

## Solid-Phase Synthesis of Oligonucleotide 5'-( $\alpha$ -*P*-Thio)triphosphates and 5'-( $\alpha$ -*P*-Thio)( $\beta$ , $\gamma$ -methylene)triphosphates

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A robust solid-phase synthesis was developed to obtain original oligonucleotides (ONs) functionalized at their 5' end with modified triphosphate (TP) moieties, in which a nonbridging oxygen atom of the  $\alpha$  phosphorus atom was replaced by a sulfur atom and the labile P–O–P linkage was changed into a methylene bridge between the  $\beta$  and  $\gamma$  phosphorus atoms. The efficient method is based on solid-supported ON assembly followed by 5'-*H*-phosphonylation, oxidation to the thiophosphate subsequently activated as a phosphoan-

hydride with diphenyl phosphoryl chloride, then nucleophilic substitution with the alkylammonium salt of pyrophosphate or its  $\beta$ , $\gamma$ -methylene analogue. After deprotection and release from the solid support under basic conditions, 5'-( $\alpha$ -*P*-thio)-TP and 5'-( $\alpha$ -*P*-thio)( $\beta$ , $\gamma$ -methylene)TP oligonucleotides were obtained in satisfactory yields, and they were isolated with high purity. These hydrolysis-resistant 5'-TP ONs will be useful in biological research to elucidate the mechanism of enzymes involved in mRNA processing and maturation.

### Introduction

Oligoribonucleotide 5'-triphosphates (TPs) are high-value molecules with wide applications in biochemistry and in molecular and cell biology. Indeed, they are good substrates for many enzymes for structural and mechanistic studies. In particular, they are used as probes to investigate enzymatic pathways involved in the maturation of pre-messenger RNAs. In bacteria, RNA turnover is managed by a Nudix protein called RNA pyrophosphohydrolase (RppH), which converts 5'-triphosphate into 5'-monophosphate; this allows access to both *endo*- and 5'-*exoribonucleases*.<sup>[1]</sup> In eukaryotes or viruses, 5'-TP RNA are substrates of a capping enzymatic complex that biologists investigate to understand the role of each enzymatic activity.<sup>[2]</sup> This leads to a high demand for structural insight into functional RNA–enzyme complexes. RNA crystallography has faced a long-standing bottleneck: production of large amounts of 5'-TP RNAs or their nonhydrolyzable analogues of the triphosphate moiety. Triphosphate analogues also have great utility for studying the mechanism and specificity of enzyme-catalyzed reactions and for investigating biochemical pathways. To address these needs, some years ago a ro-

bust solid-phase chemical synthesis of 5'-TP oligonucleotides (ONs) was developed by our group<sup>[3]</sup> and a few recent reports similarly focused on the synthesis of these compounds.<sup>[4]</sup> Moreover, modification of this procedure allowed us to achieve the first solid-phase synthesis of modified 5'-TP ONs with a methylene bisphosphonate modification pCH<sub>2</sub>pp at the 5' end of short RNA sequences that were useful to investigate the structure and functioning of *Bacillus Subtilis* RppH.<sup>[5]</sup> In the present work, we describe a practical chemical method to synthesize oligonucleotide 5'-( $\alpha$ -*P*-thio)triphosphates and 5'-( $\alpha$ -*P*-thio)( $\beta$ , $\gamma$ -methylene)triphosphates on a solid support. This phosphorothioate modification, which consists in the replacement of the nonbridging oxygen atom of the phosphate at the  $\alpha$  position of the triphosphate chain by a sulfur atom, was also combined with methylene bisphosphonate modification. Such modifications reported for dinucleotide cap analogues<sup>[6]</sup> and nucleotide triphosphate (NTP) analogues<sup>[7]</sup> have been introduced through several chemical approaches in solution.<sup>[8]</sup> Concerning solid-phase methods for the synthesis of NTPs, only a few approaches have been reported.<sup>[9]</sup> The key step in the synthesis of these triphosphate derivatives is the formation of the pyrophosphate bond, which is mainly performed by coupling a nucleophile such as phosphate, pyrophosphate, or its analogue with activated phosphate species. To achieve the solid-supported synthesis of ( $\alpha$ -*P*-thio)TP ONs, our first idea was to follow the synthetic pathway we developed for ON 5'-triphosphates,<sup>[3]</sup> but phosphate activation as a *P*-imidazolidine at the 5' end of the ON failed. Consequently, several routes were investigated, which led to the most efficient strategy depicted in Figure 1.

