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Thermolytic reagents to synthesize 5’- or 3’-mono(thio)phosphate oligodeoxynucleotides or 3'-modified oligodeoxynucleotides

Mimouna Madaoui, Albert Meyer, Jean-Jacques Vasseur and François Morvan

Institut des Biomolécules Max Mousseron (IBMM), Université de Montpellier, CNRS, ENSCM, Montpellier, France.

François Morvan https://orcid.org/0000-0001-5077-0028
Jean-Jacques Vasseur https://orcid.org/0000-0002-4379-6139

Keywords: Oligonucleotides, monophosphate, monothiophosphate, thermolytic, MALDI-TOF, Click chemistry, ·

ABSTRACT

Methylthioethanol and 2,2′-thiodiethanol were derivatized into cyanothyl-phosphoramidites and solid support and were used to synthesize 5'-, 3'-monophosphate or 5'-, 3'-monothiophosphate oligonucleotides by thermolytic treatment followed by ammonia. The corresponding 2,2′-thiodiethanol solid support was also used to release fully protected oligonucleotides from solid support without ammonia treatment, to monitor the oligonucleotide elongation on solid support by MALDI-TOF mass spectrometry without any prior chemical treatment or to synthesize 3'-pentynyl oligonucleotides in combination with a modified phosphoramidite where the cyanoethyl group was replaced by a pentynyl one.

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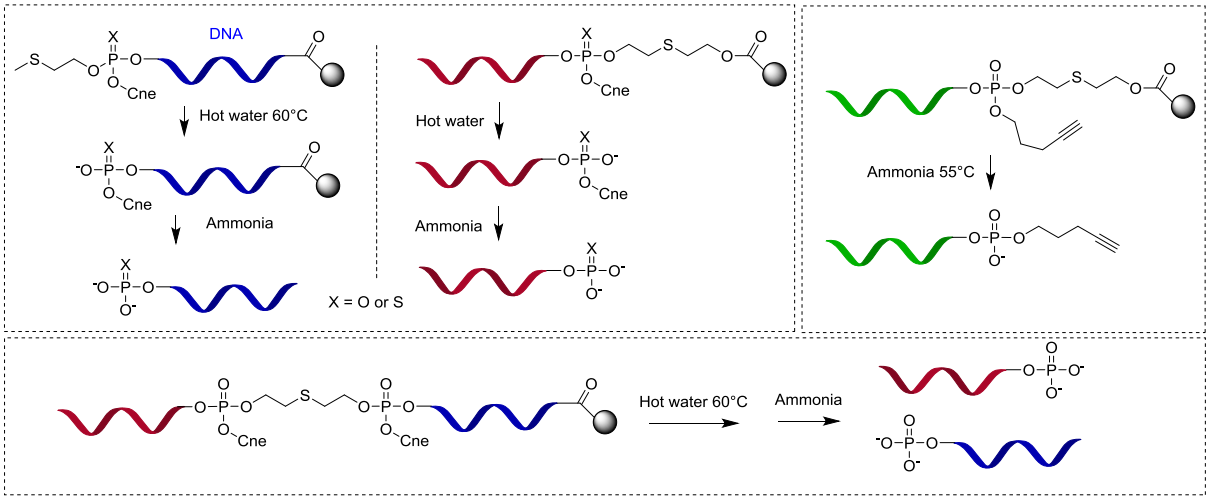
Graphical abstract



INTRODUCTION

Oligonucleotides bearing a 5'-phosphate are useful in several biological applications like conjugation[1] PCR[2] or gene construction.[3] Furthermore, the small interfering RNAs (siRNAs) required the presence of a 5'-phosphate on the target-complementary strand for RNAi activity[4-5] There are only two commercially available phosphoramidites **1**,[6] **2**[7] and also few derivatives **3**,[8] **4**,[9] **5**,[10] **6**,[11] **7**[12] and **8**[13] reported in the literature for 5'-phosphorylation (Figure 1). As an alternative 5'-phosphate oligonucleotides were also prepared by oxidation of the last nucleoside leading, during the ammonia deprotection, to the formation of a 5'-monophosphate.[14]

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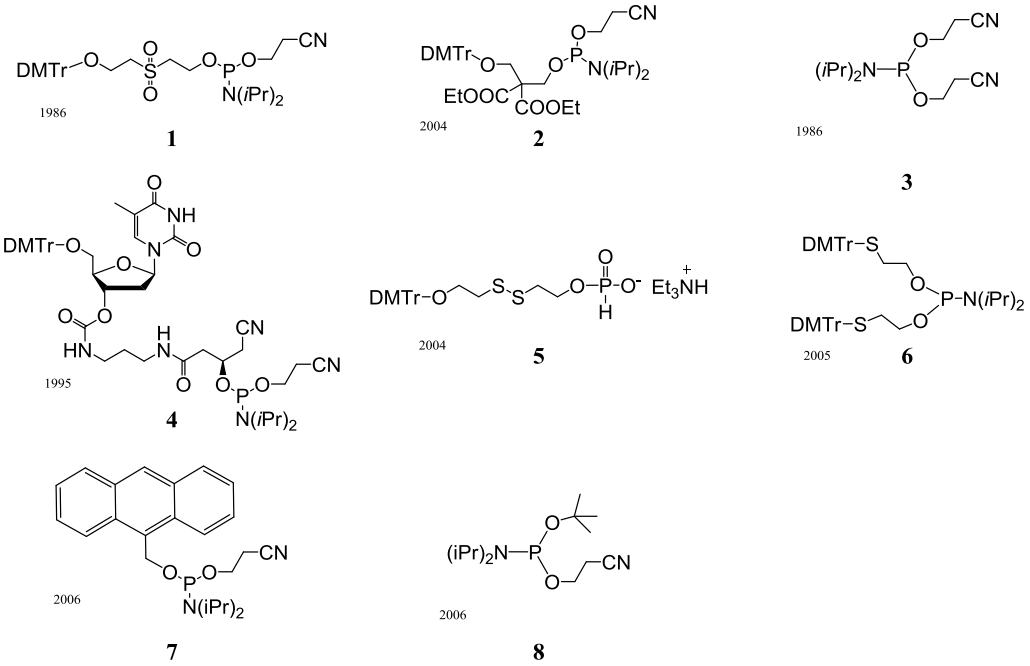

Figure 1: Structure of 5'-phosphorylating agents of oligonucleotides with the year of publication.

For the synthesis of 3'-phosphate oligonucleotides, solid supports bearing the same structure of **1** and **2** are commercially available and several solid supports have been reported in literature (for a review see [15]).

Few years ago, Beaucage et col. reported thermolytic oligonucleotide prodrugs using heat-sensitive protecting groups of phosphodiesters.[16-18] They showed that different groups can be removed by heating the oligonucleotides in water. Their half-lives vary from few minutes to several hours. Among them the 2-(methylthio)ethyl group was found to be the most heat-sensitive with a half-life below 3 min at 37 °C.[18] Along this line, we decided to use this kind of protecting group to afford 5'-phosphate or thiophosphate oligonucleotides and subsequently to use the 2,2′-thiodiethanol to synthesize either 3'- or 5'-phosphate or thiophosphate oligonucleotides as well as 3'-modified oligonucleotides.

RESULTS AND DISCUSSION

Phosphoramidite **11** and solid support **12** were prepared in two steps starting from 2,2′-thiodiethanol with first a mono dimethoxytritylation (54%) and then a phosphitylation (79%)

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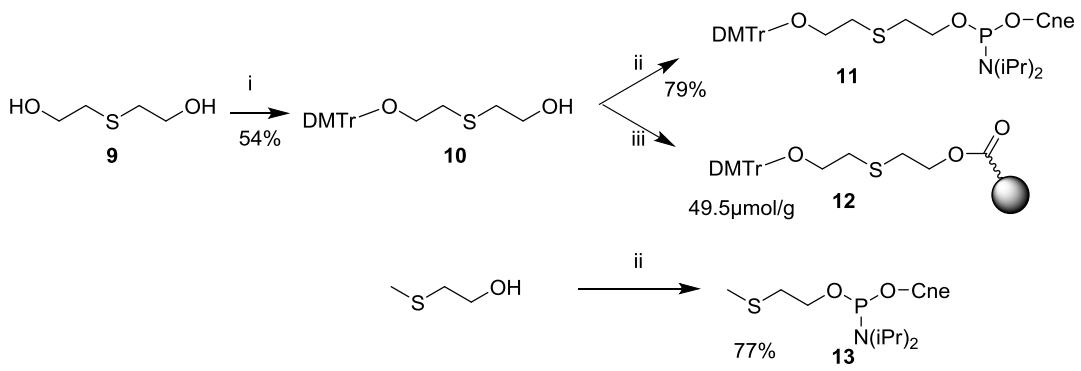
or a classic ester formation on succinate-LCAA-CPG (Scheme 1). The preparation of the phosphorylating agent **13** was straightforward since only a phosphitylation of 2-(methylthio)ethanol was required with a 77% yield. Interestingly, both reagents used to prepare these new phosphoramidites and the solid support are less expensive than 2,2′-sulfonyldiethanol and diethyl bis(hydroxymethyl)malonate required for the synthesize of **1** or **2**.



