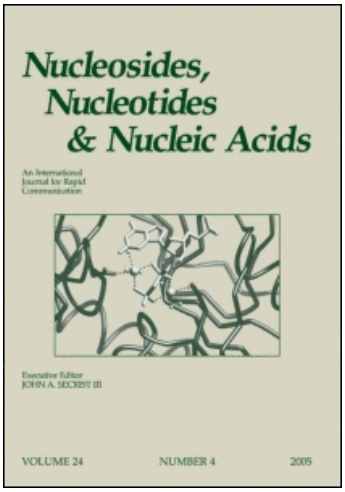

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OLIGODEOXYRIBONUCLEOTIDE PHOSPHOROTHIOATES: SUBSTANTIAL REDUCTION OF (N-1)-MER CONTENT THROUGH THE USE OF TRIMERIC PHOSPHORAMIDITE SYNTHONS

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Abstract: Use of fully protected trimeric phosphoramidite synthons in the synthesis of oligonucleotide phosphorothioate shows a substantial reduction (>85%) in (n-1)-mer content as compared to oligomers synthesized through coupling of standard phosphoramidite monomers. A 20-mer oligodeoxyribonucleotide phosphorothioate which is in phase I clinical trials was chosen as an example for the studies.

Sequence-specific modulation of gene expression for the treatment of diseases has come to reality.[1] Multiple examples of uniformly modified oligodeoxyribonucleotide phosphorothioates[2-7], in which one nonbridging oxygen atom of the internucleotide phosphate group of DNA is replaced by a sulfur atom are currently in advanced clinical trials.[8-10] Recent advances in phosphoramidite coupling chemistry[11-15] and solid phase synthesis methodology, together with current state of the art large-scale synthesizers[16], allow complete assembly of a 20-mer deoxyribonucleotide phosphorothioate at 150 mmole (1 kilogram) scale in just 8 hours. Very high average coupling efficiencies (>98.5%) have been achieved at these scales with only 1.75-fold molar amidite excess.[17]

Coupling of an activated phosphoramidite monomer to the 5'-hydroxyl of the growing chain on a solid support followed by stepwise sulfurization of the trialkylphosphite linkage is the current preferred

method for the synthesis of first generation drugs. Problems commonly encountered in this method of automated synthesis include formation of deletion sequences (internal and terminal deletionmers) which has one or more nucleotides absent.[18-20] Less than quantitative coupling efficiency, incomplete capping or removal of 5'-dimethoxytrityl group, reagent quality as well as work-up protocols could contribute to the formation of these process related impurities. In addition low levels of phosphodiester linkages are also present in the phosphorothioate (PS) oligonucleotide product. These could occur due to side reactions during sulfurization step[21] or by other mechanisms.[22] Reversed phase HPLC purification of crude oligomer takes care of DMT off capped failure sequences. However, the resolution of DMT-on full length from DMT-on failures is rather poor. In addition, PO-contining oligomers are incapable of being separated from the full length PS oligomer. Thus, there is a need to identify an alternate approach to synthesize high-quality antisense drugs.

Assuming that coupling and sulfurization inefficiencies are the main causes, a key to reduce these process-related oligonucleotides could be the use of a blockmer coupling strategy. Recently, we have demonstrated that greater than 70% reduction in (n-1)-mer content and ca. 50% reduction of phosphodiester linkages could be achieved by using dimers in some model sequences[23]. This prompted us to investigate the effect of using trimeric building blocks on the quality of synthesized oligonucleotides. Protected trinucleotides have been reported in literature for use in the controlled codon-by-codon construction of combinatorial libraries of structural genes.[24-27] However no one has used these blockmers to address the issue of improving the quality of synthesized oligonucleotides for antisense applications.

Encouraged by our positive results on the use of dimers in model compounds, we decided to investigate the use of trimers in the synthesis of a 20-mer oligodeoxyribonucleotide phosphorothioate (ISIS 2503; 5'-TCC-G-TCA-TC-GC-TCC-TCA-GG-G). This anti-cancer antisense drug is in Phase I clinical trials.[28]