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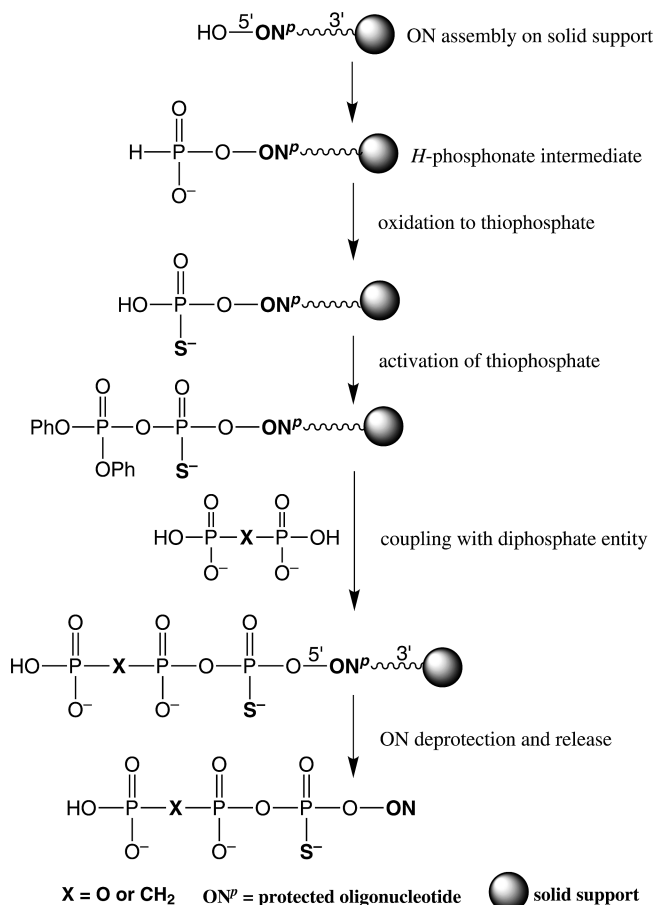


Figure 1. Synthetic scheme for the preparation of 5'-( $\alpha$ -P-thio)triphosphate and 5'-( $\alpha$ -P-thio)( $\beta,\gamma$ -methylene)triphosphate ONs on a solid support.

## Results and Discussion

After standard automated assembly of ONs on a solid support by the phosphoramidite method, the next step was the first phosphorylation at the 5' end of the solid-supported ONs. In our previous work, an *H*-phosphonate intermediate was introduced at 5'-OH by the use of a diphenyl phosphite reagent.<sup>[3]</sup> Likewise the diphenylthiophosphite analogue prepared by using a procedure described in the literature<sup>[10]</sup> was treated with 5'-OH of an hexathymidylate (T<sub>6</sub>) model anchored to the solid support to give the *H*-phosphonothioate derivative (5'-Hp<sub>S</sub> T<sub>6</sub>). Following the same scheme, the next step would have been the activation of this intermediate through amidative oxidation with carbon tetrachloride in the presence of imidazole and *N,O*-bis-trimethylsilylacetaimide (BSA) to obtain the 5'-phosphorothioimidazolide. Unfortunately, these conditions could not be applied, as CCl<sub>4</sub> cannot efficiently oxidize *H*-phosphonothioate, whereas other oxidants such as iodine, bromine, and different carbon tetrahalides are successful.<sup>[11]</sup> Small aliquots of the solid-supported 5'-Hp<sub>S</sub> T<sub>6</sub> were treated with several solutions mixing imidazole, triethylamine with CBr<sub>4</sub>, or iodine in different solvents such as pyridine, acetonitrile, or THF. After removal of the oxidative solution and after washing the support, a 0.25 M

pyrophosphate solution in DMF was applied to the synthesis column for 18 h. Then, ammonia treatment released and deprotected the ON from the solid support. The crude material was analyzed by ion-exchange chromatography (IEX)-HPLC and mass spectrometry (MALDI-TOF), and none of the tested conditions afforded the desired 5'-( $\alpha$ -P-thio)triphosphate T<sub>6</sub> (ppp<sub>S</sub>T<sub>6</sub>).

