoto and co-workers reported that modified oligodeoxynucleotides (ODNs) containing a 3-cyanovinylcarbazolenucleoside (CNVK) can cross-link with an adjacent pyrimidine base by irradiation at 366 nm for 1 s.

The photo-cross-linked ODNs revert to the original ODNs upon irradiation at 312 nm (Fig. 3).³¹ In recent studies, they have demonstrated that the photo-reversible cross-linking reaction mediated the site-specific conversion of cytosine into uracil using only UV irradiation coupled with a heating process.³²

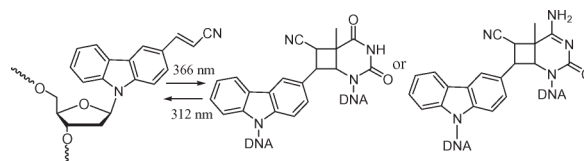


Fig. 3 Inducible cross-linking reaction *via* [2+2] cycloaddition *via* UV irradiation.

Mesmaeker and co-workers reported that ODNs labelled with ruthenium(II) complexes (Ru-ODNs) (Fig. 4) photo-cross-linked to guanine (G) through their complementary strands under visible irradiation.³³ In addition, they demonstrated that a guanine-containing Ru-ODN probe self-inhibited in the absence of specific target strands but reacted with the target strand guanine when the target strand was present. They named these conjugates 'seppuku molecules' (Fig. 4 and Fig. 5).

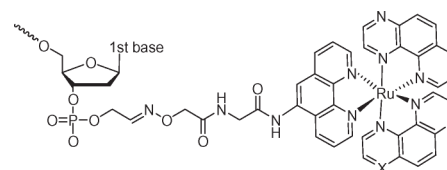


Fig. 4 Ruthenium complexes chemically tethered to the 3' end of the ODN probe.

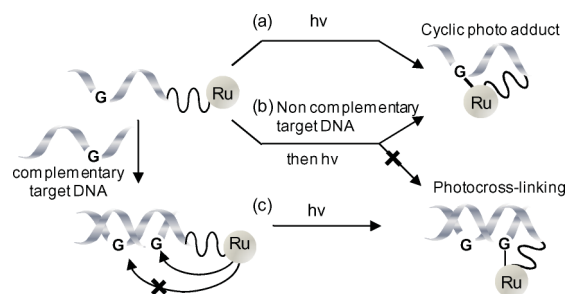


Fig. 5 Schematic representation of the photochemical behaviour of Ru-conjugated ODN (G) in the absence (a) and the presence (b, c) of G containing ODN target strands, which illustrates the 'seppuku process'.

There are several examples of cross-linking agents that are activated by oxidation. Greenberg and co-workers reported that

5-(2'-deoxyuridinyl) methyl radicals generated by photoirradiation of phenylselenide derivatives cross-linked to the opposing deoxyadenosine (dA) in DNA.^{34,35} In addition, the oxidation of phenylselenide derivatives has induced cross-linking reactions with dA *via* [2,3]-sigmatropic rearrangement.^{36,37} 2'-Deoxycytidine analogues have also formed cross-links with the opposing deoxyguanosine (dG) under the same conditions³⁸ (Fig. 6).

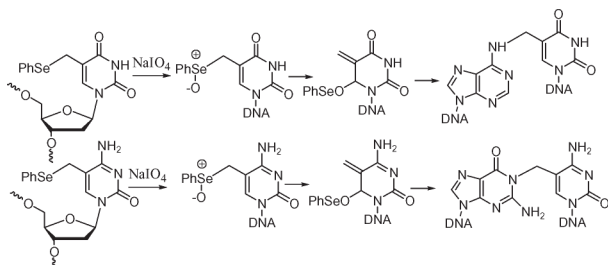


Fig. 6 Inter-strand cross-linking reactions under oxidative conditions.