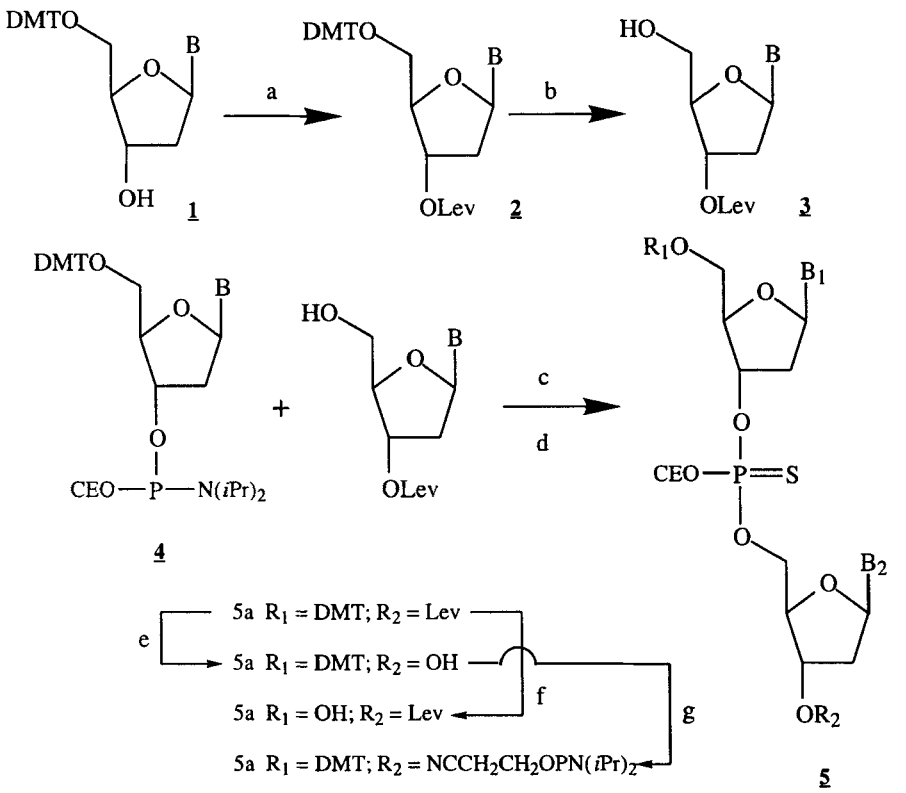
Reported methods for the synthesis of trinucleotide phosphoramidite synthons suffer from one or more limitations. For example, a recent report uses *O*-chlorophenyl group for internucleotide phosphorus protection.[26] However, from our recent experience on the large scale synthesis of an 8-mer oligonucleotide phosphorothioate using this group

leads to considerable (>4% per linkage) cleavage of internucleotide bonds. In addition, there is no report on the synthesis and use of a fully protected trinucleotide phosphoramidite synthons. Our method is characterized by use of a pair of orthogonal protecting groups for the 5'- and 3'-terminus and, as a consequence, flexibility with respect to direction of chain elongation and optimal use of synthetic intermediates. In addition we report here an efficient and inexpensive sulfurization for the synthesis of trimer building blocks.

The approach is outlined in Scheme **1**. We used β-cyanoethyl protected phosphoramidites 1 as monomer units and (*O*,*O*,*O*)-trialkyl phosphorothioate triester amidites 5d and 6 as blockmer units. For protection of the internucleotide linkage, we chose β-cyanoethyl (CE) group that is selectively removed upon treatment with base (NH_4OH), and for the 3'-*O*-protection the levulinyl group. Other groups reported in the literature like TBDMS[29] and phenoxyacetyl[24] were found to be incompatible for 3'-*O*-protection.

5'-*O*-DMT-3'-*O*-levulinyl protected nucleosides were prepared using levulinic anhydride by a modified procedure. Treatment with *p*-toluenesulfonic acid (2 equiv.) in ethylacetate/methanol (8:2 v/v) at room temperature gave after workup 3'-*O*-levulinyl nucleosides in 60-70% isolated yields (two steps) *without column chromatography*. Zinc bromide in nitromethane was also found to be an efficient and clean method[30] for the removal of the dimethoxytrityl group. Dichloromethane in place of nitromethane was a suitable alternative solvent but the reaction was considerably slow >24 h). Coupling of 1*H*-tetrazole activated phosphoramidite 4 with 3'-*O*-levulinyl nucleoside, followed by sulfurization of the resulting phosphite triester with diethyldithiocarbonate disulfide (DDD)[31-32] afforded the corresponding protected trialkyl phosphorothioate triester. Subsequent treatment with hydrazine hydrate in pyridine/glacial acetic acid followed by phosphitylation with β-cyanoethyl-*N*,*N*,*N*',*N*'-tetraisopropylphosphorbisamidite afforded dimer phosphoramidite 5 (Scheme **1**).