Taking into account the high susceptibility of *H*-phosphonothioate monoester to oxidation by iodine, the method developed by Sun et al.<sup>[12]</sup> represented an alternative to achieve the oxidative phosphorylation. The conditions used for the synthesis of NTPs via a pyridinium phosphoramidate intermediate were adapted to our synthesis route. Thus, solid-supported 5'-Hp<sub>S</sub> T<sub>6</sub> was treated with iodine in the presence of triethylamine in different solvents such as pyridine, THF, CH<sub>3</sub>CN, or a mixture of DMF and pyridine followed by the addition of pyrophosphate. IEX-HPLC analysis of the crude deprotected material showed the presence of the expected ppp<sub>S</sub>T<sub>6</sub> in various proportions as a function of the used solvent: 4% with pyridine, 12% with CH<sub>3</sub>CN, 19% with THF, and no major product was observed if the reaction was performed in DMF/pyridine. Although ppp<sub>S</sub>T<sub>6</sub> could be detected in a complex mixture, yields were too modest.

Therefore, another approach using the phosphitylation reagent of Ludwig and Eckstein<sup>[13]</sup> was developed. After elongation of a T<sub>6</sub> model on synthesizer, 5'-OH T<sub>6</sub> was functionalized with a salicylphosphite, which was further substituted by bis(tri-*n*-butylammonium) pyrophosphate by using conditions adapted from the synthesis of nucleoside 5'-( $\alpha$ -P-thio)triphosphates on a solid support.<sup>[14]</sup> Then, the oxidation reaction was performed with elemental sulfur in DMF,<sup>[15]</sup> and the cyclic triphosphate was opened by aqueous buffered treatment before ON deprotection under basic conditions. IEX-HPLC and mass spectrometry analyses of the crude mixture showed the presence of numerous species such as 5'-OH T<sub>6</sub>, 5'-pT<sub>6</sub>, 5'-p<sub>S</sub>T<sub>6</sub>, and desired ppp<sub>S</sub>T<sub>6</sub> ON **1** (16% yield calculated by integrating the HPLC peak, see Figure S3 in the Supporting Information). These data were consistent with the work reported on the synthesis of 5'-TP ONs on a solid support by using the Ludwig and Eckstein method.<sup>[16]</sup> Owing to this moderate yield, we further investigated a more effective method for the formation of the  $\alpha$ -thio TP bond.

Inspired by the synthesis of deoxynucleotide 5'-( $\alpha$ -P-thio)triphosphates by Chen and Benkovic in 40 to 65% yield,<sup>[17]</sup> we designed a similar synthetic scheme with the pyrophosphorylation of an activated thiomonophosphate at the 5' end of a solid-supported ON (Figure 1). This method was initially tested with a solid-supported T<sub>6</sub> model. The phosphorothioate monoester was generated from an *H*-phosphonate intermediate prepared as described previously.<sup>[3]</sup> Oxidation of the *H*-phosphonate (5'-Hp<sub>S</sub> T<sub>6</sub>) to phosphorothioate (p<sub>S</sub>T<sub>6</sub>) involved first its conversion into a trimethylsilyl derivative that is highly reactive for sulfuration. This was performed with a mixture of *N,O*-bis(trimethylsilyl)acetamide (BSA) and 0.2 M 3-*H*-1,2-benzodithiol-3-one-1,1-dioxide in CH<sub>3</sub>CN (Beaucage's reagent),<sup>[18]</sup>



