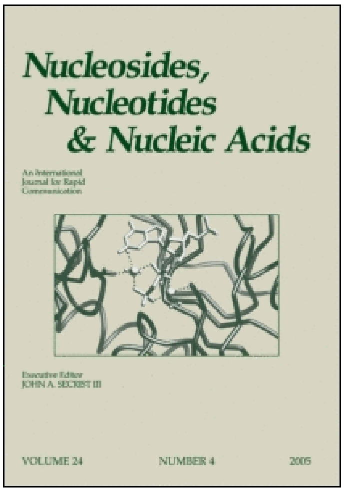

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PREPARATION OF OLIGODEOXYNUCLEOTIDE 5′-TRIPHOSPHATES USING SOLID SUPPORT APPROACH

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ABSTRACT

We report a synthetic procedure for conversion of oligonucleotides to their 5′-triphosphate derivatives with moderate yield. The oligonucleotides were synthesized on solid support using standard phosphoramidite protocols. The DMT protection group was removed and the 5′-OH was phosphitylated using 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one followed by reaction with tributyammonium pyrophosphate and iodine oxidation. After subsequent removal from support and complete deprotection, the products were isolated by anion-exchange HPLC chromatography. Structures of several 5′-triphosphate derivatives have been proven by phosphorus NMR, Mass-spectrometry and by HPLC comparison with authentic samples.

INTRODUCTION

Modified nucleoside 5′-triphosphates and their analogs are common molecular tools in biological studies and diagnostic assays. In our attempt to significantly enlarge the family of nucleoside 5′-triphosphate derivatives we have undertaken the study on synthesis of different oligodeoxyribonucleotide 5′-triphosphates. These derivatives may potentially find some applications in model biological systems and in vivo studies. In addition, they may serve as a source for preparation of γ-derivatives of oligonucleotide 5′-triphosphates (for review on the syntheses of γ-derivatives of nucleoside 5′-triphosphates, see [1]).

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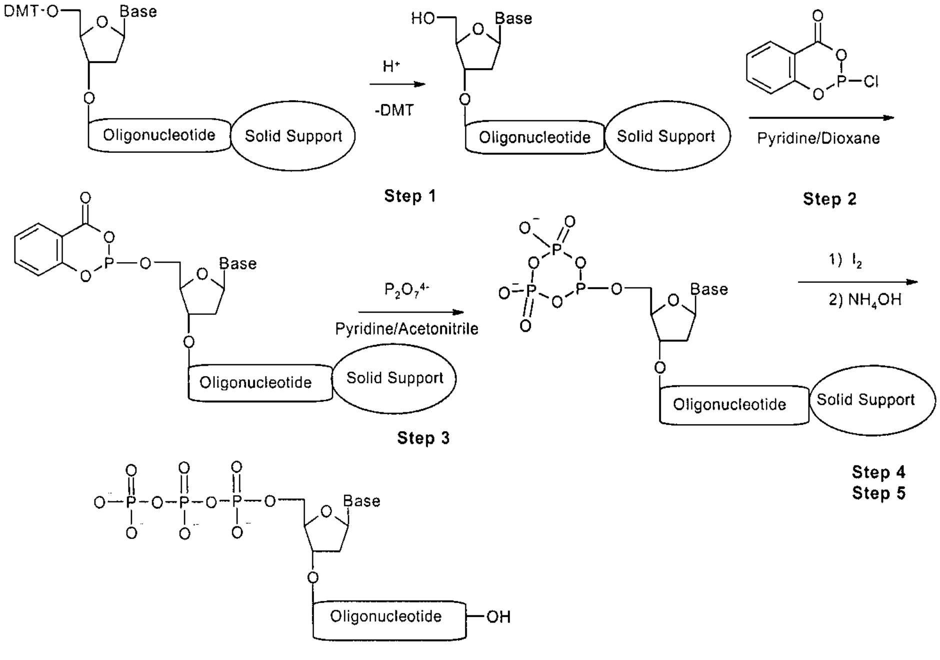
From the few known approaches for synthesis of oligonucleotide 5′-triphosphates [2–4], we have chosen trivalent phosphorus chemistry [5] on solid support [3]. If successful, this approach promises to be the most general way for the preparation of oligonucleotide 5′-triphosphates of any length, sequence or nature (DNA, RNA or their analogs).

RESULTS AND DISCUSSION

We developed a synthetic procedure allowing conversion of oligodeoxynucleotides to their 5′-triphosphate derivatives (scheme below).

