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NUCLEOSIDE H-PHOSPHONATES: VALUABLE INTERMEDIATES IN THE SYNTHESIS OF DEOXYOLIGONUCLEOTIDES

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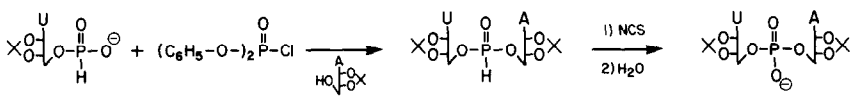
Abstract: Nucleoside H-Phosphonates are used directly in the synthesis of H-phosphonate linked deoxyoligonucleotides. A rapid and simplified procedure for the synthesis of deoxyoligonucleotides is described.

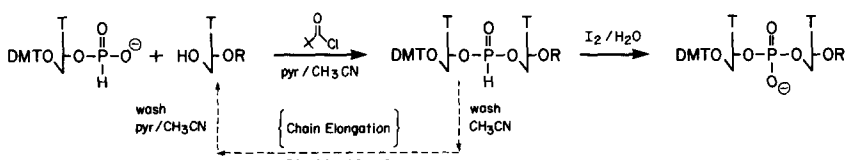
Ribonucleoside H-phosphonate 1 was introduced by Todd to prepare diribonucleotide phosphate 4 (Scheme 1)[1]. An intermediate diribonucleoside H-phosphonate (3) was isolated and oxidized with N-chlorosuccinimide (NCS, Scheme 1). After 28 years this condensation chemistry has remained unexplored in the area of deoxyoligonucleotide synthesis. The synthesis of H-phosphonate linked dinucleotides has recently been described via dinucleoside phosphite triester intermediates[2] and from aroylphosphonate protected nucleosides[3]. Described herein is the use of deoxyribonucleoside H-phosphonates (5, Scheme 2) in the direct synthesis of H-phosphonate linked deoxyoligonucleotides. The deoxyoligonucleotide H-phosphonate is easily converted to phosphodiester linked deoxyoligonucleotides by aqueous iodine oxidation[2]. The potential of this simplified procedure is demonstrated with the chemical synthesis of eicosathymidylic acid (T_{20}) and tetracontathymidylic acid (T_{40}).

Scheme 1



1 2 3 4

Scheme 2



5 6 7 8

Todd used diphenyl chlorophosphate to activate nucleoside H-phosphonate 1, presumably generating a mixed anhydride as the active phosphitylating agent[1]. We have determined that acylating agents will lead to the condensation of 5'-dimethoxytrityl-3'-thymidine H-phosphonate (5) directly with the 5'-OH of a polymer bound nucleoside (6, R=succinyl silica) to yield a dinucleoside H-phosphonate (7) (Scheme 2). Acetic anhydride, isobutyric anhydride, trimethylacetic anhydride (pivaloyl anhydride), pivaloyl chloride, pivaloyl bromide, benzoyl chloride, isobutyl chloroformate and diphenyl chlorophosphate (Todd's reagent) have been evaluated as condensing agents in the synthesis of pentathymidylic acid (T_5) by a procedure similar to that described below. The purity of each synthetic T_5 was assessed by high performance liquid chromatography (HPLC) (data not shown). The ability of pivaloyl chloride to facilitate dinucleoside H-phosphonate formation in a rapid and selective manner was distinctive . Similarly, we confirmed previous reports that aryl sulfonyl chlorides[4] and carbodiimides[5] result in poor yields of dinucleoside H-phosphonate 7.

5'-Dimethoxytrityl-3-thymidine H-phosphonate (5) was prepared as previously described[6] and characterized by ^{31}P NMR (δ -0.27 ppm, J (P-H) = 605 Hz)[7]. Reaction of 5 with 6 (R=dimethoxytrityl) in the presence of 5 eq. pivaloyl chloride led to the mixture of diastereomers 7 (δ -8.44 ppm, -7.23 ppm, J (P-H) = 716 Hz) which was oxidized to the single diester 8 (δ +1.64 ppm) with aqueous iodine. Dialkyl H-phosphonates are rapidly hydrolyzed by conc. NH_4OH[3] and are stable to mild acid[2,3]. Our experiments confirm these results. No modification of 7 was observed during 4 hrs. in a 2.5 percent dichloroacetic acid (DCA)/CH_2Cl_2 solution. These observations suggest a simple scheme for deoxyoligonucleotide synthesis.

The potential synthetic utility of 5'-dimethoxytrityl-3'-thymidine H-phosphonate (5) was evaluated by the synthesis of T_{20} and T_{40} on silica gel. The polymer support was derivatized to 25 μmole 3'-succinyl thymidine per gram of silica gel (6, R=succinyl silica) as previously described[8]. The synthetic cycle (Scheme 2) consisted of a condensation reaction with 10mM 5 (~5eq. relative to 5'-OH component) and 50mM pivaloyl chloride in anhydrous pyridine/acetonitrile (1/1) (5 min), followed by dimethoxytrityl deprotection using 2.5 percent DCA/CH_2Cl_2 (2 min). After the required number of synthetic cycles, the polythymidine H-phosphonate product was oxidized to polythymidylic acid with 0.2M I_2 in THF/Pyr/H_2O (90/5/5) (5 min.). The product was removed from the solid support (conc. NH_4OH/5 hr./55°C) and evaporated. HPLC analysis of the crude product T_{20} is shown in Fig. 1a. The product peak coeluted with a T_{20} standard prepared by the phosphoramidite method[9]. The T_{20} and T_{40} crude products were evaluated by polyacrylamide gel electrophoresis and visualized by U.V. shadowing (Fig. 1b). The analytical data presented in Fig. 1a and 1b clearly demonstrate the high yield synthesis of both T_{20} and T_{40}. T_{40} was further evaluated by 5'-end labeling with γ-^{32}P-ATP using polynucleotide T4 kinase followed by complete degradation with snake venom phosphodiesterase (data not shown). By all criteria, the synthetic T_{40} was comparable to a standard T_{40}.

