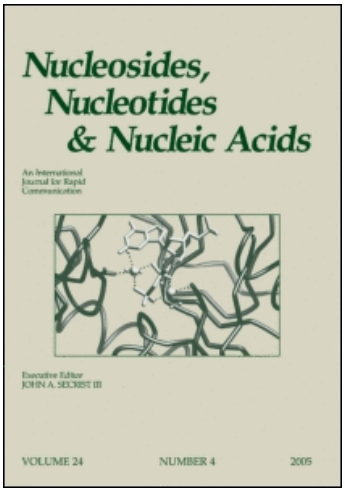

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EFFICIENCY OF COUPLING IN THE SYNTHESIS OF DEOXYRIBONUCLEOTIDE PHOSPHOROTHIOATES VIA PHOSPHOTRIESTER APPROACH UTILIZING VARIOUS ACTIVATING REAGENTS[1]

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Abstract: The synthesis of deoxyribonucleotide phosphorothioates via phosphotriester approach utilizing various coupling reagents is described.

Recently, considerable attention has been focused on the applications of oligonucleotides as therapeutic agents.[2-6] Although, initial interest in oligonucleotides was focused on their interaction with nucleic acid receptors, interest has broadened to include the use of oligonucleotides as ligands to interact with non-nucleic acid receptors like proteins as well. Thus, the creation of combinatorial libraries of oligonucleotides containing natural and unnatural motifs has greatly facilitated the exploration of these potential interactions for therapeutic purposes.[7-9]

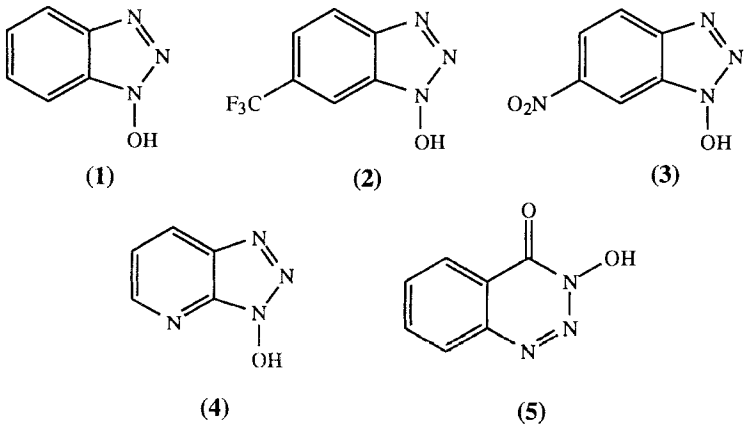
The oligodeoxyribonucleotide phosphorothioate TTGGGGTT (ISIS 5320) is a potent inhibitor of HIV infection *in vitro*. The compound was identified by combinatorial screening of a library of all possible octanucleotide sequences.[10] This octanucleotide forms a parallel stranded, tetrameric guanosine-quartet (G-quartet) structure which specifically binds to the HIV envelope glycoprotein (gp 120) and inhibits both cell-to-cell and virus-to-cell infection at submicromolar concentrations. This tetramer inhibits the infection of laboratory-derived isolates of HIV-1 and HIV-2 in a variety of phenotypically distinct, established human cell lines and a panel of biologically diverse clinical isolates in fresh human peripheral blood lymphocytes and macrophages. The compound was also active against all drug-resistance virus isolates tested. In combination with AZT, it exhibits additive to slightly synergistic anti-HIV activity. Cell-based mechanism of action studies demonstrate that the compound

inhibits the binding of infectious virus and virus-infected cells to uninfected target cells by binding to the cationic V3 loop of the envelope glycoprotein.[11]

This potential drug candidate for the treatment of AIDS is presently under pre-clinical trials. Very large quantities (several hundred grams) of this drug are needed for clinical trials and several folds more to meet market demands. Thus a key issue is the selection of a method for manufacturing the compound in multi-kilogram quantities to fully support clinical trials in the AIDS population at an acceptable cost.

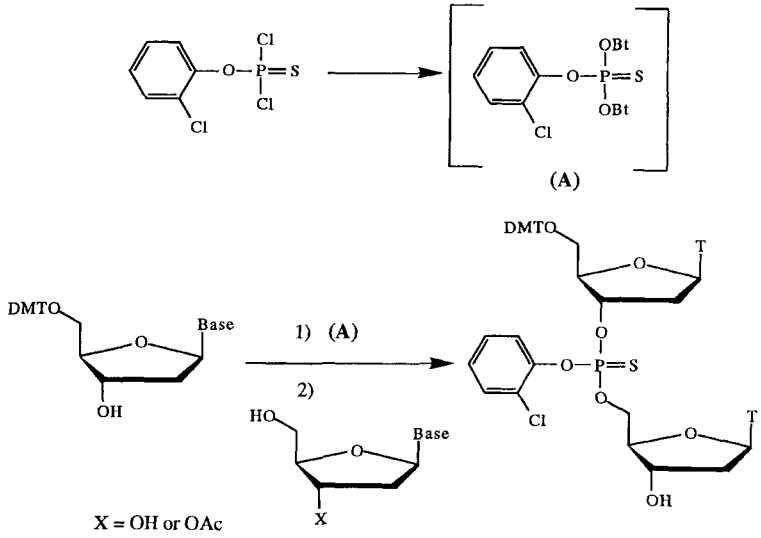
Solution-phase chemical synthesis of nucleic acids has significant potential in drug synthesis and is particularly appropriate for large-scale preparation of short sequence oligonucleotides.[12-15] Low cost reagents and operational simplicity allowing large scale, reproducible syntheses are important practical considerations. As a part of our on-going investigation on the solution phase synthesis, we were interested in the phosphotriester approach for the large scale synthesis of this octamer. Although there are several publications on the solution phase synthesis, there is no report on the synthesis of oligonucleotides in multigram quantities. Thus an industrial production of this magnitude is without precedent in oligonucleotide chemistry and because, of its commercial value, attempts to explore relative merits of its economical synthesis warrants careful consideration.

