 

*Accounts*

# Synthesis of Reactive Oligonucleotides for Gene Targeting and Their Application to Gene Expression Regulation

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Many genetic disorders have recently been identified as a major cause of diseases and the regulation of gene expression has been proposed as an attractive therapeutic strategy. Synthetic oligodeoxynucleotides (ODNs) are valuable tools that interfere with gene expression by specifically binding to target genes in a sequence-specific manner. In particular, reactive ODNs are expected to be more efficient because they covalently bind to the target genes. This review summarizes the synthesis of reactive ODNs with inducible reactivity, in particular, their applications to efficient cross-linking reactions, have been developed by the author's group. We also describe applications of the cross-linking reactions to the antisense method in cells and their impact on mutagenesis in model systems.

## Introduction

Over the last few decades, the discovery of intracellular pathways responsible for a number of disease states has led to the identification of new therapeutic targets. In particular, disorder in gene expression has now widely been accepted as a major cause for diseases like cancer. Thus, the development of new approaches to selectively inhibit or modify a target gene has become a major focus from a therapeutic viewpoint. Synthetic oligonucleotides are valuable tools that interfere with gene expression by specifically binding to target genes in a sequence-specific manner and a lot of effort has been devoted to the development of therapeutic methods. Major applications using single-stranded oligonucleotides include an mRNA-targeted antisense method,[1,2] an antigene method based on triplex formation[3–5] and more recently, a method involving small interfering RNAs (siRNAs): a new class of RNA-based oligonucleotides.[6–8] The antisense strategy aims to inhibit gene expression through the formation of a DNA–RNA hetero duplex between an mRNA target and a single-stranded oligonucleotide with a complementary sequence by Watson–Crick binding (Figure 1).

In the antigene strategy, the oligonucleotides recognize specific double-helical DNA sequences by forming Hoogsteen or reverse-Hoogsteen hydrogen bonds with purine bases on one of the duplex strands, thereby resulting in a local triple helix.[3–5] In both strategies, the function of these natural oligonucleotides relies on non-covalent hybridization; their inhibitory effect may be transient. Oligonucleotides modified with reactive appendages induce the irreversible chemical modification of the targeted sequence.[9,10] Reactive oligonucleotides with the ability to alkylate a target sequence are also involved in interstrand cross-linking, improving inhibitory activity in antisense[11,12] and antigene strategies.[13,14] In addition, the alkylation of the target DNA duplex induces site-directed mutagenesis with triplex-forming oligonucleotides (TFOs) that are conjugated to a reactive molecule.[15–20] This review focuses on cross-linking reactions in DNA duplexes, DNA–RNA hetero duplexes and triple helices, and their applications to gene expression regulation. It also reviews results that we obtained using our own cross-linking agents.

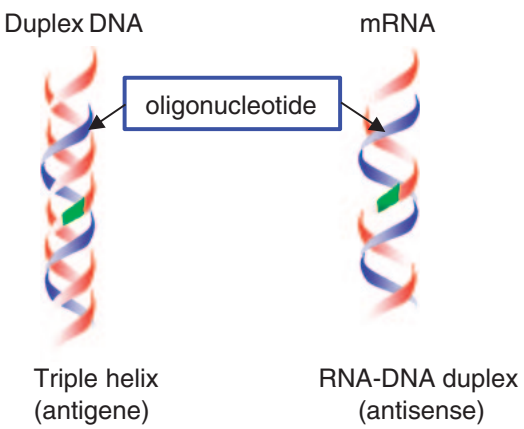


**Figure 1.** Strategy for control of genetic expression using oligonucleotides.

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## 1. Cross-Linking Reaction by Reactive Oligonucleotides

Several types of cross-linking agents have been reported in the past 15–20 years. These reagents exhibit intrinsic reactivity toward nucleophiles following a $S_N2$ mechanism, like halocarbonyl,[21–26] aziridine,[27–30] and a strained cyclopropane group[31–34] (Figure 2). Their high reactivity might be a disadvantage in biological applications. Because the alkylating group of oligonucleotides is susceptible to nucleophilic attack by numerous entities present in living systems like water, amines, and thiols, simultaneously achieving high reactivity and stability is challenging. Promising approaches using agents whose reactivity can be triggered by signals like enzymatic, chemical reactions and UV-irradiation have been reported to overcome this dilemma.

**1.1 Inducible Cross-Linking Agents by UV Irradiation via [2 + 2] Reaction.** Psoralen is a natural product used as a cross-linking reagent to react with pyrimidine bases, predominantly thymine, by forming cyclobutane linkages at the 5′-TA-3′duplex sites under UV irradiation. It is a relatively stable functional group until it is activated by UV irradiation. Psoralen conjugated oligonucleotides at the 5′-end reportedly cross-link with mRNA targets specifically and inhibit the translation of the mRNA target in vitro.[11] A psoralen covalently attached to a triple helix forming oligonucleotide (TFO) has been shown to generate interstrand cross-links between two DNA strands at specific oligonucleotide binding sequences and psoralen–oligonucleotide conjugates have been reported to block transcription upon irradiation.[35–37] Psoralen-modified TFOs have also been used for the photoinduced directed mutagenesis of specific sites in duplex DNA and targeted gene-knock out in cells (This topic will be discussed in detail in Section **3**). Thus, psoralen–oligonucleotides conjugates 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 linkers[38] and at the sugar 1′-position directly[39] to improve its flexibility when conjugated with the oligonucleotide 5′-end. Both new types of oligonucleotide conjugates show cross-linking activity upon UV irradiation (Figure 3).

Psoralen targets are limited to TpA sites and alternatives of photo-cross-linkers have been developed to overcome this limitation. Fujimoto and co-workers demonstrated that a modified oligodeoxynucleotide (ODN) containing a *p*-(carbamoylvinyl)phenol nucleoside underwent photo-cross-linking with an adjacent adenine residue upon UV irradiation.[40] They also achieved an ultrafast reversible DNA interstrand photoinduced cross-linking between a modified ODN containing a 3-cyanovinylcarbazolenucleoside ($^{CNV}K$) and an adjacent pyrimidine base upon UV irradiation.[41] Recently, the reversible photoinduced cross-linking reaction was used to select a target RNA sequence (Figure 4).[42]

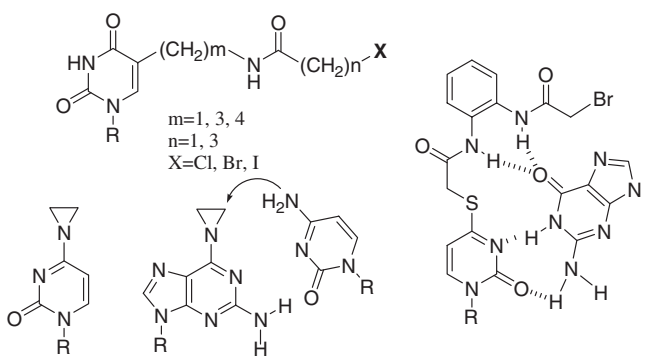



**Figure 2.** Example of the intrinsic alkylating function.

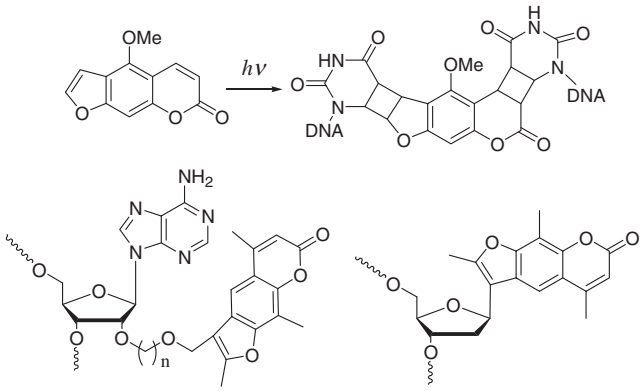


**Figure 3.** Psoralen-conjugated oligonucleotides.

Mesmaeker and co-workers reported that ODNs labeled with ruthenium(II) complexes (Ru–ODNs) (Figure 5) photo-cross-linked to guanine (G) through their complementary strand under visible irradiation (Figure 6).[43] In addition, they dem-