It has also been reported that TFO containing pyrimidine bases modified with the phenylselenide group undergo cross-linking reaction with dA or dG in the homopurine strand.³⁸

Furan-modified ODN treated with NBS yields a reactive 4-oxo-enal derivative that immediately reacts with complementary adenine and cytosine in the duplex (Fig. 7).³⁹

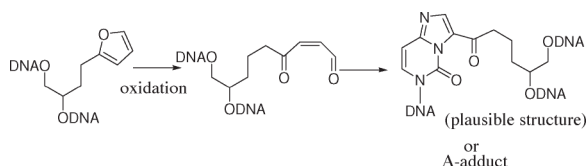


Fig. 7 Cross-linking reaction of furan-modified ODN under oxidative conditions.

Although several cross-linking agents have been reported, the application of psoralen as a cross-linking agent in cells has been reported only for reactions that are activated by UV irradiation.

Selective cross-linking reactions activated by hydrogen bond formation

Chemical stability and efficient reactivity are necessary for the applicability of cross-linking reactions to cells. In addition, a high selectivity of cross-linking reactions would be required for their development as chemical tools to induce point mutations. We have designed selective and efficient cross-linking reactions that are activated by hydrogen-bond formation in a complex between a reactive base and a target base. Our cross-linking agents are characteristic in that no external stimuli are needed for the activation except for the duplex formation between the reactive ODN and the target strand. Based on this concept, we designed two reactive bases: 2-amino-6-vinylpurine (2-AVP) and 4-amino-6-oxo-2-vinylpyrimidine (AOVPY) to be used in the cross-linking reactions.

2-Amino-6-vinylpurine (2-AVP)

We initially designed 2-AVP as a selective cytosine-specific cross-linking agent.⁴⁰⁻⁴³ In this molecule, we expected that the 4-amino

group of cytosine would be in close proximity to the vinyl group of **1** in the complex formed between the protonated nucleoside and cytosine to effectively induce covalent bond formation (Fig. 8). The ODN containing 2-AVP induced a highly efficient cross-linking reaction with cytosine at the complementary site on 2-AVP. Its stable precursor, the 2-amino-6-phenylthioethylpurine nucleoside (2-AVP (SPh)), exhibited an improved antisense inhibition through automatic activation within the complex with the target RNA. The induced alkylating system was applied to intracellular antisense inhibition in which the ODN containing 2-AVP (SME) was conjugated with polyethylene glycol and was encapsulated into nano-particles of polyion complex micelles using poly-L-lysine and showed more efficient antisense inhibition than natural antisense ODN.⁴⁴ However, we realized in subsequent studies that the reactivity of AVP depended on the target RNA sequence.⁴⁵

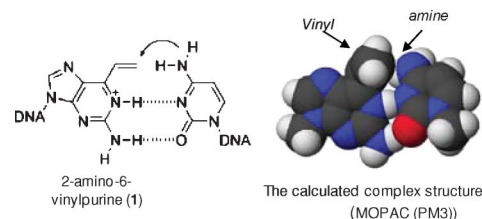


Fig. 8 Cross-linking agent designed to react selectively with cytosine.

We assumed that the lower reactivity of 2-AVP might be due to structural differences between the DNA–RNA hetero duplex and DNA–DNA homo duplex, which might influence the proximity effect between the vinyl group of 2-AVP and the amino group of cytosine.

Thus, we designed a 2'-O-methyl (2'-OMe) analogue (**2**) of 2-AVP (Fig. 9). We expected an improvement of the proximity effect towards the target base and expected that the use of the 2'-OMe backbone would enhance metabolic stability.

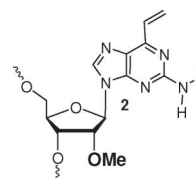
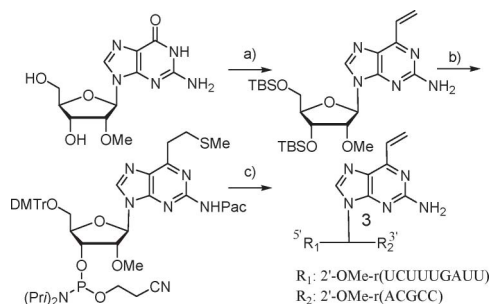


Fig. 9 Structure of the 2'-OMe analogue of 2-AVP.

