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Deoxynucleoside H-Phosphonate Diester Intermediates in the Synthesis of Internucleotide Phosphate Analogues

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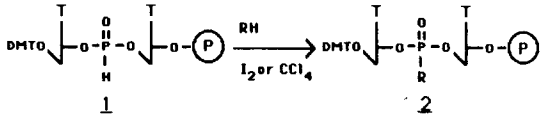
Abstract: Polymer bound deoxynucleoside H-phosphonate diesters are used as precursors to phosphoramidate, thiophosphate and phosphate triester analogues of DNA.

The synthesis of deoxyoligonucleotides (DNA) containing internucleotide phosphate analogues is becoming a field of great interest. Miller and Ts'o have shown that methyl phosphonate analogues of DNA readily pass through cell membranes and inhibit protein synthesis, presumably by interfering with mRNA translation[1,2]. Thiophosphate analogues have been used in the elucidation of certain enzyme mechanisms involving protein-nucleic acid interactions[3,4]. Phosphoramidate analogues of dinucleotides are known to bind to complementary polynucleotides and can be used for the attachment of various ligands to DNA[5]. Recently nucleoside H-phosphonates were shown to be useful in the chemical synthesis of DNA, via polynucleoside H-phosphonate diester intermediates[6,7]. Described herein is the use of polymer bound nucleoside H-phosphonate diester intermediates in the synthesis of nucleoside phosphoramidate, thiophosphate and phosphate triester analogues. Undecathymidylic acid (T_{11}) is synthesized containing from one to nine internucleotide phosphormorpholidate linkages using a rapid, simple and efficient procedure.

Oxidization of dinucleoside H-phosphonates in the presence of amines leads to the corresponding dinucleotide phosphoramidates[8,9] in high yield and the procedure appears to be general for primary and secondary amines. The dinucleotide phosphoramidates 2e and 2f (Scheme 1) were initially prepared by oxidation of the polymer bound dinucleoside H-phosphonate 1[6].

Scheme 1



1 2

RH= b) NH_3 d) n-$BuNH_2$ f) HN⟨O⟩O
a) H_2O c) $MeNH_2$ e) HN⟨⟩ g) HN⟨⟩NMe

with 0.05M I_2 in a solution of the corresponding amine (10 percent) and tetrahydrofuran (THF) for 5 min. The product amidates were removed from the solid support (50 percent conc. ammonium hydroxide (NH_4OH)/Dioxane, 8 hrs., r.t.) and evaporated. ^{31}P NMR spectra of both products show the presence of approximately equal amounts of diasteriormers[10]. The oxidation of H-phosphonate diesters to phosphoramidates is complicated by competitive hydrolysis, generating the phosphate diester 2a. Carbon tetrachloride (CCl_4) oxidation of H-phosphonate diesters to phosphoramidates[9,11] offers the advantage of low water solubility into CCl_4. All dinucleotide phosphoramidates (2b-g) were prepared with a 10 percent solution of the corresponding amine in CCl_4 (5 min)[12]. High performance liquid chromatography (HPLC) of the samples indicate a high yield of product phosphoramidate with very little competitive hydrolysis (<3 percent dithymidine phosphate diester, 2a).

The stability of the phosphoramidate linkage to conc. NH_4OH was examined for each and the results indicate the phosphoramidates 2c-g are stable to the conditions necessary for removal of the common heterocyclic N-acyl protecting groups (55°C, 5 hrs). The phosphoramidate 2b rapidly decomposes under these conditions ($t_{1/2}$ ~15 min) to a mixture of the 3' and 5' phosphoramic acid monoesters[13] (data not shown). The stability of the dinucleotide phosphoramidates (2b-g) to enzymatic degradation by exonucleases was examined using spleen phosphodiesterase and snake venom phosphordiesterase. Incubation of the dinucleotides (2a-g) with the corresponding enzyme/buffer solution at 37°C for 1 hr led to complete degradation of the phosphate diester (2a) with no detectable degradation of the phosphoramidates 2b-g (assessed via reverse phase HPLC). The observed enzymatic stability of the dinucleotide phosphoramidate 2b is contrary to the claims of Ogilvie[8] but is in agreement with those reported by Letsinger[5].

