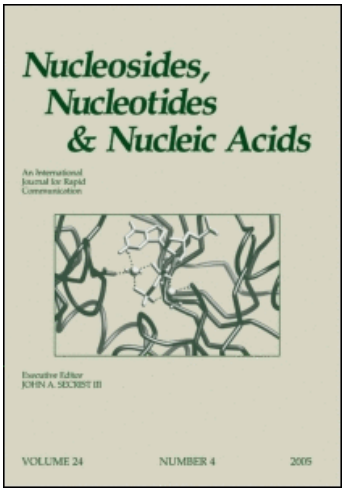


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# Influence of Diastereomeric Ratios of Deoxyribonucleoside Phosphoramidites on the Synthesis of Phosphorothioate Oligonucleotides

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# INFLUENCE OF DIASTEREOMERIC RATIOS OF DEOXYRIBONUCLEOSIDE PHOSPHORAMIDITES ON THE SYNTHESIS OF PHOSPHOROTHIOATE OLIGONUCLEOTIDES

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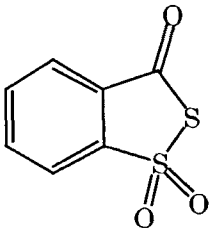
**Abstract**: Extensive investigations on the influence of diastereomeric ratios of deoxyribonucleoside phosphoramidites on stereo-reproducibility of solid phase synthesis of phosphorothioate oligodeoxyribonucleotides via the phosphoramidite approach indicate that the process is stereoreproducible and under inherent process control.



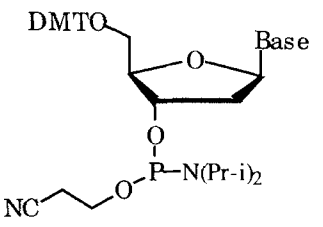
The emergence of antisense and antigene oligonucleotides as potential sequence-selective inhibitors of gene expression is evidenced by the growing number of clinical trials against a variety of diseases.[1-7] First generation antisense therapeutics utilize a uniformly modified phosphorothioate oligodeoxyribonucleotide where one non-bridging oxygen atom is formally replaced by sulfur, because natural DNA is insufficiently stable towards extra- and intracellular enzymes.[8-11]

Phosphoramidite chemistry[12-16] has been widely used for the synthesis of phosphorothioate oligonucleotides because of its potential for automation, high coupling efficiency, ease of site-specific thioate linkage incorporation, and ready scalability. Large-scale solid-supported synthesis of phosphorothioates is presently carried out by initial formation of internucleotidic phosphite linkage followed by sulfurization of the

phosphite triester to phosphorothioate using the Beaucage reagent (**1**).[17-18] For this purpose, nucleoside β-cyanoethyl phosphoramidites (**2**) are the commonly used monomer synthon.[19-20] Current state-of-the-art large-scale synthesizers[18] allow complete synthesis of a phosphorothioate oligonucleotide 20-mer at 0.15 mole scale in 10 h.[21]



Beaucage Reagent (**1**)




β-Cyanoethyl Phosphoramidites (**2**)

The resulting *O,O*-linked phosphorothioate diester linkage in the oligonucleotide is a chiral functional group. This chirality may have several ramifications with respect to using phosphorothioate oligomers as drugs. First, for a typical 20-mer there are 524,288 ($2^{19}$) possible diastereoisomers. Separation and individual quantification of this number of diastereomers is currently not feasible. Secondly, one of the best reported literature methods[22-26] for stereocontrolled synthesis of phosphorothioate oligomers is not presently useful for drug synthesis for safety reasons; that is, since net 100% enantiomeric excess is not achieved in the coupling step, the oligomeric product still consists of the same mixture of Sp and Rp diastereomers, except that the levels of all but one isomer are reduced to low individual levels. As a result, even a small change in the enantiomeric excess at a given phosphorus center could produce a huge and undetectable percentage variation in the content of one diastereomer in the final product. For this reason, and because, measuring individual diastereomer levels is beyond the capability of state of the art methods, it is not possible to validate such a stereoselective synthetic process and thereby assure that the

active pharmaceutical ingredient (API) used in a safety study represents the compositions of all lots that will subsequently be made for human use.

