Organic Process Research & Development **2002**, *6*, 798−806

Development of 2′-*O*-Methoxyethyl Phosphorothioate Oligonucleotides as Antisense Drugs under Stereochemical Control

Vasulinga T. Ravikumar* and Douglas L. Cole

Isis Pharmaceuticals, Inc., 2282 Faraday Avenue, Carlsbad, California 92008, U.S.A.

Abstract:

A systematic investigation of the stereoreproducibility of 2′-*O*-methoxyethylribonucleoside phosphoramidite-based phosphorothioate oligonucleotide synthesis reveals that 1*H*-tetrazole-catalyzed phosphoramidite coupling followed by sulfurization with phenylacetyl disulfide (PADS) is under inherent stereochemical control. Analyses of single phosphorothioate linkages in oligonucleotides as well as in dimers by ^{31}P NMR spectroscopy reveal reproducible stereochemical control in deoxy/2′-*O*-methoxyethylribo- and 2′-*O*-methoxyethylribo/deoxydinucleotide phosphorothioates and in 2′-*O*-methoxyethylribo/2′-*O*-methoxyethylribonucleotide phosphorothioate linkages. Particularly interesting is the fact that the R,S ratio of the phosphorothioate linkage is reproducible (within limits of experimental error) between syntheses and is *independent of diastereomeric composition of phosphoramidites, synthesis scale, solid support, reactor, concentration during coupling or synthesis conditions*. Activators, however, play a major role in determining the diastereomeric ratio of 2′-*O*-methoxyethyl RNA phosphorothioate internucleotide linkages.

Introduction

Phosphorothioate-linked nucleic acid analogues have found widespread application in therapeutic drug development and molecular biology.[1−6] The increased resistance to nuclease digestion displayed by these analogues has prompted their consideration for antisense therapy of a variety of diseases.[7,8] 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. Reported high-dose effects of phosphorothioate ODN include immune cell stimulation, complement activation, and introduction of blood clotting abnormalities.[9−13] Although such effects are seen only at oligonucleotide doses above those required for pharmacological activity, investigation of chemical modifications to enhance already acceptable therapeutic indices and facilitate delivery has been pursued. Chemical modification of antisense oligonucleotides can confer additional resistance to nucleases, longer serum half-life, and reduced toxicity.[14−18] Modifications can also increase affinity of an antisense oligonucleotide for its complementary target RNA, resulting in enhanced drug potency and specificity.[19−23] Among various backbone, sugar, and base modifications investigated thus far in our organization, 2′-*O*-methoxyethyl-modified oligonucleotides have been selected as second generation analogues for drug development. This modification, in addition to increasing drug potency, also appears to decrease potential acute toxicities in primates, namely increases in aPTT and complement activation.[24−26]

(1) Crooke, S. T. *Antisense Therapeutics in Biotechnology & Genetic Engineering Reviews*; Intercept Ltd: Hampshire, UK, 1998; Vol. 15, pp 121−157.
(2) Crooke, S. T. In *Basic Principles of Antisense Therapeutics in Handbook of Experimental Pharmacology: Antisense Research & Application*; Crooke, S. T., Ed.; Springer-Verlag: Berlin, Germany, 1998.
(3) Crooke, S. T. *Antisense Nucleic Acid Drug Dev.* **1998**, *8*, 115.
(4) Crooke, S. T. In *Burger's Medicinal Chemistry and Drug Discovery*; John Wiley: New York, 1995 and references therein.
(5) Monia, B. P.; Johnston, J. F.; Muller, M.; Geiger, T. H.; Fabbro, D. *Nature Med.* **1996**, *2*, 668.
(6) Crooke, S. T., Lebleu, B., Eds. *Antisense Research and Applications*; CRC Press: Boca Raton, 1993.
(7) Kisner, D. L. *12th International Roundtable on Nucleosides and Nucleotides*; September 19, 1996, La Jolla, CA.
(8) More than 15 oligodeoxyribonucleotide phosphorothioates are currently under clinical investigation for the treatment of various diseases.
(9) Cornish, K. G.; Iversen, P.; Smith, L.; Arneson, M.; Bayever, E. *Pharmacol. Commun.* **1993**, *3*, 239−247.
(10) Galbrith, W. M.; Hobson, W. C.; Giclas, P. C.; Schechter, P. J.; Agrawal, S. *Antisense Res. Dev.* **1994**, *4*, 201−206.
(11) Krieg, A. M.; Yi, A.-K.; Matson, S.; Waldschmidt, T. J.; Bishop, G. A.; Teasdale, R.; Koretzky, G. A.; Klinman, D. M. *Nature* **1995**, *374*, 546−549.
(12) Levin, A. A.; Monteith, D. K.; Leeds, J. M.; Nicklin, P. L.; Geary, R. S.; Butler, M.; Templin, M. V.; Henry, S. P. Toxicity of oligodeoxynucleotide therapeutic agents. In *Antisense Research and Applications*; Crooke, S. T., Ed.; Springer-Verlag: Heidelberg, Germany; pp 169−215, 1998.
(13) Pisetsky, D. S.; Reich, C. F. *Life Sci.* **1994**, *54*, 101−107.
(14) Crooke, S. T.; Graham, M. J.; Zuckerman, J. E.; Brooks, D.; Conklin, B. S.; Cummins, L. L.; Greig, M. J.; Guinosso, C. J.; Kornbrust, D.; Manoharan, M.; Sasmor, H. M.; Schleich, T.; Tivel, K. L.; Griffey, R. H. *J. Pharmacol. Exp. Ther.* **1996**, *277*, 923−937.
(15) Cummins, L. L.; Ownes, S. R.; Risen, L. M.; Lesnik, E. A.; Freier, S. M.; McGee, D.; Guinosso, C. J.; Cook, P. D. *Nucleic Acids Res.* **1995**, *23*, 2019−2024.
(16) Henry, S. P.; Zuckerman, J. E.; Rojko, J.; Hall, W. C.; Harman, R. J.; Kitchen, D.; Crooke, S. T. *Anticancer Drug Des.* **1996**, *12*, 1−14.
(17) Zhang, R.; Iyer, R. P.; Yu, D.; Tan, W.; Zhang, X.; Lu, Z.; Zhao, H.; Agarwal, S. *J. Pharmacol. Exp. Ther.* **1996**, *278*, 971−979.
(18) Zhao, Q.; Temsamani, J.; Iadarola, P. L.; Jiang, Z.; Agrawal, S. *Biochem. Pharmacol.* **1995**, *51*, 173−182.
(19) Altmann, K.-H.; Dean, N. M.; Fabbro, D.; Freier, S. M.; Geiger, T.; Haner, R.; Husken, D.; Martin, P.; Monia, B. P.; Muller, M.; Natt, F.; Nicklin, P.; Phillips, J.; Pieles, U.; Sasmor, H.; Moser, H. E. *Chimia* **1996**, *50*, 168−176.
(20) Freier, S. M.; Altmann, K.-H. *Nucleic Acids Res.* **1997**, *25*, 4429−4443.
(21) Freier, S. M.; Lima, W. F.; Sanghvi, Y. S.; Vickers, T.; Zounes, M.; Cook, P. D.; Ecker, D. J. Thermodynamics of Antisense Oligonucleotide Hybridization. In *Gene Regulation: Biology of Antisense RNA and DNA*; Erickson, R. P., Izant, J. G., Eds.; Raven Press: New York, 1992; pp 95−107.
(22) Matteucci, M. *Perspect. Drug Dis. Des.* **1996**, *4*, 1−6.
(23) Monia, B. P.; Lesnik, E. A.; Gonzalez, C.; Lima, W. F.; McGee, D.; Guinosso, C. J.; Kawasaki, A. M.; Cook, P. D.; Freier, S. M. *J. Biol. Chem.* **1993**, *268*, 14514−14522.
(24) Henry, S.; Stecker, K.; Brooks, D.; Monteith, D.; Conklin, B.; Bennett, C. F. *J. Pharmacol. Exp. Ther.* **2000**, *292*, 468−479.
(25) Monteith, D. K.; Levin, A. A. *Toxicol. Pathol.* **1999**, *27*, 8−13.