For this study, T<sub>6</sub> was synthesized on a solid support on a 9 μmol scale by using nine synthesis columns for a 1 μmole scale. After assembly completion, solid-supported T<sub>6</sub> in each column was subjected to 5'-functionalization to yield expected thiotriphosphate ON **3**. To allow quick access to the target ON, the total CPG beads were pooled in a 10 μmol column synthesis and were treated under basic conditions. The crude material (7624 nmol) was recovered and was purified by IEX-HPLC to provide highly pure ppp<sub>s</sub>T<sub>6</sub> ON **3** (1473 nmol, 19.3% yield) for <sup>31</sup>P NMR spectroscopy analysis. The spectrum was consistent with the formation of a hexamer bearing an α-thiotriphosphate entity. The most important signal at δ = −0.4 ppm corresponds to the five internucleosidic phosphodiester bonds. The two signals at δ = −22.5 and −6.7 ppm were assigned to β and γ phosphates of the triphosphate moiety, respectively.<sup>[13]</sup> Furthermore, the spectrum showed the expected doublet at δ = 43.8 ppm, which corresponds to two diastereoisomers (Sp and Rp) of the α phosphorus atom bearing the sulfur atom. These data are similar to the chemical shifts reported for nucleoside 5'-O-α-thiotriphosphates.<sup>[13]</sup> It is well known that replacement of an oxygen atom by a sulfur atom results in a large downfield shift in the signal for the phosphorus atom bearing the modification. Indeed, this analysis ascertained the location of the phosphorothioate group.

The above reaction conditions were further applied to the synthesis of several 5'-(α-P-thio)triphosphate RNA sequences (Table 2, ONs **4–10**), substrates of enzymes involved in mRNA maturation and processing *B. subtilis*, Dengue, or SRAS viruses. First, RNA elongation was performed from 2'-O-pivaloyloxymethyl ribonucleoside phosphoramidites (Chemgenes Corporation) by using a procedure developed in our laboratory some years ago.<sup>[20]</sup> Then, H-phosphonylation, oxidation to the phosphorothioate, activation with diphenyl chloride, substitution with pyrophosphate, and deprotection were conducted consecutively, as detailed in Scheme 3. Similar to the data obtained with the ppp<sub>s</sub>T<sub>6</sub> model, ppp<sub>s</sub>RNA ONs **4–10** were formed in yields ranging from 37 (for ON **6**) to 54% (ON **10**), as determined by their HPLC purity in the crude deprotection

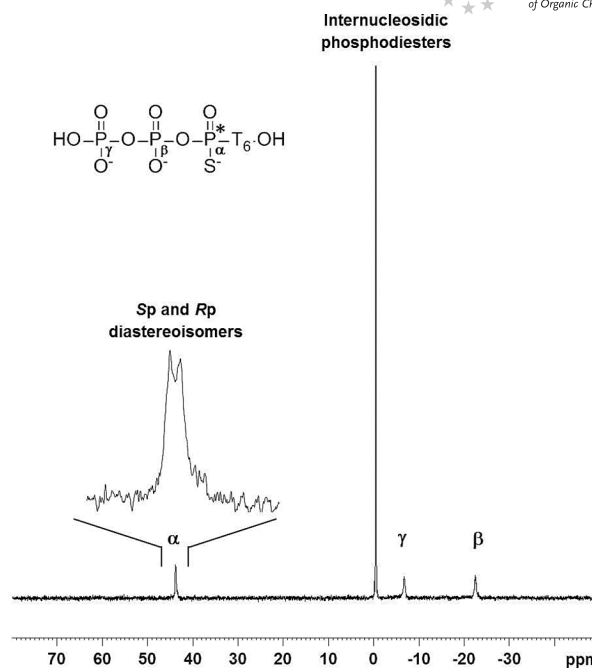


Figure 4. <sup>31</sup>P NMR spectrum of ppp<sub>s</sub>T<sub>6</sub> ON **3** (161.98 MHz, 2.4 mm in D<sub>2</sub>O, 298 K).

