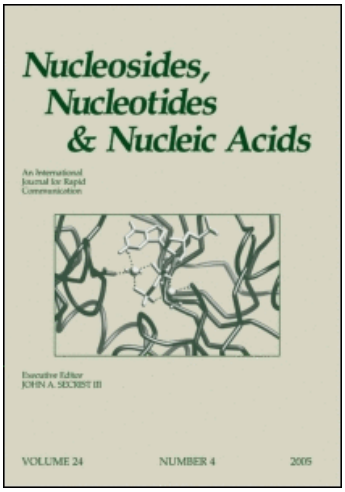

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Solid Phase Synthesis of Phosphorothioate Oligonucleotides Utilizing Diethyldithiocarbonate Disulfide (DDD) as an Efficient Sulfur Transfer Reagent

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Solid Phase Synthesis of Phosphorothioate Oligonucleotides Utilizing Diethyldithiocarbonate Disulfide (DDD) as an Efficient Sulfur Transfer Reagent

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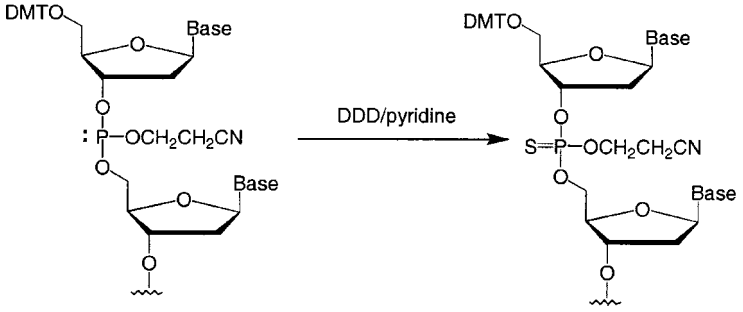
ABSTRACT

Diethyldithiodicarbonate (DDD), a cheap and easily prepared compound, is found to be a rapid and efficient sulfurizing reagent in solid phase synthesis of phosphorothioate oligodeoxyribonucleotides via the phosphoramidite approach. Product yield and quality based on IP-LC-MS compares well with high quality oligonucleotides synthesized using phenylacetyl disulfide (PADS) which is being used for manufacture of our antisense drugs.



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Key Words: Oligonucleotides; Phosphorothioates; Sulfurization; Solid phase synthesis; DDD.

Use of synthetic oligodeoxyribonucleotides or oligoribonucleotides for antisense of gene expression inhibition is a proven approach to therapy in cases where a disease originates from the expression of genes of known sequence.[1–9] An mRNA target may be bound in a highly sequence-specific fashion by hybridization with a complementary DNA, RNA, or other oligonucleotide sequence according to Watson-Crick rules. Unmodified natural oligonucleotide molecules are readily available and have low toxicities, but they do not meet the requirements for antisense drug development. Hence, oligonucleotide analogs have been investigated. Among various DNA modifications investigated, a variety of phosphorothioate oligodeoxyribonucleotides, isoelectronic analogs of natural DNA in which a non-bridging oxygen atom is replaced by sulfur, are currently marketed and in clinical trials for several diseases.[10][a]

Given approval of the first antisense drug (Vitravene^{TM}) for market and the potential for multiple systemic antisense drugs to reach market, there is a need for an efficient large scale synthesis of phosphorothioate oligodeoxyribonucleotides.[11] Deoxyribonucleoside phosphoramidite chemistry has been widely used for synthesis of phosphorothioate oligonucleotides due to its potential for automation, its high coupling efficiency, the ease of site-specific thioate linkage incorporation, and its ready scalability.[12–15] Today's state of the art DNA/RNA synthesizer allows for synthesis of phosphorothioate oligonucleotides up to 600 mmole scale, utilizing solid phase synthesis and phosphoramidite synthons. In a typical synthesis of phosphorothioate oligonucleotide, the intermediate phosphite triester linkage formed is sulfurized by a sulfur transfer reagent. It is crucial that efficient sulfur transfer occurs in each elongation cycle, and investigation of a number of sulfur transfer reagents has been reported in literature in the last few years. Among compounds reported, 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent) has been the most widely used agent due to rapid sulfurization and commercial availability.[16,17] However this reagent is expensive as its synthesis involves multiple steps and the handling of corrosive materials, hindering large scale production. This cost, combined with the oxidizing ability of its byproduct, limits its potential for large scale synthesis and has led to investigations into the use of alternate sulfur transfer agents by a number of laboratories, including ours.[18–30]

