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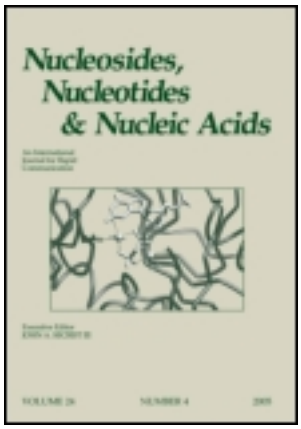

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Polysulfide Reagent in Solid-Phase Synthesis of Phosphorothioate Oligonucleotides: Greater than 99.8% Sulfurization Efficiency

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POLYSULFIDE REAGENT IN SOLID-PHASE SYNTHESIS OF PHOSPHOROTHIOATE OLIGONUCLEOTIDES: GREATER THAN 99.8% SULFURIZATION EFFICIENCY

Achim H. Krotz, Alice Hang, Dennis Gorman, and Anthony N. Scozzari ▫ *Isis Pharmaceuticals, Inc., Carlsbad, California, USA*

▫ *A solution of sulfur (0.1 M) and sodium sulfide (0.01 M) in 3-picoline, referred to as polysulfide reagent, rapidly converts trialkyl and triaryl phosphite triesters to the corresponding phosphorothioate derivatives. Greater than 99.8% average stepwise sulfurization efficiency is obtained in the solid-phase synthesis of DNA and RNA phosphorothioate oligonucleotides via the phosphoramidite approach.*

Keywords Sulfur, Polysulfide, Sulfurization, Antisense, Phosphorothioate, Oligonucleotide

INTRODUCTION

Modified oligonucleotides as modulators of gene expression are currently under intense investigation as novel therapeutic agents of high specificity through antisense mechanisms of action.[1–3] They provide a useful tool in functional genomics and target validation.[4] Phosphorothioate diester (PS) oligonucleotides, where one nonbridging oxygen of the internucleotide linkage is replaced by a sulfur atom, are much more resistant to nuclease-promoted degradation than corresponding phosphate diester (PO) oligonucleotides. They represent the first class of antisense therapeutics to get marketing approval by regulatory agencies.* A large number of oligonucleotide drugs are currently being evaluated in preclinical and clinical studies as treatment for a wide range of diseases including cancer, cardiovascular disease, autoimmune diseases, and metabolic, inflammatory, and infectious diseases.

The preparation of PS-oligonucleotides is a multistep process that may be divided into two distinct operations: solid-phase synthesis using phosphoramidite

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*In August 1998, Isis Pharmaceuticals, Inc., Carlsbad, CA and Ciba Vision, a division of Novartis AG, Switzerland, received FDA approval for VitraveneTM (fomivirsen sodium injectible) for the treatment of cytomegalovirus (CMV) retinitis in patients with AIDS.

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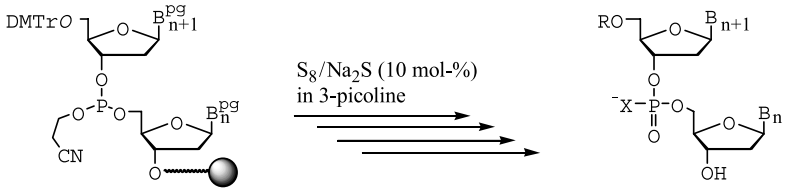

SCHEME 1 Convesion of P(III) species to phosphorate using S/Na_2S reagent.

chemistry followed by downstream processing.[5] In the first operation, a fully protected oligonucleotide is assembled stepwise in a non-stereospecific fashion from the 3′- to the 5′-terminus by repetition of a four-reaction elongation cycle (detritylation, coupling, sulfurization, capping) without isolation of intermediates. In the second operation, deprotection, cleavage from the support, purification, and isolation steps are performed.

The focus of the present investigation is the sulfurization step during solid-phase synthesis. The product of the coupling reaction prior to sulfurization is a trialkyl phosphite triester derivative (oxidation state P^{III}), which is oxidatively sulfurized to the corresponding trialkyl phosphorothioate triester (oxidation state P^V) species. Deprotection of the internucleotide linkages after the entire oligonucleotide has been assembled then affords a PS-oligonucleotide (Scheme 1). The need for a highly efficient sulfurization reaction after each coupling has led to the development of a variety of sulfurizing reagents.[6–24] Recently, during optimization of the sulfurization step using phenylacetyl disulfide (PADS)[25] we noticed that the average stepwise sulfurization efficiency between syntheses varied between 99.9% and 99.5% per linkage resulting in oligonucleotide products containing between 2 and 10% $(PO)_1$-oligonucleotide.† We found that "aged" PADS solutions in 3-picoline/acetonitrile satisfactorily yielded oligonucleotides with low PO content. The dissociation of the PADS molecule is crucial to the formation of a more reactive and more efficient (in terms of PS:PO ratio) reagent. Equilibria between acyldisulfide, sulfur, and polysulfide species, similar to those observed in *N*,*N*-dimethylacetamide,[26] may explain these observations.