mixture (Figure 5, a). After IEX-HPLC purification and desalting, the ppp<sub>s</sub>RNAs were isolated in global yields around 20% with purities higher than 95% (Figure 5, b). However, one limitation of this method was the scale up. Indeed, we could not succeed in the direct preparation of ONs **4** and **5** on a 5 μmol scale, which was required for structural studies by crystallography. If 5'-H-phosphonate formation and the following oxidation to the phosphorothioates could be achieved on a large scale, the pyrophosphorylation failed and ONs **4** and **5** could not be obtained. Consequently, nine syntheses and subsequent 5'-functionalizations were performed in parallel on a 1 μmol scale, and then the solid-supported ppp<sub>s</sub>ONs were pooled in a 10 μmol Twist column; the pool as a whole was then subjected to deprotection conditions. Moreover, reverse-

Table 2. Data for synthesized 5'-(α-P-thio)TP and 5'-(α-P-thio)(β,γ-methylene)TP oligonucleotides.

ON	5'-Sequence-3' [a]	Scale (μmol)	Yield (%) [b]	Estimated material (nmol) [c]	Isolated material (nmol) [d]	MALDI-TOF (m/z) [e] calcd.	found
<b>1</b>	ppp <sub>s</sub> T <sub>6</sub>	0.5	16	174	nd [f]	2018.19	2018.78
<b>2</b>	ppp <sub>s</sub> T <sub>6</sub>	0.5	32	157	nd [f]	2018.19	2018.53
<b>3</b>	ppp <sub>s</sub> T <sub>6</sub>	9	31	7624	1473	2018.19	2018.80
<b>4</b>	ppp <sub>s</sub> GG	5	43	2960	573	883.44	883.36
<b>5</b>	ppp <sub>s</sub> GGA	5	47	2587	546	1212.65	1212.70
<b>6</b>	ppp <sub>s</sub> AUAUUA	1	37	379	78	2099.15	2099.08
<b>7</b>	pCH <sub>2</sub> pp <sub>s</sub> AUAUUA	1	30	448	96	2097.17	2097.07
<b>8</b>	ppp <sub>s</sub> AGUUGUUAGU	1	45	397	78	3417.89	3417.29
<b>9</b>	pCH <sub>2</sub> pp <sub>s</sub> AGUUGUUAGU	1	39	384	75	3415.92	3415.15
<b>10</b>	ppp <sub>s</sub> AGUUGUUAGUCUACGUGGA	1	54	415	92	6334.61	6334.94

[a] Except for ON **1** prepared by using procedure VI.4, the other ONs were synthesized by using procedure VI.3 (see the Supporting Information). [b] Percentage yield of the modified triphosphate in the crude material, as calculated by integration of the HPLC chromatogram. [c] Total crude material released from the solid support upon ammonia treatment. [d] Isolated pure ON after HPLC purification. [e] MALDI-TOF mass spectrometry analysis performed in the negative mode. [f] Not determined because ONs **1** and **2** were not purified.





*P*-thio)triphosphate entity at their 5'-end was described. Inspired by our previous work on the synthesis of 5'-triphosphate oligonucleotides,<sup>[3]</sup> the strategy is similarly based on the use of *H*-phosphonate chemistry to introduce the first phosphate moiety at 5'-OH. After oxidation of this intermediate to the thiophosphate by using Beaucage's reagent, the pyrophosphorylation was achieved first through activation as a reactive phosphoanhydride and second through substitution with pyrophosphate or its  $\beta,\gamma$ -methylene analogue. 5'-( $\alpha$ -*P*-Thio)triphosphate and 5'-( $\alpha$ -*P*-thio)( $\beta,\gamma$ -methylene)triphosphate oligonucleotides were obtained in good yields ranging from 30 to 50%. This method was the most successful to obtain solid-supported 5'-( $\alpha$ -*P*-thio)triphosphate oligonucleotides conveniently compared to other methods involving the use of phosphoramidate or salicylphosphite intermediates. The greater availability of these original hydrolysis-resistant 5'-triphosphate RNAs represents real progress for their use in biological research to elucidate the mechanism of enzymes involved in mRNA processing and maturation.