Published on Web 10/30/2002
10.1021/op020061c CCC: $22.00

Multiple antisense 2′-*O*-methoxyethyl (2′-*O*-MOE)-modified phosphorothioate oligonucleotides have advanced to the clinic,[27] and many others are being investigated for a variety of host diseases.[28] All of these second-generation antisense drug candidates are so-called gapmers, viz. deoxynucleoside phosphorothioates flanked by 2′-*O*-MOE ribonucleoside phosphorothioates. However, all do not have same gap size; some but not all are 5−10−5 sequences. 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*-MOE-modified phosphorothioate oligoribonucleotides.[29,30]

The current route of choice for large-scale synthesis of uniform phosphorothioate oligodeoxyribonucleotides is phosphoramidite coupling, because of its potential for automation, high coupling efficiency, and ready scaleability.[31,32] Typically, oligonucleotide synthesis on scales up to 300−600 mmol is performed in a cyclic manner on the Pharmacia OligoProcess DNA/RNA synthesizer using a packed-bed column. Deoxyribonucleoside phosphoramidite coupling is highly efficient at low synthon excess (1.75−2.0 mol equiv) and coupling efficiency is very high (98.5−98.7%). The total synthesis cycle time is short (<8 h) for a 20-mer phosphorothioate. However, large-scale synthesis of 2′-*O*-MOE-modified phosphorothioate oligoribonucleotides requires optimization. In particular, understanding stereochemical control of phosphorothioate linkages between deoxy and 2′-*O*-MOE nucleosides and within uniform 2′-*O*-MOE sequences requires exploration in detail.

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. Second, stereospecific synthesis of phosphorothioate linkages[33−35] is not currently practical or useful 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 nonstereocontrolled synthesis. We disclose herein the results of our work, which clearly demonstrate that the stereochemical outcome is under inherent control.

Experimental Section

Methods and Materials. Standard phosphoramidite monomer synthons, 5′-DMT-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidites of thymidine, N^4-benzoyl deoxycytidine, N^2-isobutyryldeoxyguanosine, N^6-benzoyldeoxyadenosine, 5-methyl-2′-*O*-methoxyethyluridine, 5-methyl-2′-*O*-methoxyethylcytidine, N^6-benzoyl-2′-*O*-methoxyethyladenosine, and N^2-isobutyryl-2′-*O*-methoxyethylguanosine were purchased from Amersham-Pharmacia Biotech (Piscataway, NJ). 1*H*-Tetrazole was purchased from American International Company (Boston, MA). *tert*-Butyl hydroperoxide was purchased from Fluka Chemical Co. as a 70% aqueous solution. Phenylacetyl disulfide was purchased from H. C. Brown Labs (Mumbai, India). Anhydrous acetonitrile (<30 ppm water content) was purchased from Burdick & Jackson. Primer HL30 (loading 85−95 μmol/g) and PS 200 (loading ca. 200 μmol/g) solid supports were purchased from Amersham-Pharmacia Biotech (Piscataway, NJ). Reloaded PS 200 polystyrene solid support at a loading of 190 μmol/g was prepared in-house using DMT thymidine succinate under a slightly modified condition. ^{31}P NMR spectra were recorded on a Varian Unity Plus spectrometer at 161.9 MHz at room temperature. A minimum signal-to-noise ratio of 200 was obtained for all samples. Chemical shifts (δ) are given in ppm relative to H_3PO_4.

Automated Synthesis of Monophosphorothioate Nucleotides. Oligonucleotide and dimer syntheses were performed on a Pharmacia OligoPilot I or II or Akta DNA/RNA synthesizer by the phosphoramidite coupling method. The solid support was packed in a 1.6- or 6.3-mL stainless reactor column before use. Synthesis on Akta DNA/RNA synthesizer was performed using a glass-lined variable-scale synthesis column. Typical synthesis scales on the OligoPilot I and OligoPilot II are in the ranges of 25−35 and 150−220 μmoles, respectively. Phosphate diester linkages were incorporated via oxidation of phosphite triesters using a 15% (v/v) solution of *tert*-butyl hydroperoxide in acetonitrile at a flow rate of 5 mL/min for 15 min. Phosphorothioate linkages were introduced by sulfurization with 4 cm^3 of a 0.2 M solution of phenylacetyl disulfide in acetonitrile/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 for 4 min at a flow rate of 12.5 mL/min.[36−38] Phosphoramidites were dissolved to a nominal concentration of 0.2 M in anhydrous acetonitrile and activated with two volumes of a 0.45 M solution of 1*H*-tetrazole in acetonitrile; couplings were performed in the recycle mode with a contact time of 5 min. Activation with

(26) Monteith, D. K.; Horner, M. J.; Gillett, N. A.; Butler, M.; Geary, R.; Burckin, T.; Ushiro-Watanabe, T.; Levin, A. A. *Toxicol. Pathol.* **1999**, *27*, 307−317.

(27) ISIS 104838 is a 20-mer 2′-*O*-methoxyethyl phosphorothioate and is in phase I/II clinical studies for the treatment of rheumatoid arthritis and Crohn's disease.

(28) Multiple second-generation phosphorothioate oligonucleotides are in preclinical stages against TNFα, VLA4, ICAM, PTP-1B, c-*raf*, PKCα, etc. for the treatment of a variety of diseases such as psoriasis, cancer, diabetes, asthma, arthritis, multiple sclerosis, diabetic retinopathy, etc.

(29) Scozzari, A. N. In *Large Scale Oligonucleotide Synthesis: Production, Purification and Analysis*; IBC Conference, October 28−29, 1997, San Diego, CA.

(30) It is roughly estimated that for systemic thereapy for a chronic disease about 700−900 kg/year of drug is needed to meet the present demand.

(31) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1992**, *24*, 2223 and references therein.

(32) Iyer, R. P.; Beaucage, S. L. In *Comprehensive Natural Products Chemistry*; Barton, D. H. R., Nakanishi, K., Eds.; DNA and Aspects of Molecular Biology, Vol. 7; Kool, E. T., Ed.; Pergamon Press: Elmsford, NY, 1999; p 105.

(33) Karwowski, B.; Guga, P.; Kobylanska, A.; Stec, W. J. *Nucleosides Nucleotides* **1998**, *17*, 1747−1759.

(34) Yang, X.; Sierzcha-la, A.; Misiura, K.; Niewiarowski, W.; Sochacki, M.; Stec, W. J.; Wieczorek, M. W. *J. Org. Chem.* **1998**, *63*, 7097−7100.

(35) Stec, W. J.; Karwowski, B.; Boczkowska, M.; Guga, P.; Koziolkiewicz, M.; Sochacki, M.; Wieczorek, M. W.; Blaszczyk, J. *J. Am. Chem. Soc.* **1998**, *120*, 7156−7167.

(36) Krotz, A. H.; Carty, R. L.; Moore, M. N.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Green Chem.* **1999**, *1*, 277−282.

(37) Krotz, A. H.; Carty, R. L.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev.* **2000**, *4*, 190−193.

(38) Krotz, A. H.; Cole, D. L.; Ravikumar, V. T. *Bioorg. Med. Chem.* **1999**, *7*, 435−439.