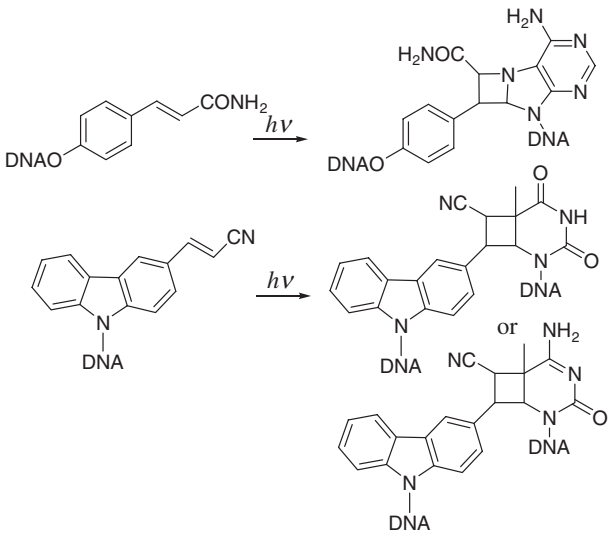


**Figure 4.** Inducible cross-linking reaction via [2 + 2] cycloaddition by UV irradiation.

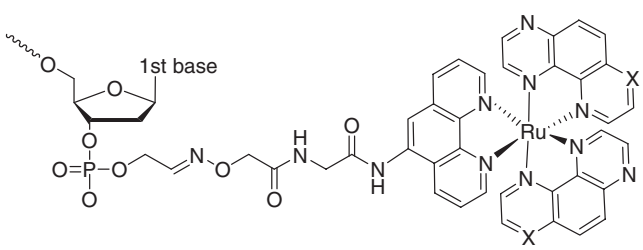


**Figure 5.** Ruthenium complexes chemically tethered to the 3′-end of the probe ODN.

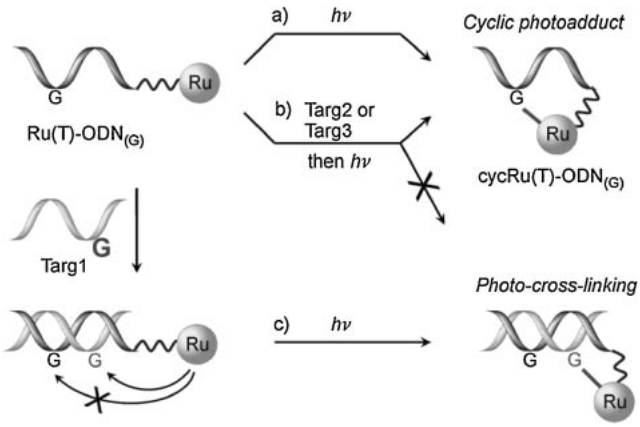



**Figure 6.** Schematic representation of the photochemical behavior of Ru(T)–ODN(G) in the absence (a) and in the presence (b, c) of G containing ODN target strands, which illustrates a "seppuku process."

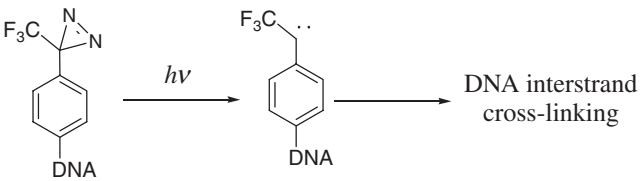



**Figure 7.** DNA interstrand photo-cross-linking.

onstrated 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."

A nucleoside analog with an aryl(trifluoromethyl)diazirine group was developed to enable interstrand cross-linking with multiple nearby bases positioned on the opposite strand in dsDNA upon near-UV irradiation through a carbene intermediate (Figure 7).[44]

**1.2 Inducible Alkylating Agents in Other Type of Reactions.** Greenberg and co-workers reported that 5-(2′-deoxyuridinyl) methyl radicals that were generated by photoirradiation of phenylselenide derivatives cross-linked to the opposing deoxyadenosine (dA) in DNA.[45,46] In addition, the oxidation of phenylselenide derivatives has induced cross-linking reactions with dA via [2,3]-sigmatropic rearrangement.[47,48] 2′-Deoxycytidine analogs have also formed cross-links with the opposing deoxyguanosine (dG) under the same conditions.[49] TFO containing phenylselenide-substituted thymidine derivatives and 5-methyldeoxycytidine at terminal position have also been reported to undergo cross-linking reaction with dA and dG in the homopurine strand (Figure 8).[49]

Madder and co-workers developed a furan-based inducible alkylating agent activated by oxidation.[50–52] The ODN incorporated furan generates a reactive 4-oxo-enal derivative by treatment with NBS and immediately reacts with complementary adenine and cytosine in duplex DNA without cross-linking to neighboring and distant bases (Figure 9).

Rokita and co-workers investigated inducible cross-linking reactions using a reactive quinone methide intermediate.[53–55] They designed a stable precursor that can generate quinone methide in the presence of fluoride anion. The oligonucleotide–*ortho*-quinone methide conjugate causes the self-adduct to form reversibly through intramolecular reaction. They also reported target-promoted alkylation through these reversible reactions. The intramolecular ODN–quinone methide adduct was proposed to induce a conformational change upon addition

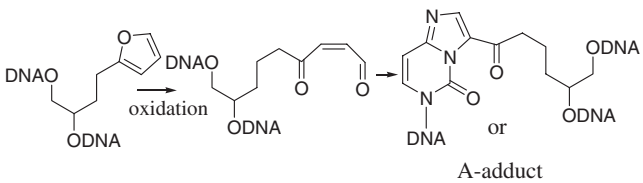



**Figure 9.** Furan-modified ODN for cross-linking reaction activated by oxidation.

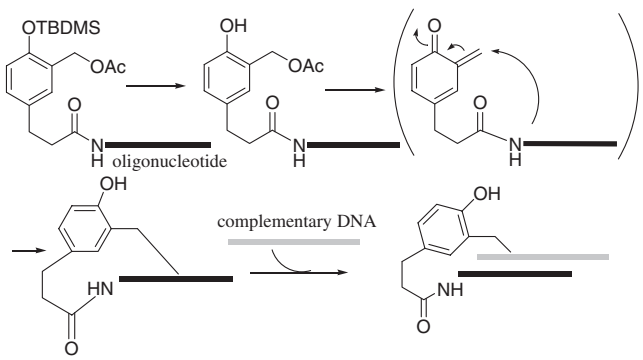



**Figure 10.** Target-promoted cross-linking reaction.

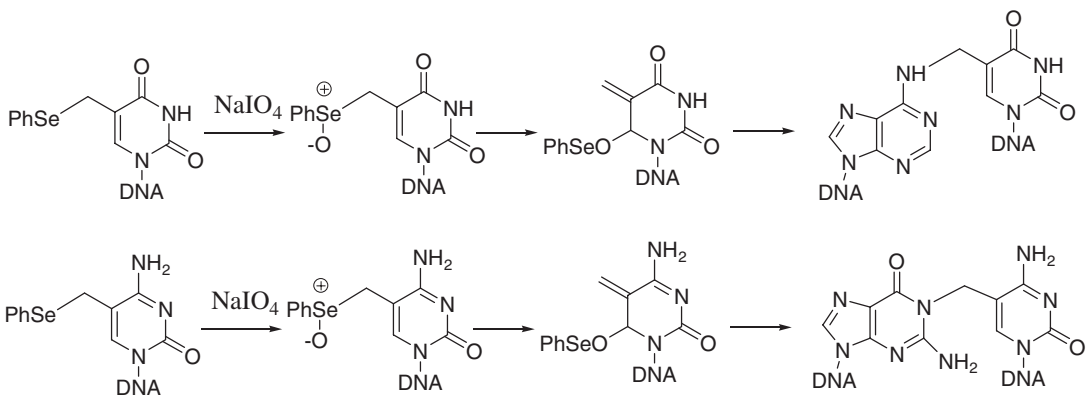



**Figure 8.** Interstrand cross-link formation under oxidative conditions.

of the complementary strand. This conformational change subsequently promoted the intermolecular transfer of quinone methide, causing the alkylation of the DNA target during duplex hybridization (Figure 10).[56,57] This strategy may provide a general approach for the target-promoted alkylation of target genes in biological systems.

## 2. Novel Cytosine Cross-Linking Agent with Inducible Reactivity during Duplex Formation

**2.1 Design, Synthesis, and Evaluation of the Reactivity.** Chemical stability and efficient reactivity toward the target site are both required for the applicability of cross-linking agents to cells. In addition, reactivity should ideally be only induced by complex formation with target genes. Our group initially designed 2-amino-6-vinylpurine nucleoside (**1**) (Figure 11) as a selective cytosine-specific cross-linking agent. In our strategy, the 4-amino group of cytosine was expected to be in close proximity with the vinyl group of **1** in the complex formed between the protonated nucleoside and cytosine to effectively induce covalent bond formation.

Because of its potential lack of stability in cells, we proposed a new strategy to induce the reactive vinyl group of 2-amino-6-vinylpurine within the duplex formation. Stable phenylsulfide-protected precursors of vinyl derivative can be oxidized to afford the corresponding sulfoxides, which then undergo elimination to regenerate the vinyl groups. It was anticipated that the vinyl groups would be regenerated by auto-activation upon duplex formation with a complementary strand (Figure 12).