The 2'-OMe ON containing 2-AVP was synthesized as described previously (Scheme 1).⁴⁶ We investigated the cross-linking reaction of the reactive 2'-OMe ON with the target DNA and RNA, labelled with fluorescein at the 5' end under acidic and neutral conditions.

Fig. 10 shows the relative reactivities of 2-AVP-containing 2'-OMe ON towards different bases at the target site of DNA and RNA. Surprisingly, 2-AVP-containing 2'-OMe ON produced the highest yields for the reaction with a thymine base in DNA and with a uracil base in RNA under acidic conditions, although it gave lower yields for reaction with the dC target (Fig. 10A).

Under neutral conditions, 2-AVP-containing 2'-OMe ON produced only the cross-linked adduct with the thymine base in the DNA target on the complementary site, and the cross-linking reactions of 2-AVP-containing 2'-OMe ON with the RNA target did not occur.



Scheme 1 Reagents and conditions: (a) (1) TBSCl, imidazole, DMF, (2) TsCl, TEA, DMAP, CH₂Cl₂, (3) (C₂H₅BO)₃, Pd(0), LiBr, dioxane–H₂O; (b) (1) CH₃SNa, CH₃CN, CH₂Cl₂, (2) PhOCH₂COCl, HBT, CH₃CN, pyridine, (3) nBu₄NF, THF, (4) DMTrCl, Py, (5) (iPr)₂NP(CI)OC₂H₄CN; (c) (1) synthesis with an automated DNA synthesizer, (2) 28% aqueous NH₃, (3) PAGE purification, (4) 2 eq. MMPP, (5) aqueous NaOH.

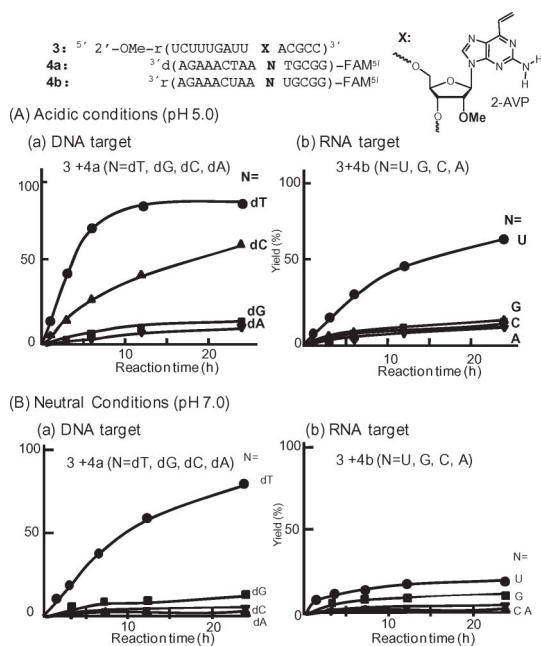


Fig. 10 Yields calculated from gel electrophoresis analysis, for a cross-linking reaction of **3** with target DNA **4a** (N = dT, dG, dC, dA) and RNA **4b** (N = U, G, C, A). Reaction was performed with 10 μM ODN **3** and 5 μM target ODN **5** in 0.1 M NaCl and 50 mM MES, (a) pH 5.0 and (b) pH 7.0, at 30 °C.

The estimates of the thermal stability obtained from the measurements of the melting temperature (T_m) of the duplex suggested that 2'-OMe ON containing 2-AVP (SMe), as a stable precursor, formed a stable duplex with the target DNA and RNA under the reaction conditions. Thus, although cross-linking reactions may have occurred in each duplex, differences in the thermal stabilities did not explain the base selectivity of the cross-linking reactions with **3**. To address this issue, we measured circular dichroism spectra to determine the conformational changes in the duplex between 2'-OMe ON containing 2-AVP (SMe) and the target DNA and RNA. The spectra were similar to that of the duplex between the 2'-OMe ON containing adenosine instead of the 2-AVP (SMe) and the target DNA and RNA. These results indicated that the 2'-OMe ON/DNA duplexes had conformations that lie between the A and B conformations, whereas the 2'-OMe

