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THIOPHOSPHORAMIDITES AND THEIR USE IN SYNTHESIZING OLIGONUCLEOTIDE PHOSPHORODITHIOATE LINKAGES

Introduction

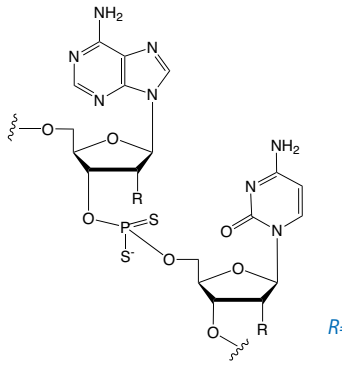
Replacing two non-bridging oxygen atoms with sulfur atoms in a DNA phosphodiester linkage creates a phosphorodithioate (PS2) linkage (Figure 1).[1] Like natural DNA, the phosphorodithioate linkage is achiral at phosphorus. Additionally, research has demonstrated that this analog is completely resistant to nuclease degradation and forms duplexes with DNA and RNA with somewhat reduced stabilities.[2] It has also been found that PS2-ODNs bind proteins with a higher affinity than their phosphodiester analogues[2-6] suggesting that PS2-ODNs may have additional utility in the form of sulfur-modified phosphate ester aptamers (thioaptamers)[3,6-8] for therapeutic and diagnostic applications. Over the past two decades, the biological interest and promise of the PS2-ODNs has spawned a variety of strategies for synthesizing, isolating, characterizing and purifying oligonucleotides containing this modified backbone.[9] The thiophosphoramidite building blocks necessary to synthesize a PS2-ODN have become commercially available after work at AM Biotechnologies (www.thioaptamer.com). Protocols to use thiophosphoramidites in popular synthesizers have been developed and optimized at AM Biotechnologies and Glen Research. Highlights are outlined in Table 1. Further information on each aspect of the synthesis process is detailed below. In addition, links to downloadable synthesis protocols can be found at the end of this report.

Dissolving Thiophosphoramidites

The structures of the nucleoside thiophosphoramidites (thioPA) are shown in Figure 2. Like normal DNA and RNA phosphoramidites, the dried solid form of each thioPA is very stable at -20°C for at least one year based on ^{31}P-NMR analysis. No reduction in reactivity for synthesizing the PS2 linkage was observed over this period. However, a few simple modifications to standard synthesis protocols are necessary when dissolving thioPAs.

(1) Unlike normal DNA phosphoramidites, the thioPAs are not completely soluble in anhydrous acetonitrile diluent. Rather, 10% anhydrous DCM (v/v) in acetonitrile is an ideal diluent for all four of the thioPAs for a final amidite concentration of 0.15 M.

FIGURE 1: PHOSPHORODITHIOATE (PS2) LINKAGE



A-PS2-C- Linkage

(2) Additionally, while normal DNA phosphoramidites are very stable in anhydrous acetonitrile at room temperature, the thioPAs are somewhat less stable in anhydrous acetonitrile containing 10% DCM; however, the coupling efficiency of all four thioPAs is not reduced after two days in solution at room temperature. Therefore, for best results the thioPA solution should be used the same day.

(3) To avoid the slight chance of some solid precipitant in the reagent bottle, the thioPA bottle on the synthesizer should be replaced with one containing acetonitrile diluent shortly after the completion of the synthesis. Flushing the synthesizer line with acetonitrile is highly recommended.

TABLE 1: RECOMMENDED SYNTHESIS CONDITIONS

Diluent: 10% DCM in Acetonitrile

Concentration: 0.15M

Activator: 0.25M ETT or 0.25M DCI

Coupling: 12-15 minutes at the 1 µmole synthesis scale

Sulfurization: Sulfurizing Reagent II (DDTT)

Cleavage and Deprotection: Ammonium hydroxide: Ethanol (3:1, V/V) containing 20mM DTT

Activators

Tetrazole has been commonly used as the activator of choice for phosphoramidite chemistry. However, unpublished studies have shown that tetrazole is not the most efficient activator for the thioPA coupling reaction. It is imperative to use an efficient activator during PS2 oligo production. DCI is emerging as an alternative activator to tetrazole. DCI is soluble in acetonitrile up to 1.1 M at room temperature and experiments with thioPAs have shown that DCI decreases the time required to achieve up to 96% coupling yield by a factor of three as compared with tetrazole. DCI is the recommended activator for thiophosphoramidites at the regular concentration of 0.25M in acetonitrile. We have also achieved equivalent coupling efficiency using 0.25M ETT in acetonitrile as activator.