Reagents and solvents: i: DMTr-Cl dry pyridine; ii: CneOPN(*i*Pr)$_2$Cl, dry CH_2Cl_2, DIEA; iii: Succinate-LCAA-CPG, EDC, DMAP, Et_3N, dry pyridine. Cne = cyanoethyl.

Scheme 1: Synthesis of 5’-phosphorylating phosphoramidites **11**, **13** and 3’-phosphorylating solid support **12**.

Synthesis of 5'-monophosphate oligonucleotides.

To evaluate the efficiency of both phosphoramidites, hexathymidylates were synthesized and **11** or **13** were coupled on it (Scheme 2). The syntheses were performed using a DNA synthesizer with a standard cycle with a 30 sec coupling time for thymidine phosphoramidite. To ensure the coupling of **11** and **13** an extended coupling time of 60 sec was applied.

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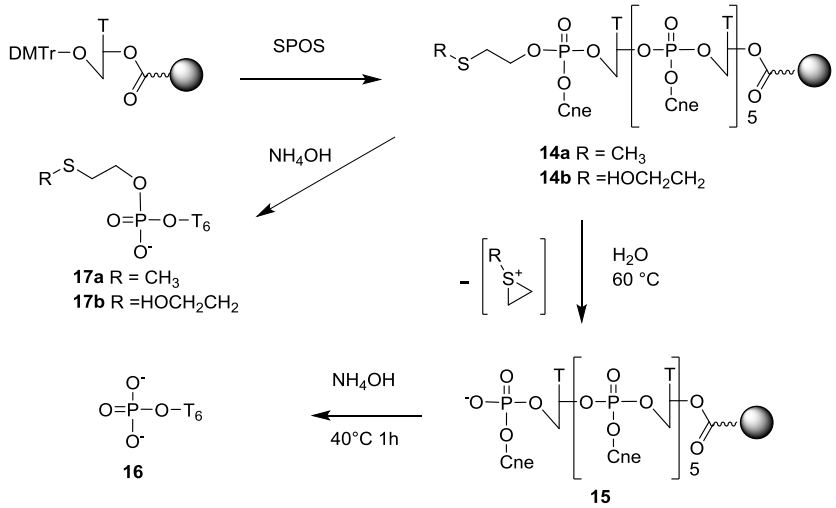

Scheme 2: Synthesis of 5'-monophosphate-T_6. SPOS Solid phase oligonucleotide synthesis: i: 3% TCA in CH_2Cl_2; ii: thymidine phosphoramidite, **11** or **13**, and benzylthiotetrazole in CH_3CN; iii: Ac_2O, *N*-methylimidazole, THF, pyridine; iv: I_2, H_2O, THF, pyridine.

After elongation, to check if the incorporation of **13** was complete, the solid-supported oligonucleotide **14a** was analyzed by MALDI-TOF MS.[19] The energy brought by the laser induces the fragmentation of the oligonucleotide at the phosphotriester levels giving ions corresponding to fully protected short-mers (Figure 2).[20] MALDI-TOF spectrum showed that **13** was well incorporated since all the ions have a 5'-phosphosdiester cyanoethyl. Indeed, due to the heat of the laser the thermolytic group was removed from the phosphotriester moiety. One can observe a partially loss of one cyanoethyl group as already reported.[20] This data confirmed the thermolytic behavior of the methylthioethyl group and the efficient coupling of **13**.

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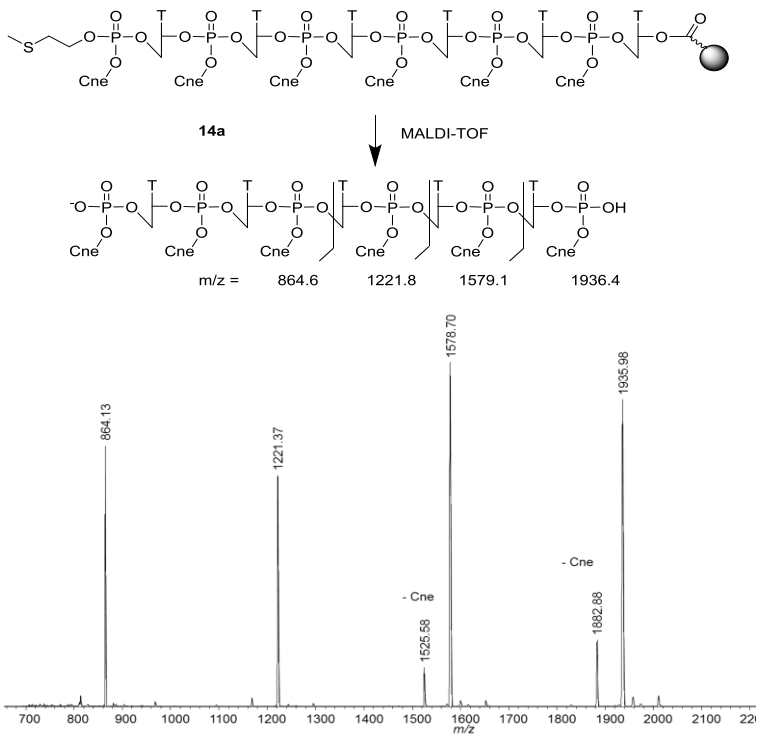

Figure 2: MALDI-TOF MS analysis of solid-supported oligonucleotide **14a**.

Then solid-supported oligonucleotide **14a** was treated with water of different temperatures (40, 50 or 60 °C) up to 80 min and finally with concentrated ammonia, (1 h 40 °C), to eliminate the cyanoethyl groups and to release the oligonucleotide from the solid support. Each sample was analyzed by C_{18} HPLC and characterized by MALDI-TOF MS (Figure 3 and S1). The full formation of 5'-PO-T_6 was obtained within 80 min at 40 °C, 40 min at 50 °C and 30 min at 60 °C. The direct treatment with ammonia mainly gave the methylthioethyl-T_6 **17a** and some 5'-PO-T_6 **16**. Interestingly, compound **17a** remained intact after additional heating with either ammonia or water. Due to the presence of a negative charge on the phosphodiester the elimination of the methylthioethyl by formation of the S-methylthiirane is no more possible. Likewise, solid supported oligonucleotide **14b** was treated with water at 60 °C for 30 min and then ammonia affording the expected 5'-PO-T_6 with the same efficacy observed for **14a** (Figure S2). These data demonstrated that both groups are thermolytic on a phosphotriester but not on a phosphodiester.

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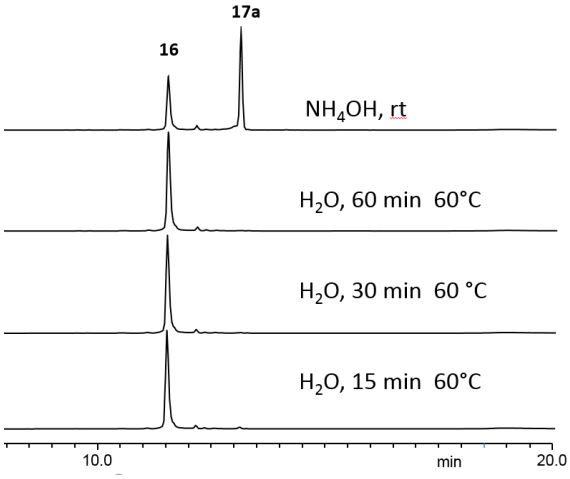

Figure 3: HPLC profiles of kinetic of deprotection with water at 60 °C then ammonia for 1 h at 40 °C or only ammonia Peak at 11.5 min is PO-T_6 **16** and that at 13.1 min is **17a**.

