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INCORPORATING DISULFIDE CROSS-LINKS AT THE TERMINUS OF OLIGONUCLEOTIDES VIA SOLID-PHASE NUCLEIC ACID SYNTHESIS

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Abstract: A new reagent has been prepared that allows disulfide cross-links to be introduced into nucleic acids during solid-phase synthesis. The disulfide is formed between thioalkyl tethers at the N^3-position of thymidines and stabilizes the termini of nucleic acid helices. This reagent should be especially useful for synthesizing small but stable nucleic acid structures.

Chemical cross-links have been introduced into nucleic acids to increase their stability and conformational homogeneity.[1] While a variety of cross-linking methods have been developed,[2] there are only a handful of techniques that allow site-specific cross-links to be engineered into nucleic acids.[3] These methods typically fall into two categories: the cross-links themselves can be introduced during oligonucleotide synthesis, or reactive functional groups can be introduced during oligonucleotide synthesis and then cross-links formed post-synthetically. Cross-links that have been introduced during oligonucleotide synthesis include (poly)ethylene glycol and stilbenedicarboxamide bridges that span terminal hydroxyl groups.[3,4] Linkers of this type have been used to stabilize small HIV-1 TAR analogs that bind Tat with high affinity[5] and an HIV-1 Rev binding element.[6]

When cross-links are introduced post synthetically reactive groups must be placed within an oligonucleotide sequence so that they will lie close to one another in the final folded or annealed structure.[7] In many instances, the cross-links between reactive groups can form rapidly and quantitatively. For example, nucleotide bases derivatized with alkylthiols have allowed disulfide bonds to be introduced at multiple locales within nucleic acids.[8] Disulfide bonds are particularly advantageous for cross-linking nucleic acids because they are chemically reversible and stable to a wide array of reagents and solvent conditions.[9] For example, Glick and co workers have shown that judicious placement of disulfide cross-links in a number of oligonucleotides[10] can increase their overall stability without perturbing native geometry (Fig. 1).[11] In addition, since disulfide cross-links need not block terminal hydroxyl groups this is currently the only cross-linking method that is compatible with standard radiolabelling techniques for oligonucleotides.

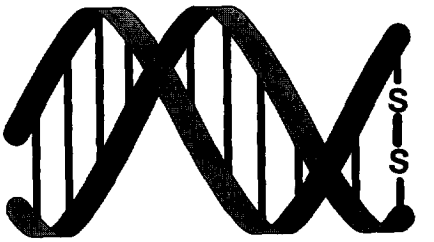

Fig. 1. Disulfide cross-link located at the terminus of an oligonucleotide. The six atom linker spans the N^3-positions of thymidines.

Unfortunately, disulfide cross-links introduced post synthetically are dependent on the proper apposition of thiol groups. If thiols are not nearby in the oligonucleotide structure, or if the locations of potential cross-linking sites are not known in advance, long reaction times, poor yields or even unwanted side-products may result. For example, we have found that thiol groups introduced at the ends of single oligonucleotides that can fold into stem-loop structures frequently form disulfide bonds with high efficiency,[10a-b] while thiol groups introduced at the ends of separate oligonucleotides that can anneal to one another sometimes do not form disulfide bonds in good yield, presumably because of energetic barriers to structure formation. In order to take advantage of the versatility of disulfide chemistry while maximizing the yield of cross-link formation we have incorporated disulfide cross-links directly into oligonucleotides during solid-phase synthesis.

The modified nucleoside **T*** allows cross-links to be efficiently introduced at the termini of any oligonucleotide, and has been synthesized and incorporated into a test oligonucleotide sequence (Fig. 2). In designing a modified nucleoside that could be used to introduce disulfide cross-links directly into an oligonucleotide sequence, it was desirable to make the chemistry amenable to both solid-phase DNA and RNA synthesis. Therefore, the free 5'- and 3'-hydroxyls were protected with *tert*-butyldimethylsilyl groups, which can easily be removed using either TBAF or TEA•3HF.[12]

