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^{31}P NMR Study of the Desulfurization of Oligonucleoside Phosphorothioates Effected by "Aged" Trichloroacetic Acid Solutions

Jacek Cieślak,[†] Cristina Ausín, Marcin K. Chmielewski,[‡] Jon S. Kauffman,[§] John Snyder,[§] Alfred Del-Grosso,[¶] and Serge L. Beaucage*

Division of Therapeutic Proteins, Center for Drug Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, Bethesda, Maryland 20892, Office of Vaccines Research and Review, Center for Biologics Evaluation and Research, Food and Drug Administration, 1401 Rockville Pike, Rockville Maryland 20852, and Lancaster Laboratories, 2425 New Holland Pike, Lancaster, Pennsylvania 17605-2425

beaucage@cber.fda.gov

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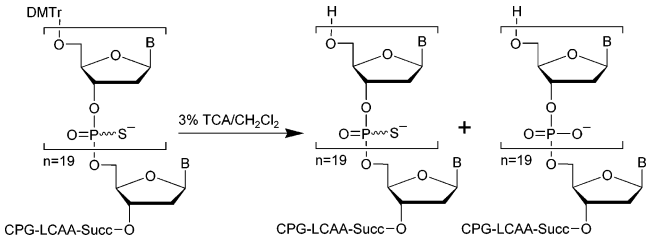


When employing phosphoramidites **1a**−**d** in the solid-phase synthesis of oligonucleoside phosphorothioates, the thermolytic 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl thiophosphate protecting group is lost to a large extent during the course of the synthesis. The resulting phosphorothioate diesters are then substantially desulfurized upon recurring exposure to a commercial solution of deblocking reagent during chain assembly. This problem is caused by the secondary decomposition product(s) of the reagent and is alleviated by using a fresh solution of the deblocking reagent prepared from solid trichloroacetic acid.

In our earlier report on the use of deoxyribonucleoside phosphoramidite **1a** in the solid-phase synthesis of oligodeoxyribonucleoside phosphorothioate d($A_{PS}T^*_{PS}T^*_{PS}C_{PS}G_{PS}T^*_{PS}A_{PS}G_{PS}C_{PS}T^*_{PS}$ $A_{PS}A_{PS}G_{PS}G_{PS}T^*_{PS}C_{PS}A_{PS}T^*_{PS}G_{PS}$C), wherein T* depicts the site-specific incorporation of **1a**, minimal (<5%) desulfurization of the deprotected oligonucleotide was observed by ^{31}P NMR spectroscopy.[1] However, when the thioated oligonucleotide was synthesized with exclusively phosphoramidites **1a**−**d**, ^{31}P NMR analysis of the deprotected oligonucleotide (**PS-oligo 1**)[2] in concd NH_4OH revealed an unacceptable level (∼20%) of phosphorothioate diester desulfurization (Figure 1).

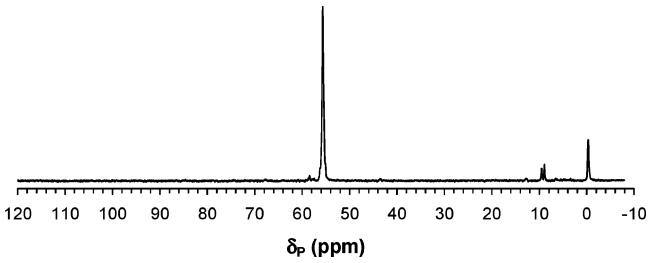
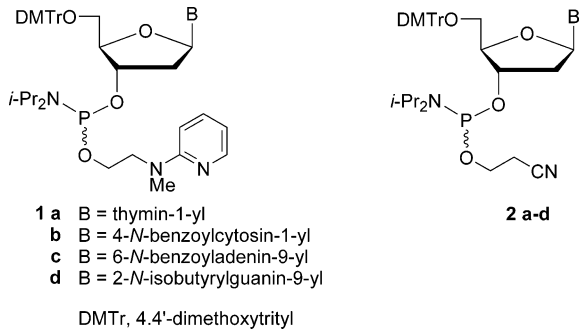