The efficacy of phosphoramidite **13** to synthesize 5'-monophosphate oligonucleotides was confirmed on a dodecanucleotide exhibiting the four nucleobases (pTAGTCAGCACTT). After elongation the solid-supported oligonucleotide was treated 1 h at 60 °C and then with ammonia 5 h at 55 °C. The reverse phase C_{18} HPLC and MALDI-TOF MS analyses confirmed the formation of the 5'-PO-12-mer ($[M-H]^-$ calcd 3699.40 found 3698.81, Figure S3).

Synthesis of 3'-monophosphate oligonucleotides

Likewise, an hexathymidylate was synthesized on the solid support **12** to obtained T_6-PO-3' (Scheme 3). The solid-supported T_6 **18** was analyzed by MALDI-TOF MS (Figure 4). Upon the laser irradiation the fully protected T_6 **19** was mainly observed (m/z 2160.97, calcd 2160.57). The ions due to the fragmentation of **18** were only visible in the background increasing 5-fold their intensity. This result confirmed the thermolytic behavior of the ethylthioethyl linker. Interestingly, this property allows to release fully protected oligonucleotides from a solid support without an ammonia treatment. Then, **18** was treated with water at 60 °C for 30 min, the supernatant containing **19** was withdrawn and the beads were washed with water and methanol. After partial evaporation **19** was analyzed by HPLC and MALDI-TOF MS confirming its formation (Figure 5, S4). A final ammonia treatment

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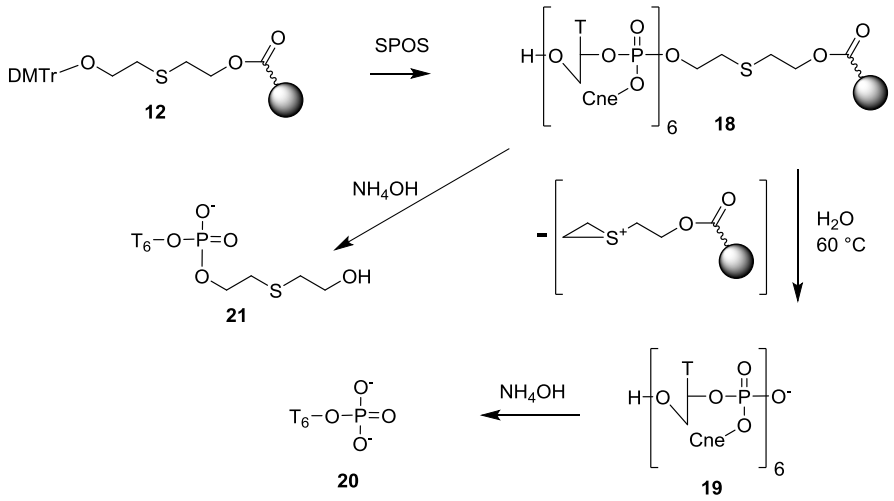
afforded the T_6-PO-3' **20**. As expected, a direct ammonia treatment of **18** essentially gave the T_6 with a 3'-ethanolthioethyl group **21**.



Scheme 3: Synthesis of 3'-monophosphate-T_6.

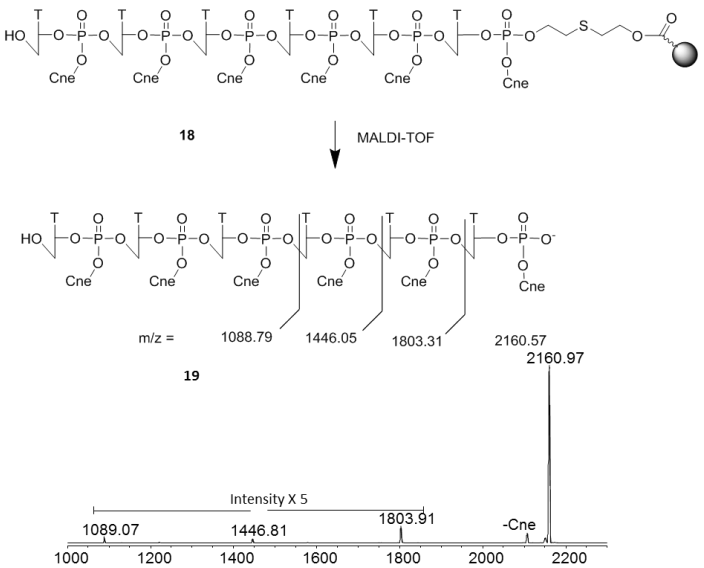


Figure 4: MALDI-TOF MS analysis of solid-supported T_6 **18**.

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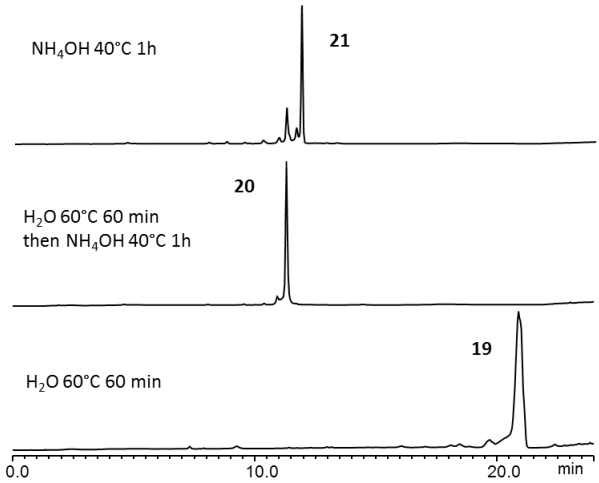

Figure 5: HPLC profiles of crude fully protected T_6 **19** (bottom), T_6-PO3' **20** (middle) and T_6-ethylthioethanol **21** (top).

A 3'-phosphate dodecamer (CAA TCC GAT GACp) was synthesized starting from solid support **12** from which the oligonucleotide was elongated by a standard cycle. The 3'-phosphate 12-mer was obtained after a first treatment with hot water (60 min 60 °C) and then with ammonia for 5 h at 55 °C. The oligonucleotide was analyzed and purified by HPLC and characterized by MALDI-TOF MS ($[M-H]^-$ m/z calcd 3693.39, found 3693.43, Figure S5).

Synthesis of 5'-monothiophosphate oligonucleotides.

Since monothiophosphate oligonucleotides are useful intermediates for conjugation through alkylation[21] we decided to synthesize them using solid support **12** or phosphoramidite **13**. A hexathymidylate model was synthesized and **13** was coupled. During the last coupling, the oxidation step was performed with the sulfurizing agent 3H-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent)[22] to give the solid-supported thionophosphotriester T_6 **22** (Scheme 4). We applied the same protocol for the deprotection than described above (H_2O 60 °C, 1 h washed with water and then ammonia 40 °C, 1h) but we were surprised to observed two peaks in HPLC, one minor at 10.8 min (21%) corresponding to the expected 5-phosphorothioate T_6 (1857.99 calcd 1858.30) and a major peak at 13.1 min (79%) with a mass corresponding to a T_6 still bearing the methylthioethyl group (1932.14 calcd 1932.40) (Figure 6 left). Surprisingly the two diastereoisomers due to the chiral phosphorothioate were not distinguished. When **22** was directly treated by ammonia, HPLC profiles showed a small peak