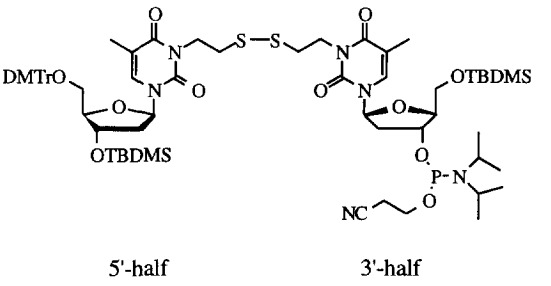

Fig. 2. Structure of modified thymidine phosphoramidite (**T***) to be incorporated at the terminus of an oligonucleotide via solid-phase synthesis.

The synthetic route to **T*** is an extension of that previously described (Fig. 3).[11c] Briefly, the 5'-half was prepared by silylating the 3'-position of **1**[13] using *tert*-butyldimethylsilyl trifluoromethanesulfonate. The 3'-half was synthesized by mono-silylation of the 5'-position of **2** followed by activation of the thiol as a hydrazinedicarboxmorpholide moiety.[14] The two halves were combined following the procedure of Wünsch and co workers to give the unsymmetrical disulfide **6**,[14b] which was then prepared for solid-phase synthesis as the phosphoramidite in good overall yield.[15]

A DNA oligonucleotide that can fold to form a short duplex with two base bulges was chosen to assess the incorporation of **T*** (Figure 4). The RNA version of this sequence has been shown to bind the Rex protein of HTLV-I with high affinity, but only when presented in the context of a longer RNA molecule.[16] In order to synthesize large amounts of a shorter, Rex-binding RNA it was first necessary to stabilize the desired duplex structure. Initial attempts at forming disulfide bonds by annealing oligonucleotides containing individual alkylthiol

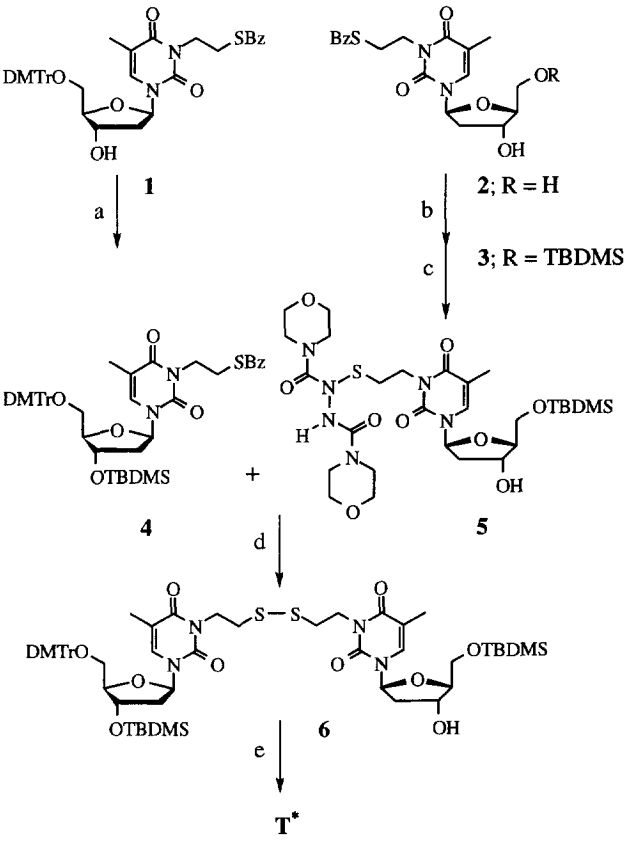

Fig. 3. Synthetic route to **T***. (a) TBDMSOTf, pyridine, imidazole (83%); (b) TBDMSCl, pyridine, imidazole (84%); (c) 1,2-hydrazinedicarboxmorpholide, LiOH•H_2O, MeOH, THF (49%); (d) LiOH•H_2O, MeOH, THF (65%); (e) Chloro-*N*,*N*-diisopropylamino-β-cyanoethylphosphine, *N*,*N*-diisopropylamine, CH_2Cl_2 (62%).