FIGURE 1. ^{31}P NMR spectrum of d($A_{PS}T_{PS}T_{PS}C_{PS}G_{PS}T_{PS}A_{PS}$-$G_{PS}C_{PS}T_{PS}A_{PS}A_{PS}G_{PS}G_{PS}T_{PS}C_{PS}A_{PS}T_{PS}G_{PS}$C) [**PS-oligo 1**] in concd NH_4OH. Phosphorothioate diesters appear as a broad signal at 56 ppm, whereas the resonance corresponding to desulfurized phosphodiesters shows at ∼0 ppm. Peak heights are normalized to the highest peak, which is set to 1 arbitrary unit.



To reconcile our seemingly contradictory findings, we set out to identify the reagent(s) responsible for causing desulfurization of phosphorothioate diesters during oligonucleotide synthesis and propose a corrective action to this problem. The resolution of this issue is important especially when the stability of thioated therapeutic oligonucleotides to extracellular and intracellular nucleases depends on the integrity of their phosphorothioate diester linkages.

As reported earlier[1] the incorporation of **1a** into an oligonucleoside phosphorothioate resulted, upon conversion of the phosphite triester to the corresponding thiophosphate triester,[3] in the immediate loss of the 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl thiophosphate protecting group from the thioated oligonucleotide. Such a rapid thiophosphate deprotection exposed phosphorothioate diester linkages to cumulative treatments with (i) 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (sulfurization step), (ii)

* To whom correspondence should be addressed. Phone: (301)-827-5162. Fax: (301)-480-3256.
[†] On leave from the Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznań, Poland.
[‡] Present address: Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61-704 Poznań, Poland.
[§] Lancaster Laboratories.
[¶] Center for Biologics Evaluation and Research.

(1) Cieślak, J.; Beaucage, S. L. *J. Org. Chem*. **2003**, *68*, 10123−10129.

(2) **PS-oligo 1** was prepared with phosphoramidites **1a**−**d**, a commercial deblocking solution (3% trichloroacetic acid in CH_2Cl_2), and commercial capping reagents (CAP A and CAP B). Specifically, CAP A and CAP B reagents were a solution of acetic anhydride in pyridine and THF, and a solution of 16% 1-methylimidazole in THF, respectively.

(3) Such a conversion was effected by treatment with 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in MeCN, see: (a) Iyer, R. P.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Am. Chem. Soc*. **1990**, *112*, 1253−1254. (b) Iyer, R. P.; Phillips, L. R.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Org. Chem*. **1990**, *55*, 4693−4699. (c) Regan, J. B.; Phillips, L. R.; Beaucage, S. L. *Org. Prep. Proced. Int*. **1992**, *24*, 488−492.

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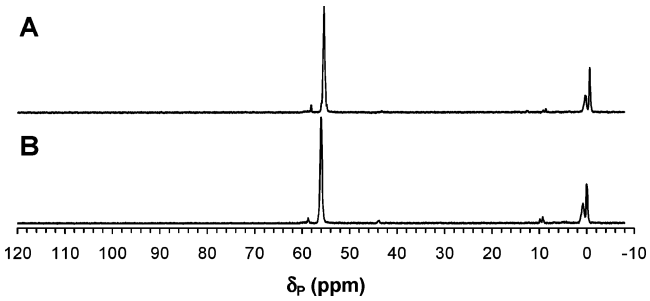


FIGURE 2. A comparative assessment of the desulfurization of thioated oligonucleotides caused by capping reagents based on ^{31}P NMR data. (A) ^{31}P NMR spectrum of fully deprotected **PS-oligo 2** (concd NH_4OH) that was synthesized with capping reagents throughout chain assembly.[5] (B) ^{31}P NMR spectrum of fully deprotected **PS-oligo 2** (concd NH_4OH) that was synthesized without employing capping reagents during chain assembly. The same commercial solution of deblocking reagent was utilized in both syntheses. Peak heights are normalized to the highest peak, which is set to 1 arbitrary unit.

acetic anhydride and 1-methylimidazole or 4-(dimethylamino)pyridine (capping step), (iii) trichloroacetic acid (TCA, 5′-deblocking step), and (iv) 1*H*-tetrazole-activated **1a**−**d** (chain elongation step).

