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A New Approach for Chemical Phosphorylation of Oligonucleotides at the 5'-Terminus

Andrei Guzaev,* Harri Salo, Alex Azhayev, and Harri Lönnberg

Department of Chemistry, University of Turku, FIN-20500 Turku, Finland

Abstract: A new efficient method for chemical 5'-phosphorylation of synthetic oligonucleotides is described. Accordingly, 2-cyanoethyl 3-(4,4'-dimethoxytrityloxy)-2,2-di(ethoxycarbonyl)propyl-1 *N,N*-diisopropyl phosphoramidite (**1**) was introduced as the 5'-terminal building block during the normal chain assembly. Conventional ammonolysis gave rise to an oligomer protected at 5'-phosphate with a dimethoxytritylated tether. At this step, the oligonucleotide may be easily separated from truncated impurities by RP HPLC. Successive detritylation and brief treatment with aqueous ammonia gave the oligonucleotide 5'-monophosphate. Alternatively, the yield of the last coupling may be quantified by detritylation of the oligonucleotide still anchored to the solid support. Usual deprotection then leads directly to the 5'-phosphorylated oligomer.

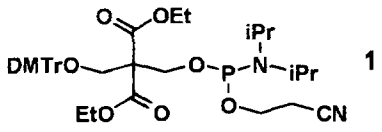
INTRODUCTION

Oligonucleotides possessing 5'-phosphate group are required as valuable tools for gene construction,[1] cloning,[2] mutagenesis,[3] the ligation chain reaction,[4] and many other biological applications. Often, they are prepared by *T4* kinase catalysed phosphorylation employing adenosine 5'-triphosphate as a phosphate source.[2]

A number of methods have been reported that allow chemical 5'-phosphorylation of pre-assembled oligonucleotide precursor. Some of them include preparation of modified nucleoside-based building blocks to be attached at the last step of the oligonucleotide synthesis.[5] Another strategy, based on non-nucleosidic building blocks, seems to be more universal, since a single reagent may be employed. A variety of approaches compatible with phosphotriester,[6] H-phosphonate,[7] methyl phosphonamidite[8] or phosphoramidite[9] chemistry have been elaborated. All of them suffer from the same shortcoming: the efficiency of the final coupling cannot be monitored by dimethoxytrityl response. In order to overcome this problem, a building block derived from (4,4'-dimethoxytrityloxyethyl) hydroxyethyl sulfone has been introduced.[10] Upon the completion of the final chain elongation step, the 5'-derivatized oligonucleotide possesses a 5'-terminal tether containing a dimethoxytrityl protecting group. This can be used to determine the coupling yield by conventional dimethoxytrityl assay. Ammonolytic deprotection, however, results in β-elimination of the *O*-phosphorylated hydroxyethyl sulfone fragment, and hence the dimethoxytrityl group is lost on release of the 5'-phosphate group. Accordingly, performing dimethoxytrityl specific isolation of the oligonucleotide is excluded.

It is well known that preparative RP-separation of oligonucleotide 5'-phosphates from the corresponding non-phosphorylated material is often unsuccessful. The use of more efficient ion exchange chromatography is, in turn, restricted by the length of the DNA fragment to be isolated. Therefore, approaches that offer a

selective isolation of the desired oligonucleotide remain to be of particular interest. A family of methods, all involving an "orthogonal" protection strategy of the 5'-terminal phosphate, has been elaborated. After the chain assembly and ammonolytic deblocking, the orthogonal protection of the 5'-phosphate [*t*-butyl,[11,12] (4-nitrophenyl)ethyl,[13] 2-(tritylthio)ethyl,[14] or 2-(triphenylsilyl)ethyl[15]] remains unchanged. Being of moderate hydrophobicity, the *t*-butyl and (4-nitrophenyl)ethyl protecting groups enable efficient separation only for relatively short oligonucleotides. In contrast, 2-(tritylthio)ethyl[14] or 2-(triphenylsilyl)ethyl[15] groups are more hydrophobic than the dimethoxytrityl group, and hence the desired oligonucleotides may be very selectively isolated by RP HPLC. After the purification, free 5'-phosphate monoester is released by treatment with an appropriate reagent: trifluoroacetic acid,[11] or trimethylsilyl chloride[12] for *t*-Bu; strong amine (DBU, TBD, and others) for (4-nitrophenyl)ethyl;[13] 2 M Bu_4NF/DMSO at 70 °C for 2-(triphenylsilyl)ethyl.[15] In this respect, 2-tritylthioethyl group[14] offers the mildest deprotection technique. To obtain the 5'-phosphate, the trityl-S bond was selectively cleaved by aqueous silver nitrate or iodine. Subsequent addition of dithiothreitol at pH 8.5 generates in both cases mercaptide ion which rapidly degrades to the target oligonucleotide 5'-phosphate and ethylene sulphide.[14]



