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Phosphorothioate Oligonucleotides with Low Phosphate Diester Content: Greater than 99.9% Sulfurization Efficiency with "Aged" Solutions of Phenylacetyl Disulfide (PADS)

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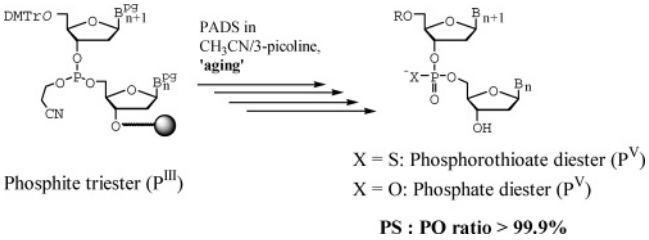
Abstract:

In this report, we describe the design of an efficient and robust sulfurization step in the production process of phosphorothioate (PS) oligonucleotides by solid-phase synthesis. Average stepwise sulfurization efficiencies >99.9% were obtained with "aged" solutions of phenylacetyl disulfide (PADS) in the solvent system acetonitrile/3-picoline (0.2 M, 1:1, v/v). Preparation of the reagent at least 1 day prior to use yields PS-oligonucleotides with low-level phosphate (PO) diester content. The use of freshly prepared PADS solutions gave oligonucleotide products with increased PO-diester content (stepwise sulfurization efficiency 99.5−99.7%). Short aging periods combined with short sulfurization contact times lead to formation of oligonucleotide 4,4′-dimethoxytrityl-*C*-phosphonate derivatives. Investigation of reagent stability and sulfurization efficiency revealed significant changes in solution composition over time caused by dissociation of the PADS molecule. The "in situ" formation of a reactive intermediate, presumably a polysulfide, appears to be crucial for optimal synthesis results.

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] Among the oligonucleotide modifications reported to date, phosphorothioate (PS) oligonucleotides, where one nonbridging oxygen of the internucleotide linkage is replaced by a sulfur atom, are the first class of antisense therapeutics to get marketing approval by regulatory agencies.[2a] A large number of PS-oligonucleotide drugs are currently being evaluated in preclinial and clinical studies as treatment for a wide range of diseases including cancer, cardiovascular disease, autoimmune diseases, diabetes, and infectious diseases.[2] Thus, there is a need to develop high-yield, economical, and robust methods for commercial scale production of high quality oligonucleotides.

Scheme 1



The manufacture of PS-oligonucleotides is a multistep process that may be divided into two distinct operations: solid-phase synthesis using phosphoramidite chemistry followed by downstream processing.[3] In the first operation, a fully protected oligonucleotide is assembled stepwise in a nonstereospecific 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.

Focus of the present investigation is the sulfurization step during solid-phase synthesis. The product of each 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 (base-catalyzed elimination of the β-cyanoethyl protecting groups as acrylonitrile) after the entire oligonucleotide has been assembled and nucleobase deprotection then affords a phosphorothioate diester oligonucleotide (Scheme 1). Addressing the increased commercial need for a highly efficient sulfurization reaction after each coupling has led to the development of a variety of sulfurizing reagents.[4]

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(1) (a) Crooke, S. T. Molecular mechanism of action of antisense drugs. *Biochim. Biophys. Acta* **1999**, *1489*, 31−43. (b) Crooke, S. T. Progress in antisense technology: The end of the beginning. *Methods Enzymol.* **2000**, *313*, 3−45. (c) Crooke, S. T. *Basic principles of antisense technology in Antisense Drug Technology: Principles, Strategies, and Applications*; Marcel Dekker: New York, 2001.

(2) (a) 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. (b) Holmlund, J. T. Applying antisense technology: Affinitak and other antisense oligonucleotides in clinical development. *Ann. N.Y. Acad. Sci.* **2003**, *1002* (Therapeutic Oligonucleotides), 244−251. (c) Dean, N. M.; Bennett, C. F. Antisense oligonucleotide-based therapeutics for cancer. *Oncogene* **2003**, *22*, 9087−9096. (d) Klasa, R. J.; Gillum, A. M.; Klem, R. E.; Frankel, S. R. Oblimersen Bcl-2 antisense: facilitating apoptosis in anticancer treatment. *Antisense & Nucleic Acid Drug Dev.* **2002**, *12*, 193−213. (e) Yu, R. Z.; Su, J. Q.; Grundy, J. S.; Geary, R. S.; Sewell, K. L.; Dorr, A.; Levin, A. A. Prediction of clinical responses in a simulated phase III trial of Crohn's patients administered the antisense phosphorothioate oligonucleotide ISIS 2302: Comparison of proposed dosing regimens. *Antisense & Nucleic Acid Drug Dev.* **2003**, *13*, 57−66. (f) Ravichandran, L. V.; Dean, N. M.; Marcusson, E. G. Use of antisense oligonucleotides in functional genomics and target validation. *Oligonucleotides* **2004**, *14*, 49−64.