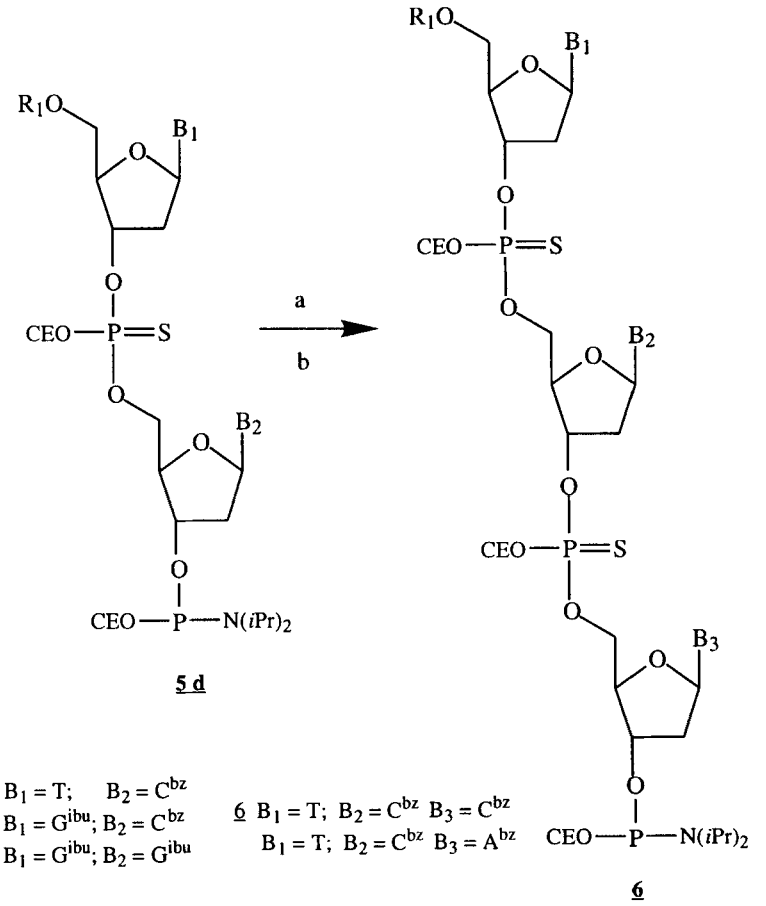
For synthesis of trimers, coupling of dimer amidite 5 with 3'-*O*-levulinyl nucleoside in presence of 1*H*-tetrazole in acetonitrile, followed by sulfurization with DDD afforded the fully protected phosphorothioate triester. Removal of levulinyl group, under identical conditions for dimers, followed by phosphitylation afforded the trimer phosphoramidite 6



Scheme 1: **a**. levulynic anhydride, rt, 5 h, **b**. pTSA, EtOAc/MeOH (8:2 v/v), **c**. 1*H*-tetrazole, CH_3CN, rt, **d**. DDD, rt, 1 h, **e**. hydrazine hydrate, pyridine/acetic acid, 0°C, **f**. $ZnBr_2$, CH_3NO_2, rt, 2 h, **g**. $NCCH_2CH_2OP[N(iPr)_2]_2$, 1*H*-tetrazole.

(Scheme 2). DDD was found to ba an efficient and inexpensive sulfurizing reagent over 3H-1,2-benzodithiol-3-one 1,1-dioxide[33-34] in the solution phase synthesis of dimer and trimer phosphorothioate triester building blocks.

Synthesis of ISIS 2503 required two trimer and three dimer building blocks (TCC, TCA, GC and GC). All the phosphoramidite blockmers were obtained in moderate yields (unoptimized) and in high purity (>99%) as judged by ^{31}P NMR in multi-gram quantities. TC dimer amidite was synthesized in ca. 300 gram scale without difficulty. TCC and TCA trimer amidites were synthesized in ca. 20 gram scale. During the phosphitylation of trimer blocks with β-cyanoethyl-*N*,*N*,*N*',*N*'-tetraisopropylphosphorbisamidite, 4-5 equivalents of phosphitylating reagent was used to drive the



Scheme 2: **a**. 1*H*-tetrazole, CH_3CN, rt, **b**. DDD, rt, 1 h, **c**. hydrazine hydrate, pyridine/acetic acid, 0°C, **d**. $NCCH_2CH_2OP[N(iPr)_2]_2$, 1*H*-tetrazole.

reaction to completion in less than 1 h. Considerable loss of β-cyanoethyl group from the internucleotide linkage was observed (^{31}P NMR) when the reaction was left for longer duration. Thus, this restricting condition becomes impractical and an alternative group in place of β-cyanoethyl is highly desirable if these building blocks are to be scaled up for the manufacture of antisense drugs.