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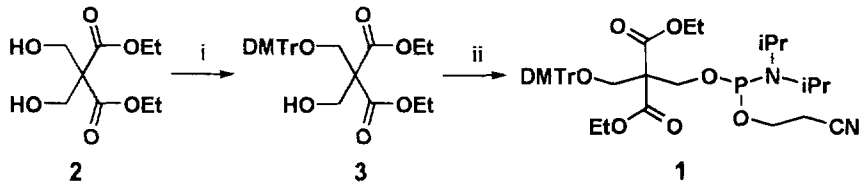
We now report on a new approach for the preparation of 5'-phosphorylated oligonucleotides by using phosphoramidite building block **1**. After the synthesis is completed, conventional ammonolysis gives rise to the DNA fragment selectively protected at 5'-phosphate group with a dimethoxytritylated tether. At this step, the modified oligonucleotide can be easily separated from truncated impurities by reversed phase HPLC. Successive detritylation and brief treatment with ammonia generate the 5'-phosphate group in more than 98% yield. Thus the method requires only those ancillary reagents that are of daily use in the DNA synthesis. Alternatively, the yield of the last coupling could be quantified by detritylation of the oligonucleotide still anchored to the solid support. Usual deprotection leads in this case directly to the target 5'- phosphorylated DNA fragment.

RESULTS AND DISCUSSION

Synthesis of the phosphoramidite building block 1. - The preparation of the building block **1** is outlined in Scheme 1. The starting diethyl 2,2-bis(hydroxymethyl)malonate, **2**, is commercially available or it can be prepared from diethyl malonate in a yield of 75%.[16] One of the hydroxy groups was 4,4'-dimethoxytritylated, and the product, **3**, was isolated by column chromatography and converted into phosphoramidite building block **1** by treatment with (2-cyanoethyl) *N,N,N',N'*-tetraisopropylphosphorodiamidite[17] in the presence of 1*H*-tetrazole. After aqueous work-up, **1** was obtained in a pure form by precipitation from toluene to hexane.



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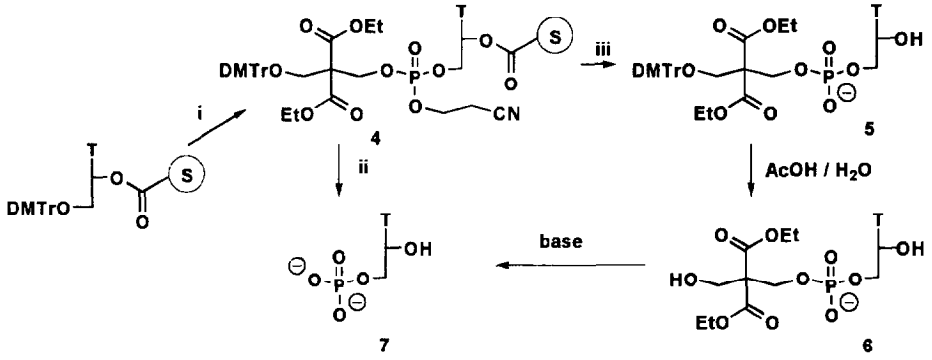
Scheme 1. i: a. DMTrCl / Py; **b.** Et_3N / H_2O; **ii: a.** (2-cyanoethyl) *N,N,N',N'*-tetraisopropylphosphorodiamidite / 1*H*-tetrazole; **b.** $NaHCO_3$ / H_2O. DMTr - 4,4'-dimethoxytrityl.

Introduction of 5'-terminal phosphate with the aid of phosphoramidite 1. - The compatibility of the phosphoramidite **1** with the established oligonucleotide synthesis, as well as the usefulness of the resulting DNA fragments in generation of oligonucleotide 5'-phosphates, was studied. First, by using standard coupling protocol commercial thymidine derivatized solid support (0.2 μmol) was detritylated, treated with **1** (0.1 M in MeCN) and excess of 1*H*-tetrazole, and oxidised with iodine solution (Scheme 2). Measurement of the coupling efficiency by conventional deprotection with 3% dichloroacetic acid in methylene dichloride showed that detritylation reaction was completed in 5 min. Coupling yield, however, was found to be satisfactory (99%). Subsequent ammonolysis gave a deoxynucleotide derivative which was compared with commercial samples of thymidine and thymidine 5'-phosphate by ion exchange and RP HPLC. According to these measurements the reaction mixture consisted of 99% thymidine 5'-phosphate, **7**, and 1% thymidine. The ratio of the starting material and product agreed with the result of the dimethoxytrityl assay.



