Highly Efficient and Selective Cross-Linking to Cytidine Based on a New Strategy for Auto-Activation within a Duplex

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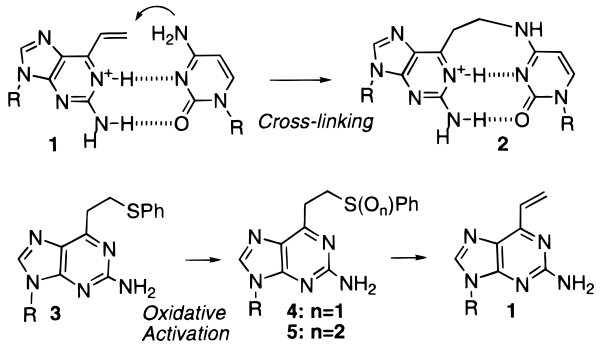
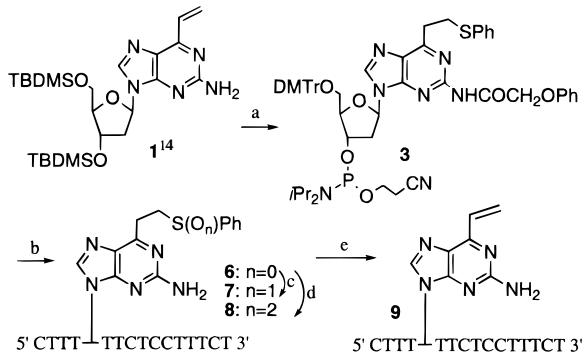
Sequence-selective cross-linking between complementary duplexes or triplexes provides an important method for improving the stability of the hybridized complexes.[1−11] This strategy has been expected to effect inhibition of gene expression in the antisense and antigene methods. The basic concept of cross-linking includes the incorporation of a reactive functional group into the oligomers, and there are a number of reports on alkylating groups, such as haloacetyl amide,[4,5,9] aziridine,[1,3,8] and psolaren derivatives.[7,10] Recently, interest in cross-linking has been expanding as a tool for site-directed chemical modification which may induce point mutation of a genetic code.[12] However, the existing aklylating agents do not seem to be satisfactory for general application, and there is an urgent requirement for new cross-linking agents that exhibit efficient and selective reactivity toward a chosen target site. The use of functional groups with intrinsic high reactivity may cause considerable instability, while those requiring ultraviolet activation may limit the target site. In our approach, we have developed a new strategy for cross-linking in which a reactive species is auto-generated within a duplex. Here we wish to report a highly efficient and selective cross-linking reaction toward cytidine with the use of derivatives of a 2-amino-6-vinylpurine nucleoside (**1**).



Scheme 1[a]



[a] (a) (1) PhSH, CH_2Cl_2 rt, 1 h, 74%, (2) $PhOCH_2COCl$, 1-hydroxybenzotriazole, 78%, (3) nBu_4NF, 76%, (4) DMTrCl, pyridine, 75%, (5) iPr_2EtN, CH_2Cl_2, $iPr_2NP(Cl)OCH_2CH_2CN$, 64%. (b) (1) synthesis with an automated DNA synthesizer, (2) 0.1 N NaOH, (3) neutralized with CH_3COOH, 70−80%, (3) 10% CH_3COOH. (c) 2 equiv MMPP (magnesium monoperphthalate), pH 10, 30 min, quantitative. (d) 10 equiv MMPP, pH 10, 1 d, quantitative, (e) 470 mM NaOH, 30 min, quantitative.

We have previously designed a 2-amino-6-vinylpurine nucleoside (**1**) as a selective alkylating agent to cytidine. The complex resembling a natural G−C pair would favor the attack of the 4-amino group of cytidine, a weak nucleophile, leading to a cross-linked product (**2**).[13] Model experiments in organic solvents and investigations with ODNs bearing **1** have demonstrated that **1** is a cytidine-selective alkylating agent.[14] To achieve high reactivity without incurring chemical stability of **1**, we next designed a new strategy in which less reactive precursors (**3**, **4**, or **5**) would be auto-activated within duplexes to generate **1**.

