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A novel reagent for the chemical phosphorylation of oligonucleotides

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Abstract—A novel phosphoramidite reagent was developed to convert terminal hydroxyl groups of oligonucleotides into phosphate monoesters. The reagent's appearance as a solid foam is advantageous for its manipulation and handling in solid-phase synthesis and improves its thermal stability.

Various applications of oligonucleotides in molecular biology, such as gene construction,[1] ligase chain amplification reactions,[2] ligase-mediated genotyping[3] and the conjugation of ligands to oligonucleotides,[4] require the presence of a 5′-terminal phosphate group. 5′-Phosphorylated oligonucleotides are commonly prepared through solid-phase synthesis wherein the phosphate group is attached to the oligonucleotide in the last coupling cycle with a speciality phosphoramidite reagent. Commercially available reagents, such as **2**[5] and **3**[6] (Fig. 1) contain two base-labile phosphate protecting groups, which are removed during the alkaline deprotection of the oligonucleotide to give the desired 5′-phosphate monoester. The cyanoethyl group is usually applied as the first phosphate protecting group. A DMT-containing group, which allows the measurement of the coupling efficiency in the last cycle, and if reagent **3** is used, the cartridge-aided purification of the DMT-ON oligonucleotide after the deprotection reaction on a reversed phase resin, is employed as the second phosphate protecting group.

One disadvantage of both reagents **2** and **3** is their appearance as viscous oils, which causes difficulties in the filling and handling of the reagents and in the visual verification of their complete dissolution in the solvent of the coupling reaction, especially when the reagents are applied in amber bottles. Reagent **3** also requires prolonged coupling and detritylation times and is incompatible with the commonly employed capping reagents of the oligonucleotide synthesis cycle. These problems were addressed by the design and the synthesis

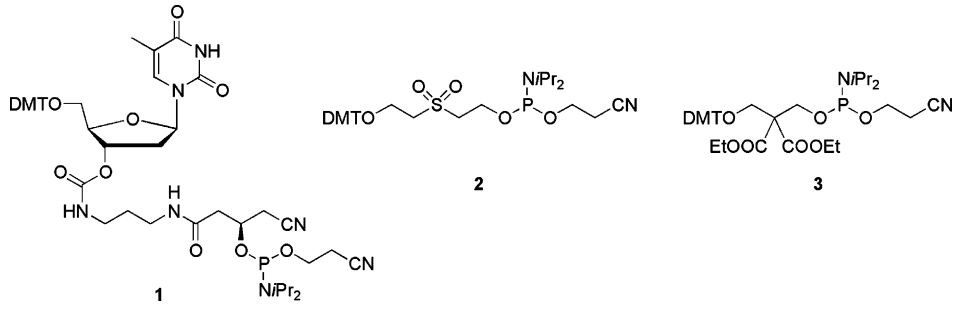

Figure 1. Novel phosphoramidite reagent **1** and commercially available reagents **2** and **3** employed for the 5′-phosphorylation of oligonucleotides.