Recently, stereo-reproducibility of phosphoramidite-based phosphorothioate oligonucleotide synthesis was examined through synthesis of singly thioate-substituted oligodeoxyribonucleotide model compounds.[27] Baseline RP-HPLC resolution of resulting Rp and Sp diastereomers allowed accurate determination of any enantiomeric excess at each phosphorothioate linkage. The results showed that phosphorothioate linkage formation is not a fully stereo-random process. All investigated stereomeric phosphorothioate diester linkages were formed with a small, reproducible excess of the R isomer (2-6% per linkage). Regardless of the synthesized sequence and position of the thioate, all Rp to Sp diastereomer ratios were between 50:50 and 60:40, indicating that the synthetic process is under inherent stereochemical control. However the study was done based on the assumption that the ratio of the starting deoxyribonucleoside phosphoramidite diastereomers was close to 50:50. Even though phosphitylation of deoxyribonucleoside to afford phosphoramidite is not a stereoselective process, purification of the crude amidite using flash silica gel chromatography or medium pressure LC may lead to enrichment of one amidite diastereomer over the other. In our experience, this has been the case, with some batches of commercial amidites having ratios of 85:15.

There are a few reports[28-31] in literature which show that 1*H*-tetrazole-activated phosphoramidite coupling in acetonitrile is a racemization process leading to a 1:1 mixture of Rp and Sp phosphate isomers even if one starts with 100% enantiomerically pure phosphoramidite. Since there was no extensive data available, we were interested in a systematic evaluation on this issue. In this paper we report our investigations on the influence of diastereomeric ratios of all eight

deoxyribonucleoside phosphoramidites in the synthesis of phosphorothioate oligonucleotides. For this study all eight deoxyribonucleoside phosphoramidite diastereomers were separated carefully using flash column chromatography. These were then individually used in the synthesis of a model 10-mer monophosphorothioate oligodeoxyribo nucleotide (TGTTXpsTATCT) using phosphoramidite chemistry. Table **1** shows the purity of each phosphoramidite used during the investigation. No attempts were made to assign stereochemistry of the individual diastereomers.

Evaluations were done using tetrazole and DCI as activators and Beaucage as the sulfurizing agent. At the end of solid-supported synthesis, the oligonucleotide was deprotected under standard conditions (30% concentrated ammonia for 12h, 55 °C), and the diastereomeric ratios of the products were determined by RP-HPLC.[27] Except for two of the cases, baseline separation of all the oligos were achieved, allowing accurate peak area integration. The results are shown in **Table 2**.

In order to assess for the influence of a purine nucleoside preceding the newly formed phosphorothioate linkage, following 10-mers TGTTXpsGATCT and TGTTXpsAATCT were synthesized with tetrazole as activator. The results are shown in **Tables 3** and **4**.

The initial studies were performed on monophosphorothioate oligonucleotide 10-mers with the phosphorothioate linkage at position 5. In order to study the influence on phosphorothioate linkages of different position in the oligomer, the 10-mer was synthesized with the phosphorothiate linkage at positions 1, 3, and 8. The results are shown in **Tables 5**, **6**, and **7**.

All these data are consistent with the theory proposed by Stec[32-33] and evidenced by Berner, *et al.*[34] for tetrazole-catalyzed mechanism of reaction of phosphoramidites with hydroxyl groups. In this mechanism, tetrazole displaces the protonated amine function to form a tetrazolide,

**Table 1.** Separation of Rp and Sp diastereomers of deoxyribonucleoside phosphoramidites.

| Amidite | Diastereomer 1 | Diastereomer 2 |
|---|---|---|
| dA-Amidite (Fr-1) | 96% | 4% |
| dA-Amidite (Fr-2) | 7% | 93% |
| dC-Amidite (Fr.1) | 100% | 0% |
| dC-Amidite (Fr.2) | 8% | 92% |
| T-Amidite (Fr-1) | 97% | 3% |
| T-Amidite (Fr-2) | 2% | 98% |
| dG-Amidite (Fr.1) | 100% | 0% |
| dG-Amidite (Fr.2) | 0% | 100% |



