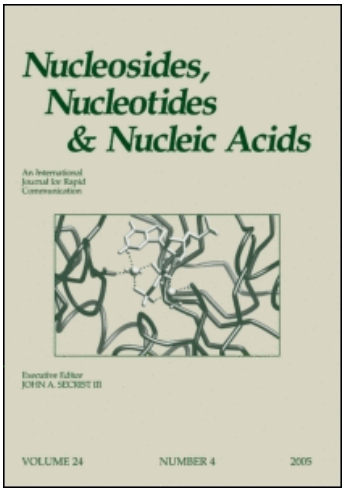


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# Diastereomeric Process Control in the Synthesis of 2'-*O*-(2-Methoxyethyl) Oligoribonucleotide Phosphorothioates as Antisense Drugs

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# Diastereomeric Process Control in the Synthesis of 2′-*O*-(2-Methoxyethyl) Oligoribonucleotide Phosphorothioates as Antisense Drugs

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## ABSTRACT

Coupling of 2′-*O*-methoxyethylsubstituted nucleoside phosphoramidites to 5′-hydroxyl group of a nucleoside or nucleotide on solid support is under stereochemical process control and is independent of scale, concentration, synthesizer, ratio of amidite diastereomers, solid support etc. However, activators and phosphate protecting groups do play a role in influencing the ratio of phosphorothioate diesters obtained by sulfurization of phosphite triesters.

*Key Words:* Coupling; Stereochemistry; Process control; Oligomerization; Activators; Phosphate protecting group.

## INTRODUCTION

Phosphorothioate-linked nucleic acid analogs have found widespread application in therapeutic drug development and molecular biology. The increased resistance to nuclease digestion displayed by these analogs has prompted their

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consideration for antisense therapy of a variety of diseases. Several antisense phosphorothioate oligodeoxyribonucleotides (ODN) are currently undergoing clinical evaluation and the first antisense drug for treatment of CMV retinitis (Vitravene™) has reached the market.

Although phosphorothioate ODN are showing excellent promise as safe and effective therapeutic agents, their profiles are not ideal. In order to increase the therapeutic value of antisense drugs various back-bone, sugar and base modifications were investigated in our organization. Among them, 2′-*O*-methoxyethyl modified oligonucleotides have been selected as second generation analogs for drug development. Multiple antisense 2′-*O*-methoxyethyl (2′-*O*-MOE) modified phosphorothioate oligonucleotides have advanced to the clinic and many others are being investigated for a variety of host diseases. All of these second generation antisense drug candidates are so called gapmers, viz. deoxynucleoside phosphorothioates flanked by 2′-*O*-MOE ribonucleoside phosphorothioates. These drugs have large potential markets and as such may demand several hundreds of kilograms of drug per indication. Thus, there is urgent need for synthesis of high quality 2′-*O*-methoxyethyl modified phosphorothioate oligoribonucleotides.

An *O*,*O*-linked phosphorothioate diester linkage is chiral. This leads to two issues. First, for a typical 20-mer, there are 524,288 ($2^{19}$) possible diastereomers. Separation and analytical control of this number of diastereomers is not feasible. Secondly, stereospecific synthesis of phosphorothioate linkages is not currently practical for development of 20-mer antisense drugs. A key to registration of second-generation 2′-*O*-MOE phosphorothioate oligonucleotide drugs is thus the demonstration of Rp/Sp ratio reproducibility at phosphorus centers in the product of non-stereocontrolled synthesis. We disclose herein the results of our work, which clearly demonstrate that the stereochemical outcome is under inherent control.



