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Phosphorylating Reagent-Free Synthesis of 5′-Phosphate Oligonucleotides by Controlled Oxidative Degradation of Their 5′-End

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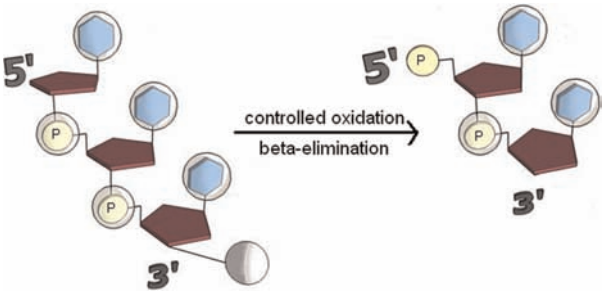
ABSTRACT



The 5′-phosphorylated oligonucleotides (5′-pONs) are currently synthesized using expensive and sensitive modified phosphoramidite reagents. In this work, a simple, cost-effective, efficient, and automatable method is presented, based on the controlled oxidation of the 5′-terminal alcohol followed by a β-elimination reaction. The latter reaction leads to the removal of the terminal 5′-nucleoside and subsequent formation of the 5′-phosphate moiety. Thus, chemical phosphorylation of oligonucleotides (DNA or RNA) is achieved without using modified phosphoramidites.

Oligonucleotides containing a 5′-terminal phosphate group have found applications in several domains, ranging from PCR processes,[1] gene construction,[2] cloning,[3] mutagenesis,[4] to conjugation reactions.[5] The small interfering RNAs (siRNAs), which have demonstrated great promise in therapeutics for selective inhibition of gene expression are short 5′-pONs.[6] The 5′-pONs are thus an important part of the biologist′s toolbox. Such oligonucleotides are routinely prepared by automated synthesis by using on-support reactions with a modified phosphoramidite reagent. The importance of this modification is highlighted by the impressive number of reagents devoted to chemical phosphorylation that have been developed,[7–9] and some of them are also commercially available.[8–10]

However, despite this wide choice, these reagents are closely related since they all belong to the phosphoramidite family. Hence, they share common disadvantages: poor

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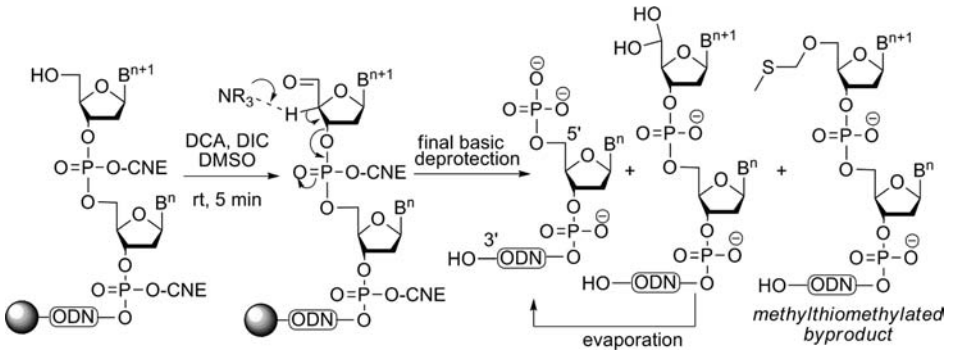
Scheme 1. Reaction Pathway[a,b]



[a] CNE = cyanoethyl; gray balls refer to controlled pore glass.
[b] The supported methylthiomethylated ONs linked to the CPG support are not shown for clarity.

stability upon dilution and production by multistep synthesis. Commercialy available reagents are expensive, and the incorporation of a phosphate group at the 5′ end of a 20-mer DNA is as expensive as the synthesis of the 20-mer moiety itself (see the Supporting Information). Considering the biological importance of 5′-pONs, there is an urgent need to develop cost-effective phosphorylation methods in order to expand their use in laboratories.

It has been shown that drugs, such as neocarzinostatin[11] and esperamicin,[12] or artificial nucleases[13] are able to cleave DNA, affording a single strand DNA terminated by a 5′-phosphate group. Further insights into the molecular mechanism reveal that the release of the 5′-pON is achieved through the oxidation of the 5′-carbon to aldehyde followed by a β-elimination reaction.[14]

Consequently, it could be envisioned that the oxidation of the 5′ primary alcohol into aldehyde in the supported oligonucleotidic chain could be an efficient and cheap method to generate 5′-pON without the use of modified phosphoramidite. Other groups have previously reported the formation of 5′-phosphate group using β-elimination reaction from an aldehydic ON, but these methods still required a modified amidite reagent and an extra step before the final basic cleavage.[15]

In this work, we report a facile method to oxidize the 5′-hydroxyl group on support-bound ONs affording the corresponding 5′-aldehyde. Subsequent treatment in basic conditions leads to the removal of the last nucleoside through a β-elimination mechanism and formation of the 5′-pON. Most interestingly, the method is fully compatible with automated DNA and RNA synthesis.

