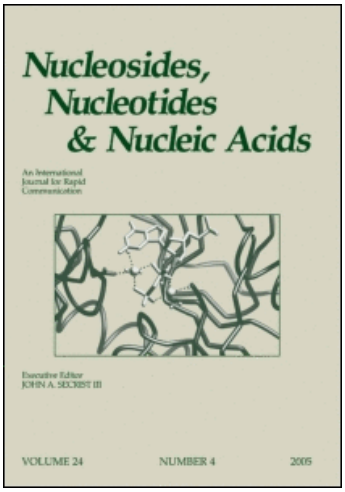

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Online publication date: 31 March 2001

To cite this Article Nagatsugi, Fumi , Kawasaki, Takeshi , Tokuda, Natsuko , Maeda, Minoru and Sasaki, Shigeki(2001) 'SITE-DIRECTED ALKYLATION TO CYTIDINE WITHIN DUPLEX BY THE OLIGONUCLEOTIDES CONTAINING FUNCTIONAL NUCLEOBASES', Nucleosides, Nucleotides and Nucleic Acids, 20: 4, 915 — 919
To link to this Article: DOI: 10.1081/NCN-100002458
URL: http://dx.doi.org/10.1081/NCN-100002458

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 20(4–7), 915–919 (2001)

SITE-DIRECTED ALKYLATION TO CYTIDINE WITHIN DUPLEX BY THE OLIGONUCLEOTIDES CONTAINING FUNCTIONAL NUCLEOBASES

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ABSTRACT

We have previously described that oligonucleotides (ODN) containing phenylsulfoxide derivative of 2-amino-6-vinylpurine nucleoside analog (**1**) are activated within duplex to form cross-link toward cytidine selectively at the target site. In this paper, we wish to report the search for more stable precursor susceptible for activation within duplex.

The cross-linking reaction between complementary duplexes or triplexes has been expected to enhance the inhibition of gene expression in the antisense (1) and antigene methods (2). Recently, this method has attracted new interest as a tool for site-directed chemical modification which may induce point mutation of a genetic code (3). However, the existing alkylating agents still need further improvement for application in either *in vitro* or *in vivo* study. For such applications, the efficiency and selectivity of reaction to the target site are necessary under the physiological condition. A dilemma is that both high reactivity and stability are required for cross-linking agents. To overcome this problem, we have designed a new strategy, in which reactive species may be auto-activated within duplex from its stable precursor.

We have previously reported that 2-amino-6-vinylpurine nucleotide (**1**) exhibited efficient and selective cross-linking to cytidine within duplex (4). Further remarkable point of **1** is that the alkylation activity can be auto-generated within

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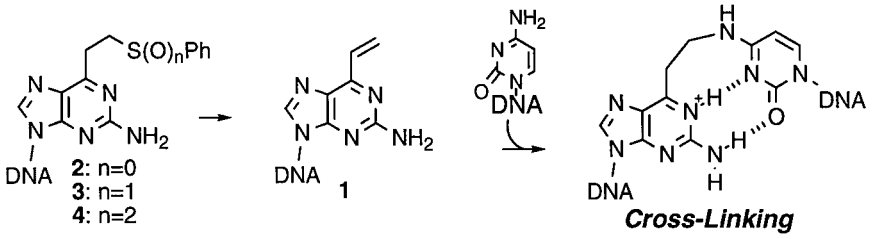
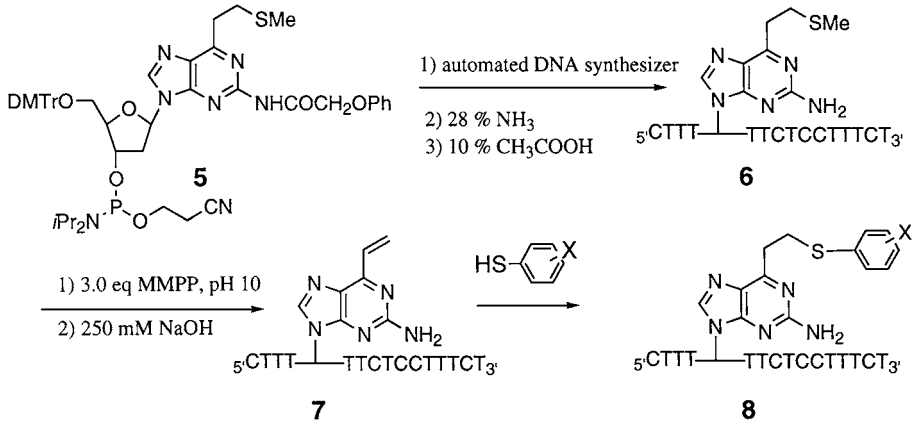


Scheme 1. The synchronous activation within duplex.

duplex from its stable precursors, phenylsulfide or phenylsulfoxide derivatives (5). Even the most stable phenylsulfide precursor gradually produced cross-linked adducts. This finding lead us to the search for a proper sulfide structure to be more efficiently activated within duplex. In this paper, we wish to report the search for more stable precursor susceptible for activation within duplex, based on the structure of substituted phenylsulfide derivatives.

