ORGANIC LETTERS

1999
Vol. 1, No. 2
311−314

Site-Specific Cross-Linking of Nucleic Acids Using the Abasic Site

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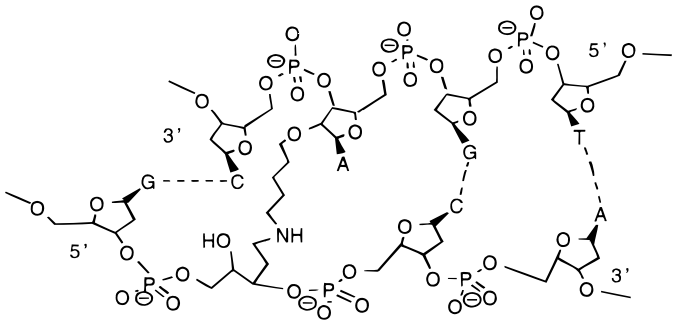
Received April 28, 1999

ABSTRACT



An efficient site-specific cross-linking reaction between two carbohydrate residues present in two complementary DNA sequences is described. One oligodeoxynucleotide, 5′d(GGCTGA*CTGCG)3′, carries an amino nucleophile tethered to the 2′-hydroxyl of an adenosine residue (A*). The target electrophile is an abasic site generated in the complementary sequence, 5′d(CGCAGDCAGCC)3′ (D represents the deoxyribose). The cross-linking reaction was carried out by a reductive amination reaction in >95% yield.

Covalent cross-linking of oligonucleotides can be classified into two categories: In the first type, one of the strands carries a reactive group which on identifying the target strand cross-links to inactivate the complementary sequence and hence the gene expression.[1] The second type of compounds involves synthesis of preformed duplexes,[2] linear linkages,[3] and circular linkages.[4] These cross-link-stabilized duplexes could be avid competitive inhibitors of nucleic acid binding proteins and thus provide an attractive way to design new therapeutics ("aptamers" [5]) targeted against viral and cellular proteins. They could also be used for isolation and purification of nucleic acid binding proteins by affinity chromatography. Such preformed cross-linked duplexes could target single-stranded mRNA via triplex formation ("antigene" [6]) and thus offer another way of developing novel therapeutic

(1) Cross-linking with concomitant alkylation: (a) Grineva, N. I.; Karpova, G. G. *FEBS Lett.* **1973**, *32*, 351. (b) Summerton, J.; Bartlett, P. A. *J. Mol. Biol.* **1978**, *122*, 145. (c) Webb, T. R.; Matteucci, M. D. *J. Am. Chem. Soc.* **1986**, *108*, 2764. (d) Cowart, M.; Gibson, K. J.; Allen, D. J.; Benkovic, S. J. *Biochemistry* **1989**, *28*, 1975. (e) Cowart, M.; Benkovic, S. J. *Biochemistry* **1991**, *30*, 788. (f) Cosstick, R.; Douglas, M. E. *J. Chem. Soc., Perkin Trans. 1* **1991**, 1035. (g) Weidner, M. F.; Millard, J. T.; Hopkins, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 9270. (h) Meyer,R. B., Jr.; Tabone, J. C.; Hurst, G. D.; Smith, T. M.; Gamper, H. *J. Am. Chem. Soc.* **1989**, *111*, 8517. (i) Povsic, T. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 9428. (j) Kido, K.; Inoue, H.; Ohtsuka, E. *Nucleic Acids Res.* **1992**, *20*, 1339. (k) Chatterjee, M.; Rokita, S. E. *J. Am. Chem. Soc.* **1990**, *112*, 6397. (l) Li, T.; Rokita, S. E. *J. Am. Chem. Soc.* **1991**, *113*, 7771. (m) Chatterjee, M.; Rokita, S. E. *J. Am. Chem. Soc.* **1991**, *113*, 5116. (n) Kirchner, J. J.; Hopkins, P. B. *J. Am. Chem. Soc.* **1991**, *113*, 4681. (o) Armstrong, R. W.; Salvati, M. E.; Nguyen, M. *J. Am. Chem. Soc.* **1992**, *114*, 3144. (p) Nagatsugi, F.; Uemura, K.; Nakashima, S.; Maeda, M.; Sasaki, S. *Tetrahedron* **1997**, *53*, 3035.

