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Novel reagents for terminal phosphorylation and thiophosphorylation of synthetic oligonucleotides

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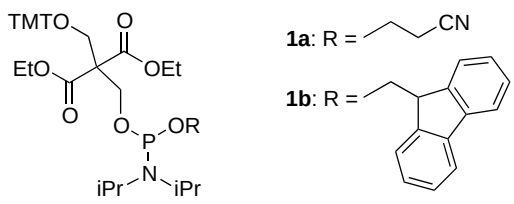
Abstract—Two novel phosphoramidite building blocks and a solid support that allow an efficient solid-phase phosphorylation or thiophosphorylation of synthetic oligonucleotides were developed. The utility of these synthetic tools was demonstrated in the preparation of 5′- or 3′-thiophosphorylated oligonucleotides, which were subsequently labeled at the termini with fluorescent reporters. © 2001 Elsevier Science Ltd. All rights reserved.

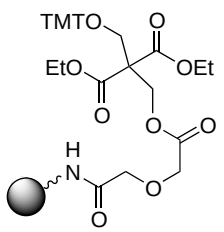
Terminal thiophosphate (PS) group provides a convenient site for regiospecific conjugation of synthetic oligonucleotides to a variety of ligands bearing an electrophilic group.[1,2] However, extensive application of this technique has been hampered by the lack of efficient methods for the terminal thiophosphorylation of synthetic oligonucleotides. Although convenient methods for the 5′-[3–5] and 3′-phosphorylation[5,6] are well documented in the literature, those dealing with the introduction of the terminal PS group are limited. The release of the terminal thionophosphomonoester employs either reductive[7] or oxidative[1] conditions that unnecessarily complicate the deprotection of oligonucleotides. Besides, both approaches suffer from substantial desulfurization of the PS moiety, which results in the corresponding oligonucleotide 5′- or 3′-phosphate (PO) in ca. 15% yield.[1] The subsequent removal of this side product by HPLC purification is not trivial.

We report here a convenient approach for the terminal phosphorylation and thiophosphorylation of synthetic oligonucleotides using phosphoramidite building blocks **1a** and **1b** and a solid support **2**. In the case of thiophosphorylation, the final deprotection under standard conditions results in the desired products with only minor desulfurization (1–1.5%). The synthesized oligonucleotides were successfully converted to their fluorescent conjugates by reacting with iodoacetamide derivatives of fluorescent dyes, fluorescein and pyrene.

Novel phosphoramidite reagents were synthesized as depicted in Scheme 1. Compound **3**[8] was treated with 4,4′,4″-trimethoxytrityl chloride (TMT-Cl) in Py/dioxane to give **4**, which was isolated on a silica gel column in 67.5% yield.[9] This was converted to the 2-cyanoethyl protected phosphoramidite **1a** under the standard conditions.[10] To obtain **1b**, compound **4** was first treated with $[(i\text{-Pr})_2\text{N}]_2\text{P–Cl}$ under basic conditions; a bisamidite obtained was treated with (9*H*-fluorene-9-yl)methanol (Fm-OH) and 1*H*-tetrazole under the reported conditions.[11,12] Compounds **1a** and **1b** were isolated by column chromatography in 86 and 68% yield, respectively. The phosphoramidite **1a** was stable at 4°C for more than 2 months. To assure similar stability of **1b**, traces of triethylamine retained in the product from the purification step should be removed by repeated co-evaporation with toluene.



1a,**b**



2

Keywords: nucleic acids; phosphorylation; phosphoramidites; solid-phase synthesis; supported reagents/reactions; labelling.

