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Synthesis and Physicochemical Studies of Partially Phosphate-Methylated Oligodeoxyribonucleotides

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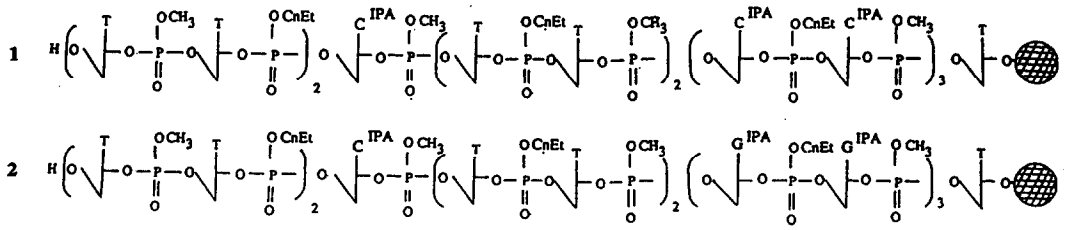
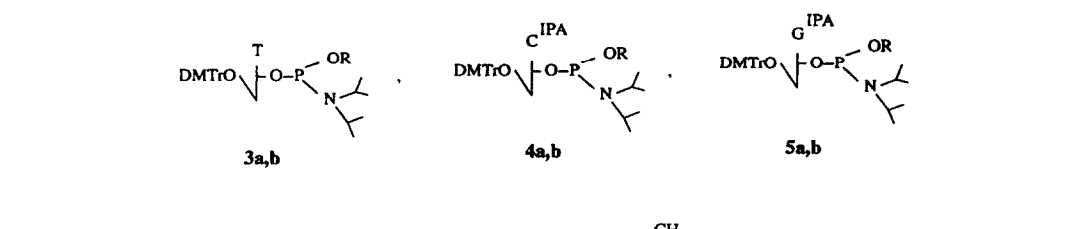
Abstract : *Partially phosphate-methylated oligodeoxyribonucleotides have been synthesized on an oxalyl-CPG derivatized support using an isopropoxyacetyl group for the protection of the exocyclic amine of the nucleic bases. Hybridization properties with the target sequence have been studied by absorption spectroscopy.*

During the past twenty years numerous studies have shown the potential usefulness of synthetic oligodeoxyribonucleotides in gene expression regulation[1]. In order to provide them with additional properties such as resistance towards nucleases, enhanced stability for binding with the target sequence, and increased cellular uptake, studies towards the design of various backbone-modified oligonucleotides have become an area of intensive research. Among them, an interesting series concerns phosphate-methylated DNA analogues. These compounds, first introduced twenty years ago[2], have recently become the field of controversial reports concerning their unexpected hybridization properties[3,4]. The results reported in References 2 and 4 concern dithymidylates and oligothymidylates methylated derivatives respectively, while those in Reference 3 describe oligomers involving the four nucleic bases. In order to develop our own opinion regarding this we decided to prepare partially phosphate-methylated oligodeoxyribonucleotides containing thymine, cytosine and guanine.

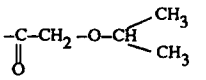
Successful solid-phase synthesis of such compounds requires a base protective group and a linker to anchor the oligonucleotide to the solid support during the chain assembly that could be cleaved under conditions that do not affect the methyl-phosphotriester. Recent reports in the literature concerning the oxalyl-CPG support[4] as well as the isopropoxyacetyl[5] group for the protection of the exocyclic amine of the nucleic bases seemed suitable to reach our goal.

In order to make the purification of the oligomers easier, we chose to prepare alternated methyl-phosphotriester-phosphodiester oligonucleotides. Starting from the dT-oxalyl-CPG derivatized support (57μmol/gram) two 16-mers **1**,**2** were assembled using the phosphoramidites monomers **3** to **5**.





