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A New Reactive Nucleoside Analogue for Highly Reactive and Selective Cross-Linking Reaction to Cytidine under Neutral Conditions

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Abstract—We have already demonstrated that the oligonucleotides DNA (ODNs) bearing a 2-amino-6-vinylpurine derivative (**1**) exhibited efficient interstrand cross-linking to cytidine selectively. In this study, a new reactive nucleoside analogue, 2-amino-6-(1-ethylsulfoxy)vinylpurine derivative (**7**), was designed based on a computational method to achieve high and selective alkylation with cytidine under neutral conditions. It has been demonstrated that the ODN (**13**) bearing 2-amino-6-(1-ethylsulfoxy)vinylpurine achieved highly selective and efficient cross-linking to cytidine under neutral conditions.

The cross-linking reaction within complementary duplexes and triplexes has been expected as a reliable strategy to ensure inhibition of gene expression in the antisense[1–7] and antigene method.[8–11] The strategy of cross-linking is based on the use of oligodeoxynucleotides (ODNs) incorporating a reactive functional, and there are a number of reports on alkylating agents such as psolaren,[7,10] haloacetyl amide[4,5,9] or aziridine[1,3,8] units, etc. Recently, the cross-linking reaction has attracted new attention as a tool for site-directed chemical reaction to nucleobases of DNA or RNA.[12] However, the existing alkylating agents do not seem to be satisfactory for general application, and there is an urgent need of new alkylating agents for application in either in vitro or in vivo study. Thus, we have established a new principle in the designing of cross-linking agents, and developed a new nucloside analogue 2-amino-6-vinylpurine derivative (**1**) that exhibited efficient and selective reactivity toward the cytidine at the target site.[13,14] The principle demonstrated with 2-amino-6-vinylpurine derivatives is characteristic in that high reactivity is exhibited by the proximity effect within the DNA hybrids without incurring chemical instability of the functional nucleoside analogue. The reactivity of **1** is also unique in that a weak nucleophilic amino group of cytidine can be selectively alkylated. High reactivity of 2-amino-6-vinylpurine skeleton to a cytosine has been successfully applied to triplex-oriented cross-linking.[15] However, application of **1** to a living system was arrested because of its need of acidic conditions. Here, we wish to report that a new derivative of 2-amino-6-vinylpurine with a double-activation structure exhibited dramatic improvement in the yield compared to that with **1**, under neutral conditions.

Previously, theoretical assumption of the reactivity between 9-methyl-6-(1-substituted vinyl)-2-aminopurine and 1-methyl-cytosine showed good agreement with the experimental data. That is, protonation to N^1 of the purine base is essential for alkylation of the 4-amino group of cytosine, and methyl or TMS substitution at the 2-position of the vinyl group decreased the reactivity.[13] We expected that introduction of an electron withdrawing group to the vinyl group would increase the reactivity. Estimation by a semi-empirical MO calculation [MOPAC (PM3)] suggested that the introduction of a methylsulfoxy or methylsulfonyl group to the vinyl group decreased the activation energy compared to the non-substituted one (Table 1, R = SOMe, SO_2Me vs R = H). Thus, the sulfoxy- or sulfonylvinyl derivatives (**2** or **4**) were expected to achieve efficient reaction even when the concentration of the N^1-protonated form of the purine analogue is low under neutral conditions. The active species (**7**) would be obtained by either chemical reaction or in situ activation within the duplex from the stable precursor (**5** or **6**) (Fig. 1).

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The synthesis of ODNs incorporating a reactive nucleoside analogue is summarized in Scheme 1. 2-Amino-6-vinylpurine (**8**) was synthesized from 2′-deoxyguanosine as described previously,[13] The vinyl group of **8** was tranformed into the dibromide, followed by displacement with ethanethiol to produce a bisethylsulfide derivative (**9**). The 2-amino group of **9** was protected with the phenoxyacetyl group,[16] followed by deprotection of the TBDMS group. The phosphoramidite precursor (**10**) was obtained in good yield by the conventional method,[17] and applied to an automated DNA synthesizer. The ODN (5′-CTTT-**X**-TTCTCCTTTCT) was cleaved from the support with 0.1 M NaOH,[18] and the solution was neutralized with CH_3COOH instantly, followed by purification by reverse-phase HPLC to give DMTr-protected ODN in good yields. The purified oligomers were treated with 10% CH_3COOH to give the deprotected ODN (**11**). Oxidation of **11** with MMPP gave a mixture of several compounds. Interestingly, mild alkaline treatment of **11** produced the ethylthiovinyl-bearing ODN (**12**) in good yield. The ODN (**12**) was treated with two equivalents of MMPP to give the corresponding ethanesulfoxyvinyl derivative (**13**). By the treatment of a large excess MMPP for 24 h, the ethanesulfonylvinyl-bearing ODN (**14**) was obtained. The structures of all ODNs were determined by MALDI-TOF measurements. The synthesis of **15** was carried out according to the reported procedure.[14]