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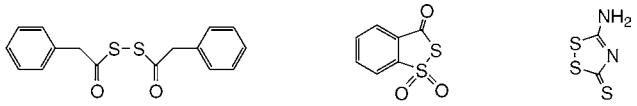
The use of phenylacetyl disulfide (PADS) in PS-oligonucleotide synthesis (in dichloroethane/2,4,6-collidine) was first reported by van Boom.[4n,o] Ravikumar provided the experimental data that subsequently led to the use of PADS in large scale manufacturing of PS-oligonucleotides.[4q,r]

In addition to PADS, to our knowledge, only 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent)[4e] and 3-amino-1,2,4-dithiazole-5-thione (ADTT, xanthane hydride)[4k] have been used in large scale (≥100 mmol) synthesis of PS-oligonucleotides (Scheme 2). However, factors such as the potent oxidizing power of byproducts[4e] formed during sulfurization, inconsistent reagent performance from lot to lot, availability, and cost of reagent have led to development of PADS as a sulfur-transfer reagent for large scale solid-phase synthesis. Here we report the results of an investigation leading to improved robustness of the sulfurization reaction allowing consistent preparation of PS-oligonucleotide with high sulfurizaton efficiency using PADS as the sulfur source.

Scheme 2



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Materials and Methods

Chemicals. Pyridine, 3-picoline, 2,6-lutidine, 2,4,6-collidine, triethyl phosphite, and deutero acetonitrile were obtained from Aldrich (Milwaukee, WI). Anhydrous acetonitrile was obtained from VWR (West Chester, PA). Standard 5′-*O*-4,4′-dimethoxytrityl-3′-*O*-cyanoethyl-*N*,*N*-diisopropyl phosphoramidite reagents of protected deoxyribonucleosides (benzoyl-dA, benzoyl-dC, isobutyryl-dG, T) (Pierce, Milwaukee), 1*H*-tetrazole (Synasia, China), dichloroacetic acid (Clariant, Germany), and PADS (Acharya Chemicals, India) were used as received.

Oligonucleotide Synthesis. Oligonucleotides were synthesized on lab scale (0.75 to 1 mmol) using an AKTA 100 solid-phase synthesizer. Primer Support dA 200 (Amersham, loading = 193 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 formulation was 0.2 M PADS in acetonitrile/3-picoline (1:1, v/v). For capping, we used a mixture of acetic anhydride/pyridine/*N*-methyl imidazole/acetonitrile. Cleavage of the oligonucleotide from the support and base deprotection was performed in concentrated ammonium hydroxide at elevated temperature (50 to 60 °C). The oligonucleotide product was analyzed by LC-MS.

Note: PADS/3-picoline solutions have an unpleasant smell. Use appropriate procedures during preparation, handling, storage, and disposal.

Analytical Methods. A. HPLC. Analytical reversed-phase HPLC for monitoring PADS stability was performed using a Waters 625 LC system equipped with a reversed-phase Luna 5 μ C18(2) column (150 mm × 4.60 mm) from Phenomenex. A linear gradient 30% to 99% acetonitrile in water over 35 min, followed by isocratic elution (35 to 50 min) was used. The detector wavelength was set at 260 nm.

B. ^{31}P NMR. NMR studies were performed using a Varian 200 MHz spectrometer. NMR tubes were washed with acetone and then rinsed with ACN before being dried in an oven at 80 °C for 24 h.

Sulfurization Effectiveness (SE) Test. The amount of sulfur available for transfer to phosphite was determined as follows:

Triethyl phosphite solution (0.25 mL, 0.25 mmol, 1 M in perdeuteroacetonitrile) and sulfurization solution (1 mL, 0.2 mmol, 0.2 M) were mixed and after a reaction time of

Table 1. **Chemical structure and sequence information of antisense oligonucleotides 1−6 and their respective biological target[a]**

oligonucleotide	sequence	biological target
ISIS 3521 (**1**)	5′-PS-d[GTTCTCGCTGGTGAGTTTCA]	PKC-α
ISIS 2302 (**2**)	5′-PS-d[GCCCAAGCTGGCATCCGTCA]	ICAM-1
ISIS 112989 (**3**)	5′-PS-[$^{Me}CAG^{Me}C$d(AGCAGAGTCTTCA)$^{Me}U^{Me}CA^{Me}U$]	clusterin
ISIS 104838 (**4**)	5′-PS-[$G^{Me}C^{Me}UG$Ad(TTAGAGAGAG)$G^{Me}U^{Me}C^{Me}C^{Me}C$]	TNF-α
ISIS 113715 (**5**)	5′-PS-[$G^{Me}C^{Me}U^{Me}C^{Me}C$d(TT^{Me}C^{Me}CA^{Me}CTGAT)$^{Me}C^{Me}C^{Me}UG^{Me}C$]	PTP-1B
ISIS 301012 (**6**)	5′-PS-[$G^{Me}C^{Me}C^{Me}U^{Me}C$d(AGT^{Me}CTG^{Me}CTT^{Me}C)$G^{Me}CA^{Me}C^{Me}C$]	apoB-100

