

Synthesis of Antisense Oligonucleotides: Replacement of 3H-1,2-Benzodithiol-3-one 1,1-Dioxide (Beaucage Reagent) with Phenylacetyl Disulfide (PADS) As Efficient Sulfurization Reagent: From Bench to Bulk Manufacture of Active Pharmaceutical Ingredient

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Abstract:

It is demonstrated that phosphorothioate oligodeoxyribonucleotides can be synthesized on scales from 1 μ mol to 150 mmol using phenylacetyl disulfide (PADS) as an efficient and economical replacement for Beaucage reagent. A 0.2 M solution of PADS in a mixture of 3-picoline and acetonitrile (1:1 v/v) as solvent with 60–120 s contact time efficiently (>99.6%) sulfurizes phosphite triesters to phosphorothioate triester linkages. Phenylacetyl disulfide reagent is inexpensive and scaleable and is currently being used by us for the manufacture of antisense phosphorothioate oligodeoxyribonucleotide active pharmaceutical ingredients (API).

Introduction:

Antisense oligonucleotides as modulators of gene expression represent an exciting new drug technology.^{1–9} Phosphorothioate oligodeoxyribonucleotides are now among the most intensively investigated nuclease-resistant antisense analogues, as evidenced by a number of ongoing clinical trials and one FDA approved drug.^{10,11} A major advantage of the antisense 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. Structurally, the phosphorothio-

ates differ from natural DNA by the replacement of one of two nonbridging oxygen atoms by a sulfur atom at each internucleotide linkage.

With the first antisense drug (Vitravene) being approved by the U.S. Food and Drug Administration and recommended by the European panel for marketing as a treatment for CMV retinitis in AIDS patients and with several systemic drugs potentially reaching the market in the next several years, the development of economical and environmentally safe methods for the synthesis of high quality oligonucleotides has become a major focus of our research.^{12–25} Typically, synthesis of oligonucleotides on scales up to 150 mmol is performed in a cyclic manner on automated solid-phase synthesizers using phosphoramidite derivatives of protected nucleosides with 4,4'-dimethoxytrityl (DMT) protection of the 5'-hydroxyl group, benzoyl protection for adenine (dA^{bz}) and cytosine (dC^{bz}), and isobutyryl protection for guanine (dG^{ibu}).

Phosphoramidite chemistry^{26–28} requires sulfurization after each coupling. It is crucial that the sulfur transfer step be

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highly efficient. During the past few years, a variety of sulfurizing reagents have been investigated.^{29–43} Among these, only 3H-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent) has been used in the large scale (≥ 150 mmol) synthesis of phosphorothioate drugs. However, the cyclic sulfoxide by-product formed during sulfurization is a potent oxidizing reagent^{29,30} and may be responsible for observed inconsistent reagent performance lot to lot. This reagent is expensive even at hundreds of kilograms scale, since the synthesis involves multiple steps and because of the handling of difficult materials that makes it not amenable to large scale production. Occasionally, the commercially available reagent is contaminated with the peracid of the precursor to the Beaucage reagent and when used, leads to objectionable levels of phosphodiester. Thus, there was an urgent need to replace this sulfurizing reagent.

Sulfurization during Solid-Phase Synthesis

In our ongoing efforts to improve the synthesis of phosphorothioate oligonucleotides we wanted to replace Beaucage reagent during the sulfurization step with a reagent that is equally or more efficient, consistent in performance, inexpensive, easy to scale-up, does not possess oxidizing properties, and is also applicable for the synthesis of 2'-O-modified oligonucleotides (second generation antisense drugs).⁴⁴

Phenylacetyl disulfide (PADS) has been reported in the literature as a sulfur transfer reagent in the synthesis of phosphorothioate oligodeoxyribonucleotides,^{42,43} 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(diisopropoxyphosphinothioyl) 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

Table 1. Synthesis parameters of cycle used on Pharmacia OligoPilot II synthesizer at 180–190 μ mol scale

step	reagent	volume (ml)	time (min)
detritylation	3% DCA/toluene	50	4
coupling	phosphoramidite (0.2M), 1H-tetrazole (0.45 m)	1.6, 5	5
sulfurization	PADS (0.2M) in 3-picoline/CH ₃ CN (1:1 v/v)	6	2
capping	Ac ₂ O/pyridine/CH ₃ CN, NMI/CH ₃ CN	1.7, 1.7	0.5

investigated. Thus, we were prompted to investigate the efficiency of PADS under a variety of conditions.

