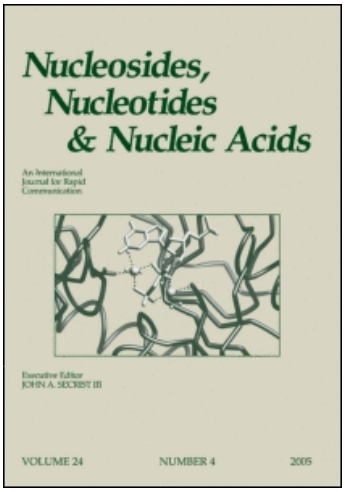

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DIASTEREOMERIC PROCESS CONTROL IN THE SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDE PHOSPHOROTHIOATES

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The emergence of antisense and antigene oligonucleotides as potential sequence-selective inhibitors of gene expression is evidenced by the growing number of ongoing clinicals trials against a variety of diseases. First generation antisense therapeutics utilize a uniformly modified oligodeoxyribonucleotide phosphorothioate where one non-bridging oxygen atom is formally replaced by sulfur, because natural DNA is unstable towards extra- and intracellular enzymes. Phosphoramidite chemistry 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. The large scale solid-supported synthesis of phosphorothioates is presently carried out by initial formation of the internucleotidic phosphite linkage followed by sulfurization of the phosphite triester to phosphorothioate using the Beaucage reagent. The resulting *O,O*-linked phosphorothioate diester linkage in the oligonucleotide is a chiral functional group. 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. In addition, the best reported methods for stereocontrolled synthesis of phosphorothioate oligomers are not presently useful for drug synthesis; 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.

Recently, stereo-reproducibility of phosphoramidite-based phosphorothioate oligonucleotide synthesis was examined extensively through synthesis of singly thioate-substituted oligodeoxyribonucleotide model compounds.[1] 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 the deoxyribonucleoside to afford the 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[2-5] in the 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 on our investigations on the influence of the diastereomeric ratios of all eight deoxyribonucleoside phosphoramidites into the synthesis of phosphorothioate oligos. 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 deoxyribo oligonucleotide (TGTTXpsTATCT) using phosphoramidite chemistry. Evaluations were done using tetrazole and 4,5-dicyanoimidazole (DCI) as activators and Beaucage as the sulfurizing agent. At the end of solid-supported synthesis, the oligonucleotide was deprotected under standard conditions

Table 1. Seperation 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 seperation of the diastereomeric amidites using flash chromatography. Isomers were seperated 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.

(30% concentrated ammonia for 12h, 55 °C), and the diastereomeric ratios of the products were determined by RP-HPLC. Except for two of the cases, baseline seperation of all the oligos were achieved, allowing accurate peak area integration. The results are shown in **Table 1**.

Similarly, the pure diastereomers were incorporated at different positions in the sequence. In all cases the ratios were close to 1:1. All these data are consistent with the theory proposed by Stec[6-7] and evidenced by Berner, *et al.*[8] for tetrazole-catalyzed mechanism of reaction of 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 tertazolides. In a slower reaction, the nucleophilic 5'-hydroxyl group then displaces tetrazole to form the phosphite triester.[9-10]

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 stereo reproducible and under inherent process control.

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