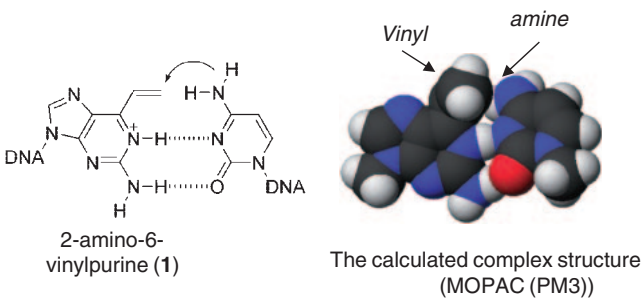



**Figure 11.** Design of a cross-linking agent to react with cytosine selectively.

The synthesis of reactive ODN is summarized in Scheme 1.[58–60] The 2-amino-6-vinylpurine (**1**) was synthesized by cross-coupling with *n*-$Bu_3Sn(CHCH_2)$ or borate ester and palladium catalyst using the tosylate derivative as a substrate. Treatment of **2** with sodium methanethiolate (MeSNa) in $CH_3CN$ gave a protected vinyl derivative. Protection of the 2-amino group and subsequent deprotection gave the diol product **3**. The amidite precursor **4** was synthesized using a standard procedure and was applied to an automated DNA synthesizer to afford the desired ODN **5**. Oxidation of the sulfide group with magnesium mono peroxyphthalate (MMPP) gave the corresponding sulfoxide **6**, and subsequent treatment with NaOH produced ODN with a vinyl group **7**. To search for the proper

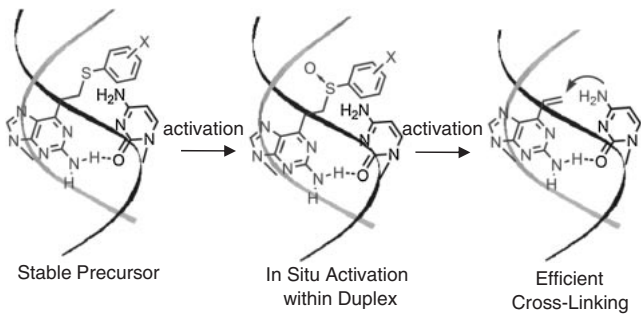
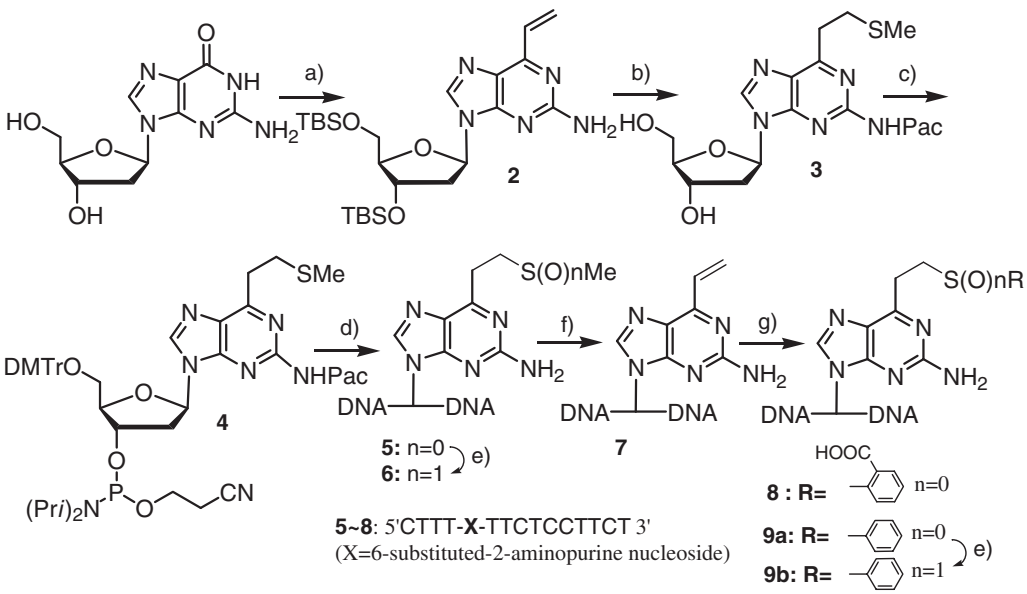



**Figure 12.** New strategy of a cross-linking agent that can be activated within a duplex DNA leading to efficient alkylation.




**Scheme 1.** Synthesis of oligonucleotides incorporating 6-substituted 2-aminopurine nucleoside. a) 1) TBSCl, imidazole, DMF, 2) TsCl, $Et_3N$, 3) *n*-$Bu_3Sn(CH{=}CH_2)$, Pd(0), LiCl, dioxane or $((CH_2{=}CH)BO)_3$, Pd(0), LiBr, LiOH, $H_2O$; b) 1) NaSMe, $CH_3CN$, 2) $PhOCH_2COCl$, pyridine, benzotriazole, 3) *n*-$Bu_4NF$; c) 1) DMTrCl, pyridine, 2) *i*-$Pr_2NP(Cl)OC_2H_4CN$, *i*-$Pr_2NEt$; d) an automated DNA synthesizer, 28% $NH_3$, AcOH; e) MMPP; f) NaOH; g) RSH, pH 7.0.

phenylsulfide structure for the in situ activation, we synthesized a series of substituted phenylsulfide derivatives by adding the corresponding thiophenol derivatives to the ODN **7** containing 2-amino-6-vinylpurine.[61,62]

The cross-linking reaction was investigated using reactive ODNs, vinyl derivative **7**, the substituted phenylsulfide derivative **8**, which exhibited the highest cross-linking yields, and phenylsulfoxide derivative **9b**. These cross-linking reactions were monitored by HPLC analysis and the efficiencies of the three functional ODNs toward cytosine within the complementary strand are compared in Figure 13. Efficient cross-linking reactions were observed for these three functional ODNs and the reaction rates decreased according to the order vinyl, phenylsulfoxide, and 2-carboxylphenylsulfide. These results demonstrated that the stable precursor, sulfide and sulfoxide derivatives might be activated in the duplex to efficiently produce the adduct.

Base-selectivity of cross-linking has been investigated using reactive ODNs **7** and **9b** and a target strand with four different bases (dC, dT, dA, and dG) at the complementary site of each reactive base. The products were analyzed by gel electrophoresis with denaturing gel (Figure 14). This suggests that the two reactive ODNs exhibit high cytosine selectivity. Site selectivity of these cross-linking reactions has also been investigated using the reactive ODNs and the target strands with dC at different target sites. Efficient cross-linking only occurred for strands in which the target C was located at a site that was complementary to the reactive bases, suggesting good site-selectivity.

Cross-linking reactions using 2-amino-6-vinylpurine were carried out under different pH conditions. The cross-linking yields were lower under neutral conditions than under acidic conditions. These data are consistent with our initial postulate stating that the protonation of 2-amino-6-vinylpurine accelerates cross-linking. However these points may be drawbacks to consider when applying these reactions to living-systems. Next, we attempted to design highly efficient cross-linking reactions under neutral conditions.

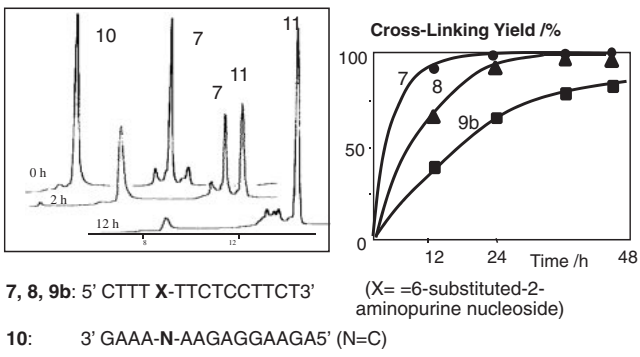



**Figure 13.** HPLC analysis of the cross-linking reaction and comparison of the cross-linking yields. 0.3 mM sample of each oligomer was used in 300 μL of $H_2O$ buffer containing 0.1 M NaCl and 50 mM MES at pH 5.0. HPLC conditions: ODS column, 1.0 mL $min^{-1}$; solvent A) 0.1 M TEAA; solvent B) $CH_3CN$, linear gradient from 0% to 30% over 20 min, 30% to 100% over 30 min, monitored at 254 nm.

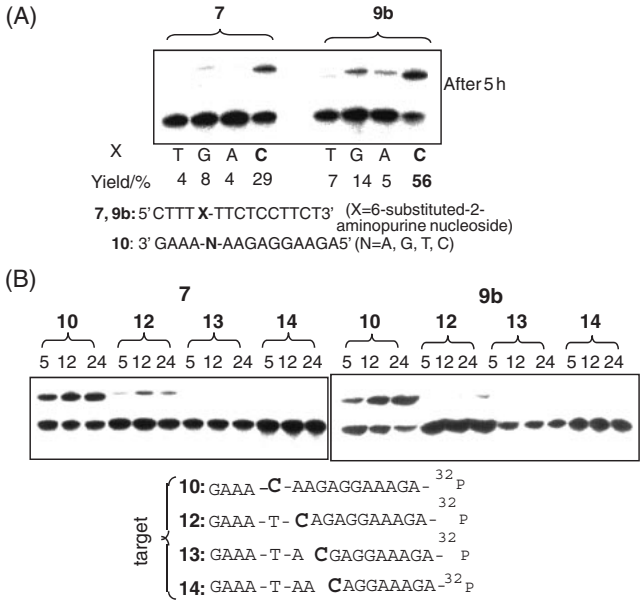
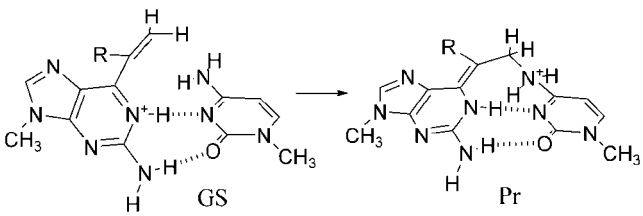



**Figure 14.** Comparison of the base selectivity and site selectivity: (A) base selectivity and (B) site selectivity. The reaction was done using ca. 2:1 mixture of active ODN **7** or **9b** and the target ODN containing $^{32}P$-labeled target as a tracer.