Investigation of PADS for Sulfurization. A series of experiments with homo-thymidine phosphorothioate 20-mer as test sequence using PADS on 1 μ mol scale were performed on ABI 394 DNA/RNA synthesizer. Analysis by ³¹P NMR indicated that certain solvents could be used to rapidly and efficiently transfer sulfur to the phosphite triester linkages attached to controlled-pore glass (CPG) solid support. This observation was also confirmed by performing a mixed sequence (20-mer) on the ABI synthesizer. Reactor design and solid support are crucially important for successful oligonucleotide synthesis. In contrast to small-scale synthesizers (e.g., ABI 394, PerSeptive Cyclone, Expedite, Beckman Oligo 1000M, Cruachem PS250 synthesizers) which typically use silica gel-based CPG solid support, medium- (0.2–3 mmol, Pharmacia OligoPilot II) and large-scale synthesis (10–250 mmol, Pharmacia OligoProcess) is most commonly performed in packed-bed column reactors using polystyrene-based polymers as solid support.

Phosphorothioate oligonucleotide **1** PS-d(TCCCGCCT-GTGACATGCATT) (ISIS 5132)⁴⁵ was synthesized on a Pharmacia OligoPilot II DNA/RNA synthesizer closely resembling the production-scale synthesizer (Pharmacia OligoProcess). Primer support T (loading 95 μ mol/g, Pharmacia) containing the 3'-terminal nucleoside of **1** was tightly packed in a steel column (volume 6 mL). Details of the synthesis cycle are given in Table 1. 3% Dichloroacetic acid in toluene was used for deblocking of the dimethoxytrityl groups from the 5'-hydroxyl group of the nucleoside.^{14,15} 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. Standard cyanoethyl-protected phosphoramidites were used during the synthesis. For sulfurization using PADS, bases such as collidine, picoline, lutidine, and pyridine were selected. At the end, the oligonucleotide was cleaved from solid support and deprotected using aqueous ammonium hydroxide at 55 °C for 18 h. The crude oligonucleotide was analyzed by ³¹P NMR using D₂O as solvent. The results are shown in Table 2.

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(44) Beaucage reagent has been found to be very inefficient for the synthesis of 2'-O-methoxyethyl modified phosphorothioate oligonucleotides on OligoPilot II synthesizers due to unacceptable levels (20–25% based on ³¹P NMR and SAX HPLC analysis) of phosphodiester contamination in the product.

(45) ISIS 5132 is targeted to the 3'-untranslated region of human *c-raf* mRNA, selectively inhibiting *c-raf* gene expression.⁴⁶ This oligonucleotide inhibits the growth of a variety of tumor types in vivo using nude mouse tumor xenografts and sequence-specific antitumor activity supports an antisense mechanism of action in vivo.⁴⁷ Clinical evaluation (Phase II) of this drug in humans as potential treatment for a variety of cancer targets is currently ongoing.