The first sulfurization reagent used in solution-phase PS-dinucleotide synthesis was sulfur.[27] However, slow reaction kinetics and poor solubility precluded its use in automated solid-phase synthesis using the commonly used cyanoethyl

†A class of process-related substances that is frequently observed in PS-oligonucleotides is a group of oligonucleotides that are identical to the main product, except it contains one (or more) phosphate (PO) diester linkage(s) instead of a PS-diester linkage randomly distributed in the sequence [$(PO)_n$-oligonucleotide, n indicates the number of PO linkages]. Removal of $(PO)_n$-oligonucleotide on preparative scale using chromatographic separation technology is difficult to achieve without significant yield loss.

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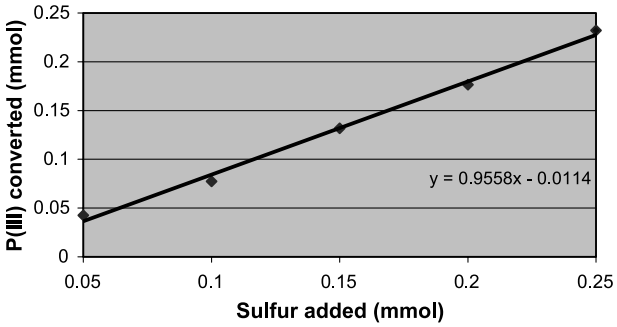

FIGURE 1 The sulfurization effectiveness of polysulfide reagent in the sulfurization of $P(OPh)_3$ is approximately 96%. Polysulfide reagent is added to $P(OPh)_3$ and the ratio of $S{=}P(OPh)_3$ to starting material is determined by HPLC.

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phosphoramidite approach.[5] Nucleophiles (e.g., sulfide ions) have an accelerating effect on the sulfurization of P(III) species, possibly by formation of polysulfides.[28–31] We tested the hypothesis that sulfur activated with sodium sulfide in a suitable solvent system may produce PS oligonucleotides with low PO content.

RESULTS AND DISCUSSION

As a starting point for the formulation design, we used the solvent from our currently preferred sulfurizing reagent system [phenylacetyl disulfide (0.2 M) in acetonitrile/3-picoline, 1:1, v/v]. Under argon, sulfur (6.4 g, 200 mmol) was dissolved in 3-picoline (500 mL), then finely ground $Na_2S \times 9H_2O$ (4.8 g, 20 mmol) was added. An immediate color change from yellow to dark red was observed. After 1 h, acetonitrile (500 mL) was added and the mixture was stirred overnight.

Since the immediate change in color upon mixing sulfur and sodium sulfide indicated a change in chemical composition we investigated the performance of the polysulfide reagent with respect to the ability to transfer sulfur using a sulfurization effectiveness test (Figure 1). Triphenyl phosphite (1 M, in acetonitrile) was reacted with polysulfide reagent and the conversion to triphenyl phosphorothioate was monitored by HPLC.[‡] Greater than 95% of sulfur is consumed in the sulfurization

[‡]Polysulfide reagent (0.1 M, 0.5–2.5 mL) was added to a $P(OPh)_3$ solution (1 M, 0.25 mL). After 15 min, a sample (0.1 mL) was removed, diluted with acetonitrile (0.9 mL), and analyzed by HPLC (injection volume 0.02 mL). HPLC conditions: Phenomenx Luna C18(2), 5 μm, 250 × 4.6 mm, solvent A: ammonium acetate (0.1 M)/1% sodium hydroxide(1 N), solvent B: acetonitrile, linear gradient from 30% to 1% A in 20 min, flow 1.5 mL/min, detector wavelength 260 nm. Retention times: $P(OPh)_3$: 11.2 min, $SP(OPh)_3$: 10.0 min. The sulfurization effectiveness is the slope of the linear regression line of a plot of *P(III) converted* [a × b × 2.34c/(2.34c + d)] versus *sulfur added* [c(S) × volume] a=volume of $P(OPh)_3$ solution, b=concentration of $P(OPh)_3$ solution, c=peak area of $SP(OPh)_3$, 2.34=extinction factor correction, d=peak area of $P(OPh)_3$, ^{31}P NMR shifts [ppm](CD_3CN): $P(OEt)_3$: 139.9, $P(OPh)_3$: 130.0, $SP(OEt)_3$: 68.5, $SP(OPh)_3$: 55.2.