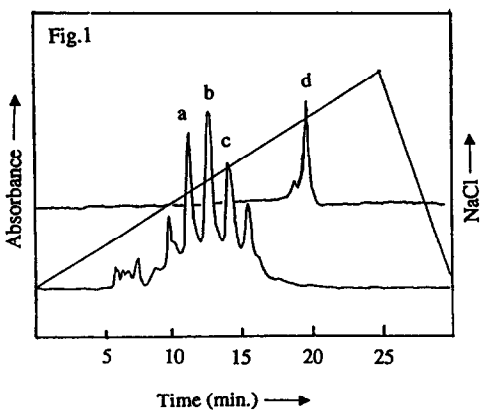
3a,b 4a,b 5a,b

a) R= CnEt, b) R = CH_3, IPA = 

One reported method[4] for the oxidation of the methylphosphite was a 2-minute treatment with 0.1 M iodine in tetrahydrofuran/pyridine/methanol [20/9/1] which proved to give methyltriester with little diester ca d[TpT]/d[T(OMe)T]=0.06. The oxidation of the cyanoethylphosphite was performed using the classical iodine/water reagent since model experiments have shown that d[T(OMe)T] is stable under these conditions[4]. After the chain elongation, an additional acid treatment was performed to remove the 5'-terminal trityl group, then at least 95% of the nucleotidic material was removed from the support after a brief treatment with 5% ammonium hydroxide in methanol (5 min). In order to determine the conditions under which the IPA as well as the remaining cyanoethyl groups could be removed from the exocyclic amines and the phosphate groups, respectively, and to get the full set of the expected dimers after nuclease degradation, we prepared the methylated dinucleosides d[T(OMe)T], d[C(OMe)C], d[T(OMe)C], d[C(OMe)T], d[G(OMe)G] and d[G(OMe)T] as well as the corresponding dinucleoside monophosphate d[TpT], d[CpC], d[CpT], d[TpC], d[GpT] and d[GpG]. We observed that complete removal of the IPA group from the cytosine needed a twenty-minute treatment at 20°C with a 5% ammonium hydroxide in methanol. Under these conditions we observed, by using two analysis systems [TLC and reversed-phase HPLC], that for d[C(OMe)C], d[T(OMe)C] and d[C(OMe)T] two major products were indentified as the phosphodiester and methylphosphotriester, respectively. The former was hydrolyzed to give deoxynucleosides by endonuclease P1 followed by alkaline phosphatase (AP) treatment while the latter remained unchanged. The phosphodiesters d[NupNu] and the phosphotriesters d[Nu(OMe)Nu] were obtained in a 0.15-0.20 ratio.

The complete deprotection of d[G^{IPA}(OMe)T] needed a sixty-minute treatment with 10% ammonium hydroxide in methanol. Unfortunately the methylphosphotriester could not withstand these conditions and in this case the yield of demethylation reached at least 35-40%. So we opted to abandon the purificaton of the guanine containing methylated oligonucleotide and to focus our study on the compound **1** involving only cytosine and thymine.

After treatment under the above mentioned conditions, purification was performed on a Mono Q ion exchange column from Pharmacia (Fig. 1). The chromatogram displayed many peaks of which the main ones (**a,b,c**) were collected separately. According to the 97% coupling yield and to the very low yield of the internucleotic bond cleavage during deprotection (not detectable for the dimers by HPLC analysis) we assumed that these three main peaks corresponded to full length 16-mers with different numbers of methylphosphotriester groups. Following this assumption, peak **a** should contain more methyl groups than the others. In order to control its length, an aliquot of the oligomer corresponding to peak **a** was submitted to a thiophenol/trietylamine treatment to remove the methyl groups from the phosphates. The progress of the reaction was followed by ion exchange analysis and after 15 hours of treatment the starting material was transformed into another product having the same retention time as the parent 16-mer involving only phosphodiesters (Fig. 1 peak **d**). Reversed-phase analysis of the methylated compounds exhibited a broad peak (Fig. 2) due to the presence of the isomers.



