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New reagent for the preparation of oligonucleotides involving a 5′-thiophosphate or a 5′-phosphate group ☆

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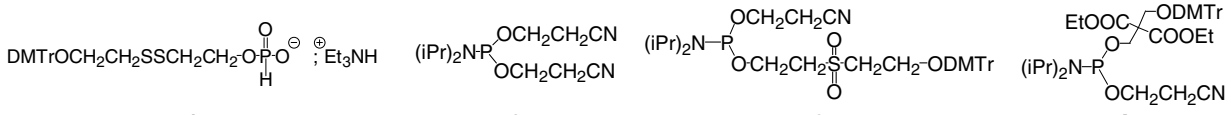
Abstract—We report here a novel, simple reagent enabling the chemical incorporation of a thiophosphate or a phosphate group at the 5′-end of oligonucleotides using very mild basic deprotection conditions. This method can be useful in the case of alkali sensitive modified oligonucleotides. This reagent also gives access to the preparation of bifunctional oligonucleotides with either a thiophosphate group at the 5′-end and a phosphate at the 3′-end, or two thiophosphate groups at both the 5′- and the 3′-ends, or a 5′-thiophosphate group and a 3′-amino-containing linker.

1. Introduction

Oligonucleotides are powerful research tools for molecular biology,[1–3] gene regulation and function analysis as well as potent therapeutic agents.[4–6] These applications imply a growing need for modified oligonucleotides involving a 5′-terminal phosphate or oligonucleotides conjugated to various ligands (labels, intercalators, groove binders as well as pendant groups aimed at increasing cellular uptake). Numerous applications require bis-derivatized oligonucleotides. In most cases this can be achieved by using oligonucleotides involving a 5′-thiol function and an amino-containing linker at the 5′-end. However, the propensity of the thiol function to dimerize can sometimes hamper the result. An alternative is to replace the thiol function by a 5′-thiophosphate group less prone to dimerization. The commercially available reagents **2**–**4** (Fig. 1) for the 5′-phosphorylation of oligonucleotides require either harsh alkali treatment **2**,[7,8] **3**[9] or a slightly complicated procedure **4**.[10] We now report the use of a novel simple reagent, the H-phosphonate derivative of 1-dimethoxytrityl 2,2′-dithiodiethanol **1** (Scheme 1). The use of this reagent offers several advantages. Firstly, the coupling yield evaluation can be monitored by a trityl cation assay. Secondly, 5′-thiophosphorylated or 5′-phosphorylated oligonucleotides can be obtained using very mild basic deprotection conditions. Thirdly, the excess of compound **1** can be recovered. Finally, this reagent can be stored at −20 °C for months without degradation. We



1 **2** **3** **4**

Figure 1. Novel H-phosphonate reagent **1** and commercially available reagents **2**–**4** employed for the 5′-phosphorylation of oligonucleotides.

Keywords: New phosphorylating reagent; 5′-Phosphorylated oligonucleotides; 5′-Thiophosphorylated oligonucleotides; Mild deprotection conditions.