[a] Nucleotides in italics indicate 2′-methoxyethyl modified ribonucleotides, ^{Me}C = 5-methyl(deoxy)cytosine, ^{Me}U = 5-methyl uridine.

10 min a ^{31}P NMR spectrum was recorded. Signals corresponding to triethyl phosphite (I_1, 139.9 ppm) and triethyl phosphorothioate (I_2, 68.5 ppm) were integrated. The sulfurization effectiveness was calculated using the following equation:

$$SE = \frac{\frac{P^{III} \times I_2}{I_1 + I_2}}{STR}$$

I_1 =Integration of phosphite resonance
I_2 =Integration of phosphorothioate resonance
P^{III} = amount of phosphite [mmol]
STR = amount of Sulfur Transfer Reagent [mmol]

Results and Discussion

A class of process-related impurities that is frequently observed in PS-oligonucleotides is a group of oligonucleotides that are identical to the main product, except they contain one (or more) phosphate (PO) diester linkage(s) instead of a PS-diester linkage anywhere in the sequence [$(PO)_n$-oligonucleotide, *n* indicates the number of PO linkages][5] (Scheme 1). Strong anion exchange chromatography, mass spectroscopy, and ^{31}P NMR spectroscopy may be used for quantitative assessment of PO-content.[5,6] In our laboratory, we use state-of-the-art high-performance liquid chromatography/mass spectroscopy (LC/MS) techniques as a specific, accurate, and sensitive means of quantitating oligonucleotides containing PO-linkages within a matrix of PS-oligonucleotides. Removal of $(PO)_n$-oligonucleotide on preparative scale using chromatographic separation technology is difficult to achieve without significant yield loss. Side reactions during sulfurization and subsequent workup are a potential source of formation of $(PO)_n$-oligonucleotides. The extent of PO-diester formation is frequently used as a quality measure of the performance of sulfurization reagents.

Recently, during process optimization of the sulfurization step of 20-mer PS-oligodeoxyribonucleotides **1** and **2** (Table 1) on laboratory scale (0.75−1 mmol) using PADS as the sulfurizing reagent, we noticed that PS-to-PO diester ratios varied between syntheses (average stepwise sulfurization efficiency between 99.9% and 99.5% per linkage) resulting in oligonucleotide products containing between 2 and 10% $(PO)_1$-oligonucleotide. We recognized the necessity of obtaining a more detailed understanding of the origin of the variability to ensure consistent oligonucleotide quality. A summary of reagent formulations and sulfurization conditions used in solid-phase synthesis and the resulting PO-diester content of the oligonucleotide product as well as the formation of DMTr-*C*-phosphonate derivatives[8] (DMTr = 4,4′-dimethoxytrityl), as determined by LC/MS, are given in Table 2. We have not observed sequence-specific effects with respect to sulfurization efficiency, therefore, analytical data from both oligonucleotides **1** and **2** have been combined. DMTr-*C*-phosphonates have been identified as signature impurities of inadequate sulfurization conditions like, for example, insufficient sulfurizing reagent or too short contact time (vide infra). Since the analytical method used for oligonucleotide analysis has not been optimized for quantitative analysis of DMTr-*C*-phosphonates, only a qualitative assessment (detected or non detected) regarding their formation has been included in Table 2. The $(PO)_1$-oligonucleotide content as function of the "aging time" of the PADS reagent is graphically depicted in Figure 1.