We recently reported on the use of phenylacetyl disulfide (PADS) as an efficient sulfur transfer agent in the solid phase synthesis of oligodeoxyribonucleotide phosphorothioates.[31,32] We now report on the use of diethyldithiocarbonate disulfide (DDD) as an efficient sulfur transfer agent for solid phase synthesis utilizing phosphoramidite chemistry. DDD is synthesized in one step in $>90\%$ yield

[a]More than 24 oligonucleotide phosphorothioates are currently under clinical investigation for the treatment of various diseases. It is roughly estimated that for a systemic dosing for treatment of a chronic disease about 700 to 900 kilograms per year of drug is needed to meet the present demand.[11]

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Table 1. Synthesis parameters of cycle used on Pharmacia OligoPilot II synthesizer.

Step	Reagent	Volume (mL)	Time (min)
Detritylation	3% DCA/toluene	50	4
Coupling	Phosphoramidite (0.2 M), 1H-tetrazole (0.45 m) in acetonitrile	1.6, 5	5
Sulfurization	DDD (0.2 M) in pyridine	8	1.5
Capping	Ac_2O/pyridine/CH_3CN, NMI/CH_3CN	1.7, 1.7	0.5

as a pale yellow crystalline solid by oxidizing the very cheap and readily available potassium salt of *O*-ethylxanthic acid in the presence of an oxidizing agent such as iodine.[33]

Initially, we synthesized homo-thymidine phosphorothioate 20-mer using DDD as sulfur transfer agent at 1.0 M concentration in pyridine. The synthesis was done at 1 μmole scale using CPG as solid support on an ABI 390 synthesizer. Analysis of crude oligonucleotide by ^{31}P NMR showed $< 0.5\%$ phosphate diester content. Encouraged, we then tested efficiency of sulfurization in solid phase synthesis of

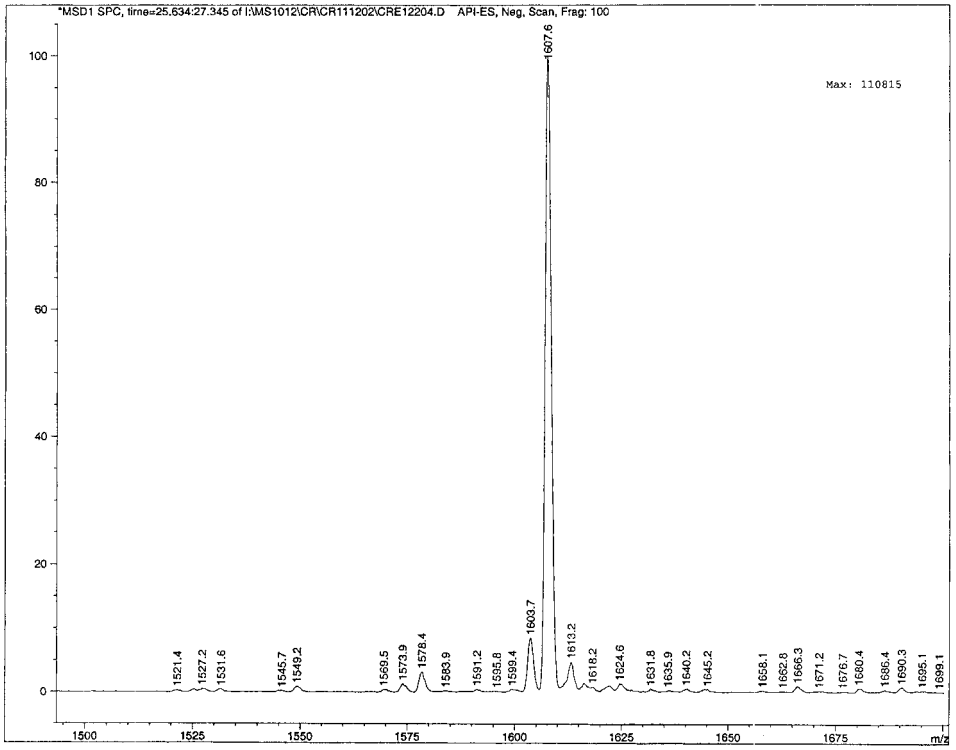

Figure 1. IP-LC-MS of the purified 20-mer phosphorothioate oligonucleotide.

