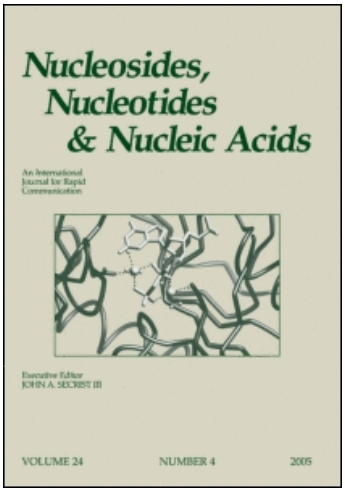

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SYNTHESIS AND PROPERTIES OF 2'-O-METHYLRIBONUCLEOTIDE METHYLPHOSPHONATE CONTAINING CHIMERIC OLIGONUCLEOTIDES

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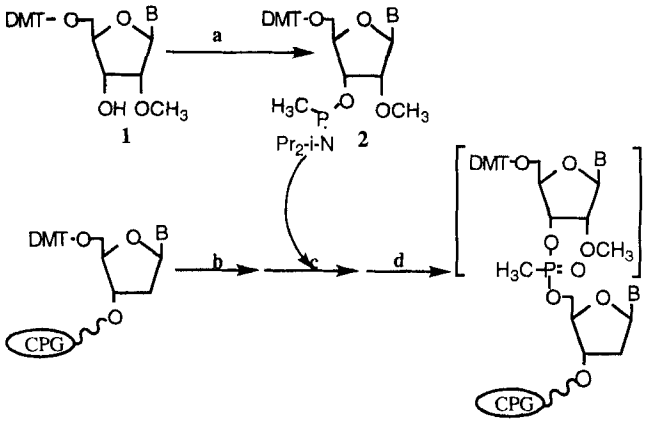
Abstract: 2'-O-methylribonucleoside methylphosphonamidites are synthesized and incorporated into oligonucleotides to obtain chimeric antisense oligonucleotides. The resulting oligonucleotide binds to their target RNA/DNA sequences efficiently and stable in a medium containing bovine serum.

Oligonucleotides (oligos) and their analogs have wide applicability in diagnostics, molecular biology and therapeutics development[1,2]. Antisense oligos were shown to be active against several targets *in vitro* and *in vivo*[3]. In order to improve cellular uptake and nuclease resistance several sugar and phosphate backbone modified oligos have been synthesized and studied[2,4]. In addition to deoxyribonucleotide analogs oligoribonucleotides have also been explored for antisense uses as they bind to an RNA target more tightly than their DNA counterpart[5]. To increase the nuclease resistance phosphorothioate analogs of RNA have been synthesized and studied[6]. However, synthesis of non-ionic methylphosphonate or methylphosphotriester oligoribonucleotides is difficult due to the unstability of such compounds because of the presence of a hydroxyl group at the 2'-position[7]. An alternative way to make oligoribonucleotides resistant to RNase is by alkylation of the 2'-hydroxyl group[8]. Synthesis of a dimer of 2'-O-methylribonucleotide methylphosphotriester was reported[9]. Recently, a preliminary report on 2'-O-methyl-ribonucleoside methylphosphonates was published[10].

Now we have developed a synthetic route for the preparation of 2'-O-methyl-ribonucleoside methylphosphonamidates (**2**) and their incorporation into oligonucleotides. The resulting new oligos containing 2'-O-methylribonucleoside analogs were examined for



Scheme 1. Scheme for the synthesis of 3'-phoshonamidites (**2**) and subsequent incorporation into oligos. a: CH_3-P(Cl)-N-i-Pr_2 and diisopropyl ethylamine; b: Dichloroacetic acid; c: Tetrazole; and d: I_2/H_2O/THF/Lutidine mixture. B could be any 2'-O-methylribonucleoside base (A, C, G or U). The new chemical modification is shown in square bracket for clarity.

exonuclease resistance, hybridization efficiency with target nucleic acids and *in vitro* translation inhibition.

The chimeric oligo (**3**) [5'-d(CTCTCGCACCCATCTCTCTC)C*U*U*C*d(T); * indicates 2'-O-methylribonucleoside methylphosphonate] and control oligo (**4**) [5'-d(CTCTCGCACCCATCTCTCTCCTTCT)] used in the study are complementary to the initiation codon region of gag RNA of HIV-1. The synthesis of 2'-O-methylribonucleoside phosphonamidites was carried out as detailed in Scheme 1. In principle, the procedure was based on the reported method for the synthesis of deoxyribonucleoside phosphonamidites and their incorporation into oligos[11,12]. The required 5'-O-dimethoxytrityl (DMT)-3'-hydroxy-2'-O-methylribonucleosides (**1**) were converted to 3'-O-methyl-N,N-diisopropyl-aminophosphonamidites (**2**) by treating **1** with methyl diisopropylchlorophosphonamidite in the presence of diisopropyl ethylamine. The overall yields of C and U methylphosphonamidites (**2**) ranged from 65-72% based on starting material **1**. These synthons were used on the DNA synthesiser to be introduced at required positions in oligos. The average coupling yields were >97%, which is comparable to the average coupling yields obtained in the synthesis of oligodeoxyribonucleoside methylphosphonates. The rest of the oligo sequences were assembled using standard

protocols for β-cyanoethyl phosphoramidite chemistry. After the completion of synthesis, oligos were deprotected and purified[11,12].

