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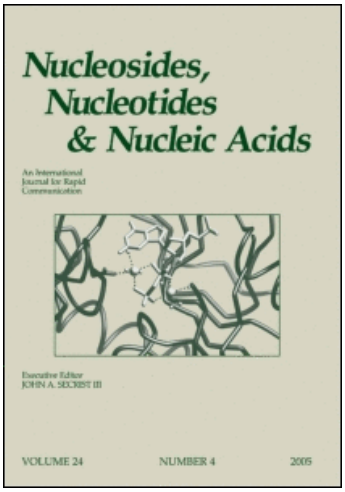

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Synthesis of Dimer Phosphoramidite Synthons for Oligodeoxyribonucleotide Phosphorothioates Using Diethyldithiocarbonate disulfide as an Efficient Sulfurizing Reagent

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Abstract: An efficient solution phase synthesis of deoxyribonucleoside phosphorothioate dimers utilizing phosphoramidite approach is described. Diethyldithiocarbonate disulfide (DDD) was found to be an efficient sulfurizing reagent in the conversion of phosphite triesters to phosphorothioate triesters.

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The antisense therapeutic principle has undergone revolutionary developments during the last few years.[1-6] This strategy is proving to be extremely promising and has thus raised the key issue of manufacturing large quantities of oligonucleotide analogs at high levels of purity. Oligodeoxyribonucleotide phosphorothioates, in which one nonbridging oxygen atom of the internucleotide phosphate group is formally replaced by sulfur, are the first class of antisense compounds to reach the clinic. Multiple compounds of 20- and 21-mer length are in clinical trials.[7-9]

Recent advances in phosphoramidite coupling chemistry[10-12] and solid-phase synthesis methodology together with current state-of-the-art large scale synthesizer allow complete synthesis of a 20-mer deoxyribonucleotide phosphorothioate at 150 mmole scale in 8 hours. Due to its high efficiency, phosphoramidite coupling followed by stepwise sulfurization of the trialky-phosphite linkage is the currently preferred method for uniformly modified phosphorothioate synthesis, providing >98.5% average coupling yields at 1.75-fold molar amidite excess.[13]

Problems commonly experienced in automated synthesis via monomeric phosphoramidite coupling include formation of a relatively small population of partial phosphodiesters as well as deletion sequences in which one or more internal

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nucleotides are absent.[14-16] Several factors, including less than quantitative coupling efficiency, incomplete capping or removal of dimethoxytrityl groups, as well as work-up protocols appear to contribute to formation of these process-related impurities. Assuming that coupling and sulfurization inefficiencies are the main causes, a key to reducing formation of these impurities could be use of a blockmer coupling strategy. Recently we have demonstrated proof of this principle by the use of dimeric phosphoramidite units and shown that greater than 70% reduction of the (n-1)-mer population and a ca 50% reduction in phosphodiester linkages could be achieved in model sequences.[14,15] Hence, there was a need to efficiently synthesize dimer building blocks.

Initially, we reported[17] synthesis of phosphorothioate triester dimers by the coupling of 1H-tetrazole activated 5'-*O*-dimethoxytritylthymidine phosphoramidite with 3'-*O*-levulinylthymidine,[18] followed by sulfurization of the resulting phosphite triester with 3H-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent)[19,20] to afford the corresponding fully protected dimer. However, for the large scale synthesis of various dimers, Beaucage reagent is not desirable for several reasons: the reagent is expensive ($5500/kg), the by-product formed after sulfurization is a potential oxidizing agent and may lead to formation of phosphate triesters, and in some cases during workup after sulfurization, we have observed loss of the dimethoxytrityl group, leading to lower yields. Hence, we explored several options for an alternative to this efficient sulfurizing reagent.

Among sulfurizing agents evaluated in our laboratory, we found diethyldithiocarbonate dilsulfide (DDD) to be an efficient and novel sulfurizing reagent for the solution phase synthesis of phosphorothioate triesters. This reagent is inexpensive ($300/kg), scaleable, and leads to very efficient formation of phosphorothioate triesters, with only 1.25 molar excess of the reagent (Fig. 1).

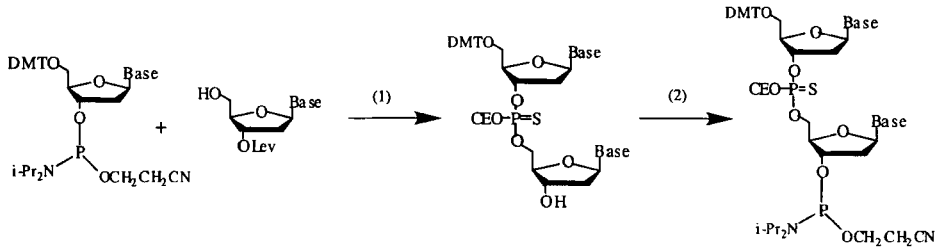

Fig. 1 (1) (a) 1H-Tetrazole, CH_3CN, rt (b) DDD (c) NH_2NH_2, Py/AcOH (2) 2-cyanoethyl *N,N,N,N*-tetraisopropylphosphorodiamidite.