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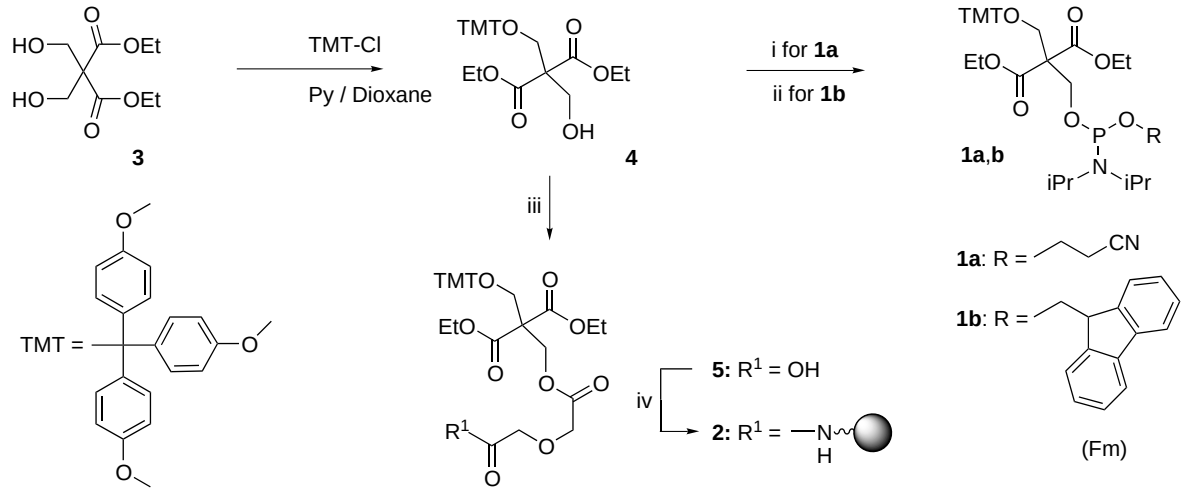
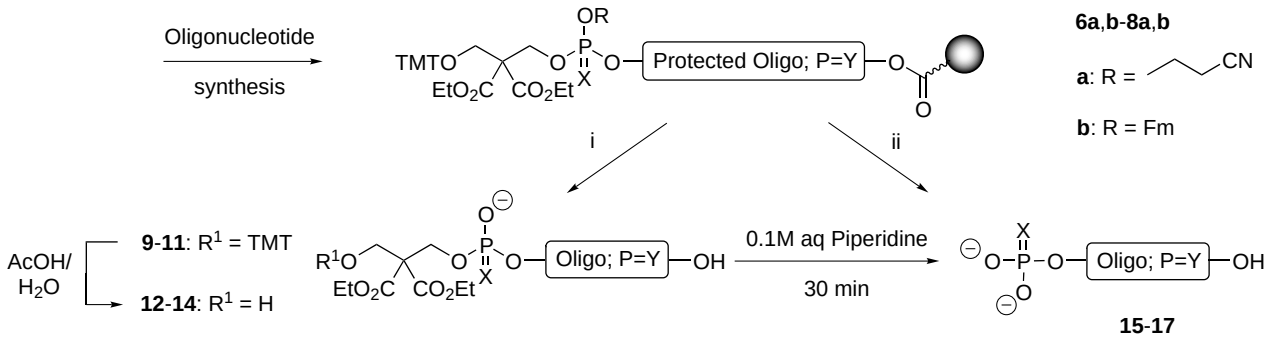
In order to synthesize the solid support **2**, **4** was treated with diglycolic anhydride[13] in pyridine/dioxane to give a monoester **5** in quantitative yield (Scheme 1). This was coupled to long chain alkylamine controlled pore glass essentially as described previously[14] to give the solid support **2** loaded at 81 μmol g^{-1}.[15]

The utility of **1a**, **1b** and **2** as oligonucleotide phosphorylation reagents was next studied. In the case of 5′-phosphorylation, protected oligonucleotides **6**–**8** were first assembled in a standard manner on 1 μmol scale using 0.1 M solutions of **1a** and **1b** in MeCN for the last coupling step (Scheme 2). To achieve the 3′-phosphorylation, the synthesis of oligonucleotides **18**–**20** was carried out on the solid support **2** (Scheme 3). The solid support-bound **6** and **18** were assembled using the conventional oxidation with I_2 solution. Oligonucleotide phosphorothioates **7** and **19** were synthesized using 3*H*-1,2-benzodithiol-3-one 1,1-dioxide as a sulfur-transfer agent.[16] To synthesize oligonucleotides **8** and **20**, the sulfurization was carried out within the last and the first elongation cycles, respectively, while all internucleosidic linkages were oxidized with *t*-BuOOH (10% in MeCN; 10 min).