**2.2 Design and Execution of Efficient Cross-Linking under Neutral Conditions.** We designed a new reactive 2-amino-6-(1-ethylsulfinylvinyl)purine derivative **16**, which was expected to undergo highly selective alkylation with cytosine under neutral conditions according to computations. Introducing an electron-withdrawing substituent on the vinyl group was expected to increase its reactivity. Semiempirical MO calculations using PM3 implemented in MOPAC suggested that introducing a methylsulfinyl substituent on the vinyl group decreased the activation energy compared to unsubstituted group (Table 1, R = SOMe vs. R = H).[63]

Thus, sulfinylvinyl derivative **16** was expected to achieve efficient reaction even when the concentration of the N1-protonated form of the purine analog was low under neutral conditions. In a previous study,[62] substituents on the sulfide group were shown to affect the efficiency of the inducible

**Table 1.** Estimated Heat of Formation (kcal $mol^{-1}$)[a)]




| Compd. | R | GS | TS | $\Delta G$ | Pr |
|---|---|---|---|---|---|
| **15** | H | 185.7 | 208.7 | 23.0 | 205.4 |
| **16** | SOMe | 159.2 | 177.7 | 18.5 | 174.5 |
| **17** | SMe | 186.3 | 210.4 | 24.1 | 189.3 |

a) MOPAC96 (PM3). GS: ground state, TS: transition state, Pr: product. Structures of ground state and product of the complex are schematically shown.

cross-linking reaction. We designed a series of substituted bis-sulfides identifying appropriate analogs susceptible to activation within the duplex (Figure 15).

The synthesis of ODNs incorporating functionalized nucleoside analogs is summarized in Scheme 2. The vinyl group of **2** was transformed into the corresponding dibromo derivative then treatment with alkanethiol reagents produced different bis-alkylsulfide derivatives.

After protection of the 2-amino group and deprotection of the hydroxy groups, the phosphoramidite precursors **19** were synthesized using a conventional method, then transferred to an automated DNA synthesizer to generate ODNs. Purified ODNs containing bis(alkylthio) derivatives **20** were treated under mild alkaline conditions to produce the alkylthiovinyl bearing ODNs **21** (Scheme 3). After oxidation with MMPP, the resulting mixtures were directly used in cross-linking reactions. Figure 16 shows a representative result for the reaction between an ODN bearing the ethylsulfinylvinyl nucleoside **22a** and DNA targets having a different base at the position that was complementary to the reactive base under neutral conditions.

These results clearly show that 2-amino-6-(1-ethylsulfinylvinyl)purine **22a** exhibits highly selective and efficient cross-linking activity toward cytosine at the target site under neutral conditions. Figure 17 compares cross-linking yields obtained with ODNs bearing alkylsulfinylvinyl nucleotides. Except for the octane thiol derivative, sulfoxide-substituted vinyl derivatives exhibited similar reactivities toward cytosine.

The stable bis-sulfide derivatives were oxidized with MMPP and the mixtures were directly used for cross-linking reactions. The cross-linking yields of the functionalized ODNs

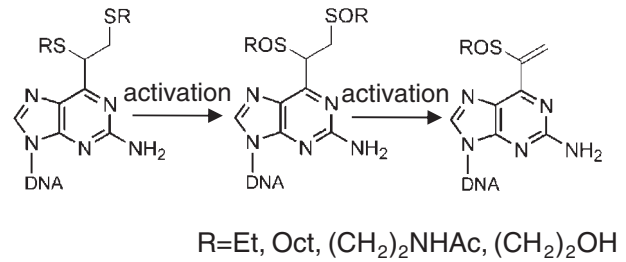
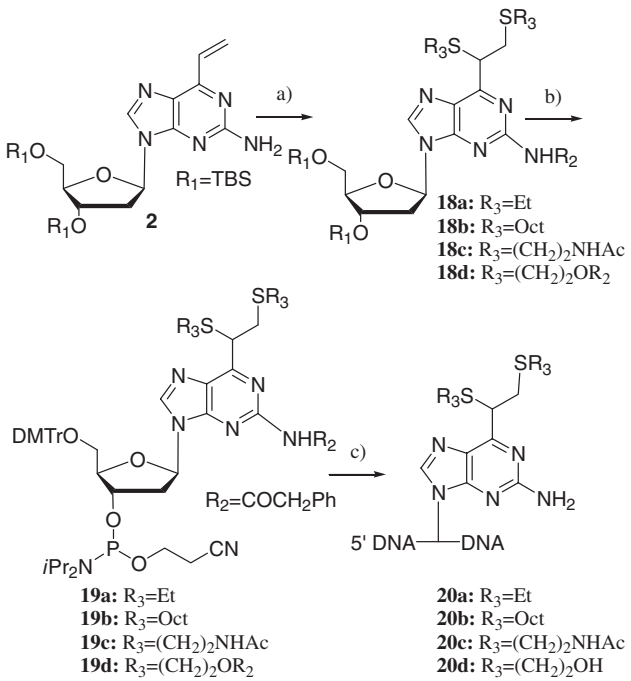
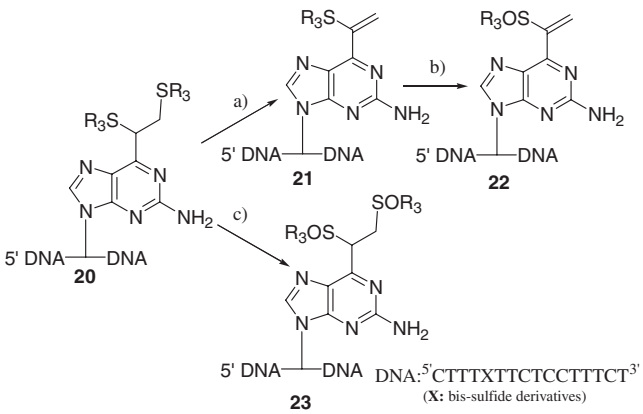



**Figure 15.** New cross-linking agents activated under neutral conditions.




**Scheme 2.** Synthesis of bis-sulfide derivatives incorporation into the ODN. a) 1) $Br_2$ aq, $CHCl_3$, 2 h, 2) DBU, $CH_2Cl_2$, 1 h, then RSH; b) 1) $PhOCH_2COCl$, 1-hydroxybenzotriazole, pyridine–$CH_3CN$, 2) $Et_3N$, HF, 73%, 3) DMTrCl, pyridine, 4) *i*-$Pr_2EtN$, $CH_2Cl_2$, *i*-$Pr_2NP(Cl)OCH_2CH_2CN$, 44%; c) 1) synthesis with an automated DNA synthesizer, 2) 0.1 M NaOH for R: Et, Oct, $(CH_2)_2NHAc$, 0.1 M NaOH in the presence of $HSCH_2CH_2OH$ for R: $CH_2CH_2OH$, 3) 10% $CH_3COOH$.




**Scheme 3.** Synthesis of double activated ODN. a) 0.5 M NaOH, 24 h; b) 2 equiv MMPP pH 10, 30 min; c) 6 equiv MMPP, pH 10, 1 d.

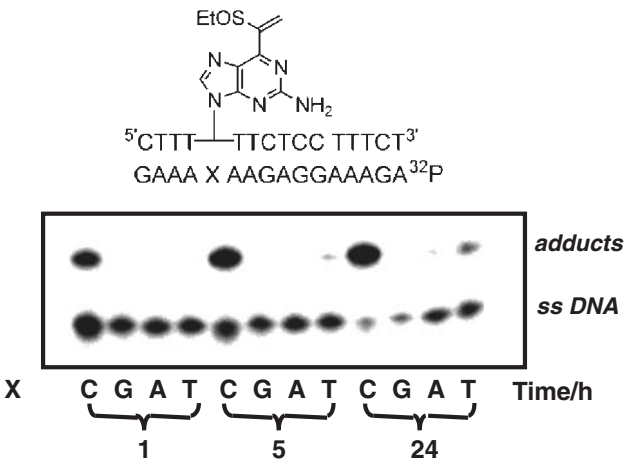



**Figure 16.** Base selectivity of the cross-linking using ethyl sulfinylvinyl nucleoside under neutral conditions. Cross-linking was done using using 10 μM reactive ODN **22a**, 1 μM target oligomer **10** in the buffer containing 100 mM NaCl, 50 mM MES, pH 7.0, 37 °C, and analyzed by electrophoresis with 19% denaturing polyacrylamide gel at the indicated time. Yields were determined by quantification of the bands on the gels by BAS 2500 using the imaging plate.