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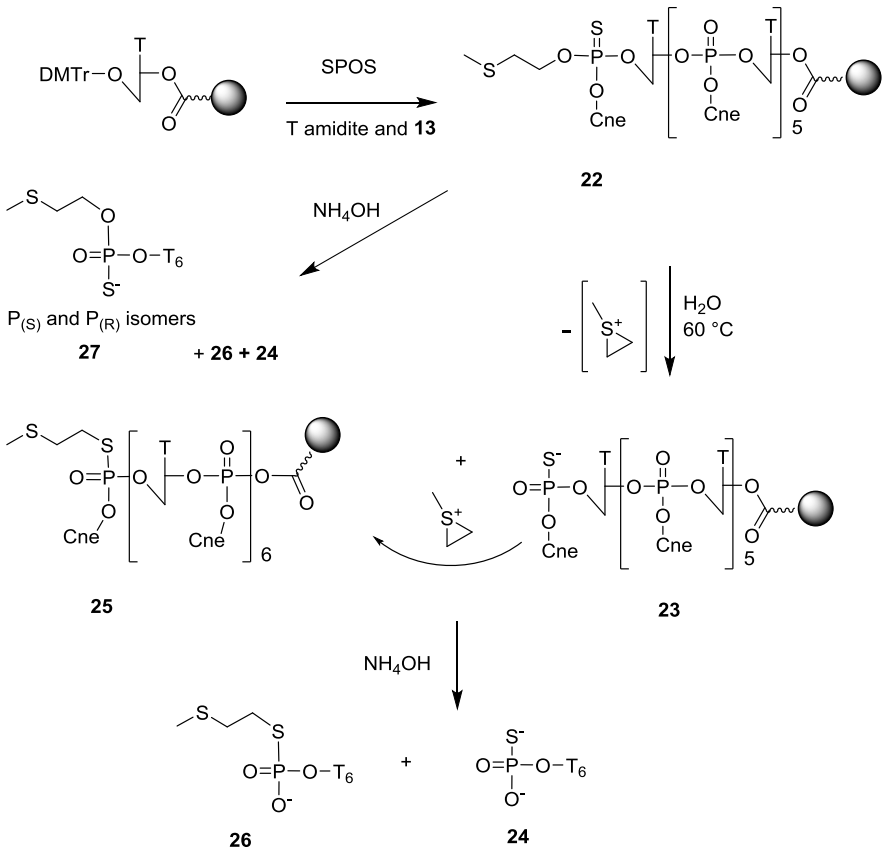
(7.9%) at 10.8 min and three major peaks one at 13.1 min (35.4%), like previously observed, and two peaks with very close retention times (13.4 min 29.7% and 13.5 min 27.6%) (Figure 6 right). MS analysis of the three isolated peaks between 13.1 and 13.5 min showed only one species corresponding to the T_6 with the pendant group. The two peaks of highest retention time correspond to the T_6 with a chiral phosphorothioate diester bearing the methylthioethyl group **27** due to the elimination of the cyanoethyl. The previous peak at 13.1 min is the T_6 with a thiolate methylthioethyl **26**. The explanation for its formation is that the solid-supported thiophosphatediester derivative **23** partially reacted with the S-methyl thiirane, formed in situ, to give **25**. It has been reported in literature that thiophosphodiesters are also able to performed alkylation even if they are less reactive than monothiophosphates.[23] This side reaction was not observed during of the formation of a phosphorothioate diester by thermolysis in solution.[18] It appeared that the methylthiolethyl group on a thiolate function was not able to be eliminated. Ammonia treatment finally gave the expected 5'-PS-T_6 **24** and its thiolate derivative **26**.



Scheme 4: 5'-thiophosphate-oligonucleotide.

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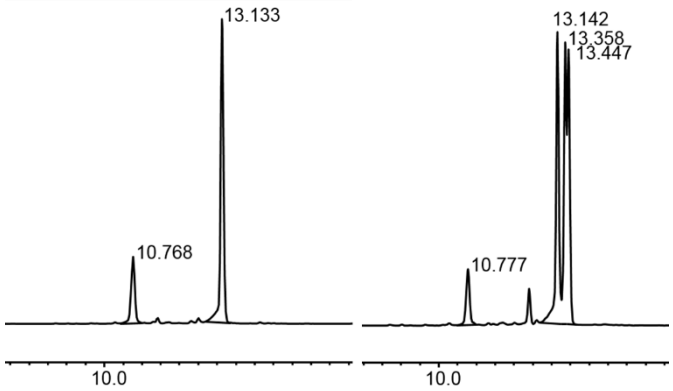
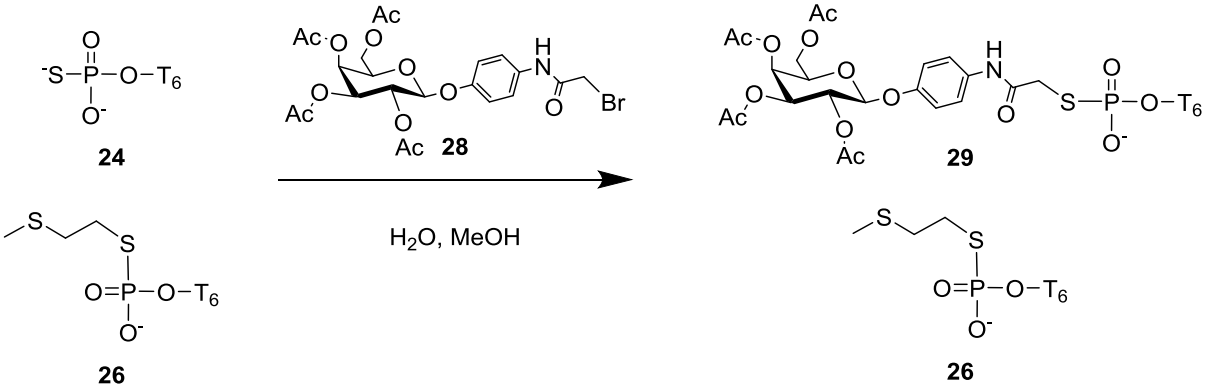


Figure 6. HPLC chromatograms of **22** (left) after treatment with hot water (1h 60 °C) and then ammonia and (right) only ammonia deprotection.

To confirm the formation of the mixture of PS-T_6 **24** and methylthioethyl thiolate T_6 **26**, tetra-*O*-acetyl-galactoside phenyl bromoacetamide **28**[24] was added for conjugation through thiol-alkylation (Scheme 5).[25] After 4h, the mixture was analyzed by HPLC and MALDI-TOF MS. The HPLC profiles showed the almost disappearing of the peak of PS-T_6 with the appearance of a peak at 18.3 min while the peak of **26** at 13.1 min remained unchanged (Figure 7). MS analysis confirmed the formation of the Ac4galacto-PS-T_6 conjugate **29** ([M-H]$^-$ found 2337.61, calcd 2337.69) and still the presence of **26**.



Scheme 5: Conjugation of PS-T_6 with galactoside bromoacetamido derivative **28**.

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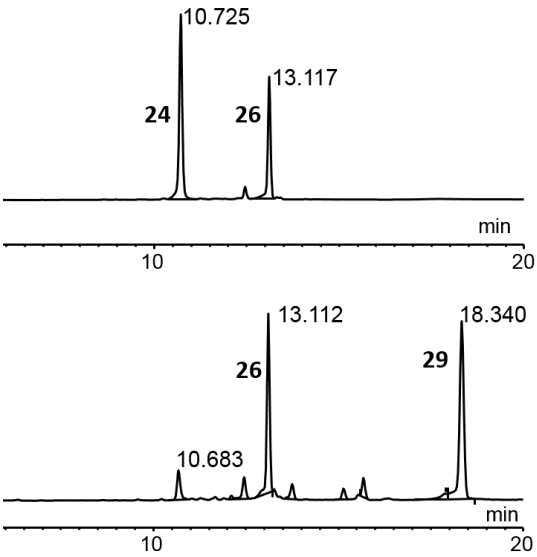

Figure 7. HPLC profiles of (top) starting mixture of PS-T_6 **24** and MeSEtSP-T_6 **26** and (bottom) after addition of tetraacetyl galactoside phenol bromoacetamido **28** affording the conjugate **29**.

Several attempts were done to reduce the amount of the side reaction: use of a large quantity of water under stirring, percolation during 1 h with hot water (70 °C) to wash off the methyl-thiirane, but the formation of the PS-T_6 stayed below 27%. We may assume that the reaction of the solid-supported PS-T_6 with the methyl-thiirane occurred very rapidly due to their close vicinity in the pores of the CPG. In order to trap the methyl-thiirane, we decided to perform the elimination in presence of thiophosphate as scavenger. Hence **22** was treated with a 0.250 or 0.125 M Na_3PO_3S aqueous solution in 1.0 M TEAAC buffer pH 7 at 60 °C for 60 min under stirring. After wash with water, conc. ammonia was added (1 h, 40 °C). HPLC profiles showed the formation of the PS-T6 **24** with only a very low amount of **26** and **27**. MS analysis confirmed for both concentrations of PO_3S used, the formation of **24** (1858.06 and 1858.51 calcd 1858.30 Figure 8, S7).