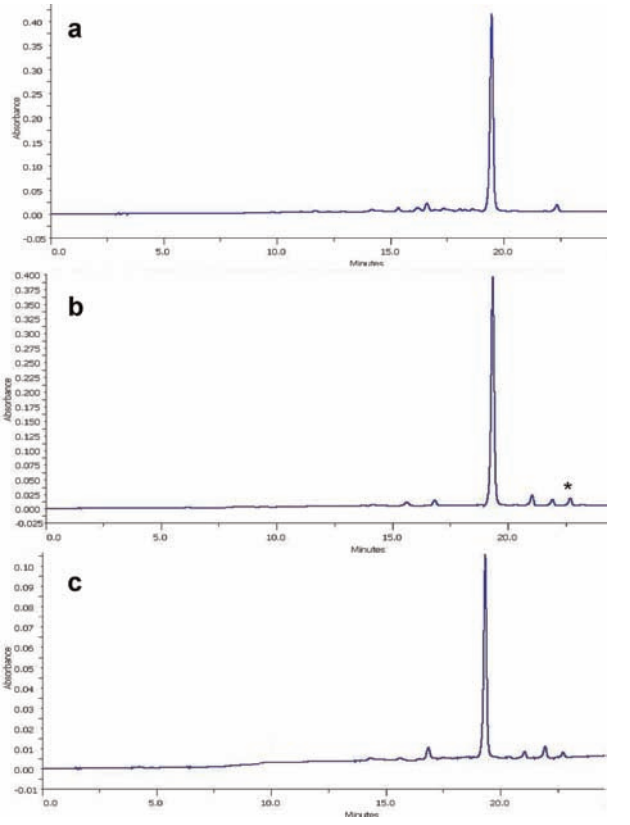


Figure 1. RP-HPLC chromatogrammes of crude reaction mixture of $^{5'}pT_3$ obtained using (a) commercialy phosphorylation reagent, (b) the present method, and (c) coinjection of both mixtures. Methylthiomethylated adduct is indicated by an asterisk (vide infra).

A large number of reagents have been reported for oxidizing alcohol to aldehyde. Preliminary assays using hypervalent iodine reagent such as IBX or Dess–Martin periodinane gave unacceptable amounts of byproducts. We then turned toward Moffat's reagent, which consists of

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Table 1. Phosphorylation Yields for DNA Oligonucleotides (Entries 1−15) and RNA Oligonucleotides (Entries 16−21)

		ESI-MS data (MW)[b]		
entry	synthesized ON[a]	expected	observed	yield (%)[c]
1	(T) pT GTT	1259.2	1258.7	82
2	(A) pTT TTT TTT TTT	3362.5	3362.8	80
3	(T) pTT TTT TTT TTT	3362.5	3363.0	84
4	(G) pTT TTT TTT TTT	3362.5	3363.0	80
5	(C) pTT TTT TTT TTT	3362.5	3362.9	81
6	(T) pTCT CCT TCC CT	3272.5	3272.9	77
7	(C) pTCT CCT TCC CT	3272.5	3272.9	82
8	(T) pATT TAC TAA AT	3392.6	3392.9	83
9	(T) pGAC GAT CGT TA	3434.6	3435.2	72
10	(T) pCCT CTC TTT CTC TCT TTC	5370.8	5372.2	85
11	(T) pCCA TAT CCA ATT CAC ATA CTC	6346.1	6347.3	87
12	(T) pCAG CTA GAC CAT GCA	4623.8	4625.0	72
13	(T) pCAT ACA TGA ACA TAC ACT A	5826.0	5827.0	80
14	(T) pCGA CAT CGA CAT CGC A	4912.8	4914.0	76
15	(T) pTCA GAT ACT TAG CAT GGA CA A CA	7117.2	7119.4	69
16[d]	(U) pUU UUU UUU UUU	3384.3	3384.5	81
17[d]	(C) pUU UUU UUU UUU	3384.3	3384.5	79
18[d]	(U) pUCU CCU UUC CUC UCU UUC U	5824.6	5848.5[e]	82
19[d]	(U) pGUG UGU GC	2621.3	2623.1	72
20[d]	(C) pAUA CAU CCA AUU	3802.5	3804.9	89
21[d]	(U) pGGU UAG UUG UGG UA	4582.5	4584.0	60

[a] Base in parentheses refers to the oxidized 5′-nucleobase of the starting ODN released during reaction. [b] Obtained after deconvolution. [c] Determined from RP-HPLC profiles of the crude reaction mixture. [d] Oligoribonucleotides. [e] Only Na^+ adducts were observed.

a mixture of a carbodiimide derivatives and an acid in DMSO. It has been successfully used for the synthesis of 5′-aldehydic nucleosides.[16] Preliminary experiments revealed that for handling purposes, diisopropylcarbodiimide (DIC) and dichloroacetic acid (DCA) were the best candidates.

