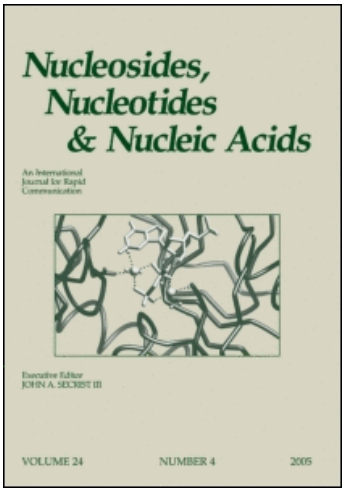

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5'-PHOSPHORYLATION OF OLIGONUCLEOTIDES WITH PHOSPHOROUS ACID IN AUTOMATED DNA SYNTHESIS

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Abstract: Coupling of phosphorous acid in automated DNA synthesis using H-phosphonate methodology leads to 5'-5' linked dimers and 5'-H-phosphonates. The yield is dependent on the phosphorous acid concentration, chain length of the oligomer, and pore size of the support. 5'-Phosphate oligomers are obtained from the H-phosphonate oligomers by silylation and oxidation.

5'-Phosphorylated oligodeoxynucleotides are required in molecular biology in ligations of DNA fragments for gene construction[1,2]. 5'-Phosphorylated oligomers can also serve as intermediates in covalent coupling of reporter groups to DNA[3,4]. The most commonly used method for the preparation of oligonucleotide-5'-phosphates is the enzymatic phosphorylation of the 5'-hydroxyl species using T4 polynucleotide kinase and ATP[5]. More convenient methods of chemical phosphorylation have recently been studied with several reagents compatible with either phosphotriester[6,7], phosphoramidite[8,9], or H-phosphonate methodologies[10]. In this report, we study the use of phosphorous acid in automated DNA synthesis as a means of preparing 5'-phosphorylated oligomers.

Results and Discussion

A solution of phosphorous acid (0.02, 0.2, 0.5 and 1.0 M) in pyridine/acetonitrile was used in the final H-phosphonate coupling cycle with pivaloyl chloride as the activating agent after the preparation of a T_{10} oligomer[11]. Following conventional oxidation and cleavage from the support, analysis of the reaction products by denaturing polyacrylamide gel electrophoresis showed the formation of two new species (see Figure 1). At 0.02 M H_2PO_3, the predominant product showed lower mobility, running at twice the molecular weight of T_{10}. At 0.2 - 0.5 M H_2PO_3, the yield of this product decreased

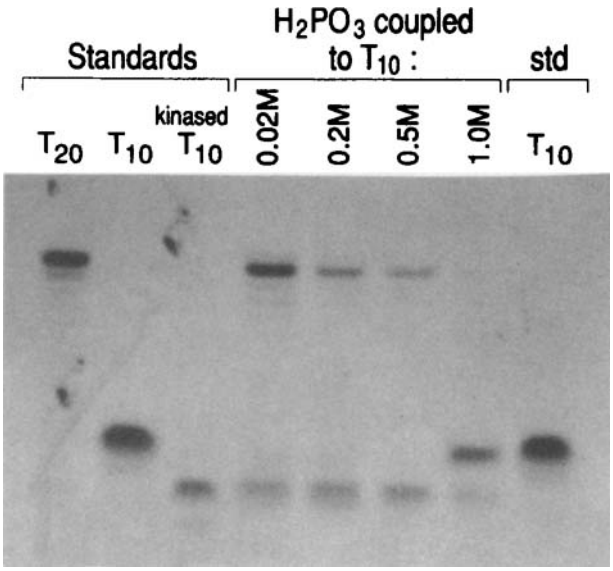


Figure 1. PAGE (20%, 7 M urea, UV shadow) of the products of the coupling of H_2PO_3 (0.02, 0.2, 0.5, and 1.0 M) to T_{10} in automated DNA synthesis

and it was replaced with a new band of slightly higher mobility than the T_{10} . At 1 M H_2PO_3, little or no reaction was observed.