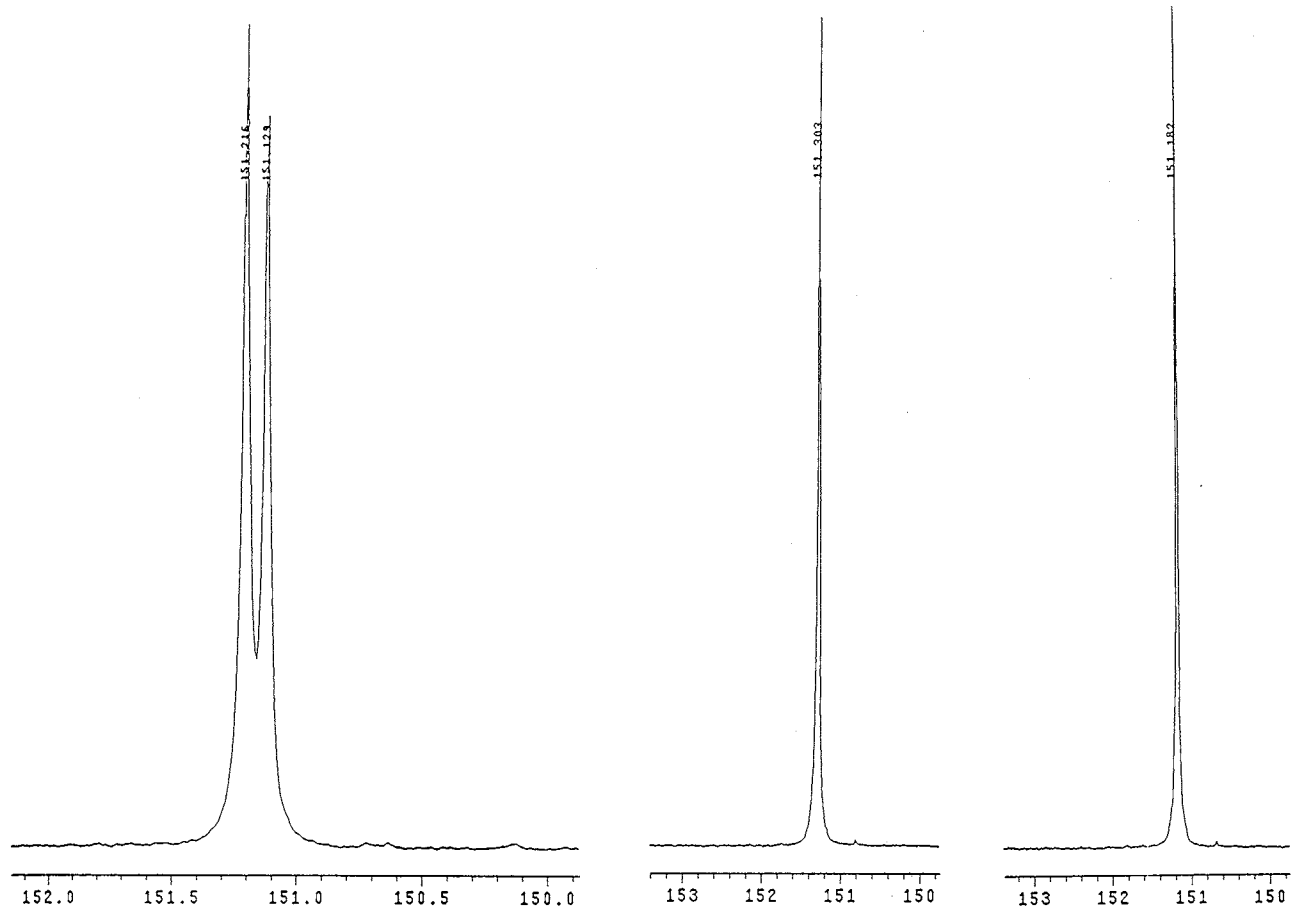


Figure 1. **^{31}P NMR($CDCl_3$) of racemic and individual diastereomers of 5′-*O*-DMT-2′-*O*-methoxyethyl-5-methyluridine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite.**

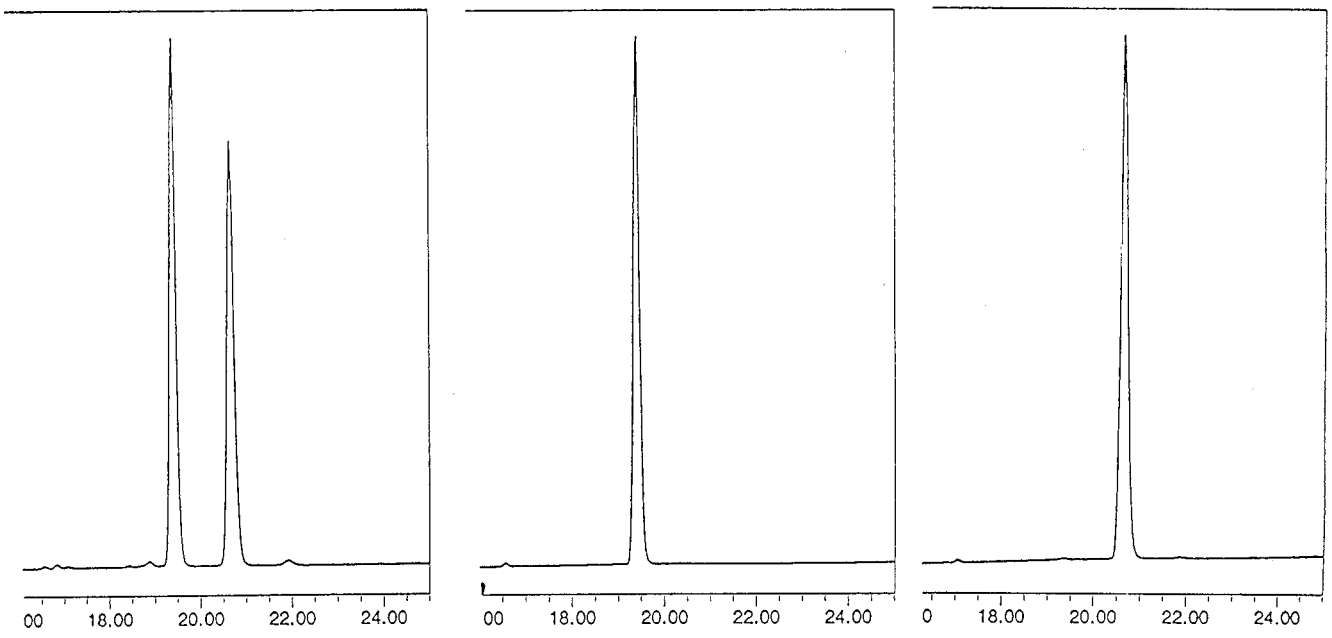


Figure 2. **HPLC analysis of racemic and individual diastereomers of 5′-*O*-DMT-2′-*O*-methoxyethyl-5-methyluridine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite.**

pyridinium trifluoroacetate was carried out with a 0.22 M solution in acetonitrile in combination with 0.11 M solution of *N*-methylimidazole. Similarly, 4,5-dicyanoimidazole (DCI) was used as a 0.8 M solution in acetonitrile for activating phosphoramidites. Capping was performed using 4 mL of a 1:1 v/v mixture of acetic anhydride in acetonitrile (1:4 v/v) and *N*-methylimidazole-pyridine in acetonitrile (2:3:5 v/v/v) for a contact time of 1 min. Final detritylation at the end of synthesis was performed on column before deprotection and cleavage.