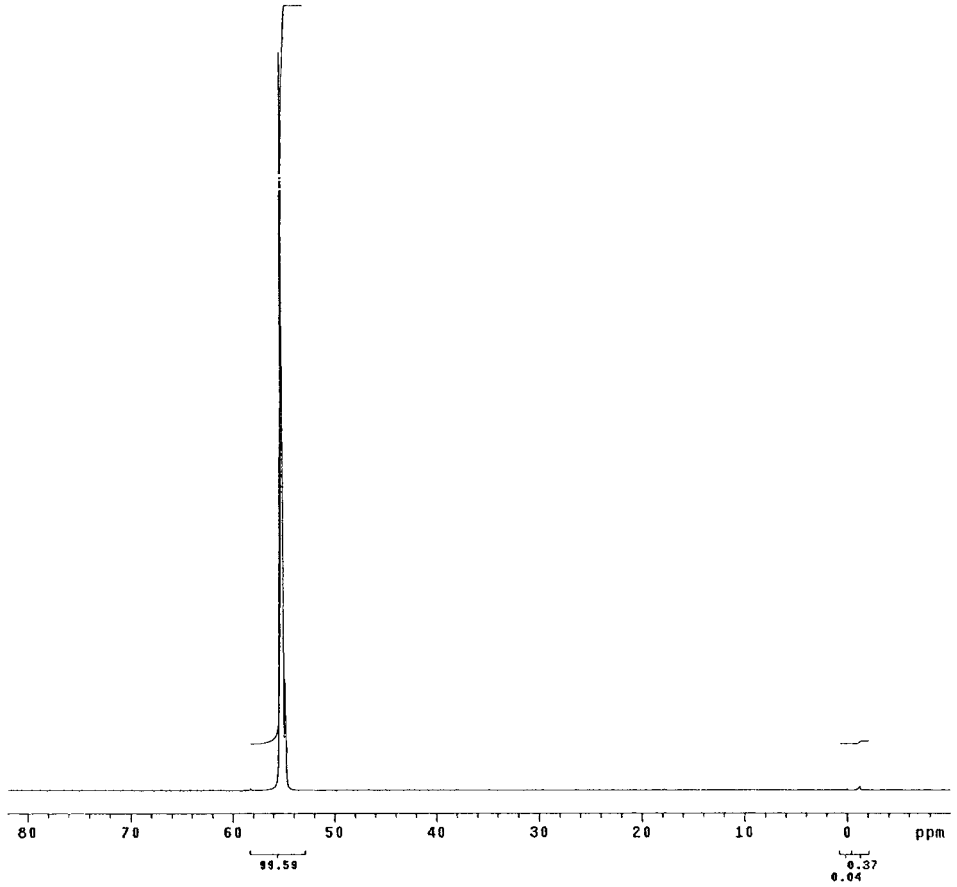

Figure 2. ^{31}P NMR(D_2O) of the 20-mer phosphorothioate.

mixed deoxyoligonucleotide 20-mer phosphorothioate, ISIS 5132 (5′-TCC-CGC-CTG-TGA-CAT-GCA-TT-3′), an anti-cancer compound that is a potent and selective inhibitor of c-raf kinase expression. The synthesis was done on 180–190 μmole scale using a Pharmacia OligoPilot II DNA/RNA synthesizer on HL30 Primer support (90 μmole/gram loading) and standard cyanoethyl phosphoramidites (0.2 M solution in acetonitrile). We had noted earlier that the rate and efficiency of sulfurization are dependent on the solvent system. Hence, several experiments were performed on this scale by varying solvents (acetonitrile, dichloromethane, dichloroethane, tetrahydrofuran), bases (pyridine, lutidine, *sym*-collidine, 2-picoline, 3-picoline), sulfurization time (60, 120, 300 sec), solvent to base ratio and using different concentrations (0.2, 0.5, 0.8, 1.0 M) of the sulfurizing reagent. Based on data obtained from these experiments and taking into considerations some scalability issues, it was found that a 0.2 M solution of DDD in pyridine with 60–90 seconds contact time gave an optimal sulfurization efficiency of $>99.6\%$.