(2) Preformed duplexes: (a) Devadas, B.; Leonard, N. J. *J. Am. Chem. Soc.* **1990**, *112*, 3125. (b) Ferentz, A. E.; Keating, T. A.; Verdine, G. L. *J. Am. Chem. Soc.* **1993**, *115*, 9006 and references cited. (c) Osborne, S. E.; Voelker, J.; Stevens, S. Y.; Breslauer, K. J.; Glick, G. D. *J. Am. Chem. Soc.* **1996**, *118*, 11993 and references cited. (d) Huang, H.; Hopkins, P. B. *J. Am. Chem. Soc.* **1993**, *115*, 9402.

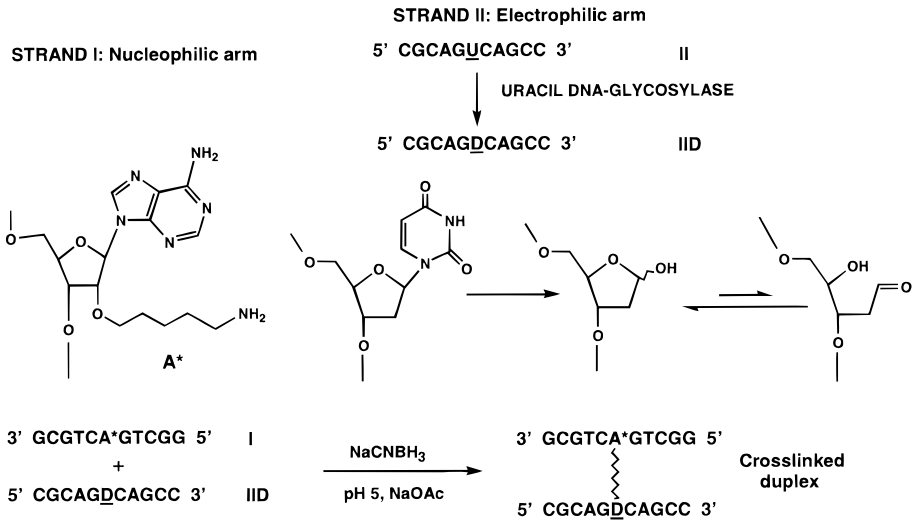
(3) Linearly linked oligonucleotides: (a) Richardson, P. L.; Schepartz, A. *J. Am. Chem. Soc.* **1991**, *113*, 5109. (b) Horne, D. A.; Dervan, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 2435. (c) Froehler, B. C.; Terhorst, T.; Shaw, J.-P.; McCurdy, S. N. *Biochemistry* **1992**, *31*, 1603. (d) Ono, A.; Chen, C.-N.; Kan, L.-S. *Biochemistry* **1991**, *30*, 9914.

(4) Circular oligonucleotides: Kool, E. T. *Annu. Rev. Biophys. Biomol. Struct.* **1996**, *25*, 1 and references cited.

(5) Nucleic acid interactions involving non-nucleic acid receptors: Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermaas, E. H.; Toole, J. J. *Nature* **1992**, *355*, 564.

10.1021/ol9906209 CCC: $18.00
Published on Web 06/05/1999

Scheme 1



agents. Finally, cross-linking the duplexes at specified sites might stabilize thermodynamically unfavorable, yet biologically relevant, intermediate structures.

We report herein an efficient site-specific cross-linking reaction of the second type between two complementary DNA sequences. This cross-linking reaction involves an amino nuclephile tethered from the 2′-position[7] and an electrophile at the 1′-position of an abasic site. The abasic site was conveniently generated by the use of uracil-DNA glycosylase that has been cloned and overexpressed[8] on a uracil-containing oligodeoxynucleotide.