reaction. For comparison, aged PADS reagent has a sulfurization effectiveness of approximately 45%. It should also be noted that the sulfurization of $P(OPh)_3$, a compound that reacts only very slowly with sulfur in toluene, is complete in seconds.[32]

To evaluate the efficiency of the polysulfide reagent in solid-phase synthesis, we prepared 20-mer (PS)-oligodeoxyribonucleotide **1** [PS-d(5′-GCCCAAGCTGG-CATCCGTCA)] on 0.75-mmol scale.[#] **1** Selectively inhibits the formation of intercellular adhesion molecule-1 (ICAM-1) and is currently evaluated as an anti-inflammatory agent in advanced clinical trials in various indications.[33]

Using standard synthesis conditions, 3 molar equivalents of S (calculated based on nucleoside loading of the support) and a 3.2-min contact time (not optimized), crude DMTr-on **1** was obtained in high coupling efficiency (79% full-length) and greater than 99.8% sulfurization efficiency. The relative PS content (97.3%) of the oligonucleotide and the number of sulfurization steps n is used to calculate stepwise sulfurization efficiency (SSE) using the following formula:

$$(\mathrm{SSE})^{n} = \text{relative PS content}$$

HPLC analysis and mass spectral data are shown in Figure 2. Phosphite triester intermediates that fail to oxidize to the corresponding phosphorothioate triester react during the subsequent acid-induced (dichloroacetic acid) detritylation with the DMTr cation or its equivalent in an Arbuzov-type reaction. This leads to formation of DMTr-*C*-phosphonate mono- and diesters resulting in oligonucleotides modified with a DMTr-*C*-phosphonate moiety located internally or at the 5′ terminal hydroxyl group.[34] The absence of any detectable amounts of DMTr-*C*-phosphonate derivatives, signature impurities of incomplete sulfurization, is evidence for the high reactivity of the polysulfide reagent.

Next, we modified the initial formulation slightly to account for the high reactivity and the high sulfur transfer effectiveness. Reducing the sulfur concentration to 0.1 M gave the same amount of transferable sulfur per column volume as an aged PADS solution (0.2 M PADS), allowing us to use the same synthesizer program. Instead of Na_2S nonahydrate we used anhydrous Na_2S (0.01 M) to limit the amount of moisture introduced into the system.

To demonstrate the applicability of the polysulfide reagent in solid-phase synthesis of RNA-derivatives, we prepared 20-mer oligonucleotide ISIS 116847 (**2**). Second generation antisense oligonucleotide **2** [PS-d(5′-$^{Me}C^{Me}UG^{Me}C^{Me}U$AG^{Me}C^{Me}

[#]Oligonucleotides were synthesized on lab-scale (0.75 to 1 mmol) using an AKTA 100 solid-phase synthesizer. Primer Support 200 (Amersham, loading=approx. 200 μmol/g) was used as solid support. For detritylation we used dichloroacetic acid in toluene (10%). Standard phosphoramidites (0.2 M in acetonitrile) and 1*H*-tetrazole (0.45 M in acetonitrile) were used for coupling. The sulfurization reagent was prepared from sulfur and sodium sulfide (see text). For capping we used a mixture of acetic anhydride/pyridine/*N*-methyl imidazole/acetonitrile. Cleavage of the oligonucleotide ("DMT-on" mode) from the support and base deprotection was performed in conc. ammonium hydroxide at elevated temperature (50 to 60°C) for approximately 12 h. The crude oligonucleotide product was analyzed by LC-MS.

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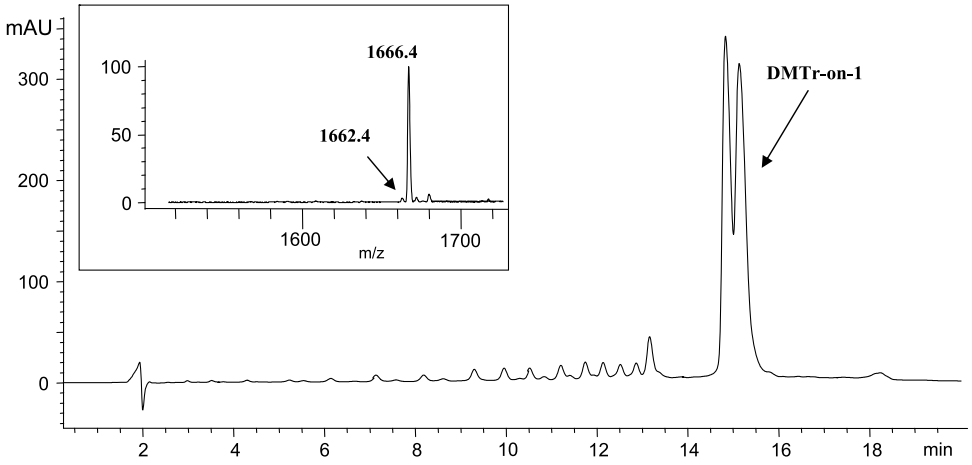