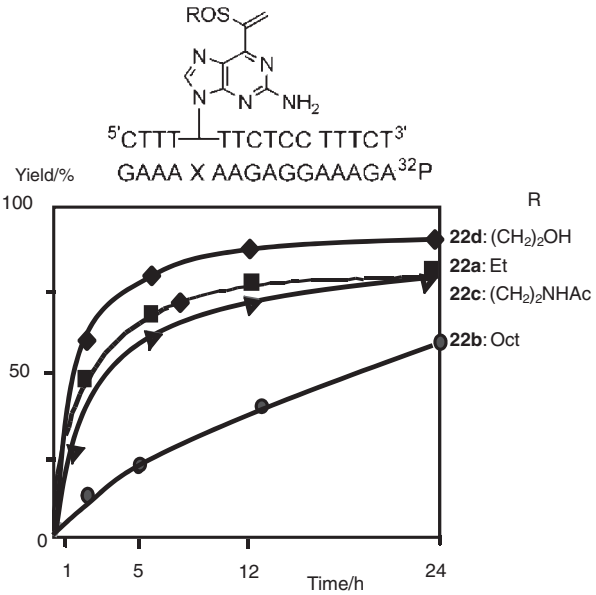



**Figure 17.** Comparison of cross-linking reactivity with vinyl sulfoxide derivatives under neutral conditions.

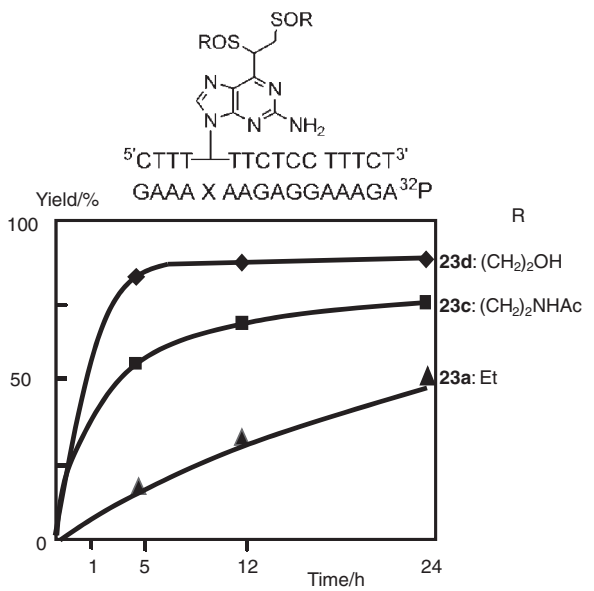



**Figure 18.** Comparison of cross-linking reactivity with bis-sulfoxide derivatives under neutral conditions.

(Figure 18) showed that the reactivity increased according to the substituent order hydroxylethyl (**23d**) > acetamidoethyl (**23c**) > ethyl (**23a**). These results suggested that the bis-(hydroxyethyl) sulfoxide derivative might be activated efficiently within the duplex and exhibit high cross-linking reactivity under neutral conditions.

**2.3 Application of 2-Amino-6-vinylpurine to Cross-Linking Reaction during Triple Helix Formation.** Triplex-forming oligonucleotides (TFOs) bind with homopurine–homopyrimidine regions in duplex DNA in a sequence-specific manner (Figure 19).

Triplex-based approaches are an attractive means to achieve targeted gene regulation and manipulation in vitro and in vivo.

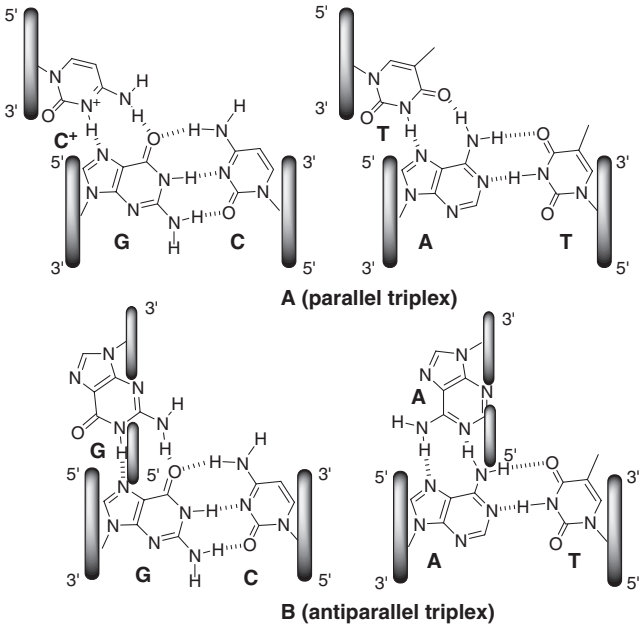



**Figure 19.** Schematic illustration of triple helix formation.

Cross-linking reactions within triplexes have been used to ensure triplex formation and subsequent inhibition of gene expression in the antigene method.

We designed new nucleoside derivatives containing butyl or ethyl spacer between the sugar and the 2-amino-6-vinylpurine to induce cross-linking within triple helices (Figure 20). The 2-amino-6-vinylpurine was expected to react with cytosine and adenine by positioning itself in close proximity to the target bases.

These nucleoside derivatives were synthesized using a coupling reaction between 2-amino-6-chloropurine and sugar derivatives **27** containing an ethyl or a butyl spacer. The vinyl group was introduced by palladium-catalyzed cross-coupling in the presence of $Bu_3Sn(CHCH_2)$ and treatment with MeSNa produced the protected reactive bases. The conventional method produced the amidite precursors **29**, which were applied to the DNA synthesizer to give the desired TFO (Scheme 4).

The ODNs **30** were then smoothly converted to vinyl containing ODNs **31** by oxidation with MMPP and elimination under alkaline conditions. Cross-linking between functionalized ODNs and the target duplex was carried out and the resulting adducts were analyzed by denaturing gel electro-

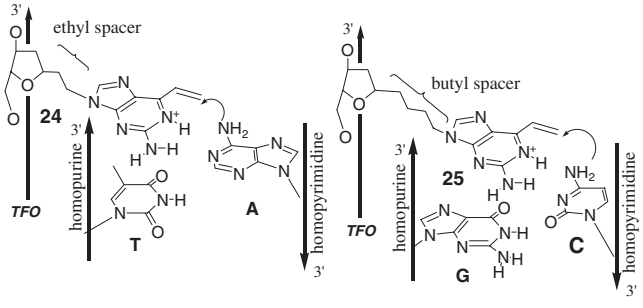
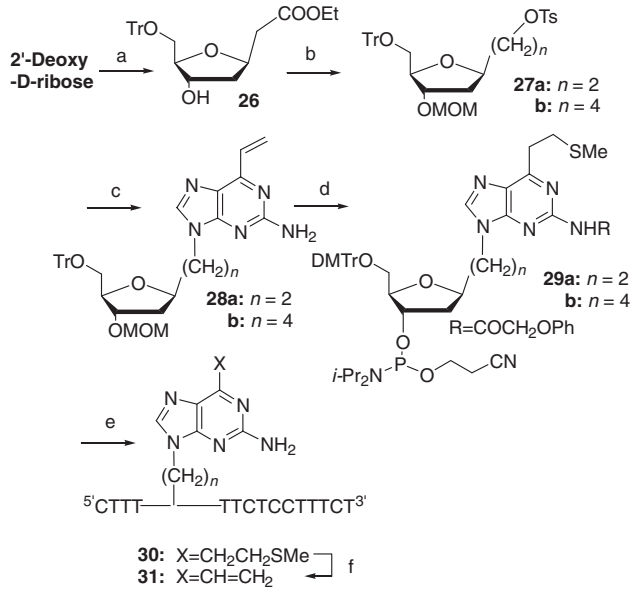



**Figure 20.** Design of novel cross-linking agents to achieve selective reaction to the flipping cytidine within the triplex.

phoresis (Figure 21). The results clearly showed that the ODN with ethyl spacer **31a** only reacted with adenine, whereas the ODN with a butyl spacer **31b** only reacted with cytosine in the pyrimidine strand upon triple helix formation.[64,65] This demonstrates that nucleoside derivatives with a butyl or an ethyl spacer between sugar and 2-amino-6-vinylpurine selectively react with cytosine or adenine by positioning themselves in close proximity to the target bases upon triple helix formation.




**Scheme 4.** a) 1) TrCl, DMAP, pyridine, 2) *t*-BuOK, THF, $(EtO)_2P(O)CH_2CO_2Et$; b) **27a**: 1) *i*-$Pr_2NEt$, MOMCl, 2) DIBAL, $CH_2Cl_2$, 3) TsCl, pyridine; **27b**: 1) *i*-$Pr_2NEt$, MOMCl, 2) DIBAL, $CH_2Cl_2$, 3) NaH, $(EtO)_2P(O)CH_2CO_2Et$, THF, 4) $H_2$, Pd–C, EtOH, 5) LAH, THF, 6) TsCl, pyridine; c) 1) 2-amino-6-chloropurine, *t*-BuOK, DMSO, 2) *n*-$Bu_3SnCH{=}CH_2$, $(Ph_3P)_2PdCl_2$, dioxane; d) 1) MeSNa, $CH_3CN$, 2) $PhOCH_2COCl$, 1-HBT, $CH_3CN$, pyridine, 3) $BF_3 \cdot Et_2O$, $Me_2S$, 4) DMTrCI, pyridine, 5) *i*-$Pr_2NEt$, $CH_2Cl_2$, *i*-$Pr_2NP(Cl)OC_2H_4CN$; e) 1) automated DNA synthesizer, 2) 28% $NH_3$, 3) 10% AcOH; f) 1) 3.0 equiv MMPP, pH 10, 2) 470 mM NaOH.