In this context, multiple exposures of the phosphorothioate diesters produced during synthesis of d($A_{PS}T^*_{PS}T^*_{PS}C_{PS}G_{PS}T^*_{PS}A_{PS}G_{PS}C_{PS}T^*_{PS}A_{PS}A_{PS}G_{PS}G_{PS}T^*_{PS}C_{PS}A_{PS}T^*_{PS}G_{PS}C$) to either 3*H*-1,2-benzodithiol-3-one 1,1-dioxide or 1*H*-tetrazole-activated deoxyribonucleoside phosphoramidites were inconsequential in terms of desulfurization considering that less than 5% of desulfurized phosphorothioate diesters was determined from ^{31}P NMR analysis of the thioated oligonucleotide.[1] Consequently, we focused our attention on the reagents used in the capping and 5′-deblocking steps to determine which of these were accountable for the extensive desulfurization of **PS-oligo 1**.

To assess whether the capping reagents can desulfurize phosphorothioate diesters, solid-phase synthesis of **PS-oligo 2** was achieved by using commercial deoxyribonucleoside phosphoramidites **2a**−**d**, instead of **1a**−**d**, and performing a thiophosphate deprotection step[4] immediately after each sulfurization reaction, with or without a capping step during chain assembly.[5] ^{31}P NMR analysis of fully deprotected **PS-oligo 2** revealed desulfurization of phosphorothioate diesters to the extent of ∼35%, regardless of whether capping reagents were used or not during synthesis (Figure 2). These data indicate that the capping reagents do not induce significant desulfurization of phosphorothioate diester linkages. The data also suggest that the reagent used for the 5′-deblocking step in solid-phase oligonucleotide synthesis is most likely responsible for desulfurizing **PS-oligo 1** and **PS-oligo 2**.

The variability in the level of desulfurization of phosphorothioate diesters observed during the synthesis of

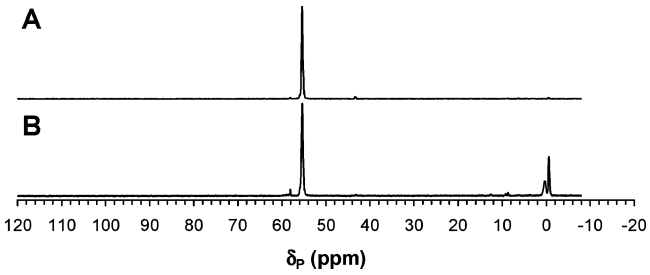


FIGURE 3. A comparative assessment of the desulfurization of thioated oligonucleotides caused by an aged commercial solution of the 5′-deblocking reagent based on ^{31}P NMR data. (A) ^{31}P NMR spectrum of fully deprotected **PS-oligo 2** (concd NH_4OH) that was synthesized with a freshly prepared deblocking solution and capping reagents[7] throughout chain assembly. (B) ^{31}P NMR spectrum of fully deprotected **PS-oligo 2** (concd NH_4OH) that was synthesized with an aged commercial deblocking solution and capping reagents[7] during chain assembly. Peak heights are normalized to the highest peak, which is set to 1 arbitrary unit.

PS-oligo 1 and **PS-oligo 2** relates to both the ratio of phosphorothioate triester/diester groups in each oligonucleotide and the age of the deblocking reagent (3% TCA in CH_2Cl_2) that was stored under normal laboratory conditions. The amount of phosphorothioate diester desulfurization increases with the age of the deblocking reagent.[6] For example, when **PS-oligo 2** is synthesized with a 2-month old commercial solution of deblocking reagent, the extent of phosphorothioate diester desulfurization decreases to ∼8% (data shown in the Supporting Information). Phosphorothioate diester desulfurization is further decreased to less than 1% when the deblocking solution is freshly prepared from solid TCA (Figure 3).