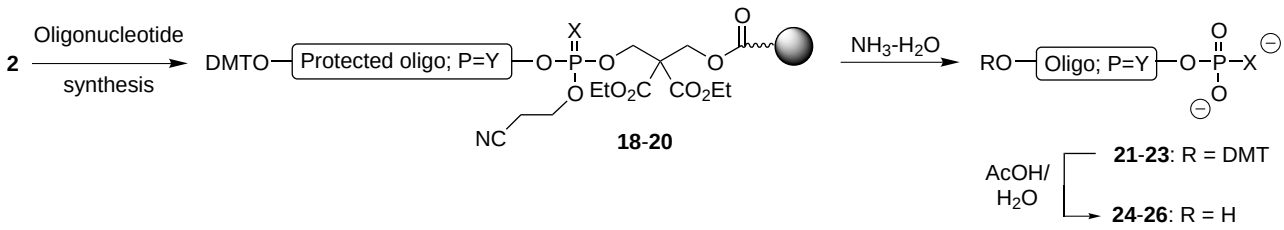
When the syntheses were complete, the solid support-bound material was deprotected with concentrated aqueous ammonium hydroxide under the standard con-



Scheme 1. (i) $[(i\text{-Pr})_2N]_2P\text{–}O(CH_2)_2CN$, 1*H*-tetrazole, MeCN (1 h, rt); (ii) 1, $[(i\text{-Pr})_2N]_2P\text{–Cl}$, $(i\text{-Pr})_2NEt$, CH_2Cl_2 (45 min, –70 to 20°C); 2, Fm-OH, 1*H*-tetrazole (2 h, rt); (iii) diglycolic anhydride, Py, dioxane; (iv) 1, 2,2′-dithiobis(5-nitropyridine), Ph_3P/DMAP/MeCN/1,2-dichloroethane; 2, long chain alkylamine CPG.



Scheme 2. i: Concentrated $NH_3\text{–}H_2O$; **ii**: 1, $Cl_2HCCOOH$ (3% in CH_2Cl_2, 45 s); 2, concentrated $NH_3\text{–}H_2O$. **6**, **9**, **12**, **15**: Oligo = T_{12}, X = Y = O; **7**, **10**, **13**, **16**: Oligo = 5′-ATGCAT$_2$CTGC$_5$A$_2$G$_2$A-3′, X = Y = S; **8**, **11**, **14**, **17**: Oligo = 5′-ATGCAT$_2$CTGC$_5$A$_2$G$_2$A-3′, X = S, Y = O.

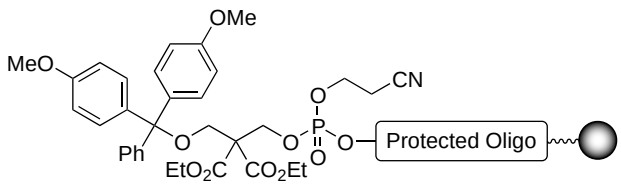


Scheme 3. Oligo = 5′-TGCATC$_5$AG$_2$C$_2$AC$_2$AT-3′; **18**, **21** and **24**: X = Y = O; **19**, **22** and **25**: X = Y = S; **20**, **23** and **26**: X = S, Y = O.

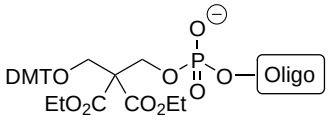
ditions (2 h/rt for **6** or 6 h/55°C for **7**, **8** and **18**–**20**). For **6**–**8**, this led to **9**–**11**, which were purified by reverse-phase HPLC and characterized. Oligonucleotides **9**–**11** were detritylated with 10% aqueous AcOH to give **12**–**14**, which were subsequently converted to **15**–**17** by a 30 min treatment with 0.1 M aqueous piperidine. Alternatively, **6**–**8** were detritylated on the synthesizer and then deprotected with concentrated aqueous ammonium hydroxide to give crude **15**–**17**. The deprotection of **18**–**20** released the oligonucleotides **21**–**23**. The deprotection mixtures and the final products were analyzed by reverse-phase HPLC–ESMS and anion-exchange HPLC.