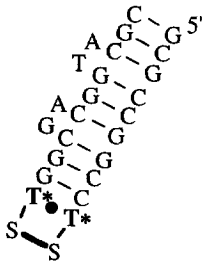

Fig. 4. Oligonucleotide used to examine the incorporation of T*.

moieties were unsuccessful both because the short duplex was unstable and because side products corresponding to other nucleic acid structures accumulated. These difficulties were largely obviated by demonstrating that the T* phosphoramidite could be incorporated into an oligonucleotide with an average coupling efficiency >98% (based on trityl cation release).[17] Following standard deprotection of the base protecting groups, the oligonucleotide was treated with TEA•3HF for 90 min at 65 °C.[12] The DNA was desalted by precipitation with *n*-butanol, then purified by 20% denaturing (8 M urea) PAGE to give an overall yield of 55% (based on a 0.2 μmol synthesis). The relative base composition of the modified oligonucleotide was determined by HPLC analysis of an enzymatic digestion.[18] As seen in Figure 5, five well resolved peaks were present on the chromatogram and correspond to the four standard nucleosides and T* (based on co-injection with authentic material).[19] In addition, the oligonucleotide was radiolabelled with polynucleotide kinase at its 5' termini and then analyzed on a 20% denaturing (8 M urea) gel in the absence and presence of DTT (Fig. 6). In the absence of DTT the oligonucleotide migrated as a single band that corresponded in size to a 24 residue DNA. However, in the presence of DTT the original band was not present and two faster migrating bands (one with higher intensity than the other) were apparent. The relative sizes of these bands was consistent with the interpretation that the disulfide cross-link had been reduced to yield two single-stranded DNA oligonucleotides 10- and 14-bases in length. The disparity in band intensity is likely due to the unequal incorporation of ^{32}P during the radiolabelling procedure. Surprisingly, the majority of the label is incorporated at the 5' terminal hydroxyl adjacent to T*, indicating that polynucleotide kinase does not discriminate against this modified residue.[11c,19]

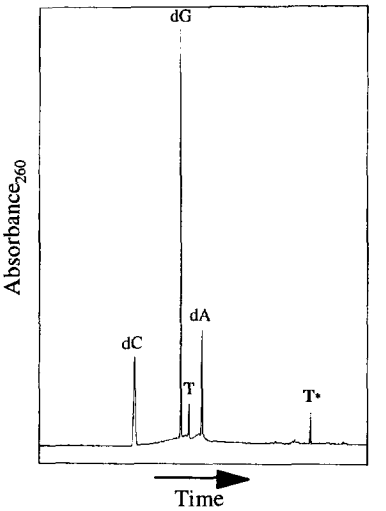

Fig. 5. HPLC chromatogram of the enzymatic digested oligonucleotide containing T*.

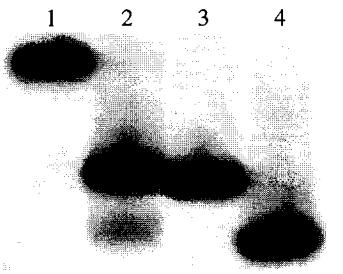

Fig. 6. Denaturing PAGE analysis of radio-labeled oligonucleotide containing T*. Lane 1: oligonucleotide containing T* - DTT; Lane 2: oligonucleotide containing T* + DTT; Lane 3: d(TGGCGACGGTACGC); Lane 4: d(GCGCCGGCCT).

This report demonstrates that a disulfide cross-link can be incorporated at the terminus of oligonucleotides during solid-phase synthesis. This method complements the chemistry previously reported by Glick[10] and co workers for post synthetically introducing disulfide cross-links into nucleic acid structures, and is more robust than the chemistry reported by Cook and co workers for introducing non-nucleotide disulfide loops during synthesis,[4e,5] as products can be conveniently radiolabelled. Since a wide variety of nucleic acid shapes have been identified that can bind with high affinity and specificity to biomedically relevant protein targets,[20] and since disulfide cross-links have been shown to be uniquely compatible with nucleic acid structure and function,[11] this method should prove useful for engineering small, stable nucleic acids that can be synthesized in high yield and potentially serve as drug leads.

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