ON/RNA duplexes had A conformations.⁴⁷ ODN containing 2'-deoxy 2-AVP cross-linked to the cytosine base in the DNA/DNA duplex was in the B conformation,⁴⁵ hence the change in selectivity towards the thymine base when we used 2'-OMe ON might be due to the difference in the duplex conformation. 2'-OMe ON containing deoxy 2-AVP reacted with the cytosine in RNA at the complementary site of 2-AVP. These results suggest that the change in selectivity might be due to extremely local structural changes that affect the closeness between the vinyl group of the AVP and the target base. However, it is not clear why the base selectivities of the cross-linking reactions using 2-AVP in 2'-OMe ON changed.

To understand the cross-linking reaction better, we isolated the cross-linked nucleoside by enzymatic hydrolysis. The purified cross-linked adduct was digested with nuclease P1, snake venom phosphodiesterase and alkaline phosphatase to produce eight nucleosides together with the adduct (**5**). Analysis of the HPLC purified adduct (Fig. 11) by electron-spray ionisation time-of-flight mass spectrometry (ESI-TOF MS) and ¹H NMR spectroscopy demonstrated that cross-linking occurred with either the 2- or 4-oxygen.

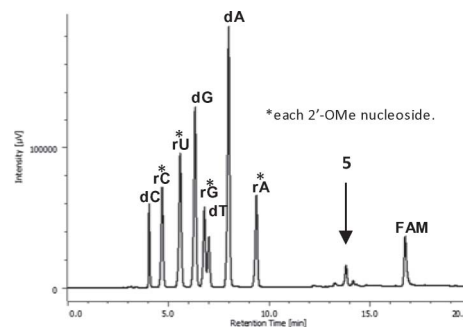


Fig. 11 HPLC analysis of the enzymatic hydrolysate. HPLC conditions: ODS column 1 mL min⁻¹; solvent A, 0.1 M TEAA buffer, solvent B, CH₃CN, B: 5–15%/10 min, 15–40%/20 min.

We anticipated that 2-AVP in the 2'-OMe ON might react with the thymine base, because of the proximity effect, to form hydrogen bonds (Fig. 12A). Accordingly, we assumed that the cross-linking reaction of the 2-AVP : 4-thiothymine complex would have a higher reactivity because of the high nucleophilicity of the sulfur atom (Fig. 12B). In contrast, the 2-thiothymine base would inhibit the cross-link formation because of the steric repulsion between the 2-thio group⁴⁸ and the 2-amino group of the AVP (Fig. 12C).

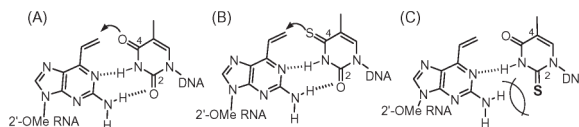


Fig. 12 Speculative complex between 2-AVP and thymine and 2- or 4-thiothymine in the duplex.

Fig. 13 compares the reactivities of **3** towards DNA bearing dT, 4-thio- and 2-thiothymine. We observed rate enhancement for the reaction with the 4-thiothymine base, and the reaction with the 2-thiothymine base was inhibited. These results agreed with our assumption that the cross-linking reaction of the 2-AVP derivative in the 2'-OMe ON might have occurred with the O4 of the thymine base at the target site.

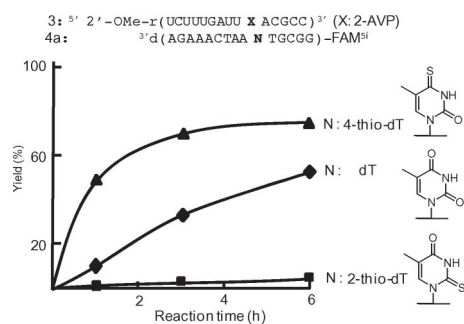


Fig. 13 Cross-linking capability of **3** for the target DNA with thymine or thiothymine derivatives at the complementary site. The reaction was performed with 10 μM ODN (**3**) and 5 μM target ODN in 0.1 M NaCl and 50 mM MES, pH 7.0 at 30 $^{\circ}\text{C}$.