The tetrathymidylate bound to CPG-solid support was chosen for the model reaction because the expected $^{5'}pT_3$ product is easily resolved by RP-HPLC from the parent T_4. The reaction mixture was neither stirred nor heated to ensure a future technological transfer toward automated DNA synthesizer. Different reaction conditions (i.e., different ratios and concentrations of DIC and DCA reagents and reaction times) were studied. It was found that the best DIC/DCA ratio is 6:1 (concentrations in reactant of 0.6 and 0.1 M for DIC and DCA, respectively). Use of higher concentrations did not enhance the yields (see the Supporting Information). We observed a ca. 90% yield for conversion to $^{5'}pT_3$ in less than 30 min at rt. Moreover, the putative aldehyde intermediate (Scheme 1) could be trapped by reacting it with an oxyamine derivative (see the Supporting Information). It was also observed that heating of the reaction mixture to 45 °C leads to a decrease in reaction yield.

The β-elimination reaction was concomitant to the final deprotection step of the support-bound ONs and gave identical results irrespective of conditions used (i.e., 30% NH_4OH, MA (40% aq $MeNH_2$), 50 mM K_2CO_3 in MeOH). Evaporation to dryness was found necessary to achieve complete conversion of the hydrated aldehyde to 5′-pON (Scheme 1 and the Supporting Information).

The structure of the resulting product was confirmed by satisfactory ^{31}P NMR and mass data (see the Supporting Information). In particular, the ^{31}P NMR spectrum showed the appearance of a new peak at 3.83 ppm attributed to the phosphomonoester group in addition of the peak corresponding to the phosphodiester bonds at −1.0 ppm.[17] The HPLC profile of a coinjection of $^{5'}pT_3$ synthesized using commercialy available phosphoramidite[9,10c] and the present method showed a single peak (see Figure 1), thus suggesting that the two products are identical.

Whatever the sequence and the conditions used, a small amount (2−5%) of 5′-methylthiomethylated product (Scheme 1) was also isolated[18] (see the Supporting Information). This byproduct was obtained by reaction between the 5′-alcohol of the CPG-bound ON and the $[CH_3—S{=}CH_2]^+$ cation formed upon elimination from the DCI adduct of DMSO.[16a] It should be noted that this unavoidable impurity could interfere with some applications.

In order to assess the chemospecificity of this reaction, the possibility of nucleobase degradation was evaluated. Purines,[19] and particulary contiguous guanines,[20] are

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especially prone to oxidation. Various ONs containing the four different nucleobases as well as guanine tracks were prepared, subjected to the aforementionned oxidation conditions, deprotected, purified, and subjected to enzymatic digestion by alkaline phosphatase and nuclease P1 enzymes. RP-HPLC profiles of the resulting mixture were identical to those obtained from nonoxidized ONs (see the Supporting Information), thus suggesting the absence of nucleobase degradation.

The method reported therein was then adapted for automated ONs synthesizer. The results were initially poorly reproducible because of the high viscosity of DMSO solutions, which prevented regular flow through the fine tubing of the synthesizer. This was resolved by adding 20% acetonitrile to DMSO. DIC and DCA solutions were found stable for weeks upon dilution in DMSO. Bottles were clipped on unused amidite positions, and reagents were mixed in the main valve block by simultaneous argon positive pressure (see the Supporting Information for modified script). Several 5′-phosphorylated DNA and RNA sequences were then synthesized to validate the method developed in this work (see Table 1). The nature of the released 5′-base was found to have no influence on reaction yields (Table 1, consider entries 2−5, entries 6−7, and entries 16−17), and for practical reasons, T (or U) was used as final nucleobase. Yields were determined from crude reaction mixture by UV monitoring of RP-HPLC analysis. They are consistently good for short sequences (ca. <15 bases). For some longer sequences (see entries 14, 15, and 21), slighty lower yields were obtained.

The present method uses particulary cheap and stable reagents contrary to the commercialy available phosphoramidites. It is fully automatable and does not need an extra step before nor after final basic deprotection. Therefore, we believe this method could be valuable in particular for high throughput synthesis of 5′-pONs or for their synthesis in bulk quantities.[21]

With the exception of Kool's work on direct ON iodination,[22] little work has been done on direct functionalization of ONs. Modification of ONs at their 5′-end is almost always achieved by use of the modified phosphoramidite. We hope our work will constitute a new approach for the production of modified ODN. Notably, we are currently working on the use of the aldehydic intermediate as a versatile key compound toward other modified ONs.

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Supporting Information Available. Enlarged version of Figure 1. Reaction yield optimization experiments (reagent concentrations and reaction time). Evidence for aldehydic intermediate and methylthiomethyl byproduct formation. Enzymatic degradation experiments. Estimation of costs for the two methods. Modified script for ABI3400 synthesizer. ESI-MS and RP-HPLC analysis for phosphorylated ONs. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.