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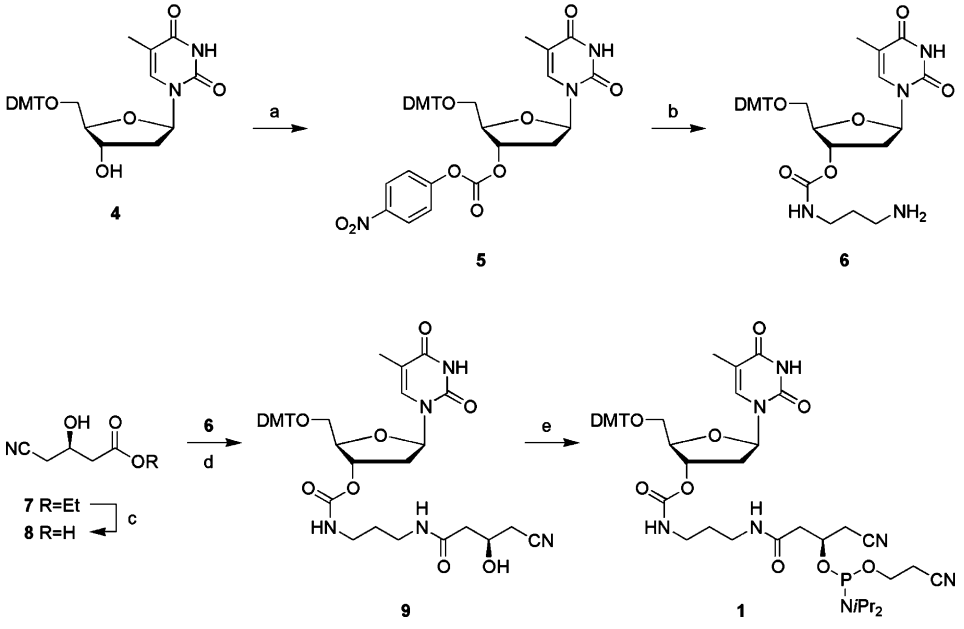
of the novel phosphoramidite **1**. The structure of reagent **1** is based on two cyanoethyl protecting groups, wherein one of the cyanoethyl groups is substituted in the α-position with a DMT-protected thymidine moiety that confers solidity on the product and allows the monitoring of the coupling reaction through the Vis-spectroscopic measurement of the dimethoxytrityl cation in acidic solution. It can be expected that the α-substituted cyanoethyl group would be cleaved more rapidly through base-induced β-elimination than the unmodified cyanoethyl group due to the positive inductive effect of the substituent.

Phosphoramidite reagent **1** was prepared from the commercially available 5′-*O*-DMT-thymidine **4** in four steps (Scheme 1). In the first step, **4** was converted into the 4-nitrophenyl carbonate **5** (62%) by treatment with 4-nitrophenyl chloroformate in pyridine.[7,8] The carbonate **5** was then added, under high dilution, to a mixture of 1,3-diaminopropane and triethylamine in CH_2Cl_2 to generate the carbamate **6** in 69% yield.[9] Saponification of the chiral ester **7** was accomplished by treatment with 1 M NaOH solution. After neutralization with phosphate buffer the crude acid **8** was coupled with amine **6** in aqueous solution by treatment with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole hydrate (HOBt) following the Nozaki procedure.[10,11] The resulting alcohol **9** was converted into the phosphoramidite reagent **1** by treatment with chloro-(2-cyanoethyl) diisopropylaminophosphane and *N*,*N*-diisopropylethylamine (DIPEA).[12] Reagent **1** was obtained in 63% yield and appears as a colorless solid foam that can be easily manipulated and weighed into bottles in small portions. It dissolves readily in acetonitrile, the most common solvent for phosphoramidite couplings, and its dissolution can be easily monitored by visual inspection. This property of reagent **1** is particularly valuable as it avoids the tedious handling of very viscous liquids, which is a common feature of other phosphorylation reagents. The solid reagent can be stored at 4 °C for prolonged periods.

Reagent **1** was applied in the preparation of the 5′-phosphorylated oligonucleotides **10**–**12** (Table 1). The automated solid-phase syntheses of the oligonucleotides were performed on 1 μmol scale employing a 0.1 M solution of phosphoramidite **1** in acetonitrile in the last coupling step. A standard synthesis cycle protocol (DMT-ON) was used with a coupling time of 90 s. Cleavage of the oligonucleotides from the support was accomplished by treatment with concd ammonia at room temperature for 1 h. Removal of the protecting groups was conducted through heating of the resulting ammonia solutions at 55 °C overnight. Anion exchange HPLC and MALDI–TOF MS analysis of crude oligonucleotides **10**–**12** confirmed the successful and nearly quantitative phosphorylation of the oligonucleotides (Table 1).

A second synthesis of the 5′-phosphorylated oligonucleotide **10** was performed as described above in DMT-OFF mode. The column effluents from the last and the last but one detritylation step and from the respective washing steps with acetonitrile were collected and photometrically analyzed for the orange dimethoxytrityl cation at 480 nm. A coupling efficiency for phosphoramidite reagent **1** of 97.8% was inferred. Finally, the required reaction time for the complete removal of the phosphate protective groups was investigated. The 5′-phosphorylated oligonucleotide **10** was prepared as above in DMT-ON mode. Aliquots of



Scheme 1. Synthesis of chemical phosphorylation reagent **1**. Reagents and conditions: (a) 4-nitrophenyl chloroformate, pyr, CH_2Cl_2, rt, 16 h, 62%; (b) 1,3-diaminopropane, Et_3N, CH_2Cl_2, rt, 16 h, 69%; (c) 1 M NaOH, rt, 2 h; (d) EDC, HOBt, phosphate buffer, DMF, THF, rt, 16 h, 64%; (e) $iPr_2NP(Cl)OCH_2CH_2CN$, DIPEA, CH_2Cl_2, rt, 3 h, 63%.