The PO-oligonucleotide content of the product and the presence or absence of DMTr-*C*-phosphonates seemed to depend on the solvent composition of the PADS formulation, the age of the solution, the amount of reagent added, and the contact time during solid-phase synthesis. The time between reagent preparation and start of solid-phase synthesis was a most critical factor that has not been considered in the past. One of the prominent features of PADS solutions in the currently recommended formulation **F1** (0.2 M, 3-picoline/acetonitrile 1:1, v/v) is the gradual change in color of the solution from pale yellow to amber, dark amber, green-brown, and dark green to black over a period of 1−2 days. Since this is obviously due to a change in chemical composition, possibly involving dissociation of the PADS molecule, we initially focused our efforts on preparing formulations and developing synthesis procedures in which the integrity of the PADS molecule was maintained. To our surprise, formulations using solvents in which PADS was very stable, e.g., acetonitrile, performed inferior to formulations containing an organic base as a cosolvent. We also found that *freshly prepared* PADS formulations using 3-picoline as a cosolvent performed suboptimally during oligonucleotide synthesis. For example, PADS solution in acetonitrile (0.4 M) mixed on-line with the same volume of 3-picoline just prior to entering the solid-phase synthesis

(5) Phosphate diester content [$(PO)_1$, $(PO)_2$, $(PO)_3$, etc.] in *n*-mer oligonucleotides is statistically distributed according to the terms of the binomial expansion series $(x + y)^n$, where *x* is the decimal fraction of all linkages that are phosphorothioates, *y* is the decimal fraction of all linkages that are phosphate diesters, and $y = 1.000 - x$. Bergot, B. J; Egan, W. Separation of synthetic phosphorothioate oligodeoxynucleotides from their oxygenated (phosphodiester) defect species by strong-anion-exchange high-performance liquid chromatography. *J. Chromatogr., A* **1992**, *599*, 35−42.

(6) SAX chromatography and LC/MS analysis provide the relative ratio of $(PO)_n$-oligonucleotides with respect to all-PS oligonucleotides. ^{31}P NMR spectroscopy provides a relative ratio of phosphate diester linkages to phosphorothioate diester linkages. For example, a 2% $(PO)_1$ content of a 20-mer PS-oligonucleotide (by SAX or LC/MS) corresponds to 0.1% PO content by ^{31}P NMR.

Table 2. **PADS formulations used in laboratory scale syntheses of PS-oligodeoxyribonucleotides 1 and 2 and formation of $(PO)_1$-oligonucleotides and DMTr-*C*-phosphonates**

entry	pads formulation	aging time [h]	contact time [min]	$(PO)_1$ content [%, by LC/MS]	DMTr-*C*-phosphonates[a]
1	PADS in ACN (0.4 M), on-line mixing with 3-picoline	0	2.2	10.9	det
2	0.2 M in ACN/3-picoline	0.5−6	3.1	6.5, 6.9, 10.8	nd
3	0.2 M in ACN/3-picoline	0.5−6	2.2	9.0	det
4	0.1 M in ACN/3-picoline	0.5−6	2.2	6.7	det
5	0.2 M in toluene/3-picoline	0.5−6	2.2	10.9	det
6	0.2 M in toluene/3-picoline	0.5−8	6.7	9.7	det
7	0.4 M in ACN/3-picoline	5−11	3.1	4.2	nd
8	0.2 M in ACN/3-picoline	6−12	3.1	7.2	nd
9	0.2 M in ACN/3-picoline	12−18	3.1	5.6	nd
10	0.2 M in ACN/3-picoline	18−24	3.1	5.2	nd
11	0.2 M in ACN/3-picoline	22−28	3.1	3.2	nd
12	0.2 M in ACN/3-picoline	24−30	10	3.4	nd
13	0.2 M in ACN/3-picoline	24−30	3.1	3.7, 2.6, 2.2, 2.2, 2.1	nd
14	0.1 M in ACN/3-picoline	24−30	3.1	2.6	nd
15	0.1 M in ACN/3-picoline	24−30	2.2	4.0	nd
16	0.2 M in ACN/3-picoline	24−30	2.0	2.0	nd
17	0.05 M in ACN/3-picoline	24−30	3.1	3.0	det
18	0.2 M in ACN/3-picoline	24−30	1.5	2.1	nd
19	0.4 M in ACN/3-picoline	31−37	3.1	2.2	nd
20	0.1 M in ACN/3-picoline	48−54	3.1	3.4, 2.1, 2.0	nd
21	0.4 M in ACN/3-picoline	52−58	3.1	2	nd

[a] det: detected. nd: not detected.

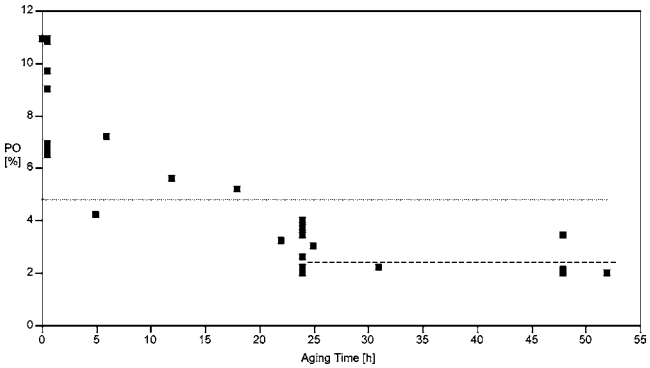


Figure 1. **Graphical depiction of phosphate diester contents of PS-oligonucleotides 1 and 2 synthesized with different sulfurization conditions.**