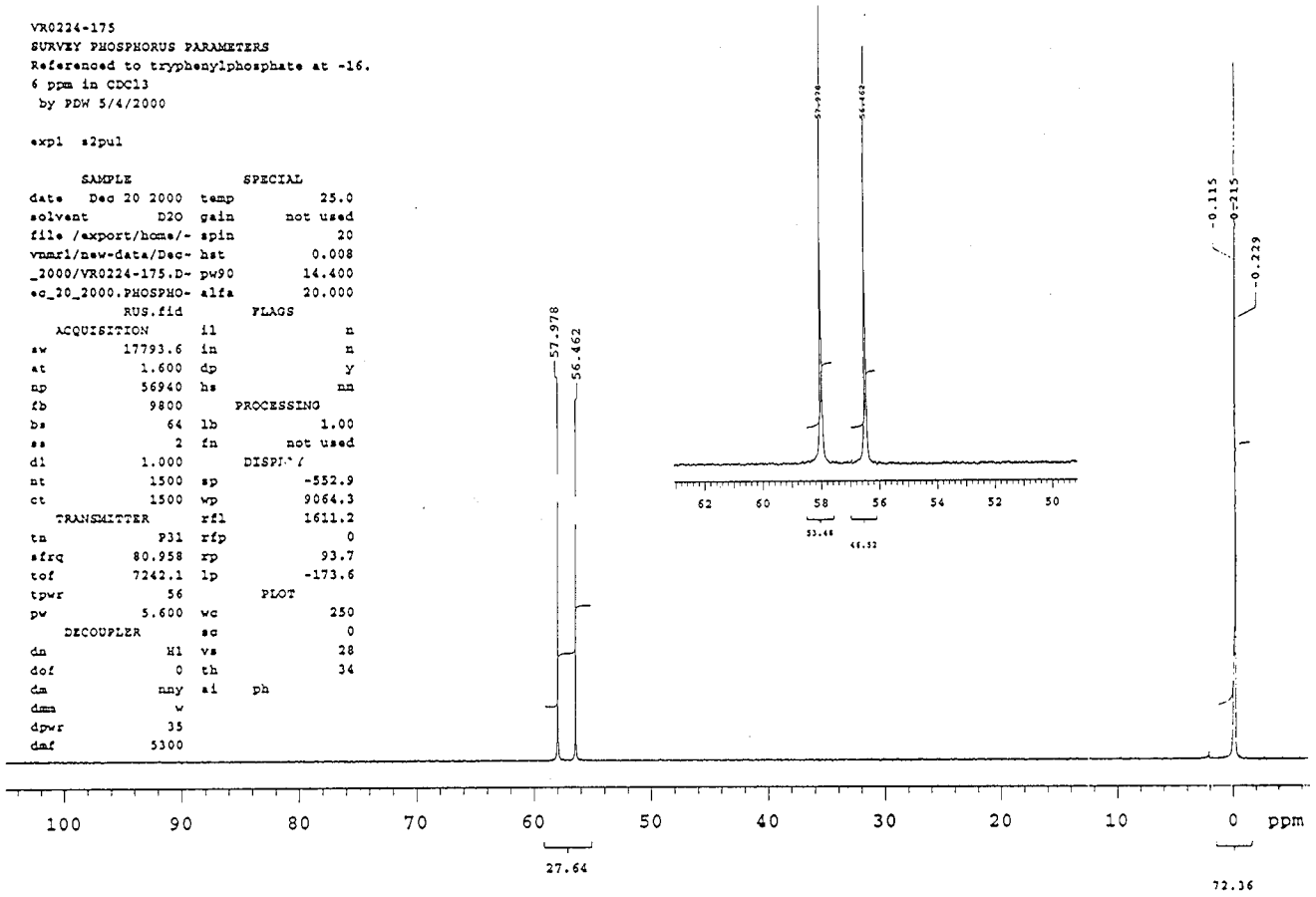


Figure 3. **^{31}P NMR(D_2O) of a typical 5-mer phosphate/phosphorothioate oligonucleotide.**

Deprotection and Analysis of Monophosphorothioate Nucleotides by ^{31}P NMR Spectroscopy. Following chain assembly the support-bound DMT-off oligonucleotide/dimer (300 mg) was treated with concentrated ammonium hydroxide (NH_4OH, 10 cm^3) for 12 h at 55 °C. The products were filtered and the filtrate was 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.

Results and Discussion

The phosphoramidite approach for automated synthesis of phosphorothioate analogues of DNA and modified RNA involves repetitive formation of chiral phosphite triester intermediates, followed by oxidative sulfurization to phosphorothioate triester linkages. Although sulfurization can be accomplished using a variety of sulfur-transfer reagents including 3*H*-1,2-benzodithiol-3-one 1,1-dioxide,[39,40] phenylacetyl disulfide (PADS) has become very popular for the development of antisense drugs due to its very high sulfurization efficiency and inexpensive nature.[41,42]

Table 1. **Analysis of 5′-MOE U^{me} ps TTTT-3′, oligomer using 2′-*O*-MOE U^{me} phosphoramidite diastereomers**

			^{31}P NMR (D_2O)	
expt	MOE U^{me} amidite	scale (μmol)	ppm	diastereomer ratio
0841-090	fraction 1	111	57.59/56.57	57.50/42.50
0841-091	fraction 1	115	57.90/56.86	57.95/42.05
0841-092	fraction 1	138	57.59/56.56	57.27/42.73
0841-093	fraction 1	123	57.60/56.55	57.58/42.42
0841-094	fraction 2	116	57.60/56.58	57.59/42.41
0841-095	fraction 2	115	58.11/57.07	57.67/42.33
0841-096	fraction 2	120	57.55/56.58	57.93/42.07
0841-097	fraction 2	126	57.55/56.58	57.89/42.11
0224-139	racemic	167	57.56/56.51	57.68/42.32
0224-135	racemic	176	57.57/56.54	57.82/42.18
0224-189	racemic	168	57.56/56.59	57.41/42.59
0224-190	racemic	164	57.58/56.60	57.47/42.53

There are several reports[43−48] in the literature including one from our laboratory which show that 1*H*-tetrazole-

(39) Iyer, R. P.; Phillips, L. R.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Org. Chem.* **1990**, *55*, 4693.
(40) Iyer, R. P.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Am. Chem. Soc.* **1990**, *112*, 1253.
(41) Cheruvallath, Z. S.; Carty, R. L.; Moore, M. N.; Capaldi, D. C.; Krotz, A. H.; Wheeler, P. D.; Turney, B. J.; Craig, S. R.; Gaus, H. J.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev.* **2000**, *4*, 199−204.
(42) Cheruvallath, Z. S.; Wheeler, P. D.; Cole, D. L.; Ravikumar, V. T. *Nucleosides Nucleotides* **1999**, *18*, 1195−1197.
(43) Iyer, R. P.; Yu, D.; Ho, N.; Tan, W.; Agrawal, S. *Tetrahedron: Asymmetry* **1995**, *6*, 1051−1054.
(44) Charubala, R.; Stengele, K. P.; Pfleiderer, W. *Nucleosides Nucleotides* **1989**, *8*, 1007−1010.
(45) Stec, W. J.; Zon, G. *Tetrahedron Lett.* **1984**, *25*, 5279−5282.
(46) Stec, W. J.; Zon, G.; Gallo, K. A.; Byrd, R. A. *Tetrahedron Lett.* **1985**, *26*, 2191−2194.
(47) Cheruvallath, Z. S.; Wyrzykiewicz, T. K.; Cole, D. L.; Ravikumar, V. T. *Nucleosides Nucleotides* **1999**, *18*, 1191−1194.
(48) Cheruvallath, Z. S.; Sasmor, H.; Cole, D. L.; Ravikumar, V. T. *Nucleosides, Nucleotides Nucleic Acids* **2000**, *19*, 533−543.

