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EFFICIENCY OF SULFURIZATION IN THE SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDE PHOSPHOROTHIOATES UTILIZING VARIOUS SULFURIZING REAGENTS

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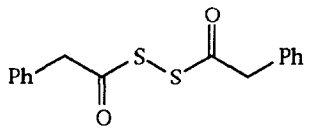
Abstract: The synthesis of fully modified oligodeoxyribonucleotide phosphorothioates utilizing various sulfurizing reagents is described.

The chemical synthesis of oligodeoxyribonucleotides has undergone revolutionary developments during the last decade. In particular, modifications at the internucleotidic linkage have been widely exploited owing to the potential therapeutic effect of these antisense DNA oligomers in various types of viral diseases. The inspiration and promise of synthetic oligonucleotides as antisense therapeutics is amply self-evident from the volumes of publications reported in recent times.[1-9] Among the modifications reported so far, uniformly modified phosphorothioate oligodeoxyribonucleotides have been the first class of compounds to reach the clinic. Although antisense strategy looks promising, a key question has been the feasibility of creating therapeutic oligonucleotides. The development of phosphoramidite chemistry and its elaboration into an automated technology have greatly enhanced the ease with which oligonucleotides are synthesized and consequently their availability. However, the cost of synthesizing these oligonucleotides are still on the higher side and several process related improvements have to be performed before becoming affordable to patients.

In a typical solid-supported synthesis of oligonucleotide phosphorothioate utilizing phosphoramidite chemistry, the first nucleoside attached to a solid-support is detritylated, followed by coupling with another nucleosidic monomer to afford phosphite triester. This step is then followed by sulfurization to afford the phosphorothioate. If sulfurization reaction is not quantitative then the unsulfurized phosphite triester will be cleaved in the subsequent acidic detritylation step leading to considerable reduction in the full length content.[10] Also during the capping step a small fraction of undesired randomly distributed phosphodiesters are formed,[11] which in some instances have close mobility with the parent drug substance thereby rendering the purification process difficult. In order to investigate this crucial step during the synthetic cycle, we became interested in investigating the efficiency of different sulfurization reagents

used in the synthesis of phosphorothioates.

Excluding elemental sulfur which has suitability problem in automation, five sulfurization reagents[11-16] have been shown to be effective in the synthesis of phosphorothioates using phosphoramidite chemistry on solid support *viz.* Phenylacetyl disulfide (**1**), Tetraethylthiuram disulfide (TETD) (**2**), Dibenzoyl tetrasulfide (**3**), 3H-1,2-Benzodithiol-3-one 1,1-dioxide (**4**), and Bis(*O*,*O*-diisopropoxyphosphinothioyl) disulfide (**5**).



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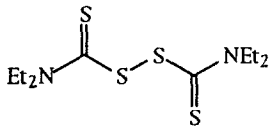

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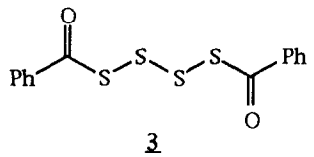

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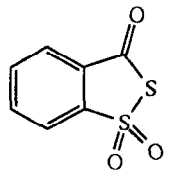

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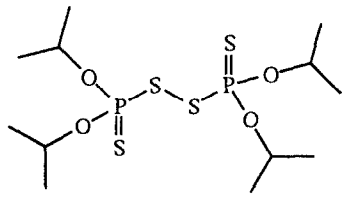

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The efficiencies of sulfurizing reagents **1-5** are comparable on a research scale (≤ 1 μmol). However, in our hands application of different reagents on larger scale always led to different amounts of isolated crude products. In order to obtain a quantitative comparison between these reagents, we designed a synthetic cycle incorporating sulfurization step followed by a standard iodine oxidation. This will convert all unsulfurized species to phosphodiesters which can determined by ^{31}P NMR.