Gel mobility of the upper band suggested it to be the product of a dimerization reaction between two neighboring oligomers resulting in a 5'-5'-linked T_{20}. This was confirmed by the enzymatic analysis: Treatment with polynucleotide kinase and ATP, or with alkaline phosphatase or with spleen phosphodiesterase resulted in no reaction, confirming that the compound has no free 5'-hydroxyls or 5'-phosphates. Reaction with terminal deoxynucleotidyl transferase and 3'-deoxythymidine-5'-triphosphate (ddTTP) resulted in a product which exhibited the same mobility as T_{22}, indicating that the dimer must have two 3' ends. Finally, independent synthesis of this dimeric species using the retrothymidine monomer 3'-O-DMT-5'-cyanoethyl diisopropylamino phosphoroamidite[12] resulted in the 3'-T_{10}-OPO_3-T_{10}-3' which exhibited identical electrophoretic mobility and enzymatic reactivity as the product of phosphorous acid coupling. Such 5'-5'-linked oligodeoxynucleotides have recently been investigated for their ability to form hairpin parallel duplexes[12] and suggested as duplex DNA binding agents[13]. This dimerization reaction of two oligonucleotide chains on solid support is also related to the recently

published synthesis of branched structures which contain vicinal 3'-5' and 2'-5' phosphodiester linkages[14] and occur as intermediates in RNA splicing.

Increasing the phosphorous acid concentration leads to increased formation of a second species which shows the same gel mobility as a 5'-phosphorylated T_{10}, obtained from T_{10} with polynucleotide kinase and ATP. However, the high mobility compound does not react with spleen phosphodiesterase or alkaline phosphatase or polynucleotide kinase, indicating the absence of a free 5'-hydroxyl or 5'-phosphate. This observation suggested it to be a 5'-H-phosphonate, since H-phosphonate mono esters are known to require more stringent oxidation conditions than phosphonate diesters[15]. Additional evidence for the structure of this high mobility species was provided by chemical means. As outlined in Figure 2, following coupling of phosphorous acid, reaction with either bis(trimethylsilyl) acetamide (BSA) to form the silyl phosphite[15,16], or coupling with hydroxypropionitrile to form the phosphonate diester[17], allowed isolation of the 5'-phosphate oligomer after conventional oxidation and cleavage. Both these results are consistent with intermediate formation of a terminal H-phosphonate with phosphorous acid as outlined in the reaction scheme in Figure 3.

We have also used capillary gel electrophoresis[18] for the analysis of the oligomeric reaction products. As can be seen in Figure 4, T_{10}, enzymatically 5'-phosphorylated T_{10}, and the 5'-H-phosphonate-T_{10} product of the phosphorous acid coupling were cleanly separated on a 10% polyacrylamide gel filled capillary. On the other hand, the 5'-phosphorylated T_{10} and the 5'-H-phosphonate T_{10} could not be separated by electrophoresis on a conventional slab gel. The high sensitivity and excellent resolution of the acrylamide gel capillary make it an invaluable tool for precise analyses of oligonucleotides. The 5'-H-phosphonate (ii) unexpectedly ran as a doublet indicating some inhomogeneity which we cannot as yet explain. However, direct oxidation of the H-phosphonate T_{10} (Figure 2) caused a disappearance of both peaks and formation of a single 5'-phosphorylated product peak.

The formation of the dimer on coupling with phosphorous acid occurred even as the initial chain length was increased to T_{20}, T_{40}, T_{60}, and T_{80} (see Figure 5). In each case, the upper band migrated at a size consistent with the oligodeoxynucleotide dimer and was formed in good yield even at the highest chain lengths. Separations of these products using polyacrylamide gel capillary electrophoresis have been published elsewhere[19]. The dimerization reaction appears to be only slightly dependent upon the chain length of the oligomer. The % dimer drops from 90 to 50% when the length increases from T_{10} to T_{40}. Good yields (~50%) of dimer are still formed even at chain lengths as high as T_{80}. The high yield of the T_{10}-T_{10} dimer (20 mM H_2PO_3) may indicate a high effective molarity[20] of the unreacted 5'-ends of the oligomer on the solid support. Alternatively, it may be that access of the active species is limited by slow mass transfer at the surface of the support. To examine this question further, we tried several different CPG supports.