Further investigation is needed to clarify the origin of the selectivity change from the cytosine base in the DNA/DNA duplexes with the thymine base in the 2'-OMe ON/DNA and RNA duplexes observed in this study.

We evaluated the antisense effects of the reactive ONs by a translation assay of the cell lysates. The ONs were first incubated for 5 h with the target mRNA of firefly luciferase under acidic conditions (pH 5.0), and then subjected to translation reactions with wheat germ extract for 2 h at 30 $^{\circ}\text{C}$. Fig.14 summarizes the antisense inhibitory effects. The 2'-OMe ONs with the non-reactive control sequences (**6a**, **7a**) and the cytosine targeted sequence (**6b**) containing 2-AVP did not demonstrate antisense inhibition. 2'-OMe ON (**6c**) containing deoxy 2-AVP, which reacted with cytosine in an RNA target *in vitro*, inhibited luciferase production. In contrast, the 2-AVP-containing 2'-OMe ON (**7b**) with the uracil-targeting sequence showed a higher antisense inhibition. The effect of the antisense inhibition by 2-AVP-containing 2'-OMe ONs agreed with the chemical reactivity in the RNA target under acidic conditions. These results suggest that the cross-linking reactions using reactive 2'-OMe ONs may be responsible for enhanced antisense inhibition.

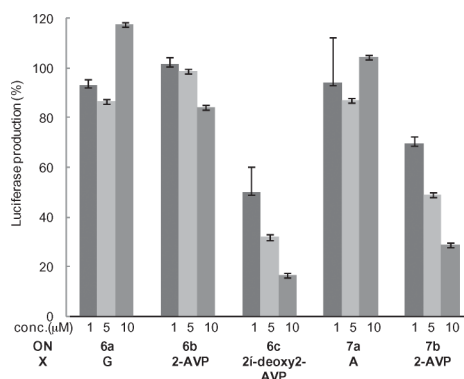


Fig. 14 Antisense inhibitory effects on luciferase production in an assay without cells. The extent of luciferase production relative to that in the control, performed by translation in the absence of ON, is shown in the ordinate.

4-Amino-6-oxo-2-vinylpyrimidine (4-AOVPY)

As mentioned above, we have demonstrated that 2-AVP undergoes a highly selective cross-linking reaction with a target base by

forming a complex with hydrogen bonds. Accordingly, we designed 4-amino-6-oxo-2-vinylpyrimidine (4-AOVPY: **8**) as a reactive base. We expected that the cross-linking agent **8a** (Fig. 15) would form a complex with guanine by two hydrogen bonds.

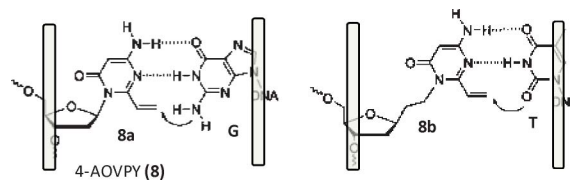
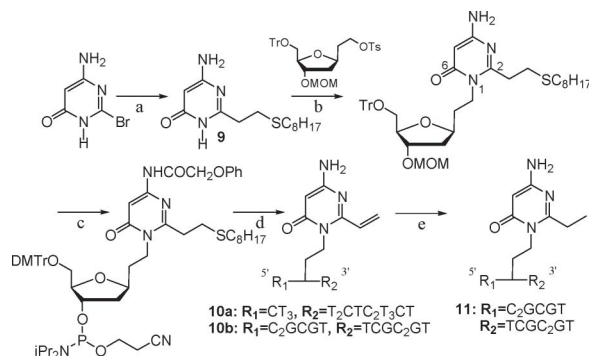


Fig. 15 Design of the novel cross-linking agents **8a** and **8b**.

Nucleoside derivatives containing an ethyl spacer between the sugar and 2-AVP react selectively with adenine by positioning themselves in close proximity to the target bases during the triple helix formation.⁴⁹ Accordingly, we designed a novel nucleoside derivative **8b** (Fig. 15) containing an ethyl spacer between the sugar and the 4-AOVPY motif and expected it to react selectively with thymine.