☆ Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2004.06.067

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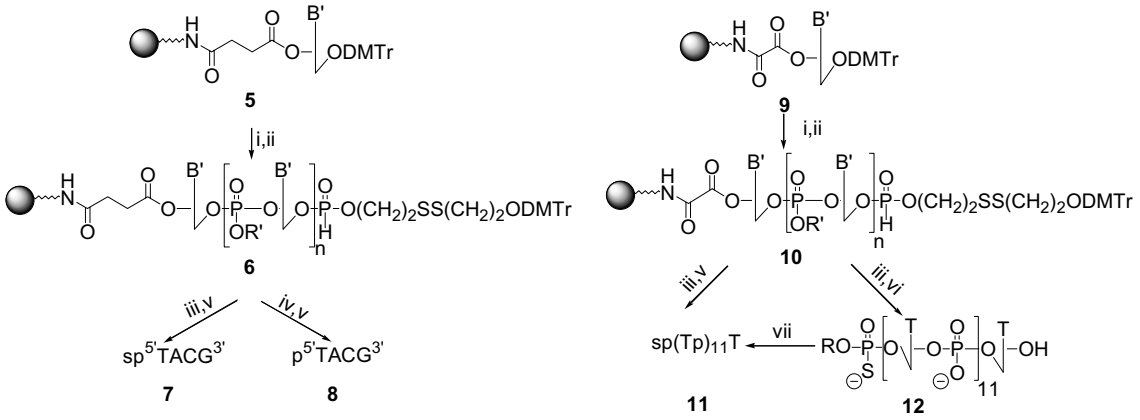
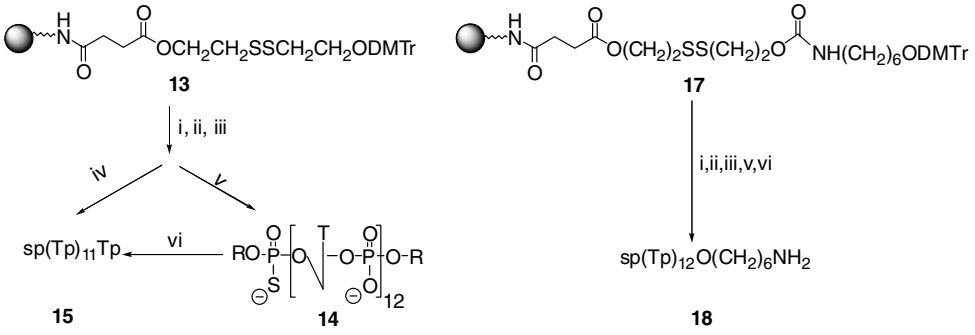
Scheme 1. Synthesis of the novel reagent **1**. Reagents: (i) DMTrCl; (ii) 2-chlorobenzo-1,3-dioxa-2-phosphorin-4-one; (iii) Et_3NH^+, HCO_3^-, 1 M.



Scheme 2. Synthesis of 5′-functionalized oligonucleotides. Reagents and conditions: (i) oligonucleotide chain elongation; (ii) **1**, PivCl, CH_3CN/Pyr (1:1, v/v) 2.5 min; (iii) S_8, CS_2/Pyr (3:2, v/v) 20 min; (iv) I_2, Pyr/H_2O (98:2, v/v) 1 h; (v) concd NH_4OH, one night, 50 °C; (vi) 10 M methanolic ammonia, 1 h, rt; (vii) DTT (0.5%, w/v) in 0.5% aqueous DIEA, 30 min. Abbreviations: DMTr = Dimethoxytrityl; B′ = protected nucleic bases (A, T, G or C); R = –$CH_2CH_2SSCH_2CH_2OH$; R′ = –CH_2CH_2CN. Gray ball = 3-aminopropyl-CPG support.



Scheme 3. Synthesis of bifunctionalized oligonucleotides. Reagents and conditions: (i) oligonucleotide chain elongation; (ii) **1**, PivCl, CH_3CN/Pyr (1:1, v/v) 2.5 min; (iii) S_8, CS_2/Pyr (3:2, v/v) 20 min; (iv) concd NH_4OH, one night, 50 °C; (v) 10 M methanolic ammonia 4 h, rt; (vi) DTT (0.5% w:v) in 0.5% aqueous DIEA, 30 min. Abbreviations: DMTr = Dimethoxytrityl, R = $CH_2CH_2SSCH_2CH_2OH$. Gray ball = 3-aminopropyl-CPG support.

now report its synthesis (Scheme 1) and its application to the preparation of mono- and bifunctional oligonucleotides (Schemes 2 and 3).

2. Synthesis of the phosphorylating agent 1

The synthesis of compound **1** was performed following a two-step procedure (Scheme 1). First the 2,2′-dithiodiethanol was dimethoxytritylated as previously reported[11] and then after purification the second hydroxyl function was phosphorylated by treatment with 2-chlorobenzo-1,3,2-dioxaphosphorin-4-one (1.5 equiv).[12] See Supporting Information for the experimental procedure and characterizations. The use of this reagent enables the preparation of either 5′-thiophosphorylated or 5′-phosphorylated oligonucleotides that can be performed as follows (Scheme 2).

3. Synthesis of the 5′-modified oligonucleotides

To test various conditions for the deprotection step, we chose two different oligonucleotides. A short oligonucleotide, a tetramer involving the four nucleic bases protected by the classic protective groups (benzoyl group for the exocyclic amino functions of the cytosine and the adenine and isobutyryl group for the amino function of the guanine) was chosen in order to facilitate the separation between the oligonucleotide involving the hydroxyl function and the oligonucleotides involving the 5′-terminal phosphate or the 5′-thiophosphate groups.

