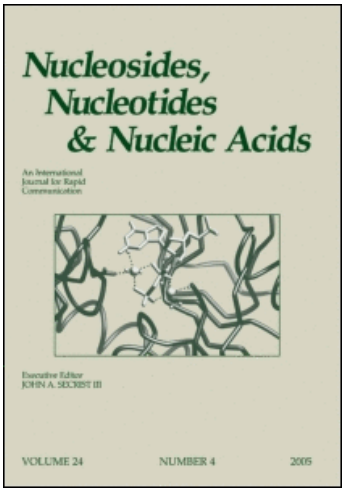

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USE OF PHENYLACETYL DISULFIDE (PADS) IN THE SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDE PHOSPHOROTHIOATES

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Abstract: Investigations into the use of phenylacetyl disulfide (PADS) as an efficient sulfur transfer agent in the solid phase synthesis of oligodeoxyribonucleotide phosphorothioates showed that under suitable solvent conditions, this relatively inexpensive reagent rapidly and efficiently sulfurizes internucleotide phosphite linkages.

The ability to use short segments of antisense oligonucleotides to bind specific mRNA sequences through Watson-Crick base pairing, resulting in sequence-specific inhibition of gene expression, has led to the possibility of their being used as novel therapeutic agents.[1-8] A major advantage of this strategy is its potential specificity of action. In principle, an antisense oligonucleotide can be designed to target a single gene within the human genome, creating a specific therapeutic for any disease for which a causative or contributory gene is known. Among the DNA modifications reported to date for use in antisense drug design, phosphorothioates, where one non bridging oxygen of the internucleotide phosphate is formally replaced by a sulfur atom, are the first class to undergo human clinical trials.[9-10] Ongoing phosphorothioate clinical trials against several disease targets has necessitiated manufacture of very large quantities of oligonucleotide active pharmaceutical ingredient (API).[11] Clinical trial and future market demands have stimulated effort towards developing cost efficient large scale synthesis of these complex bio-molecules. In the last few years a number of groups including our own have investigated issues related to fast and efficient synthesis, automation, and large scale

purification. This effort has culminated in the routine synthesis of 20-mer oligodeoxyribonucleotide phosphorothioates at 150 mmole scale using only 1.75-fold molar excess of amidites in less than 10 h total synthesis time.[12]

The current chemistry of choice, the phosphoramidite route, requires sulfurization after each coupling.[13-15] Hence, it is crucial that the sulfur transfer step be highly efficient. During the last few years, a variety of sulfurizing reagents have been investigated.[16-27] Among these, 3H-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent) is most widely used because of its rapid sulfurization and commercial availability. However, the sulfoxide by-product formed during sulfurization is a potent oxidizing reagent[16,17] and may be responsible for inconsistent reagent performance lot to lot. In addition, the synthesis of this reagent involves multiple steps and handling of difficult materials that makes it not amenable to large scale productions. Thus, there is an urgent need to replace this expensive reagent.

Phenylacetyl disulfide (PADS) has been reported in the literature as a sulfur transfer reagent in the synthesis of deoxyribonucleotide phosphorothioates,[28-29] but under the reported conditions it was found to be inefficient. While investigating a range of potential sulfur transfer reagents, we noticed that rate and efficiency of sulfurization are very dependent on the solvent system. Thus, bis(diisopropxy phosphinothioyl)disulfide was efficient in pyridine whereas Beaucage reagent performed well in acetonitrile. This was also true for some of the other sulfur transfer reagents we investigated. Thus, we were prompted to investigate the efficiency of PADS under a variety of conditions.