Results shown in Figure 3 demonstrate that an aged TCA solution elicits the desulfurization of phosphorothioate diesters during synthesis.

Interestingly, the use of commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites (**2a**−**d**) in the solid-phase synthesis of oligonucleoside phosphorothioates has, in our hands, typically produced variable amounts (2−5%) of desulfurized phosphorothioate diesters. One might then question the stability of 2-cyanoethyl thiophosphate triesters when repeatedly exposed to the capping reagents during synthesis.[8] To address this concern, a thioated poly-dT oligonucleotide (20-mer) was synthesized with phosphoramidite **2a**, a freshly prepared deblocking solution, and commercial capping reagents. Upon completion of the synthesis, the solid-phase-linked oligonucleotide was immersed in a solution of 10% CH_3I in MeCN to convert any phosphorothioate diesters to the corresponding *S*-methyl phosphorothiolate triesters. Removal of the remaining 2-cyanoethyl thiophosphate

(4) This step was carried out by exposing the solid-phase-linked oligonucleotide to a solution of 5% TMG (1,1,3,3-tetramethylguanidine) in MeCN. TMG is a strong base inducing the production of phosphorothioate diesters via β-elimination of 2-cyanoethyl phosphorothioate triesters.

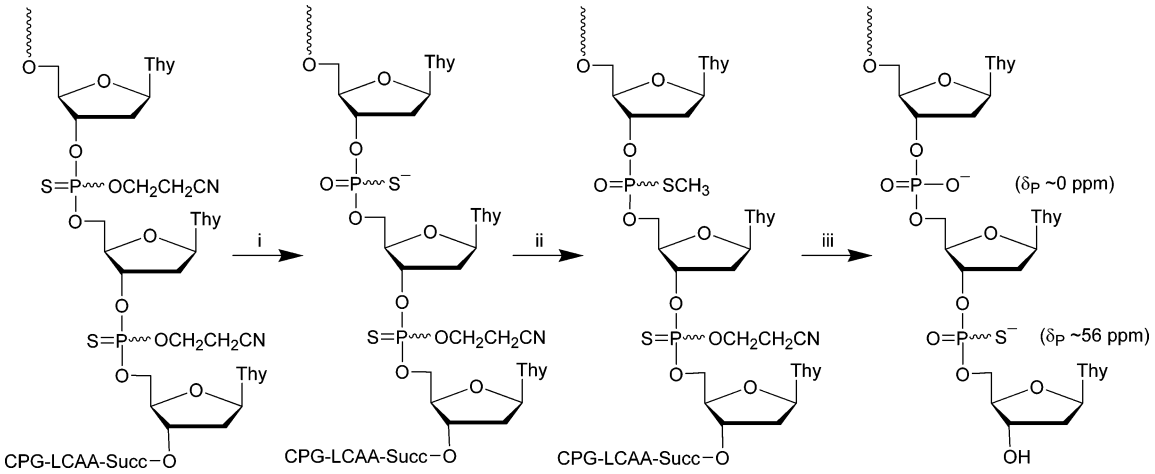
(5) A commercial deblocking solution (3% TCA in CH_2Cl_2) and commercial capping reagents (CAP A and CAP B) were used in the synthesis of **PS-oligo 2**. The CAP B reagent was a solution of 16% 1-methylimidazole in THF.

(6) An aged commercial solution of deblocking reagent is arbitrarily defined as one that has been stored, as received, under normal laboratory conditions over a period of time exceeding 6 months.

(7) The CAP B reagent used in this experiment was a solution of 16% 1-methylimidazole in THF.