reactor column (Table 2, entry 1) and solutions prepared immediately prior to solid-phase synthesis (entries 2−6) yielded PS-oligonucleotides with increased $(PO)_1$-diester content (6.5−10.9%). Increasing the contact time from 2.2 to 6.7 min for toluene/3-picoline formulations (entries 5 and 6) did not reduce the PO content. Reducing the contact time from 3.1 to 2.2 min led to formation of oligonucleotide DMTr-*C*-phosphonate mono and diesters which are typically not seen when sulfurization is performed under optimized conditions. After several fruitless attempts to reduce PO-formation by preventing PADS dissociation, we decided to go the other way. We found that when "aged" for periods of at least 1 day prior to solid-phase synthesis, PADS solutions in acetonitrile/3-picoline satisfactorily yielded oligonucleotides with low PO content without formation of additional process-related side products. When the entire set of 29 laboratory scale syntheses (details in Table 2) is considered, the mean PO content is 4.8% (solid line in Figure 1) with a standard deviation of 3.0% and actual values ranging from 2.0 to 10.9%. Figure 1 clearly shows the correlation between "aging time" and "PO content": short (<20 h) aging times result in higher $(PO)_1$ content, long (>20 h) aging times result in lower $(PO)_1$ content. Considering only those syntheses with a PADS aging period of >20 h the mean PO content drops to 2.5% (dashed line in Figure 1) with a standard deviation of 0.7% and actual values ranging from 2.0 to 4.0%. Among those, only entry 17 contained appreciable amounts of DMTr-*C*-phosphonates which was not surprising as the amount of PADS reagent was cut to 25% (or 1.6 molar equiv. of PADS) of the typical amount. As shown below, the amount of "available sulfur" in this experiment was below the stoichiometric amount required.

Upon the basis of these findings on laboratory scale, we applied the "aging" concept on large-scale production. We were extremely pleased to find that "aged" PADS exceeded our expections in terms of sulfurization efficiency and reproducibility. Figure 2 illustrates the favorable sulfurization properties of 'aged' PADS solutions (aging time 1 to 6 days) when used in large scale solid-phase synthesis (160 to 750 mmol) of PS-oligonucleotides **2** to **6**. The mean PO-diester content is 1.75% corresponding to an average stepwise sulfurization efficiency of >99.9%, with a standard deviation of 0.5% and actual values ranging from 1.0 to 2.7%. Extended "aging" periods did not lead to further reduction of the PO-content.

During this process optimization, a new interesting class of process-related oligonucleotide impurities was identified which required additional control of process conditions to

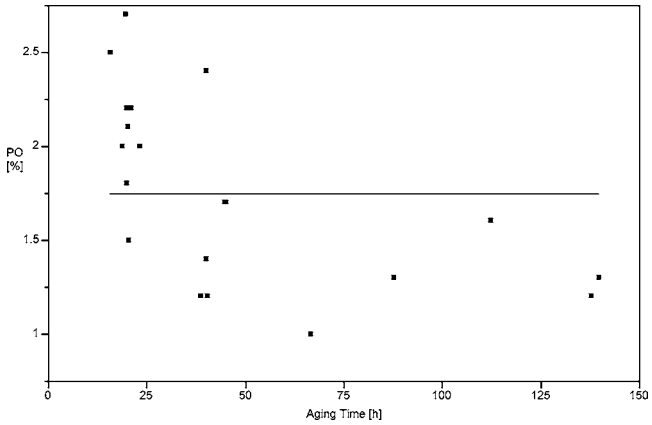


Figure 2. **Graphical depiction of phosphate diester contents of PS-oligonucleotides 2−6 produced on scales ranging from 160 to 750 mmol using "aged" PADS solutions.**