First, the oligonucleotides were synthesized on solid support using standard phosphoramidite protocols. Next, the DMT protecting group was removed (step 1) and the 5′-OH was phosphitylated using 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (step 2) followed by reaction with pyrophosphate (step 3) and iodine oxidation (step 4). After subsequent removal from support and complete deprotection (step 5) the products were analyzed by anion exchange HPLC chromatography. Most of the syntheses were performed on 1 μmol of respective deoxynucleoside polymer support. However, in order to obtain a sufficient amount of oligonucleotide 5′-triphosphate for ^{31}P NMR the syntheses of pppdCpdC and pppdApdCpdTpdGpdT were made on a 15 μmol scale.

We successfully converted several short oligodeoxynucleotides and deoxynucleosides to their 5′-triphosphate derivatives. For pppdT and pppdA (prepared from



Scheme.

dT-CPG or dA-CPG, respectively) the products were identified by comparison with authentic samples using ion-exchange HPLC. For compounds pppdTpdT, pppdTpdTpdT, pppdApdCpdTpdGpdT, pppdCpdC the authentic samples were unavailable. However, HPLC analyses of these reaction mixtures showed a significant peak (or peaks) with a longer retention time than those for the respective 5′-OH oligonucleotide precursors. The 15 μmol syntheses of pppdCpdC and pppdApdCpdTpdGpdT (HPLC analyses, Fig. 1) provided a sufficient amount of material to record ^{31}P NMR spectra (Fig. 2). The signals of tripolyphosphate chains and phosphodiester bonds are clearly seen in both NMR spectra. In addition, a respective molecular ion was detected in Mass-spectrum of pppdCpdC (see experimental section). All the data obtained thus far are in agreement with proposed structures for 5′-triphosphate derivatives.

The overall isolated yields of the desired compounds are moderate and vary from experiment to experiment (∼15–30%). We believe the reason for this inconsistency is due to poor experimental reproducibility of the manual syringe technique used in our synthetic procedure. It is acknowledged in oligonucleotide synthesis that manual coupling often lacks reproducibility compared to machine-based oligonucleotide synthesis (where the optimization of the overall process is readily achievable). Several unidentified by-products are also clearly seen in the HPLC profiles. In the case of the synthesis of pppdApdCpdTpdGpdT, one of the major products has been identified as pdApdCpdTpdGpdT indicating that the pyrophosphorylation reaction (step 3) did not go to completion.

We tested the above procedure for the synthesis of longer oligonucleotide 5′-triphosphate derivatives of $d(T)_9$ and $d(T)_{15}$. The HPLC analyses of both reaction mixtures showed the presence of oligonucleotide derivatives possessing several additional ionized groups compared to precursor 9-mer or 15-mer. The longer retention times are in agreement with the presence of oligomers possessing 5′-polyphosphate chain(s). The structures of these derivatives are under investigation.

The presented data demonstrates that short oligonucleotide 5′-triphosphates can be prepared with moderate yields using phosphorus (III) chemistry on solid support. It is also clear from this work that overall chemical procedure is not fully optimized. Optimization of this multiple step process is in progress.

EXPERIMENTAL SECTION

^{31}P NMR spectra were recorded on a Bruker AM 500 MHz instrument. Mass-spectrum was obtained on a Hewlett-Packard 1100 MSD electrospray Mass-spectrometer. HPLC analyses were performed on a Beckman Gold HPLC system using a 4.6 × 250 mm Dionex DNA-Pac PA-100 column and gradient of 1M NaCl (from 0 to 50% in 40 min) in 20 mM Na-phosphate buffer (pH 7.2) at a flow rate of 1 mL/min.