The analysis revealed quantitative introduction of the non-nucleosidic moiety to **9**–**11** and the terminal PO residues to **15** and **24**. Similarly, 3′-terminal thiophosphorylation resulted in quantitative modification of oligonucleotides to give **22** and **23** contaminated with a minor amount of desulfurized oligonucleotides. To test the feasibility of **1a**, **1b** and **2** for the terminal thiophosphorylation, the extent of desulfurization was determined. As shown by HPLC–ESMS, the content of mono-desulfurized oligonucleotides in crude samples of **10**, **11**, **16**, **17**, **22** and **25** adhered to the limits characteristic for the standard phosphorothioate synthesis (ca. 0.5–0.6% per linkage). The extent of desulfurization was slightly higher in **23** and **26** (ca. 1.5% with respect to the intact oligonucleotides). This might reflect a partial desulfurization resulting from the exposure of the 3′-terminal PS moiety to 19 cycles of oxidation. In comparison with the reported methods,[1] these results demonstrate a dramatic improvement in the purity of the oligonucleotides. More importantly, the method reported here does not require any additional postsynthetic procedures for the deprotection of the terminal PS group.

All terminally phosphorylated oligonucleotides, **15**–**17** and **24**–**26**, were isolated by HPLC in yields typical for the scale of synthesis employed (68% for **15** and 35–45% for **16**, **17** and **24**–**26**). The high yields and purity of these compounds indicated that the release of the terminal PO and PS groups was most likely governed by the mechanism and the kinetics reported for the earlier generation of the phosphoramidite **1** and the solid support **2**.[4–6]



27



28

The practical utility of **1a**, **1b** and **2** was significantly improved by using the TMT protection. When the more common 4,4′-dimethoxytrityl (DMT) group was used, the detritylation of a non-nucleosidic moiety in analogs of **27** on solid phase required treatment with TFA (2% in CH_2Cl_2) and dichloroacetic acid (DCA; 3% in CH_2Cl_2) for 40 s and 5 min, respectively.[4–6] In contrast, when **2** and **6**–**8** were detritylated under the standard conditions (3% DCA in CH_2Cl_2), the removal of the TMT protection was complete in 45 s, i.e. as fast as that of the DMT group from a 5′-nucleoside in solid support-bound, 5′-DMT protected oligonucleotides. This assured the compatibility of **1a**, **1b** and **2** with the conventional protocol of the detritylation on automated synthesizers and simplified their use in the routine preparation of synthetic phosphorylated oligonucleotides.

In contrast to detritylation on solid phase, the removal of the DMT group from phosphate and base-deprotected oligonucleotides **28** in solution using 80% aqueous AcOH proceeded in a normal fashion.[4,5] This prompted us to verify whether the stability of the TMT group was sufficient for the safe isolation of the TMT-On oligonucleotides in high yields and purity. The greater lability of the TMT group was indeed reflected in the fact that the detritylation of **9**–**11** required only 10% aqueous AcOH for 30 min. However, no detectable loss of the TMT group was observed during the ammonolysis and subsequent evaporation and purification of **9**–**11**. This suggests that the TMT group displays sufficient stability required for the safe processing of TMT-On oligonucleotides.

The presence of terminal PS groups in oligonucleotides **17** and **26**, was further verified by alkylation with fluorescent labeling reagents, iodoacetamides **29** and **30** (Scheme 4).[1] The products, **31**–**34**, demonstrated UV–vis

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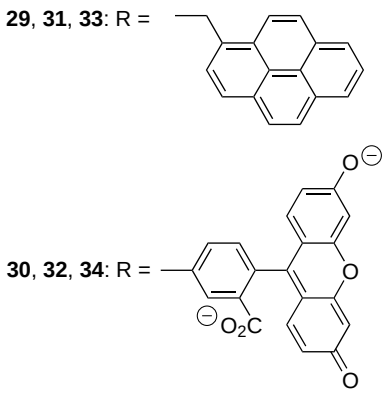