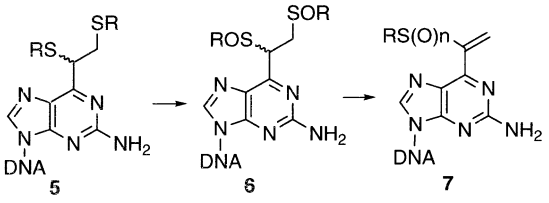
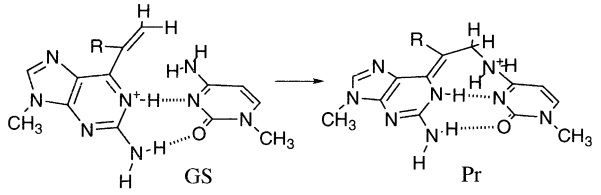


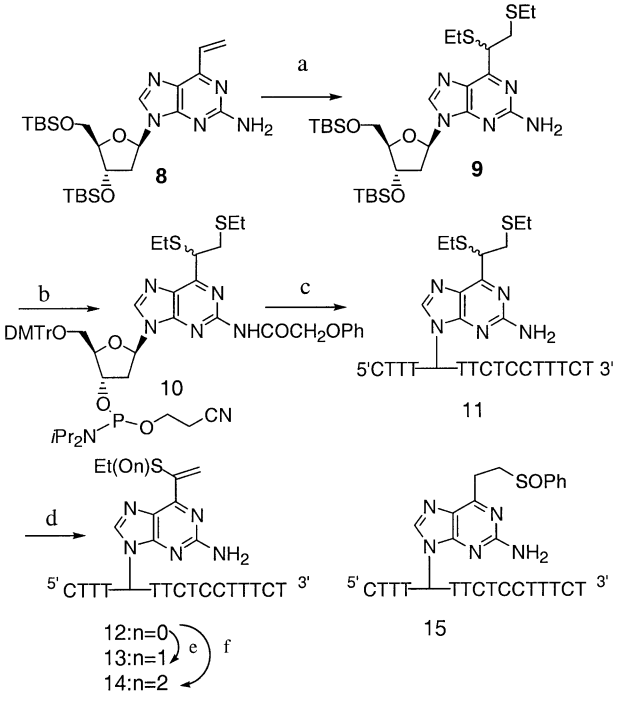
Figure 1. In situ activation to sulfonylvinyl derivative (**7**).

Table 1. Estimated heat of formation (kcal/mol[a])



Compd	R	GS	TS	ΔG	Pr
1	H	185.7	208.7	23.0	205.4
2	SOMe	159.2	177.7	18.5	174.5
3	SMe	186.3	210.4	24.1	189.3
4	SO_2Me	123.0	141.70	18.7	140.1

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



Scheme 1. (a) (1) Br_2 aq, $CHCl_3$, 0 °C, 2 h, 65%; (2) DBU, $CHCl_3$, 0 °C, 1 h, then EtSH, 83%, (b) (1) $PhOCH_2COCl$ 1-hydroxy-benzotriazole, pyridine–CH_3CN, 95%; (2) nBu_4NF, 73%; (3) DMTrCl, pyridine, 86%; (4) iPr_2EtN, CH_2Cl_2, $iPr_2NP(Cl)OCH_2CH_2CN$, 44%; (c) (1) synthesis with an automated DNA synthesizer; (2) 0.1 N NaOH; (3) neutralized with CH_3COOH, 20–30%; (3) 10% CH_3COOH; (d) 0.5 M NaOH, 24 h; (e) 2 equiv MMPP (magesium monoperphthalate), pH 10, 30 min, quant; (f) 6 equiv MMPP, pH 10, 1 day, quant.