Scheme 2. i: a) DCA/CH_2Cl_2; b) 1/1*H*-tetrazole; c) $J_2/Py/H_2O/THF$; **ii:** a) DCA/CH_2Cl_2; b) $NH_3 \cdot H_2O$; **iii:** $NH_3 \cdot H_2O$; **base:** NH_3-H_2O, $MeNH_2$, 1,3-propanediamine. S - solid support.

Next question was to clarify which step of the deprotection procedure is responsible for the conversion of the 5'-tethered deoxynucleotide into the 5'-phosphate. First, it was found that the product pattern significantly depending on whether the oligonucleotide carries a 5'-terminal dimethoxytrityl group when subjected to ammonolysis (Scheme 2). The formation of **7** was observed only when the solid support **4** was detritylated before the final deprotection. In contrast, when **4** having the 5'-OH group still dimethoxytritylated was released and deprotected with either conc. aqueous ammonia, methylamine, or 1,3-propanediamine, the conjugate **5** was obtained. Its structure was established as follows: (i) treatment with all 3 amines gave products that coeluted on RP column; (ii) the products obtained by ammonia and methylamine deprotection of **4** (10-25 μmol) were shown to exhibit ^1H NMR signals of thymidine and the nonnucleosidic moiety **3**. The stability of the ester groups in **5** towards ammonolysis and aminolysis is rather unexpected and contrasts to the known behaviour of the alkyl ester function under similar conditions.[18] Detritylation of **5** with 80% aq. AcOH for 30 min led, in 91% yield, to compound **6** which was characterised by ^1H and ^{31}P NMR.

The phosphodiester **6** was found to be stable under neutral conditions. However, when treated with diluted base, it was rapidly converted into thymidine 5'-phosphate **7**. In order to estimate the half-life of this reaction in the basic media, several experiments were carried out by using decreasing concentrations of different aqueous amines: ammonia, methylamine, and *n*-butylamine. The reaction was too fast to be monitored by RP HPLC in 1.0 and 0.1 M aq. solutions of these amines at ambient temperature (>95% conversion in 15

min). In 0.01M aq. *n*-butylamine, the half-life was found to be about 15 min, and a number of intermediates were detected in the reaction mixture. None of them was accumulated at a marked extent, and the reaction was smoothly completed in 3 h. Independent of the amine employed, the yield of **7** was repeatedly > 99.5%.

Synthesis of the oligodeoxynucleotide 5'-phosphates. - Since these results seemed to offer a convenient method for the 5'-terminal phosphorylation of oligonucleotides, they were verified by applying the phosphoramidite **1** in the DNA synthesis. A protected oligonucleotide 5'-GAACATCATGGTCGT-3' (**8**) was assembled on the commercial solid support, and the phosphoramidite **1** was attached to its 5'-terminus to give **9**. When standard protocol was employed (0.1 M **1** in acetonitrile, coupling time 25s), the coupling yield was found to be greater than 98% and comparable with commercial phosphoramidites. As control samples, non-phosphorylated oligonucleotide (**10**) and oligonucleotide 5'-phosphate (**11a**) of the same sequence were prepared. To synthesise the latter, the reported method[10] using commercial Phosphoralink™ reagent (Applied Biosystems)[19] was adopted. Upon conventional deprotection, the crude reaction mixtures were analysed by ion exchange and/or RP HPLC. The retention times observed are collected in the Table 1.

Table 1. HPLC Retention Times (in minutes) of the Oligonucleotides Prepared.

Oligo[a]	5'-DMT-**10**	**10**	**11a**[c,d]	**13**	**14**	**11b**[d,e]
RP[b]	26.2	21.2	20.3	25.8	22.9	20.3
Ion exchange[b]	--	23.5	28.6	--	23.5	28.6

a. For the structures, consult *Scheme 2*; **b.** For the conditions, see experimental section; **c.** Prepared by reported method;[10] **d.** Coelute when coinjected; **e.** Prepared by using **1**.