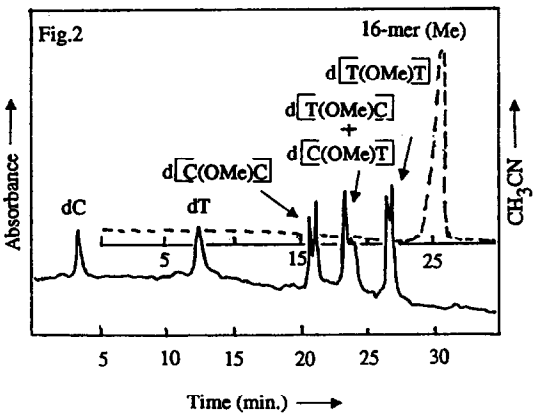


Figure 1: Ion exchange analysis of the crude deprotected compound **1** at $\lambda = 254$ nm on a Mono Q HR5/5 column from Pharmacia using 10^{-2}M KH_2PO_4 pH 6.8 buffer in 20% CH_3CN and a linear gradient of NaCl (0 to 0.6 M in 25 min) with a flow rate of 1 ml/min. Peak **d** corresponds to the product obtained after treatment of peak **a** with thiophenol/triethylamine. Figure 2: Reversed-phase analysis on a Lichrocart column (125 x 4 mn) packed with 5 µm Lichrospher RP 18 from Merck with a gradient of CH_3CN [0% CH_3CN for 5 Min, then 0 to 40% CH_3CN in 40 min] in 0.1 M aqueous triethylammonium acetate pH 7, with a flow rate of 1 ml/min at $\lambda = 260$ nm. Full line: the base composition analysis of the compound corresponding to peak **a** in Figure 1 after degradation with P1 from Penicilium citrinum and A.P. The dotted line represents the 16-mer corresponding to peak **a** in Fig. 1.

The nuclease degradation of the methylated oligomer **a** by P1 and AP showed the concomitant presence of the expected methylated dimers d[T(OMe)T], d[C(OMe)C], d[C(OMe)T], d[T(OMe)C] and of

the monomers dT and dC (due to demethylation) with approximately 25-30% of monomers (Fig. 2) when perfectly alternated methyltriester-phosphodiester should give only methylated dimers. Theses results showed that the iso-propoxyacetyl and the cyanoethyl groups were fully removed. Each methylated dimer appears as a mixture of two peaks corresponding to the two diastereoisomeric forms. By reversed-phase analysis the elution order of the compounds corresponding to peaks **a,b,c** (Figure 1) is the reverse of that obtained by ion exchange analysis. But the differences between retention times are small (data not shown). Moreover nuclease degradation of the oligomers corresponding to the peaks **b** and **c** led to the same compounds as those obtained with the compound **a** with an increasing amount of monomers comparative to the amount of dimers. The [monomers]/[dimers] ratio increases in the following order **a<b<c**. These results are in accordance with our hypothesis that the compound corresponding to peak **a** (Figure 1) contains more methyl groups than the other compounds. In order to compare the binding properties of the partially methylated oligonucleotide **a** with those of the unmodified parent, their interactions with the complementary sequence d-5'TCAGGGGGGAAAAGAAAATTTT 3' 22-mer were studied by absorption spectroscopy. For both oligomers the addition of increasing concentrations of 22-mer at 4°C in the presence of 10^{-2} M sodium cacodylate buffer pH7, 0.1 M NaCl and 10^{-2} M $MgCl_2$ led to a 1:1 duplex. The melting temperatures were determined for 1:1 duplex (1μM for each strand) in 10^{-2}M sodium cacodylate buffer, pH 7, in the presence of 10^{-1}M NaCl and 10^{-2}M $MgCl_2$. The obtained Tm values were 47°C for the duplex formed with the 16-mer methylated oligomer and 56°C for the duplex formed with the parent 16-mer diester respectively.

A 16-mer oligopyrimidine sequence partially methylated on the phosphate groups (5 or 6 methyl groups out of 8 expected for the perfectly alternated oligomer) has been prepared using the isopropoxyacetyl group for the cytosine protection and phosphoramidite chemistry on an oxalyl-CPG derivatized support. Our results showed that the esterification of some of the phosphate by a methyl group had a destabilizing effect on the hybridization properties of the modified oligomer with its complementary sequence through comparison with the unmodified 16-mer parent .

Acknowledgments

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