New phosphorylating reagents for deoxyribonucleosides and oligonucleotides

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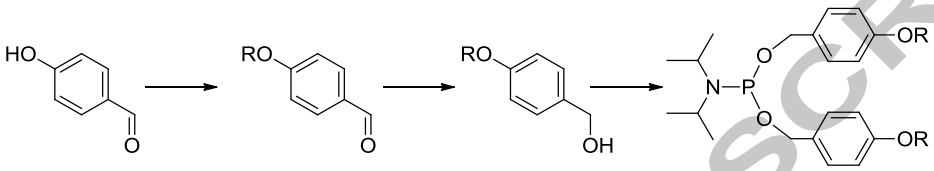

3 steps
more than 55% overall yields

R= 4-monomethoxytryphenylmethyl, tryphenylmethyl.

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New phosphorylating reagents for deoxyribonucleosides and oligonucleotides

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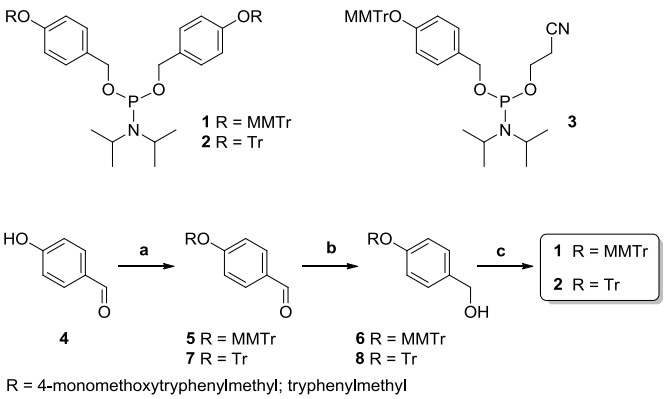
ABSTRACT

New phosphorylating reagents **1** and **2** were prepared in three steps from 4-hydroxybenzaldehyde. They showed good efficiency in the solid phase synthesis of 5′-phosphate monoester nucleosides. End-phosphate DNA sequence synthesis demonstrated the efficiency of the new reagents (**1** and **2**) according to the general procedure of automated DNA synthesis. The oxidation of P(III) to P(V) and the removal of benzyl protecting groups were achieved in a single step by treatment with a 0.02 M I_2/pyridine/H_2O solution. Due to this *one-pot* treatment, it is possible to use the phosphorylating reagents (**1** and **2**) for the synthesis of base-sensitive ODNs. The reagents **1** and **2** are unique among phosphorylating reagents.

Synthetic oligonucleotides (ONs) have found numerous applications as tools and diagnostics in molecular biology. In this context, 5'-phosphate ONs are requisite valuable tools for gene construction, cloning, mutagenesis, the ligation chain reaction and many other biological applications.[1] Over the years, a number of methods have been reported that allow chemical 3'-/5'-phosphorylation for obtaining monoester derivatives of nucleosides, ONs and other polyols.[2] While the coupling efficiency for some of these reagents cannot be easily monitored because the reagent is devoid of marker groups, others require elevated temperature conditions that generally are incompatible with the synthesis of modified ONs and oligodeoxyribonucleotides (ODNs). In fact, this aspect is crucial for the preparation of ODNs functionalized with thermo- and base-sensitive groups (e.g., phosphotriester groups).[3] In 2005, Ausìn C. et al.[4] reported a new phosphorylating reagent useful for generating phosphate monoesters in the presence of phosphorothioate triester functions with minimal cleavage of the functions (<2%). In this work, the phosphorus protecting group was mercaptoethanol, and its removal required very peculiar basic conditions (*ca* 5 bar $MeNH_2$ gas for 30 minutes), which are borderline with the stability of ester and phosphotriester functions. The synthesis of a phosphorylating agent with removable protecting groups in very mild conditions, to be compatible with thermo- and base-sensitive functions, is still in great demand.

In this work, we have focused our attention on the development of new phosphorylating reagents that are totally compatible with the preparation of functionalized base-sensitive oligonucleotides. The key point of our approach, which is based on phosphoramidite chemistry, is the design of a new phosphorous protecting group that is removable during the oxidation step of P(III) to P(V) by treatment with a conventional *oxidizer solution* (I_2/pyridine/H_2O). The phosphorylating agents **1** and **2** (Scheme 1) were designed to satisfy the above requirements and were prepared similarly to **3,** which was the subject of a patent[5] based on a new phosphorylating reagent for the synthesis of ODN 5'-phosphate.