## EXPERIMENTAL

The experimental approach chosen was to synthesize dimers and short oligonucleotides about 5-mer in length in which a single phosphorus center was replaced by a phosphorothioate linkage. Oligonucleotide syntheses were performed on a Pharmacia OligoPilot I or II or Akta DNA/RNA synthesizer by the phosphoramidite method. Phosphate diester linkages were incorporated via oxidation of phosphite triesters using a 15% (v/v) solution of *tert*-butyl hydroperoxide in $CH_3CN$ for 15 min. Phosphorothioate linkages were introduced by sulfurization with a 0.2 M solution of phenylacetyl disulfide in $CH_3CN$/3-picoline (1:1 v/v) for a contact time of 2 min. Detritylation was effected by treatment with a 3% (v/v) solution of dichloroacetic acid in toluene. Phosphoramidites were dissolved to a nominal concentration of 0.2 M in anhydrous $CH_3CN$ and activated with two volumes of a 0.45 M solution of 1*H*-tetrazole in $CH_3CN$. Activation with PTFA was carried out with a 0.22 M solution in $CH_3CN$ in combination with 0.11 M solution of *N*-methylimidazole. Similarly, 4,5-dicyanoimidazole (DCI) was used as a 0.8 M solution in $CH_3CN$ for activating phosphoramidites. Final detritylation at the end of synthesis was performed on column before deprotection and cleavage. Following



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chain assembly the support-bound DMT-off oligonucleotide/dimer (300 mg) was treated with concentrated ammonium hydroxide ($NH_4OH$, 10 mL) for 12 h at 55°C. The products were filtered and the filtrate evaporated under reduced pressure. The residue was dissolved in deuterium oxide (0.5 mL) and carefully transferred to a 5 mm NMR tube for analysis. Good separation of two phosphorothioate diastereomer signals was observed, and a minimum signal-to-noise ratio of 200 was obtained for all samples analyzed (Fig. 1).

**Assignment of Stereochemistry.** The *Rp* and *Sp* configurations of 2′-*O*-methoxyethyl modified phosphorothioate linkages were tentatively assigned based on earlier investigation of an analogous molecule viz. 2′-*O*-methyl oligoribonucleotide phosphorothioates. Thus, the up field shift in the $^{31}P$ NMR signal of 2′-*O*-methoxyethyl modified phosphorothioate diester linkage was assigned the *Sp* configuration and downfield shift signal was assigned the *Rp* configuration. Work on assigning the absolute configuration is under progress.



**Factors That Could Influence Stereochemistry.** The stereochemical outcome of phosphorothioate linkages could potentially be influenced by factors such as diastereomeric composition of phosphoramidites, scale, synthesizer, solid support, bases (purines vs. pyrimidines), concentration, equivalents of amidites used, incoming monomer (MOE vs. deoxy), 5′-terminal nucleoside on solid support, phosphate protecting group, and activators.

**Diastereomeric Composition of Phosphoramidites.** The two diastereoisomers of commercially available 5′-*O*-DMT-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite of 5-methyl-2′-*O*-methoxyethyluridine were cleanly separated using flash silica gel chromatography on 15 g scale and used for investigation (Figs. 2 and 3). A pentamer (5′-MOE me Ups TTTT-3′) was synthesized using diastereomer **I** and diastereomer II as well as racemic mixture. Each experiment was repeated four times and in all cases the ratio (Rp:Sp = 58:43 ppm) was highly reproducible and consistent.

***Table 1.*** Analysis of phosphorothioate oligomers using various phosphate protecting group in presence of tetrazole as activator.

| Oligomer | Scale (μmole) | MOE meU amidite | $^{31}P$ NMR ($D_2O$) | |
|---|---|---|---|---|
| | | | PPM | Diastereomer ratio |
| 5′-MOE meU ps TTTT-3′ | 167 | CNE | 57.56/56.51 | 57.68/42.32 |
| 5′-MOE meU ps TTTT-3′ | 134 | Me | 57.73/56.61 | 60.65/39.35 |
| 5′-MOE meU ps TTTT-3′ | 165 | APOE | 57.56/56.56 | 63.13/36.87 |
| 5′-MOE meC ps TTTT-3′ | 168 | CNE | 57.88/56.36 | 52.97/47.03 |
| 5′-MOE meC ps TTTT-3′ | 140 | Me | 57.97/56.39 | 56.22/43.78 |
| 5′-MOE meC ps TTTT-3′ | 148 | APOE | 57.94/56.36 | 59.07/40.93 |



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***Table 2.*** Analysis of phosphorothioate oligomers using various activators.