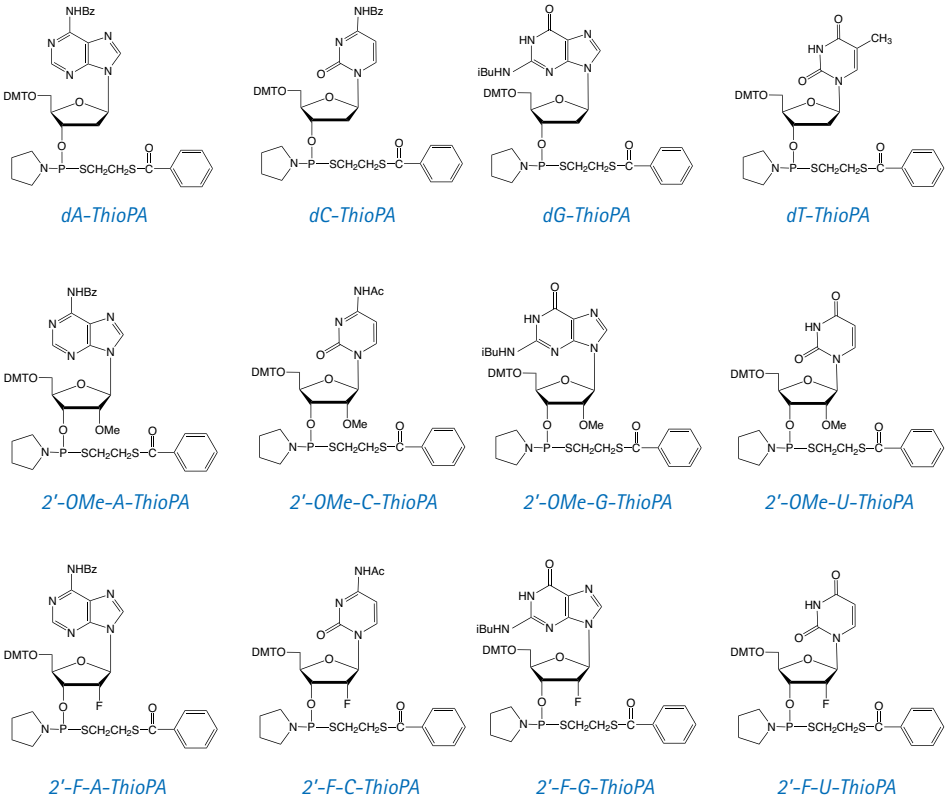
Sulfurizing Reagent

Beaucage Reagent is a very popular sulfurizing agent for the synthesis of phosphoromonothioate linkages using normal phosphoramidites. When Beaucage Reagent was used with the thioPAs to synthesize PS2 linkages, it was observed that the by-product formed in the sulfurization reaction oxidizes the thiophosphite triester. This oxidation leads to phosphoromonothioate by-products, thus lowering the desired product yield and complicating the purification of the desired product. DDTT is another sulfurizing agent primarily used to synthesize RNA phosphoromonothioates; however, it can be used to synthesize DNA phosphoromonothioates as well. Comparing DDTT efficiency to Beaucage Reagent in sulfurizing PS2 linkages, it was found that DDTT has slightly better sulfurizing reactivity than Beaucage Reagent; and, importantly, DDTT reduces the formation of phosphoromonothioate linkages during the synthesis of PS2 linkages thus increasing product yield.

Cleavage and Deprotection

Upon completion of the automated synthesis, the support was removed from the synthesizer and dried with argon. The support was transferred into a 4 mL sealable vial where 1 mL of concentrated ammonia:ethanol (3:1, v:v) mix containing 20 mM DTT was added to the vial. The vial was sealed and incubated at 55 °C for 15-16 h. After the vial was removed from the oven and cooled to room temperature, the solution was transferred to a larger vial and 4~5 mL of distilled water was added. Solvents were removed by lyophilization.

FIGURE 2: THIOPHOSPHORAMIDITES (THIOPA)



Protocols

Links to our preferred protocols can be found on our web site:

(http://www.glenresearch.com/Technical/ABI_394_PS2_Protocol.PDF)

(http://www.glenresearch.com/Technical/Expedite_8909_PS2_DCI_Protocol.PDF)

References

1. J. Nielsen, W.K.D. Brill, and M.H. Caruthers, *Tetrahedron Letters*, 1988, **29**, 2911-2914.
2. L. Cummins, D. Graff, G. Beaton, W.S. Marshall, and M.H. Caruthers, *Biochemistry*, 1996, **35**, 8734-41.
3. X. Yang, and D.G. Gorenstein, *Curr Drug Targets*, 2004, **5**, 705-15.
4. W.S. Marshall, and M.H. Caruthers, *Science*, 1993, **259**, 1564-70.
5. J.L. Tonkinson, et al., *Antisense Research and Development*, 1994, **4**, 269-278.
6. X. Yang, et al., *Bioorg Med Chem Lett*, 1999, **9**, 3357-62.
7. X. Yang, et al., *Ann N Y Acad Sci*, 2006, **1082**, 116-9.
8. X. Yang, et al., *Nucleic Acids Res*, 2002, **30**, e132.
9. W.T. Wiesler, W.S. Marshall, and M.H. Caruthers, *Methods Mol Biol*, 1993, **20**, 191-206.