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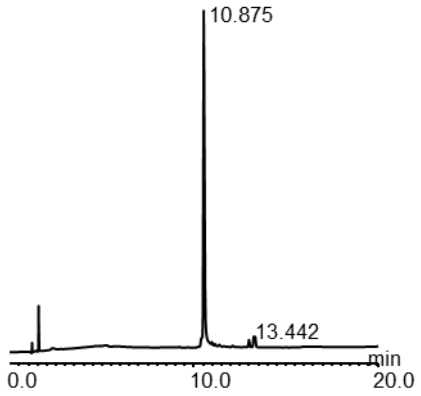

Figure 8. HPLC profile of crude PS-T_6 using 0.25 M Na_3PO_3S as scavenger in 1 M TEAAC pH 7, 1 h at 60 °C and then NH_4OH 1 h 40 °C.

Synthesis of 3'-monothiophosphate oligonucleotides

An heptathymidylate was synthesized on solid support **12** with a sulfurizing step during the first coupling and then regular iodine water oxidizing for the others (Scheme 6). The resulting solid-supported T_7 **30** was treated with hot water 60 °C or with 0.125 M Na_3PO_3S aqueous solution in 1.0 M TEAAC buffer pH 7 at 60 °C for 60 min under stirring. The supernatants were withdrawn and after evaporation treated with ammonia as well as the solid supports. For a reference, **30** was treated with cold ammonia for 3 h and then heated at 40 °C for 1 h giving the T_7-PS with the 2-ethanol-2′-thioethyl group **35**, which was eluted as a single peak (12.24 min, Figure 9 left, S8). HPLC and MS analyses confirmed the presence of the T_7-PS **32** in the supernatants and showed the presence of T_7-PS with the linker either on the phosphorothioate **35** or on the phosphorothiolate **34** in the solution resulting from the treatment of the solid supports with ammonia. The presence of **35** indicated that the treatment for the release was too short. More **35** was obtained in the case of the treatment with the thiophosphate solution. The same treatments were applied at 70 °C for 60 min and in this case none **35** was observed when the solid supports were treated with water and then ammonia (Figure 9 right) and just a few for the treatment with PO_3S solution. The supernatant containing **31** was treated with ammonia affording **32** (11.18 min, Figure 9 middle). The amount of T_7-PS **32** isolated from the supernatant represented about 60% of the total for the treatment with water and only 33% when the aqueous solution with PO_3S was used. Indeed, due to the large amount of salts, a size exclusion chromatography (Nap10) must be applied and some compound was lost. To sum up, for the synthesis of 3’-phosphorothioate oligonucleotides the scavenger (PO_3S) was

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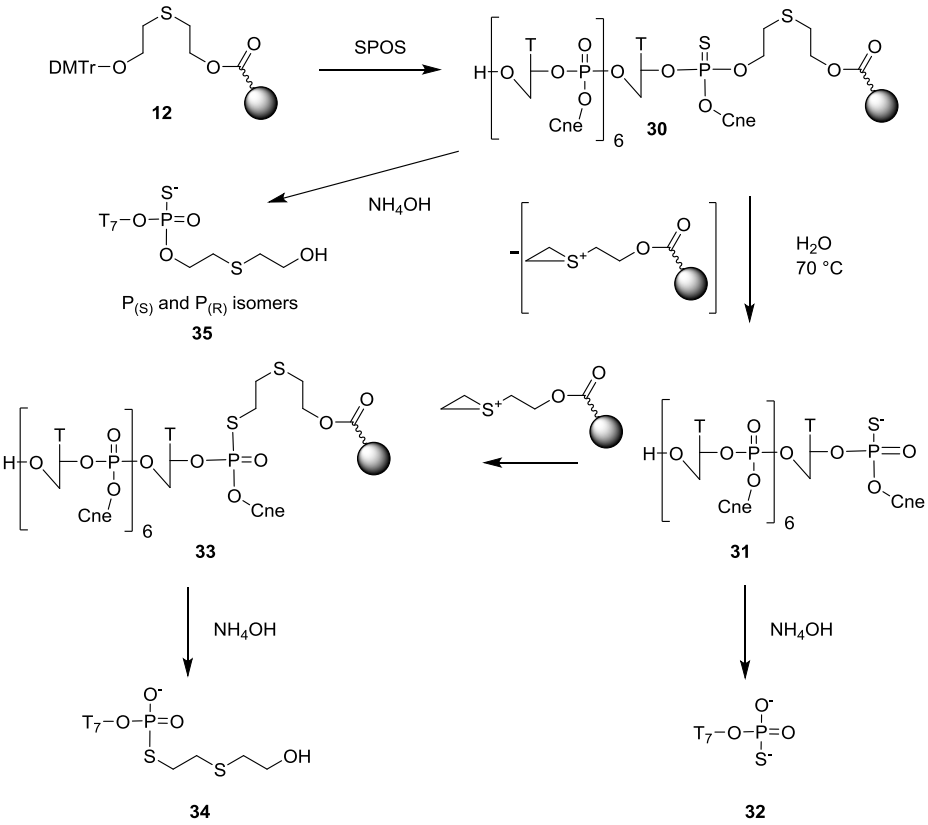
not helpful. This data confirmed that the reaction of the phosphorothioate diester with the solid-supported thiirane derivative is fast but the dispersion of the protected oligonucleotide **31** in the solution allowed to isolate the expected T_7-PS **32** at the end with a 60% yield.



Scheme 6: Synthesis of 3'-thiophosphate-heptathymidylate.

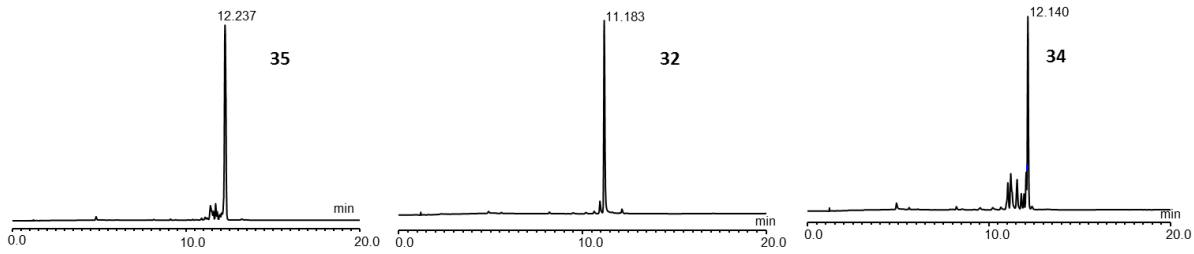


Figure 9. HPLC profiles of **30** treated with (left) NH_4OH 3h 40 °C then 1 h 40 °C affording T_7-POS-OEtSEt-OH **35**, (middle) with water 1 h at 70 °C and then NH_4OH 1 h 40 °C affording T_7-PS **32**, (right) solid support from the previous treatment treated with NH_4OH 1 h 40 °C affording T_7-POOS-EtSEt-OH **34**.

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Monitoring of solid-supported oligonucleotide synthesis by MALDI-TOF MS

Since the ethylthioethyl linker is able to be cleaved upon laser irradiation, we studied the direct monitoring of the elongation of a solid-supported 24-mer by MALDI-TOF MS without any prior chemical treatment. The oligonucleotide TAC ACT TGA CTC GTC ATA GGC TCG was synthesized starting from solid support **12** and few beads were withdrawn every three couplings starting from the 6-mer. The beads were suspended in a THAP matrix solution and deposited on the stainless steel plate. The MS analysis showed for each sample the formation of the major ion corresponding to the fully protected oligonucleotide with the 3'-cyanoethyl phosphodiester from 6 to 18 nucleotides showing the efficient couplings (Figure 10). The detection of the ion corresponding to the 18-mer (7708.0) was difficult but still possible while those of the 21-mer (9284.4) and the 24-mer (10558.4) were not possible. The ion M-53 corresponding to the loss of one cyanoethyl group was often seen. In our case, the accuracy of the ion mass is usually lower than that of an oligonucleotide in solution but enough to give a clear information on the coupling efficiency. So thanks to the use of solid support **12**, it is possible and rapid to monitor, by MALDI-TOF MS, the elongation of oligonucleotides up to 18-mer without the need to deprotect a sample.