| Oligomer | Scale (μmole) | Activator (pKa [$H_2O$]) | $^{31}$P NMR ($D_2O$) PPM | Diastereomer ratio |
|---|---|---|---|---|
| 5′-MOE meU ps TTTT-3′ | 172 | ETT(4.28) | 57.54/56.52 | 69.61/30.39 |
| 5′-MOE meU ps TTTT-3′ | 167 | 1*H*-Tetrazole(4.8) | 57.56/56.51 | 57.68/42.32 |
| 5′-MOE meU ps TTTT-3′ | 162 | PTFA(5.2) | 57.51/56.52 | 51.19/48.81 |
| 5′-MOE meU ps TTTT-3′ | 171 | 4,5-DCI(5.5) | 57.54/56.53 | 45.16/54.84 |
| 5′-MOE meU ps TTTT-3′ | 159 | Im Tf(6.9) | 57.52/56.58 | 27.51/72.49 |
| 5′-MOE meC ps TTTT-3′ | 172 | ETT(4.28) | 57.80/56.34 | 65.21/34.79 |
| 5′-MOE meC ps TTTT-3′ | 168 | 1*H*-Tetrazole(4.8) | 57.88/56.36 | 52.97/47.03 |
| 5′-MOE meC ps TTTT-3′ | 173 | PTFA(5.2) | 57.83/56.35 | 49.52/50.40 |
| 5′-MOE meC ps TTTT-3′ | 173 | 4,5-DCI(5.5) | 57.82/56.34 | 41.86/58.14 |
| 5′-MOE meC ps TTTT-3′ | 167 | Im Tf(6.9) | 57.82/56.39 | 30.31/69.69 |

**Synthesizer, Scale, and Support for Oligonucleotide Synthesis.** Multiple dimers were synthesized on different scales using different synthesizers (OligoPilot I (30 μmole), OligoPilot II (ca 250 μmole) and Akta OligoPilot (1000 μmole). In all cases the ratios were highly reproducible indicating that synthesizers and scale do

***Table 3.*** Diastereomeric reproducibility of phosphorothioate linkages during oligonucleotide synthesis.

| Oligomer | Scale (μmole) | $^{31}$P NMR ($D_2O$) PPM | Diastereomer ratio |
|---|---|---|---|
| 5′-MOE G ps TTTT-3′ | 149 | 57.69/56.32 | 49.17/50.83 |
| 5′-MOE G ps TTTT-3′ | 148 | 57.68/56.32 | 49.27/50.73 |
| 5′-MOE G ps TTTT-3′ | 154 | 57.68/56.33 | 49.55/50.45 |
| 5′-MOE G ps TTTT-3′ | 160 | 57.68/56.32 | 49.68/50.32 |
| 5′-MOE G ps MOE meU po TTT-3′ | 148 | 58.22/56.02 | 49.02/50.98 |
| 5′-MOE G ps MOE meU po TTT-3′ | 158 | 58.25/56.01 | 49.17/50.83 |
| 5′-MOE G ps MOE meU po TTT-3′ | 147 | 58.26/56.02 | 49.33/50.67 |
| 5′-MOE G ps MOE meU po TTT-3′ | 140 | 58.21/56.01 | 49.46/50.54 |
| 5′-MOE G ps MOE meC po TTT-3′ | 149 | 59.52/55.31 | 49.65/50.35 |
| 5′-MOE G ps MOE meC po TTT-3′ | 151 | 59.48/55.36 | 49.99/50.01 |
| 5′-MOE G ps MOE meC po TTT-3′ | 147 | 59.51/55.31 | 49.39/50.61 |
| 5′-MOE G ps MOE meC po TTT-3′ | 162 | 59.56/55.23 | 49.71/50.29 |
| 5′-dA ps MOE meU po TTT-3′ | 159 | 56.80/56.01 | 57.18/42.82 |
| 5′-dA ps MOE meU po TTT-3′ | 167 | 56.80/55.99 | 57.05/42.95 |
| 5′-dA ps MOE meU po TTT-3′ | 161 | 56.76/55.98 | 57.85/42.15 |
| 5′-dA ps MOE meU po TTT-3′ | 164 | 56.80/55.99 | 57.79/42.21 |
| 5′-dG ps MOE meU po TTT-3′ | 169 | 56.64/56.07 | 47.57/52.43 |
| 5′-dG ps MOE meU po TTT-3′ | 152 | 56.68/56.09 | 47.14/52.86 |
| 5′-dG ps MOE meU po TTT-3′ | 157 | 56.65/56.07 | 47.19/52.81 |
| 5′-dG ps MOE meU po TTT-3′ | 169 | 56.63/56.07 | 47.50/52.50 |





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not play any role in determining the ratio of phosphorothioate diastereomer formation. In addition, three different solid supports were evaluated and found to play no role also.