Encouraged by our initial experiments on the synthesis of homo-thymidine phosphorothioate 20-mer using PADS under new conditions, we tested the sulfurization efficiency in the solid phase synthesis of a mixed deoxyoligonucleotide sequence (5'-TCC-CGC-CTG-TGA-CAT-GCA-TT, ISIS 5132). Several experiments (ca. 75 syntheses) were performed at 180-190 μmole scale using a Pharmacia OligoPilot II DNA/RNA synthesizer on polystyrene support (90 μmole/gram loading) by changing solvents (dichloromethane, dichloroethane, acetonitrile), bases (pyridine, *sym*-collidine, lutidine, 2-picoline, 4-picoline, 3-picoline), sulfurization time (600, 480, 360, 100, 60 sec), solvent to base ratio (100:0, 75:25, 50:50, 25:75, 0:100), concentration (2.0, 1.0, 0.8, 0.6, 0.5, 0.4, 0.2, 0.1M), and column volume (1.5, 1.0, 0.5). Based on the data obtained from these

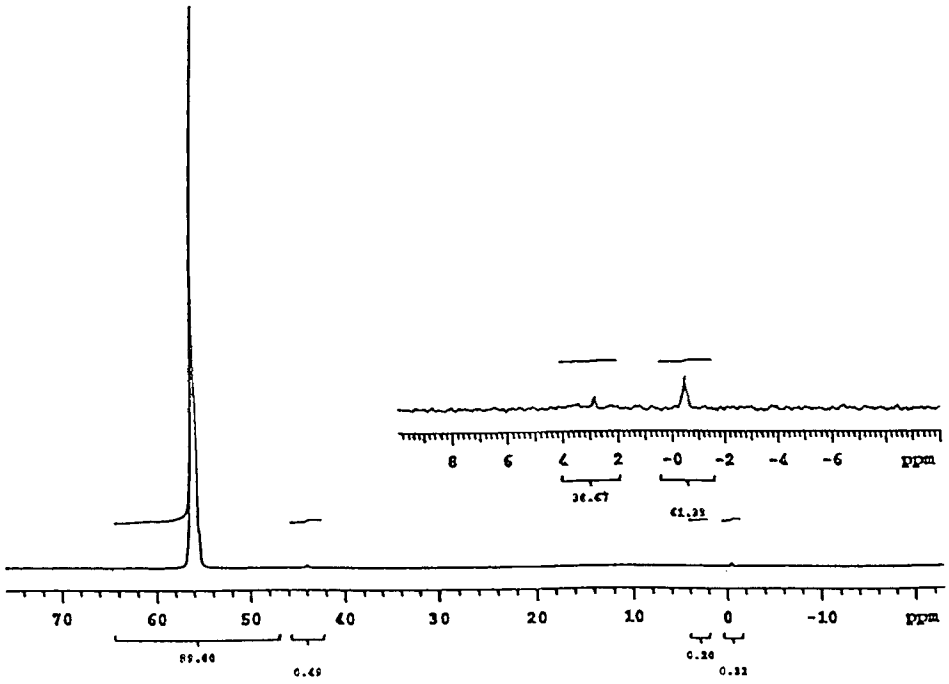

Fig. 1: ^{31}P NMR of the crude oligonucleotide synthesized.

experiments, it was found that a 0.2M solution of PADS (6 molar equivalents) in 1:1 (v/v) CH_3CN:3-picoline for 60 sec gave a sulfurization efficiency of >99.6%.

The oligonucleotide phosphorothioate synthesized under these optimized conditions was analysed by ^{31}P NMR (Fig. 1), capillary gel electrophoresis (CGE)[30] (Fig. 2), SAX-HPLC (Fig. 3), and by measuring crude yield by UV. The following table compares sulfur transfer and synthesis efficiencies of Beaucage reagent and that of with PADS.