The phosphorylating agents **1** and **2** were prepared in three steps from 4-hydroxybenzaldehyde (Scheme 1) in 55% overall yield.[6,7]



Scheme 1. a. MMTrCl (or TrCl for **7**), DIEA in DCM, 6 h; **b.** $NaBH_4$ in THF, 6 h; **c.** *i*-Pr_2NPCl_2, DIEA in DCM, 1.5 h.

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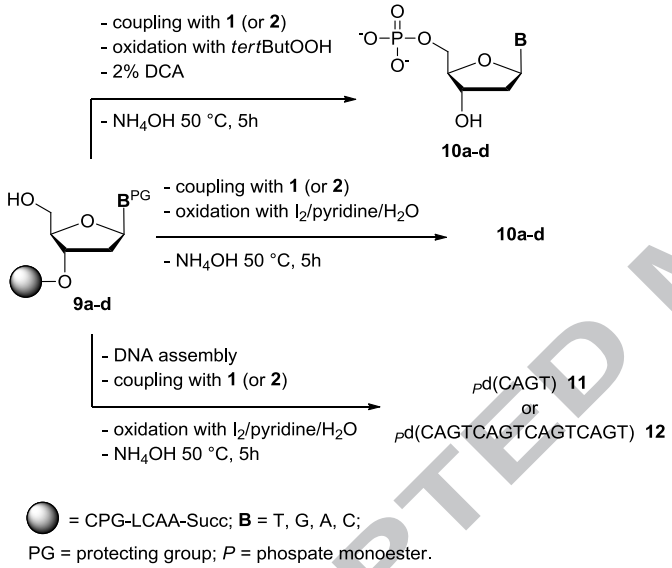
The commercially available 4-hydroxybenzaldehyde **4** was first reacted with 4-monomethoxytritylchloride (MMTrCl) or tritylchloride (TrCl) in dry DCM and in the presence of DIPEA, affording **5** and **7** in 82% and 75% yields, respectively. After reduction with $NaBH_4$ in THF, alcohols **6** and **8** were obtained in 80% and 85% yields, respectively. **7** (or **8**) was then reacted with *i*-Pr_2NPCl_2 and DIPEA in anhydrous DCM, and after silica gel chromatography, **1** (or **2**) was obtained in a good yield (82% for **1**[6] and 80% for **2**[7]). The identities of all desired products were confirmed by NMR and ESI-MS analyses.

The conditions for the optimal coupling efficiency of **1** (or **2**) were first investigated by performing manual synthesis of 5′-phosphate monoester derivatives. For this purpose, CPG anchored the 3'-O-succinyl (**9a-d**, Scheme 2) contained in a classic tube for the synthesis of DNA, which was mixed with 0.25 M 1H-tetrazole in MeCN (*activator solution*) for 2 minute and then with a solution of 0.1 M phosphoramidite **1** (or **2**) in MeCN for 2 minutes. This process was repeated three times, performing all operations with the same solvents, instrumentation and water-free conditions.



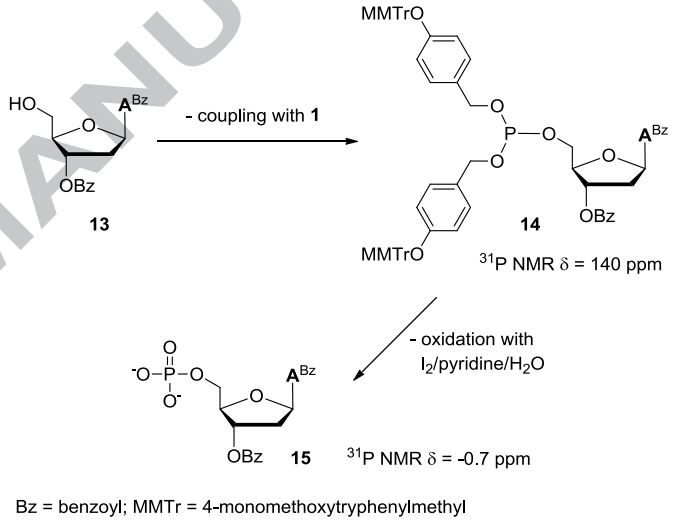
Scheme 2. Synthesis of 5'-phosphate nucleoside monoesters and end-phosphate ODNs.