Table 2. Sulfurization efficiency of PADS under various conditions

concn (M)	solvent	time (min)	column vol	P = S	P = O
1	1:1 (v/v) collidine:CH ₃ CN	5		99.63	0.37
1	1:1 (v/v) 2-picoline:CH ₃ CN	5		99.34	0.66
1	1:1 (v/v) utidine:CH ₃ CN	5		99.50	0.50
1	1:1 (v/v) picoline:CH ₃ CN	5		99.48	0.52
1	1:1 (v/v) picoline:CH ₃ CN	3		99.68	0.32
0.8	1:1 (v/v) 3-picoline:CH ₃ CN	5	0.5	98.91	0.52
0.4	1:1 (v/v) 3-picoline:CH ₃ CN	5	0.5	98.46	0.57
0.2	1:1 (v/v) 3-picoline:CH ₃ CN	5	0.5	97.27	0.31
0.2	1:1 (v/v) 3-picoline:CH ₃ CN	2	1	99.21	0.33
0.2	1:1 (v/v) 3-picoline:CH ₃ CN	1	1	99.01	0.40
0.1	1:9 (v/v) 3-picoline:CH ₃ CN	2	1	97.79	0.47
0.1	1:9 (v/v) 3-picoline:CH ₃ CN	1	1	98.0	1.0
0.1	1:1 (v/v) pyridine:CH ₃ CN	1	1	85.39	1.44
0.1	1:1 (v/v) 3-picoline:CH ₃ CN	1	1	99.15	0.24

Table 3. Comparison of sulfur transfer efficiencies of Beaucage and PADS for the synthesis of oligonucleotide 1 at 180–190 μmol scale

reagent	crude yield mg/μmol	crude full length (%)	purified full length ⁴⁸ (n - 1) (%)	P = S:P = O (³¹ P NMR)	P = S:P = O (SAX HPLC)
Beaucage	6.8	72	91–92 (2.3)	99.4:0.6	99.5:0.5
PADS	7.3	73	91–92 (2.2)	99.6:0.4	99.6:0.4

On the basis of the data obtained, it was clear that sulfurization was efficient using PADS in a mixture of acetonitrile and 3-picoline. Further optimization of conditions was attempted by varying contact time, concentration of the reagent, and the number of equivalents used (estimated by column volume). Except for the sulfurization step, identical conditions were used on the same oligonucleotide **1** at the same scale (180 μmol). The results are summarized in Table 2. It was found at the end of analysis that a 0.2 M solution of PADS in 1:1 (v/v) acetonitrile:3-picoline with a contact time of 60 s gave a sulfurization efficiency of >99.6%. The phosphorothioate oligonucleotide synthesized under these optimized conditions was further purified by C₁₈ reversed phase HPLC that allowed facile separation of the 5'-O-DMT- on oligonucleotide from the capped failure sequence. Extensive analysis of the purified material was performed using capillary gel electrophoresis (CGE), SAX-HPLC, ³¹P NMR, and by measuring crude yield by UV. Table 3 compares the sulfur transfer and synthesis efficiencies of PADS and Beaucage reagent.

Table 4. Analytical data of phosphorothioate oligonucleotide **2** synthesized using PADS on 150 mmol scale on OligoProcess synthesizer

reagent	purified yield g/mmol	crude full length (%)	purified full length ⁴⁸ (n - 1) (%)	P = S:P = O
PADS	3.12	72	92–93 (2.3) 97 (2.3) (after fractionation)	³¹ P NMR 99.8:0.2 SAX HPLC 99.8:0.2 LC-MS 99.8:0.2

The results clearly demonstrate that PADS is an efficient sulfur transfer reagent in the synthesis of phosphorothioate oligodeoxyribonucleotides, fully equivalent in performance to Beaucage reagent. To verify the efficiency of this reagent for different sequences, this optimized condition was used to synthesize five other phosphorothioate oligodeoxyribonucleotides (5'-GCC-CAA-GCT-GGC-ATC-CGT-CA, **2**; 5'-GTT-CTC-GCT-GGT-GAG-TTT-CA, **3**; 5'-TCC-GTC-ATC-GCT-CCCT-CAG-GG, **4**; 5'-GCG-TTT-GCT-CTT-CTT-GCG, **5**; 5'-GTG-CTC-ATG-GTG-CAC-GGT-CT, **6**) (20 to 21-mer in length) and again found to be an efficient sulfur transfer reagent.

