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OLIGODEOXYNUCLEOSIDE METHYLPHOSPHONATES: SYNTHESIS AND ENZYMIC DEGRADATION

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Abstract The automated synthesis of oligodeoxynucleosides containing methylphosphonate linkages has been improved by using nucleoside methylphosphonamidites as starting materials. It was found that two adjacent methylphosphonate linkages protect an oligomer from degradation by snake venom phophodiesterase or spleen phophodiesterase.

Oligodeoxynucleotides complementary to viral RNA may be used to inhibit viral replication (1,2). Uncharged derivatives, for example with methylphosphonate internucleoside linkages, have similar properties and may have advantage in nuclease resistance and enhanced cell penetration (3,4).

Synthesis of the internucleoside methylphosphonate linkage was first achieved using activated nucleoside methylphosphonates but these proved much slower in coupling reactions than the usual phosphates (5-11). Attempts to convert the methyl phosphite intermediate of the conventional phosphoramidite method to methylphosphonates using the Arbusov reaction also proved inefficient (12). Two reports of the use of nucleoside methylphosphonamidites for the solution phase synthesis of dimers give very different coupling times probably due to the different activating agents used (9,13).

We wish to report the use of the nucleoside methylphosphonamidites 2a-d in an automated, solid phase oligonucleotide synthesis using the same program as for phosphoramidites and with the same coupling efficiency.

Methylchloro-N,N-diisopropylaminophosphine, 1, was prepared by reaction of methyldichlorophosphine (51mmol) with diisopropylamine (102 mmol) in ether at 15° under nitrogen. After removal of salt by filtration and evaporation of solvent, 1 was obtained as an oil (48 mmol, 95% of theory) that was characterized by ^{1}H and ^{31}P NMR (see Table) and was stable at -20° for at least eight weeks. The product was reacted with the usual protected nucleosides in dichloromethane containing N,N-diisopropylethylamine at room temperature for 10-20 min. Aqueous work up and precipitation from ethyl acetate using pentane at -30° to -40° gave 2a-d as white solids in 80-90% yield. Products were pure by tlc on silica in CH_2Cl_2:EtOAc:Et_3N(9:9:2) and were characterized by ^{1}H and ^{31}P NMR (See Table for ^{31}P data).

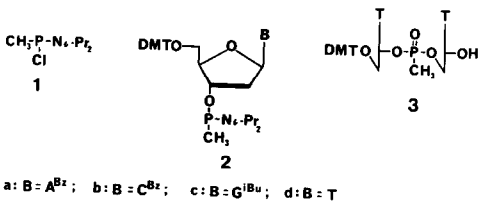

TABLE ^{31}P Chemical Shift Values (a)

Compound	δ	Compound	δ
1	137.43	2c	121.93, 120.19
2a	122.99, 121.36	2d	121.00, 120.67
2b	121.00, 119.70	3	35.9

(a) Downfield chemical shifts in dichloromethane from an internal reference of 85% phosphoric acid.

These products were used in an automated DNA synthesiser (Biosearch 8600) using the same conditions and program used for standard phosphoramidite reagents. Nucleotides were dissolved in acetonitile at a concentration of 33 mg/ml and activated with tetrazole (16). Synthesis on prepacked CPG support (Biosearch) was performed using a coupling time of 1 minute.

Coupling efficiency was followed by the dimethoxytrityl assay and was found to be the same as for control syntheses run in parallel using phosphoramidites.

After the last coupling, the product was detritylated then cleaved from the support with NH_4OH at room temperature for 2 hrs and deblocked using ethylenediamine: ethanol (1:1) at room temperature for 4 hrs. (10). This basic treatment caused about 1% degradation of the internucleoside phosphonate group in a model study assayed by HPLC.

The dimer 3, was made and shown by ^{31}P NMR to have a methylphosphonate linkage (Table). Hydrolysis with piperidine (14) followed by reverse phase HPLC gave only a peak for thymidine and a faster moving peak of equal area assumed to be thymidine 5'- and 3'- methylphosphonates.

Comparison of the unpurified heptamer TpTpTpTpTpTpT containing a single methylphosphonate linkage (15) with a control compound $T(pT)_6$ by HPLC on Partisil SAX showed the two to have very similar profiles (Figure 1). As expected, due to its smaller charge, the phosphonate ran faster (midway between a 5-mer and 6-mer in the control). To show that this was the desired heptamer and not a failure sequence the position of the phosphonate was varied along the chain. All compounds showed similar HPLC profiles and retention times as well as satisfactory trityl assays for all couplings. Hence the presence of the methylphosphonate does not inhibit subsequent extension of the oligomer.

Similar results were obtained using the other nucleoside phosphonamidites 2a-c and also when increasing numbers of phosphonates were included in the chain. We have made a variety of hetero sequences including a 19-mer and 21-mer complementary to strategic regions of the human immunodeficiency virus, each with four methylphosphonate linkages situated to provide maximum protection against exonucleases (see below and Figure 2).

Enzyme digestion Degradation of TpTpTpTpTpTpT from the 3'-end with snake venom phosphodiesterase followed by HPLC on Partisil SAX gave peaks corresponding to T, pT and pTpT in the expected ratio. Hence, the enzyme can jump over a single methylphosphonate linkage as has been found for a single phosphotriester linkage (17). Increasing the number of phosphonates progressively slows down degradation by the enzyme and two consecutive phosphonates increase $t_{1/2}$ by two orders of magnitude. Similar results were found with spleen phosphodiesterase which degrades from the 5'-end. Thus good protection against both enzymes can be afforded by two consecutive phosphonate linkages at each end of the oligomer.

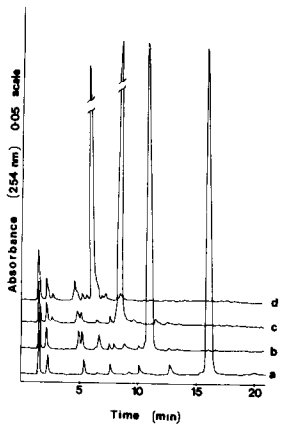

Figure 1

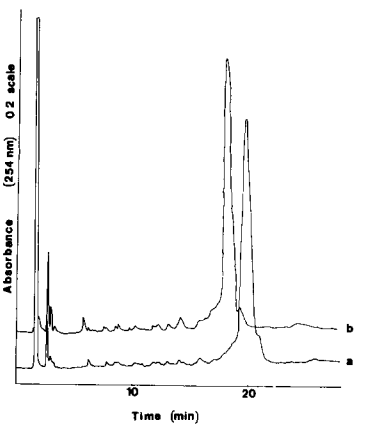

Figure 2

Figure 1 HPLC on Partisil SAX of (a)TpTpTpApGpCpA; (b)TpTpTpApGpCpA; (c)TpTpTpApGpCpA; (d)TpTpTpTpGpCpA Gradient 3.4 to 105 mM phosphate (pH 6.8) in 20% acetonitrile in 30 min at 3 mL/min.

Figure 2 HPLC as in Figure 1 using 3.4 to 376 mM phosphate in 40 min.
(a)CpCpApApTpTpCpTpGpApApApApTpGpGpApTpApApA (b)ApApTpTpCpTpGpApApApApTpGpGpApTpApApA

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