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SYNTHESIS OF OLIGODEOXYNUCLEOSIDE METHYLPHOSPHONOTHIOATES

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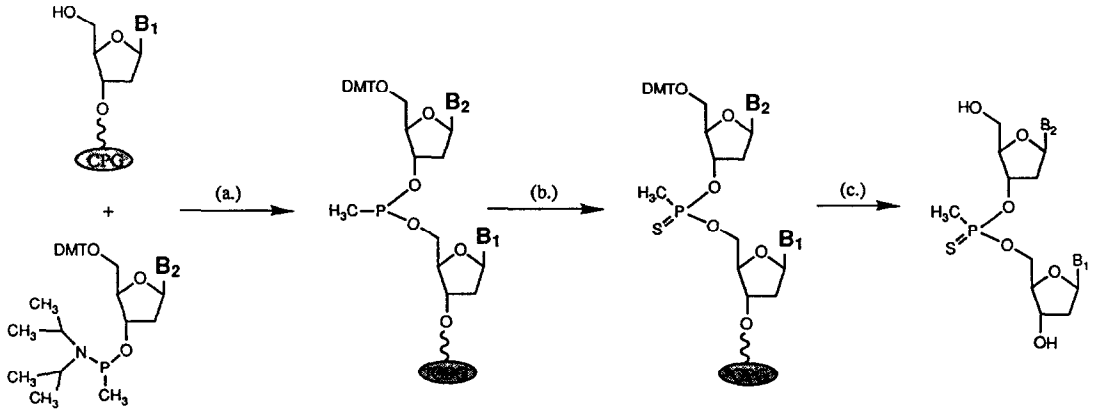
Oligodeoxynucleoside methylphosphonothioates have been synthesized using nucleoside methylphosphonamidites as synthons and phosphoramidite coupling cycle, followed by oxidation of intermediate methylphosphite internucleoside linkage with 3H-1,2-Benzodithiol-3-one 1,1dioxide.

Oligodeoxynucleotides and analogs containing non-ionic internucleotide linkages, such as alkylphosphonates (1,2) and alkyl phosphotriesters (3,4) are useful as therapeutic agents in addition to their other biochemical properties. These analogs are chiral, form stable duplexes with complementary nucleic acids, have increased nuclease resistance and are known to penetrate cell membranes easily.

Oligodeoxynucleotide alkylphosphonothioates have not been studied in detail because of the unavailability of established synthetic procedures. Attempts have been made to synthesize only dinucleotides containing a methylphosphonothioate linkage. The first report (5,6) describes the use of methylphosphonothioic dichloride and obtained dinucleotide methylphosphonothioate in 56% yield. Dinucleoside methylphosphonothioates have also been synthesized in solution, in 60-70% yield, by using a reagent obtained in situ by treating methylphosphonothioic dichloride with 1-hydroxy-6-trifluoromethyl benzotriazole (7). Recently, synthesis of dinucleotides containing stereospecific methylphosphonothiate linkage in solution, in 50-60% yield, have been reported (8). Here we report the method for automated synthesis of oligodeoxynucleotide methylphosphonothioates with average efficiency of greater than 97%.

The synthetic steps involved in assembling oligodeoxynucleoside methylphosphonothioates are listed in *Scheme 1*. Nucleoside methylphosphonamidites (2) were used as synthons to synthesize

Scheme 1



(a) Tetrazole/CH_3CN (b) 3H-1, 2-Benzodithiol-3-one 1,1-dioxide/CH_3CN (c) 2% v/v Dichloroacetic acid in CH_2Cl_2

oligonucleotides on solid support by using a normal coupling cycle for phosphoramidite chemistry. After each coupling, the intermediate methylphosphite internucleoside linkage was oxidized with 1% solution of 3H-1,2-

benzodithiol-3-one 1,1-dioxide (9) in acetonitrile for 5 minutes, to generate methylphonphonothioate internucleoside linkages. After the assembly of the required oligonucleotide, deprotection was carried out by using ethylenediamine-ethanol (1:1, v/v) for 6 hours at room temperature.

Several dinucleotides and oligonucleotides containing methylphosphonothioate linkages have been synthesized, deprotected and analyzed. Dinucleotides TT and AG containing methylphosphonothioate linkages have been synthesized and deprotected by the same procedure as described above. Reversed phase HPLC analysis of both dinucleotides showed two peaks, because of the diastereomeric nature of the methylphosphonothioate linkage *(Figure 1A)*. Dinucleotide TT containing methylphosphonate linkage eluted earlier than dinucleotide TT containing methylphosphonothioate linkage suggesting the increased hydrophobic nature of methylphosphonothioate linkages over methylphosphonate linkages. The identity of methylphosphonothioate linkage was further confirmed by ^{31}P NMR. Dinucleotide TT containing methylphosphonothioate linkage gave a peak at 95.9 ppm. In comparison, dinucleotide TT containing methylphosphonate linkage gave a peak at 37.3 ppm (13).