We investigated cross-linking reaction with the use of functional ODNs having various sulfide structures. Syntheses of the ODNs incorporating substituted phenylsulfide derivatives are summarized in Scheme 2.

The phosphoramidite precursor of methylsulfide derivative was synthesized as described previously (6b) and applied to an automated DNA synthesizer. Synthesized ODN was obtained in a satisfactory yield and purified by HPLC. The sulfide-protected ODN was smoothly converted to vinyl-bearing ODN by oxidation with magnesium monoperphthalate (MMPP) following an alkaline condition (6). A series of substituted phenylsulfide containing ODNs were obtained by the addition of corresponding thiophenol derivatives to vinyl-bearing ODN **7**. The structures of the ODNs were confirmed by MALDI-TOF mass measurements.



Scheme 2. The synthesis of phenylsulfide-bearing ODNs.

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Table 1. Relationship Between the Substituent and Cross-Linking Yield

Run	X	*Yield (%)
1	4-NO_2	9
2	4-Br	14
3	4-H	25
4	4-OH	30
5	4-NH_2	44
6	2,4-Me_2	28
7	3,4-$(MeO)_2$	37
8	2,4,6-Me_3	42
9	2-COO^-	60

*) Reaction time was 24 hr. Yield was determined by gel electrophorsis. Reaction was done 7 μM ODNs (**8**), 3 μM target ODN (**9**) including labeld with ^{32}P at 5′ end as a tracer in 0.1 M NaCl, 50 mM MOPS, pH 5.0, 30°C.
8: $^{5'}$ CTTT-**G**-TTCTCCTTTCT$^{3'}$.
9: $^{3'}$ GAAA-**C**-AAGAGGAAAGA5′.
*G = 2-amino-6-substituted purine.

Interstrand cross-linking reaction between the ODNs **8** and the target DNA labeled at the 5′ end with ^{32}P (**9**, 5′AGAAAGAGAACAAAG) was done at 30°C, and analyzed by gel electrophoresis (20% denaturing gel). Table 1 has shown the yields of adduct using several phenylsulfide bearing-ODNs after 24 hours.

Introduction of electron-withdrawing group on the phenylsulfide decreased the yield of adduct, whereas the yield was increased by introduction of electron-donating group. It is interesting to note that the 2-carboxyphenylsulfide derivative produces the adduct in a similar yield with phenylsulfoxide derivative. In addition, we found that the cross-linking yields linearly correlated to the HOMO energy of sulfide bond, suggesting that the phenylsulfide derivatives might be activated within duplex by some chemical reaction relating to the sulfide HOMO (Fig.1). Next, we have investigated about some mechanistic studies of this cross-linking reaction with the ODNs having phenylsulfoxide or 2-carboxyphenylsulfide derivative. The cross-linking yield with ODN was not affected in the absence or presence of O_2, but increased in the presence of H_2O_2. On the other hand, the cross-linking yield with all sulfoxide derivatives was not affected by H_2O_2. These results have suggested that the oxidative activation is the rate-determining step for this cross-linking reaction, but that O_2 is not the oxidant. In addition, reagents, such as glutathione, that may affect electron-transfer, inhibited the cross-linking reaction using **2**-carboxy-sulfide bearing ODN (**8**, X = 2-COO^-). Probably, we suspected that the oxidative activation of sulfide ODNs might include electron transfer mechanism within the duplex. Further investigation is a currently under way in the elucidation of the synchronous-activation mechanism.

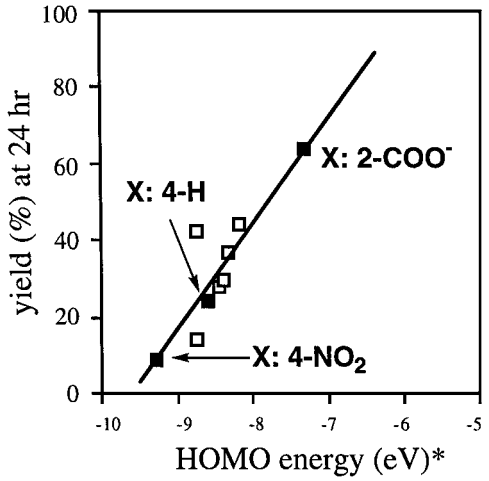

Figure 1. Relationship between the HOMO energy of the sulfide bond and the yield of the cross-linking. *Calculated by MOPAC (PM3).

In conclusion, we have successfully demonstrated more highly efficient cross-linking toward cytidine by functional nucleobases capable of activation within a duplex. In particular, the 2-carboxy-phenylsulfide bearing-ODN (**8**, X = 2-COO^-) produced cross-linking product in high yield without any chemical activation process within duplex. The sulfide-bearing ODNs are very stable and have inducible high reactivity only to form the complex with the target DNA. These facts suggested that these ODNs might apply to use living system. Furthermore, applications to triplex formation and to the antisense method are now ongoing.

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