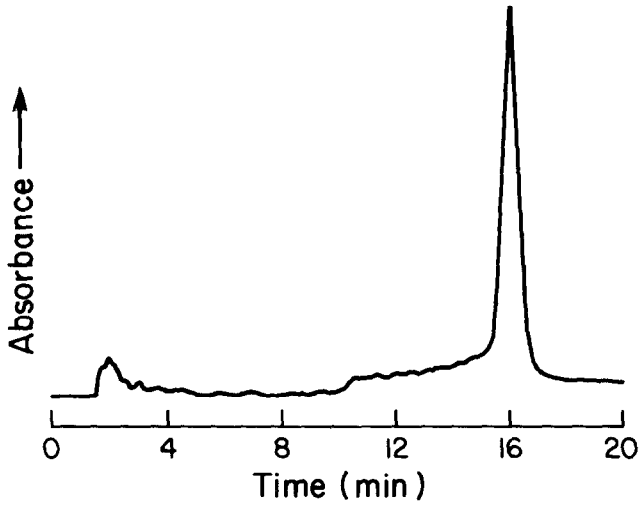


Fig. 1a - HPLC profile of synthetic T_{20}, Zorbax-NH_2, flow rate = 2 ml/min, linear gradient (30 min), 0.1 M KH_2PO_4/20 percent CH_3CN (pH = 6.3) to 0.4 M KH_2PO_4 20 percent CH_3CN (pH = 6.3) Absorbance at 254 nm

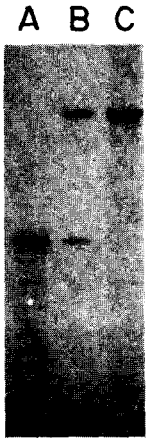


Fig. 1b - Polyacrylamide gel electrophoresis
A - Synthetic T_{20}
B - T_{20} and T_{40} standards
C - Synthetic T_{40}

The syntheses reported above used 5 equivalents of pivaloyl chloride relative to mononucleoside H-phosphonate (5) in a 5 min. condensation reaction. The effect of shorter condensation time was evaluated with the synthesis and HPLC analysis of T_5. The results presented in Fig. 2 demonstrate that the condensation reaction is complete within 1 min.

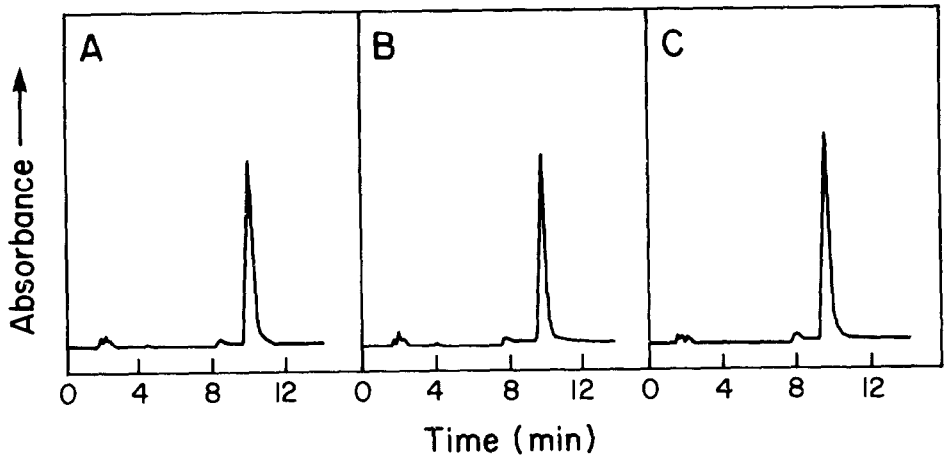


Fig. 2. - HPLC profile of synthetic T_5.
A - 5 min. condensation reaction, B - 2.5 min. condensation reaction, C - 1 min. condensation reaction
Zorbax-NH_2, flow rate = 2.5 ml/min, linear gradient (15 min), 25mM KH_2PO_4/15 percent CH_3CN (pH = 4.8) to 250mM KH_2PO_4/15 percent CH_3CN (pH = 4.8)

When the experiment is repeated using 10 equivalents of pivaloyl chloride a lower yield of product T_5 is observed (data not shown). These results suggest that competitive acylation is yield limiting, therefore a capping step is not required in the synthetic protocol.

Mononucleoside H-phosphonates have been shown to be useful in the direct synthesis of dinucleoside H-phosphonates. Pivaloyl chloride activation of monoňucleoside H-phosphonates yields selective phosphitylation over acylation and rapid rates of dimer formation. Dinucleoside H-phosphonates are stable to mild acid, allowing for the rapid and simple synthesis of H-phosphonate linked deoxyoligonucleotides, and are easily converted to native phosphate diesters by I_2/H_2O oxidation. The experiments reported above used the mononucleoside H-phosphonate in a concentration of 10mM, significantly lower than the mononucleoside concentration typically used in phosphoramidite and phosphotriester chemistry. These results demonstrate the potential for deoxyoligonucleotide synthesis using nucleoside H-phosphonate intermediates in a rapid, simple and reagent efficient synthetic protocol.

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