Subsequently, the resins were washed with MeCN and then treated with a 1.1 M solution of *tert*-butyl-hydroperoxide (*tert*ButOOH) in decane for 4 minutes. After the oxidation treatment, the resins were washed with MeCN and then submitted to a spectrophotometric test of the trityl cation for calculation of the reaction yield. The resultant yields were on average 95%. The treatment of the resins with 2% DCA in DCM allowed the removal of the MMTr group. Finally, treatment with a solution of 28% aqueous ammonia at 50 °C for 5 hours allowed the deprotection and detachment of the desired nucleotides from the supports. RP-HPLC analyses of the crude detached materials were performed. The HPLC profiles of **10a-d** were identical to those of 5′-monophosphate nucleosides obtained from commercial sources.[8]

Surprisingly, when the oxidation step was performed with the classic *oxidizer solution* (0.02 M I_2/pyridine/H_2O) for 10 minutes, the MMTr cation tests on aliquots of weighted resin provided values close to zero. The treatment of the resulting support with a solution of 28% aqueous ammonia at 50 °C for 5 hours led to the 5'-end phosphorylated nucleosides (**10a-d**), as shown by HPLC analysis.[8] In this case, the oxidation treatment led not only to the oxidation of phosphite to phosphate but also the removal of the 4-MMTr-benzyl group. We have explained this behaviour with a one-pot mechanism of oxidation/deprotection similar to that reported by Filippov, D. V. et al.[9] The same results were also observed for the reagent **3**.

To further assess the goodness of **1** and **2** as end-phosphorylating reagents, the preparation of an ON 5′-phosphate model was undertaken (Scheme 2). Specifically, the solid-phase syntheses of $_p$d(CAGT) and $_p$d(CAGTCAGTCAGTCAGT) were performed according to an automated synthesis protocol. ODNs were released from the support by NH_4OH treatment, and RP-HPLC analyses indicate that the use of **1** or **2** led to the formation of ODN 5′-phosphate in yields exceeding 90%. MALDI-TOF analysis confirmed the expected structures of **11** and **12**.

The removal of phosphate protecting groups in very mild conditions was then evaluated in an *ad hoc* experiment (Scheme 3) by the use of nucleotide model **13** (3'-O-benzoyl-N^6-benzoyl-2'-deoxyadenosine). Condensation of **1** with **13** in the presence of 1H-tetrazole in MeCN, followed by silica gel chromatography, afforded the nucleoside phosphite triester **14** (Scheme 3) in 75% yield.[10]



Scheme 3. Synthesis of dA 5'-monophospate derivative **15**: oxidation and deprotection of phosphorus in a one-pot reaction with I_2/pyridine/H_2O.

Treatment of **14** with 0.02 M I_2/pyridine/H_2O in THF was performed. The reaction was monitored by ^{31}P-NMR, and after only a few minutes (<5 minutes), the disappearance of the typical signal of the phosphite at 140 ppm and the appearance of its monoester phosphate at –0.7 ppm were observed. The crude material was then purified by silica gel chromatography, and 2'-deoxy adenosine-5'-monophosphate nucleoside **15** (Scheme 3) was isolated in 60% yield. As expected, ^{31}P-NMR analysis showed the diagnostic variation of the phosphorus chemical shift from 140 to –0.7 ppm, and ^{1}H,^{13}C NMR and ESI-MS analyses confirmed the expected structure of **15**.

Conclusion

New phosphorylating reagents **1** and **2** were prepared in good yields starting from 4-hydroxybenzaldehyde (Scheme 1), and their efficiency in the synthesis of 5′-phosphate monoester nucleosides (**10a-d**, Scheme 2) has been demonstrated. Phosphorylating reagents **1** and **2** are also efficient in the preparation of end-phosphate ODNs (**11** and **12** Scheme 2). Thus, following the classic procedure of automated DNA synthesis, the end-phosphate DNA sequence models were synthesized in very good yields. The removal of phosphate protective groups in very mild conditions by an *ad hoc* experiment on nucleoside model **13**

was demonstrated (Scheme 3). The oxidation to phosphate and the removal of phosphate protecting groups were achieved by a *one-pot* treatment with a 0.02 M I_2/pyridine/H_2O solution (*oxidizer solution*). In light of these results, the new phosphorylating agents **1** and **2** are compatible with base-sensitive functions and would be very useful for the synthesis of phosphate monoesters endowed with base-labile functions (esters, phosphotriester). This makes the reagents **1** and **2** unique among all phosphorylating reagents.