To further explore the scope of this oxidation procedure, T_{11} was prepared containing from 1 to 9 phosphormorpholidate linkages. The synthesis of polythymidine H-phosphonate on control pore glass was carried out using a standard synthetic protocol[6,7]. The strategy outlined in Scheme 2 demonstrates the synthesis of T_{11} containing nine phosphormorpholidate linkages followed by one phosphate diester linkage. This strategy was used for all T_{11} products containing 3' phosphoramidate linkages. The products were removed from the solid support with conc. NH_4OH (2 hr/r.t.) and evaporated. Polyacrylamide gel electrophoresis (PAGE) and U.V. shadowing indicated a major product band with each. The band was cut,

crushed, eluted with H_2O and isolated by reverse phase HPLC (C_{18}). The autoradiogram shown in Figure 1 (Panel A, lanes 9–0) is derived from 5'-end labeling of these products with T4 polynucleotide kinase and γ-^{32}P ATP followed by PAGE (17 percent polyacrylamide/7M urea). The change in electrophoretic mobility of the different T_{11} products is very pronounced, each additional morpholidate resulting in an increase in mass and a decrease in charge. It is interesting to note that the electrophoretic mobilities of the different T_{11} products do not follow a strict mass/charge ratio dependence. The products containing many phosphoramidate linkages (high mass/charge ratio) have greater mobility than would be predicted based upon the mobilities of the T_{11} products with fewer phosphoramidates. Treatment of each of these polythymidine phosphormorpholidates with 85 percent formic acid (95°C, 15 min) generates T_{11} containing all diester linkages (Figure 1, Panel B, lanes

3'-T-ODMT [T_1]

Repeated 5'-DMT deprotection and repeated Nucleoside H-phosphonate condensation

1 2 3 4 5 6 7 8 9

3'-T-p-T-p-T-p-T-p-T-p-T-p-T-p-T-p-T-ODMT [T_{10}]

Oxidation of the polynucleoside H-phosphonate to a polynucleotide phosphormorpholidate with CCl_4 (10 min)

1* 2* 3* 4* 5* 6* 7* 8* 9*

3'-T-p-T-p-T-p-T-p-T-p-T-p-T-p-T-p-T-ODMT [T_{10}]

5'-DMT deprotection and Nucleoside H-phosphonate condensation

1* 2* 3* 4* 5* 6* 7* 8* 9* 10

3'-T-p-T-p-T-p-T-p-T-p-T-p-T-p-T-p-T-p-T-OH [T_{11}]

5'-DMT deprotection followed by oxidation of the final product with aqueous I_2.

SCHEME 2

Synthesis of T_{11} containing nine phosphormorpholidate linkages (3' end) followed by one phosphate diester linkage (5' end).
* indicates phosphormorpholidate linkage.