Oligonucleotide phosphorothioate S-d(TTG-CTT-CCA-TCT-TCC-TCG-TC), targeted to the E2 mRNA of papillomavirus for the treatment of genital warts[17] was chosen as a model. The synthesis was carried out on a 10 μmol scale (twice and simultaneously in each case on the same instrument), using a DNA synthesizer (Applied Biosystems Model 394B) and modified the standard cycle according to the literature suggestions for various reagents. These modifications include changes in delivery time, coupling time and an additional washing of the support (before and after sulfurization) with a solvent used for preparation of the corresponding sulfurizing reagent. Table **1** shows the sulfurization conditions utilized for various reagents. Attempted oxidation of the above synthesized CPG-bound fully-protected oligonucleotide,

using standard oxidation solution (0.1M iodine in THF/pyridine/water/, 8:4:2, v/v/v), within 30 minutes (reaction time equivalent to the total exposure to iodine), removed only 0.6% of the sulfur atoms (^{31}P NMR).[18]

Table 1. Sulfurization using various reagents.

Reagent	Solvent	Concentration	Sulfurization Time
(1)	Dichloroethane / Collidine, 4/1, v/v	0.17 M	300 s
(2)	CH_3CN	0.5 M	900 s
(3)	THF	0.4 M	60 s
(4)	CH_3CN	0.2 M	180 s
(5)	Pyridine	0.2 M	220 s

Reagents **1**, **4** and **5** were used without any additional purification. Reagents **2** and **3** were purified prior to use by crystallization (twice) from ethyl alcohol and ethyl acetate, respectively. At the end of synthesis, the products were deprotected under standard conditions (30% NH_4OH, 20 h, at 55°C) and isolated by ethanol precipitation. 500 O.D. of each sample was dissolved in 600 µl of D_2O and analyzed by ^{31}P NMR.[19] All scannings were performed under identical conditions at room temperature and the results are summarized in Table **2**.

Table 2. ^{31}P NMR analysis of the synthesized oligomers.

Reagent	Synthesis I P = S / P = O	Synthesis II P = S / P = O	Average sulfurization efficiency (ASE)[20]
(1)	95.87 / 3.17	95.11 / 3.31	99.78
(2)	87.48 / 10.37	87.65 / 10.20	99.30
(3)	45.53 / 51.55	---	96.80
(4)	95.94 / 2.09	94.7 / 2.86	99.75
(5)	95.18 / 3.50	95.71 / 3.04	99.76

Based on the above table it appears that reagents (**1**) and (**5**) are equally effective as reagent (**4**) for sulfurization. However in considering the utility of a sulfurization reagent for large scale synthesis of antisense oligonucleotides, efficiency is not the only criterion for consideration. A careful judgment has to be made between the time, cost,[21] efficiency and ease of removal of undesired phosphodiesters during purification. Reagent (**4**) has already been widely used in very large scale synthesis and our present study may suggest other useful information in

reducing the cost of antisense drugs. Further work on the optimization of time, concentration of reagent and scale are in progress.

Acknowledgments

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19 In all cases, signals corresponding to the phosphorothioate (PS)(δ 56 ppm) and phosphate (PO)(δ -0.3 ppm) diesters were integrated after signal to noise ratio reached value ≤ 700.
20 ASE values were calculated based on the equation ASE =(PS [%]/100)$^{1/19}$. The PS value was obtained from integrated value of ^{31}P NMR spectrum of the crude oligonucleotide product and the integrated values all signals present in the spectrum are equal to 100%.
21 At current rate the cost of various reagents are as follows: Beaucage : $ 10/g ($ 2.00 / mmole); Van Boom : $ 1.80/g ($ 0.54 / mmole); TETD : $ 0.02/g ($ 0.006 / mmole); Stec : $ 4/g ($ 1.70 / mmole). The cost of Reese reagent was roughly calculated to be $ 2.75/g ($ 0.93 / mmole).

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