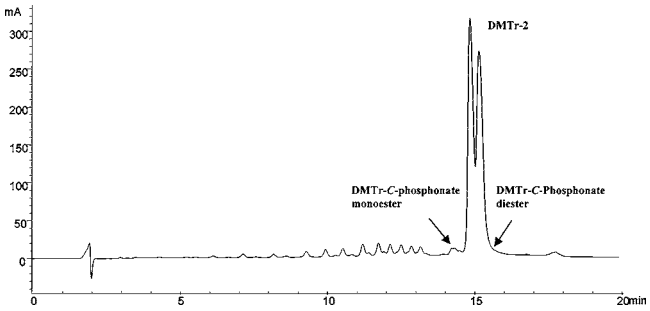
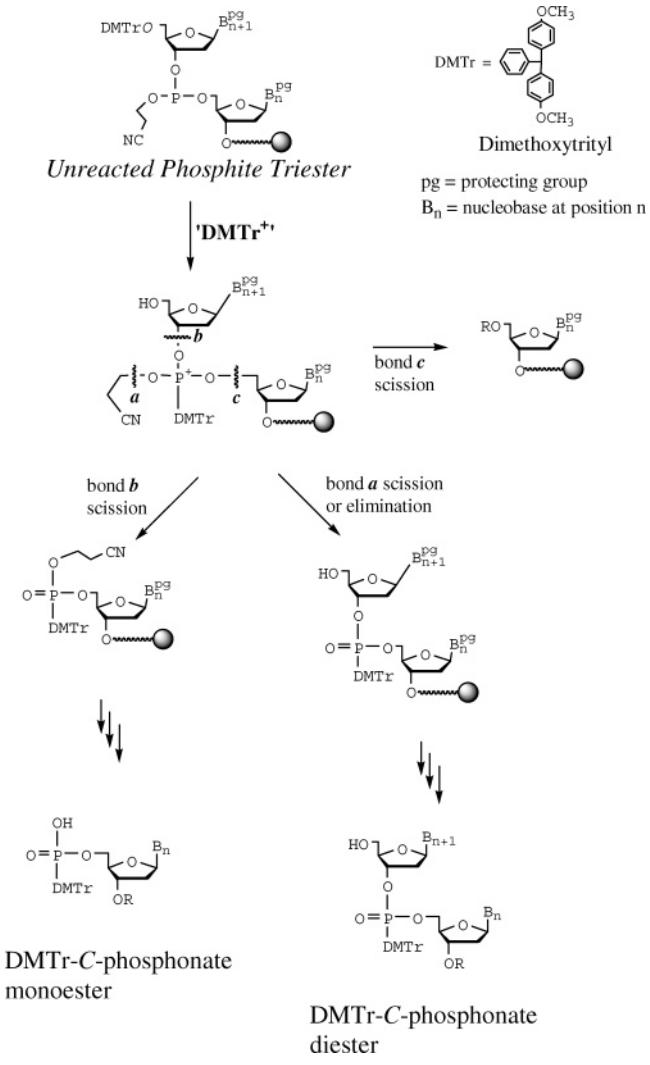


Figure 3. **HPLC trace of crude oligonucleotide 2 (Table 1, entry 17). Suboptimal sulfurization conditions lead to formation of DMTr-*C*-phosphonate monoesters and DMTr-*C*-phosphonate diester (coeluting with the main peak).**

Scheme 3



eliminate their formation. There appears to be no published example of DMTr-*C*-phosphonate formation during PS-oligonucleotide synthesis using β-cyanoethyl phosphoramidites.[7] Knowledge of the chemical structure of actual or potential process-related impurities as well as a better understanding of the origins and mechanisms leading to their formation is crucial in the design and execution of both lab scale and commercial scale synthesis. It leads to improvements in oligonucleotide quality and yield, facilitates process optimization and troubleshooting, and forms a solid foundation for a science-based assessment of a synthetic process. Incomplete sulfurization during solid-phase synthesis of PS-oligonucleotides using phosphoramidite chemistry was identified as the cause of formation of two new classes of process-related oligonucleotide impurities containing a DMTr-*C*-phosphonate moiety.[8] Phosphite triester intermediates that failed 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 leading 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 hydroxy group (Scheme 3). Figure 3 illustrates the LC/MS detection of DMTr-*C*-phosphonates in crude product **2** obtained from a synthesis that intentionally used less of sulfurization reagent at a lower concentration (Table 1, entry 17). Several overlapping DMTr-*C*-phosphonate monoesters are partially resolved from the main peak; the most abundant full length DMTr-*C*-phosphonate diester is detected by mass spectrometry under the main peak [*m*/*z* 1734.2 (−4 charge state, 1734 (calcd)]. Trace quantities of shorter DMTr-*C*-phosphonate diesters have also been detected. The analytical data that led to the proposed structures shown in Scheme 3 and a mechanistic rationale have been published recently.[8] DMTr-*C*-phosphonate derivatives are not detected when optimized sulfurization conditions are employed.