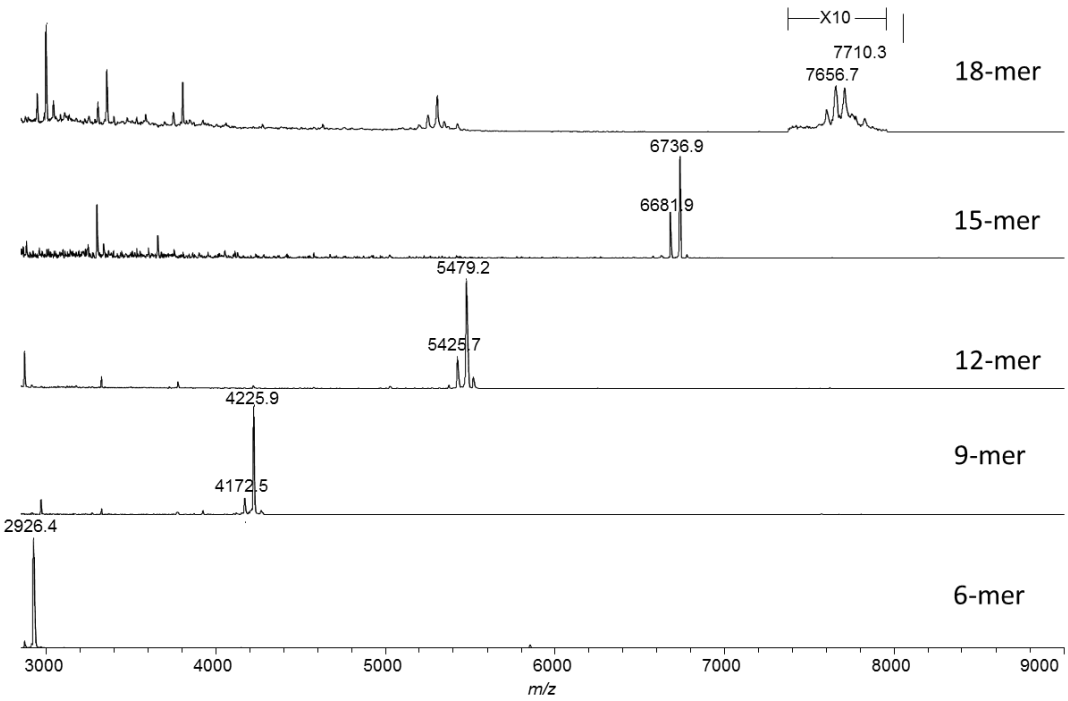

Figure 10. MALDI-TOF spectra of solid-supported oligonucleotides with a ethylthioethyl linker between the oligonucleotide and the CPG beads TGA CTC GTC ATA GGC TCG.

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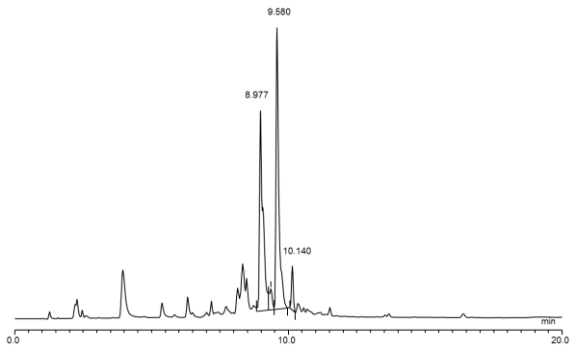
Expected $[M-H]^-$: 6-mer 2926.4; 9-mer 4224.5; 12-mer 5480.4; 15-mer 6730.4 and 18-mer 7708.0. For the 18-mer the intensity was increased 10-fold.

To analyze solid-supported oligonucleotides without deprotection, we previously used a solid support with a photolabile linker between the CPG beads and the oligonucleotide.[26-27] In that case, upon laser irradiation the photolabile linker was broken releasing the fully protected 3'-phosphodiester oligonucleotide. The current solid support **12** is much more accessible and cheaper than the previous one.[28]

Tandem synthesis.

Thanks to the ethylthioethyl phosphoramidite **11**, it is possible to synthesize in the same row a 3'-phosphate and a 5'-phosphate oligonucleotides starting from a commercially available solid support with a succinyl linker between the nucleoside and the LCAA-CPG. To this end, the oligonucleotide CTACT**11**GTTCAC was synthesized with the introduction of phosphoramidite **11** in the middle of the sequence. After elongation the solid-supported oligonucleotide was treated with water at 60 °C for 1 h and then with ammonia for 5 h at 40 °C. HPLC profiles of the crude showed the presence of two main peaks at 8.98 min and 9.58 min that were characterized by MALDI-TOF MS as CTACT-PO (1516.93 for 1516.97 calcd) and PO-GTTCAC (1845.87 for 1846.18 calcd) respectively (Figure 11). The CTACT (1437.02 for 1437.00 calcd) at 10.14 min was also present at a low level due to some loss of 3'-phosphate. No loss of 5'-phosphate was observed (1766.20 calcd).



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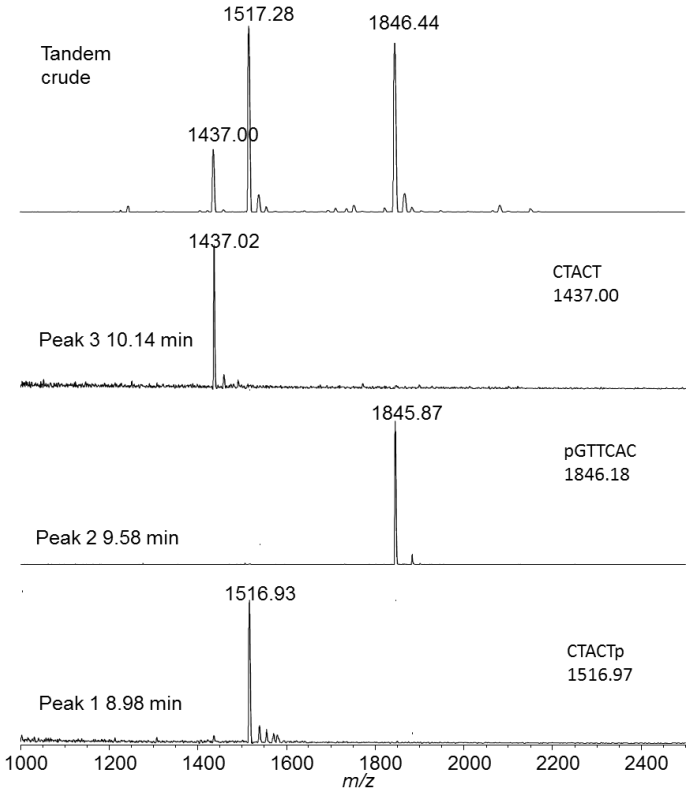

Figure 11: HPLC profile of crude tandem and MALDI-TOF MS characterization

Synthesis of 3'-modified oligonucleotides.

