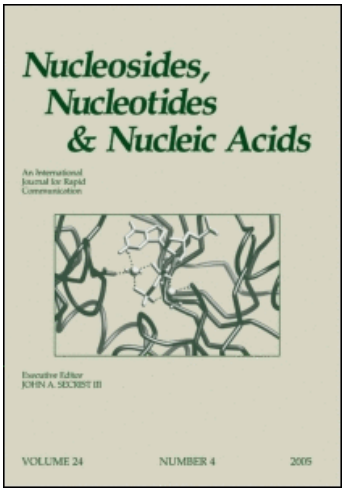

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IMPROVED IMPURITY PROFILE OF PHOSPHOROTHIOATE OLIGONUCLEOTIDES THROUGH THE USE OF DIMERIC PHOSPHORAMIDITE SYNTHONS

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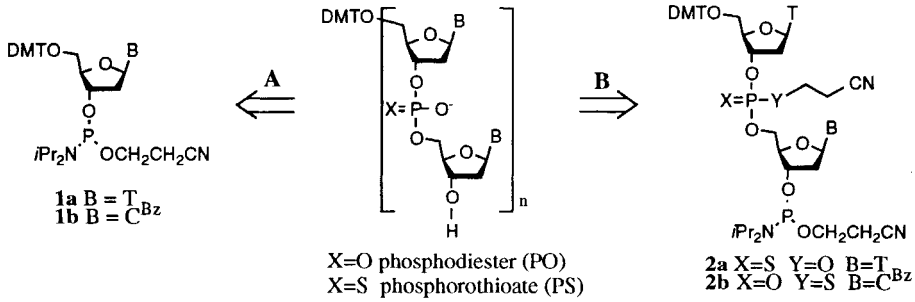
ABSTRACT. Phosphorothioate oligonucleotides synthesized through assembly of dimeric phosphoramidite synthons show a significantly improved impurity profile compared to oligomers synthesized through coupling of standard monomer phosphoramidites. A greater than 70% reduction of the (n-1)-mer population and a ca. 50% reduction of phosphodiester linkages has been achieved.

Multiple examples of the first generation of antisense drugs, uniformly modified oligodeoxyribonucleoside phosphorothioates, are currently in advanced clinical trials.[1] Phosphoramidite coupling[2] followed by stepwise sulfurization of the trialkylphosphite linkage is the preferred method for large scale phosphorothioate oligonucleotide synthesis, providing 98.5+% coupling yields at 1.5 fold molar amidite excess and corresponding high product purities. Problems commonly experienced in automated oligophosphorothioate synthesis *via* mononucleotide phosphoramidite coupling include formation of a population of shorter deletion sequences ((n-1)-, (n-2)-mers, etc.).[3] The occurrence of phosphodiester (PO) linkages at a low level is mainly due to side reactions during the sulfurization step.[4]



Assuming that coupling and sulfurization inefficiencies are the main causes of (n-1)-mer and PO formation, a key to reducing both side products could be the use of a block-

mer coupling strategy.[5] We have investigated this hypothesis by comparing the impurity profiles of model phosphorothioate oligomers T_{19} and $(TdC)_9T$ synthesized through monomer (**A**) and dimer (**B**) assembly, respectively.[6]

We used standard 5'-(4,4'-dimethoxytrityl) protected amidites **1** as monomeric building units and (*O,O,O*)- and (*O,O,S*)-trialkyl phosphorothioate dimer amidites **2a** and **2b** as dimeric building blocks.[2] Coupling of 1*H*-tetrazole activated amidite **1a** with 3'-*O*-levulinyl thymidine[5f] in CH_3CN, followed by sulfurization with 3*H*-1,2-benzodithiol-3-one-1,1-di-oxide[4] afforded the corresponding trialkyl phosphorothioate. Subsequent treatment with hydrazine hydrate in pyridine/glacial acetic acid[5f] allowed selective deprotection of the 3' terminus. Phosphitylation with *O*-β-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphorodiamidite afforded dimer phosphoramidite **2a** in high overall yield. **2a** allows direct comparison of the impurity profiles of the oligomers $(T)_{19}$ and $(\underline{T_2})_9T$ as the oligonucleotides at the end of the solid phase synthesis are virtually identical. *S*-CE protected dinucleoside synthons like **2b**[7] are readily accessible through phosphotriester chemistry in solution.[8] The *O*-CE group is selectively removed from (*O,O,O*)-trialkyl phosphorothioates upon treatment with base through β-elimination.[9a] In case of the *S*-CE group deprotection through β-elimination and hydrolytic deprotection leading to undesired (PO) linkages are competing reaction pathways.[9b]