The cross-linking reaction (Scheme 1) was performed by introducing a nucleophilic arm at the 2′-position in the middle of strand I 5′d(GGCTGA*CTGCG)3′ using a 2′-*O*-pentylamino linker. This was achieved by the introduction of the 2′-*O*-pentylamino side chain by a direct alkylation of adenosine and utilizing the resultant phosphoramidite.[7] In the complementary strand, 5′d(CGCAGUCAGCC)3′, a single deoxyuridine moiety was placed in the middle of the sequence. These two strands form a normal duplex with a B form geometry.[9] Upon treatment with uracil-DNA glycosylase, an abasic site is generated from the single strand II to give the oligonucleotide IID, 5′d(CGCAGDCAGCC)3′ where D stands for deoxyribose. This glycosylase enzyme removes uracil from DNA strands by hydrolytic cleavage of the uracil-bearing glycosidic bond that produces an aldehydo-abasic site. Repair at the lesion is affected by the subsequent action of other endonucleases. The aldehydo-abasic site exists predominantly as a mixture of α- and β-hemiacetals[10] with a small population of open aldehyde form (Scheme 1).

When strand I and strand IID were mixed in equimolar proportion under reductive amination conditions (pH 5, NaOAc buffer, $NaCNBH_3$), a cross-link was formed between the two strands. The reaction was observed to be complete (nearly quantitative yield) in about 3 h as judged by reverse phase, anion exchange HPLC and denatured polyacrylamide gel (PAGE) analysis. The cross-linked product had a longer retention time than the starting oligonucleotides in HPLC analyses; in PAGE analysis a slower migrating band (lane 3, Figure 1; lanes 1 and 2 represent strands II and I, respectively) was observed. The structure of the resultant cross-link is shown in Figure 2.

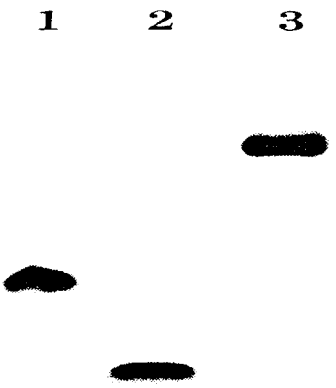


Figure 1. Gel analysis of the cross-linked duplex and the starting oligonucleotides.

The cross-linked product maintains Watson−Crick base pairing as judged by NMR and optical hypochromicity studies. In proton NMR experiments, the imino protons of the bases which are diagnostic of the base pairing were

(6) Giovannangeli, C.; Montenay-Garestier, T. M.; Rougee, M.; Chassignol, M.; Thuong, N. T.; Hélène, C. *J. Am. Chem. Soc.* **1991**, *113*, 7775.
(7) Manoharan, M.; Guinosso, C. J.; Cook, P. D. *Tetrahedron Lett.* **1991**, *32*, 7171.
(8) (a) Lindahl, T.; Ljungquist, S.; Siegert, W.; Nyberg, B.; Sperens, B. *J. Biol. Chem.* **1977**, *252*, 3286. (b) Duncan, B. K.; Chambers, J. A. *Gene* **1984**, *28*, 211. This enzyme is presently available commercially from GIBCO/BRL.
(9) (a) Withka, J. M.; Wilde, J. A.; Bolton, P. H.; Mazumder, A.; Gerlt, J. A. *Biochemistry*, **1991,** *30*, 9931. (b) Goljer, I.; Withka, J. M.; Kao, J. Y.; Bolton, P. H. *Biochemistry* **1992**, *31*, 11614.
(10) Manoharan, M.; Ransom, S. C.; Mazumder, A.; Gerlt, J. A.; Wilde, J. A.; Withka, J. A.; Bolton, P. H. *J. Am. Chem. Soc.* **1988**, *110*, 1620.