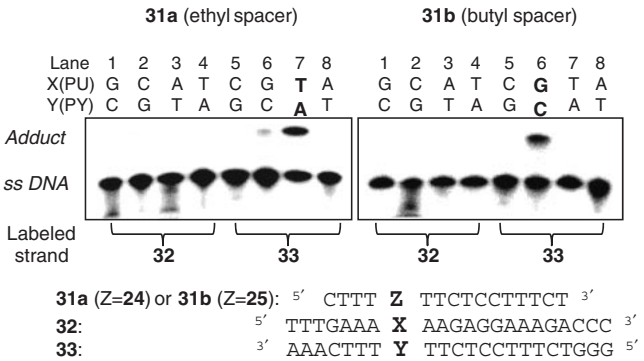



**Figure 21.** Comparison of the cross-linking reactivity by auto-radiogram of gel electrophoresis (15% denaturing gel). **A** with **31a** (Z = **24**), **B** with **31b** (Z = **25**). The reaction was done using 10 μM of ODNs (**31a**: Z = **24** or **31b**: Z = **25**), 1 μM target duplex **32**, **33** in a buffer including 10 mM cacodylate, 0.25 mM spermine, 100 mM NaCl, pH 4.5 at 30 °C. The reaction mixture contained either 5′-$^{32}$P-labeled **32** or **33** as a tracer. The reaction was stopped by the addition of formamide after 20 h.

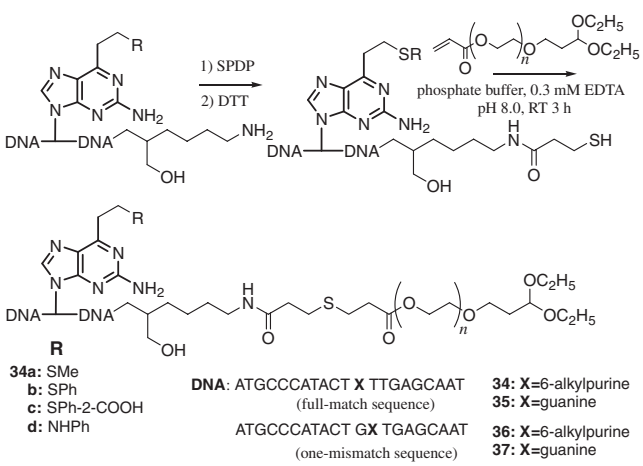
## 3. Evaluation of Cross-Linking Reactions in Cells

We developed a novel cross-linking agent that was activated during duplex formation, and reacted it with cytosine selectively in an attempt to use it in living systems. In addition, we demonstrated that 2-amino-6-vinylpurine containing TFOs exhibited a highly selective reactivity toward cytosine or adenine in pyrimidine strands during triple helix formation. This high selectivity is useful for site-specific modifications. Thus, we investigated the potential for reactive TFO to induce the mutations at cross-linking sites. These results are described in this section.

**3.1 Antisense Effect in Cells Using Inducible Cross-Linking Reaction.**[66] ODNs are thought to inhibit gene expression in cells, but they have not been as efficient as expected, because of their susceptibility to enzymatic degradation and limited cell permeability. In our approach, we used polyion-complex (PIC) micelles of PEG–ODN conjugates to protect ODNs from enzymatic digestion and promote their efficient delivery into cells.[67]

PEG-conjugated functional ODNs were prepared as shown in Scheme 5 and mixed with poly-L-lysine to form PIC micelles.

The intracellular antisense activity of the functionalized ODN–PEG conjugates was evaluated using a dual-luciferase reporter assay. Antisense effects are shown in Figure 22. ODNs containing sulfide derivatives of 2-aminopurine showed greater antisense effects than the natural antisense ODN conjugated with PEG. As discussed in Section **2.1**, 2-carboxy sulfide derivative showed high cross-linking reactivity in vitro, but ODN which contained this derivative **34c** exhibited similar antisense effects to natural ODN in cells. These results suggested that derivatives, which were reactive in vitro may be unstable in cells and instantly lose their reactivity. On the other hand, less antisense effect was observed when an aniline




**Scheme 5.** Synthesis of the antisense PEG conjugates in which 2-amino-6-alkylpurine is incorporated.

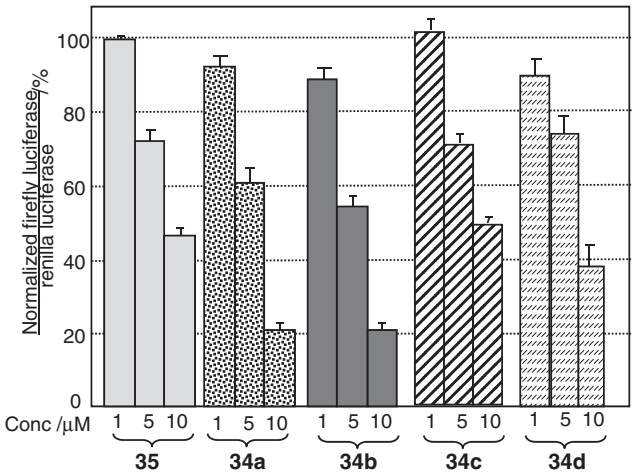



**Figure 22.** Antisense effects with PEG–ODN/PLL PIC micelles against gene expression of firefly luciferase in cultured HuH-7 cells. Normalized ratios of firefly-luciferase activity to that of renilla luciferase are shown in the ordinate.

containing ODN was used as an un-reactive control. The enhancement of antisense inhibition may therefore be attributed to the cross-linking ability of the reactive ODN.

The impact of one mismatched site on antisense effects of natural antisense ODNs was compared to its impact for reactive ODNs (Figure 23). The natural ODN with one mismatched site **37** (X = G) exhibited similar antisense inhibition as the fully matched ODN **35** (X = G). In marked contrast, reactive ODN **36b** in which the 2-aminopurine derivative was adjacent to the target cytosine, was much less efficient than the fully matched ODN, demonstrating the greater ability of reactive ODNs to discriminate single nucleoside differences in cells compared to natural antisense ODNs. Thus, sulfide precursors of 2-amino-6-vinylpurine derivatives may be useful for further in vivo applications.

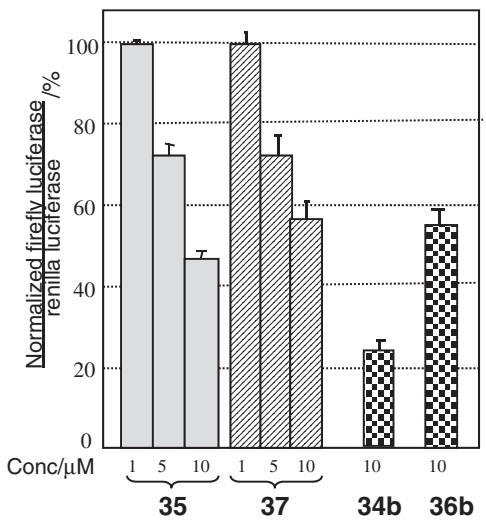



**Figure 23.** Comparison of effects of one-mismatched site on the antisense effects. Antisense effects were assayed similarly as described in the footnote of Figure 22.

**3.2 Targeted Mutagenesis Using 2-Amino-6-vinylpurine Containing TFOs.**[68,69] Psoralen-linked TFOs were used for photoinduced site-directed mutagenesis in reporter genes (Figure 24).[15–20]

Replication and repair of targeted adducts can result in mutagenesis at the target site. Thus, the alkylation of nucleobases using functionalized TFOs may be of interest for site-directed mutation. We investigated the use of 2-amino-6-vinylpurine containing TFOs to target mutations at specific sites in shuttle vector plasmids, that replicates in mammalian cells. In our previous study, we discovered that a TFO containing 2-amino-6-vinylpurine at the internal position selectively reacted with the cytosine within the pyrimidine strand, and that a TFO bearing 2-amino-6-vinylpurine at the terminal position reacted with adenine. Thus we prepared two oligomer sequences to verify this result. One sequence contained **25** at the internal position and was designed to react with cytosine. The other sequence had **25** at the external position and was expected to react externally with the triplex. The TFOs were designed to form triplexes with target sequences embedded within supF mutation marker genes (Figure 25), which were carried by shuttle vector plasmids. Except for the 2-amino-6-vinylpurine derivative, the TFOs were constructed with 2′-*O*-methoxy (2′-OMe) nucleosides to increase pyrimidine triplex stability. The extent of reaction between reactive TFOs and target reporter plasmid was determined through a restriction enzyme protection assay. The covalent reaction site of reactive TFO was contained in the recognition sequence for the restriction enzyme and covalent linkage of the TFOs to the target blocked cleavage by the enzyme. The results of these assay demonstrated equivalent protection from enzyme digestion with reactive TFOs (about 50%). This clearly shows that reactive TFOs exhibit reactivity to the target duplex DNA in vitro.