Large-Scale Synthesis of a Phosphorothioate Oligonucleotide. Next, we scaled up the synthesis of **2** another 800-fold to 150 mmol scale on Pharmacia's OligoProcess synthesizer. Three syntheses were performed using PADS for sulfurization and 3% DCA/toluene for detritylation. The total synthesis time was shortened to 9 h for a 20-mer phosphorothioate oligonucleotide at this large scale. Even better performance of sulfurization (as measured by anion-exchange HPLC, ³¹P NMR and LC-MS) was observed compared to OligoPilot II syntheses. The oligonucleotide was purified in the usual manner using C₁₈ reversed phase HPLC; appropriate fractions were collected, detritylated, precipitated and lyophilized. An average of 468 g of purified drug was obtained per 150 mmol synthesis. Analysis was then performed on the purified active pharmaceutical ingredient (API). Table 4 shows the data obtained from the 150 mmol scale synthesis. Again, high quality oligonucleotide was obtained using PADS for sulfurization. Figures 1, 2, and 3 show CGE, SAX, and ³¹P NMR analyses of the phosphorothioate oligonucleotide synthesized, respectively.

Impact of Water during Sulfurization Step. During our investigation we noticed that PADS gave significantly more consistent results than Beaucage reagent. This may be due to the absence of oxidizing by-products when using PADS. In addition, the quality of 3-picoline used was not very high, possibly having higher levels of water than the acetonitrile used. This prompted us to investigate the impact of water during the sulfurization step. Although it is generally accepted that the presence of water in most sulfur transfer reagents leads to increased levels of phosphate diester linkages (possibly due to reaction of water with the initially formed phosphonium salt intermediates), we were encouraged to find a single report of the use of pyridinium and trialkylammonium tetrathionates in which it was claimed³⁸ that presence of water led to no increase in unwanted oxidation products.

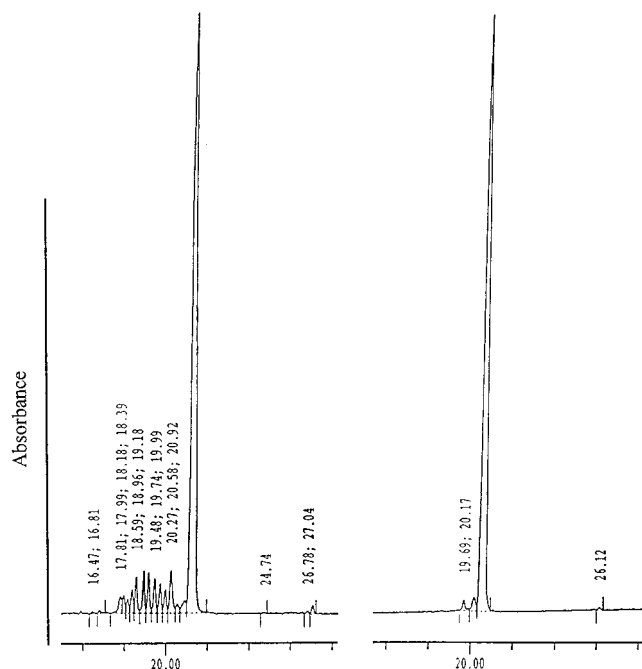


Figure 1. CGE Analysis of phosphorothioate oligonucleotide synthesized.