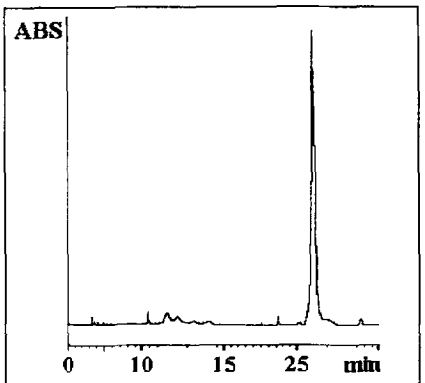


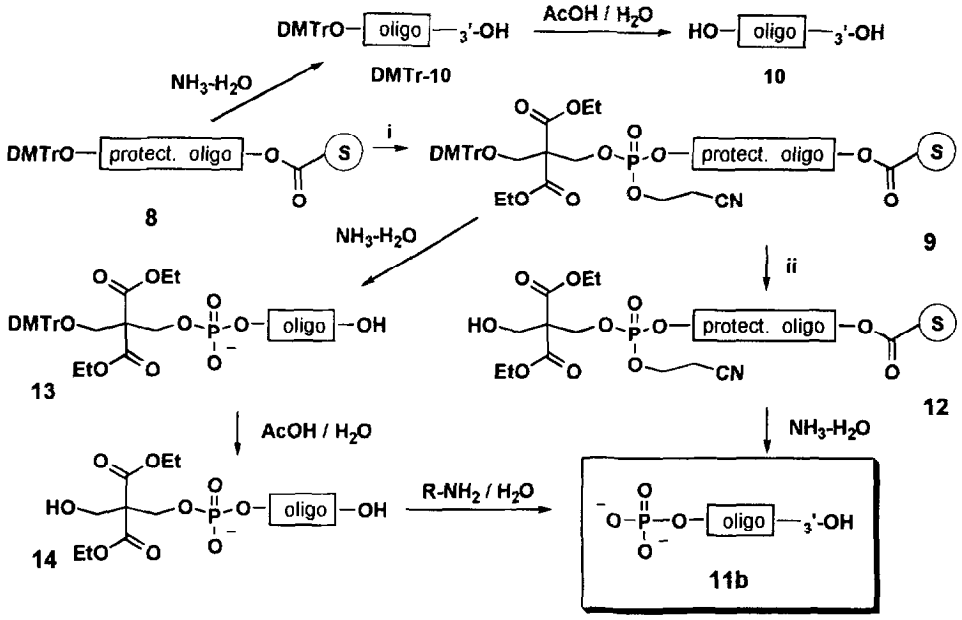
Fig. 1. RP HPLC profile of crude **13**.

The deprotection of the anchored oligonucleotide **9** led generally to the same results observed with **4** (Scheme 3). If the synthesis was carried out in a DMT-Off mode (final detritylation for 5 min), the partially protected oligonucleotide **12** underwent conversion to the 5'-phosphorylated derivative **11b** during the base deprotection. In contrast, ammonolysis of **9** led to a stable DMT-On derivative, **13**, which was isolated by RP HPLC (Fig. 1).

It has to be emphasised that the detritylation of **13** under aqueous conditions does not markedly differ from that of underivatized oligonucleotides. This contrasts to exceptional resistance of the dimethoxytrityl protected **9** and its analogues towards treatment with dichloroacetic acid in methylene dichloride, as mentioned above. Conventional detritylation (80% aq. AcOH; 20 min) converts **13** into the corresponding alcohol **14**, which was found to be stable under neutral or slightly acidic conditions (pH 5-7) for several days. Oligonucleotide **14** could be rechromatographed by either RP (Fig. 2) or ion exchange chromatography. This procedure, however, is not routinely required.

When treated with diluted base, **14** was rapidly converted into 5'-phosphorylated oligonucleotide **11b**. Again, the use of aqueous ammonia, methyl- or *n*-butylamine in a concentration higher than 0.1M led to formation of **11b** in a yield of 98.5 to 99.5% within 15 min (Fig. 3). The reaction mixtures contained 0.5 to

1.5% of an unidentified oligonucleotide of lower negative charge and higher hydrophobicity. Oligonucleotides prepared by both routes of deprotection (Scheme 3) were found to be identical to the authentic **11a**.



Scheme 3. i: a. 1/1*H*-tetrazole; **b.** I_2 / H_2O / Py / THF; **ii:** 3% dichloroacetic acid / CH_2Cl_2. R = H, CH_3; *n*-C_4H_9.

Thus, in a routine preparation of oligonucleotide 5'-phosphates, it is more than sufficient to purify and detritylate **9**, followed by treatment of crude **14** with concentrated ammonia for 15 min. Upon evaporation of the reaction mixture, the desired product, **11b**, may be isolated by HPLC.

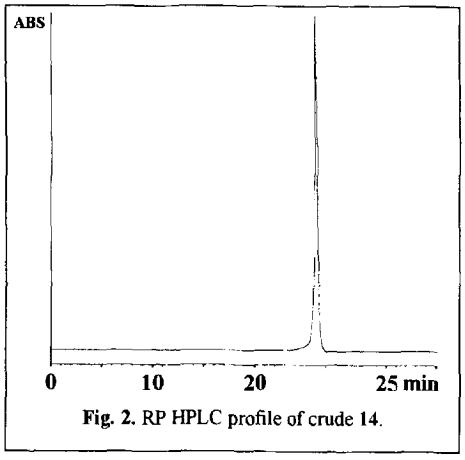


Fig. 2. RP HPLC profile of crude 14.