Table 1. 5′-Phosphorylated oligonucleotides **10**–**12** prepared with the phosphoramidite reagent **1**

Oligonucleotide	AX-HPLC[a]		MALDI–MS		OD_{260}
	R_t (min)	Purity (%)	Calculated	Found	
5′-*p*-d(TTT TTT TTT T) **10**	15.62[b]	90.8	3059.9	3065.4	79.1
5′-*p*-d(CTC TCA GCG AGC CTC AA) **11**	9.93[c]	83.1	5192.3	5188.7	125.7
5′-*p*-d(TTG AGG CTC GCT GAG AG) **12**	9.63[c]	67.6	5343.9	5328.8	116.4

[a] Anion exchange HPLC conditions: Dionex DNAPac PA100 column (4×250 mm) eluting with a linear gradient at 85 °C with a flow rate of 1.5 mL/min, detection at $\lambda = 260$ nm, buffer A = 25 mM trizma hydrochloride/1 mM EDTA/10%CH_3CN, pH 7.5, buffer B = 25 mM trizma hydrochloride/1 mM EDTA/10%CH_3CN/1 M NaCl, pH 7.5.
[b] Gradient 1: 10–46% buffer B in 22.00 min.
[c] Gradient 2: 36–80% buffer B in 22.00 min.

the controlled pore glass (CPG) support containing the oligonucleotide were treated with concd ammonia solution or with AMA solution (concd ammonia/40% aq methylamine 1/1, v/v) using various temperatures and times. Anion exchange HPLC analysis of the ammonia solutions indicated that complete deprotection was achieved within 2 h in concd ammonia at 55 °C or within 15 min in AMA solution at 65 °C.

In conclusion, the novel phosphoramidite **1**, prepared in five steps from a common thymidine intermediate, is employed as an efficient reagent for the 5′-phosphorylation of oligonucleotides. The reagent's appearance as a solid foam is advantageous for its manipulation and handling in solid-phase synthesis and improves its thermal stability.

