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Selective Cross-Linking to the Adenine of the TA Interrupting Site within the Triple Helix

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Abstract—The triplex-forming oligonucleotide incorporating the new nucleoside derivative (**2**) that connects the 2-amino-6-vinylpurine moiety to the 2-deoxyribose unit with an ethyl spacer has exhibited highly selective cross-linking reaction to the adenine of the TA interrupting site within the triple helix.

Triplex-forming oligonucleotides (TFOs) can bind to homopurine-homopyrimidine regions in duplex DNA in a sequence-specific manner.[1,2] It has recently been reported that a single insertion of an interrupting site such as a TA or a CG pair can be recognized by TFO having new nucleoside analogues,[3] although a general method for recognition of any duplex sequences has not appeared to date.[4] Triplex-based approaches are an attractive means to achieve targeted gene regulation[5–7] and gene manipulation[8–10] both in vitro and in vivo. Cross-linking reaction within the triplexes has been used to ensure triplex formation and subsequent inhibition of gene expression in the antigene method.[11] Furthermore, covalent modification of a single base has been expected to alter the recognition mode of the base, and would be useful as a new biotechnology.[8–10] However, only a limited number of cross-linking agents are currently available, which include alkylating agents to N^7 of guanine as well as photo-reactive psolarens to a thymine of an AT pair.[12] For cross-linking agents within triplexes to become a useful tool for gene manipulation, they must exhibit high selectivity at the base level. Thus, there is an urgent need for the development of a reliable concept on which a new cross-linking molecule may be designed to attain desired base selectivity.

Recently, we have demonstrated that the new nucleoside derivative (**1**) with a butyl spacer between the sugar part and the 2-amino-6-vinylpurine[13] skeleton achieves triplex-forming cross-linking with high selectively toward the cytosine of the G-C target site (Fig. 1A).[14] The high selectivity to cytosine in the pyrimidine strand would be due to a complex between the protonated form of 2-amino-6-vinylpurine moiety (**1**) and a cytosine which is flipped out from the GC pair.

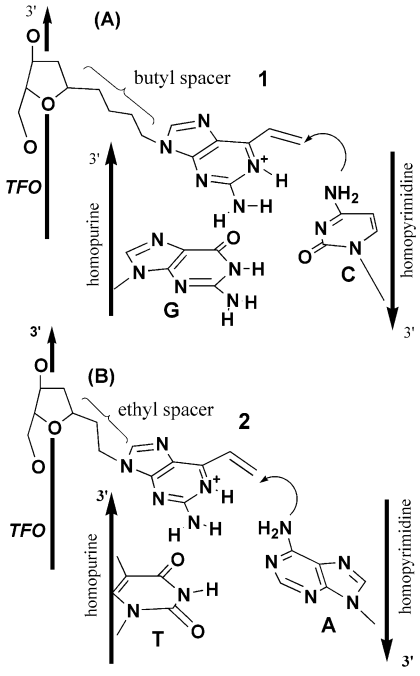

Figure 1. The reaction of **1** with the flipping cytosine (A) leading to the design of the new cross-linking agent **2** to the adenine within the TA interrupting site (B).

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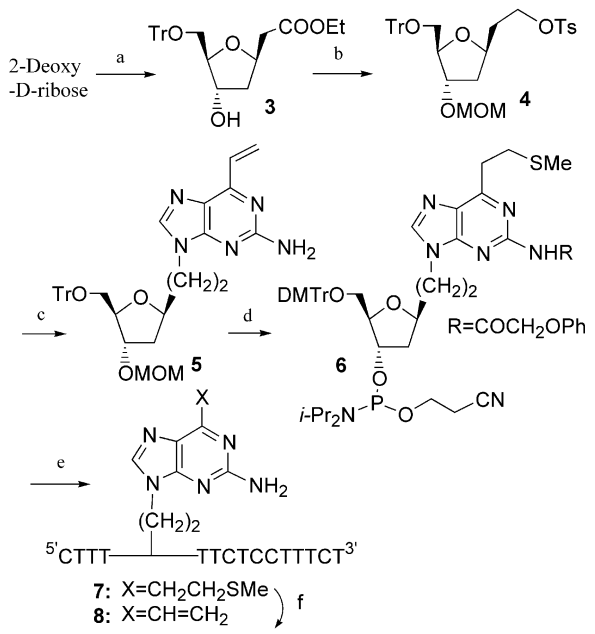
Then, we proposed the new concept in which a flipping base can be selectively reacted by the cross-linking agent. As an application of this concept, we have designed a new nucleoside derivative (**2**) having an ethyl spacer between the sugar part and the 2-amino-6-vinylpurine skeleton as a cross-linking agent to an adenine at the TA base pair. Here, we wish to demonstrate the validity of the new concept by achieving the first selective cross-linking at the TA interrupting site.