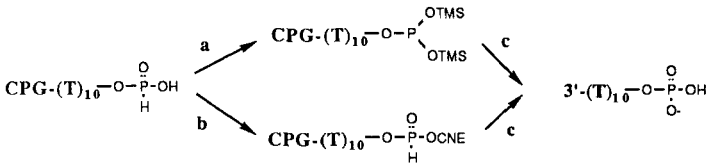


Figure 2. Reaction of the 5'-H-phosphonate to yield 5'-phosphorylated oligomer. a: 20% (v/v) BSA. b: 0.3 M $HOCH_2CH_2CN$, 0.1 M piv Cl. c: 0.2 M I_2, then NH_4OH (6 hrs, 55° C).

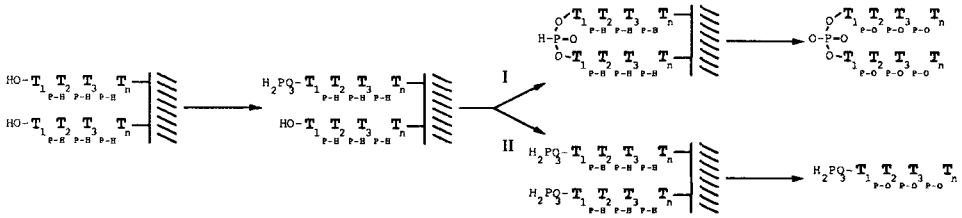


Figure 3. Reaction scheme of the coupling of H_2PO_3 in the final cycle of automated DNA synthesis showing competitive pathways leading to dimeric (I) and monomeric (II) products.

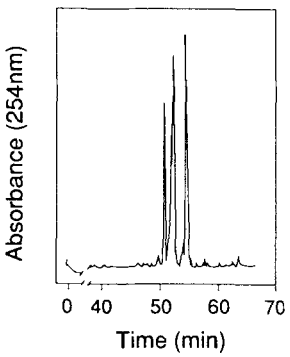


Figure 4. Electrophoretic separation using polyacrylamide gel capillary (10%, 7 M urea, 150 μm i.d. x 50 cm, 10 kV) of enzymatically 5'-phosphorylated T_{10} (i, 51 min), 5'-H-phosphonate T_{10} (ii, 53 min), and T_{10} (iii, 56 min).

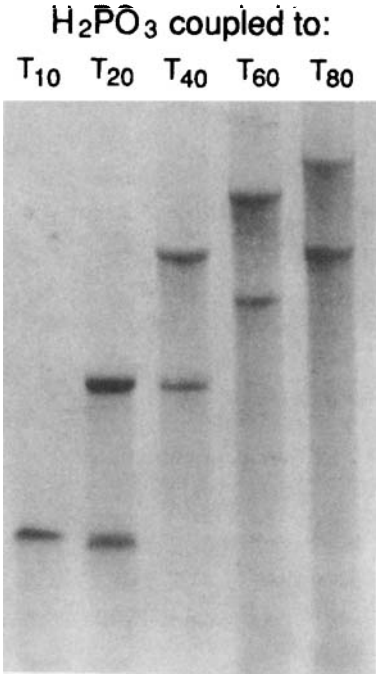
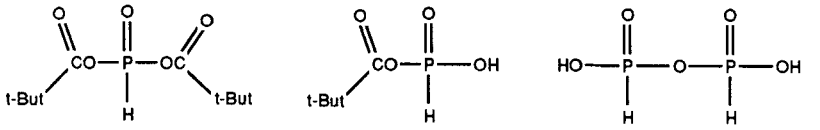


Figure 5. PAGE (15%, 7 M urea, UV shadow) of the products of the coupling of H_2PO_3 (20 mM) to support bound T_{10}, T_{20}, T_{40}, T_{60}, and T_{80}.