References and Notes

1. (a) Wosnick, M. A.; Barnett, R. W.; Vicentini, A. M.; Erfle, H.; Elliott, R.; Sumner-Smith, M.; Mantei, N.; Davies, R. W. *Gene* **1987**, *60*, 115–127; (b) Carter, J. B.; Pulaski, S. P.; Theriault, N. Y. *BioTechniques* **1988**, *6*, 470–474.
2. Barany, F. *PCR Meth. Appl.* **1991**, *1*, 5–16.
3. Landegren, U.; Kaiser, R.; Sanders, J.; Hood, L. *Science* **1988**, *241*, 1077–1080.
4. (a) Chu, B. C. F.; Wahl, G. M.; Orgel, L. E. *Nucl. Acids Res.* **1983**, *11*, 6513–6529; (b) Fedorova, O. S.; Savitskii, A. P.; Shoikhet, K. G.; Ponomarev, G. V. *FEBS Lett.* **1990**, *259*, 335–337.
5. (a) Horn, T.; Urdea, M. S. *Tetrahedron Lett.* **1986**, *27*, 4705–4708; (b) Horn, T.; Allen, J. S.; Urdea, M. S. *Nucleosides Nucleotides* **1987**, *6*, 335–340.
6. (a) Guzaev, A.; Salo, H.; Azhayev, A.; Lönnberg, H. *Tetrahedron* **1995**, *51*, 9375–9384; (b) Guzaev, A.; Lönnberg, H. *Tetrahedron* **1999**, *55*, 9101–9116.
7. A similar reaction to prepare compound **5** is described by Li, H.; Miller, M. J. *Tetrahedron Lett.* **2000**, *41*, 4323–4327.
8. 5′-*O*-DMT-thymidine **4** (5.02 g, 9.22 mmol) was co-evaporated with pyridine (2×20 mL), dissolved in pyridine (25 mL) and treated dropwise under ice cooling with a solution of 4-nitrophenyl chloroformate (1.95 g, 9.68 mmol) in CH_2Cl_2 (25 mL). The mixture was stirred overnight at room temperature. The solution was concentrated to dryness and the remainder was redissolved in CH_2Cl_2 (20 mL). This solution was added slowly to vigorously stirred diethyl ether (150 mL). The resulting precipitate was filtered off and discarded. The filtrate was poured into stirred hexanes (500 mL) to give a colorless precipitate that was filtered and dried to provide 4.35 g (62%) of the carbonate **5** as a colorless powder.
9. The carbonate **5** (4.15 g, 5.85 mmol) was dissolved in CH_2Cl_2 (15 mL) and added dropwise to a stirred and ice-cooled solution of 1,3-diaminopropane (2.16 g, 29.3 mmol) and triethylamine (4.1 mL, 29.3 mmol) in CH_2Cl_2 (50 mL). The mixture was stirred at room temperature overnight, then diluted with CH_2Cl_2 (100 mL) and washed with aq satd $NaHCO_3$ solution (5×100 mL) and brine (100 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to provide a yellow foam, which was purified via flash chromatography eluting with CH_2Cl_2/EtOH/Et_3N 80:15:5 (v/v/v) to give 2.60 g (69%) of the amine **6** as a colorless amorphous solid. R_f 0.08 (CH_2Cl_2/EtOH/Et_3N 80:15:5, v/v/v). ^1H NMR (CD_3CN, 300 MHz): δ 7.50–7.41 (m, 3H), 7.38–7.25 (m, 7H), 6.90 (d, $J = 8.8$ Hz, 4H), 6.26 (dd, $J = 5.9$, 2.6 Hz, 1H), 6.10–6.04 (m, 1H), 5.35–5.29 (m, 1H), 4.08 (d, $J = 2.6$ Hz, 1H), 3.85 (br s, 2H), 3.78 (s, 6H), 3.40 (dd, $J = 10.5$, 3.4 Hz, 1H), 3.31 (dd, $J = 10.5$, 2.9 Hz, 1H), 3.16 (q, $J = 6.5$ Hz, 2H), 2.65 (t, $J = 6.7$ Hz, 2H), 2.50–2.30 (m, 2H), 1.57 (quintet, $J = 6.7$ Hz, 2H), 1.42 (s, 3H). ^{13}C NMR (CD_3CN, 75 MHz): δ 164.0, 159.0, 156.1, 150.8, 145.0, 136.0, 135.8, 130.3, 128.3, 128.2, 127.3, 113.4, 110.9, 86.9, 84.4, 84.2, 75.2, 64.0, 55.2, 38.7, 38.3, 37.6, 32.2, 11.4.
10. Nozaki, S. *Chem. Lett.* **1997**, 1–2.
11. Ethyl (*R*)-4-cyano-3-hydroxybutanoate **7** (0.702 g, 4.47 mmol) was treated with 1 M NaOH (9 mL, 9 mmol) and the resulting mixture was stirred at room temperature for 2 h. 1.5 M NaH_2PO_4 (9 mL) was added and the pH of the solution was adjusted to 5.7 with concd HCl. After addition of HOBt·H_2O (70 mg, 0.46 mmol) and DMF (9 mL) the mixture was treated with a solution of the amine **6** (1.35 g, 2.09 mmol) in THF (4 mL). The pH was again adjusted to 6.0 with diluted HCl (concd HCl/water 1:10, v/v) and EDC (0.801 g, 4.18 mmol) was added. The reaction mixture was stirred overnight, then diluted with water (100 mL) and extracted with EtOAc (3×50 mL). The organic phase was dried over Na_2SO_4 and concentrated. The crude product was purified via flash chromatography eluting with EtOAc/hexanes/acetonitrile 70:25:5 (v/v/v) to provide 1.01 g (64%) of alcohol **9** as a colorless foam. R_f 0.43 (EtOAc/hexanes/EtOH 70:25:5, v/v/v) ^1H NMR (CD_3CN, 300 MHz): δ 9.09 (br s, 1H), 7.50–7.40 (m, 3H), 7.38–7.23 (m, 7H), 6.90 (d, $J = 8.8$ Hz, 4H), 6.68–6.61 (m, 1H), 6.27 (dd, $J = 6.2$, 2.3 Hz, 1H), 5.86 (t, $J = 6.2$ Hz, 1H), 5.34–5.28 (m, 1H), 4.19 (quintet, $J = 6.5$ Hz, 1H), 4.12–4.08 (m, 1H), 3.78 (s, 6H), 3.41 (dd, $J = 10.5$, 3.4 Hz, 1H), 3.32 (dd, $J = 10.5$, 2.9 Hz, 1H), 3.20 (quintet, $J = 6.4$ Hz, 2H), 3.11 (dt, $J = 7.2$, 6.4 Hz, 2H), 2.63 (dd, $J = 16.8$, 5.0 Hz, 1H), 2.53 (dd, $J = 16.8$, 6.2 Hz, 1H), 2.45–2.30 (m, 4H), 2.18 (s, 1H), 1.61 (quintet, $J = 6.4$ Hz, 2H), 1.43 (s, 3H). ^{13}C NMR (CD_3CN,