The 2′-deoxy nucleoside derivative of 2-amino-6-vinylpurine (**1**) was synthesized from 2′-deoxyguanosine as described previously,[14] protected as phenylsulfide, and then transformed to the amidite precursor (**3**). An example of the synthesis of functionalized ODNs incorporating a 6-substituted 2-aminopurine nucleoside is illustrated in Scheme 1. The sulfide functional group of **6** was converted to the sulfoxide form (**7**) as a sole product by oxidation with 2 equiv of magnesium monoperphthalate (MMPP). The transformation of **6** to the sulfone form (**8**) was also a quantitative reaction with an excess of MMPP. Both **7** and **8** were smoothly transformed to **9**, incorporating the 2-amino-6-vinylpurine nucleoside under mild alkaline conditions. The structures of the ODNs (**6**−**9**) were confirmed by UV and MALDI-TOF mass measurements.[15]

The cross-linking was investigated with the functionalized ODNs (**6**−**9**) and the target ODN (**10**) in the presence of ^{32}P-labeled **10** as a tracer and analyzed by gel electrophoresis with

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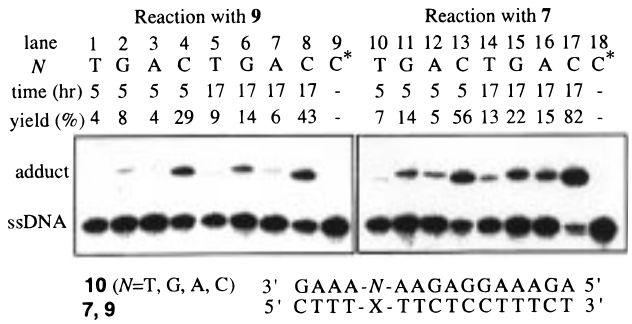

Figure 1. Comparison of the base selectivity. Lanes 1−9, the reaction with **9**; lanes 10−18, the reaction with **7**. The reaction was done using 7 μM ODNs (**7** or **9**), 3 μM target oligomer including **10** labeled with ^{32}P at 5′ end as a tracer in 0.1 M NaCl, 50 mM MOPS, pH 5.0, 33 °C. (*) Control in the absence **9** or **7**.

20% denaturing gel.[16] All of the functionalized ODNs produced a higher molecular weight band, indicating the formation of a cross-linked adduct. Figure 1 illustrates the reactivity of the ODNs (**7** and **9**) toward the different target site (**10**, N = C, G, A, or T). The highest yields to cytidine (lanes 4 and 8) were obtained with the ODN **9** incorporating the 2-amino-6-vinylpurine nucleoside, clearly demonstrating cytidine selectivity. The reactivity is decreased in the order of C > G > A ≫ T, which is in good agreement with the result obtained in an organic solvent.[14d] It should be noted that **7** incorporating the sulfoxide-substituted nucleoside underwent the reaction more efficiently than **9** while retaining similar cytidine selectivity (lanes 4, 8 vs 13, 17).

The reactivities of the four functionalized ODNs (**6**−**9**) toward the ODN bearing cytidine at the target site (**10**, N = C) are compared in Figure 2. The yields of the adduct with **9** were almost steady between ca. 40 and 50% after 5 h. In contrast, **7** produced the adduct in over 80% yield after 12 h, demonstrating the remarkably efficient cross-linking reaction. The ODN (**8**) incorporating the sulfone-substituted nucleoside gave the adduct in a somewhat slower reaction rate than **9**. It is interesting that the yield with the ODN (**6**) incorporating the sulfide-substituted nucleoside gradually increased and reached ca. 15% after 36 h.