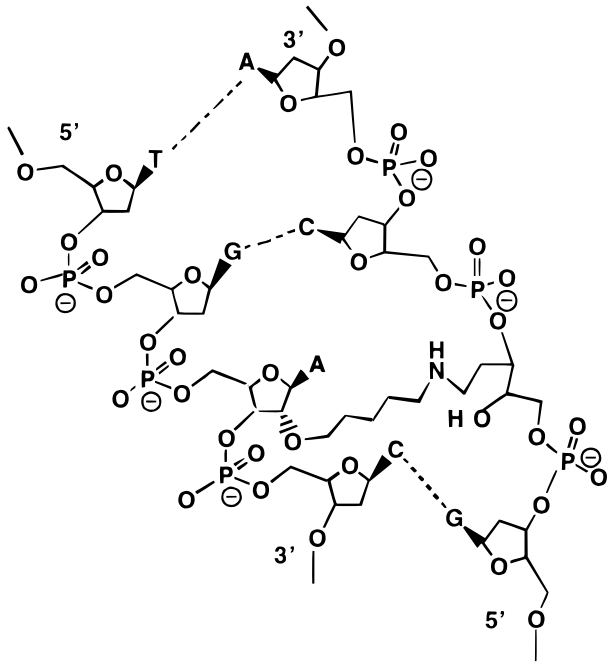


Figure 2. Chemical structure of the cross-link in the duplex.

examined. At 20, 40, and 50 °C, the normal hybridization between the strands was evident from the appearance of imino proton resonances (N-1 of G and N-3 of T) in a 90:10 H_2O/D_2O solution (Figure 3). However, at 60 °C the imino protons completely disappeared, indicating a fast duplex to coil transition. This phenomenon was completely reversible as expected. In contrast, the equimolar mixture of precursor strands I and IID **(**without reductive amination) did not show any base pairing above 25 °C under the same conditions. ^{31}P NMR showed one signal shifted 0.4 ppm downfield from the rest of the signals, indicating a novel linkage around the middle phosphate. The absence of any significant dispersion of shifts in the ^{31}P NMR spectrum represents a normal undistorted duplex (data not shown).

Molecular modeling with energy minimization revealed (Figure 4) that the newly formed linker (shown with an asterisk) does not cause any strain in the duplex. The cross-linked duplex is chemically stable[11] and had a T_m of 76 °C (0.1 M NaCl) as measured by UV hypochromicity compared to the parent duplex (formed between I and II) with a T_m value of 48 °C (Figure 5). The abasic site containing duplex (I and IID) melted near 30 °C with a biphasic curve. Thus, the cross-link stabilizes the abasic duplex by increasing T_m by 46 °C and for the native duplex by 28 °C.

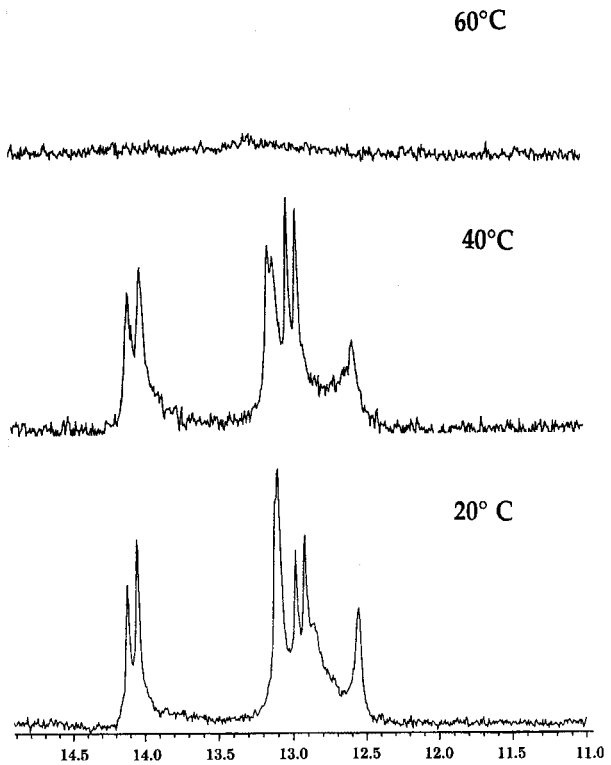


Figure 3. Variable temperature base pair protons NMR.

