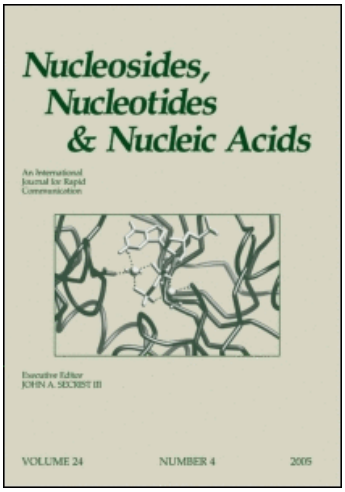

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A Short, Novel, and Cheaper Procedure for Oligonucleotide Synthesis Using Automated Solid Phase Synthesizer

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A Short, Novel, and Cheaper Procedure for Oligonucleotide Synthesis Using Automated Solid Phase Synthesizer

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ABSTRACT

Dimethylthiuram disulfide (DTD) has been developed as an efficient thiolation reagent during automated synthesis of oligonucleotides using phosphoramidite chemistry. Simultaneous thiolation and capping was accomplished by mixing DTD with capping solution B, which saved 20% of solvent consumption and compressed the four-step synthesis cycle to three. Large-scale (1 mmol) synthesis of phosphorothioate oligonucleotides has been demonstrated with excellent yield and purity.

Key Words: Antisense; Sulfur-transfer reagent; Dimethylthiuram disulfide; Oligonucleotide synthesis.

INTRODUCTION

The use of antisense oligonucleotides represents a powerful new strategy in the development of therapeutic agents that act by the selective inhibition of gene expression. With increasing number of antisense products in clinic and successful outcome of these clinical trials, large-scale production of oligonucleotides at low-cost would become mandatory. Therefore, developing low-cost approaches for oligonucleotide

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synthesis are of obvious importance and widespread interest. Automated synthesis on solid-supports using phosphoramidite chemistry has been the most successful method for the large-scale synthesis of oligonucleotide-based drugs.[1] Detritylation, coupling, oxidation and capping are the four key steps during automated synthesis of oligonucleotides. During synthesis of phosphorothioate oligonucleotides oxidation of phosphite P(III) into phosphorothioate triester P(V) is accomplished via a sulfur-transfer step. Elemental sulfur was first used for this step, which is poorly soluble in organic solvents commonly used for automated synthesis. Also the rate of conversion of P(III) into P(V) product is not fast and facile with elemental sulfur. For these reasons, many sulfur-transfer reagents have been developed during past decade.[2] Among a dozen reagents described in the literature, 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent)[3] and phenylacetyl disulfide (PADS)[4] are the two most notable and widely used reagents at the present time on large-scale. Both are soluble in organic solvents and produce efficient and rapid sulfurization kinetics. Although these two reagents are currently used on industrial scale, they have certain limitations. For example, Beaucage reagent is expensive, long-term stability in solution is not optimal and the cyclic sulfoxide by-product formed during sulfurization is an efficient oxidizing agent which increases the levels of phosphodiesters during packed-bed automated synthesis. Sulfurization with PADS is accomplished in combination with 3-picoline. Unfortunately the smell of this mixture is obnoxious and upon standing it changes color to a dark solution. Thus, our investigation for an alternative sulfur-transfer reagent that is cheap and user friendly led us to dimethylthiuram disulfide (DTD) as a reagent of choice.

CHEMISTRY

In 1991, Vu and Hirschbein first reported on the application of tetraethylthiuram disulfide (**1**) as a sulfur-transfer reagent.[5] We found **1** attractive as a sulfur-transfer reagent for three reasons. First, ease of synthesis and low cost. Second, proven utility for small-scale oligonucleotide synthesis and third its atom efficiency.[6] Based on these criterions, we embarked on an efficient synthesis of **1** and its analogs using a common approach. Herein, we report one-pot synthesis of disulfides **1**–**4** in excellent yield (> 90%; Sch. 1) starting from alkylamine and carbon disulfide which are both cheap and commercially available on industrial scale. The products are isolated as stable pale yellow crystalline solid that is highly soluble in organic solvents.[a]

[a]Preparation of dimethylthiuram disulfide (**4**): NaOH (80 g, 2 mol) was dissolved in water (500 mL) and the solution was cooled to 0°C. THF (200 mL), methylamine (40% in water, 170 mL, 2 mol) and carbon disulfide (120 mL, 2 mol) were added over a period of 30 min to aq. NaOH solution while stirring at 0°C. Crushed ice (1.5 Kg) was added to the reaction mixture, followed by acetic acid (300 mL). Hydrogen peroxide (30%, 100 mL, 1 mol) was added gradually while stirring over 15 min while maintaining the reaction temperature below 5°C. Heptane (800 mL) was added to the reaction mixture and stirred for additional 30 min. The product was filtered off and washed with aq. acetic acid (2%, 5×200 mL) and heptanes (2×200 mL). The product was air dried until constant weight (~1 day) to furnish off-white solid (205 g, 97%). M.p. 98–100°C (dec.)