Oligomerization: Synthesis of the 20-mer oligodeoxyribonucleotide phosphorothioate 5'-TCC-G-TCA-TC-GC-TCC-TCA-GG-G (ISIS 2503) using monomer amidites 4 and blockmer synthons 5d and 6 were

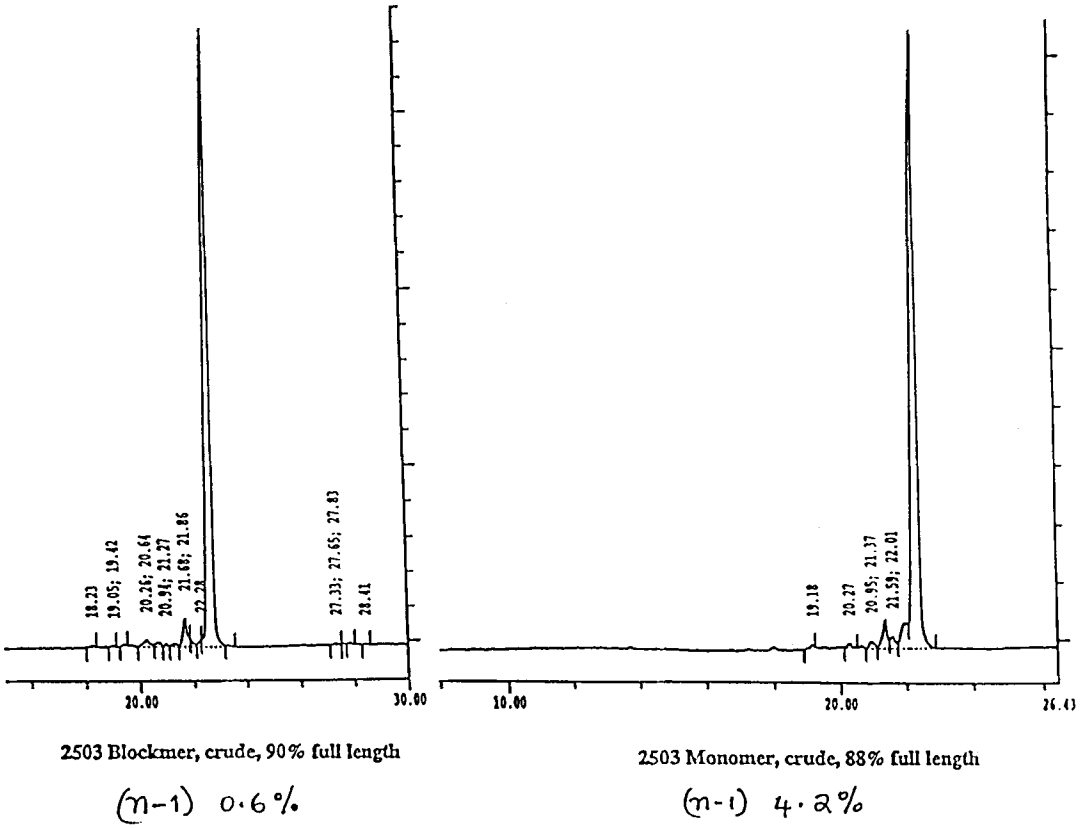


Fig. 1: CGE analysis of crude oligomer synthesized using monomer and blockmer.

performed on a 1 μmole scale on an ABI 394 DNA/RNA synthesizer.[35] The CPG-bound DMT-on oligomer was treated with 30% ammonium hydroxide for 1 h at room temperature, followed by heating of the solution at 60°C for 18 h. The incubated solution was cooled, concentrated and purified by reversed phase HPLC on a C18 column using standard gradient conditions. Fig. 1 shows the capillary gel electrophoresis analysis[36-38] of the crude oligomer products synthesized using monomers and blockmers.

Based on CGE, the amount of (n-1)-mer in monomer based synthesis and trimer based synthesis are 4.2% and 0.6% respectively and represents a substantial reduction in the (n-1)-mer content of the synthesized oligonucleotide. This result clearly demonstrates that most of the (n-1)-mer population is indeed formed during the chain elongation reactions and that

only a small portion of it is due to other factors.[18-20] In summary, we have shown experimentally that use of trimer and dimer building blocks in the solid phase synthesis of oligodeoxyribonucleotide phosphorothioates leads to substantial reduction of (n-1)-mer content leading to high quality of purified active pharmaceutical ingredients.

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35. Column dG-CPG (I mmol) from Glen Research, Sterling, VA. Standard detritylation, capping and 1*H*-tetrazole solutions (Applied Biosystems). Sulfurizing solution was 0.2M 3H-1,2-benzodithiol-3-one 1,1-dioxide (R.I. Chemical, Orange, CA) in anhydrous CH_3CN. Amidites were used as 0.1M solutions in CH_3CN. Coupling time 200 s, sulfurization time 900s (not optimized).

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38. Conditions for CGE: P?ACE System 5000, eCAP ssDNA 100 Gel Capillary (47 cm), tris/borate/7M urea buffer (all Beckman), running voltage 14.1 kV, injection 1 s @ 3 kV, temp 40°C.

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