We prepared the reactive nucleobase shown in Scheme 2. We attempted to perform a coupling reaction between the nucleobase (**9**) and various D-ribose derivatives under a variety of conditions to synthesize **8a**; however, we did not obtain the target compound. The ODN containing **8b** was synthesized as described previously (Scheme 2).⁵⁰



Scheme 2 Reagents and conditions for the synthesis of reactive ODN **10**: (a) (1) $n\text{Bu}_3\text{SnCH}_2$, $(\text{PPh}_3)_2\text{PdCl}_2$, DMF, (2) $\text{C}_8\text{H}_{17}\text{SH}$; (b) BuLi, dioxane, reflux; (c) (1) $\text{PhOCH}_2\text{COCl}$, 1-HBT, pyridine, CH_3CN , (2) $\text{BF}_3 \cdot \text{Et}_2\text{O}$, Me_2S , CH_2Cl_2 , (3) DMTrCl , pyridine, (4) $i\text{Pr}_2\text{NP}(\text{Cl})\text{OC}_2\text{H}_4\text{CN}$, $i\text{Pr}_2\text{NET}$, CH_2Cl_2 , 63%; (d) (1) synthesis with an automated DNA synthesizer, (2) 28% aqueous NH_3 , (3) 3.0 eq. of MMPP, pH 10. (4) 400 mM NaOH; (e) NaBH_4 .

We investigated cross-linking reactions between the reactive vinylated ODN **10a** and either DNA or RNA targets, which were labelled with fluorescein at the 5' end, under neutral conditions.

Fig. 16 shows the relative reactivity of **10a** towards different bases at the DNA and RNA target sites. Cross-linking reaction between **10a** and either dT or U on the target DNA and RNA sites was extremely rapid, producing adducts with these targets in over 50% yields after just 1.5 h.

Although ODN **10a** bearing 4-AOVPY contained a significant amount of thymine in the strand, it was very stable as a single strand. This property indicated that the cross-linking reactions using ODN containing 4-AOVPY occurred selectively by forming a complex to thymine or uracil base in the duplex. The ODN

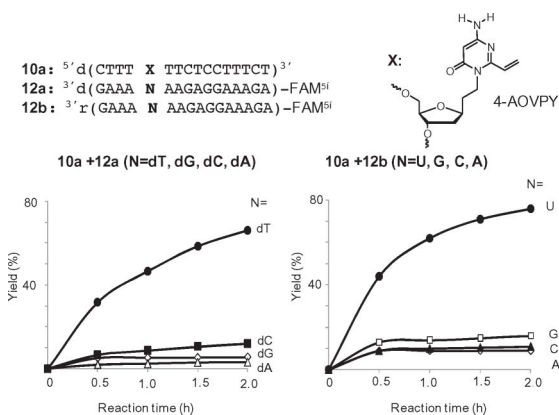


Fig. 16 Yields calculated by gel electrophoresis analysis, for cross-linking reaction of **10a** with target DNA **12a** (N = dT, dG, dC, dA) and RNA **12b** (N = U, G, C, A). Reaction was performed using 10 μ M ODN **10a** and 5 μ M target ODN **12** in 0.1 M NaCl and 50 mM MES, pH 7.0, 30 $^{\circ}$ C.

containing 4-AOVPY with another sequence (**10b**) (d(CCG CGT X TCG CCG): X = 4-AOVPY) also exhibited similar cross-linking reactivity with ODN (**10a**).