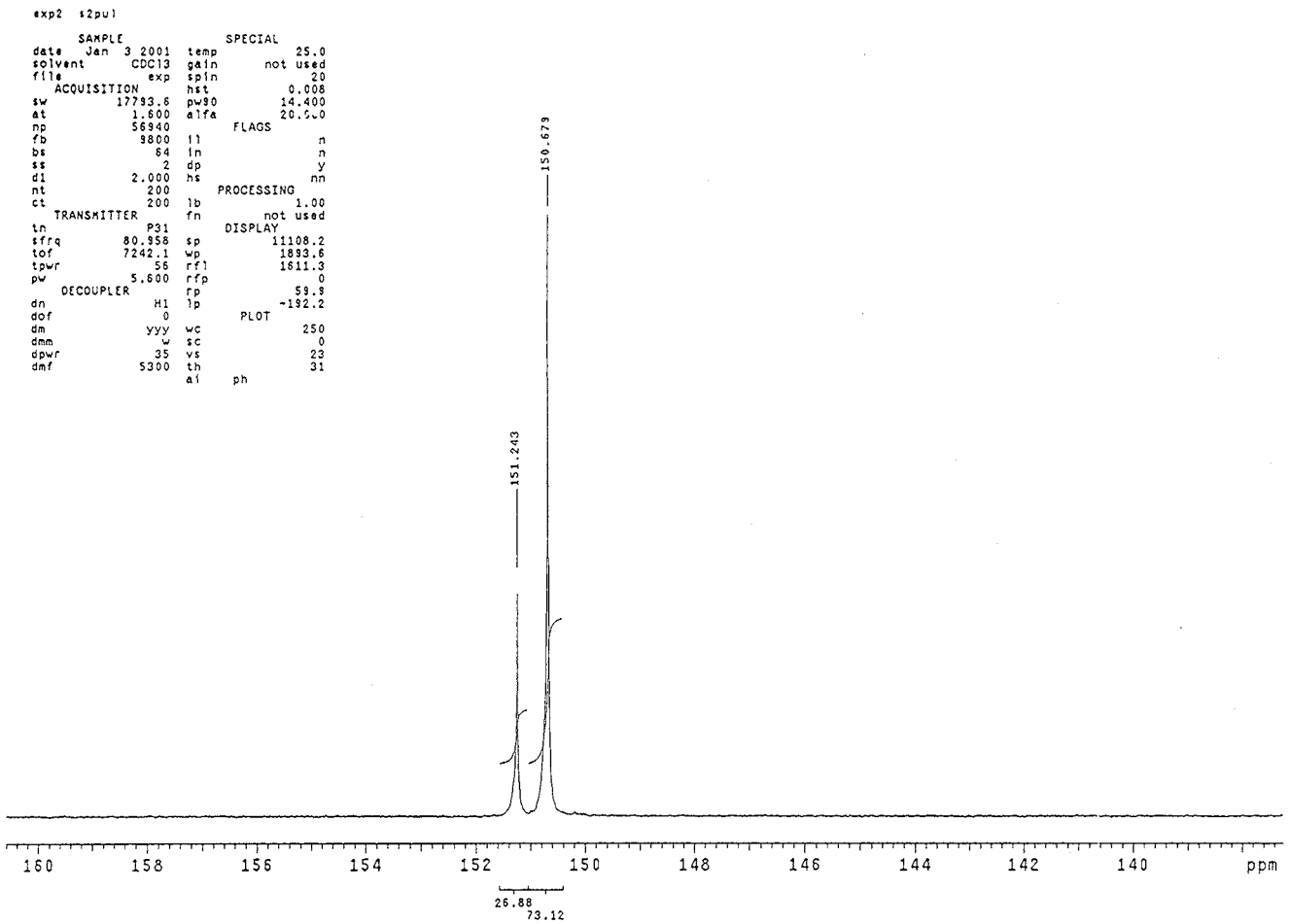


Figure 4. **^{31}P NMR($CDCl_3$) of racemic 5′-*O*-DMT-*N*^{2}-isobutyryl-2′-*O*-methoxyethylguanosine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite.**

Table 2. **Analysis of 5-mers using diastereomerically enriched 2′-*O*-MOE G amidite**

			^{31}P NMR (D_2O)	
expt	oligomer	scale (μmol)	ppm	diastereomer ratio
0750-84	5′-MOE G ps MOE U^{me} po TTT-3′	148	58.22/56.02	49.02/50.98
0750-85	5′-MOE G ps MOE U^{me} po TTT-3′	158	58.25/56.01	49.17/50.83
0750-86	5′-MOE G ps MOE U^{me} po TTT-3′	147	58.26/56.02	49.33/50.67
0750-87	5′-MOE G ps MOE U^{me} po TTT-3′	140	58.21/56.01	49.46/50.54
0750-88	5′-MOE G ps TTTT-3′	149	57.69/56.32	49.17/50.83
0750-89	5′-MOE G ps TTTT-3′	148	57.68/56.32	49.27/50.73
0750-90	5′-MOE G ps TTTT-3′	154	57.68/56.33	49.55/50.45
0750-91	5′-MOE G ps TTTT-3′	160	57.68/56.32	49.68/50.32
0750-92	5′-MOE G ps MOE C^{me} po TTT-3′	149	59.52/55.31	49.65/ 50.35
0750-93	5′-MOE G ps MOE C^{me} po TTT-3′	151	59.48/55.36	49.99/50.01
0750-94	5′-MOE G ps MOE C^{me} po TTT-3′	147	59.51/55.31	49.39/50.61
0750-95	5′-MOE G ps MOE C^{me} po TTT-3′	162	59.56/55.23	49.71/50.29

activated deoxynucleoside phosphoramidite coupling is a racemic process leading to a nearly 1:1 mixture of Rp and Sp phosphorothioate diastereomers even if one starts with 100% enantiomerically pure phosphoramidite. However, we hypothesized that if chiral atoms or relatively bulky groups such as 2′-*O*-methoxyethyl are present in the vicinity of the phosphorus center, the stereochemical outcome of coupling reaction could be influenced substantially. Also, the impact of sulfurization with phenylacetyl disulfide (PADS) on the stereochemical outcome has not been investigated and reported, although it has been shown that thiolation of phosphites with 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent) occurs with retention of configuration. Thus, there are many factors potentially contributing to the final chirality of synthesized 2′-*O*-methoxyethyl phosphorothioate oligonucleotides.

To minimize our workload and to determine the relative importance of the factors contributing to final chirality of phosphorothioate linkages, we investigated the diastereomeric compositions of phosphoramidites as the first order of

Table 3. **Analysis of 5-mers using various phosphoramidites**

expt	oligomer	scale (μmol)	^{31}P NMR (D_2O) ppm	^{31}P NMR (D_2O) diastereomer ratio
0224-168	5′-MOE A ps TTTT-3′	164	57.57/56.34	53.75/46.25
0224-169	5′-MOE A ps TTTT-3′	163	57.60/56.28	53.96/46.04
0224-170	5′-MOE A ps TTTT-3′	157	57.57/56.30	53.41/46.59
0224-171	5′-MOE A ps TTTT-3′	157	57.58/56.33	53.79/46.21
0224-172	5′-MOE A ps MOE A po TTT-3′	160	57.99/56.46	53.45/46.55
0224-173	5′-MOE A ps MOE A po TTT-3′	176	58.01/56.46	52.91/47.09
0224-174	5′-MOE A ps MOE A po TTT-3′	171	57.97/56.47	52.95/47.05
0224-175	5′-MOE A ps MOE A po TTT-3′	159	57.98/56.46	53.48/46.52
0224-136	5′-MOE U^{me}psMOE U^{me} poTTT-3′	166	57.89/56.24	56.08/43.92
0750-1	5′-MOE U^{me}psMOE U^{me} poTTT-3′	163	57.95/56.30	56.78/43.22
0750-2	5′-MOE U^{me}psMOE U^{me} poTTT-3′	166	57.99/56.31	56.20/43.80
0750-3	5′-MOE U^{me}psMOE U^{me} poTTT-3′	154	57.99/56.30	56.52/43.48
0750-70	5′-dA ps MOE U^{me} po TTT-3′	159	56.80/56.01	57.18/42.82
0750-71	5′-dA ps MOE U^{me} po TTT-3′	167	56.80/55.99	57.05/42.95
0750-72	5′-dA ps MOE U^{me} po TTT-3′	161	56.76/55.98	57.85/42.15
0750-73	5′-dA ps MOE U^{me} po TTT-3′	164	56.80/55.99	57.79/42.21
0750-74	5′-dG ps MOE U^{me} po TTT-3′	169	56.64/56.07	47.57/52.43
0750-75	5′-dG ps MOE U^{me} po TTT-3′	152	56.68/56.09	47.14/52.86
0750-76	5′-dG ps MOE U^{me} po TTT-3′	157	56.65/56.07	47.19/52.81
0750-77	5′-dG ps MOE U^{me} po TTT-3′	169	56.63/56.07	47.50/52.50
0750-80	5′-dC ps MOE U^{me} po TTT-3′	151	56.70/56.06	54.33/45.67
0750-81	5′-dC ps MOE U^{me} po TTT-3′	155	56.76/56.09	54.81/45.19
0750-82	5′-dC ps MOE U^{me} po TTT-3′	165	56.78/56.10	54.39/45.61
0750-83	5′-dC ps MOE U^{me} po TTT-3′	156	56.76/56.09	54.84/45.16