The following oligonucelotides containing one methylphosphonothioate linkage have been synthesized.

[1] TTTTTT [2.] TTTTTT [3.] TTTTTT

□ indicates position of methylphosphonothioate linkage

The above oligonucleotides were synthesized using nucleoside ß-cyanoethyl phosphoramidite on an automated machine (Millipore, Model 8700) by using standard coupling cycle and oxidation of intermediate phosphite linkage with iodine reagent after each coupling. At the site of methylphosphonothioate linkage, coupling was carried out using nucleoside methylphosphonamidite followed by oxidation with 1% solution of 3H-1,2-benzodithiol-3-one 1,1-dioxide. Deprotection was carried out using ethylenediamine-ethanol (1:1; v/v) for 6 hours

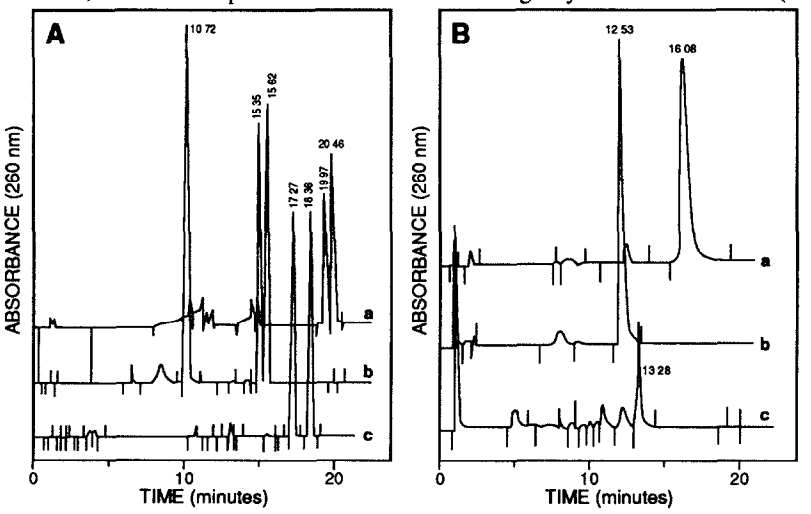


Figure 1. (A) Reversed phase HPLC profile of dinucleotides (a) TT and (c) AG containing methylphosphonothioate internucleotide linkages and (b) dinucleotide TT containing methylphosphonate internucleotide linkage. Peak RT - 10.7 min is of thymidine nucleoside. (B) Ion exchange HPLC profile of (a) oligonucleotide 1, (b) oligonucleotide 2 and (c) oligonucleotide 3 (for HPLC conditions see ref 11).

at room temperature. Analysis of oligonucleotides **1**, **2**, and **3** on ion exchange HPLC showed that oligonucleotides **2** and **3** eluted earlier than oligonucleotide **1**, indicating that oligonucleotides **2** and **3** have one less charge than oligonucleotide **1** because of non-ionic methylphosphonothioate linkage *(Figure 1B)*

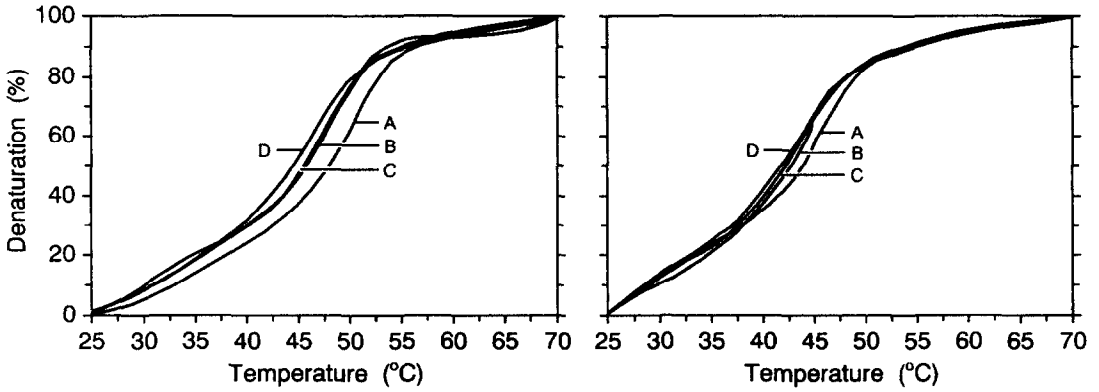


Figure 2. Melting temperature curves of oligonucleotides (A) -4, (B) -5, (C) -6 and (D) -7 with complementary olilgoribonucleotide (31-mer) in 100 mM NaCl concentration (left hand side block) and in 50 mM NaCl (right hand side block). (for experimental condition see reference 12).