We estimated the thermal stabilities by measuring the melting temperatures (T_m) of the duplex between the target DNA or RNA and (**11**) (X = 4-amino-6-oxo-2-ethylpyrimidine), which was an inactive structural analogue of 4-AOVPY, prepared by reduction of **10b** with NaBH₄. Table 1 summarizes the results. T_m values of **11** (4-AOVPY (Et)) and DNA or RNA duplexes in all combinations were lower than those with natural ODN (**11**) containing dA instead of 4-AOVPY (Et) by more than 10 $^{\circ}$ C. These results indicated that the duplex was destabilized by 4-AOVPY (Et) with all bases at the complementary site, but the ODN containing 4-AOVPY formed a stable duplex with the target DNA or RNA under the reaction conditions (at 30 $^{\circ}$ C). Therefore, the cross-linking reactions with ODN containing 4-AOVPY may have occurred in each duplex, and the differences in the cross-linking reactivities to each base was not a result of the duplex stability.

The purified cross-linked adduct was digested with snake venom phosphodiesterase I and bacterial alkaline phosphatase to yield a new product and natural nucleosides. The analysis of the HPLC-purified product (Fig. 17) by ESI-TOF MS and ¹H NMR spectroscopy shows that the selective cross-linking reaction between **10** and thymine occurred at the complementary target, positioned at O2 in thymine.

Thus, nucleoside derivative **8b** may have selectively reacted with thymine because of the proximity effects induced by the

Table 1 T_m values of the 4-AOVPY (Et) in ODN with target DNA and RNA^a

X	DNA		RNA	
	Y	$T_m/^{\circ}$ C	Y	$T_m/^{\circ}$ C
A	T	60.9 \pm 0.4	U	57.7 \pm 1.6
4-AOVPY(Et)	A	49.7 \pm 0.4	A	39.8 \pm 0.6
4-AOVPY(Et)	G	50.0 \pm 0.1	G	39.8 \pm 3.0
4-AOVPY(Et)	C	46.8 \pm 0.6	C	36.2 \pm 0.5
4-AOVPY(Et)	T	50.3 \pm 0.3	U	39.8 \pm 0.4

^a UV-melting profiles measured using 1.0 μ M each of the strand in 100 mM NaCl and 50 mM MES buffer, pH 7.0.

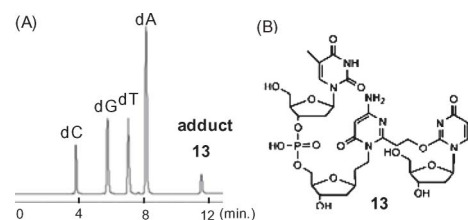


Fig. 17 (A) HPLC analysis of the enzymatic hydrolysate. HPLC conditions: ODS column 1 mL min⁻¹; solvent A, 0.1 M TEAA buffer, solvent B, CH₃CN, B: 5–15%/10 min, 15–40%/20 min. (B) Speculative structure of the adduct.

hydrogen bonds between **8b** and thymine at the complementary site. However, it was unclear whether compound **13** is the primary product of the cross-linking reactions. Further studies are necessary to determine the cross-linking reaction pathways.

Conclusions

Cross-linking reactions to target genes induced by external stimulus have great potential for efficient regulation of gene expression. We developed a new concept for inducing cross-linking reactions, invoked by the formation of a complex with a target base in its near proximity, without UV irradiation or any chemical reactions. For this purpose, we designed two reactive bases: 2-AVP and 4-AOVPY. Both bases exhibited highly efficient and selective cross-linking to thymine base at the complementary site. The strict specificities of the reactivities with 2-AVP and 4-AOVPY are advantageous over other cross-linking agents. These features may provide opportunities for developing new chemical tools to induce selective site-directed mutagenesis. Our cross-linking agents are comparatively stable as single strands under neutral conditions, but many reactive nucleophiles, such as thiol groups, exist in cells. Thus, the design of more stable precursors of 2-AVP and 4-AOVPY seems to be necessary for intracellular applications. We previously reported that the sulfide protected derivatives of deoxy 2-AVP showed higher antisense effects in intracellular conditions.⁴⁴ We expected that the sulfide protected derivatives of 2'-OMe 2-AVP and 4-AOVPY have the potential to provide effective antisense activity by inducible alkylation in a cell. Now further study of the cellular applications is being conducted.

Induced cross-linking reactions have great potential for manipulation of gene expression in cells. Hence, extensive research on the applications of cross-linking agents in cells may provide efficient strategies for controlling gene expression.

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