## Experimental Section

**General Procedure for the Oxidation of 5'-*H*-Phosphonate ON by Using 3*H*-1,2-Benzodithiol-3-one 1,1-Dioxide:** Prior to oxidation, the synthesis column containing the solid-supported 5'-*H*-phosphonate ON<sup>[3]</sup> was flushed with argon and activated 3 Å molecular sieves were added to two glass syringes (five beads each) connected to the column. The oxidation solution containing 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (40 mg, 0.2 mmol) and *N,O*-bis-trimethylsilylacetamide (0.4 mL, 1.64 mmol) in anhydrous CH<sub>3</sub>CN (1.6 mL) was pushed back and forth through the column by using two syringes for 1 min and then left to react for 5 min at 30 °C. The mixture was removed from the column, and the solid support was washed with CH<sub>3</sub>CN (4 × 2 mL, HPLC grade) and flushed with argon. Then, 0.1 M TEAB (2 mL) was pushed back and forth through the column and left to react for 15 min at 30 °C. The solution was removed from the column, and the solid support was washed with anhydrous CH<sub>3</sub>CN (8 × 2 mL) and then dried under a stream of argon.

**General Procedure for Pyrophosphorylation through Activation with Diphenyl Phosphoryl Chloride:** A 0.1 M diphenyl phosphoryl chloride solution (0.4 mL, 1.93 mmol) in anhydrous pyridine (1.6 mL) was manually passed with a glass syringe filled with five beads of activated 4 Å molecular sieves through the column containing the 5'-thiomonophosphate ON still anchored to the solid support. Using another syringe also containing five beads of activated 4 Å molecular sieves, the solution was pushed back and forth for 1 min and left to stand for 4 min at room temperature. The solution was quickly removed from the column, and the solid support was washed with anhydrous CH<sub>3</sub>CN (4 × 2 mL) and then flushed with argon. A fresh 1 M solution of bis(tri-*n*-butylammonium) pyrophosphate (360 mg, 0.53 mmol) or bis(tri-*n*-butylammonium) methylenediphosphonic acid (358 mg, 0.53 mmol) in anhydrous pyridine (250 µL) was applied to the column by using two 1 mL plastic syringes. The solution was passed through the column and left to stand for 2 h at 30 °C. The solution was removed from the column, and the solid support was washed with anhydrous CH<sub>3</sub>CN (3 × 2 mL) and then dried with a stream of argon.

**General Procedure for the Deprotection and Release of Oligonucleotides 5'-( $\alpha$ -*P*-thio)TP or 5'-( $\alpha$ -*P*-thio)( $\beta,\gamma$ -methylene)TP:** The solid-

supported ON 5'-( $\alpha$ -*P*-thio)TP or 5'-( $\alpha$ -*P*-thio)( $\beta,\gamma$ -methylene)TP in a Twist column (0.5 µmol to 1 µmol scale) was treated with a 1 M solution of DBU in anhydrous CH<sub>3</sub>CN (2 mL) for 3 min. Then, the solution was removed, and the solid support was washed with anhydrous CH<sub>3</sub>CN (8 × 2 mL) and then flushed with argon for 1 min. 30% Aqueous ammonia solution was applied to the column in three batches (1.5, 1, and 0.5 mL) for 30 min each. The three ammonia fractions were collected into a 50 mL round-bottomed flask and were left to react at 30 °C for 1.5 h. Then, isopropylamine (15% of total volume: 0.45 mL) was added to the solution, and the mixture was evaporated under reduced pressure with a bath at a maximum of 30 °C until the volume was reduced to approximately 0.3 mL. The mixture was coevaporated with Milli-Q water (3 × 1 mL) following the same protocol. The residue was dissolved in Milli-Q water (1.5 mL divided into three portions for flask rinse: 0.8, 0.4, and 0.3 mL) and transferred to a 2 mL Eppendorf vial, then lyophilized from water.

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