Among the few protecting groups available, the levulinyl group was chosen to protect the 3'-hydroxyl of the nucleoside. This group is stable to coupling and sulfurization conditions and easily removed under mild conditions (NH_2NH_2, Py, AcOH) in a short time (<1 h). All dimers synthesized had undetectable levels of phosphate triesters as determined by ^{31}P NMR.

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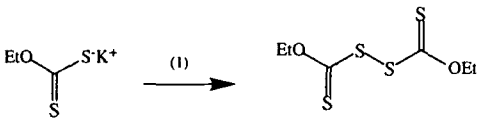
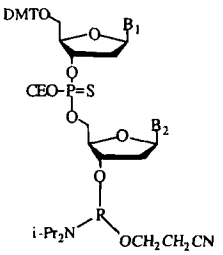
DDD is synthesized in one step in >90% yield as a pale yellow crystalline solid by oxidizing the potassium salt of *O*-ethylxanthic acid with an oxidizing agent such as iodine (Fig. **2**).[21-23] The reagent is soluble in most organic solvents



Diethyldithiocarbonate disulfide

Fig. **2** (1) Iodine, KI, water.

used for nucleotide synthesis (CH_3CN, CH_2Cl_2, THF). Fig **3** shows the various dimers synthesized using DDD as sulfurizing reagent. Dimers (2) and (3) have been synthesized at 300 gram scale without difficulty and in high quality. The synthesis of dimer (8) was performed using 5-methyldeoxycytidine as the nucleoside.



(1) B_1 = T; B_2 = T
(2) B_1 = T; B_2 = C^{bz}
(3) B_1 = G^{ibu}; B_2 = T
(4) B_1 = G^{ibu}; B_2 = C^{bz}
(5) B_1 = A^{bz}; B_2 = T
(6) B_1 = A^{bz}; B_2 = C^{bz}
(7) B_1 = T; B_2 = G^{ibu}
(8) B_1 = $^{m}C^{bz}$; B_2 = T

Fig. **3** Dimers Synthesized Using DDD.

Typical Experimental Procedure: To a stirred solution of 3'-*O*-levulinylthymidine (16.2 g; 47.7 mmole) and 1*H*-tetrazole (16.71 g; 238.5 mmole) in dry acetonitrile (250 ml) at room temperature under argon was added a solution of 5'-*O*-(4,4'-dimethoxytrityl)thymidine-3'-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (42.3 g; 56.8 mmole) in dry acetonitrile (120 ml). After stirring for 1 h, the sulfurizing reagent (DDD) (16.33 g; 59.6 mmol) was added as a solid all at once. After 45 minutes, the reaction mixture was concentrated under reduced pressure, the residue partioned between dichloromethane (350 ml) and saturated sodium bicarbonate solution (300 ml). The aqueous layer was back extracted with dichloromethane (150 ml) and the combined organic layers were dried and concentrated (91%). The crude material was used in the next step.

Removal of 3'-O-levulinyl group: The fully protected dimer (15 g, 14.76 mmole) was treated with a mixture of hydrazine hydrate (4.6 ml, 147.6 mmol), pyridine (31 ml) and acetic acid (18 ml) at room temperature. After 30 minutes, crushed ice was added and left aside for 1 h. The reaction mixture was extracted with dichloromethane (400 ml), dried and concentrated. The crude product was purified

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by flash chromatography using silica gel and dichloromethane/methanol as eluents. Yield 11.68 g (86%). ^{31}P NMR ($CDCl_3$): 66.4, 66.6.

Synthesis of Dimer amidite: Under argon, a solution of 1*H* -tetrazole (0.192 g; 27 mmol) and 2-cyanoethyl-*N,N,N',N'*-tetra-isopropylphosphorodiamidite (18 g, 60 mmol) in dry CH_2Cl_2 (200 ml) is added to a solution of DMT TpsT-OH dimer (30 mmol) in CH_2Cl_2 (100 ml). After amidite formation is complete (1.5 h), the solution is extracted twice with aqueous $NaHCO_3$ (1M). The organic phase is dried over Na_2SO_4 and the solvent is removed under reduced pressure. The residue is dissolved in CH_2Cl_2 (300 ml) and hexane (600 ml) is added. A colorless oil separates. After 1h, the mixture is decanted. The remaining oil is dissolved in 5% $MeOH/CH_2Cl_2$ (200 ml), and filtered through a layer of silica (12 cm). The compound is eluted with the same solvent (ca. 800 ml). The solvent is evaporated, the residue dissolved in CH_2Cl_2 (150 ml) and treated with hexane (300 ml). After 1 h the supernatent is decanted, the remaining oil washed with hexane and dried: (85%). ^{31}P NMR ($CDCl_3$): 67.5, 67.8, 149.7, 150 ppm.

In summary we have shown that DDD is an efficient and useful sulfurizing reagent for the large scale solution phase synthesis of phosphorothioate triester dimers.

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