Mechanistic Considerations

PADS Formulations in Inert Solvents. Phenylacetyl disulfide (PADS) is commercially available and inexpensive.[4q] In its pure form, it is a white solid, obtained from pheny-

(7) The preparation of DMTr-*C*-phosphonate derivatives of oligodeoxyribonucleotides has been described. The adventitious formation of DMT-*C*-phosphonates during oligonucleotide synthesis using methyl phosphoramidites as possible side reaction was suggested by Stec et al. Stec, W. J.; Zon, G.; Egan, W.; Byrd, R. A.; Phillips, L. R.; Gallo, K. A. Solid-Phase Synthesis, Separation, and Stereochemical Aspects of P-Chiral Methane- and 4,4′-Dimethoxytriphenylmethanephosphonate Analogues of Oligodeoxyribonucleotides. *J. Org. Chem.* **1985**, *50*, 3908−3913.

(8) Capaldi, D. C.; Gaus, H. J.; Carty, R. L.; Moore, M. N.; Turney, B. J.; Decottignies, S. D.; McArdle, J. V.; Scozzari, A. N.; Ravikumar, V. T.; Krotz, A. H. Formation of 4,4′-Dimethoxytrityl-*C*-Phosphonate Oligonucleotides. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4683−4690.

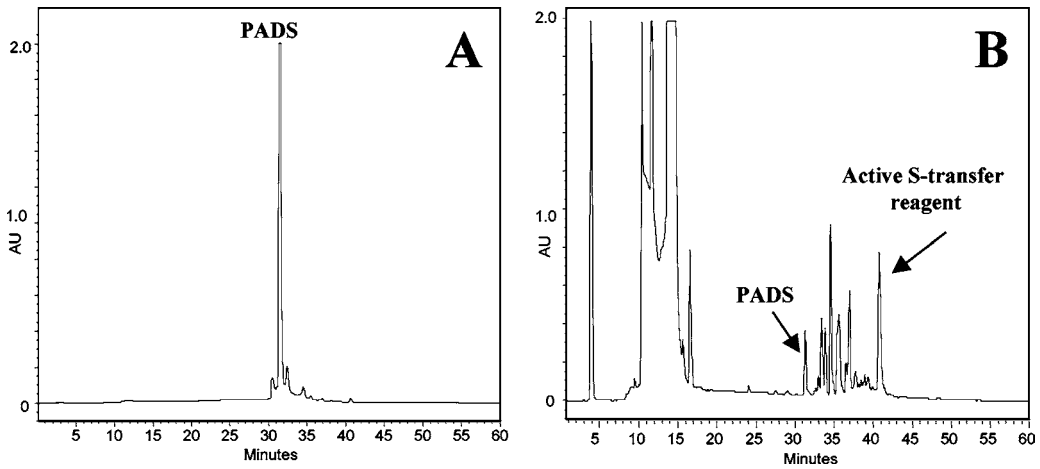


Figure 4. **HPLC trace of (A) PADS solution in acetonitrile after 42 h and (B) PADS solution in acetonitrile/3-picoline after 42 h. Note the degradation of the PADS molecule and the formation of a new active S-transfer reagent.**

lacetyl chloride and sodium disulfide. It dissolves readily in organic solvents, e.g., acetonitrile (750 mg/mL at room temp., 450 mg/mL at 7 °C) or toluene. PADS solutions in these inert solvents are stable for long periods of time at room temperature as indicated by visual inspection and by HPLC analysis. The proportional decrease in peak area of PADS upon addition of $P(OEt)_3$ is indicative of its ability to transfer one of its sulfur atoms onto a P(III) species. The formation of trialkyl phosphorothioates and phenylacetyl sulfide, without formation of Arbuzov type side reactions, has been described by van Boom (PADS in 1,2-dichloroethane). However, the sulfurization efficiency of those formulations turned out to be inadequate for efficient solid-phase synthesis of PS-oligonucleotides.

PADS Formulations Containing Organic Bases. Early on in the development of the PADS reagent, we found that the addition of organic bases as a cosolvent improved the performance of the reagent.[4q] In screening tests for the solvent and cosolvent the combination of acetonitrile and 3-picoline gave remarkably high PS-to-PO ratios. The current sulfurization formulation is a 0.2 M solution of PADS in acetonitrile/3-picoline (1:1, v/v) (**F1**). The multiple, gradual color changes of these PADS formulations provide a visual indication of changes of chemical composition that might explain the apparent correlation between age of solution and its performance in solid-phase oligonucleotide synthesis. HPLC analysis of **F1** showed that the peak corresponding to PADS (t_R 31.5 min) decreased over time (ca 50% in 12 h), while several new peaks appeared in the chromatogram. In a less polar formulation of toluene/3-picoline (1:1, v/v), the rate of degradation of PADS is ca. 5 times slower (50% in 64 h). Addition of $P(OEt)_3$ to **F1** resulted in a decrease in the PADS peak and in a decrease in a newly formed peak at t_R 41 min (Figure 4). Evidently, another reactive sulfurizing species had formed upon "aging". Using preparative HPLC or flash chromatography, both methods include exposure of **F1** to excess water, we isolated the substance giving rise to the newly formed "reactive" peak. Physical (appearance, solubility, melting point, HPLC retention time) and chemical (reactivity towards $P(OEt)_3$) properties were consistent with the isolated substance being sulfur. When it is considered that even at higher PADS concentrations precipitation of sulfur has not been observed (the solubility of sulfur in acetonitrile/3-picoline 1:1 is 2.7 mg/mL), it seems reasonable to assume that sulfur is not the only reactive species in "aged" PADS. This is supported by the fact that commercial sulfur in acetonitrile/3-picoline does not perform as well as PADS. Equilibria between acyl disulfide, sulfur, and polysulfide species, similar to those observed in *N*,*N*-dimethylacetamide,[9] might best explain the observations.