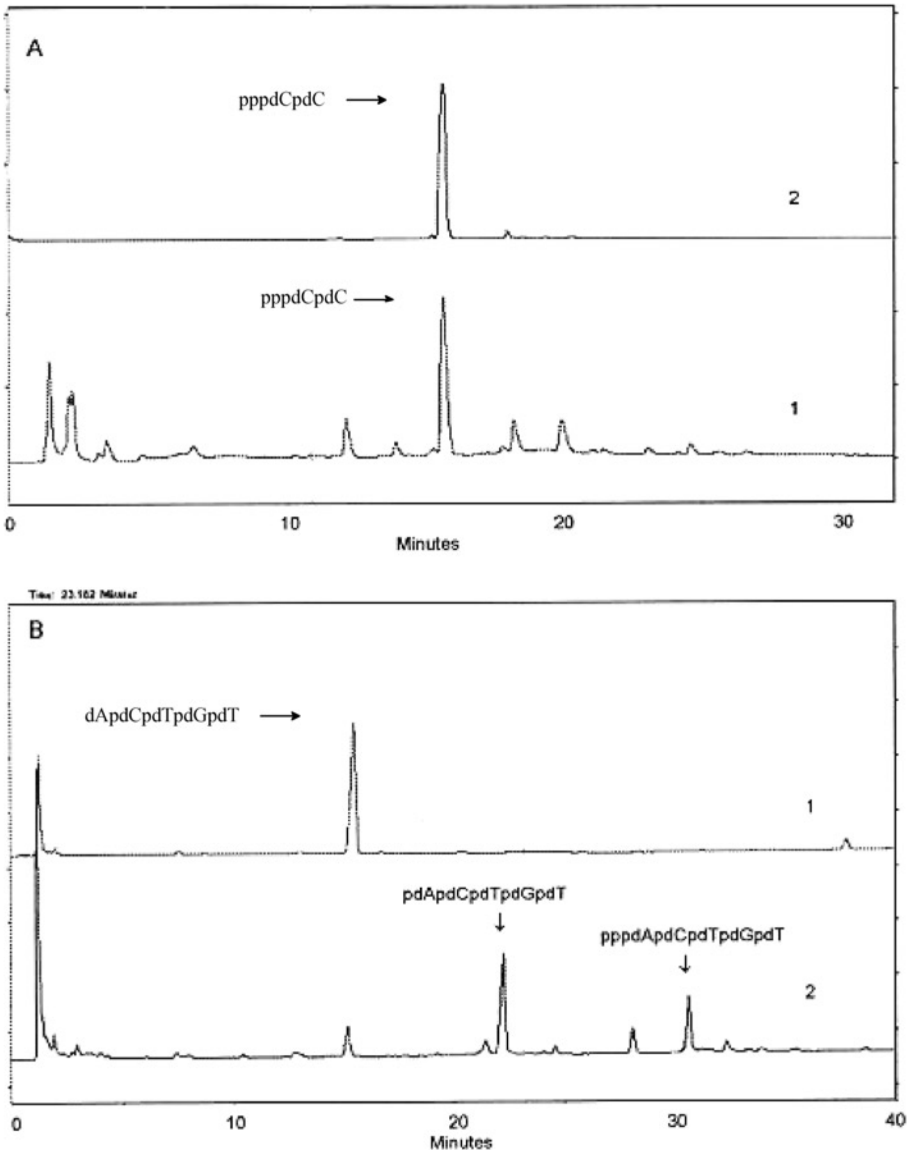


Figure 1. (A) Preparation of pppdCpdC. Anion-exchange HPLC analyses (see experimental section) of the reaction mixture after ammonia treatment (trace 1) and pppdcpdC isolated by preparative chromatography on DEAE Sephadex A-25 (trace 2). (B) Preparation of pppddApdCpdTpdGpdT. Anion-exchange HPLC analyses of precursor dApdCpdTpdGpdT (trace 1) and the reaction mixture after ammonia treatment (trace 2).

CPG solid support, phosphoramidite monomers, and oxidizer solution were purchased from Glen-Research, Inc. All other chemicals were purchased from Aldrich and were used without further purification. However the presence of water (must be <40 ppm) in all solvents was carefully controlled. Solvents were stored under Argon.