**Table 2.** Separation of diastereomers of Monophosphorothioate-substituted (PS) Oligonucleotides

| X | Activator | RT(min) | | Rp | Sp |
|---|---|---|---|---|---|
| A-Amidite (racemic) | Tetrazole | 53.94 | 54.81 | 56.59 | 42.64 |
| A-Amidite (Fr.1) | Tetrazole | 53.96 | 54.81 | 56.58 | 43.42 |
| A-Amidite (Fr.1) | DCI | 51.93 | 53.83 | 51.17 | 48.82 |
| A-Amidite (Fr.2) | Tetrazole | 54.18 | 55.01 | 56.64 | 43.35 |
| A-Amidite (Fr.2) | DCI | 53.74 | 54.60 | 56.91 | 43.08 |
| C-Amidite (racemic) | Tetrazole | 53.24 | 54.42 | 53.65 | 46.34 |
| C-Amidite (racemic) | DCI | 53.42 | 54.57 | 55.14 | 44.85 |
| C-Amidite (Fr.1) | Tetrazole | 53.41 | 54.54 | 53.81 | 46.18 |
| C-Amidite (Fr.1) | DCI | 53.10 | 54.22 | 56.03 | 43.96 |
| C-Amidite (Fr.2) | Tetrazole | 53.44 | 54.62 | 53.98 | 46.01 |
| C-Amidite (Fr.2) | DCI | 53.29 | 54.41 | 53.30 | 46.69 |
| T-Amidite (racemic) | Tetrazole | 54.22 | 55.52 | 55.43 | 44.56 |
| T-Amidite (racemic) | DCI | 54.36 | 55.66 | 57.75 | 42.24 |
| T-Amidite (Fr.1) | Tetrazole | 54.25 | 55.57 | 55.40 | 44.59 |
| T-Amidite (Fr.1) | DCI | 54.16 | 55.46 | 58.68 | 41.31 |
| T-Amidite (Fr.2) | Tetrazole | 54.27 | 55.59 | 55.87 | 44.12 |
| T-Amidite (Fr.2) | DCI | 54.55 | 55.55 | 58.83 | 41.16 |
| G-Amidite (Fr.1) | Tetrazole | 52.87 | 53.37 | 48.21 | 51.79 |
| G-Amidite (Fr.1) | DCI | 53.07 | 53.73 | 50.05 | 49.95 |
| G-Amidite (Fr.2) | Tetrazole | 53.21 | 53.86 | 47.40 | 52.60 |
| G-Amidite (Fr.2) | DCI | 53.09 | 53.74 | 50.08 | 49.92 |

Fr.1 is the faster moving amidite during the separation of the diastereomeric amidites using flash chromatography. Isomers were separated using ODS Hypersil column (5 Mm, 100 x 4.6 mm) and a linear gradient of buffer A and buffer B (0% B for 5 min; 0-15% B for 65 min). Buffer A, 0.1 M TEAA, pH 7; buffer B, acetonitrile. Assignments of Rp and Sp were done based on the elution order as reported in the literature.

**Table 3.** Separation of diastereomers of Monophosphorothioate-substituted (PS) Oligonucleotides

| X | Activator | RT(min) | | Rp | Sp |
|---|---|---|---|---|---|
| A-Amidite (Fr.1) | Tetrazole | 51.13 | 51.47 | 57.28 | 42.72 |
| A-Amidite (Fr.2) | Tetrazole | 51.52 | 52.14 | 55.24 | 44.76 |
| T-Amidite (Fr.1) | Tetrazole | 51.85 | 52.47 | 52.50 | 47.50 |
| T-Amidite (Fr.2) | Tetrazole | 51.54 | 52.81 | 52.75 | 47.25 |

The 10-mer model monophosphorothioate oligonucleotide was TGTTXpsGATCT.

**Table 4.** Separation of diastereomers of Monophosphorothioate-substituted (PS) Oligonucleotides

| X | Activator | RT(min) | | Rp | Sp |
|---|---|---|---|---|---|
| C-Amidite (Fr.1) | Tetrazole | 52.24 | 53.34 | 57.03 | 42.97 |
| C-Amidite (Fr.2) | Tetrazole | 51.76 | 52.58 | 56.41 | 43.59 |
| G-Amidite (Fr.1) | Tetrazole | 51.79 | 52.11 | 43.13 | 56.87 |
| G-Amidite (Fr.2) | Tetrazole | 51.16 | 51.38 | 49.73 | 50.27 |

The 10-mer model monophosphorothioate oligonucleotide was TGT TXpsA ATCT.



**Table 5.** Separation of diastereomers of Monophosphorothioate-substituted (PS) Oligonucleotides

| X | Activator | RT(min) | | Rp | Sp |
|---|---|---|---|---|---|
| G-Amidite (Fr.1) | Tetrazole | 54.83 | 55.58 | 48.06 | 51.94 |
| G-Amidite (Fr.2) | Tetrazole | 53.98 | 54.57 | 48.20 | 51.80 |

The 10-mer model monophosphorothioate oligonucleotide was TXpsTTCTATCT.