Thanks to the solid support **12** and a nucleoside phosphoramidite bearing a functional group instead of the cyanoethyl one it is possible to easily introduce a modification at the 3' end of oligonucleotides. To illustrate this option, a short oligonucleotide TAACCAGT was elongated on solid support **12** starting with the incorporation of the thymidine phosphoramidite **36** bearing a pent-4-ynyl group and then commercially available nucleoside phosphoramidites using standard phosphoramidite oligonucleotide chemistry (Scheme 7). After elongation the solid-supported 8-mer **37** was analyzed by MALDI-TOF MS showing the efficient synthesis with the formation of the ion corresponding to fully protected 8-mer-pentynyl **38**. Then **37** was treated with water (60 °C, 1 h) and conc. ammonia (40 °C, overnight) or directly with conc. ammonia. After evaporation the 3'-pentynyl 8-mers **39** were analyzed by HPLC and characterized by MALDI-TOF MS (Figure 12, S9). HPLC profiles showed a slightly cleaner synthesis of **39** when a simple ammonia treatment was applied than when a hot water treatment was applied first. Then a 12-mer ACA GGT GTATGT pentynyl was synthesized according the same protocol and directly deprotected with conc. ammonia affording the

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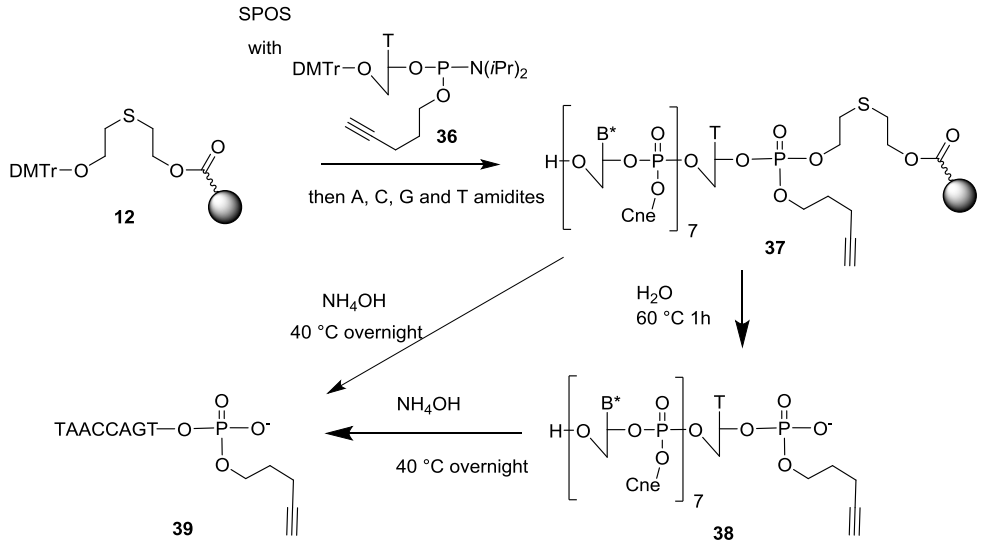
expected 3'-pentynyl-12-mer with high efficiency as confirmed by HPLC and MS analysis and characterization (Figure S10).



Scheme 7. Synthesis of 3'pentynyl oligonucleotide.

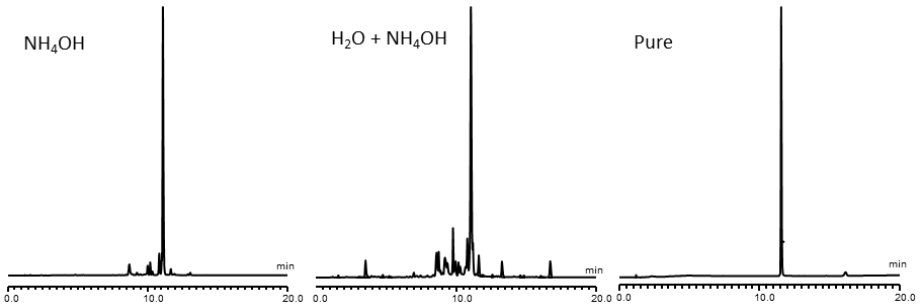


Figure 12. HPLC profiles of 8-mer-pentynyl obtained after left) a direct ammonia treatment middle) 1h at 60 °C water then ammonia, right) pure **39**.

CONCLUSIONS

In conclusion, methylthioethanol was easily and efficiently converted into its phosphoramidite **13** in one step. It allows the synthesis of 5'-mono-phosphate and 5'-monothiophosphate oligonucleotides. The thermolytic methylthioethyl group was removed by heating the solid supported oligonucleotide at 60 °C for 1h in water, after washing the protecting groups (cyanoethyl, benzoyl, isobutyryl) were removed by standard ammonia treatment. In the case

of 5'-thiophosphate it was compulsory to perform the elimination of the thermolytic group in presence of thiophosphate to trap the methylthiirane formed in situ.

2,2′-Thiodiethanol was converted into its monodimethoxytrityl derivative and then into its phosphoramidite **11** or solid support **12**. Phosphoramidite **11** allowed the synthesis of 5'-monophosphate oligonucleotides with the same efficiency as **13** but it synthesis is more expensive since a DMTr group must be introduced. However, one advantage is that it could be introduce into an oligonucleotide affording by thermolysis and then ammonia treatment two shorter oligonucleotides with for one a 3'-monophosphate and for the other a 5'-monophosphate.

The solid support **12**, with the thiodiethanol motif, has several applications: 1) it allows the formation of 3'-monophosphate or 3'-thiomonophosphate; 2) it allows the release of fully protected oligonucleotides from the solid support by treatment with water at 60°C without ammonia treatment; 3) it allows to monitor the synthesis of solid-supported oligonucleotides (up to a length of 18 nucleotides) by MALDI-TOF MS without any prior chemical treatment; 4) it allows to introduce at the 3'-end of an oligonucleotide a modification using a phosphoramidite where the cyanoethyl group is replaced by the expected modification (e.g. pentynyl group).

EXPERIMENTAL SECTION

General Methods: All commercial chemicals were reagent grade and were used without further purification. The DNA synthesis reagents and phosphoramidites were commercially available. Flash column chromatography was performed on silica gel 60 (40–63 μm). ^{1}H NMR (400–600 MHz), ^{13}C NMR spectra (101-151 MHz), and ^{31}P NMR (162 MHz) were recorded in the stated solvents at room temperature, unless otherwise specified.

MALDI-TOF MS studies were performed on a Shimadzu Assurance equipped with a 337 nm nitrogen laser. Spectra were recorded, in negative mode, using 2,4,6-trihydroxyacetophenone (THAP) with 10% of ammonium citrate as a matrix in water CH_3CN, 1:1, v/v. Liquid samples were mixed with the matrix as 1:1 or 1:5 v/v ratio and 1μL of the solution was deposited on the stainless steel plate for drying. For the analyses performed with the solid-supported oligonucleotides, few CPG beads were suspended with the matrix and then deposited on the stainless steel plate for drying.

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1-Dimethoxytrityl-2,2'-thiodiethanol 10: 2,2'-Thiodiethanol (500 µL, 5 mmol) was solubilized in anhydrous pyridine (50 mL) and DMT-Cl (1.7 g, 5 mmol) was added on three parts at 0 °C, over 20 min. Orange solution was stirred for 1 h at room temperature. Pyridine was evaporated to ¾ and a saturated solution of $NaHCO_3$ (30 mL) was added, aqueous layer was extracted with CH_2Cl_2 (3 x 30 mL). Organic layer was dried over Na_2SO_4 and solvent evaporated under vacuum. Crude was purified by flash chromatography on silica gel using cyclohexane with increasing AcOEt from 0 to 60% to **10** as a colorless oil (1.15 g, 54 %). Rf: 0.6 Cyclohexane/AcOEt (3:7 v/v), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46-7.21 (m, 9H, Ar), 6.84 (d, *J* = 8.8 Hz, 4H, Ar), 3.79 (s, 6H, OCH_3), 3.67 and 3.66 (2t, *J* = 5.8 Hz, 2H, HO$\underline{CH_2}$), 3.31 (t, *J* = 6.7 Hz, 2H, DMTrOCH_2), 2.69 (t, *J* = 5.8 Hz, 2H, DMTr$OCH_2\underline{CH_2}$), 2.68 (t, *J* = 6.7 Hz, 2H, SCH_2), 2.17 (t, 5.8 Hz, 1H, OH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.7, 145.1, 136.3, 130.2, 128.3, 127.9, 126.9, 113.3, 86.6, 63.5, 60.6, 55.3, 35.9, 31.9. HRMS (ESI/QTOF): $[M+Na]^+$ Calcd for $C_{25}H_{28}O_4SNa$, 447.1606, found 447.1608.