Sulfurization Effectiveness (SE) Test. Since the change in color of PADS formulations indicated a change in chemical composition, we investigated the performance of such solutions with respect to the ability to transfer sulfur over a period of 8 days using a sulfurization effectiveness test. Triethyl phosphite (in perdeuteroacetonitrile) was reacted with PADS solution (aged for 0−193 h at room temperature, in perdeuteroacetonitrile/3-picoline 1:1, v/v), and the conversion of trialkyl phosphite to trialkyl phosphorothioate triester was monitored by ^{31}P NMR spectroscopy. We used 0.8 molar equiv of PADS (calculation is based on the solution preparation 0.2 M) relative to triethyl phosphite. The product ratio was determined 10 min after mixing of the two solutions by ^{31}P NMR. For comparison, the contact time for sulfurization during solid-phase synthesis is approximately 3 min. The sulfurization effectiveness (SE), as defined in the Materials and Methods section, is plotted against the age of the sulfurizing solution (Figure 3). Freshly prepared PADS solution in ACN/3-picoline (0.8 equiv) converts 80% of the triethyl phosphite to the corresponding triethyl phosphorothioate indicating 100% activity of the reagent. As expected, only one of the two sulfur atoms of PADS is available for oxidative sulfurization. After an aging time of 7.5 h only 68% available substrate is consumed. This value drops to 45% after 24 h and stays constant at that level for several days (Figure 5). NMR spectra of the same sample recorded 1 and 3 h later indicated additional sulfur transfer, 5 to 15% and 25%, respectively. Interestingly, these data and the data obtained from oligonucleotide synthesis show that despite having approximately 50% less sulfur "available"

(9) Robert, J.; Anouti, M.; Paris, J. Formation of acyl disulfide ions from the reaction of sulfur with thiocarboxylate ions, and reactivity towards acyl chlorides in *N*,*N*-dimethylformamide. *J. Chem. Soc., Perkin Trans. 2* **1997**, 473−478.

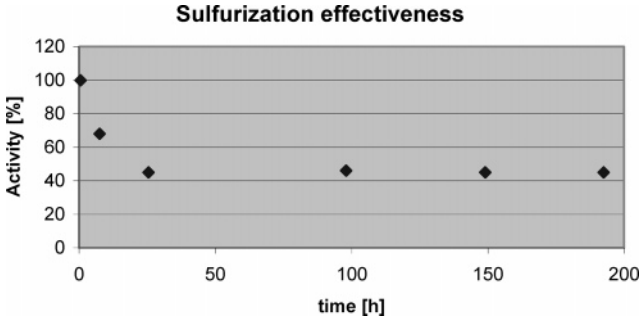


Figure 5. **Sulfurization effectiveness of F1 as function of time.**

for sulfurization, the PO-content is much lower compared to a freshly prepared PADS reagent. Furthermore, DMTr-*C*-phosphonates have not been detected with "aged" reagent at a contact time of 1.5 min, whereas in the case of freshly prepared reagent we find DMTr-*C*-phosphonates at contact times of 2.2 min. From a mechanistic viewpoint, we believe that this further supports the notion that a more reactive sulfurizing agent is being formed over time upon dissolution of PADS in a base-containing solvent. Although questions regarding the chemical nature of the putative reactive species remain, the better understanding of the performance of the sulfurization reagent has led to a more robust process and has opened the door for further process improvements.

Summary

Process optimization of the sulfurization step in solid-phase synthesis of PS-oligonucleotides using PADS as the sulfur source has been performed. From the viewpoint of sulfurization efficiency and robustness of a manufacturing process, an "aging" period of the reagent (0.2 M PADS in acetonitrile/3-picoline, 1:1) of at least 1 day prior to solid-phase synthesis has been adopted. It is demonstrated that average stepwise sulfurization efficiencies >99.9% are obtained with "aged" PADS reagent. The "in situ" formation of a reactive intermediate appears to be crucial for optimal synthesis results.

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