Table 4. **Analysis of dimers synthesized on OligoPilot I**

expt	dimer	scale (μmol)	^{31}P NMR (D_2O) ppm	^{31}P NMR (D_2O) diastereomer ratio
0225-152	5′-MOE G ps MOE U^{me}-3′	30	58.05/56.32	47.04/52.96
0225-159	5′-MOE G ps dG-3′	30	57.41/56.76	51.60/48.40
0225-148	5′-MOE G ps MOE C^{me}-3′	32	58.87/55.89	49.99/50.01
0225-167	5′-dC ps MOE U^{me}-3′	30	57.84/56.45	55.82/44.18

Table 5. **Analysis of dimers synthesized on OligoPilot II**

expt	dimer	scale (μmol)	^{31}P NMR (D_2O) ppm	^{31}P NMR (D_2O) diastereomer ratio
0224-82	5′-MOE G ps MOE U^{me}-3′	274	57.55/56.27	47.24/52.76
0224-142	5′-MOE G ps dG-3′	156	57.44/56.78	51.60/48.40
0224-84	5′-MOE G ps MOE C^{me}-3′	328	58.73/55.44	49.14/50.86
0750-146	5′-dC ps MOE U^{me}-3′	144	56.80/56.17	56.04/43.96

Table 6. **Analysis of dimers synthesized on Akta OligoPilot**

expt	dimer	scale (μmol)	^{31}P NMR (D_2O) ppm	^{31}P NMR (D_2O) diastereomer ratio
0750-190	5′-MOE G ps MOE U^{me}-3′	1000	57.95/56.32	48.07/51.93
0750-189	5′-MOE G ps dG-3′	1000	57.39/56.77	51.53/48.47
0750-188	5′-MOE G ps MOE C^{me}-3′	1000	58.96/55.74	49.24/50.76
0750-191	5′-dC ps MOE U^{me}-3′	1000	56.75/56.17	55.37/44.63

business. To determine whether 1*H*-tetrazole-activated coupling of 2′-*O*-methoxyethyl-3′-*O*-(2-cyanoethyl) phosphoramidites is a racemization process we wanted to use pure diastereomers for our studies. Thus, the two diastereomers of 5′-*O*-DMT-2′-*O*-methoxyethyl-5-methyluridine-3′-*O*-(2-cyanoethyl) phosphoramidite (15 g) were cleanly and quantitatively separated using flash silica gel chromatography. ^{31}P NMR and reversed-phase HPLC (RP-HPLC) analysis of racemic and individual diastereomers of the phosphoramidite are shown in Figures 1 and 2. The earlier eluting isomer was termed "fraction 1", and the late eluting isomer was termed "fraction 2".

The experimental approach chosen was to synthesize short oligonucleotides about 5-mer in length in which a single phosphorus center was replaced by a phosphorothioate linkage. Oligonucleotides were synthesized using HL-30 thymidine Primer Support on an OligoPilot II DNA/RNA synthesizer using phenylacetyl disulfide as sulfurizing agent. The DMT group on the final base at the 5′-terminus was removed on the synthesizer column. After standard deprotection (30% concentrated ammonium hydroxide, 55 °C) the oligonucleotide was analyzed by RP-HPLC. Multiple analyses using different types of column packing materials (phenyl, cyano, C18, C8, C4), gradients, and carrier solvents failed to resolve the two diastereomers. At this stage we thought the oligomer might be sufficiently long to limit selectivity; thus, we tried synthesizing dimers using the same protocol used for 5-mers. Yet, even in the latter case resolution was not achieved under multiple conditions. Ultimately, we resorted to ^{31}P NMR for analysis of the phosphorothioate linkages. Good separation of the two diastereomer signals was observed, even in the case of 5-mers (Figure 3). A minimum signal-to-noise ratio of 200 and a standard deviation of 2−3% was obtained for all samples analyzed. Table 1 shows the ratios of phosphorothioate diastereomers obtained using the diastereomerically pure 2′-*O*-methoxyethyl-5-methyluridine phosphoramidite.

In addition to the above example, we also investigated commercially available 5′-*O*-DMT-N^2-isobutyryl-2′-*O*-meth-

Table 7. **Analysis of 5-mers using different supports synthesized on OligoPilot II**

expt	oligomer	support	^{31}P NMR (D_2O) ppm	^{31}P NMR (D_2O) diastereomer ratio
0224-139	5′-MOE U^{me} ps TTTT-3′	HL 30	57.56/56.51	57.68/42.32
0750-149	5′-MOE U^{me} ps TTTT-3′	PS 200	57.62/56.55	57.52/42.48
0750-150	5′-MOE U^{me} ps TTTT-3′	reloaded PS 200	57.62/56.54	56.86/43.14
0224-136	5′-MOE U^{me}psMOE U^{me} TTT-3′	HL 30	57.89/56.24	56.08/43.92
0750-151	5′-MOE U^{me}psMOE U^{me} TTT-3′	PS 200	57.99/56.23	55.81/44.19
0750-152	5′-MOE U^{me}psMOE U^{me} TTT-3′	reloaded PS 200	57.99/56.23	55.40/44.60
0750-80	5′-dC ps MOE U^{me} po TTT-3′	HL 30	56.70/56.06	54.33/45.67
0750-147	5′-dC ps MOE U^{me} po TTT-3′	PS 200	56.78/56.07	54.38/45.62
0750-148	5′-dC ps MOE U^{me} po TTT-3′	reloaded PS 200	56.78/56.07	54.23/45.77

Table 8. **Analysis of 5′-MOE C^{me} ps TTTT-3′ oligomer using 5-ethylthio-1*H*-tetrazole at various concentrations**

expt	concentration (M)	scale (μmol)	^{31}P NMR (D_2O) ppm	^{31}P NMR (D_2O) diastereomer ratio
0749-150	0.05	111	57.72/56.38	64.11/35.89
0749-148	0.1	113	57.82/56.40	64.68/35.32
0749-146	0.2	114	57.76/56.38	65.01/34.99
0749-72	0.4	165	57.73/56.38	64.33/35.67
0762-66	0.5	172	57.80/56.34	65.21/34.79
0749-71	0.8	165	57.74/56.38	65.12/34.88
0749-70	1.2	157	57.76/56.38	64.72/35.28

Table 9. **Analysis of 5′-MOE A ps TTTT-3′ oligomer using 5-ethylthio-1*H*-tetrazole at various concentrations**

	concentration (M)	scale (μmol)	^{31}P NMR (D_2O) ppm	^{31}P NMR (D_2O) diastereomer ratio
0749-151	0.05	113	57.44/56.36	60.78/39.22
0749-149	0.1	114	57.45/56.36	62.26/37.74
0749-147	0.2	112	57.38/56.37	60.79/39.21
0749-73	0.4	167	57.40/56.42	62.12/37.88
0762-64	0.5	167	57.51/56.32	61.42/38.58
0749-74	0.8	167	57.45/56.42	61.52/38.48
0749-75	1.2	168	57.44/56.42	61.48/38.52

oxyethylguanosine-3′-*O*-(2-cyanoethyl) phosphoramidite by ^{31}P NMR spectroscopy (Figure 4). Analysis of the spectrum reveals that the two diastereomers are present approximately in the ratio of 3:1. Hence, we decided not to further enrich the ratio of these two diastereomers. Table 2 shows the ratios of phosphorothioate diastereomers obtained using 2′-*O*-methoxyethylguanosine phosphoramidite.