2-methylthioethyl 2-cyanoethyl *N,N*-diisopropylphosphoramidite 13: To a solution of 2-methylthioethanol (174 µL, 2 mmol) in anhydrous CH_2Cl_2 (15 mL), DIEA (491 µL, 2.2 mmol) and molecular sieve (4 Å) were added. The mixture was stirred under argon atmosphere for 1 h. Cyanoethyl-*N,N*- diisopropylphosphoramido chloridite (491 µL, 2.2 mmol) was added at 0° C and the solution was stirred for 2 h at room temperature. Then water (1 ml) was added and after 15 min a solution of saturated $NaHCO_3$ was added (30 mL). Aqueous solution was extracted twice (2 x 30 mL) with CH_2Cl_2. Organic layers were combined, dried over Na_2SO_4 and solvents were evaporated under vacuum. The crude was purified by silica gel chromatography (Cyclohexane/AcOEt/10 %NEt_3) from 1/0 to 4/5 to afford phosphoramidite product **13** (449 mg; 77 %). Rf: 0.66 Cyclohexane/AcOEt/NEt_3 (45:45:10, v/v/v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.87 – 3.74-3.67 (m, 4H, PO$\underline{CH_2}CH_2CN$, MeS$\underline{CH_2}$), 3.60 – 3.52 (m, 2H, CHiPr), 2.68 (t, *J* = 7.0 Hz, 2H, PO$\underline{CH_2}CH_2S$), 2.61 (td, *J* = 6.4, 2.2 Hz, 2H, CH_2CN), 2.10 (s, 3H, CH_3S), 1.15 (d, *J* = 7.2 Hz, 6H, CH_3), 1.14 (d, *J* = 7.1 Hz, 6H, CH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 117.65, 62.53, 58.46, 43.05, 35.05, 24.54, 20.37, 15.84. ^{31}P NMR (162 MHz, $CDCl_3$) δ 147.9. HRMS (ESI/QTOF): $[M+H]^+$ Calcd for $C_{12}H_{26}N_2O_2SP$ 293.1453, found = 293.1448

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1-Dimethoxytrityl-2,2'-thiodiethanol 2-Cyanoethyl *N,N*-diisopropylphosphoramidite 11: Same protocol than for **13** starting from of 1-dimethoxytrityl-2,2'-thiodiethanol **10** (606 mg, 1.4 mmol) affording 693 mg 79 %. Rf: 0.67 Cyclohexane/AcOEt/NEt_3 (50:40:10, v/v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45-7.20 (m, 9H, Ar), 6.83 (d, *J* = 8.8 Hz, 4H, Ar), 3.88 – 3.65 (m, 4H, $POCH_2$), 3.79 (s, 6H, CH_3O), 3.65 – 3.52 (m, 2H, CHiPr), 3.27 (t, *J* = 6.8 Hz, 2H, $DMTrOCH_2$), 2.72 (t, *J* = 6.9 Hz, 4H, CH_2SCH_2), 2.61 (t, *J* = 6.6 Hz, 2H, CH_2CN), 1.18 (d, *J* = 8.8 Hz, 6H, CH_3), 1.17 (d, *J* = 8.8 Hz, 6H, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.64, 145.16, 136.43, 130.18, 128.34, 127.94, 126.89, 117.73, 113.26, 86.44, 63.60, 63.16, 58.67, 55.37, 43.23, 33.71, 32.64, 24.62, 20.53. ^{31}P NMR (162 MHz, $CDCl_3$) δ 147.97. HRMS (ESI/QTOF): $[M+H]^+$ Calcd for $C_{34}H_{46}N_2O_5SP$ 625.2865 found = 625.2852

1-Dimethoxytrityl-2,2'-thiodiethanol succinyl Solid support 12: 1-Dimethoxytrityl-2,2'-thiodiethanol **10** (32 mg, 76 µmol) and DMAP (9 mg, 76 µmol) were co-evaporated three times with anhydrous pyridine, succinate-CPG (500 Å, 500 mg) was added and mixture was co-evapored twice with anhydrous pyridine. Anhydrous pyridine (2 mL), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 73 mg, 380 µmol) and anhydrous triethylamine (32 µL, 228 µmol) were added. The heterogeneous mixture was shaken for 48 h at room temperature. CPG beads were filtered, washed with CH_2Cl_2 and dried under vacuum. Solid support was treated with in CapA/CapB (1:1, v/v, 5 mL) solution and shaken for 2 h. CPG was filtered, washed with CH_2Cl_2 and dried under vacuum to afford support with a loading of 49.5 µmole/g measured by trityl assay. General procedure for titration of support: 20.0 mg of support was treated with 10.0 mL of APTS solution (0.1 % in CH_3CN), 1.0 mL of the resulting solution was diluted with 9.0 mL of APTS solution. Concentration of DMTr cation was calculated from the reading of the absorption at 498 nm with an extinction coefficient of 70 000 $L.mol^{-1}.cm^{-1}$ for the $DMTr^+$ cation.

General procedure for Oligonucleotide Solid Support Synthesis: Oligonucleotides were synthesized on a 1 µmol scale with a DNA synthesizer (394 ABI) using standard phosphoramidite chemistry. For the coupling step, benzylmercaptotetrazole (BMT, 0.3 M in anhydrous CH_3CN) was used as the activator, commercially available nucleoside phosphoramidites (with benzoyl for A and C and isobutyryl for G, 0.075 M in anhydrous CH_3CN) were introduced with 30 s coupling times, and phosphoramidites **11**, **13** and **36** (0.1

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M in anhydrous CH_3CN) were introduced with 60 s coupling times. The capping steps were performed with acetic anhydride in commercially available solutions (Cap A: phenoxyacetyl anhydride, pyridine, THF 10:10:80 v/v/v and Cap B: 10% *N*-methylimidazole in THF) for 20 s. Oxidation was performed with a commercially available solution of iodide (I_2, 0.1 M, THF/pyridine/water 90:5:5, v/v/v for 15 s). Sulfurization was performed with a fresh solution of Beaucage reagent (0.05 M in anhydrous CH_3CN) for 60 sec. Detritylation was performed with 3% TCA in CH_2Cl_2 for 65 s.

General procedure for thermolytic elimination or cleavage from solid support and deprotection:

5'-phosphorylation: Solid-supported oligonucleotide was dispersed in H_2O (1.0 mL) under magnetic stirring and heated at 60 °C for 60 min. Solid support was filtered and treated with concentrated ammonia solution for 1 h at 40 °C for homothymidylates or overnight at 40 °C for heterooligonucleotides. The supernatant was evaporated and 5'-monophosphate-oligonucleotides were analyzed.

3'-phosphorylation: Solid-supported oligonucleotide CPG was dispersed in H_2O (1 mL) under magnetic stirring and heated at 60 °C for 60 min. Solid support was filtered and the filtrate was treated with concentrated ammonia solution for 1 h at 40 °C for homothymidylates or overnight at 40 °C for heterooligonucleotides. The solution was evaporated and 3'-monophosphate-oligonucleotides were analyzed.

5'-thiophosphorylation: Solid-supported oligonucleotide was dispersed in an aqueous solution of 0.25 M Na_3PO_3S, 1.0 M triethylammonium acetate (TEAAc) + 10 µL AcOH pH 7 (1.0 mL) under magnetic stirring and heated at 60 °C for 60 min. Solid support was filtered and treated with concentrated ammonia solution for 1 h at 40 °C. The supernatant was evaporated and 5'-thiomonophosphate-oligonucleotides were analyzed.

3'-thiophosphorylation: Solid-supported oligonucleotide CPG was dispersed in H_2O (1 mL) under magnetic stirring and heated at 70 °C for 60 min. Solid support was filtered and the

filtrate was treated with concentrated ammonia solution for 1 h at 40 °C. 3'-thionomonophosphate-oligonucleotides were analyzed.

Tandem synthesis: Oligonucleotide was synthesized on commercially available solid support according to the general procedure during the synthesis phosphoramidite **11** was introduced in the sequence. Solid-supported oligonucleotide was dispersed in H_2O (1.0 mL) under magnetic stirring and heated at 60 °C for 60 min. Water was mainly evaporated under vacuum and concentrated ammonia solution was added. After overnight treatment at 40 °C. The solution was withdrawn and evaporated affording the 5'- and 3'-monophosphate-oligonucleotides.

3'-pentynyl-oligonucleotides: Oligonucleotide was synthesized on solid support **12** starting by coupling of phosphoramidite pentynyl thymidine **36**[29] and then commercially available nucleosides according to the general procedure. After elongation solid-supported oligonucleotides were treated with concentrated ammonia solution overnight at 40 °C. After evaporation 3'-pentynyl oligonucleotide was obtained.

Supporting Information (see footnote on the first page of this article): NMR spectra of **10**, **11**, and **13**; HPLC profiles and MALDI-TOF mass spectra.

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