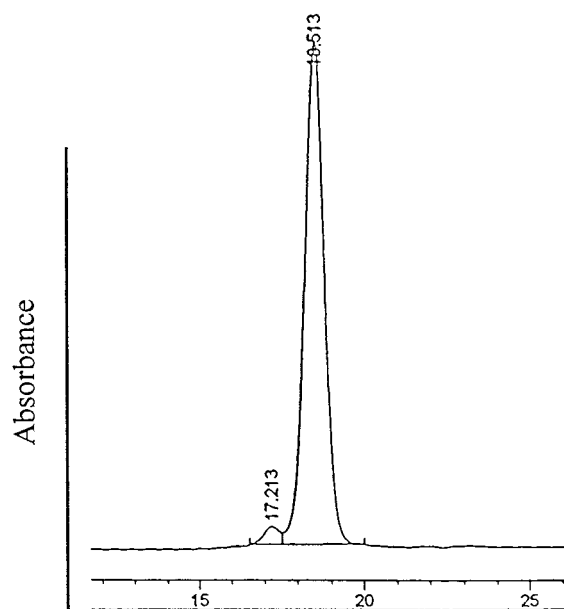


Figure 2. Anion-exchange HPLC analysis of phosphorothioate oligonucleotide synthesized.

The 20-mer phosphorothioate oligonucleotide **2** was chosen as an example. All syntheses were performed on Pharmacia Oligopilot II DNA/RNA synthesizer using cyanoethyl phosphoramidite synthons (0.2 M solution in acetonitrile). Pharmacia HL 30 Primer support dA was used in all experiments. Amidites and tetrazole solutions were prepared using anhydrous acetonitrile (~ 10 ppm) and were dried further by addition of activated 4 Å molecular sieves (~ 50 g/litre). Acetonitrile of varying water content was prepared by adding known volumes of purified water to anhydrous acetonitrile; the final water content of each solvent mixture was estimated by Karl Fischer titration. These solvent mixtures were used both for preparing PADS solution

and as the wash solvent during each step on a cycle. At the end of synthesis trityl-on support-bound oligo was treated with 30% aqueous ammonium hydroxide solution for 16 h at 55 °C to effect release from support and base and phosphate deprotection. Yield (expressed in mg of oligonucleotide/ μ mol of support),⁴⁹ capillary gel electrophoresis (CGE), and ³¹P NMR data were collected for each synthesis. Increasing the water content in PADS solution from 10 ppm (control experiment) to 1200 ppm resulted in an unacceptable decrease in total yield, although the quality of the material produced was unchanged. Particularly interesting was the observation that the presence of water did not lead to an increase in the amount of phosphate diester produced.

Stereo-Reproducibility during Sulfurization in the Synthesis of Phosphorothioate Oligonucleotides Using Phenylacetyl Disulfide (PADS). During each oligonucleotide synthesis cycle, nucleophilic attack of the 5'-terminal hydroxyl on a tetrazole-activated nucleoside phosphoramidite resulted in formation of a chiral phosphite triester intermediate, which was then sulfurized with a sulfur transfer reagent. The 1*H*-tetrazole-catalyzed activation of phosphoramidite took place with epimerization at the phosphorus center. Hence, an initial enantiomeric excess present in phosphoramidite monomer should not influence the ratio of phosphite triester diastereoisomers formed, although bulky activator chiral groups in proximity to the phosphorus atom have been shown to influence the stereochemical outcome of the coupling reaction. We were interested in investigating the influence of PADS on the net stereo-reproducibility of phosphorothioate linkages formed.

The experimental approach was to synthesize mixed-sequences with a single phosphorothioate linkage. Shortmers (10-mers and 8-mers) were chosen as model oligonucleotides as the literature reports baseline RP-HPLC separation of the resulting diastereomers may be achieved, allowing ready determination of the product diastereomer ratio. Oligonucleotides were synthesized via phosphoramidite coupling chemistry using tetrazole as activator. The oxidizing agent used for formation of phosphodiester linkages was 10% *t*-BuOOH/CH₃CN. A 0.2 M solution of phenylacetyl disulfide (PADS) in 1:1 (CH₃CN/3-picoline) was used for sulfurization. After synthesis, the oligonucleotides were deprotected using standard conditions (30% concentrated ammonia for 12h, 55 °C). The crude oligonucleotides were then analyzed by reversed phase HPLC using a Hypersil-OD column (5 μ m, 100 \times 4.6 mm). Table 5 shows the stereo-reproducibility of phosphorothioate linkages formed when using PADS and clearly demonstrates that regardless of the monophosphorothioate DNA oligonucleotide base sequence synthesized

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(48) 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 the DMT-on peak results in less than a 5% loss of full-length material.