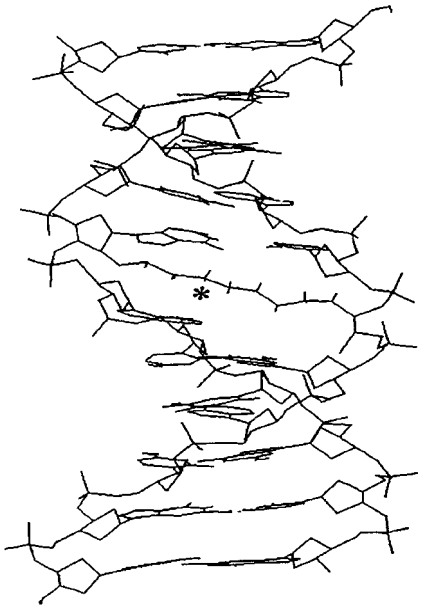


Figure 4. Molecular modeling of cross-linked duplex.

In summary, we have demonstrated site-specific cross-linking of DNA oligonucleotides employing an abasic site. In addition to the enzymatic route described above, chemical means[12] exist to generate abasic sites in other chemically

(11) We examined the chemical stability of the cross-linked DNA and double-stranded DNA duplex containing an abasic site (mixture of I and IID). While the cross-linked DNA is stable to treatment with 1 M piperidine at 90 °C for 30 min, the duplex containing the abasic site was cleaved and yielded three products (I, plus two fragments from IID, presumably 5′d-(CGCAGp)3′ and 5′ (pCAGCC)3′)) as analyzed by HPLC and PAGE.

(12) Péoch, D.; Meyer, A.; Imbach, J. L.; Rayner, B. *Tetrahedron Lett.* **1991**, *32*, 207. (b) Groebke, K.; Leumann, C. *Helv. Chim. Acta* **1990**, *73*, 608. (c) Iocono, J. A.; Gildea, B.; McLaughlin, L. W.*Tetrahedron Lett.* **1990**, *31*, 175.

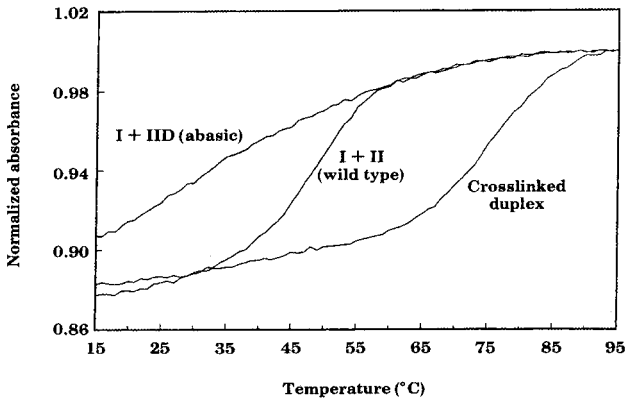


Figure 5. T_m analyisis of cross-linked duplex.

modified oligonucleotides such as phosphorothioates. Thus, the 2′- to 1′-linkage shown here is of general use. The length of the nucleophilic arm can be varied and can potentially be tailored to cross-link the whole family of nucleic acid structures and conformations. We are currently setting up biological tests to probe the utilities of the linkage achieved. There remains the interesting question of whether the cross-linked abasic site is a substrate for DNA repair endonucleases.[13]

Acknowledgment. We are grateful to Dr. Thomas W. Bruice for his contributions, Professor John A. Gerlt for the gift of uracil-DNA glycosylase, Dr. Rich Griffey for NMR analysis, Dr. Elena Lesnik for Tm measurements, Dr. V. Mohan for molecular modeling, and Dr. Sue Freier for helpful discussions.

OL9906209

(13) Modified abasic sites as substrates for endonucleases: cf. Sanderson, B. J.; Chang, C.-N.; Grollman, A. P.; Henner, W. D. *Biochemistry* **1989**, *28*, 3894.