Acknowledgments

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6. Synthesis of **1** (Scheme 1): The 4-hydroxybenzaldehyde **4** (0.8 g, 6.5 mmol) was reacted with 4-monometoxytriphenylmethyl (1.0 g, 3.2 mmol) in 10 mL of anhydrous DCM in the presence of DIEA (2.2 mL, 12.9 mmol). After 6 hours at r.t., the reaction was quenched by dilution with DCM (100 mL), and the organic phase was washed three times with a solution of 0.1 M NaOH (3x100 mL). The organic phase was dried with $MgSO_4$, and then the solvent was removed under vacuum. The crude material was then purified on a column of silica gel (70 g) suspended in hexane/EtOAc 70:30 (v/v) with 1% of TEA, leading to product **5** (1.05 g, 82%). ^{1}H NMR (400 MHz, 25 °C, δ, ppm in $CDCl_3$): δ 9.76 (s, 1H), 7.57-7.25 (complex signals, 14H), 6.86 (d, J = 8.5 Hz, 2H), 6.82 (d, J = 8.0 Hz, 2H), 3.77 (s, 3H). ^{13}C NMR (100 MHz, 25 °C, δ, ppm in $CDCl_3$): δ 190.7, 161.9, 158.7, 143.8, 134.9130.6, 130.2, 128.3, 127.8, 127.3, 120.3, 113.1, 91.1, 55.0. Product **5** (1.05 g, 2.66 mmol) was subsequently treated with 0.25 g of $NaBH_4$ (6.60 mmol) in THF (10 mL) for 6 hours at r.t. The mixture was diluted with DCM (3x100 mL), and the organic phase was washed three times with water (100 mL). The organic phase was dried with $MgSO_4$, and then the solvent was removed under vacuum. The crude solid thus obtained was purified on a column of silica gel (70 g) suspended in hexane/EtOAc 60:40 (v/v) with 1% of TEA. From the column was recovered 0.845 g of clean desired product **6** (2.13 mmol, 80%). ^{1}H NMR (400 MHz, 25 °C, δ, ppm in $CDCl_3$): δ 7.49 (d, J=6.0 Hz, 4H), 7.37-7.22 (complex signals, 8H), 6.98 (d, J = 7.2 Hz, 2H), 6.79 (d, J = 7.2 Hz, 2H), 6.70 (d, J = 6.8 Hz, 2H), 4.45 (s, 2H), 3.75 (s, 3H). ^{13}C NMR (100 MHz, 25 °C, δ, ppm in $CDCl_3$): δ 158.4, 155.8, 144.5, 135.6, 133.3, 130.4, 128.6, 127.6, 127.4, 126.9, 120.6, 112.8, 90.0, 64.8, 55.0. Then, 0.40 g (1.01 mmol) of product **6** was reacted with N,N-diisopropyldichlorophosphoramidite (124 μL, 0.67 mmol) in the presence of DIEA (348 μL, 2.68 mmol) in DCM (7 mL). After 1.5 h, the reaction was quenched by dilution with DCM, and the organic phase was washed three times with cold water. The organic phase was dried with $MgSO_4$ and the solvent removed under vacuum. The material was purified with column chromatography of silica gel in hexane/EtOAc 85:15 (v / v) with 2% of TEA. From the column was recovered 0.74 g of clean desired product (**1**, 87%). ^{1}H NMR (400 MHz, 25 °C, δ, ppm in $CDCl_3$): δ 7.60 (d, J = 7.6 Hz, 8H), 7.42 (d, J = 8.8 Hz, 4H), 7.15-6.98 (complex signals, 16H), 6.82 (d, J = 7.6 Hz, 4H), 6.61 (d, J = 8.8 Hz, 4H), 4.48 (m, 4H), 3.55 (m, 2H), 3.20 (s, 6H), 1.06 (d, J = 6.8 Hz, 12H). ^{13}C NMR (100 MHz, , 25 °C, δ, ppm in $CDCl_3$): δ 158.8, 155.9, 145.1, 135.7, 132.8, 130.8, 127.9, 127.7, 127.4, 126.8, 120.9, 113.0, 90.5, 65.1, 54.3, 42.9, 24.3. ^{31}P NMR (162 MHz , 25 °C, δ, ppm in $CDCl_3$): d 147.8. ESI-MS calcd. [M] 921.09; found 945.30 $[MNa]^+$.
