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Solid-Phase Synthesis Of Oligonucleoside Methylphosphonate 5'-Phosphates

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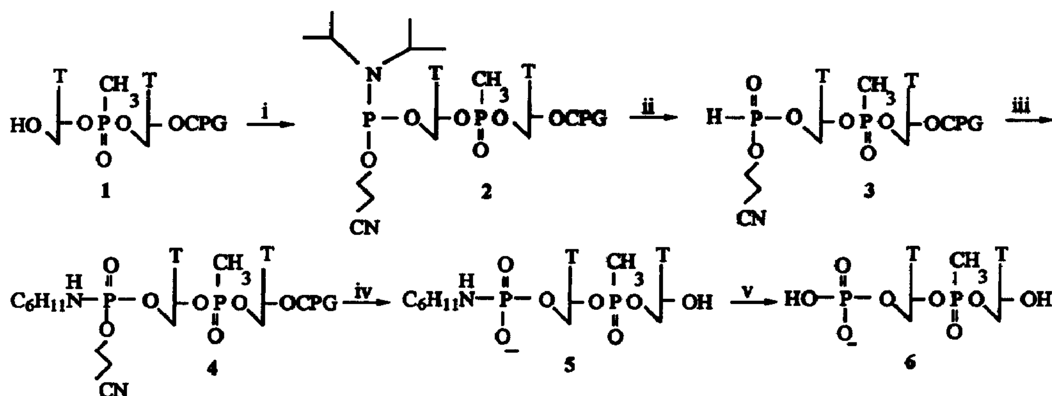
Abstract: A method for the chemical 5'-phosphorylation of oligonucleoside methylphosphonates on solid support is described.

The potential of oligonucleotide based therapy in the treatment of certain cancers, viral diseases and genetic disorders has led to an intense effort in designing nuclease resistant oligonucleotide analogues that can also hybridize to their complementary nucleic acid targets in a sequence specific manner.[1] Two of the most promising and widely studied oligonucleotide analogues, to date, are the oligonucleoside methylphosphonates and the phosphorothioates.[2]

Methylphosphonate oligonucleotides are stable to degradation by serum or cellular nucleases and can significantly inhibit viral gene expression in number of cell-culture systems.[3] Recently, the oligomers were conjugated with psoralens to increase their efficacy as photoactivated antisense/antigene modulators of gene expression.[4] The psoralens, with suitable linkers, were conjugated to the 5'-phosphate of the oligomers via a nuclease resistant phosphoramidate linkage.[4b] Because there is no method available for chemical phosphorylation of methylphosphonate oligonucleotides, the 5'-phosphate precursors used in these studies were prepared by enzymatic phosphorylation with T4 polynucleotide kinase and ATP. The enzymatic phosphorylation is inconvenient and impractical for large scale preparations and requires a phosphodiester linkage at the 5'-end of the oligomer.[5] Moreover, in contrast to the nuclease resistant methylphosphonate backbone,[6] this phosphodiester linkage is susceptible to degradation inside the cells.[7] Most of the standard 5'-phosphorylating reagents and methods,[8] designed for solid-phase DNA synthesis, require moderately harsh alkaline conditions to remove phosphate protecting groups. Under these alkaline conditions, methylphosphonate backbone is easily degraded by hydrolysis.[9] It would therefore, be useful to develop, chemical phosphorylation procedures for methylphosphonate oligonucleotides that eliminate the terminal phosphodiester requirement and are conveniently carried out on solid support. This report describes a simple and mild method for the solid phase phosphorylation of methylphosphonate oligonucleotides (Scheme 1).

A <u>TT</u> methylphosphonate dinucleotide **1** was first prepared (1-5 μmole scale) on LCAA-CPG support.[10] While still bound on the support, the detritylated dinucleotide served as the starting point for the intended phosphorylation. The support was treated with a 0.4 M solution of 2-cyanoethyl N, N-

diisopropylchlorophosphoramidite in methylene chloride in the presence of diisopropylethylamine to give the phosphoramidite **2** which was readily converted to the cyanoethyl H-phosphonate intermediate



Scheme 1. i: 0.4 M 2-cyanoethyl N,N-diisopropylchlorophosphoramidite in diisopropylethylamine-methylene chloride (1:9) 0.5 h; ii: 0.4 M 1H-tetrazole in 20% water-acetonitrile, 0.1 h; iii: 10% cyclohexylamine in carbon tetrachloride-pyridine (1:1) 2 h; iv: ethylenediamine-95% ethanol (1:1) 6 h; v: 80% acetic acid, 48 h or 10% isoamyl nitrite in water/acetic acid/ethanol 45^0 C, 5 h.