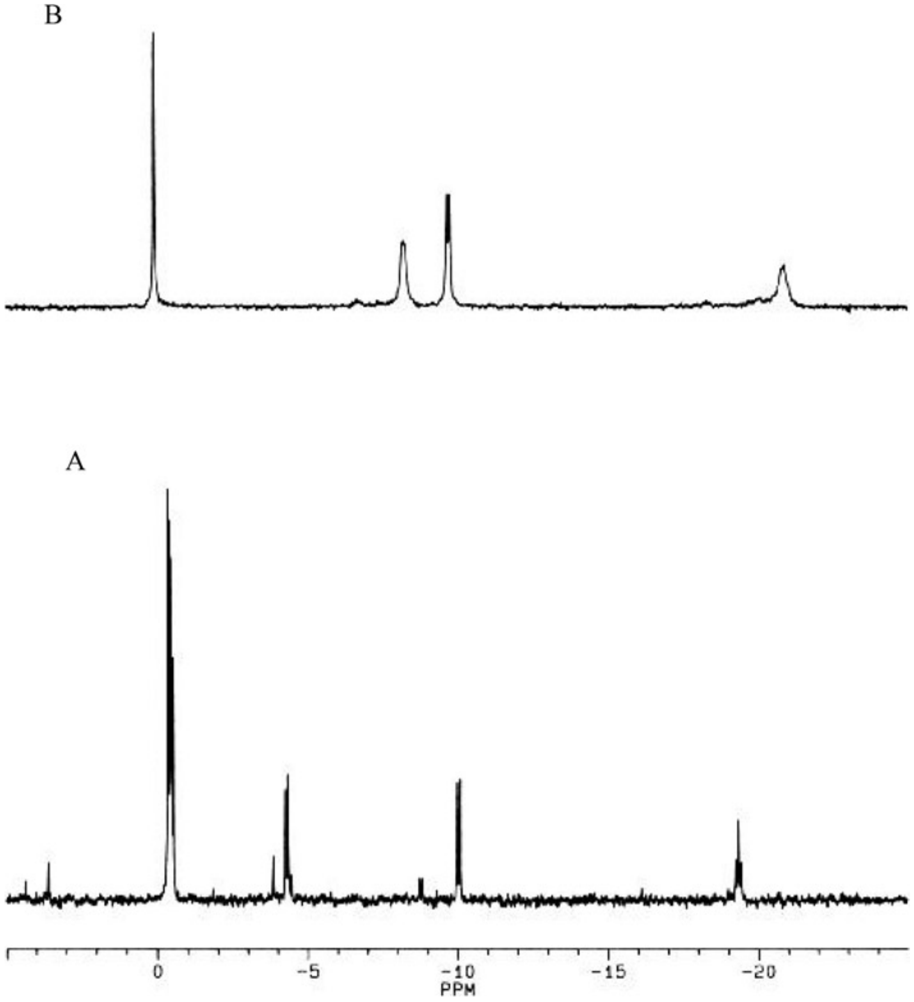


Figure 2. ^{31}P NMR spectra of isolated pppdApdCpdTpdGpdT (A) and pppdCpdC (B).

General Procedure for 5′-triphosphorylation of Nucleosides and Oligonucleotides using Manual Syringe Technique

1 umol scale synthesis

Syntheses of oligonucleotides were performed on a PerSeptive Biosystems Expedite Model 8909 synthesizer using a standard phosphoramidite protocol. The DMT group was removed at the end of oligonucleotide synthesis, the column was washed with acetonitrile and dried with Argon flow for 5 min.

The column was washed with dry dioxane (2 × 10 mL) and 1 mL of a freshly prepared 1 M solution of 2-chloro-4H-1,3,2-benzodioxa-phosphorin-4-one in dioxane:pyridine (4:1) was pushed through the column. After 2 min another 1 mL portion of the phosphyitylating solution was pushed through the column and the reaction was allowed to continue for 5 min. The last step was repeated and the reaction continued for 10 min more. The column was washed with 10 mL of dioxane, 10 mL of acetonitrile, followed by 2 mL of 0.5 M tributylammonium pyrophosphate in acetonitrile:pyridine (1:1), then with 0.5 mL portions each minute (20 times). Next, the column was washed with 10 mL of acetonitrile and 10 ml of the oxidizer solution was pushed through the column over 5 min. The column was washed with 10 mL of 70% pyridine in water, 2 × 10 mL of acetonitrile, 2 × 10 mL of dichloromethane and dried with air flow.

The polymer support was removed from the column, transferred into a 4 mL screw cap vial and treated with 1 mL of concentrated ammonium hydroxide for 1 h at room temperature (for phenoxyacetyl protected nucleosides and oligothymidylates) or 15 h at 55°C (for benzoyl and isobutyryl protected nucleosides). The solution was separated from the polymer support and the polymer support washed twice with 1 mL of concentrated ammonia. The solvent was evaporated and the recovered product analyzed by HPLC.

15 umol scale synthesis

The 15 umol scale syntheses were performed by analogy to the 1 umol procedure except that 5-fold amounts of reagents were used.

pppdT, *pppdA*, *pppdTpdT*, *pppdTpdTpdT*, *pppd*$(T)_9$ *and pppd*$(T)_{15}$: Syntheses were performed on a 1 umol scale. The reaction mixtures were analyzed by anion-exchange HPLC. (Products were not isolated).

pppdCpdC: Synthesis was performed on a 15 umol scale and the reaction mixture was separated by ion-exchange chromatography on DEAE Sephadex A-25 in a gradient of 0.8M TEAB (pH 7.5). The fractions containing pppdCdpC were combined and the TEAB removed by repetitive co-evaporation with methanol under reduced pressure. The product was re-purified by HPLC on a Pharmacia Resource Q anion exchange column (1 × 5 cm) in a gradient of 1 M LiCl in water. Fractions containing pppdCdpC were combined, evaporated, the product precipitated from acetone, washed twice with acetone and dried under high vacuum to give 5.3 mg of pppdCdpC as a lithium salt. The NMR spectrum of pppdCdpC was recorded in deuterium oxide. Mass-spectrum: m/z 755 $(M\text{-}H)^-$.

pppdApdCdpdTpdGpdT: Synthesis was performed on a 15 umol scale. The products were separated by ion-exchange chromatography on DEAE Sephadex A-25 in a gradient of 1.5 M LiCl. Fractions containing pppdApdCdpdTpdGpdT were combined, evaporated, the product precipitated from acetone, washed twice with acetone and dried under high vacuum to give 20.5 OD (at 260 mn) of the pppdApdCdpdTpdGpdT as lithium salt. The ^{31}P NMR spectrum of pppdApdCdpdTpdGpdT was recorded in deuterium oxide.

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