75 MHz): δ 171.1, 163.8, 159.0, 156.0, 150.7, 145.1, 135.8, 135.7, 130.3, 128.3, 128.2, 127.3, 118.3, 113.4, 110.8, 86.9, 84.4, 84.2, 75.3, 64.8, 64.0, 55.2, 41.5, 37.8; 37.5, 36.1, 29.5, 25.2, 11.4.

12. A solution of the alcohol **9** (0.950 g, 1.26 mmol) and *N*,*N*-diisopropylethylamine (2.2 mL, 12.6 mmol) in CH_2Cl_2 (15 mL) was treated with chloro-(2-cyanoethyl) diisopropylaminophosphane (0.312 g, 1.32 mmol) and stirred at room temperature for 3 h. The reaction mixture was concentrated to dryness and the remainder was redissolved in EtOAc (100 mL). The resulting solution was extracted with 10% aq Na_2CO_3 solution (2×50 mL) and brine (50 mL). The organic phase was dried over Na_2SO_4 and concentrated to dryness. The crude product was purified via flash chromatography eluting with EtOAc/CH_2Cl_2/acetonitrile 70:23:7 (v/v/v) to provide 0.758 g (63%) of the phosphoramidite reagent **1** as a colorless foam. R_f 0.25 (EtOAc/CH_2Cl_2/acetonitrile 70:23:7, v/v/v) ^{1}H NMR (CD_3CN, 300 MHz): δ 9.11 (br s, 1H), 7.55–7.42 (m, 3H), 7.40–7.25 (m, 7H), 6.90 (d, $J = 9.1$ Hz, 4H), 6.67–6.60 (m, 1H), 6.27 (dd, $J = 6.3$, 2.0 Hz, 1H), 5.90 (t, $J = 6.2$ Hz, 1H), 5.35–5.27 (m, 1H), 4.50–4.38 (m, 1H), 4.11–4.07 (m, 1H), 3.90–3.69 (m, 8H), 3.67–3.50 (m, 2H), 3.40 (dd, $J = 10.4$, 3.7 Hz, 1H), 3.31 (dd, $J = 10.4$, 3.1 Hz, 1H), 3.27–3.05 (m, 4H), 2.80–2.30 (m, 8H), 1.60 (quintet, $J = 6.3$ Hz, 2H), 1.44 (s, 3H) 1.25–1.15 (m, 12H). ^{13}C NMR (CD_3CN, 75 MHz): δ 169.6, 164.0, 159.3, 156.2, 151.0, 145.3, 136.0, 135.9, 130.5, 128.5, 128.4, 127.5, 119.2, 117.8, 113.6, 111.1, 87.1, 84.6, 84.4, 75.4, 67.9, 67.6, 67.5, 67.3, 64.3, 59.0, 58.8, 55.4, 43.7, 43.6, 43.5, 42.3, 42.1, 38.2, 37.8, 36.5, 29.9, 25.2, 24.4, 24.3, 20.4, 11.7. ^{31}P NMR (CD_3CN, 121.5 MHz): δ 150.2, 150.1.