FIGURE 2 HPLC chromatogram (260 nm) of crude DMTr-on **1**. The full-length content is 79%. Chromatographic resolution (split peaks) of diastereomers of DMTr-on PS-oligonucleotides is frequently observed. Insert: the average mass spectrum of the main peak indicates 2.7% $(PO)_1$-**1** (m/z 1662.4, − 4 charge state) with respect to the main peak (m/z 1666.4, − 4 charge state) corresponding to greater than 99.8% sulfurization efficiency.

$CT^{Me}CTGGA^{Me}U^{Me}U^{Me}UGA$)] is a chimerical PS-oligonucleotide composed of 10 deoxyribonucleotides flanked on either side by five 2′-*O*-methoxyethyl modified ribonucleotides. **2** Selectively inhibits PTEN (phosphatase and tensin homolog) gene expression. It has been demonstrated that suppression of PTEN expression products may represent a therapeutic approach for the treatment of type-2 diabetes.[35]

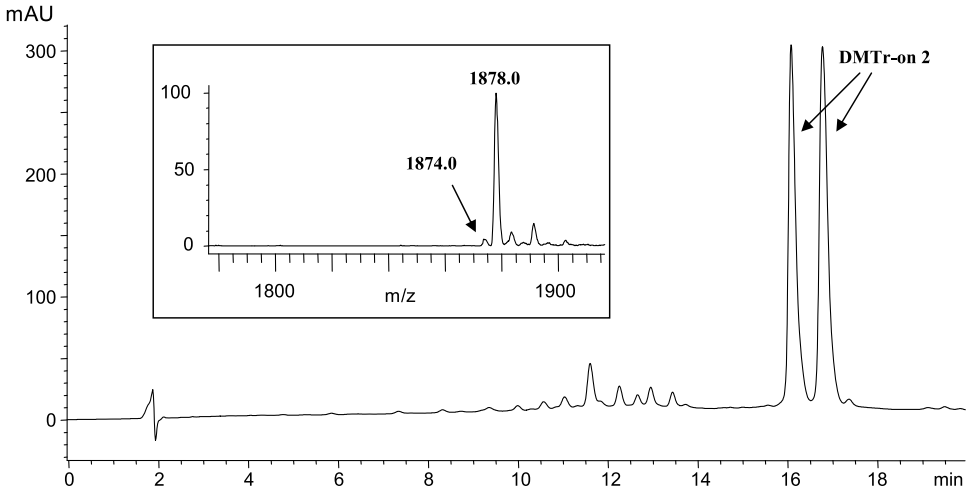

FIGURE 3 HPLC chromatogram (260 nm) of crude DMTr-on **2**. The full-length content is 80%. Insert: the average mass spectrum of the main peak indicates 4.1% $(PO)_1$-**2** (m/z 1874, − 4 charge state) with respect to the main peak (m/z 1878, − 4 charge state) corresponding to 99.8% sulfurization efficiency.

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Using standard synthesis conditions, 3 molar equivalents of S (calculated based on nucleoside loading of the support) and a 3.2-min contact time crude DMTr-on **2** was obtained in high coupling efficiency (80% full-length) and 99.8% sulfurization efficiency (relative $(PO)_1$-**2** content 4.1%). HPLC analysis and mass spectral data are shown in Figure 3.

In summary, it has been demonstrated that elemental sulfur may be used as the sulfur source in solid-phase phosphorothioate oligonucleotide synthesis when a suitable activator and a suitable solvent are chosen. The polysulfide reagent, prepared from sulfur (0.1 M), sodium sulfide (0.01 M), and 3-picoline, is an excellent reagent for solid-phase synthesis of PS-oligonucleotides via the phosphoramidite approach. Average stepwise sulfurization efficiencies greater 99.8% are obtained. The reagent is easy to prepare and its cost is extremely low.

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