In order to test the possibility of generating the 5′-terminal thiophosphate and phosphate groups under very mild basic conditions, dodecathymidylates were also synthesized on an oxalyl support.[13]

After the chain elongation on support **5** or **9**, an additional detritylation step was performed. The cartridge containing the oligonucleotide on the support was removed from the synthesizer and the new compound **1** (20 equiv) was added manually with a syringe in the presence of pivaloyl chloride (40 equiv). After 2.5 min of reaction, the reaction mixture was removed and the support washed with a pyridine/acetonitrile mixture (1:1, v/v). [It must be noted that after hydrolysis of the reaction mixture with the TEAB buffer, the excess of reagent **1** can be recovered by silica gel purification.] At this step it is possible to measure the coupling efficiency by performing a trityl cation assay on an aliquot of the support. It is then possible to perform either a sulfurization step by treatment with elemental sulfur in a CS_2/pyridine mixture (50 mg/2.5 mL) for 20 min to give a 5′-terminal thiophosphate group after deprotection of the oligonucleotide or an oxidation step by treatment with a 0.1 M solution of iodine in pyridine/water (98:2, v/v) for 1 h to give a 5′-terminal phosphate group after deprotection of the oligonucleotide.

Different results were obtained depending on the deprotection conditions used. The following observations have been made. Treatment with concentrated aqueous ammonia overnight at 50 °C without a reducing agent to cleave the disulfide bridge (classical conditions used for the deprotection of the oligonucleotides involving the four nucleic bases protected with conventional groups) led directly to oligonucleotides involving either the thiophosphate **7** or the phosphate **8** at the 5′-end or to the dodecathymidylate **11** involving a thiophosphate group at the 5′-end as indicated by the mass analysis (Scheme 2 and Table 1). This result is in accordance with a report describing the instability of the disulfide bond under harsh basic conditions.[14] Analyses performed by both reversed-phase and ion-exchange chromatography using the corresponding unmodified oligonucleotide as reference confirmed that both oligonucleotides involving a 5′-terminal thiophosphate and a 5′-terminal phosphate group were obtained with a good yield (>90%) confirming the efficiency of the procedure (an example is shown in the Supporting Information, Fig. 2). When mild basic deprotection conditions were used, such as treatment with a 10 M methanolic ammonia solution at room temperature for 1 h, different behaviour was observed. The oligonucleotide **12** obtained showed increased retention time as compared to that of the corresponding oligonucleotide **11** obtained after deprotection with concentrated aqueous ammonia. Mass analysis confirmed that this oligonucleotide involved one 2,2′-dithiodiethanol group. (The dimethoxytrityl group was lost. Only a small per cent of the dimethoxytritylated oligonucleotide, with a higher retention time, was obtained.) The presence of this lipophilic chain can help for the purification of the 5′-modified oligonucleotides (it is known that it is difficult, except for very short oligonucleotides, to separate 5′-hydroxylated oligonucleotides from the corresponding 5′-phosphorylated or thiophosphorylated oligonucleotides). Treatment of oligonucleotide **12** with DTT in the presence of 0.5% aqueous DIEA led to the expected fully deprotected oligonucleotide **11** (Scheme 2).

4. Synthesis of bifunctionalized oligonucleotides

To test the possibility of obtaining bifunctional oligonucleotides involving either a thiophosphate group at the 5′-end and a phosphate group the 3′-end or a thiophosphate group at the 5′-end and an aminohexyl linker at the 3′-end, oligothymidylates were also synthesized on our previously reported supports **13**[11] and **17**[15] (Scheme 3).

Since these supports involve both a succinyl linker, more stable than the oxalyl anchor in basic medium, and a disulfide bond, two strategies can be followed for the deprotection of the bifunctional oligonucleotides. In the case of the preparation of the oligonucleotide involving a 5′-thiophosphate and a 3′-phosphate groups we have monitored the deprotection by treatment with a 10 M methanolic ammonia solution at room temperature. The complete release from the support of the oligonucleotide **14** involving a 2,2′-dithiodiethanol group at each end (Table 1) was obtained after a 4-h treatment. The full release from the support was assessed by additional treatment of the support with concentrated aqueous ammonia for 1 h at room temperature. After purification