Neither the monomers of the sulfide-substituted (**3**), the sulfoxide-substituted (**4**), nor the sulfone-substituted nucleoside (**5**) formed adducts with cytidine in the model experiment in organic solvents. Furthermore, the functionalized ODNs (**6**−**8**) were stable and were almost unchanged in the buffer. These facts have indicated that the vinyl group might be generated within the duplex in the proximity of the target cytidine and followed by the cross-linking. It turned out that such activation has been

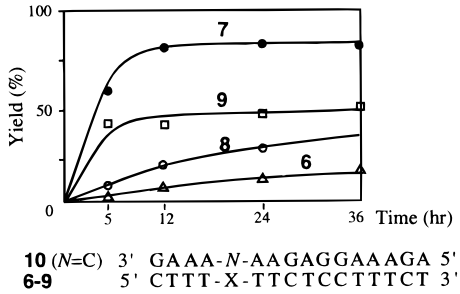

Figure 2. Comparison of the cross-linking reactivity. The reaction was done under the same condition as described in Figure 1, and stopped by adding formamide at the indicated time. Yields were determined by quantitating the bands on the gels by BAS2500 using the imaging plate. The yield of each reaction is indicated with the following marks: **6**: △, **7**: ●, **8**: ○, **9**: □.

most effectively achieved within the ODN (**7**) incorporating the sulfoxide-substituted nucleoside. The vinyl functional group of **9** might suffer an undesired nucleophilic reaction, thereby causing lower yield. Although actual activation mechanisms of the functional ODNs (**7**−**9**) for cross-linking reaction are uncertain, the present results have clearly supported the validity of the new strategy.

In a preparative reaction using equimolar amounts of **7** and **10** (N = C), the two peaks were transformed to a single peak in an almost quantitative yield after 24 h at room temperature. The isolated peak was proved to be the cross-linked product by the MALDI-TOF mass measurement (calculated, 9768.76; found, 9770.97).[17] Further proof of cross-linking at cytidine was evidenced by observing peaks corresponding to the authentic adduct (**2**) in addition to A, G, T, and C in an expected ratio in the HPLC analysis after the hydrolysis with snake venom phophodiestrease and alkaline phosphatase (*Escherichia coli* C75). The cross-linked adduct between **9** and **10** was also confirmed by the same method.

In conclusion, we have successfully demonstrated the validity of the new strategy by achieving a highly efficient and selective cross-linking reaction toward cytidine without incurring chemical instability by using the derivatives of a 2-amino-6-vinylpurine nucleoside (**1**). To our knowledge, the sulfoxide-substituted nucleoside **4** is the best alkylating agent toward cytidine so far reported from the viewpoints of reactivity and selectivity as well chemical stability. The new cross-linking motifs based on **1** will be generally useful in the antisense strategy as well as for site-directed chemical modification of a cytidine within a selected target. Further work is now ongoing in the search for sulfide structures which are more susceptible to the activation within the duplex.

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(15) Retention times of oligomers (HPLC conditions: nacalai tesque COSMOSIL 5C18-MS; buffer A: 0.1 M TEAA, B: CH_3CN. B: 10% to 30%/20 min, linear gradient; flow rate, 1 mL/min) and MALDI-TOF MS (negative mode) of the ODNs were as follows: **6** at 17.0 min, MS m/z 4875.15 (M^{-1}, calcd 4873.84), **7** at 14.0 min, MS m/z 4890.95 (M^{-1}, calcd 4889.83), **8** at 14.3 min, MS m/z 4904.17 (M^{-1}, calcd 4905.83), **9** at 13.0 min, MS m/z 4761.10 (M^{-1}, calcd 4763.82).

(16) Melting temperature of the duplex between **6** and **10** (N = C) at 0.2 μM is 35 °C, indicating the majority of the ODNs in the duplex form under the reaction conditions. The reactions proceeded under physiological conditions at pH 6.8 at a somewhat slower rate than at pH 5.0.

(17) Retention times in the HPLC[15] of **7**, **10**, and the adduct were as follows: **7** at 14.0 min, **10** at 9 min, and the adduct at 13 min.