Towards the end of our work, a new polystyrene solid support was introduced to the market by Amersham Pharmacia Biotech, viz. Primer PS200 with a loading

Table 2. Comparison of oligonucleotide synthesized using PADS and DDD.

Reagent	Crude yield (mg/μmole)	Crude full length (%) (RP HPLC)	Purified full length[a] (n−1) (%) (CGE)	Purified full length[b] (n−1) (%) (IP-LC-MS)	P = S:P = O (^{31}P NMR)	P = S:P = O (IP-LC-MS)
PADS	6.60	72	91–92 (2.3)	82.4 (2.3)	99.63:0.37	99.64:0.36
DDD	6.56	73	91–92 (2.2)	85.5 (1.7)	99.63:0.37	99.63:0.37

[a]The 91% full-length is typical when the entire DMT-on peak is collected. Much higher purity (~96–97% full length) is easily achieved by efficient fractionation of the DMT-on peak. Fractionation of DMT-on peak results in less than a 5% loss of full-length material.
[b]The purified full length by IP-LC-MS is less than CGE values since the former analytical tool is efficiently able to distinguish and quantify process related species such as the depurinated oligonucleotides that co-migrate in CGE.

of 200 μmole/gram. Since we decided to switch to this support for manufacture of our drugs, we were interested in evaluating the efficiency of DDD towards synthesis of phosphorothioate oligonucleotides using PS200 solid support.

Affinitak™ (ISIS 3521, 5′-GTT-CTC-GCT-GGT-GAG-TTT-CA -3′), a 20-mer phosphorothioate oligodeoxyribonucleotide targeted towards protein kinase C-α which is in phase III clinical trials for treatment of non-small cell lung cancer was chosen as an example. Synthesis was performed in triplicate on 240–250 μmole scale using a Pharmacia OligoPilot II DNA/RNA synthesizer. Primer support dA containing the 3′ terminal nucleoside of the oligonucleotide was tightly packed in a steel column (volume 6.3 mL). Details of the synthesis cycle are given in Table 1. 3% Dichloroacetic acid in toluene was used for deblocking of dimethoxytrityl groups from the 5′ hydroxyl group of the nucleoside.[34,35] Unlike dichloromethane, which gives conductivity-based or UV-based DMT yields, no conductivity-based detritylation yields were obtained when toluene was used as solvent for deblocking.

The solid-support bound phosphorothioate oligonucleotide synthesized under these optimized conditions was dried overnight under high vacuum to determine the crude yield,[36] then treated with a solution of triethylamine:acetonitrile (1:1, v/v) at room temperature for a period of 6 h to deprotect cyanoethyl group,[37] support washed thoroughly with acetonitrile, incubated with concentrated ammonium hydroxide at 55°C for 14 h. The cooled solution was filtered, support washed with a solution of ethanol:water (1:1, v/v) and the combined solutions concentrated under vacuum. The solid residue was reconstituted in water and triethyamine (1 mL) was added to keep the solution basic. The crude oligonucleotide was further purified by C_{18} reversed phase HPLC that allowed facile separation of 5′-*O*-DMT-on oligonucleotide from the capped failure sequence. To evaluate the comparison between two sulfur transfer reagents under identical conditions, all fractions containing DMT group were collected, and detritylated using standard conditions. Extensive analysis of the purified material was performed using IP-LC-MS (Fig. 1), capillary gel electrophoresis (CGE), and ^{31}P NMR (Fig. 2). Table 2 compares the sulfur transfer and synthesis efficiencies of PADS and DDD reagent.

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CONCLUSION

As evident from the data presented in this paper, a solution of DDD in pyridine allows sulfurization of phosphite triesters to phosphorothioate triesters from 1 μmole to 250 μmole scale providing phosphorothioate oligonucleotides in high yield and purity. Multiple analytical methods showed equivalence to oligonucleotides synthesized using PADS reagent. In conclusion, we have unequivocally proven that DDD reagent compares well with PADS, without compromising yield or quality of the antisense oligonucleotide products.

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