**Concentration of Activator.** 5-ethylthio-1*H*-tetrazole (ETT) was chosen as an example. Various concentrations (0.05, 0.1, 0.2, 0.4, 0.5, 0.8, 1.2 M) were chosen for the synthesis of pentamer (5′-MOE meC ps TTTT-3′). Once again, no difference in ratio of phosphorothioate diastereomers was observed.

**Phosphate Protecting Group and Activators.** Three different phosphate protecting groups were used for the investigation viz. β-cyanoethyl (CNE), methyl (Me) and (2′-acetoxy)-2-phenoxyethyl (APOE). Table 1 shows the influence of protecting group on diastereoselectivity of phosphorothioate linkages. Similarly, five different activators 5-ethylthio-1*H*-tetrazole (ETT), 1*H*-tetrazole, pyridinium trifluoroacetate (PTFA), 4,5-dicyanoimidazole (DCI), and imidazolium triflate (Im Tf) were chosen for investigation. Table 2 demonstrates the influence of activators during the coupling of 2′-*O*-methoxyethyl substituted phosphoramidites to form phosphorothioate linkages.

**Reproducibility of Diastereomeric Composition of Phosphorothioate Linkages.** An important question to ask is: Do we synthesize batch-to-batch similar

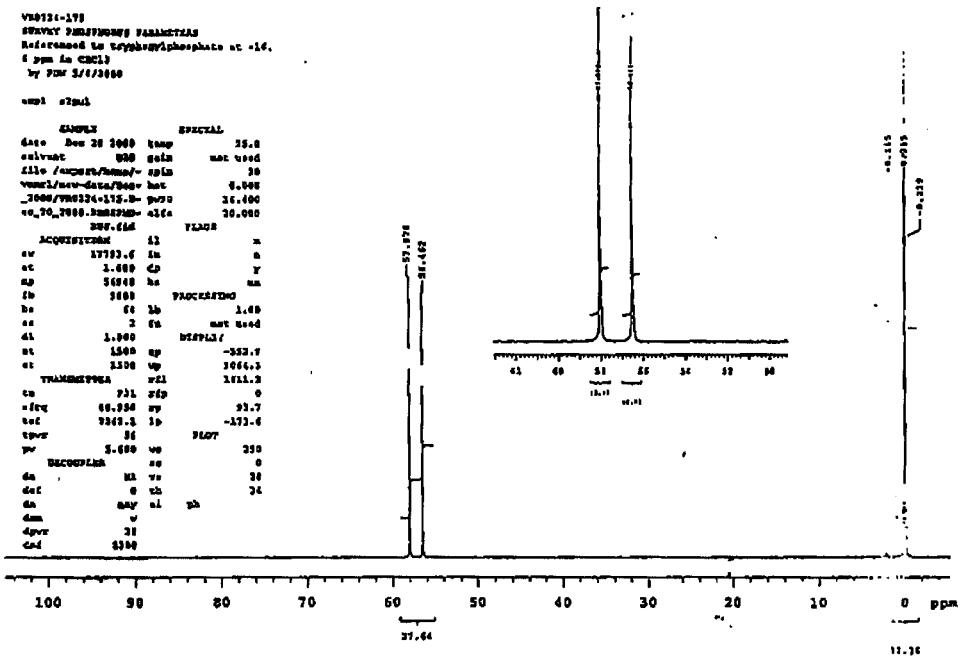


*Figure 1.* $^{31}P$ NMR($D_2O$) of a typical 5-mer phosphate/phosphorothioate oligonucleotide.