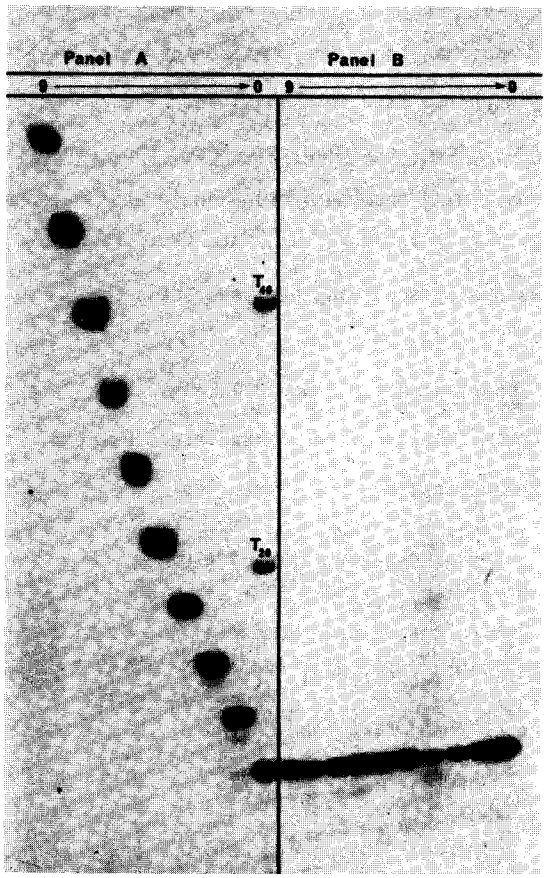


FIGURE 1. Autoradiogram derived from PAGE of T_{11} phosphormorpholidates. Lane number equals the number of phosphormorpholidate linkage

9-0). T_{40} and T_{20} standards are included in Panel A, lane 0 for comparison of electrophoretic mobility.

Nucleoside H-phosphonate diesters have been used as precursors to thiophosphate analogues[14]. This conversion is accomplished in high yield (>98 percent) directly from the polymer bound dinucleoside H-phosphonate diester (1) by treatment with a solution of 0.1M S_8 in Triethylamine (TEA)/carbon disulfide (1/9, 5 min). Triesters of oxygen are prepared by reaction of the nucleoside H-phosphonate diester (1) with a 10 percent solution of the corresponding alcohol (MeOH or n-BuOH) in N-methyl imidazole/TEA/CCl_4 (5/5/90). This reaction is very susceptible to competitive hydrolysis and care must be taken to assure anhydrous conditions.

The results presented above demonstrate that deoxynucleoside H-phosphonates are valuable precursors to a variety of internucleotide phosphate analogues. Oxidation of a polynucleoside H-phosphonate in the presence of amines leads to the corresponding polynucleotide phosphoramidate in high yield. The H-phosphonate method of DNA synthesis is a simple, fast and reagent efficient procedure that can now be applied to the rapid synthesis of DNA containing internucleotide phosphate analogues. This method should provide an easy and efficient route to a variety of internucleotide phosphate analogues of DNA containing the naturally occurring deoxynucleotides.

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References:

1) Blake, K., Murakami, A., Spitz, S., Glave, S., Reddy, M., Ts'o, P. and Miller, P., Biochemistry 24, 6139 (1985).
2) Smith, C., Aurelian, L., Reddy, M., Miller, P. and Ts'o, P., Proc. Natl. Acad. Sci. 83, 2787 (1986).
3) Potter, B. and Eckstein, F., J. Biol. Chem. 259, 14243 (1984).
4) Brody, R., Adler, S., Modrich, P., Stec, W., Leznikowski, Z. and Frey, P., Biochemistry 21, 2570 (1982).
5) Letsinger, R., Bach, S. and Eadie, J., Nucl. Acids Res. 14, 3487 (1986).
6) Froehler, B. and Matteucci, M., Tetrahedron Lett. 27, 469 (1986).
7) Froehler, B., Ng, P. and Matteucci, M., Nucl. Acids Res. 14, 4444 (1986).
8) Nemer, M. and Ogilvie, K., Tetrahedron Lett. 21, 4149 (1980).
9) Atherton, F., Openshaw, H. and Todd, A., J. Chem. Soc. 660 (1945).
10) ^{31}P NMR spectra were obtained in $CDCl_3$ and chemical shifts are reported relative to 5 percent phosphoric acid/D_2O (ext. std.); 2e δ-9.1, -8.8 ppm; 2f δ -7.9, -7.6 ppm.
11) Zwierzak, A., Synthesis 507 (1975).
12) A saturated solution of ammonia or methyl amine in Dioxane/CCl_4 (1/4) was used to prepare the corresponding phosphoramidates.
13) Tomasz, J., Nucleosides and Nucleotides 2, 51 (1983).
14) Fujii, M., Ozaki, K., Kume, A., Sekine, M. and Hata, T., Tetrahedron Lett. 26, 935 (1986).

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