(49) Experience has taught us that yields expressed in terms of weight/ μ mole of support are more reliable than those expressed in terms of optical density/ μ mole.

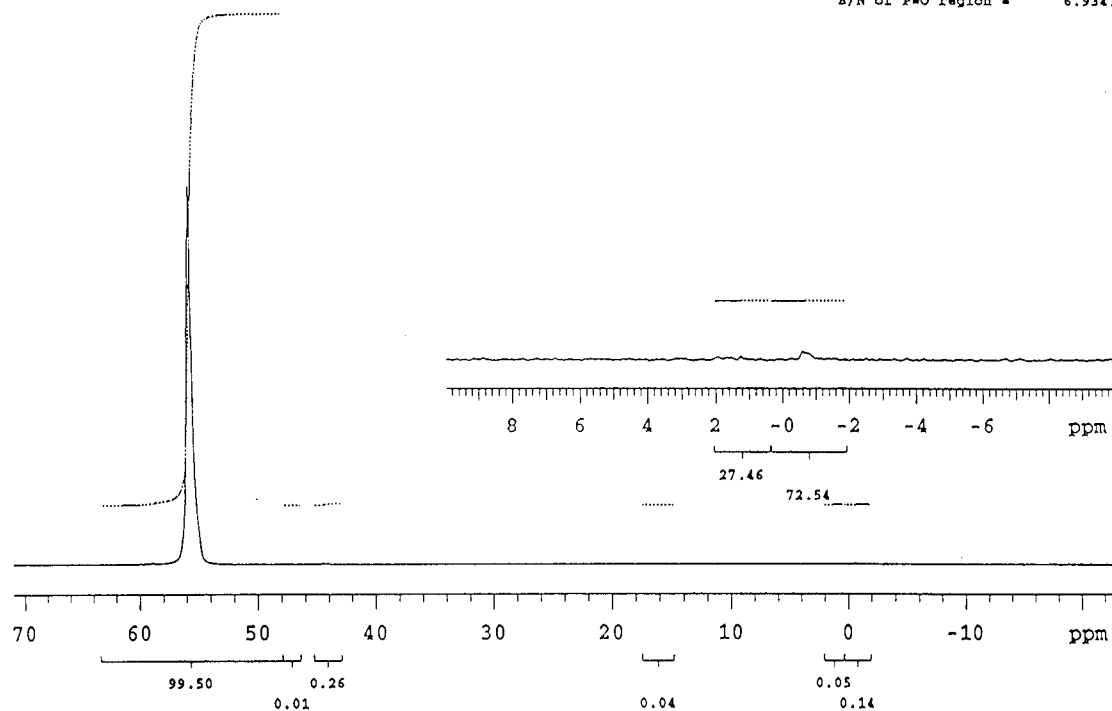


Figure 3. ^{31}P NMR analysis of phosphorothioate oligonucleotide synthesized.

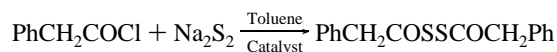
Table 5. Separation of diastereomers of monophosphorothioate-substituted (PS) oligonucleotides using PADS

