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An Improved Method for the Synthesis of N3'→P5' Phosphoramidate Oligonucleotides

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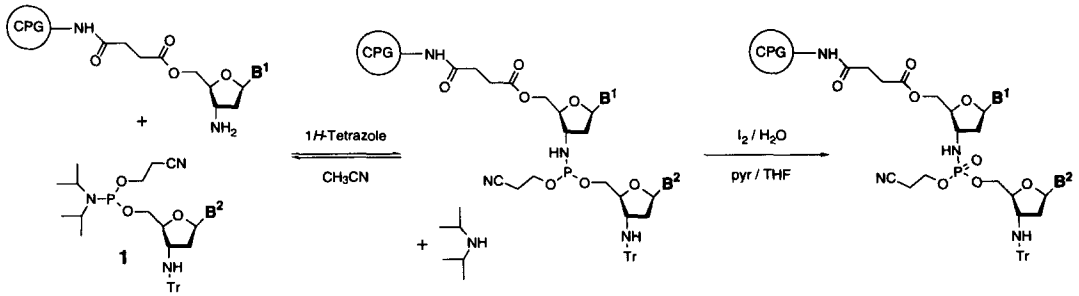
Abstract: A new method for the synthesis of N3'→P5' phosphoramidate oligonucleotides is demonstrated. This phosphoramidite amine-exchange method generates cycle yields in the 92-95% range, and provides overall yields that are 3-6 times higher than the previously published oxidative phosphorylation method. Copyright © 1996 Elsevier Science Ltd

Oligonucleotides are promising drug candidates in the fields of antisense and antigene therapies and may also play a crucial role as diagnostic and analytical agents.[1] The synthesis and characterization of oligonucleotide N3'→P5' phosphoramidates, wherein the 3'-oxygen of the 2'-deoxyribose is replaced by a 3'-amine, was recently described.[2,3] These analogs form very stable, sequence-specific complexes with single-stranded DNA, RNA, and double-stranded DNA, and may prove to be potent therapeutic and diagnostic agents.[2-5]

We report here a new, automated solid-phase method for the synthesis of N3'→P5' phosphoramidate oligonucleotides based on a phosphoramidite amine-exchange reaction.[6,7] The key step in this method is the exchange of a solid support-bound 3'-amino (oligo)nucleotide for the diisopropylamino group of the 5'-(*N*,*N*-diisopropylamino 2-cyanoethyl) phosphoramidite-3'-(trityl)amino-2',3'-dideoxynucleoside monomer, **1**. The resulting internucleotide phosphoramidite is then oxidized to the stable protected phosphoramidate (Scheme 1).

Scheme 1. Phosphoramidite amine-exchange reaction.



The synthesis cycle, which builds the oligomer in the 5' to 3' direction, is outlined in Scheme 2. The cycle begins with detritylation of the 3'-(trityl)amino nucleoside bound to aminopropyl-CPG

via a 5'-succinyl linker (1 μmol). The trityl group is used for protection of the 3'-amine because it is stable to the weakly acidic coupling solution, as well as the oxidation reagent, yet is completely removed by a 60 second flow of 3% dichloroacetic acid in CH_2Cl_2. After thoroughly washing the support-bound (oligo)nucleotide with acetonitrile (6 x 10 sec), the resulting free amine is coupled to the next base using an alternating flow of 0.5 M 1*H*-tetrazole in acetonitrile (200 equivs.) and 0.1 M 5'-phosphoramidite, **1** (15 equivs.; A^{Bz}, C^{Bz}, G^{Ibu}, T), in acetonitrile[8] followed by a 5 minute wait. The internucleotide phosphoramidite is immediately oxidized with a solution of 0.1 M iodine in 2% water/20% pyridine/THF (0.8 mL) for 2 minutes. The support-bound oligonucleotide is thoroughly washed with acetonitrile (6 x 10 sec) and the coupling and oxidation steps are repeated (double couple/ox). In accordance with equilibrium theory for an amine-exchange reaction, this couple-oxidize-couple-oxidize approach is more efficient than a single coupling, even when using twice the equivalents of monomer for the single coupling. Presumably, the first oxidation "locks in" the equilibrium concentration of desired phosphoramidite from the first coupling as the stable phosphoramidate and then any unreacted amino groups can engage in a new equilibration with monomer during the second coupling.

Scheme 2. Phosphoramidite amine-exchange cycle for the synthesis of N3'→P5' phosphoramidate oligonucleotides.