Scheme 4. For **17**, **31** and **32**: Oligo = 5′-pS-ATGCAT$_2$CTGC$_5$A$_2$G$_2$A-3′; for **26**, **33** and **34**: Oligo = 5′-TGCATC$_5$AG$_2$C$_2$AC$_2$AT-pS-3′.

spectra consistent with the presence of appropriate dyes. As evidenced by reverse-phase HPLC, treatment of the conjugates **31**–**34** with concentrated aqueous ammonium hydroxide did not result in the loss of the reporter group. This suggests that the conjugation at the terminal PS group results in the negatively charged *S*,*O*-dialkyl phosphorothiolate moiety.

In conclusion, the use of the phosphoramidite building blocks **1a** and **1b** and the solid support **2** improves the methodology for the terminal phosphorylation of synthetic oligonucleotides. Moreover, it allows a straightforward preparation of thiophosphorylated oligonucleotides in high purity and their subsequent site-specific conjugation with thiophilic reporter groups.

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9. Compound **3** (11.5 g, 55.0 mmol) was treated with TMT-Cl (19.2 g, 52.0 mmol) in Py (16 mL) and dioxane (100 mL) overnight and the solvent was evaporated. The residue was dissolved in CH_2Cl_2 (500 mL), washed with $NaHCO_3$ (5% aq., 3×100 mL), brine (2×100 mL), dried over Na_2SO_4 and evaporated. Purification on a silica gel column using a step gradient of ethyl acetate (0–15%) in toluene gave **4** (8.2 g, 67.5%) as an oil. HR MALDI MS: calcd for $C_{31}H_{36}O_9$ ($M+H^+$) 553.2442, found 553.2438. ^{1}H NMR ($CDCl_3$): δ 7.29 (6H, d, $J=8.8$ Hz), 6.83 (6H, d, $J=8.8$ Hz), 4.26–4.11 (6H, m), 3.80 (9H, s), 3.62 (2H, s), 2.11 (1H, t, $J=5.8$ Hz), 1.24 (6H, t, $J=7.0$ Hz). ^{13}C NMR ($CDCl_3$): δ 169.3, 158.5, 136.1, 129.8, 113.2, 86.0, 63.7, 61.9, 61.6, 60.6, 55.2, 14.1.
10. 1*H*-Tetrazole (0.45 M in MeCN, 1.1 mL, 0.5 mmol) was added to **4** (553 mg, 1.0 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropyl phosphorodiamidite (332 mg, 1.1 mmol) in CH_2Cl_2 (5 mL). The solution was stirred for 2 h, and $NaHCO_3$ (5% aqueous, 5 mL) was added. The mixture was diluted with brine (15 mL) and extracted with CH_2Cl_2 (3×40 mL). The extracts were dried over Na_2SO_4 and evaporated. The residue was purified on a silica gel column eluting with a gradient from 0:95:5 to 30:65:5 ethyl acetate/hexane/triethylamine (TEA) to give **1a** (650 mg, 86.3%) as a colorless oil. HR FABMS: found m/z 752.8309; $C_{40}H_{53}N_2O_{10}P$ requires 752.8301. ^{1}H NMR ($CDCl_3$): δ 7.40–7.25 (6H, m), 6.9–6.65 (6H, m), 4.3–4.0 (6H, m), 3.77 (9H, s), 3.65 (2H, s), 3.8–3.4 (4H, m), 2.55 (2H, m), 1.40–1.00 (18H, m). ^{13}C NMR ($CDCl_3$): δ 168.6, 168.4, 158.3, 136.5, 130.0, 117.3, 113.0, 85.6, 63.9, 63.6, 62.1, 61.9, 61.4, 61.3, 61.1, 60.9, 55.1, 43.1, 42.9, 24.6, 24.4, 20.5, 14.2. ^{31}P NMR ($CDCl_3$): δ 147.4.