The conformational change of the triplex during the cross-linking of **1** with cytosine has been estimated by MM3 calculation, and it has been suggested that only a slight flipping of the cytosine may cause satisfactory proximity between the vinyl and the amino groups, and that almost no distortion is observed for the backbone geometries of the triplex (Fig. 1A). The new cross-linking agent **2** connects the 2-amino-6-vinylpurine skeleton to the sugar part with an *ethyl spacer* (Fig. 1B). A similar MM3 estimation has shown that a small flipping of the adenine would be enough to bring the amino group of the adenine into proximity of the vinyl group of **2**, without distortion of the triplex geometry (Fig. 1B). It has been also suggested that hydrogen bonds between the three components, T, A and the 2-amino-purine moiety would be maintained during the reaction. In contrast, the reactions of **2** with GC, CG or AT base pair were found to need a large conformational change. Thus, it was anticipated that **2** would react selectively with the adenine of the TA interrupting site.

The synthesis of the ODNs incorporating functionalized nucleoside analogue (**2**) is summarized in Scheme 1. The sugar part (**3**) was synthesized by β-selective C-glycoside formation via Wittig–Horner–Emmons reaction from 2-deoxy-D-ribose (Scheme 1).[15] The alcohol of **3** was protected with a methoxymethyl (MOM) group, and the following reduction and tosylation gave **4**. The coupling reaction between 2-amino-6-chloropurine[16] and **4** yielded the desired product as a major isomer, which was used for the Pd(II)-catalyzed cross-coupling reaction with nBu$_3$SnCH=CH$_2$ to afford 2-amino-6-vinylpurine



Scheme 1. (a) TrCl, DMAP, pyridine 52%; (2) t-BuOK, $(EtO)_2P(O)CH_2CO_2Et$, THF, 68%; (b) (1) i-Pr$_2$NEt, MOMCl, 96%; (2) LAH, THF, 96%; (3) TsCl, pyridine, 72%; (c) (1) 2-amino-6-chloropurine, t-BuOK, DMSO, 73%; (2) n-Bu$_3$SnCH=CH$_2$, Pd(PPh$_3$)$_4$, dioxane, 65%; (d) (1) MeSNa, CH$_3$CN, 73%; (2) PhOCH$_2$COCl, 1-HBT, CH$_3$CN, pyridine, 89%; (3) BF$_3$–Et$_2$O, Me$_2$S, 63%, (4) DMTrCl, pyridine, 91%; (5) i-Pr$_2$NEt, CH$_2$Cl$_2$, i-Pr$_2$NP(Cl)OC$_2$H$_4$CN, 97%; (e) (1) synthesis with an automated DNA synthesizer; (2) 28% NH$_3$, 20–30%, (3) 10% CH$_3$COOH; (f) (1) 3 equiv MMPP pH 10; (2) 0.5 M NaOH.

derivative (**5**). After protection of the vinyl group with methylsulfide and the 2-amino group with phenoxyacetyl, the conventional procedure produced the phosphoramidite precursor (**6**) in good yield. The sulfide-protected ODN (**7**) was obtained by applying **6** to an automated DNA synthesizer in good yield after purification with RP-HPLC. The ODN (**7**) was then smoothly converted to **8** by oxidation with magnesium monoperphthalate (MMPP) following elimination under an alkaline condition. The structure of ODN was confirmed by MALDI-TOF mass measurements.