1) 3% Cl_2CHCO_2H in CH_2Cl_2 (60 sec), then CH_3CN wash (6 x 10 sec)
2) Monomer + 1*H*-Tetrazole in CH_3CN (5 min)
3) I_2, H_2O oxidation (2 min), then CH_3CN wash (6 x 10 sec)
4) Monomer + 1*H*-Tetrazole in CH_3CN (5 min)
5) I_2, H_2O oxidation (2 min), then CH_3CN wash (6 x 10 sec)
6) Repetition of steps 1-5
7) 3% Cl_2CHCO_2H in CH_2Cl_2 (60 sec), then CH_3CN wash (6 x 10 sec)
8) Concentrated aqueous NH_3 (55°C for 8-12 h)

The synthesis cycle is repeated until the desired sequence is fully assembled, then the resulting oligonucleotide is cleaved from the support and deprotected in concentrated aqueous ammonia (1 mL) at 55 °C for 8-12 hours. The N3'→P5' phosphoramidate oligonucleotides are generally synthesized "trityl-off" because ammoniolytic removal of the cyanoethyl protecting groups renders the phosphoramidate oligomer relatively unstable to the acidic detritylation conditions.[7] We continue to investigate alternative strategies to enable hydrophobic purification of the assembled phosphoramidate oligomers. These oligonucleotides can be synthesized with either a terminal 3'-amino group by using the described monomers, **1**, or a terminal 3'-hydroxyl group by using commercially available 5'-(*N*,*N*-diisopropylamino 2-cyanoethyl) phosphoramidite-3'-*O*-dimethoxytrityl 2'-deoxynucleosides for the final cycle.[9] Chimeric phosphodiester- or phosphorothioate-N3'→P5' phosphoramidate oligonucleotides can also be synthesized by choosing the appropriate monomer and oxidation or sulfurization, respectively.

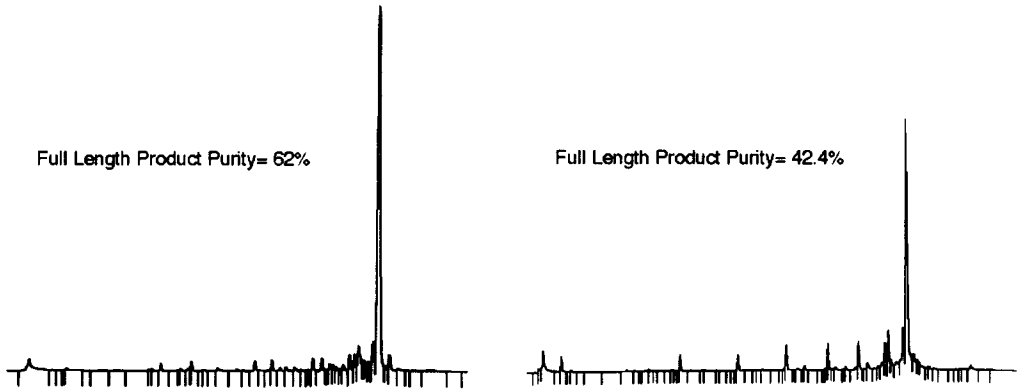
Due to the slight nucleophilicity of the terminal 3'-(trityl)amine of the growing oligonucleotide chain and despite the trityl group's steric hindrance, acetic anhydride/*N*-methylimidazole capping has proven to be problematic. Coupling yields as determined by measurement of released trityl cation can therefore only be approximated, but actual cycle yields appear to be in the 92-95% range based on crude ODU's isolated and anion exchange HPLC purity. Table 1 gives some representative examples of phosphoramidate sequences and yields using the new phosphoramidite amine-exchange method with some comparisons to results achieved with the previously reported oxidative phosphorylation method.[3]

Table 1. Comparison of sequences and yields of N3'→P5' phosphoramidate oligonucleotides using either the phosphoramidite amine-exchange method or the oxidative phosphorylation method.

Sequence	A_{260} units	Product Purity[a]	Method
5'-AAA-AAA-AAA-AAA-AAA-3'[b]	100.8	39.25%	Amine-Exchange
5'-AAA-AAA-AAA-AAA-AAA-3'[c]	85.5	8.2%	Oxidative phosphorylation
5'-TTT-TTT-TTT-TTT-TTT-3'[b]	74	56%	Amine-Exchange
5'-TTT-TTT-TTT-TTT-TTT-3'[c]	48.8	28.5%	Oxidative phosphorylation
5'-AAC-GTT-GAG-GGG-CAT-3'[b]	100	37.8%	Amine-Exchange
5'-AAC-GAG-TTG-GGG-CAT-3'[b]	96	42.4%	Amine-Exchange
5'-TTC-TCT-CTC-TA-3'[b]	70.8	62%	Amine-Exchange

(a) Product purity is the area-% of the main peak measured by anion-exchange chromatography. (b) Terminal 3'-amine. (c) Terminal 3'-hydroxyl.