3 with aqueous tetrazole.[11] Since cyanoethyl phosphate protecting group is cleaved under milder alkaline conditions on a phosphotriester than on a phosphodiester backbone,[12] **3** was first converted to **4** by oxidation with cyclohexylamine in carbon tetrachloride-pyridine.[13] Exposure of phosphoramidate **4** with ethylenediamine-ethanol for 6 h[14] resulted in the cleavage of the dinucleotide from the support with concomitant removal of the cyanoethyl protecting group to furnish the diester **5**. The acid sensitive phosphoramidate linkage was next cleaved with 80% acetic acid at room temperature for 48h or by treatment with 10% isoamyl nitrite in water/acetic acid/ethanol (1/4/5) at 45^0 C for 5h[13a,15] to give the 5'-phosphorylated product **6** which appeared as a mixture of two distereoisomers on reversed phase HPLC[16] (^{31}P NMR: 3.23, 3.65). The presence of the phosphate group was confirmed when compound **6** on overnight treatment with bacterial alkaline phosphatase in 50 mM Tris buffer (pH 7) gave the dephosphorylated product T͟T͟. HPLC analysis of the reaction mixture showed complete disappearance of **6** and the appearance of two new peaks with retention times corresponding to those of the T͟T͟ dinucleotide.[17]

Additional support to the efficacy of the phosphorylation procedure was obtained by synthesis of a chimeric methylphosphonate pentamer TpT͟T͟T͟T͟ containing a phosphodiester linkage at the 5'-end.[18] The oligomer appeared as a single peak with retention time of 21.3 min on reversed phase HPLC (Figure 1, A). The pentamer was then enzymatically 5-end labelled with ATP and T_4 polynucleotide kinase to furnish after desalting,[19] the desired phosphorylated oligomer pTpT͟T͟T͟T͟ (B). This product migrating with a retention time of 19.9 min was indistinguishable from the chemically phosphorylated

crude pentamer pTpTTTT (C) prepared in 80% yield by the procedure described above. The minor peak eluting with a retention time of 21.3 min in C and accounting for ~ 8% of the total product, corresponds to the unphosphorylated starting material. Finally, oligomer A when phosphorylated with [γ-^{32}P]ATP and T4 polynucleotide kinase migrated as a single band on 20% polyacrylamide gel electrophoresis

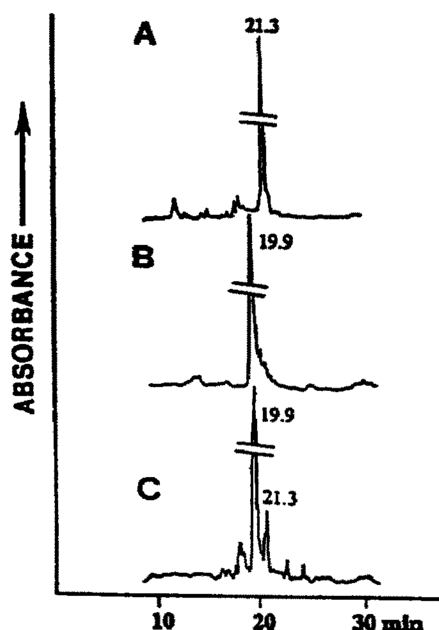


Figure 1. Reversed phase HPLC[16] profiles of the crude methylphosphonate oligonucleotides: (A) TpTTTT (B) enzymatically phosphorylated pTpTTTT (C) chemically phosphorylated pTpTTTT.

with the same gel mobility (UV shadowing) as the chemically phosphorylated oligomer C (data not shown).

In conclusion, the simple and convenient method described herein promises to be an effective chemical route to methylphosphonate 5'-phosphates. Although the phosphorylation reaction was carried out with thymidine oligomers, the chemistry described above should also be compatible with other nucleosides. Further, solid-phase attachment of reporter groups on methylphosphonate oligonucleotides via phosphoramidate linkages is currently under investigation and will be reported soon.

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16. All analytical HPLC was carried on a Beckman 5m Ultrasphere column (4.6 X 25 cm) under a 25 min. linear gradient of 0-30% acetonitrile in 0.1 M TEAA buffer (pH 7.0) at a flow rate of 1ml/min. Peaks were detected on a Beckman diode-array UV detector operating at 260 nm wavelength.
17. TT dinucleotide (t_R: 17.99 min, 18.41 min); dinucleotide phosphate **6** (t_R: 14.94 min, 15.57 min); dinucleotide phosphoramidate **5** (t_R: 23.35 min, 23.66 min).
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19. Desalting was carried out on a Waters Sep-Pak[R] C-18 reversed-phase cartridge.

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