Table 1. Oligonucleotide characterizations

Oligonucleotides	Formula	Mass spectrometry analysis		Chromatography analysis (Rt)	
		Theoretical	Experimental	By reversed phase[a]	By ion exchange[b]
7	$C_{39}H_{51}O_{24}N_{15}P_4S$	1269.87	1269.21 ± 0.73	16 min 30 s	24 min 6 s
8	$C_{39}H_{51}O_{25}N_{15}P_4$	1253.82	1253.71 ± 0.17	15 min 54 s	20 min 36 s
11	$C_{120}H_{158}O_{84}N_{24}P_{12}S$	3684.45	3684.98 ± 1.40	10 min 6 s	19 min 30 s
12	$C_{124}H_{166}O_{85}N_{24}P_{12}S_3$	3820.68	3820.68 ± 0.89	12 min 36 s	—
14	$C_{128}H_{175}O_{89}N_{24}P_{13}S_5$	4036.90	4036.42 ± 0.80	13 min 48 s	—
15	$C_{120}H_{159}O_{87}N_{24}P_{13}S$	3764.43	3765.05 ± 0.60	10 min	21 min
18	$C_{126}H_{172}O_{87}N_{25}P_{13}S$	3863.60	3863.69 ± 1.33	10 min 6 s	20 min 36 s

[a] Licrospher 100 RP 18 (5 μm) column (25 × 4 mm), (Merck). Linear gradient of CH_3CN (0–8% in 8 min then 8–13% in 24 min for oligonucleotides **7**, **8** and 10–30% in 20 min for oligonucleotides **11**, **12**, **14**, **15** and **18**) in 0.1 M TEAA, pH = 7, buffer with a flow rate of 1 mL/min.

[b] Mono-Q HR 5/5 (Pharmacia) Linear gradient of NaCl (0–0.3 M in 30 min for oligonucleotides **7**, **8** and 0.2–0.5 M for oligonucleotides **11**, **12**, **14**, **15**, **18**) in TRIS 25 mM, pH = 8, buffer containing 10% MeOH with a flow rate of 1 mL/min.

of oligonucleotide **14** by reversed-phase chromatography, an additional treatment with DTT in the presence of 0.5% aqueous DIEA gave the expected fully deprotected oligonucleotide **15** as confirmed by mass spectrometry analysis.

Alternatively, the deprotection step can be directly performed by using a methanolic ammonia solution containing DTT. A pentadecathymidylate **16** involving two thiophosphate groups at both the 5′- and the 3′-ends was obtained by replacing the first oxidation step by a sulfurization step as previously reported[11] and by using a methanolic ammonia solution containing the reducing agent at room temperature for 1 h for the deprotection step (calculated mass for $C_{150}H_{194}O_{107}N_{30}P_{16}S_2$: 4693.08. Found: 4692.63 ± 1.48). Following the same deprotection strategy the fully deprotected oligonucleotide **18** involving both a 5′-thiophosphate and a 3′-aminohexyl linker was obtained as confirmed by mass spectrometry analysis. To assess the quality of oligonucleotide **18** it was successively reacted with {4-[(4-dimethylamino)-phenyl]azo} benzoic acid, succinimidyl ester and 5-iodoacetamidyl fluorescein. After reversed-phase chromatography the mass spectrometry analysis confirmed the integrity of the bis-labelled oligonucleotide **19**. The UV–visible spectrum of the bis-labelled oligonucleotide is shown in the Supporting information (Fig. 3).

Altogether, these results show that the newly reported reagent **1** could be a useful tool for the phosphorylation and the thiophosphorylation of base sensitive modified oligonucleotides. Furthermore, the use of this new reagent, in association with our previously reported supports[11,15] could also allow the preparation of various bifunctional oligonucleotides under very mild basic conditions. These modified oligonucleotides can involve either two thiophosphate (or phosphate) groups at each end of the oligonucleotide or a thiophosphate group at one end and a phosphate group at the other or a thiophosphate group at the 5′-end and an aminohexyl linker at the 3′-end thus allowing the coupling of various ligands.

Supplementary material

Experimental procedures for the synthesis of the H-phosphonate derivative **1**. Procedure for the reaction of compound **1** with the 5′-terminal hydroxyl function of oligonucleotides. Reversed-phase analysis of the crude oligonucleotide **7** (Fig. 2). UV–visible spectrum of oligonucleotide **19** (Fig. 3) is available on-line with this article.

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