Figure 1: Anion-exchange chromatograms of crude N3'→P5' phosphoramidate oligonucleotides synthesized by the phosphoramidite amine-exchange method.
(a) 5'-TTCTCTCTCTA-3'-NH_2 (b) 5'-AACGAGTTGGGGCAT-3'-NH_2



Anion exchange chromatography was performed on a Dionex PA-100 column (4 x 250 mm) using a gradient of 0 to 50% B over 40 min at 1 mL/min. Buffer A = 0.01 M NaOH/0.01 M NaCl/H_2O; Buffer B = 0.01 M NaOH/1.5 M NaCl/H_2O.

The crude N3'→P5' phosphoramidate oligonucleotides are purified by anion-exchange

chromatography[10] and the fractions are analyzed (anion-exchange chromatography), pooled, and concentrated to about one-third the original volume. After precipitating the product two times from 1 M aqueous NaCl by addition of three volumes of ethanol, the oligonucleotide is dissolved in water, quantified at 260 nm, and analyzed by anion-exchange chromatography and capillary electrophoresis. The anion-exchange chromatograms of two crude N3'→P5' phosphoramidate oligonucleotides synthesized by the new method are shown in Figure 1.

The phosphoramidite amine-exchange method was used to synthesize a wide variety of oligonucleotide sequences on the research scale and proved to be both reliable and reproducible. The yields were consistently higher than those obtained from the previously reported oxidative phosphorylation method. We intend to report soon a full paper on this method, as well as a more efficient and economical version of this method for both research and large scale N3'→P5' phosphoramidate oligonucleotide syntheses. The large scale method utilizes significantly less equivalents of 5'-phosphoramidite monomer.

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References and Notes

1. Uhlman, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 544-584.
2. Gryaznov, S.; Chen, J.-K. *J. Am. Chem. Soc.* **1994**, *116*, 3143-3144.
3. Chen, J.-K.; Schultz, R. G.; Lloyd, D. H.; Gryaznov, S. M. *Nucleic Acids Res.* **1995**, *23*, 2661-2668.
4. Gryaznov, S. M.; Lloyd, D. H.; Chen, J.-K.; Schultz, R. G.; DeDionisio, L. A.; Ratmeyer, L.; Wilson, W. D. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 5798-5802.
5. Gryaznov, S.; Skorski, T.; Cucco, C.; Nieborwska-Skorska, M.; Chiu, C. Y.; Lloyd, D.; Chen, J.-K.; Koziolkiewicz, M.; Calabretta, B. *Nucleic Acids Res.* **1996**, *24*, 1508-1514.
6. The phosphoramidite amine-exchange method has previously been used to form from 1 to 3 P3'→N5' phosphoramidate oligonucleotide linkages (Bannwarth, W. *Helv. Chim. Acta* **1988**, *71*, 1517-1527 and Gryaznov, S. M.; Letsinger, R. L. *Nucleic Acids Res.* **1992**, *20*, 3403-3409).
7. The phosphoramidite amine exchange approach has been recently described (Schultz, R. G.; and Gryaznov, S. M. *Nucleic Acids Res.* **1996**, *24*, 2966-2973) for the synthesis of 2'-fluoro-2'-deoxynucleotide N3'→P5' phosphoramidates in which the basicity of the 3'-amine is considerably lower than in this reported chemistry where the 2' position is unfunctionalized.
8. The 0.1 M phosphoramidite/acetonitrile solutions are stable on the synthesizer for 1 week, with the exception of G^{Ibu}, which is stable for 1 day only.
9. 5'-(*N,N*-diisopropylamino 2-cyanoethyl) phosphoramidite-3-*O*-dimethoxytrityl 2'-deoxynucleosides are available from Glen Research. A 90 s detritylation time with 3% dichloroacetic acid should be used.
10. Preparative anion exchange chromatography is performed on a Pharmacia Mono Q 10/10 column (2.6 x 10 cm) at 1 mL/min using a gradient of 20 to 50% B over 60 minutes, followed by holding at 50% B for 10 minutes. Buffer A is 0.01 M NaOH/0.01M NaCl/H_2O and Buffer B is 0.01 M NaOH/1.5 M NaCl/H_2O.

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