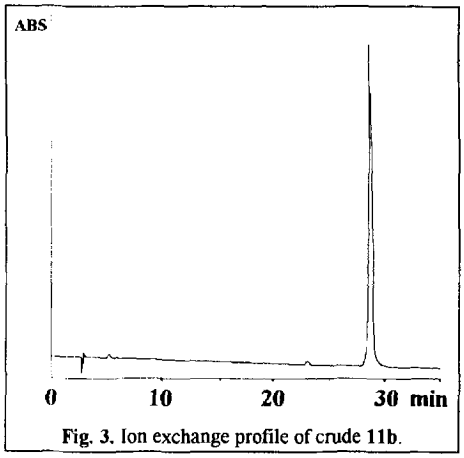
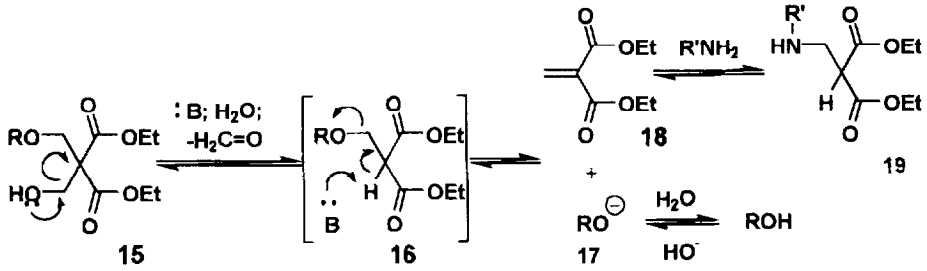


Fig. 3. Ion exchange profile of crude 11b.

On mechanism of the 5'-terminal phosphate deprotection. - The conversion of **6**,**12**,**14** to **7**,**11b** proceeds very easily in aqueous amines. No data on the base catalysed reactions of **2** or its monoethers has

been reported. Our attempts to rationalise the reactivity of **6**,**12**,**14** revealed that **2** and **3** are extremely sensitive to the basic conditions. For example, **3** disappeared quantitatively within 30 min when treated with a catalytic amount of *n*-butylamine (0.1 eq.) in aqueous pyridine. The only UV-absorbing product detected mixture was 4,4'-dimethoxytrityl alcohol (characterised by ^{1}H and ^{13}C NMR). *O*,*O*-Alkylidene derivatives of **2** are, in turn, known to be smoothly hydrolysed with strong alkali to the corresponding dicarboxylates the 1,3-dioxane moiety remaining intact.[20] Similarly, dimercapto analogues of **2** are, in both free and cyclic disulphide form, resistant to the treatment with 1,2-ethanediamine.[21] These observations illustrate that the presence of at least one unprotected hydroxy group greatly destabilises the 1,3-propanediol fragment of **2** and its derivatives.

Mechanistically the phenomenon could be explained as a two-step process. First, a retrograde aldol reaction of **15** that generate an intermediate **16**, may occur under basic conditions (Scheme 4).[22]



Scheme 4. R = H, DMTr, oligonucleotide; R' = H, CH_3, *n*-Bu.

One can expect that being a reversible reaction, the process should be facilitated by the presence of primary amines or ammonia which are able to trap the released formaldehyde. Compound **16** should, in turn, be extremely susceptible to the β-elimination reaction leading to **17** which is in the present case an oligonucleotide 5'-phosphate. The other product may then tentatively be assigned as a diethyl methylenemalonate, **18**. Finally, the amine might be expected to undergo conjugate nucleophilic 1,4-addition to **18** to give **19**.[23]

The stability of **6**,**12**,**14** under acidic conditions is expected: derivatives of **2** are known to stand weak acids.[24] Only when concentrated solutions of aqueous mineral acids are applied, **2** undergoes ester bond hydrolysis followed by decarboxylation.

Conclusions. - The non-nucleosidic building block **1** allows the efficient preparation of oligonucleotide 5'-phosphates by employing only the procedures and ancillary reagents adopted in the routine DNA synthesis. The precursor of oligonucleotide 5'-phosphate contains a dimethoxytrityl protecting group and thus can be easily isolated by RP HPLC. Subsequent treatments give rise to the target oligonucleotide 5'-phosphate in a high yield within less than 1h.