(8) The CAP B reagent contains 10% or 16% 1-methylimidazole, or 6.5% 4-(dimethylamino)pyridine in THF depending on the commercial formulation used. 1-Methylimidazole or 4-(dimethylamino)pyridine may induce β-elimination of the 2-cyanoethyl group from phosphorothioate triesters, thus exposing the resulting phosphorothioate diesters to aged TCA through each 5′-deblocking step and causing unwanted desulfurization of these thioated diesters.

SCHEME 1. Conversion of Phosphorothioate Diesters to Phosphate Diesters via *S*-Methylation[a]



[a] Reagents and conditions: (i) commercial Cap A and Cap B solutions, 60 s; (ii) 10% CH_3I in MeCN, 3 h, 25 °C; (iii) concd NH_4OH, 24 h, 55 °C. Key: Thy, thymin-1-yl; CPG-LCAA-Succ, succinyl long-chain alkylamine controlled-pore glass.

protecting group and release of the oligonucleotide from the solid support were achieved by treatment with concd NH_4OH over a period of 24 h at 55 °C. Such conditions are necessary to convert *S*-methyl phosphorothiolate triesters to phosphate diesters (Scheme 1). Thus, by comparing the area of the ^{31}P NMR signal corresponding to phosphate diesters (~0 ppm) with that of the phosphorothioate diesters (~56 ppm), one can determine the loss of the 2-cyanoethyl thiophosphate protecting group occurring under solid-phase synthesis conditions. Typically, the 2-cyanoethyl thiophosphate protecting group was lost to the extent of ~5−13% (Scheme 1, data shown in the Supporting Information) depending on the capping reagents being used.[9]

The adequacy of the *S*-methylation reaction when evaluating the loss of the 2-cyanoethyl thiophosphate protecting group was further tested through solid-phase synthesis of the above thioated poly-dT oligonucleotide (20-mer) under conditions identical with those described for the synthesis of **PS-oligo 2** (vide supra).[10] Following treatment with 10% CH_3I in MeCN and extended exposure to concd NH_4OH at 55 °C (Scheme 1), ^{31}P NMR analysis of the thioated 20-mer indicated a complete lack of phosphorothioate diester linkages characterized by the absence of a signal at ~56 ppm (data shown in the Supporting Information). This control experiment demonstrated that the *S*-methylation of phosphorothioate diesters is suitable for accurately assessing any loss of the 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl thiophosphate protecting group during solid-phase oligonucleotide synthesis. Indeed, when phosphoramidite **1a** was used in the preparation of a 20-mer thioated poly-dT,[10] ~80% of the 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl thiophosphate protecting group was lost during synthesis (data shown in the Supporting Information) on the basis of ^{31}P NMR analysis of the *S*-methylation of the 20-mer.

The desulfurization of phosphorothioate diesters caused by aged solutions of deblocking reagent is peculiar although it is known that aqueous solutions of less than 30% TCA are not recommended for storage. Under these conditions, TCA decomposes to $CHCl_3$, HCl, CO_2, and CO.[11] It is also known that $CHCl_3$, when exposed to air and light, decomposes mainly to $COCl_2$, Cl_2, and HCl.[12] One might then argue that a solution of 3% TCA in CH_2Cl_2 could also generate these decomposition products over time, and that exposure of phosphorothioate diesters to $COCl_2$ and Cl_2 may result in the desulfurization of these species. Incidentally, the conversion of phosphorothioate diesters to the corresponding phosphate diesters upon reaction with I_2 has been described in the literature.[13] To assess whether the formation of $COCl_2$ could contribute to desulfurization of phosphorothioate diesters, the solid-phase synthesis of **PS-oligo 2** was achieved by using a freshly prepared deblocking solution and capping reagents[7] throughout chain assembly. While still bound to the solid support, the oligonucleotide was treated with a solution of 0.1% $COCl_2$ in freshly prepared deblocking reagent for 10 min at 25 °C. Following release of the oligonucleotide from the solid support and its complete deprotection under standard basic conditions, ^{31}P NMR analysis of phosgene-treated **PS-oligo 2** showed significant desulfurization (~20%) of phosphorothioate diesters (data shown in the Supporting Information) and thus identified $COCl_2$, one of TCA's secondary decomposition products, as being responsible for desulfurizing thiophosphate diesters.[14] Actually, phosgene was detected in both

(9) The 2-cyanoethyl thiophosphate protecting group was lost to the level of ~5% when the CAP B solution was 10% or 16% 1-methylimidazole in THF, and to the extent of ~13% when the CAP B solution was 6.5% 4-(dimethylamino)pyridine in THF.