Reagent	Crude Yield OD/μmole	Crude Full Length (%)	Full Length after purification (n-1) (%)	P=S : P=O (^{31}P NMR)	P=S : P=O (SAX HPLC)
Beaucage	115-120	72	91-92 (2.3)	99.4 : 0.6	99.5 : 0.5
PADS	118-125	73	91-92 (2.2)	99.6 : 0.4	99.6 : 0.4

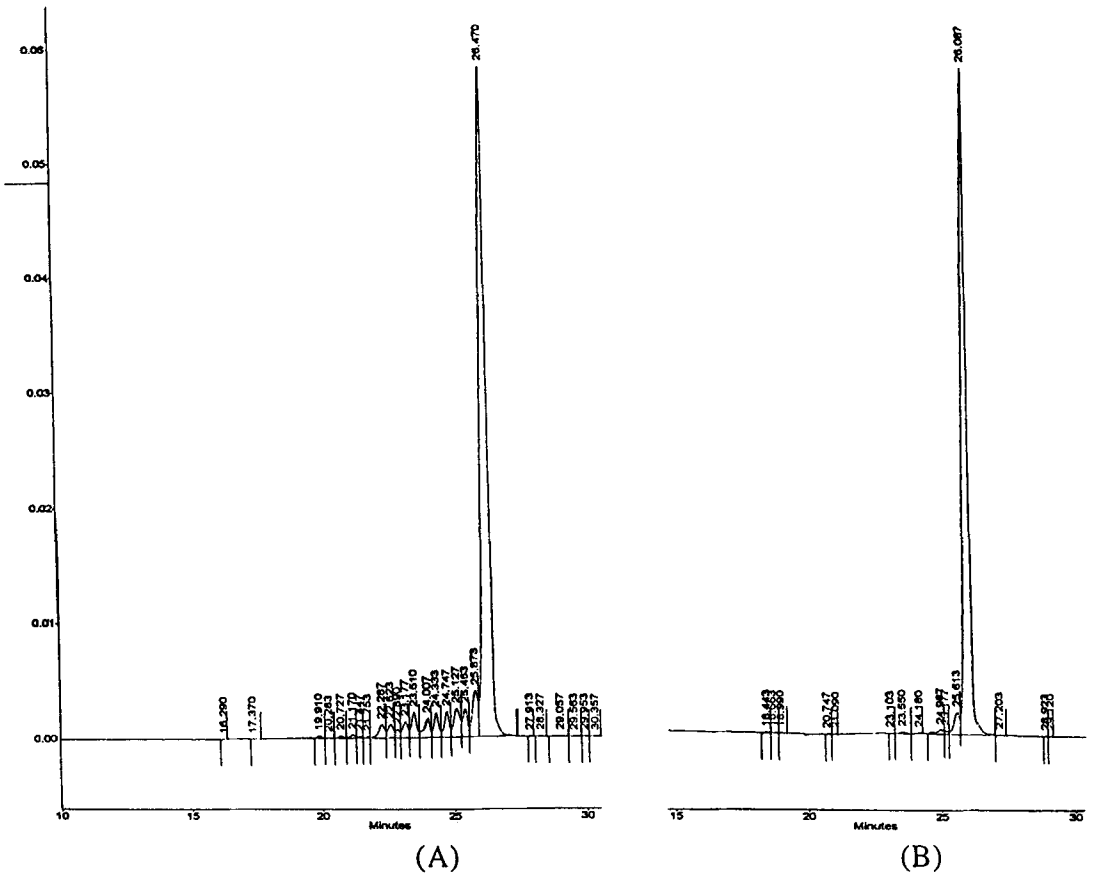

Fig. 2: Capillary gel electrophoresis of the crude (A) and reversed phase purified (B) oligonucleotide.