no.	oligonucleotide sequence (5'-3')	Rp/Sp	t_R (min)
1	TTApsCTTTTTT	59.39/40.61	56.18/57.37
2	TTTTTTTCpsCT	57.08/43.39	56.20/57.55
3	TTTTTCpsCTTTT	59.04/40.96	56.56/58.46
4	TCpsCTTTTTTT	57.16/42.84	55.91/57.74
5	TTGCTTpsCC	55.68/44.26	51.54/52.27
6	TTGCTpsTCC	50.82/49.18	50.93/52.23q
7	TTGCpsTTCC	50.48/49.52	49.16/50.23
8	TTpsGCTTCC	57.92/42.08	49.99/51.51
9	TpsTGCTTCC	55.55/44.45	51.51/52.96
10	CCATCTpsTC	55.41/44.59	52.19/52.61
11	CCATCpsTTC	59.37/40.63	51.95/52.58
12	CCATpsCTTC	55.53/44.47	50.26/51.49
13	CCapsTCTTC	55.01/44.99	50.67/50.94
14	CCpsATCTTC	55.53/44.47	55.17/50.99
15	CpsCATCTTC	57.39/42.61	51.10/51.43
16	TCCTCGpsTC	55.99/44.01	49.09/49.75
17	TCCTCpsGTC	58.12/41.88	48.61/49.27
18	TCCTpsCGTC	52.66/47.34	48.95/49.38
19	TCCpsTCGTC	53.19/46.81	48.34/49.14
20	TCpsCTCGTC	52.88/47.12	47.17/47.44
21	TpsCCTCGTC	52.33/47.67	45.67/46.35

and position of the phosphorothioate linkage, all product Rp/Sp diastereomer ratios were between 40:60 and 60:40.

Economics of Sulfurization. Unlike synthesis of Beaucage reagent, that of phenylacetyl disulfide involves a single step and is easily scaleable. The synthesis of PADS involves reaction of phenylacetyl chloride with sodium disulfide under phase transfer conditions using hexadecyltributylphosphonium bromide as catalyst. Thus, an aqueous solution of sodium disulfide is prepared by heating a mixture of sulfur

and sodium sulfide in water at 90 °C for 30 min with stirring. To this solution was added dropwise a solution of phenylacetyl chloride and phase transfer catalyst dissolved in toluene at 0 °C. The reaction mixture is then worked up and crystallized first from methanol and then from ether/hexane to afford colorless crystals of PADS. A single batch of 50–75 kg of PADS has been synthesized without any difficulty.^{50,51}



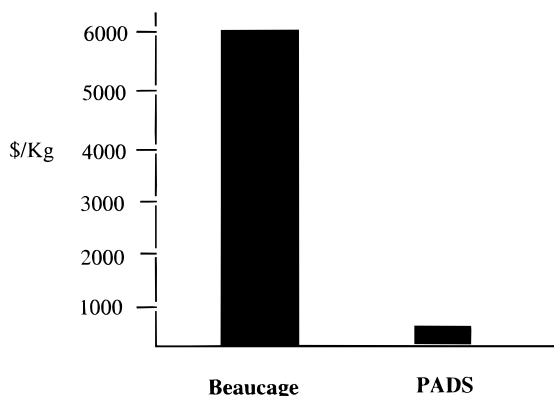
For the successful development of antisense drugs, the cost of raw materials and of oligonucleotide synthesis has to be kept low. The following chart clearly shows that the raw material cost of phosphorothioate oligodeoxyribonucleotide drugs could be substantially reduced by using the PADS reagent.

Conclusions

As evident from the data presented in this paper, a solution of phenylacetyl disulfide in 3-picoline/acetonitrile allows sulfurization of phosphite triesters to phosphorothioate triesters from 1 μmol to 150 mmol scale, providing phosphorothioate oligonucleotides in high yield and purity. Multiple analytical methods showed equivalence to oligonucleotides synthesized using Beaucage reagent. In conclusion, we have unequivocally proven that Beaucage reagent can be replaced with PADS, without compromising yield or quality of the antisense oligonucleotide products.

(50) Kodomari, M.; Fukuda, M.; Yoshitomi, S. *Synthesis* **1981**, 637.

(51) PADS is now commercially available from different vendors.



Comparison of current kilogram scale costs for sulfur transfer reagents.