Testing various CPG supports (500 Å (CPG, Inc.), 1000 Å (Peninsula), and 2000 Å (Peninsula) mean pore diameters) gave similar results, indicating that the relative yield of the dimer and monomer is relatively insensitive to changes in pore diameter or surface area of the support. In theory, dimerization with phosphorous acid could be used to characterize the effects of solvent, chain length, pore size, loading, and type of support upon the reactivity and accessibility of the 5'-ends of the growing oligonucleotide chain. Steric crowding has been proposed as the cause of decreased yields of oligomers greater than 100 bases in length[21]. Also, the effects of support linkages and spacer length have been well studied[22,23]. Our observation of efficient dimerization of a T_{80} using 500 Å CPG suggests that pore filling does not inhibit coupling of adjacent oligomers. Dimerization and H_2PO_3 coupling may exhibit different steric requirements.

It is likely that the structure of the activated species[24] changes as the H_2PO_3 concentration is increased, and product formation may not simply be a function of the relative rates of Pathways I and II (Figure 3). In automated synthesis, the pivaloyl chloride concentration is fixed at 75 mM, and as higher concentrations of H_2PO_3 are mixed in the synthesizer the initial reaction products may change (compounds A, B, and C below). We propose that at 20 mM H_2PO_3, species A predominates and favors dimerization products; at 0.2-0.5 M H_2PO_3, species B is formed which yields the terminal H-phosphonate.



A B C

At 1 M H_2PO_3, unreactive compound C is the predominant product. We can only speculate as to the validity of this hypothesis.

In conclusion, we report that the reaction of phosphorous acid in H-phosphonate DNA synthesis produces a novel 5'-5' dimerization reaction in competition with formation of 5'-H-phosphonate. This dimerization occurred at chain lengths as high as 80 bases, and partitioning was dependent upon the concentration of H_2PO_3 and oligomer chain length. Results of this study present interesting questions about the possible uses of such dimerization reactions in the investigation of structures and reactivities of the active species in H-phosphonate DNA synthesis. Also, it is possible that such reactions present a new method of studying the properties of the oligomer support.

Experimental Methods

Solvents and reagents were obtained from Aldrich, Sigma, or Burdick and Jackson. Oligodeoxynucleotides were synthesized using H-phosphonate[11] (Milligen Biosearch 8600 DNA Synthesizer) or phosphoramidite[25] (Milligen 7500 DNA Synthesizer) methodologies. Reaction products were examined and purified by electrophoresis (15-20% polyacrylamide gel, 7 M urea, visualization by UV shadowing). Enzymatic reactions employing alkaline phosphatase[26], spleen phosphodiesterase[27], T4 polynucleotide kinase[28], or terminal deoxynucleotidyl transferase[29] followed published protocols. Phosphorous acid (Aldrich) was evaporated twice from anhydrous pyridine, dissolved in 50% pyridine/acetonitrile, and coupled using a conventional automated synthetic cycle[11]. Acrylamide gel capillaries (10%, 7 M urea, 150 μm x 50 cm, 10kV) were prepared and run using published procedures[18].

In the silylation/oxidation of the 5'-H-phosphonate, bis(trimethylsilyl)acetamide (100 μL, Aldrich) was mixed with 5'-H-phosphonate T_{10}-CPG suspended in pyridine/acetonitrile (0.5 mL, 10 min), then mixed with I_2 (0.5 mL, 0.2 M, THF/H_2O/CH_3CN (8:1:1), 1 hr). Coupling of hydroxypropionitrile (Aldrich) was accomplished by treating a suspension of the 5'-H-phosphonate-T_{10}-CPG with the alcohol (10 μL, 0.5 mL of 0.1 M pivaloyl chloride in pyridine/CH_3CN, 2 min). After washing (pyridine/CH_3CN, CH_3CN), the material was oxidized with I_2 as above.

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