Assignment of Stereochemistry. The Rp and Sp configurations of 2′-*O*-MOE-modified phosphorothioate linkages were tentatively assigned on the basis of earlier investigation of an analogous molecule, viz. 2′-*O*-methyl oligoribonucleotide phosphorothioates.[49−51] Thus, the upfield shift in the ^{31}P NMR signal of the 2′-*O*-MOE-modified phosphorothioate diester linkage was assigned the Sp configuration, and the downfield shift signal was assigned the Rp configuration. Work on assigning the absolute configuration is under progress.

Tables 1 and 2 clearly demonstrate that the presence of relatively bulky groups such as 2′-*O*-methoxyethyl does not deter racemization nor does it influence final chirality of phosphorothioate linkage in a measurable manner. Thus, 1*H*-tetrazole-catalyzed activation of phosphoramidites takes place with epimerization at the phosphorus center[48] similar to that of the deoxyribonucleotide phosphoramidites. Consequently, the initial enantiomeric excess present in the phosphoramidite monomer does not influence the ratio of the formed isomers. This conclusion was further substantiated where phosphorothioate dimers were synthesized using 2′-*O*-methoxyethylguanosine phosphoramidite and analyzed by ^{31}P NMR (data presented in Supporting Information). A typical example of ^{31}P NMR of a monophosphorothioate linkage in a 1:1 diastereomeric ratio is shown in Figure 3. An important point to be observed in Tables 1 and 2 is the reproducibility of diastereomeric composition of phosphorothioate linkage of the same oligonucleotide synthesized multiple times. Tables 1 and 2 also show that it is unnecessary to enrich the phosphoramidite diastereomers for further investigation. Hence, commercially available amidites were used as received for further experiments.

The second most important question to ask is how reproducible is the ratio of a given phosphorothioate linkage in a given oligomer when synthesized several times? Tables 1 and 2 clearly demonstrate that the stereochemistry of phosphorothioate linkage is under complete control even when repeated several times. *It should be kept in mind that it is not the absolute numbers that matter but the relative ratios between multiple syntheses and between different bases*.

Having demonstrated the stereochemical control with 2′-*O*-methoxyethyl-5-methyluridine and 2′-*O*-methoxyethylguanosine phosphoramidites, we extended the above conclusion to other phosphoramidites also, as shown in Table 3 (additional examples are included in Supporting Information). These tables also reveal that coupling of deoxynucleoside phosphoramidites to the 5′-hydroxyl of 2′-*O*-methoxyethylribonucleosides is also a racemic process leading to a 1:1 mixture of phosphorothioate linkages. Thus, coupling of 2′-*O*-MOE to deoxy, deoxy to 2′-*O*-MOE, and 2′-*O*-MOE to 2′-*O*-MOE all lead to inherent net sterochemical control. We have already shown that coupling of deoxy phosphoramidites to 5′-hydroxyl of deoxynucleoside/tide followed by sulfur-

(49) Guo, M.; Yu, D.; Iyer, R. P.; Agreawal, S. *Bioorg. Med. Chem. Lett*. **1998**, *8*, 2539−2544.
(50) Iyer, R. P.; Guo, M.; Yu, D.; Agrawal, S. *Tetrahedron Lett*. **1998**, *39*, 2491−2494.
(51) Iyer, R. P.; Yu, D.; Agrawal, S. *Bioorg. Med. Chem. Lett*. **1994**, *4*, 2471−2474.

Table 10. **Analysis of monophosphorothioate oligomers using different activators**

			^{31}P NMR (D_2O)	
expt	oligomer	activator	ppm	diastereomer ratio
0762-62	5′-MOE U^{me} ps TTTT-3′	ETT	57.54/56.52	69.61/30.39
0224-139	5′-MOE U^{me} ps TTTT-3′	1H-tetrazole	57.56/56.51	57.68/42.32
0750-156	5′-MOE U^{me} ps TTTT-3′	PTFA	57.51/56.52	51.19/48.81
0750-153	5′-MOE U^{me} ps TTTT-3′	DCI	57.54/56.53	45.16/54.84
0762-112	5′-MOE U^{me} ps TTTT-3′	ImTf	57.52/56.58	27.51/72.49
0762-63	5′-MOE U^{me}psMOE U^{me} TTT-3′	ETT	57.90/56.25	69.00/31.00
0224-136	5′-MOE U^{me}psMOE U^{me} TTT-3′	1H-tetrazole	57.89/56.24	56.08/43.92
0750-157	5′-MOE U^{me}psMOE U^{me} TTT-3′	PTFA	57.92/56.24	49.07/50.93
0750-154	5′-MOE U^{me}psMOE U^{me} TTT-3′	DCI	57.86/56.23	45.44/54.56
0762-113	5′-MOE U^{me}psMOE U^{me} TTT-3′	ImTf	57.88/56.28	25.82/74.18
0750-80	5′-dC ps MOE U^{me} po TTT-3′	ETT	56.70/56.06	54.33/45.67
0750-158	5′-dC ps MOE U^{me} po TTT-3′	1H-tetrazole	56.60/56.03	55.28/44.72
9761-32	5′-dC ps MOE U^{me} po TTT-3′	PTFA	56.45/56.09	55.63/44.37
0750-155	5′-dC ps MOE U^{me} po TTT-3′	DCI	56.64/56.00	54.51/45.49
0761-38	5′-dC ps MOE U^{me} po TTT-3′	ImTf	56.47/56.10	54.87/45.13
0750-70	5′-dA ps MOE U^{me} po TTT-3′	ETT	56.80/56.01	57.18/42.82
0762-2	5′-dA ps MOE U^{me} po TTT-3′	1H-tetrazole	56.72/55.98	56.59/43.41
0761-32	5′-dA ps MOE U^{me} po TTT-3′	PTFA	56.46/56.01	55.27/44.73
0762-3	5′-dA ps MOE U^{me} po TTT-3′	DCI	56.72/55.98	57.49/42.51
0761-39	5′-dA ps MOE U^{me} po TTT-3′	ImTf	56.49/56.03	55.36/44.64
0762-65	5′-MOE A ps MOE A po TTT-3′	ETT	57.94/56.42	63.37/36.63
0224-172	5′-MOE A ps MOE A po TTT-3′	1H-tetrazole	57.99/56.46	53.45/46.55
0762-4	5′-MOE A ps MOE A po TTT-3′	PTFA	57.94/56.44	50.60/49.40
0762-5	5′-MOE A ps MOE A po TTT-3′	DCI	57.79/56.46	45.62/54.38
0762-117	5′-MOE A ps MOE A po TTT-3′	ImTf	57.86/56.46	33.41/66.59
0762-64	5′-MOE A ps TTTT-3′	ETT	57.51/56.32	61.42/38.58
0224-168	5′-MOE A ps TTTT-3′	1H-tetrazole	57.57/56.34	53.75/46.25
0762-6	5′-MOE A ps TTTT-3′	PTFA	57.50/56.35	49.32/50.68
0762-1	5′-MOE A ps TTTT-3′	DCI	57.44/56.38	41.32/58.68
0762-116	5′-MOE A ps TTTT-3′	ImTf	57.53/56.32	32.27/67.73
0762-66	5′-MOE C^{me}ps TTTT-3′	ETT	57.80/56.34	65.21/34.79
0762-72	5′-MOE C^{me}ps TTTT-3′	1H-tetrazole	57.88/56.36	52.97/47.03
0762-70	5′-MOE C^{me}ps TTTT-3′	PTFA	57.83/56.35	49.52/50.48
0762-68	5′-MOE C^{me}ps TTTT-3′	DCI	57.82/56.34	41.86/58.14
0762-108	5′-MOE C^{me}ps TTTT-3′	ImTf	57.82/56.39	30.31/69.69
0762-67	5′-MOE C^{me}ps MOE C^{me}poTTT-3′	ETT	59.09/55.81	69.82/30.18
0762-73	5′-MOE C^{me}ps MOE C^{me}poTTT-3′	1H-tetrazole	58.94/55.81	55.30/44.70
0762-71	5′-MOE C^{me}ps MOE C^{me}poTTT-3′	PTFA	59.01/55.81	49.57/50.43
0762-69	5′-MOE C^{me}ps MOE C^{me}poTTT-3′	DCI	58.87/55.80	41.72/58.28
0762-109	5′-MOE C^{me}ps MOE C^{me}poTTT-3′	ImTf	58.94/55.85	30.12/69.88