EXPERIMENTAL

General. - Diethyl 2,2-bis(hydroxymethyl)malonate and 4,4'-dimethoxytrityl chloride (recrystallised before use) were purchased from Aldrich, reagents for oligonucleotide synthesis from Cruachem, and {2-[2-(4,4'-dimethoxytrityloxyethyl) sulfonyl] ethyl} (2-cyanoethyl) *N*,*N*-diisopropylphosphoramidite (Phosphoralink™) from Applied Biosystems. Adsorption column chromatography was performed on Silica gel 60 (Merck). NMR spectra were recorded on Jeol GX-400 spectrometer operating at 399.8 and 161.9 MHz for ^{1}H and ^{31}P,

respectively. Either $CDCl_3$ or DMSO-d_6 were used as solvents, with either TMS as internal (^1H) or H_3PO_4 as external (^{31}P) standard.

HPLC techniques. - The oligonucleotides were analysed and isolated by RP chromatography (column: Nucleosil 300-5C18, 4.0x250 mm, Macherey-Nagel; buffer A: 0.05 M NH_4OAc; buffer B: 0.05 M NH_4OAc in 65% MeCN; flow rate 1.0 mL min^{-1}; a linear gradient from 5 to 60% B in 30 min was applied for dimethoxytrityl protected oligonucleotides, otherwise from 0 to 30% B in 30 min). Oligonucleotides which did not contain dimethoxytrityl group were analysed by ion exchange chromatography (column: SynChropack AX-300, 4.6x250 mm; 6.5 μm, SynChrom, Inc.; buffer A: 0.05 M KH_2PO_4 in 50% formamid; buffer B: A + 0.6 M $(NH_4)_2SO_4$; flow rate 1.0 mL min^{-1}; a linear gradient from 25 to 85% B in 35 min. Semipreparative RP HPLC was carried out on a Hypersil® ODS column (5 μm; 10×250 mm) by using 0.05M aq. NaOAc as buffer A, 80% aq. MeCN as buffer B, and water as C at a flow rate 4.5 mL × min^{-1}. Compound **5** was isolated employing a linear gradient from 30 to 90% B in 30 min. Desalting was performed on the same column eluted initially with buffer A (10 min), then with water (10 min). The desalted products were obtained eluting the column with a mixture of B and C (60:40% for **5** and 30:70 for **6**, correspondingly).

Oligodeoxyribonucleotide synthesis. - The protected oligonucleotides were assembled on an Applied Biosystems 392 DNA Synthesizer using commercial solid support, phosphoramidite chemistry, and recommended protocols for 0.2 and 10 μmol scale. A modified synthetic cycle was used for the preparation of **4** in 25 μmol scale. Accordingly, a mixture of 1*H*-tetrazole and phosphoramidite solutions was delivered to the column for 34 s. All the steps involving the delivery of MeCN, oxidiser, or capping mixture to column as well as reversed flush steps were reprogrammed for the time longer as much as twice than that employed in ABI 10 μmol protocol. The detritylation step was carried out by using two consequent "#14 (acid solution) to column" steps (2×80 s), separated by a trityl flush step (7 s). Phosphoramidite **1** was used as 0.1 M solution in dry MeCN, with unaltered coupling time. The oligonucleotides were deprotected with conc. aqueous NH_3 (2h at room temperature, then 7h at 55°C), analysed and isolated by HPLC. For 5'-DMTr-**10** and **13**, dimethoxytrityl group was cleaved with 80% aq. AcOH for 20 min at ambient temperature, followed by analysis on both RP- and ion exchange columns and RP-isolation of **10** and **14**.

5'-Phosphate deprotection experiments. - Aliquots of purified **14** (20 AU/mL; 50 μL) were diluted to 250 μL with aqueous solution of ammonia or appropriate amine, giving the following final concentrations: ammonia: 25%, 1.0, 0.1 M; methylamine: 1.0, 0.1 M; *n*-butylamine: 1.0, 0.1, 0.01 M. Samples (50 μL) of the reaction mixtures were taken after 15 and 30 min, evaporated to dryness, dissolved in water (250 μL) and analysed by RP HPLC comparing retention times with those of **14** and **11a**. Additional samples (50 μL) were withdrawn from the reaction in 0.01 M aqueous *n*-butylamine at 60, 120, and 180 min. The aliquots were treated and analysed as described above.