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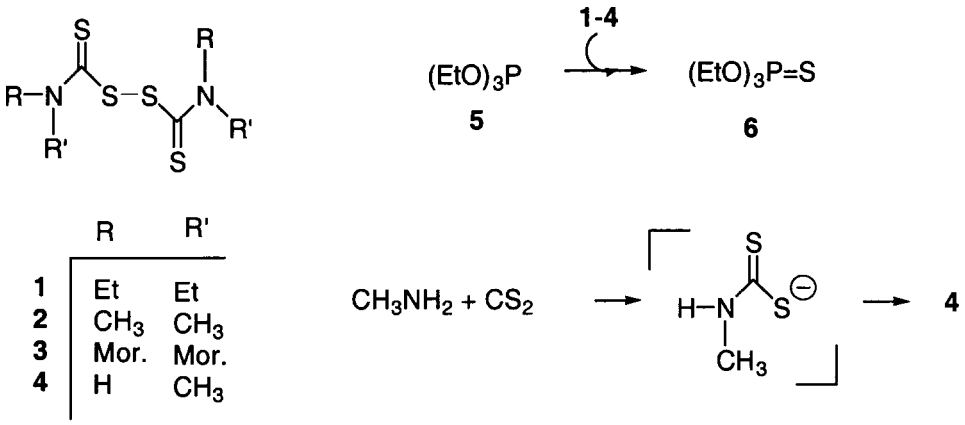

Scheme 1. Synthesis and structure of thiuram disulfide analogs.

Disulfides **1**–**4** were tested for their ability to convert triethyl phosphite **5** into triethyl phosphorothioate **6** in CD_3CN solution at room temperature. The conversion was monitored by ^{31}P NMR spectroscopy. Reaction of disulfide **1** with **5** furnished the expected phosphorothioate (68 ppm) as a major product and 18% minor products (16–18 ppm). Similarly reaction of disulfides **2** and **3** with **5** furnished significant amount of undesirable phosphate products (16–18 ppm). Interestingly, the efficacy of DTD (**4**) as a sulfur-transfer agent was excellent with >99% product formation. Both PADS and Beaucage reagent gave identical results, confirming that conversion of **5** into **6** was a reliable method for quick screening of various sulfur-transfer reagents.

Next, stability and solubility of DTD was tested in various solvents for extended period of time. DTD is highly soluble in THF (1 M) and relatively less in CH_3CN (0.3 M). Addition of base to a solution of DTD rapidly degraded the product. Whereas, addition of an acid such as 4-chlorophenol stabilized a 0.3 M DTD in THF for 3-days. Similarly addition of acetic acid to a solution of DTD significantly increased the stability of the reagent (>30-days) without compromising the sulfur-transfer ability. The exceptional stability of DTD in presence of acetic acid encouraged us to mix it with capping solution B, which contains acetic anhydride in CH_3CN hoping that oxidation and capping steps could be performed simultaneously. The shorter protocol (3-steps vs. 4-steps) may result in reducing solvent wash, time savings in overall synthesis cycle and make DTD solution stable.

Therefore, synthesis of ISIS-14803, a 20-mer phosphorothioate oligonucleotide was undertaken using the new 3-step protocol on OligoPilot II synthesizer at 173–193 μmol scale. The first two reaction cycles, detritylation (3% DCA in toluene) and coupling (0.2 M amidite with 1-*H* tetrazole) were unchanged. In order to accomplish oxidation and capping in one step, 0.3 M DTD was added to 20% acetic anhydride in CH_3CN (capping B solution), and capping solution A was left unaltered (*N*-methylimidazole/pyridine/CH_3CN, 2:3:5, v/v). The contact time during synthesis for combined step was 4 min and 2 column volume of solution was utilized for the reaction. Rest of the synthesis cycle and procedures are similar to literature

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Table 1. Data on crude oligonucleotides.

S-transfer reagent	Oligo	Scale (μ mol)	OD/ μ mol	Trityl-on %	P = 0%	CH_3CN use (L)	Synthesis Time (min)
PADS	14803	173	148	74.7	7.0	2.5	470
DTD	14803	169	149	76.2	5.1	2.0	350
PADS	Affinitak™	193	151	72.0	8.7	5.4	540
DTD	Affinitak™	193	152	73.0	4.9	4.5	450

ISIS 14803 Sequence: CTGCTCATGGTGCACGGTCT.
Affinitak™ Sequence: GTTCTCGCTGGTGAGTTTCA.

method.[1] Identical sequence was made as a control using PADS for sulfurization. The data on crude oligonucleotide made by DTD and PADS as sulfur-transfer reagent is summarized in Table 1. Clearly, the data indicates that there was no obvious difference between the two syntheses. Subsequent purification of two oligonucleotides further confirmed that both were equally pure and furnished the expected yield.

The synthesis was repeated with DTD on larger scale (1 mmol) using another 20-mer phoshorothioate oligonucleotide (Affinitak™). Same oligonucleotide using PADS was also synthesized as a control. The data in Table 1 indicates that DTD works as good as PADS on larger scale. More importantly, the overall solvent (CH_3CN) consumption for synthesis with DTD is reduced by 20% compared to the synthesis done with PADS due to elimination of a synthesis/wash cycle. The results indicate that quality and yield of the product is not compromised when DTD is employed in a three-step mode as described above. The products were analyzed by HPLC, CGE, ^{31}P NMR and LC MS method.

CONCLUSIONS

In summary, use of DTD as a sulfur-transfer reagent may provide following advantages over PADS and other conventional reagents.

- DTD is inexpensive compared to other sulfur-transfer reagents ($10/Kg).
- Overall usage of CH_3CN is reduced by 20% which is a significant cost savings.
- Due to reduced solvent consumption, there is less waste solvent to dispose.
- The total synthesis time was reduced by 25% which increases the throughput.
- The atom efficiency with DTD is excellent due to its small size.
- Use of DTD is safe and convenient on large-scale.

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