In mutagenesis experiments, we used one shuttle vector to detect mutations at the terminal adenine, and another shuttle vector to detect mutations at the terminal adenine and internal cytosine simultaneously. These vectors contained the supF gene, an amber suppressor tyrosine tRNA gene of *Escherichia coli*, which serves as a mutation reporter at target sites. In our experiments, triplex formation and target modification were carried in vitro, and the adducted plasmids were subsequently introduced into cells. The mutation frequencies caused by reactive TFOs ranged from 0.19 to 0.35% for individual preparations (Table 2).

On the other hand, the mutation did not occur when an unreactive SMe containing TFO was used (≪0.02%). These results provide evidence for reactive TFO-directed mutagenesis within cells. The mutation spectra indicated that almost all

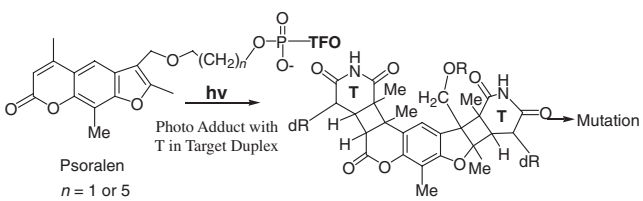



**Figure 24.** Induced mutations with use of the psoralen-linked TFO.

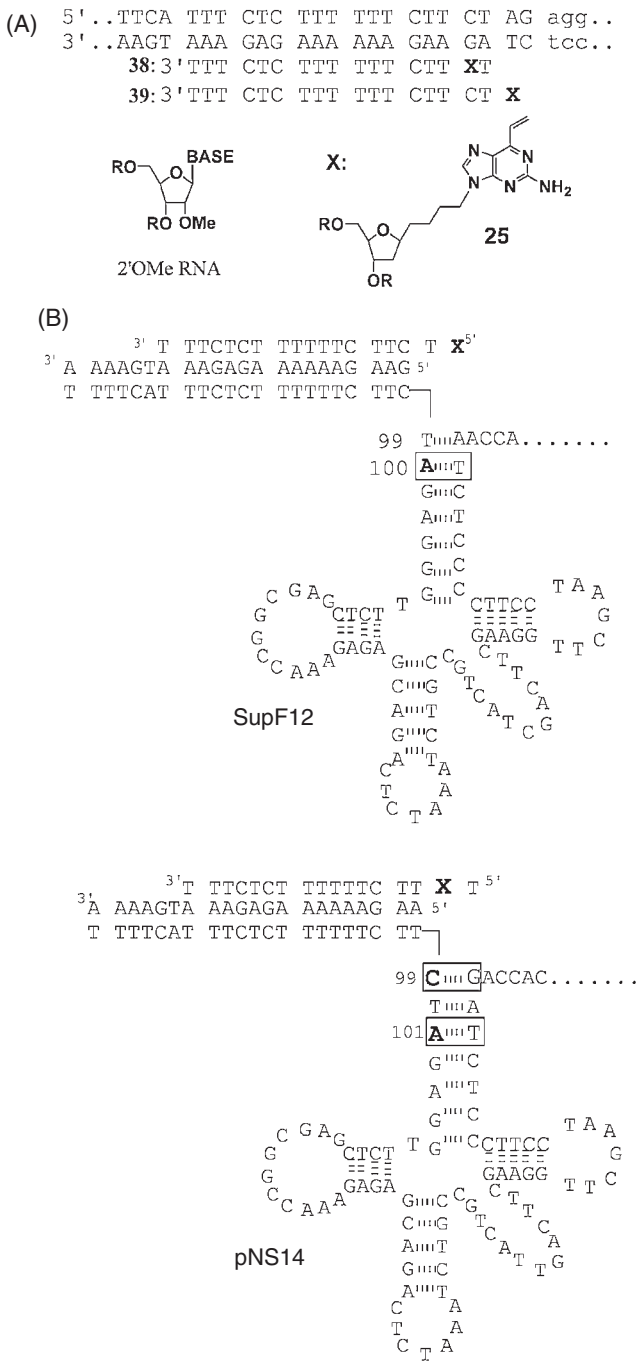



**Figure 25.** (A) Triplex target sequence and TFOs and (B) schematic diagram of the supF12 and pNS14 gene embedded in the pre-tRNA region.

mutations occurred at target adenine or cytosine depending on the TFO conjugate involved. The 2′-OMe–TFO designed to react with adenine exclusively exhibited A → G mutations in both plasmids (Figure 26). On the other hand, the TFO designed to react with cytosine induced various types of mutation, albeit only at the target cytosine. Mutagenesis mechanisms were unclear but the mutations were clearly introduced selectively at cross-linking sites during replication and repair in mammalian cells.

**Table 2.** Mutation Frequency of Reactive TFO-Treated Plasmid in SVXP12BE Cells[a)]

| TFO | Plasmid | No. of colonies | | Mutation frequency/% |
|---|---|---|---|---|
| | | mutant | total | |
| **38** | pNS14 | 10 | 3052 | 0.33 |
| **39** | pNS14 | 8 | 3926 | 0.20 |
| **39** | supF12 | 15 | 4381 | 0.34 |
| con | pNS14 | 0 | 6665 | 0 |

a) Numbers represent the frequency of mutations seen in supF gene. Mutations in this gene yield white colonies among the wild type blue colonies.

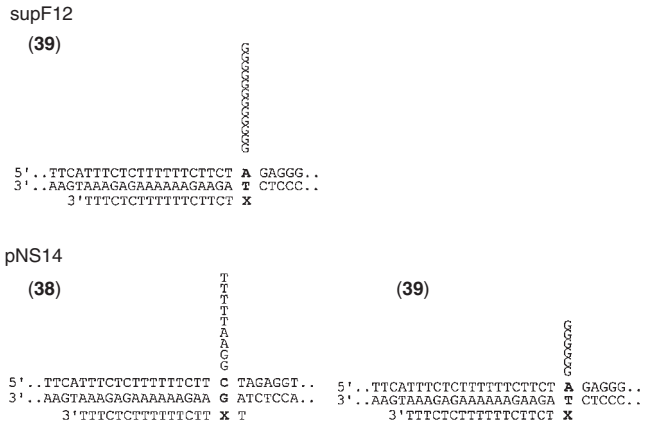



**Figure 26.** Spectra of supF gene mutations produced in SVXP12BE cells.

## Conclusion

Reactive ODNs are of great potential to increase the efficiency of gene expression inhibition through antisense and antigene methods. In addition, chemical modification of nucleobases may change their recognition properties, thereby potentially inducing mutation gene. Selective cross-linking reactions can chemically cause point mutations. However, only psoralen ODN conjugates have been applied to direct mutagenesis to specific sites in cells by photoactivation. The development of new cross-linking agents, which possess selective and efficient reactivity toward target genes, is needed for gene manipulation application. We exploited this new concept to design functional groups whose reactivity can be induced in the proximity of the target base. ODNs containing 2-amino-6-vinylpurine derivatives have been shown to efficiently and selectively cross-link with cytosine at the complementary site. Their stable phenylsulfide precursors were selectively activated in the target duplex to form cross-linked products with cytosine. In addition, we showed that an inducible PEG–ODN alkylation system promoted efficient and selective antisense activity against luciferase production through the delivery system of PIC micelles in an intracellular environment. To the best of our knowledge, this is the first example of an effective cross-linking reaction in cells without UV irradiation. Furthermore, we demonstrated that TFOs conjugated with 2-amino-6-vinylpurine derivatives caused specific point mutations in model cell systems. These reactive ODNs may be developed as chemical tools for gene manipulation at the molecular level.

**References**


1 T. Da Ros, G. Spalluto, M. Prato, T. Saison-Behmoaras, A. Boutorine, B. Cacciari, *Curr. Med. Chem.* **2005**, *12*, 71.

2 T. Aboul-Fadl, *Curr. Med. Chem.* **2005**, *12*, 2193.

3 L. D. Curcio, D. Y. Bouffard, K. J. Scanlon, *Pharmacol. Ther.* **1997**, *74*, 317.

4 B. P. Casey, P. M. Glazer, *Prog. Nucleic Acid Res. Mol. Biol.* **2001**, *67*, 163.

5 M. Faria, C. Giovannangeli, *J. Gene Med.* **2001**, *3*, 299.

6 F. Takeshita, T. Ochiya, *Cancer Sci.* **2006**, *97*, 689.

7 I. Drude, V. Dombos, S. Vauleon, S. Muller, *Mini-Rev. Med. Chem.* **2007**, *7*, 912.

8 T. I. Novobrantseva, A. Akinc, A. Borodovsky, A. de Fougerolles, *Curr. Opin. Drug Discovery Dev.* **2008**, *11*, 217.