The cross-linking was investigated under neutral conditions with the functionalized ODNs (**11**–**14** and **15**) and the target ODN (**16**) in the presence of ^{32}P-labeled **16** as a tracer, and analyzed by gel electrophoresis with 19% denaturing gel. The formation of a higher molecular weight band is indicative of the cross-linked adduct, and the yield of the adduct can be obtained by quantification of both the lower and higher bands. The reactivities of the four functionalized ODNs (**12**–**14** and **15**) toward the ODN bearing cytidine at the target site (**16**, N = C) are compared in Fig. 2. The ODN (**11**) did not give the adduct at all, and the reactivity of ODN (**12**) incorpor-

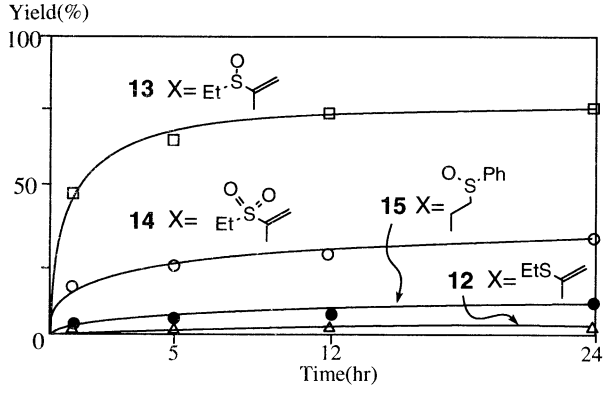
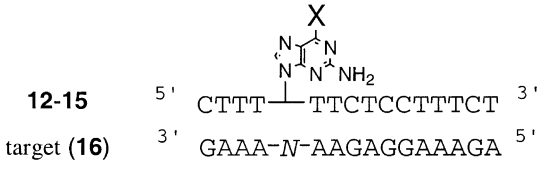


Figure 2. Comparison of the cross-linking reactivity. Cross-linking was done using using 10 μM reactive ODN (**12**–**15**), 1 μM target oligomer (**16**) in the buffer containing 100 mM NaCl, 50 mM MES, pH 7.0, 37 °C, and analyzed by electrophoresis with 19% denaturing polyacrylamide gel at the indicated time. Yields were determined by quantification of the bands on the gels by BAS 2500 using the imaging plate.



ating the ethylthiovinyl nucleoside is negligible. In contrast, the ODN (**13**) incorporating the ethylsulfoxyvinyl nucleoside exhibited the most efficient reaction, and the yield was as high as 50% at 1 h and reached 80% after 12 h. Although the phenylsulfoxide analogue (**15**) produced the adduct in over 80% yield under acidic conditions, only slow reaction was observed under the neutral condition. Thus, it has been proved that the new ethylsulfoxyvinyl nucleoside may be applicable under physiological conditions. The ODN (**14**) with an ethylsulfonylvinyl analogue gave the adduct in a lower yield than the ODN (**13**), which differs from the theoretical assumption that has suggested similar ΔG values for both the methylsulfoxy- and methylsulfonylvinyl (Table 1). As the primary product after the addition reaction is only slightly more stable than the transition state in the reaction with the sulfonylvinyl compound, a reverse reaction to the starting structure might impede the promotion to adduct formation.

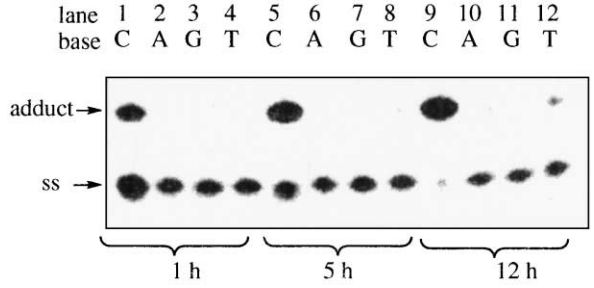


Figure 3. Base selectivity of the cross-linking. Reaction conditions are recorded in the caption of Figure 2.

Figure 3 illustrates high cytidine selectivity of the ODN (**13**) bearing the ethylsulfonylvinyl nucleoside in the reaction with the target ODN (**16**, N = C, G, A, or T). In all cases, adduct formation was observed only to the ODN (**16**) with C at the target site.

Finally, we have successfully demonstrated that 2-amino-6-(1-ethylsulfoxyvinyl)purine exhibited selective and efficient cross-linking to a cytidine at the target site under neutral conditions. As this new reactive motif is stable under the reaction conditions, 2-amino-6-(1-ethylsulfoxyvinyl)purine will be useful in the antisense strategy as well as for site-directed chemical modification of a cytidine within a selected target. Application of the 2-amino-6-(1-ethylsulfoxyvinyl)purine motif to TFOs for triplex cross-linking is now under study.

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