11. A solution of chloro bis[(*N*,*N*,-diisopropyl)amino]-phosphite (827 mg, 3.1 mmol) in dry CH_2Cl_2 (10 mL) was added dropwise to a mixture of **4** (1430 mg, 2.6 mmol) and *N*-ethyl-*N*,*N*-diisopropylamine (439 mg, 3.4 mmol) in dry CH_2Cl_2 (10 mL) under magnetic stirring at −30°C. The reaction mixture was warmed to room temperature, and the stirring was continued for 1 h. Fm-OH (712 mg, 3.6 mmol) and 1*H*-tetrazole (0.45 M in MeCN; 5.6 mL, 2.5 mmol) were added. The resulting mixture was kept at rt for 2 h. Aqueous $NaHCO_3$ (5%; 5 mL) was added, the emulsion was diluted with brine (25 mL) and extracted with ethyl acetate (3×50 mL). Extracts were washed with brine (3×25 mL), dried over Na_2SO_4 and evaporated. The residue was dissolved in toluene (50 mL), applied on a silica gel column, and separated eluting with a step gradient from 0:99:1 to 25:74:1 ethyl acetate/hexane/TEA. The fractions were evaporated, co-evaporated with dry toluene (5×100 mL), and dried on an oil pump to give **1b** (1548 mg, 68.1%) as a white solid foam. HR FABMS: found m/z 727.3486; $C_{39}H_{54}NO_{10}P$ requires 727.3485. ^{1}H NMR ($CDCl_3$): δ 7.8–7.5 (4H, m); 7.5–7.15 (10H, m); 6.9–6.7 (6H, m); 4.50–4.22 (2H, m); 4.20–4.05 (4H, m); 4.05–3.85 (2H, m); 3.72 (9H, s); 3.80–3.40 (5H, m); 1.30–1.00 (18H, m). ^{31}P NMR ($CDCl_3$): δ 147.4. HR FABMS: found m/z 727.3486; $C_{39}H_{54}NO_{10}P$ requires 727.3485. ^{1}H NMR ($CDCl_3$): δ 7.8–7.5 (4H, m); 7.5–7.15 (10H, m); 6.9–6.7 (6H, m); 4.50–4.22 (2H, m); 4.20–4.05 (4H, m); 4.05–3.85 (2H, m); 3.72 (9H, s); 3.80–3.40 (5H, m); 1.30–1.00 (18H, m). ^{31}P NMR ($CDCl_3$): δ 147.4.
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15. A mixture of **4** (1.11 g, 2.0 mmol), 1,4-dioxane-2,6-dione (0.70 g, 6.0 mmol), Py (5 mL), and dioxane (5 mL) was kept overnight at rt. The solvent was evaporated and the residue was dissolved in ethyl acetate (50 mL). The solution was washed with 2 M aq. triethylammonium acetate (5×10 mL) and water (5×10 mL) and dried over Na_2SO_4. The extract was evaporated to give crude monoester **5** (1.54 g; triethylammonium salt) as a solid foam in quantitative yield. Crude **5** (0.99 g, 1.28 mmol) and Ph_3P (0.40 g, 1.5 mmol) were dissolved in 1,2-dichloroethane (5 mL). To this was added a solution of 2,2′-dithiobis(5-nitropyridine) (0.47 g, 1.5 mmol) and DMAP (0.18 g, 1.5 mmol) in 1,2-dichloroethane (5 mL). The mixture was shaken for 15 min and filtered. The precipitate was washed on filter with 1,2-dichloroethane

(5 mL), and the combined filtrates were added to alkylamine CPG (5.98 g; 119 μmol g^{-1}, 0.71 mmol). The suspension was shaken for 1 h and filtered. The solid support was washed with 1,2-dichloroethane (3×10 mL), treated with Ac_2O/*N*-methylimidazole/THF (10:10:80) for 30 min, washed with 1,2-dichloroethane (5×10 mL) and MeCN (5×10 mL), and dried to give **2**. An aliquot of **2** was treated with TFA (2% in CH_2Cl_2), and the concentration of the released TMT cation was determined colorimetrically at 486 nm to give the loading of 81 μmol g^{-1}.

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