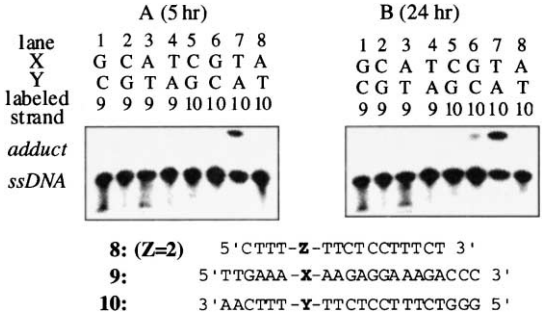


Figure 2. Cross-linking selectivity with the TFO **8**: (A) after 5 h, (B) after 24 h. The reaction was done using 10 μM of ODNs (**8**: Z=**2**), 1 μM of target duplex **9**–**10** in a buffer including 10 mM cacodylate, 0.25 mM spermine, 100 mM NaCl, pH 4.5±0.3 at 30 °C. The reaction mixture contained either 5′-^{32}P-labeled **9** or **10** as a tracer. The reaction was stopped by the addition of formamide after 20 h, and analyzed by gel electrophoresis with 15% denaturing polyacrylamide gel.

The cross-linking was investigated with the functionalized ODNs (**8**) and the target duplexes (**9**–**10**). In order to define the strand of the reaction, one of the two strands (**9** or **10**) labeled with ^{32}P at the 5′-end was used as a tracer. The results were analyzed by gel electrophoresis with 15% denaturing gel, in which the cross-linked products were identified as less-mobility bands relative to the unreacted radiolabeled single strand (Fig. 2). The less-mobility bands were observed in lane 7 (Fig. 2A and B), clearly indicating that the ODN (**8**) bearing **2** reacted only with the adenine within the pyrimidine strand. The product yield was estimated by quantification of each band to be ca. 50% after 24 h. Intermediary formation of the triplex has been supported by the facts that the reaction did not take place in the presence of the competitive TFO **8** (Z=G instead of **2**) or in the absence of **9**. Thus, high selectivity of **8** to the adenine in

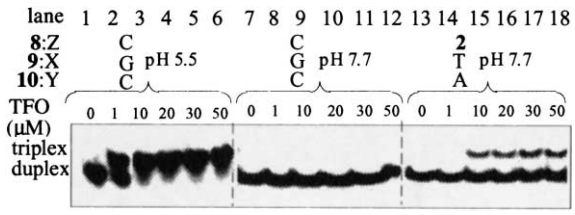


Figure 3. Gel-shift analysis of triplexes with 15% non-denaturing polyacrylamide gel at pH 5.5 and 7.7. The triplex formation was performed at pH 4.5 for 24 h under the same condition as described in Figure 2, and was analyzed by gel electrophoresis at indicated pH.

the pyrimidine strand at the TA base pair of the triplex has been proven.

We next examined the effect of cross-link to the TA interrupting site on the stability of the triplex by gel-shift assay with non-denaturing gel (Fig. 3). The triplexes containing C-GC base triplet were observed at the concentrations higher than 1 μM of the TFO at pH 5.5 (lanes 2–6), whereas they dissociated at pH 7.7 (lanes 7–12). The less-mobility bands in lanes 15–18 showed the same gel-shift with the triplexes of lanes 2–6, suggesting that the triplex is stabilized by the interstrand cross-link between **8** and **10**.[17] In the investigation with the monomer derivative of the vinyl compound (**5**) in organic solvents, no adduct was formed with the adenosine derivative.[18] The cross-linking reaction with **2** and adenine may be effected by close proximity within the triplex as expected.

In conclusion, a highly selective cross-linking reaction to the adenine at the TA interrupting site within the triplex has been achieved with the use of the TFO incorporating the new cross-linking agent **2**. It should be noted that only a minor change from a butyl to an ethyl spacer caused a drastic change in base selectivity, and the validity of the design concept has been confirmed. The new cross-liking reagent **2** is the first example toward the TA interrupting site within the triplex. We have recently reported that the ethylsulfoxide substitution on the vinyl group of the 2-amino-6-vinylpurine skeleton improved its reactivity to a greater extent at pH 7.[13d] Such modification of **2** would effect more efficient reactivity within the triplex at pH 7, and produce a useful cross-linking agent for application to site-directed modification of an adenine within a selected target.

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