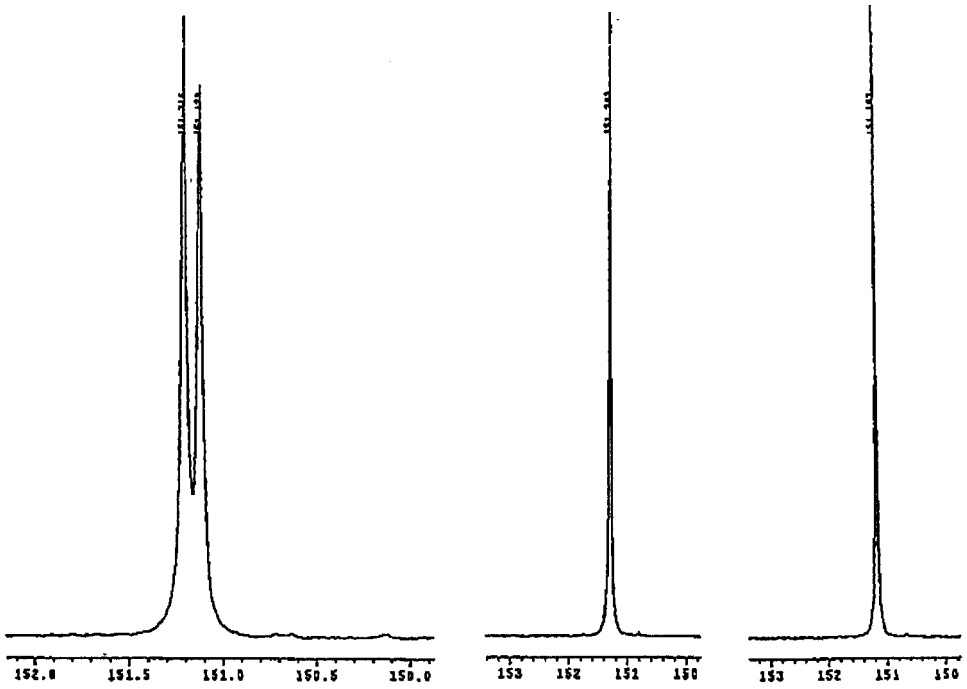


*Figure 2.* $^{31}P$ NMR($CDC1_3$) of racemic and purified 5′-*O*-DMT-2′-*O*-methoxyethyl-5-methyluridine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite.

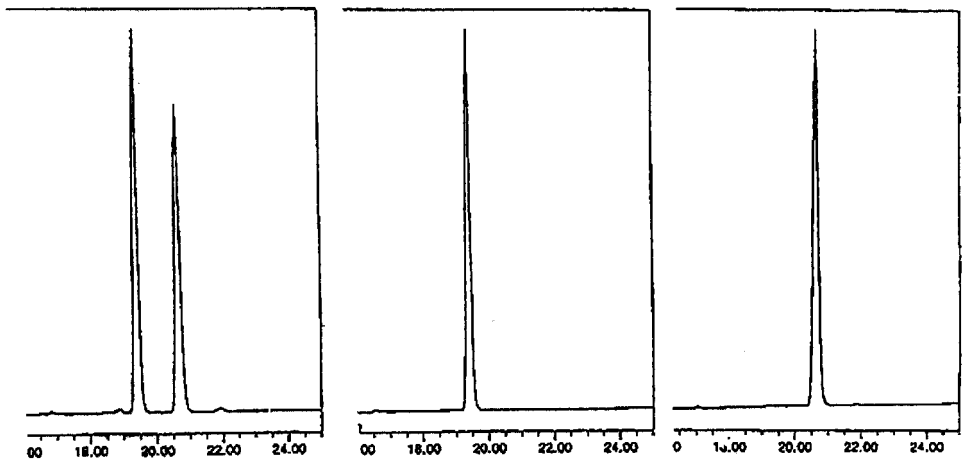


*Figure 3.* HPLC analysis of racemic and purified 5′-*O*-DMT-2′*O*-methoxyethyl-5-methyluridine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite.





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composition of phosphorothioate linkages under a given set of conditions? Table 3 clearly demonstrates that stereochemistry is highly reproducible and is under inherent process control during the synthesis of 2′-*O*-methoxyethyl phosphorothioate oligonucleotides.

## CONCLUSION

Based on the extensive investigation performed, we can conclude that except phosphate protecting group and activators, all other factors have undetectable role in influencing the outcome of 2′-*O*-methoxyethyl substituted phosphorothioate linkages.





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