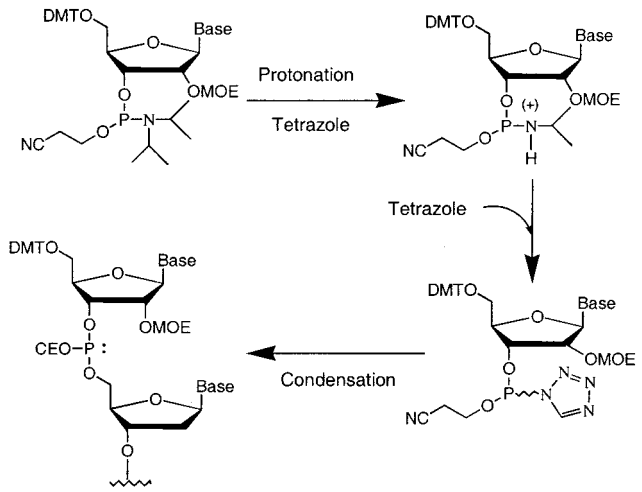
ization using phenylacetyl disulfide leads to stereocontrolled phosphorothioate linkages.[48]

It is well-known that different synthesizers work differently, viz. the benchtop ABI 390Z DNA/RNA synthesizer uses an argon gas-sparged reactor, whereas Amersham Pharmacia Biotech OligoPilot I and II and Akta synthesizers use a packed-bed stainless steel column with no room for agitation. Since OligoProcess is the only true large-scale synthesizer available and a large-scale version of OligoPilot and is also used for the routine manufacture of antisense drugs for market (Vitravene) as well as for clinical trial evaluations by us and by others, we were more interested in finding whether these synthesizers contributed to any influence in stereoselectivity. Also, a closely related question to ask is does the stereoselectivity of a given phosphorothioate linkage vary with respect to scale? Tables 4, 5, and 6 present the data of dimers obtained from different synthesizers. These tables clearly demonstrate that scales and synthesizers do not influence stereochemical outcome of the phosphorothioate linkage.

It is also well-known that development of solid supports for the synthesis of oligonucleotides has gone through several changes, viz. from silica-based controlled-pore glass to dextran-coated HL 30 primer support to polymeric high-loaded polystyrene solid support. Thus, the obvious question to ask is whether the solid support contributes to influencing the ratio of phosphorothioate linkages. Table 7 representing the data shows that solid supports also do not influence the stereochemistry of phosphorothioate linkages in any measurable manner.

To demonstrate the stereochemical homogeneity, solid support at the end of synthesis was sampled from the top, middle, and bottom of the 6-mL synthesizer column and analyzed (data presented in Supporting Information). No difference in ratio was observed.

Another variable that could potentially influence the diastereomeric ratio is the concentration of reactants during coupling. Since 5-ethylthio-1*H*-tetrazole has a wide range of solubility, it was chosen instead of 1*H*-tetrazole. Tables 8 and 9 clearly show that concentration of reactants during



coupling does not have any measurable impact on the stereochemical outcome.

The phosphoramidite approach has undergone revolutionary changes in terms of its applicability/suitability for development of antisense drugs. One such area is the activator used for coupling of phosphoroamidites. Multiple activators have been evaluated[52] for synthesis of oligonucleotides as an alternative to 1*H*-tetrazole due to its potential explosive nature. As a replacement we believe pyridinium trifluoroacetate (PTFA)[52] and 4,5-dicyanoimidazole (DCI)[53] are the best alternatives for large-scale synthesis of antisense drugs. Hence the final question to ask is does the activator contribute to the stereochemical outcome of phosphorothioate linkages?

The interesting data from Table 10 indicates that activators have much greater influence on the stereochemical ratio of phosphorothioate linkages than any other variable. It appears from the table that (a) the 2′-*O*-methoxyethyl group of the nucleoside on the 5′-end of the growing chain on solid support has very little influence on stereochemical ratio of phosphorothioate linkage, (b) the 2′-*O*-methoxyethyl group of the incoming phosphoramidite greatly influences the stereochemical ratio, and (c) the size or pK_a of activator or both are playing a significant role towards the diastereomeric ratio. Hence, one must be attentive to potential stereochemical differences resulting from the choice of activator used for manufacture of antisense drugs. It is perfectly fine to use 1*H*-tetrazole or DCI or PTFA as the activator for the synthesis of 2′-*O*-methoxyethyl oligonucleotide phosphorothioates. However, once an activator is chosen and used to make a batch of drug (active pharmaceutical ingredient) for clinical trial use, potential effects (i.e., toxicological profile or therapeutic outcome) resulting from a different pool of diastereomers should be considered prior to switching activators for further batches.

Conclusions

All the above data are consistent with the theory proposed by Stec[54,55] and evidenced by Berner, et al.[56] for the tetrazole-catalyzed mechanism of reaction of deoxyribonucleotide phosphoramidites with hydroxyl groups. In this mechanism, tetrazole displaces the protonated amine function to form a tetrazolide, which undergoes further rapid reaction with tetrazole to give a mixture of epimeric tetrazolides. In a slower reaction, the nucleophilic 5′-hydroxyl group then displaces tetrazole to form the phosphite triester.[57,58] The phosphite intermediate then undergoes sulfurization to give phosphorothioate triesters.

A systematic evaluation of the various plausible contributors to the stereochemistry reveals that racemization is a rapid process, no single factor (except activator) contributes in a measurable way to the net stereochemical outcome of phosphorothioate linkage formation. In addition, all couplings followed by sulfurization with PADS lead to nearly 1:1 ratio of Rp and Sp phosphorothioate diesters, due to racemization during coupling, indicating that the overall synthetic process is stereoreproducible and under inherent process control.

Acknowledgment

We thank Mark Andrade and Patrick D. Wheeler for their valuable help.

Supporting Information Available

Additional figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review July 4, 2002.

OP020061C

(52) Eleuteri, A.; Capaldi, D. C.; Krotz, A. H.; Cole, D. L.; Ravikumar, V. T. *Org. Process Res. Dev.* **2000**, *4*, 182−189 and references therein.
(53) Vargeese, C.; Carter, J.; Yegge, J.; Krivjansky, S.; Settle, A.; Kropp, E.; Peterson, K.; Pieken, W. *Nucleic Acids Res.* **1998**, *26*, 1046−1050.
(54) Stec, W. J.; Zon, G. *Tetrahedron Lett.* **1984**, *25*, 5279−5282.
(55) Wilk, A.; Uznanski, B.; Stec, W. J. *Nucleic Acids Symp. Ser.* **1991**, *24*, 63−66.
(56) Berner, S.; Mühlegger, K.; Seliger, H. *Nucleic Acids Res.* **1989**, *17*, 853−864.
(57) Dahl, B. H.; Nielsen, J.; Dahl, O. *Nucleic Acids Res.* **1987**, *15*, 1730−1743.
(58) Ghosh, M.; Ghosh, K.; Cohen, J. S. *Antisense Res. Dev.* **1992**, *2*, 11−14.