(10) A freshly prepared solution of deblocking reagent and commercial capping reagents[7] were employed in this experiment.

(11) *The Merck Index*, 13th ed.; Merck & Co., Inc.: Whitehouse Station, NJ, 2001; p 1716.

(12) Perrin, D. D.; Armarego, W. L. F.; Perrin, D. R. In *Purification of Laboratory Chemicals*, 2nd ed.; Pergamon Press Ltd.: Oxford, UK, 1980; pp 167−168.

(13) (a) Burgers, P. M. J.; Eckstein, F. *Biochemistry* **1979**, *18*, 450−454. (b) Connolly, B. A.; Potter, B. V. L.; Eckstein, F.; Pingoud, A.; Grotjahn, L. *Biochemistry* **1984**, *23*, 3443−3453. (c) Cummins, J. H.; Potter, B. V. L. *Chem. Commun*. **1985**, 800−802. (d) Cosstick, R.; Eckstein, F. *Biochemistry* **1985**, *24*, 3630−3638. (e) Capaldi, D. C.; Gaus, H.; Krotz, A. H.; Arnold, J.; Carty, R. L.; Moore, M. N.; Scozzari, A. N.; Lowery, K.; Cole, D. L.; Ravikumar, V. T. *Org. Proc. Res. Dev*. **2003**, *7*, 832−838. A tentative mechanism for the phosgene-mediated desulfurization of phosphorothioate diesters is proposed in the Supporting Information (p S14).

fresh and aged solutions of 3% TCA in CH_2Cl_2, using GC-MS techniques (data shown in the Supporting Information). As expected, the concentration of $COCl_2$ was much higher (>3-fold) in aged TCA solutions than in freshly prepared solutions (data not shown).

The implications of our findings are far-reaching in terms of the many variables involved in the desulfurization of oligonucleoside phosphorothioates, and in regard to the interpretation and accuracy of desulfurization data reported in the scientific literature. The age of commercial deblocking solution (3% TCA in CH_2Cl_2) is accountable for the desulfurization of phosphorothioate diesters that have been generated as a consequence of the loss of either thermolytic or 2-cyanoethyl thiophosphate protecting groups during synthesis. Given that the age of a commercial deblocking solution at the time of use is difficult to determine considering that the period of time between its manufacturing date and its distribution to the end user may vary considerably, it is therefore recommended to prepare the deblocking solution from solid TCA prior to use to ensure the integrity of phosphorothioate diesters.

The nature and concentration of a capping formulation are variables affecting the premature removal of the base-labile 2-cyanoethyl thiophosphate protecting group from oligonucleotides during synthesis. To minimize desulfurization of the resulting phosphorothioate diesters induced by aged deblocking solutions, it is suggested to (i) select a capping formulation containing 1-methylimidazole, instead of 4-(dimethylamino)pyridine,[9] (ii) limit to a minimum the total exposure time of 2-cyanoethyl thiophosphates to such a formulation,[15] and (iii) use a freshly prepared solution of 5′-deblocking reagent.