**Table 6.** Separation of diastereomers of Monophosphorothioate-substituted (PS) Oligonucleotides

| X | Activator | RT(min) | | Rp | Sp |
|---|---|---|---|---|---|
| C-Amidite (Fr.1) | Tetrazole | 53.85 | 54.35 | 54.11 | 45.89 |
| C-Amidite (Fr.2) | Tetrazole | 52.32 | 53.02 | 55.26 | 44.74 |

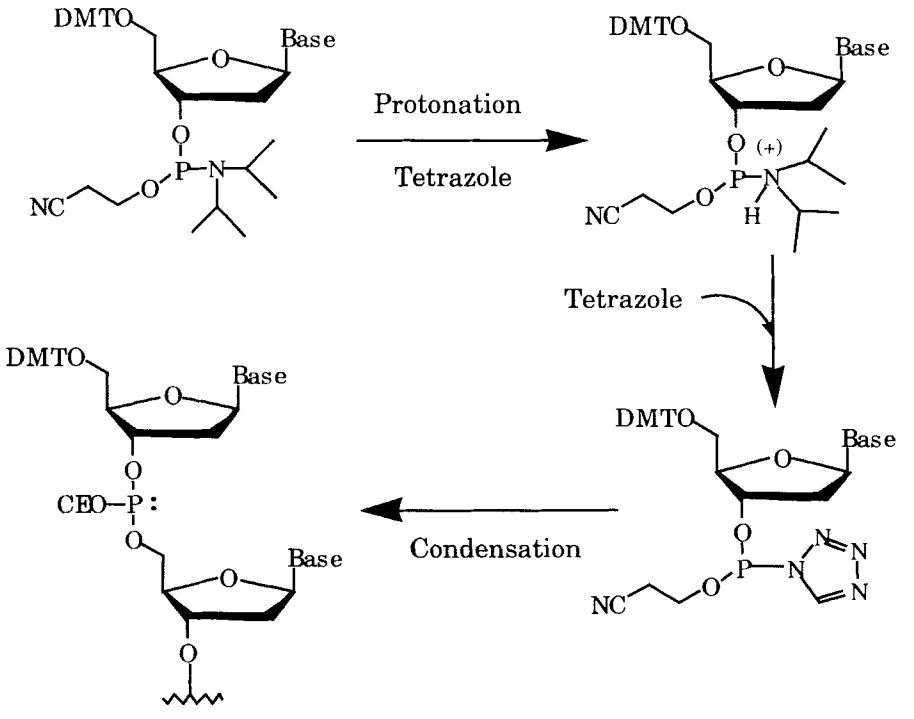
The 10-mer model monophosphorothioate oligonucleotide was TGTTCTATXpsT.

**Table 7.** Separation of diastereomers of Monophosphorothioate-substituted (PS) Oligonucleotides

| X | Activator | RT(min) | | Rp | Sp |
|---|---|---|---|---|---|
| A-Amidite (Fr.1) | Tetrazole | 54.0 | 54.0 | nd | nd |
| A-Amidite (Fr.2) | Tetrazole | 54.0 | 54.0 | nd | nd |

The 10-mer model monophosphorothioate oligonucleotide was TGTTCTXpsTCT. nd=these two 10-mers were not separable using the same conditions used to separate the other 10-mers. All the compounds above were also analyzed and matched the expected values by mass spectroscopy. Isomers were separated using ODS Hypersil column (5 Mm, 100 x 4.6 mm) and a linear gradient of buffer A and buffer B (0% B for 5 min; 0-15% B for 65 min). Buffer A, 0.1 M TEAA, pH 7; buffer B, acetonitrile.

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.[35-36]




In summary, based on the above wealth of information, it can be concluded that a range of diastereomeric phosphoramidite compositions, all 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.

## Stereo-reproducibility during Sulfurization in the Synthesis of Phosphorothioate Oligonucleotides using Phenylacetyl Disulfide

During each oligonucleotide synthesis cycle, nucleophilic attack of the 5'-terminal hydroxyl on a tetrazole-activated nucleoside phosphoroamidite results in formation of a chiral phosphite triester intermediate, which is then sulfurized with a sulfur transfer reagent. The 1*H*-tetrazole-catalyzed activation of phosphoramidite takes place with epimerization at the phosphorous center as shown above. Hence, an initial enantiomeric excess present in phosphoramidite monomer should not influence the ratio of phosphite triester diastereoisomers formed, though bulky activator chiral groups in proximity to the phosphorous atom have been shown to influence the stereochemical outcome of the coupling reaction. Over the last few years a variety of sulfurizing agents have been investigated for synthesis of phosphorothioate oligonucleotides. Among these, the Beaucage reagent is the most widely used, due to its rapid sulfurization kinetics and commercial availability. We reported earlier on the stereo-reproducibility of the phosphoramidite method for synthesis of phosphorothioate oligonucleotides using Beaucage reagent for sulfur transfer. With Beaucage reagent, however, the by-product formed during sulfurization is a potent oxidizing agent. Also, Beaucage reagent is costly to synthesize even at large scale (multiple steps and low yields). Recently, we reinvestigated the use of phenylacetyl disulfide (PADS) as a sulfur transfer reagent in the synthesis of phosphorothioate oligonucleotides and found that under suitable solvent conditions, this inexpensive compound rapidly and efficiently sulfurizes internucleotide phosphite triester linkages.