Optimised procedure for isolation and final deprotection of oligodeoxynucleotide 5'-phosphates. - A pre-assembled oligonucleotide **9** was ammonolysed to give **13** which, after removal of ammonia by evaporation, was isolated by RP HPLC (Fig. 1). Dimethoxytrityl protection was cleaved as specified above, and **14** was purified by RP HPLC (Fig. 2). The fraction collected (*cca.* 0.4 mL) was diluted with conc. ammonia (2 mL),

left at room temperature for 15 min, and evaporated to dryness. The residue was dissolved in water (1 mL), analysed by ion exchange chromatography (Fig.3) and subjected onto RP column. Pure **11b** was collected and found to be homogeneous and chromatographycally identical (Table 1) to that prepared by the method reported in literature.[10]

Diethyl 2-hydroxymethyl-2-(4,4'-dimethoxytrityloxymethyl)malonate 3. - Diethyl 2,2-bis(hydroxymethyl) malonate **2** (8.46 g, 38.4 mmol) was dried by coevaporation with pyridine (3x50 mL) and dissolved in a mixture of dry pyridine (50 mL) and dioxane (50 mL). 4,4'-Dimethoxytrityl chloride (7.80 g, 23 mmol, 0.6 eq.) was added to the stirred solution in small portions within 2h. The reaction mixture was stirred for 4h, after which the reaction was stopped by adding methanol (0.5 mL) The resulting solution was neutralised with triethylamine (3.6 mL) and evaporated *in vacuo* to an oil. The residue was dissolved in methylene dichloride (200 mL), washed with water (3x50 mL), dried over Na_2SO_4, and evaporated to dryness. The crude mixture obtained was separated on a silica gel column (40x150 mm) eluting with a gradient of methanol in CH_2Cl_2 from 0 to 5%. Collected fractions were evaporated to dryness to give 8.77g (73%) of pure **3** as a white foam. ^{1}H NMR (δ, ppm): 7.40-7.38 (m, 2H, arom); 7.31-7.18 (m, 7H, arom); 6.84-6.80 (m, 4H, arom); 4.22 (sx, 2H, J^2_{AB} = 10.7 Hz, $J^3_{H^AMe}$ = 7.1 Hz, CH_3-C***H***^{A}H^{B}O-C=O); 4.17 (sx, 2H, J^2_{AB} = 10.7 Hz, $J^3_{H^BMe}$ = 7.1 Hz, CH_3-CH^A***H***^{B}O-C=O); 4.13 (br. s., 2H, -C***H***$_2$OH); 3.77 (s., 6H, 2xC***H***$_3$O); 3.63 (s., 2H, -C***H***$_2$ODMTr); 1.22 (tr., 6H, J^3 = 7.1 Hz 2xC***H***$_3$). IR (λ, cm^{-1}): 3535 (νOH); 1732 (νC=O); 1251 (νC-O-C); 1608, 1582, 1509 (arom.). Anal. Found: C, 68.73; H, 6.50%. Calcd. for $C_{30}H_{34}O_8$: C, 68.95; H, 6.56%.

(2-Cyanoethyl) [2,2-bis(ethoxycarbonyl)-3-(4,4'-dimethoxytrityloxy)propyl-1] N,N-diisopropyl phosphoramidite 1. - Alcohol **2** (650 mg, 1.25 mmol), predried by coevaporation with MeCN, and (2-cyanoethyl) *N,N,N',N'*-tetraisopropylphosphorodiamidite[17] (563 mg, 1.87 mmol) were dissolved in dry MeCN (1.5 mL). 1*H*-Tetrazole (2.92 mL of 0.45 M solution in dry MeCN; 1.31 mmol) was added, and the mixture was stirred for 20 min in dry atmosphere at ambient temperature. Aqueous $NaHCO_3$ (5%; 25 mL) was added, and the mixture was extracted with methylene dichloride (100 mL). Extract was washed with saturated NaCl, dried over Na_2SO_4, and evaporated to a white foam. It was dissolved in toluene (3 mL) and added dropwise to dry hexane (75 mL) at room temperature. To remove solid impurities, the solution was filtered, the filtrate obtained was kept for 2h at -25°C in dry atmosphere. The solvent was decanted from the product precipitated as a colourless oil. A fresh portion of hexane (50 mL) was added to the precipitate, the mixture was shaken, and left at room temperature to settle down. The solvent was decanted, and the residue was dried *in vacuo* to give **1** (850 mg, 94 %) as a colourless oil. ^{31}P NMR (δ, ppm): 148.3. T.l.c. (Kieselgel 60 F_{254}, MeOH-CH_2Cl_2, 1:49): R_f 0.7.

Thymidine 5'-[3-(4,4'-dimethoxytrityloxy)-2,2-di(ethoxycarbonyl)propyl-1]phosphate (5).