In light of the findings reported herein, one might question the accuracy of our results published earlier on the synthesis of thioated DNA oligonucleotides through the use of deoxyribonucleoside phosphoramidites functionalized with thermolytic groups for phosphorus protection.[1,16] In these studies, the 5′-deblocking solution was not freshly prepared from solid TCA prior to use and may have resulted in unwanted desulfurization of thermolytically generated phosphorothioate diesters. Consequently, ^{31}P NMR analyses of these oligonucleoside phosphorothioates may have revealed more desulfurized material than expected, had a freshly prepared solution of TCA been used for the iterative 5′-deblocking step. To substantiate this assertion, **PS-oligo 1** was resynthesized with phosphoramidite **1a**−**d** and a freshly prepared solution of 3% TCA in CH_2Cl_2 as a deblocking reagent. ^{31}P NMR analysis of deprotected **PS-oligo 1** in concd NH_4OH revealed about 1% desulfurization of phosphorothioate diesters (Figure 4). This analysis is in sharp contrast with that shown in Figure 1, but consistent with the fact that an aged solution of TCA is to blame for the desulfurization of phosphorothioated diester groups (vide supra).

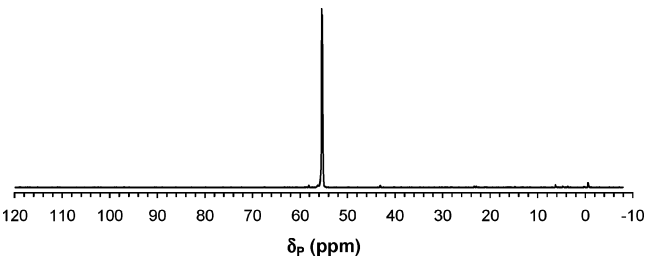


FIGURE 4. Solid-phase synthesis of **PS-oligo 1** with a freshly prepared deblocking solution and capping reagents. ^{31}P NMR spectrum of fully deprotected **PS-oligo 1** in concd NH_4OH. Peak heights are normalized to the highest peak, which is set to 1 arbitrary unit.

Further characterization of **PS-oligo 1** by polyacrylamide gel electrophoresis (PAGE) demonstrated that the coupling efficiency of **1a**−**d** was as efficient as that of **2a**−**d** when comparing the relative intensity of the bands corresponding to oligonucleotides shorter than full-length for each oligonucleotide (data shown in the Supporting Information).

Our findings underscore the importance of properly selecting capping reagents and conditions, and using freshly prepared TCA deblocking solutions when optimizing the preparation of therapeutic oligonucleotides functionalized with phosphorothioate diester linkages.

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Supporting Information Available: Experimental Section including materials and methods; synthesis and characterization of deoxyribonucleoside phosphoramidites **1a**−**d**; solid-phase synthesis, deprotection and analysis of oligonucleoside phosphorothioates; *S*-methylation of phosphorothioate diester groups; phosgene-mediated desulfurization of phosphorothioate diesters; GC-MS analysis of phosgene in TCA solutions; rationale for limiting investigations to the use of TCA as a deblocking reagent; ^{31}P NMR spectra of **1a**−**d**; polyacrylamide gel electrophoresis analysis of **PS-oligo 1** (Chart 1); ^{31}P NMR spectrum of **PS-oligo 2** that was synthesized with a two-month old commercial solution of deblocking reagent (Chart 2); ^{31}P NMR spectra of thioated poly-dT (20-mers) that were *S*-methylated and then treated with concd NH_4OH (Charts 3−5); ^{31}P NMR spectrum of phosgene-treated **PS-oligo 2** (Chart 6); mass spectrum of phosgene detected in the GC-MS analysis of an aged commercial solution of deblocking reagent (Chart 7); and mass spectrum of phosgene obtained from the GC-MS analysis of a commercial phosgene solution (Chart 8). This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) We limited the scope of our investigations to the use of 3% TCA in CH_2Cl_2 because of the popularity of this deblocking solution in the solid-phase synthesis of oligonucleotides and their phosphorothioated analogues (see the Supporting Information for a detailed discussion).

(15) Although the exposure time of 2-cyanoethyl thiophosphates to capping reagents has not been specifically addressed in this report, it is nonetheless safe to speculate that the loss of the 2-cyanoethyl group will be commensurate to its contact time with the capping reagents.

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