The results demonstrate that PADS is an efficient sulfur transfer reagent in the large scale synthesis of oligodeoxyribonucleotide phosphorothioates, fully equivalent in performance to Beaucage reagent. To verify the efficiency of this reagent for different sequences, this optimized conditon were used to synthesize four other oligodeoxyribonucleotide phosphorothioates (5'-GCC-CAA-GCT-GGC-ATC-CGT-CA, ISIS 2302; 5'-GTT-CTC-GCT-GGT-GAG-TTT-CA, ISIS 3521; 5'-TCC-GTC-ATC-GCT-CCT-CAG-GG, ISIS 2503; 5'-GCG-TTT-GCT-CTT-CTT-CTT-GCG, ISIS 2922) (20 to 21-mer in length) and again found to be an efficient sulfur transfer reagent. In addition, during our investigation we noted that PADS gave significantly more reproducible results than Beaucage reagent. This may be due to the absence of oxidizing

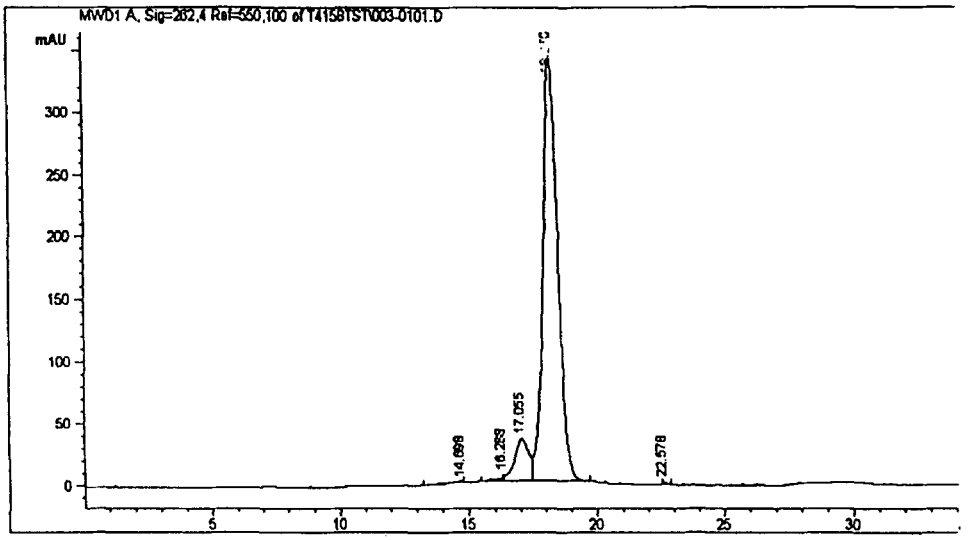

Fig. 3: SAX HPLC analysis of the reversed phase purified oligonucleotide.

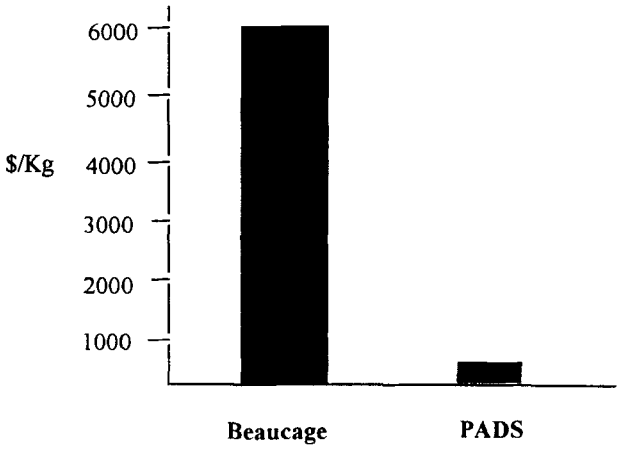

Fig. 4: Comparison of current kilogram scale costs for the materials.

by-products when using PADS. In addition, a solution of PADS in acetonitrile/3-picoline (1:1, v/v) stored at room temperature for two weeks gave results similar to those from a freshly prepared solution.[31]

Another major advantage of PADS over Beaucage reagent is its cost. The following chart clearly shows that the raw material cost of oligodeoxyribonucleotide phosphorothioate drugs could be substantially reduced by using this reagent.[32-33]

In conclusion, we have demonstrated that phenylacetyl disulfide, if used under proper conditions, is a very effective replacement for Beaucage reagent in the synthesis of phosphorothioate oligonucleotides.

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