Experimental Section

^{31}P NMR spectra were recorded on a Unity-400 spectrometer (Varian) operating at 161.9 MHz. Capillary gel electrophoresis^{52,53} was performed on a eCAP ssDNA 100 gel capillary (47 cm) on a P/ACE system 5000 using Tris/borate/7 M urea buffer (all Beckman), running voltage 14.1 kV, temp 40 °C. Electrospray ionization mass spectrometry was performed using a Hewlett-Packard 59987A electrospray quadrupole mass analyzer using negative polarity. Anion-exchange high performance liquid chromatography (SAX HPLC) of DMT-off oligonucleotide was performed on a Resource Q column (1 mL) from Pharmacia, temp 60 °C, flow rate 1 mL/min, $\lambda = 260$ nm, mobile phases: NaCl (1 M), sodium phosphate (0.1 M) pH 11.5 (A), NaCl (2 M), sodium phosphate (0.1 M) pH 11.5 (B), gradient 100% A for 5 min, 100 to 0% A from 5 to 40 min, t_{R} (all-PS) 18.5 min, t_{R} (mono-PO) 17.2 min.

Phosphorothioate Oligodeoxyribonucleotide Synthesis.

(a) Applied Biosystems 394 DNA/RNA synthesizer, commercial columns with CPG support (Glen Research), scale 1 μmol , standard 5'-O-DMT cyanoethyl phosphoramidites (0.2 M in CH_3CN), 1*H*-tetrazole (0.45 M in CH_3CN), coupling time: 25 s, (b) Pharmacia OligoPilot II, HL 30 Primer polystyrene support T or dA or dG (loading 90 ± 10 $\mu\text{mol/g}$), scale 160–180 μmol , standard 5'-O-DMT cyano-

ethyl phosphoramidites (0.2 M in CH_3CN ; 2.0 equiv), 1*H*-tetrazole (0.45 M in CH_3CN), coupling time: 5 min, 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (0.5 M in CH_3CN) for 60 s, phenylacetyl disulfide (0.2 M in 3-picoline/ CH_3CN , 1:1 v/v) for 60–90 s.

Work Up. Primer support (~100 mg) was treated with 2 mL of NH_4OH (30%) for 15 h at 60 °C, filtered and rinsed with ethanol/water (1/1, v/v), and the combined solutions were evaporated to dryness under vacuum. CPG support was treated with 2 mL of NH_4OH (30%) for 1 h at room temperature, filtered, and rinsed with 1 mL NH_4OH (30%), and the combined solutions were kept at 60 °C for 15 h, followed by evaporation to dryness under vacuum. The residue was dissolved in 200 μL of water.

HPLC Analysis and Purification. Analysis and purification of oligonucleotides by reversed phase high performance liquid chromatography (RP-HPLC) was performed on a Waters Nova-Pak C_{18} column (3.9×300 mm) using a Waters HPLC system (600E system controller, 996 photodiode array detector, 717 autosampler). For analysis an acetonitrile (A)/0.1 M triethylammonium acetate gradient was used: 5–35% A from 0 to 10 min, then 35–40% A from 10 to 20 min, then 40–95% A from 20 to 25 min, flow rate = 10 mL/min/50% A from 8 to 9 min, 9 to 26 min at 50%, flow rate = 1.0 mL/min, t_{R} (DMT-off) 10–11 min, t_{R} (DMT-on) 14–16 min. The DMT-on fraction was collected and was evaporated in a vacuum, redissolved in 50 μL water and the DMT group removed as described below.

Dedimethoxytritylation. An aliquot (30 μL) was transferred into an Eppendorff tube (1.5 mL), and acetic acid (50%, 30 μL) was added. After 30 min at room temperature sodium acetate (2.5 M, 20 μL) was added, followed by cold ethanol (1.2 mL). The mixture was vortexed and cooled in dry ice for 20 min. The precipitate was spun down with a centrifuge, the supernatant was discarded, and the precipitate was rinsed with ethanol and dried under vacuum.

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