9 S. Sasaki, *Eur. J. Pharm. Sci.* **2001**, *13*, 43.

10 S. Sasaki, F. Nagatsugi, *Curr. Opin. Chem. Biol.* **2006**, *10*, 615.

11 J. M. Kean, A. Murakami, K. R. Blake, C. D. Cushman, P. S. Miller, *Biochemistry* **1988**, *27*, 9113.

12 J. T. Levis, P. S. Miller, *Antisense Res. Dev.* **1994**, *4*, 223.

13 C. Helene, N. T. Thuong, A. Harel-Bellan, *Ann. N.Y. Acad. Sci.* **1992**, *660*, 27.

14 C. Giovannangéli, N. T. Thuong, C. Héléne, *Nucleic Acids Res.* **1992**, *20*, 4275.

15 K.-H. Kim, P. E. Nielsen, P. M. Glazer, *Nucleic Acids Res.* **2007**, *35*, 7604.

16 M. P. Knauert, J. M. Kalish, D. C. Hegan, P. M. Glazer, *Mol. Ther.* **2006**, *14*, 392.

17 J. M. Kalish, P. M. Glazer, in *Therapeutic Oligonucleotides: Transcriptional and Translational Strategies for Silencing Gene Expression*, ed. by Y. S. Cho-Chung, A. M. Gerwirtz, C. A. Stein, **2005**, Vol. 1058, p. 151.

18 M. M. Seidman, P. M. Glazer, *J. Clin. Invest.* **2003**, *112*, 487.

19 A. Majumdar, P. A. Muniandy, J. Liu, J. Liu, S. Liu, B. Cuenoud, M. M. Seidman, *J. Biol. Chem.* **2008**, *283*, 11244.

20 H. Li, P. S. Miller, M. M. Seidman, *Org. Biomol. Chem.* **2008**, *6*, 4212.

21 T. J. Povsic, P. B. Dervan, *J. Am. Chem. Soc.* **1990**, *112*, 9428.

22 T. J. Povsic, S. A. Strobel, P. B. Dervan, *J. Am. Chem. Soc.* **1992**, *114*, 5934.

23 J. C. Tabone, M. R. Stamm, H. B. Gamper, R. B. Meyer, Jr., *Biochemistry* **1994**, *33*, 375.

24 R. S. Coleman, E. A. Kesicki, *J. Org. Chem.* **1995**, *60*, 6252.

25 R. S. Coleman, R. M. Pires, *Nucleic Acids Res.* **1997**, *25*, 4771.

26 K. B. Grant, P. B. Dervan, *Biochemistry* **1996**, *35*, 12313.

27 T. R. Webb, M. D. Matteucci, *Nucleic Acids Res.* **1986**, *14*, 7661.

28 T. R. Webb, M. D. Matteucci, *J. Am. Chem. Soc.* **1986**, *108*, 2764.

29 M. Cowart, S. J. Benkovic, *Biochemistry* **1991**, *30*, 788.

30 M. W. Reed, A. Wald, R. B. Meyer, *J. Am. Chem. Soc.* **1998**, *120*, 9729.

31 E. A. Lukhtanov, I. V. Kutyavin, H. B. Gamper, R. B. Meyer, Jr., *Bioconjugate Chem.* **1995**, *6*, 418.

32 E. A. Lukhtanov, M. A. Podyminogin, I. V. Kutyavin, R. B. Meyer, H. B. Gamper, *Nucleic Acids Res.* **1996**, *24*, 683.

33 E. A. Lukhtanov, I. V. Kutyavin, V. V. Gorn, M. W. Reed, A. D. Adams, D. D. Lucas, R. B. Meyer, Jr., *J. Am. Chem. Soc.* **1997**, *119*, 6214.

34 E. A. Lukhtanov, A. G. Mills, I. V. Kutyavin, V. V. Gorn, M. W. Reed, R. B. Meyer, *Nucleic Acids Res.* **1997**, *25*, 5077.

35 D. Praseuth, A. L. Guieysse, C. Hélène, *Biochim. Biophys. Acta, Gene Struct. Expression* **1999**, *1489*, 181.

36 S. Diviacco, V. Rapozzi, L. Xodo, C. Hélène, F. Quadrifoglio, C. Giovannangeli, *FASEB J.* **2001**, *15*, 2660.

37 M. Faria, C. D. Wood, M. R. H. White, C. Hélène, C. Giovannangeli, *J. Mol. Biol.* **2001**, *306*, 15.

38 M. Higuchi, A. Kobori, A. Yamayoshi, A. Murakami, *Bioorg. Med. Chem.* **2009**, *17*, 475.

39 A. Kobori, K. Takaya, M. Higuchi, A. Yamayoshi, A. Murakami, *Chem. Lett.* **2009**, *38*, 272.

40 Y. Yoshimura, Y. Ito, K. Fujimoto, *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1299.

41 Y. Yoshimura, K. Fujimoto, *Org. Lett.* **2008**, *10*, 3227.

42 Y. Yoshimura, T. Ohtake, H. Okada, K. Fujimoto, *ChemBioChem* **2009**, *10*, 1473.

43 S. Le Gac, S. Rickling, P. Gerbaux, E. Defrancq, C. Moucheron, A. Kirsch-De Mesmaeker, *Angew. Chem., Int. Ed.* **2009**, *48*, 1122.

44 Z. Qiu, L. Lu, X. Jian, C. He, *J. Am. Chem. Soc.* **2008**, *130*, 14398.

45 M. M. Greenberg, *Org. Biomol. Chem.* **2007**, *5*, 18.

46 I. S. Hong, M. M. Greenberg, *J. Am. Chem. Soc.* **2005**, *127*, 3692.

47 I. S. Hong, H. Ding, M. M. Greenberg, *J. Am. Chem. Soc.* **2006**, *128*, 485.

48 I. S. Hong, M. M. Greenberg, *J. Am. Chem. Soc.* **2005**, *127*, 10510.

49 X. Peng, I. S. Hong, H. Li, M. M. Seidman, M. M. Greenberg, *J. Am. Chem. Soc.* **2008**, *130*, 10299.

50 S. Halila, T. Velasco, P. De Clercq, A. Madder, *Chem. Commun.* **2005**, 936.

51 K. Stevens, M. Op de Beeck, S. Figaroli, A. Madder, *Collect. Symp. Ser.* **2008**, *10*, 210.

52 K. Stevens, A. Madder, *Nucleic Acids Res.* **2009**, *37*, 1555.

53 Q. Zhou, P. Pande, A. E. Johnson, S. E. Rokita, *Bioorg. Med. Chem.* **2001**, *9*, 2347.

54 P. Pande, J. Shearer, J. Yang, W. A. Greenberg, S. E. Rokita, *J. Am. Chem. Soc.* **1999**, *121*, 6773.

55 H. Kang, S. E. Rokita, *Nucleic Acids Res.* **1996**, *24*, 3896.

56 W. F. Veldhuyzen, P. Pande, S. E. Rokita, *J. Am. Chem. Soc.* **2003**, *125*, 14005.

57 Q. Zhou, S. E. Rokita, *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 15452.

58 F. Nagatsugi, Y. Ogata, S. Imoto, S. Sasaki, *Heterocycles* **2007**, *73*, 493.

59 F. Nagatsugi, K. Uemura, S. Nakashima, M. Maeda, S. Sasaki, *Tetrahedron* **1997**, *53*, 3035.

60 F. Nagatsugi, K. Uemura, S. Nakashima, M. Maeda, S. Sasaki, *Tetrahedron Lett.* **1995**, *36*, 421.

61 F. Nagatsugi, T. Kawasaki, D. Usui, M. Maeda, S. Sasaki, *J. Am. Chem. Soc.* **1999**, *121*, 6753.

62 T. Kawasaki, F. Nagatsugi, M. M. Ali, M. Maeda, K. Sugiyama, K. Hori, S. Sasaki, *J. Org. Chem.* **2005**, *70*, 14.

63 F. Nagatsugi, N. Tokuda, M. Maeda, S. Sasaki, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2577.

64 F. Nagatsugi, Y. Matsuyama, M. Maeda, S. Sasaki, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 487.

65 F. Nagatsugi, D. Usui, T. Kawasaki, M. Maeda, S. Sasaki,

*Bioorg. Med. Chem. Lett.* **2001**, *11*, 343.
66 M. M. Ali, M. Oishi, F. Nagatsugi, K. Mori, Y. Nagasaki, K. Kataoka, S. Sasaki, *Angew. Chem., Int. Ed.* **2006**, *45*, 3136.
67 M. Oishi, F. Nagatsugi, S. Sasaki, Y. Nagasaki, K. Kataoka, *ChemBioChem* **2005**, *6*, 718.
68 F. Nagatsugi, S. Sasaki, *Biol. Pharm. Bull.* **2004**, *27*, 463.
69 F. Nagatsugi, S. Sasaki, P. S. Miller, M. M. Seidman, *Nucleic Acids Res.* **2003**, *31*, e31.

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