**Table 8**. Separation of diastereomers of Monophosphorothioate-substituted (PS) Oligonucleotides using PADS

| No. | Oligonucleotide sequence (5'-3') | Rp / Sp | RT (min) |
|---|---|---|---|
| 1 | TTApsCTTTTTT | 59.39 / 40.61 | 56.18 / 57.37 |
| 2 | TTTTTTTCpsCT | 57.08 / 43.39 | 56.20 / 57.55 |
| 3 | TTTTCpsCTTTT | 59.04 / 40.96 | 56.56 / 58.46 |
| 4 | TCpsCTTTTTTT | 57.16 / 42.84 | 55.91 / 57.74 |
| 5 | TTGCTTpsCC | 55.68 / 44.26 | 51.54 / 52.27 |
| 6 | TTGCTpsTCC | 50.82 / 49.18 | 50.93 / 52.23 |
| 7 | TTGCpsTTCC | 50.48 / 49.52 | 49.16 / 50.23 |
| 8 | TTpsGCTTCC | 57.92 / 42.08 | 49.99 / 51.51 |
| 9 | TpsTGCTTCC | 55.55 / 44.45 | 51.51 / 52.96 |
| 10 | CCATCTpsTC | 55.41 / 44.59 | 52.19 / 52.61 |
| 11 | CCATCpsTTC | 59.37 / 40.63 | 51.95 / 52.58 |
| 12 | CCATpsCTTC | 55.53 / 44.47 | 50.26 / 51.49 |
| 13 | CCApsTCTTC | 55.01 / 44.99 | 50.67 / 50.94 |
| 14 | CCpsATCTTC | 55.53 / 44.47 | 55.17 / 50.99 |
| 15 | CpsCATCTTC | 57.39 / 42.61 | 51.10 / 51.43 |
| 16. | TCCTCGpsTC | 55.99 / 44.01 | 49.09 / 49.72 |
| 17 | TCCTCpsGTC | 58.12 / 41.88 | 48.61 / 49.27 |
| 18 | TCCTpsCGTC | 52.66 / 47.34 | 48.95 / 49.38 |
| 19 | TCCpsTCGTC | 53.19 / 46.81 | 48.34 / 49.14 |
| 20 | TCpsCTCGTC | 52.88 / 47.12 | 47.17 / 47.44 |
| 21 | TpsCCTCGTC | 52.33 / 47.67 | 45.67 / 46.35 |



We describe our investigations concerning the influence of this new sulfur transfer reagent on the net stereo-reproducibility of phosphorothiate linkages formed.

The experimental approach was to synthesize mixed-sequence oligodeoxynucleotides with a single phosphorothioate linkage. Shortmers (10-mers and 8-mers) were chosen as model oligonulceotides as the literature reports baseline RP-HPLC separation of the resulting diastereomers may be achieved, allowing ready determination of the product diastereomer ratio. Oligonucleotides were synthesized via phosphoramidite coupling chemistry using tetrazole as activator. The oxidizing agent used for formation of phosphodiester linkages was 10% *t*-

BuOOH/$CH_3CN$. A 0.2M solution of phenylacetyl disulfide (PADS) in 1:1 ($CH_3CN$/3-picoline) was used for sulfurization. After synthesis, the oligonucleotides were deprotected using standard conditions (30% concentrated ammonia for 12h, 55 °C). The crude oligonucleotides were then analyzed by reversed phase HPLC using a Hypersil-OD column (5 μm, 100x4.6 mm). **Table 8** shows the stereo-reproducibility of phosphorothioate linkages formed when using PADS and Beaucage reagent.

**Conclusions:** Regardless of the mono-phosphorothioate DNA oligonucleotide base sequence synthesized and position of the phosphorothioate linkage, all product Rp/Sp diastereomer ratios were between 40:60 and 60:40.



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in Phase II stages, and several are in Phase I trials. In all about 20 drugs are being evaluated against various disease targets.

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