Oligomer syntheses were performed on a 1 μmol scale on an ABI 394 DNA/RNA Synthesizer.[10] T_{19} and (Tp(*O*-CE) $dC^{Bz})_9T$ oligomers were cleaved from the resin and deprotected with conc. NH_4OH. The CPG-bound (Tp(*O/S*-CE)$dC^{Bz})_9T$ oligomer was treated with anhydr. *t*$BuNH_2$/pyridine (1:5, *v/v*) for 20 h at r. t., filtered, rinsed with CH_3CN and then treated with 30% NH_4OH for 1 h at r. t., followed by heating at 60 °C for 2 h. Table 1 summarizes the results of the CGE, ^{31}P NMR and ion exchange HPLC analyses. The PO content decreases in the order $(T)_{19} > (\underline{T_2})_5(T)_9 \cong (T)_8(\underline{T_2})_5T > (\underline{T_2})_9T$. Consistent with our hypothesis we observe a reduction of PO linkages of ca. 50%. Strong anion exchange (SAX) HPLC of the DMT-off oligomers (Fig. 1) shows increasing all-PS and decreasing $(PO)_1(PS)_{17}$ oligomer content consistent with the NMR results. A similar reduction of (PO) linkages was not apparent in oligomers $(TdC)_9T$, $(\underline{TdC})_5$ $(TdC)_4T$, $(TdC)_4(\underline{TdC})_5T$ and $(\underline{TdC})_9T$ possibly due to a small extent of hydrolysis of the *S*-CE group. Quantitative CGE analysis of the crude oligomer products showed increased full length oligomer content in oligomers synthesized with **2a** or **2b** demonstrating a high coupling efficiency of the dimer synthons. Even more importantly, the CGE profiles show a more than 70% reduced (n-1)/[n+(n-1)] ratio in case of $(\underline{T_2})_9T$ and $(\underline{TdC})_9T$ (<0.7%)[11] compared to $(T)_{19}$ and $(TdC)_9T$ (2.1% and 2.0%), respectively. Two typical CGE traces are shown in Fig. 1. These results demonstrate experimentally that most of the (n-1)-mer population is indeed formed during the chain elongation reactions and that only a small

Table 1. Analysis of T_{19} and $(TdC)_9T$ phosphorothioates.

oligo	ACE (%) (per nucl.)[1]	n/(n-1) [1]	(PO) content (±0.1)[2]	$(PO)_1(PS)_{17}$:$(PS)_{18}$ ratio [3]
T_{19}	99.4 (99.4)	97.9:2.1	0.9	20:80
$(TdC)_9T$	98.9 (98.9)	98.0:2.0	0.8	20:80
$T_8(\underline{T_2})_5T$	99.0 (99.3)	99.0:1.0	0.6	12:88
$(TdC)_4(\underline{TdC})_5T$	99.1 (99.3)	99.1:0.9	1.2	20:80
$(\underline{T_2})_5T_9$	98.8 (99.1)	98.8:1.2	0.7	13:87
$(\underline{TdC})_5(TdC)_4T$	99.0 (99.3)	98.8:1.2	0.6	16:84
$(\underline{T_2})_9T$	98.9 (99.5)	>99.3:0.7	0.5	12:88
$(\underline{TdC})_9T$	98.8 (99.4)	>99.4:0.6	1.3	21:79

[1] from CGE [2] from ^{31}P NMR [3] from SAX

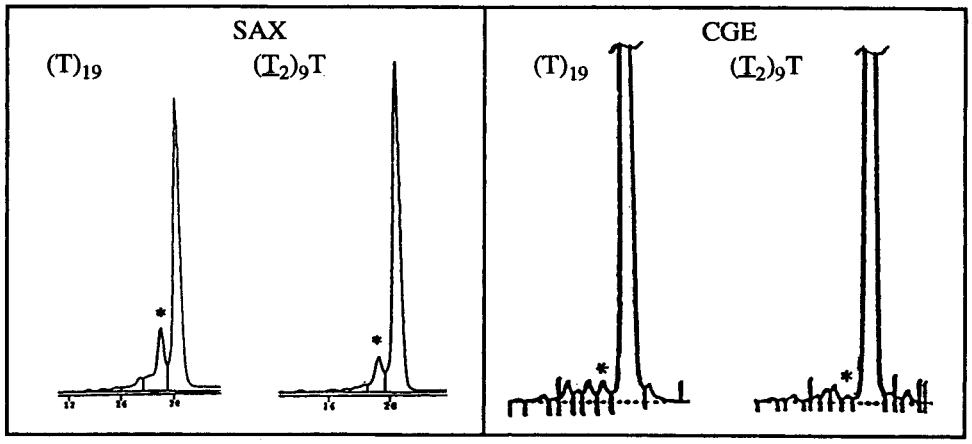


Fig. 1. Impurity profiles of T_{19} phosphorothioates. (left) SAX HPLC, notice the reduced $(PS)_{17}(PO)_1$ content (*). (right) CGE analysis, notice the reduced (n-1)-mer content (*).

portion of it is due to other factors.[3] Oligomers $(\underline{T_2})_5(T)_9$ (1.2 %) and $(\underline{TdC})_5(TdC)_4T$ (1.2 %) or $(T)_8(\underline{T_2})_5T$ (1.0 %) and $(TdC)_4(\underline{TdC})_5T$ (0.9%) have a reduced (n-1)-mer content in proportion to the number of dimer synthons used in the synthesis. No significant preference for the formation of (n-1)-mers at either the 3' or 5' side of the oligomer is observed.