Method A. Phosphoramidite **1** (1.0 M in MeCN) was coupled to thymidine derivatized controlled pore glass in a 10 μmol scale. After the completeness of the synthesis the solid support **4** was dried and treated with conc. aq. NH_3 for 2 days at room temperature. The liquid phase was evaporated to dryness *in vacuo*, the residue was dissolved in 30% aq. MeOH and separated by semipreparative RP HPLC. The fraction containing the product was evaporated to an oil, and the residue was desalted on the same column.. The solvent was evaporated, the residue was coevaporated with MeCN (3×5 mL) and, finally, dried *in vacuo* to give 10.2 mg (80%) of **5**

(sodium salt) as a white powder. ^{1}H NMR (DMSO-d_6): 7.94, 1H, d. J^4_{HMe} = 1.0 Hz (H-6); 7.27-7.37, 4H, m.; 7.17-7.24, 5H, m.; 6.92-6.85, 4H, m. (arom.); 6.22, 1H, dd., J^3 = 5.8, 7.8 Hz (H-1'); 4.35-4.30, 1H, m., J^2 = 9.8 Hz, J^3 = 2.9 Hz (H-5'); 4.30-4.25, 2H, m. (H-5" and H-3'); 4.01, 4H, q. J^3 = 6.9 Hz, (2×C***H***$_2$CH$_3$); 3.83, 1H, m. (H-4'); 3.73, 6H, s. (2×CH$_3$O); 3.65, 2H, m. (CH$_2$-O-P); 3.48, 1H, d., J^2_{AB} = 8.8 Hz (C***H***$_A$H$_B$-ODMTr); 3.44, 1H, d., J^2_{AB} = 8.8 Hz (CH$_A$***H***$_B$-ODMTr); 2.12, 1H, m., and 2.02, 1H, m., $J^2_{2',2''}$ = 13.2 Hz (H-2' and H-2"); 1.84, 3H, d., J^4_{MeH} = 1.0 Hz (C^5-CH$_3$); 1.06, t., J^3 = 7.1 Hz, and 1.05, t., J^3 = 7.0 Hz, totally 6H, (2×CH$_2$C***H***$_3$).

Method B. The solid support **4** (25 μmol) was prepared analogously to the method A and treated with 40% aq. methylamine (25 mL) for 2h at room temperature. The reaction mixture was worked up, and the product was isolated as described above to give 18.3 mg (86.2%) of **5** as a white powder which was identical to that prepared by method A as evidenced by ^{1}H NMR and analytical RP HPLC.

Method C. Deprotection of the solid support **4** (0.2 μmol) with 50% aq. 1,3-propanediamine (75 μL) for 12 h at room temperature followed by evaporation of the liquid phase gave the single product, **5**, which coeluted with an authentic sample prepared by method A when coinjected on analytical RP HPLC.

Thymidine 5'-[3-hydroxy-2,2-di(ethoxycarbonyl)propyl-1]phosphate (6). Compound **5** (23.7 mg, 28 μmol) was treated with 80% aq. AcOH (25 mL) for 30 min at room temperature. The reaction mixture was evaporated *in vacuo*, coevaporated with water (3×5 mL), and partitioned between water (5 mL) and CH_2Cl_2 (15 mL). The organic layer was discarded, and the aqueous phase was extracted with CH_2Cl_2 (3×5 mL). At this step, the aqueous phase contained a detritylated product in >97% purity along with contaminating 4,4'-dimethoxytrityl alcohol. Accordingly, the homogeneous product was isolated by semipreparative RP HPLC within the desalting protocol.. Evaporation and final drying *in vacuo* gave sodium salt of **6** (13.9 mg, 91%) as a white powder. ^{1}H NMR (DMSO-d_6): 8.31, 1H, s. (NH); 7.81, 1H, d., J^4_{HMe} = 1.0 Hz, (H-6); 6.22, 1H, dd., $J^3_{1'2'}$ = 6.1, $J^3_{1'2''}$ = 7.8 Hz (H-1'); 4.28, 1H, m. (H-3') 4.19-4.06, m., J^2 = 9.8 Hz, J^3 = 2.9 Hz (H-5', H5"), 4.10, q., J^3 = 7.1 Hz and 4.09 q., J^3 = 7.1 Hz (2×C***H***$_2$CH$_3$) totally 6H; 3.88, 1H, d., and 3.86, 1H, d., J^2_{AB} = 12.1 Hz (CH^{A}H^{B}OH); 3.79 1H, m. (H-4'); 3.65, 2H, m. (CH$_2$-O-P); 2.11, 1H, m., and 2.04, 1H, m., $J^2_{2',2''}$ = 13.3 Hz, $J^3_{2'1'}$ = 7.8 Hz, $J^3_{2'3'}$ = 5.5 Hz, $J^3_{2''1'}$ = 6.1 Hz, $J^3_{2''3'}$ = 2.7 Hz, (H-2' and H-2"); 1.81, 3H, d., J^4_{MeH} = 1.0 Hz (C^5-CH$_3$); 1.15, t., J^3 = 7.1 Hz, and 1.14, t., J^3 = 7.1 Hz, totally 6H, (2×CH$_2$C***H***$_3$). ^{31}P NMR (DMSO-d_6): -2.20.

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