7. Synthesis of **2** (Scheme 1): Starting from **4**, the same procedure was followed using the same molar ratios and the same purification conditions used to synthesize **1**. The phosphoramidite **2** was obtained in an 80% yield. ^{1}H NMR (400 MHz, 25 °C, δ, ppm in $CDCl_3$): δ 7.53 (d, J = 7.1 Hz, 12H), 7.35-7.23 (complex signals, 18H), 7.01 (d, J = 8.4 Hz, 4H), 6.70 (d, J = 8.5 Hz, 4H), 4.54 (m, 4H), 3.64 (m, 4H), 1.17 (d, J = 6.8 Hz, 12H). ^{13}C NMR (100 MHz, 25 °C, δ, ppm in $CDCl_3$): δ 155.6, 144.2, 132.2, 132.2, 129.0, 127.8, 127.5, 127.2, 90.3, 65.2, 65.0, 43.0, 42.9, 24.7, 24.6 ^{31}P NMR (162 MHz , 25 °C, δ, ppm in $CDCl_3$): δ 147.3. ESI-MS calcd. [M] 861.39; found 885.20 $[MNa]^+$.
8. See *Supporting Information* file.
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10. Synthesis of **15** (Scheme 3): 35 mg (0.076 mmol) of 3'-O-benzoyl-N^6-benzoyl-2'-deoxyadenosine **13** was dissolved in 300 μL of DCM, after which 500 μL of activator solution was added (1H-tetrazole 0.25 M), and finally a solution of 76 mg of **1** (0.083 mmol) dissolved in 500 μL of DCM was added. After 30 min, the reaction was completed as shown from TLC [hexane/EtOAc 40:60 (v/v)] control. The solvent was removed under vacuum, and the crude material was purified by column chromatography on silica gel in hexane/EtOAc 50:50 (v/v) with 2% of TEA. From the column was recovered 73 mg of clean desired product **14** (75%). ^{1}H NMR (400 MHz, 25 °C, δ, ppm in $CDCl_3$): δ 8.84 (s, 1H), 8.39 (s, 1H), 8.10 (d, J =8.4 Hz, 2H), 8.05 (d, J = 8.5 Hz, 2H), 7.66-7.17 (complex signals, 30H), 6.94 (m, 4H), 6.76 (d, J = 9.0Hz, 4H), 6.73 (d, J = 9Hz, 4H), 5.57 (d, J = 5.6Hz, 1H), 4.40 (m, 1H), 4.03 (complex signals, 2H), 3.75 (m, 1H), 2.63 (m, 2H). ^{13}C NMR (100 MHz, , 25 °C, δ, ppm in $CDCl_3$): δ 165.8, 158.5, 156.2, 152.8, 151.7, 149.5, 144.5, 141.2, 135.7, 133.6, 132.8, 129.8, 128.9, 128.7, 128.2, 128.1, 127.7, 127.0, 120.8, 112.9, 90.2, 84.6, 84.2, 76.1, 64.7, 64.5, 64.4, 64.3, 60.4, 55.1. ^{31}P NMR (162 MHz, 25 °C, δ, ppm in $CDCl_3$): δ 140.4. ESI-MS calcd. [M] for $C_{78}H_{66}N_5O_{11}P$ = 1279.45; found 1303.69 $[MNa]^+$.
3 mL of oxidizer solution (0.02 M I_2/pyridine/H_2O) was added to 70 mg (0.05 mmol) of **14** and stirred. The reaction was followed by ^{31}P NMR by suspending a small amount of crude material in $CDCl_3$. Five minutes after the addition of the oxidizer solution, the disappearance of the signal at 140 ppm (phosphite triester) and the appearance of a typical signal of a phosphate monoester at -0.7 ppm were observed. The solvent was removed under vacuum, and the crude material was purified by silica gel chromatography in DCM/MeOH 80:20 (v/v). From the column was recovered 19 mg of 3'-O-benzoyl-N^6-benzoyl-2'-deoxyadenosine-5'-monophosphate desired product **15** (60%). ^{1}H NMR (400 MHz, 25 °C, δ, ppm in $CDCl_3$): δ 8.89 (s, 1H), 8.78 (s, 1H), 8.13-7.36 (complex signals, 4H), 7.77-7.43 (complex signals, 4H), 6.69 (dd, J = 8.4 and 6.1 Hz, 1H), 5.69 (m, 1H), 4.46 (m, 1H), 4.02 (m, 2H), 3.01 (m, 1H), 2.79 (dd, J = 14.0, 4.0 Hz, 1H). ^{13}C NMR (100 MHz, , 25 °C, δ, ppm in $CDCl_3$): δ 167.6, 154.1, 153.6, 151.4, 144.5, 135.1, 134.4, 131.0, 130.1, 130.0, 129.8, 124.1, 86.2, 84.0, 77.6, 66.8, 39.2. ^{31}P NMR (162 MHz , 25 °C, δ, ppm in $CDCl_3$): δ – 0.7. ESI-MS calcd. [M] for $C_{24}H_{22}N_5O_8P$ = 539.12; found 562.66 $[MNa]^+$; 578.83 $[MK]^+$.

1. New phosphorylating reagents
2. New phosphate protecting group
3. Synthesis of Nucleotides and Oligonucleotides by Solid phase synthesis