These results demonstrate that the use of dimer building blocks **2a** and **2b** in the solid phase synthesis of phosphorothioate oligomers leads to largely reduced (n-1) mer content and in case of **2a** also reduces the amount of phosphodiester linkages formed

during the sulfurization step by ca. 50 %. Amidite coupling dependent and, to a much smaller extent, coupling independent factors contribute to the final (n-1)-mer content.

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References and Notes

1. D. L. Kisner, *12th International Roundtable on Nucleosides and Nucleotides*, September 19, **1996**, La Jolla, CA, USA.
2. L. C. McBride, M. H. Caruthers, *Tetrahedron Lett.* **1983**, *24*, 245.
3. a) J. Temsamani, M. Kubert, S. Agrawal, *Nucleic Acids Res.* **1995**, *23*, 1841-1844; b) K. L. Fearon, J. T. Stults, B. J. Bergot, L. M. Christensen, A. M. Raible, *Nucleic Acids Res.* **1995**, *23*, 2754-2761; c) R. P. Iyer, D. Yu, Z. Jiang, S. Agrawal, *Nucleosides & Nucleotides* **1995**, 14, 1349-1357.
4. R. P. Iyer, W. Egan, J. B. Regan, S. L. Beaucage, *J. Am. Chem. Soc.* **1990**, *112*, 1253-1254.
5. a) N. J. Cusack, C. B. Reese, J. H. van Boom, *Tetrahedron Lett.* **1973**, *24*, 2209-2212; b) R. Crea, A. Kraszewski, T. Hirose, K. Itakura, *Proc. Natl. Acad. Sci. USA* **1978**, *75*, 5765-5769; c) R. L. Letsinger, E. P. Groody, N. Lander, T. Tanaka, *Tetrahedron* **1984**, *40*, 137-143; d) E. S. Werstiuk, T. Neilson, *Can. J. Chem.* **1976**, *54*, 2689-2696; e) E. Ohtsuka, S. Tanaka, M. Ikehara, *J. Am. Chem. Soc.* **1978**, *100*, 8210-8213; f) G. Kumar, M. S. Poonian, *J. Org. Chem.* **1984**, *49*, 4905-4912; g) A. Wolter, J. Biernat, H. Köster, *Nucleosides & Nucleotides* **1986**, *5*, 65-77; h) K. Miura, K. Sawadaishi, H. Inoue, E. Ohtsuka, *Chem. Pharm. Bull.* **1987**, *35(2)*, 833-836; DNA containing one or more PS linkages: i) J. E. Marugg, C. van den Bergh, M. Tromp, G. A. van der Marel, W. J. van Zoest, J. H. van Boom, *Nucleic Acids Res.* **1984**, *12*, 9095-9110; j) B. A. Connolly, B. V. L. Potter, F. Eckstein, A. Pingoud, L. Grotjahn, *Biochemistry* **1984**, *23*, 3443-3453; k) R. Cosstick, F. Eckstein, *Biochemistry* **1985**, 24, 3630-3638.
6. Nomenclature: T_{19} denotes a nonadecathymidylate phosphorothioate, $(T)_{19}$ and $(TdC)_9T$ indicate monomer assembly, $(\underline{T_2})_9T$, $(\underline{TdC})_9T$ indicate dimer assembly, $\underline{TdC}$ indicates *S*-CE protection.
7. Cruachem, Inc., Glasgow, Scotland.
8. X. Liu, C. B. Reese, *J. Chem. Soc. Perkin Trans. 1*, **1995**, 1685-1694.
9. a) N. D. Sinha, J. Biernat, J. McManus, H. Köster, *Nucleic Acids Res.* **1984**, *12*, 4539. b) R. Cosstick, D. M. Williams, *Nucleic Acids Res.* **1987**, *15*, 9921-9932.
10. Columns: dT-CPG (1μmol) from Glen Research. Standard detritylation, capping and 1*H*-tetrazole solutions (Applied Biosystems). Amidites (0.1 M in CH_3CN), coupling time 200 s. Sulfurization: 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (0.2 M in CH_3CN, R. I. Chemical, Orange, CA), 900 s (not optimized).
11. Precise quantitation below 1% is difficult due to lack of baseline resolution. The actual value may be significantly lower.