We have also synthesized the following oligonucleotides containing two, three and four methylphosphonothioate linkages at 3'-end.

[4] 5'ACACCCAATTCTGAAAATGG3'

[5] ACACCCAATTCTGAAAA TGG

[6] ACACCCAATTCTGAAA ATGG

[7] ACACCCAATTCTGAA AATGG

☐ indicates position of methylphosphonothioate linkage

The above oligonucleotides were synthesized on a 1μmole scale using phosphoramidite approach. Oligonucleotide **4** was synthesized using nucleoside ß-cyanoethyl phosphoramidite, followed by oxidation with iodine reagent after each coupling. For assembly of oligonucleotides **5**, **6** and **7**, the first two, three and four couplings respectively were carried out using nucleoside methylphosphonamidites, followed by oxidation with 3H-1,2-benzodithiole-3-one 1,1-dioxide. The rest of the couplings were then carried out using nucleoside ß-cyanoethyl phosphoramidites followed by oxidation with iodine reagent and was deprotected as described above. Purification was carried out using polyacrylamide gel electrophoresis (20% containing 7M urea).

Oligonucleotides **4**, **5**, **6** and **7** have been studied for their nuclease resistance against a 3'-exonuclease (10). $T_{1/2}$ of digestion of oligonucleotides **4**, **5**, **6** and **7** was 44, 210, 264 and 401 seconds respectively. The hyperchromicity change was 22.5, 23.5, 18 and 15.5 percent for oligonucleotides **4**, **5**, **6** and **7** respectively.

Duplex stability of oligonucleotides **4**, **5**, **6** and **7** with complementary RNA have been studied *(Figure 2)*. Melting temperatures of the duplex were 51.2°C, 47.8°C, 48°C and 47.1°C in 100 mM NaCl and 46.3°C, 44.1°C, 44.1°C and 43.6°C in 50 mM NaCl respectively for oligonucleotides **4**, **5**, **6** and **7**.

Various oligonucleotides and their analogs containing methylphosphonothioates linkages at 3'-end are being studied for their gene regulation activity in cells.

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10 0.4 A_{260} units of oligonucleotide was lyophilized, suspended in 0.5 ml of buffer (10 mM Tris 10 mM $MgCl_2$, pH 8.5), mixed with 5 μL (1.5×10^{-3} units) of snake venom phosphodiesterase. The mixture was inclubated at 37°C and A_{260} was recorded over 30 minutes.
11 HPLC was carried out using Waters 600E gradient system, 486 variable wavelength detector, and 720 data module. For reversed phase HPLC, column used was Novapak C_{18} (Waters), buffers (A) 100 mM NH_4OAc, (B) buffer A containing 80% CH_3CN; gradient 0% buffer B for 2', 0-60% buffer B over 30 min; flow 1.5 ml min^{-1}; detector 260 nm. For ion exchange HPLC column used was Partisil SAX (Whatman), buffers (A) 1 mM KH_2PO_4 (pH 6.3) (B) 300 mM KH_2PO_4 (pH 6.3), both containing 60% $HCONH_2$; gradient 0% B for 2', 0-20% B over 25'min, flow 3 mL min^{-1}, detector 280 nm.
12 Melting curves were recorded on a Perkin Elmer Lambda-UV/VIS spectrometer equipped with a temperature programmer. Oligonucelotides 4, 5, 6 and 7 were mixed with complementary oligoribonucelotide 5'GUUAUCCAUUUUCAGAAUUGGGUGUCGACAU3' at equimolar concentration (0.2 μm each) in buffer (100 mM NaCl, 10 mM Na_2HPO_4 pH 7.4 or 50 mM NaCl, 5 mM Na_2HPO_4 pH 7.4) The duplex was heated to 80° C, then cooled down to room temperature. The duplex was then heated from 25°C to 70°C at a rate of 1°C/min and A_{260} was recorded against temperature.
13 NMR Data was recorded on a Varian Gemini 300 Spectrometer in D_2O solutions using phosphoric acid as the external reference.