The incorporation of the methylphosphonate linkages into the oligos was examined by ion exchange HPLC[12] and it was confirmed that the oligo **3** has four charges less than oligo **4** as deduced from retention times. This suggests that the incorporation of the modified monomers does not prevent subsequent addition and extension of the oligo chain. The thermal melting study of the modified 25 mer, **3** with complementary nucleic acid strands demonstrated efficient hybridization with both RNA and DNA targets[13]. Typical melting curves are shown in figure 1. The oligo **3** gave Tms of 68.7 and 63.7°C with RNA and DNA complementary strands, respectively. Under the same conditions the unmodified control oligo **4** showed Tms of 71.8 and 65.7°C with RNA and DNA complementary strands, respectively. The lower Tm values of 2-3°C obtained with the new oligo **3** compared to the control oligo **4** with both the target nucleic acids could be due to decreased concentration of the binding species because of the presence of nonbinding S_p-diastereomers arising from the methyl substitution on phosphate[14]. This indicates that the new modification introduced in the oligo does not substantially destabilise the duplex formed between the oligo and its complementary RNA or DNA target. The % hyperchromicity values obtained with oligo **3** (15 and 19.2 with RNA and DNA strands respectively) compared to the unmodified oligo **4** duplex (18.1 and 22.3 with RNA and DNA strands respectively) further confirmed the validity of the Tm values obtained.

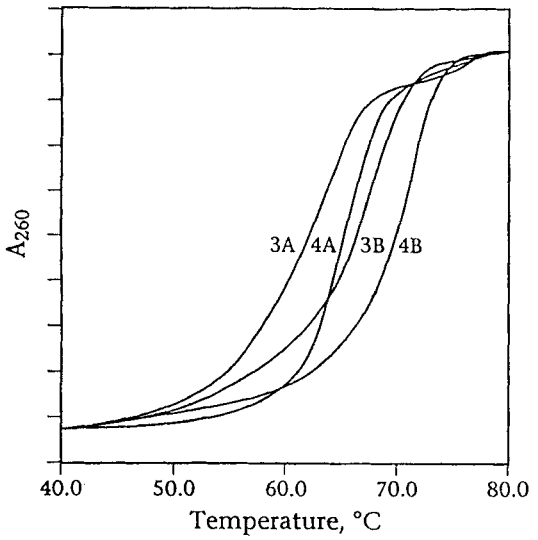


Figure 1. Melting curves of chimeric (**3**) and control (**4**) oligos with **A**: DNA, and **B**: RNA complementary strands in 10 mM disodium hydrogen phosphate, pH 7.4 and 100 mM sodium chloride buffer.

The presence of the 2'-O-methylribonucleoside methylphosphonate group at the 3'-end confers stability to the oligo against exonucleases. The normal oligo **4** was digested to half of its initial concentration ($T_{1/2}$) in about 100 seconds by snake venom phosphodiesterase (SVPD), a 3'-exonuclease[12]. However, the $T_{1/2}$ of the oligo **3** was measured to be up to 800-900 seconds under the same experimental conditions. This indicates that the modification is resistant to the exonuclease action. A more comprehensive

way to examine oligo stability *in vitro* is by incubating in serum. The stability of oligos **3** and **4** in 10% fetal bovine serum[15] at different time intervals is shown in figure 2. The majority of the oligo **3** was intact up to 4 hours compared to the control oligo **4**. The densometric analysis of the autoradiogram showed that more than 95% of the unmodified oligo **4** was digested by bovine serum nucleases within one hour. However, at least 40-50% of the oligo **3** with four modifications at 3'-end was intact for up to 4 hours.

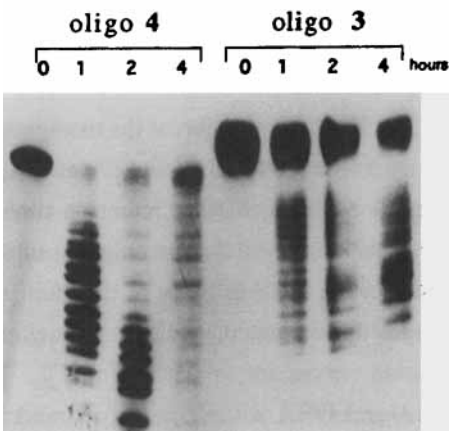


Figure 2. Stability of the oligos **3** and **4** in cell culture medium containing of 10% fetal bovine serum.

The ability to inhibit translation in a cell free system by oligos **3** and **4** was studied[15]. In wheat germ extract, containing RNase H, both of the oligonucleotides arrested protein synthesis compared to the control. The results showed 10-15% higher inhibition with unmodified oligo **4** than with the modified oligo. This result correlates well with the lower duplex stability of oligo **3** observed in melting studies. However, increased stability to nucleases is more critical in the *in vivo* rather than *in vitro* situation .

We conclude that the synthesis of 2'-O-methylribonucleoside methylphosphonate oligos can be carried out using an automated DNA synthesizer. The oligo containing this modification hybridizes to both RNA and DNA single strand targets efficiently, has increased stability in cell culture medium containing 10% fetal bovine serum and inhibits translation *in vitro*. Currently we are investigating the cellular pharmacology and gene regulation activity of oligo **3** and similar oligos against HIV-1.

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