Among the three kinds of coupling chemistries, viz. phosphoramidite, H-phosphonate and phosphotriester, the last approach is more suited for solution phase synthesis of oligonucleotides. However, the phosphotriester coupling chemistry is not as efficient as the phosphoramidite approach, and in an effort to increase the coupling yield, we were interested in investigating large scale synthesis of dimer **6** utilizing various activating reagents. The deoxyribonucleotide phosphorothioate dimer **6** could be synthesized by starting with thymidine (X = OH) as the nucleoside to form 5'-DMT-TpsT-OH-3', then acylate the free 3'-hydroxyl group and finally remove the dimethoxytrityl group. Alternatively, the dimer **6** could be prepared by starting with 3'-O-acetyl thymidine and reduce one step in the synthesis. The advantage of the latter method is the elimination of 3'-3' coupled product and hence better yield, but on the other hand, 3'-O-acetylthymidine is more expensive than thymidine.



(1) (2) (3)

(4) (5)

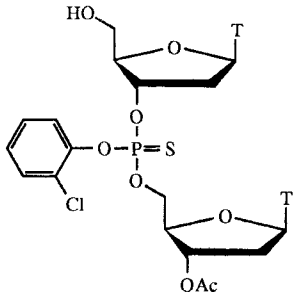


Scheme 1

Table 1. Yields of the dimers synthesized.

Coupling Reagent	Dimer[a]	Yield (%)[b]
(1)	5'-DMT-T - T-OAc-3'	82
(2)	5'-DMT-T - T-OAc-3'	86
(3)	5'-DMT-T - T-OAc-3'	82
(4)	5'-DMT-T - T-OAc-3'	72
(5)	5'-DMT-T - T-OAc-3'	77
(1)	5'-DMT-T - T-OH-3'	61
(2)	5'-DMT-T - T-OH-3'	63
(3)	5'-DMT-T - T-OH-3'	58
(4)	5'-DMT-T - T-OH-3'	45
(5)	5'-DMT-T - T-OH-3'	51

[a]Obtained as a mixture of diastereomers [b]Isolated yield



(6)

The efficiency of coupling utilizing five different reagents viz. 1-hydroxybenzotriazole (**1**),[16,17] 1-hydroxy-6-(trifluoromethyl)benzotriazole (**2**),[18,19] 1-hydroxy-6-nitrobenzotriazole (**3**),[19,20] 1-hydroxy-7-azabenzotriazole (**4**),[21,22] and 3-hydroxy-1,2,3-benzotriazin-4(3H)-one (**5**)[23-24] were investigated in the synthesis of homodimers of thymidine. Scheme **1** shows a typical example of the synthesis of dimer and Table **1** shows the yields of dimers utilizing the above activating reagents.

In conclusion, based on the above results it appears that 1-hydroxy-6-(trifluoromethyl)benzotriazole (**2**) is a better activating agent and gives higher yields than other reagents. In addition, since the cost of thymidine ($1.50/g) is much cheaper compared to 3'-O-acetylthymidine ($50/g), and also the yield of 5'-DMT-TpsT-OH-3' is acceptable (63%), it is preferable to synthesize 5'-DMT-TpsT-OH-3', then acylate the 3'-hydroxyl group than synthesize 5'-DMT-TpsT-OAc-3'. Finally, although formation of 3'-3' coupling products with various reagents was not thoroughly investigated, based on tlc, it appears that use of thymidine gave lower yields because of incomplete reaction and not because of symmetrical dimer formation.

Typical Experimental Procedure: To a stirred solution of anhydrous 1-hydroxybenzotriazole (4.86 g; 36 mmole) in anhydrous dioxane (12 ml) in a 250 ml flask under argon was added anhydrous pyridine (6.4 ml; 79.2 mmole) at room temperature. Then a solution of 2-chlorophenylphosphorodichloridothioate[25,26] (3.93 g; 15 mmole) in anhydrous dioxane (60 ml) was added over a period of 15 min. at room temperature. Stirring was continued for 5 h. The pyridinium hydrochloride salt was filtered under argon and the clear solution of bis-phosphorylating reagent was used as a stock solution. 5'-DMT Thymidine (4.9 g; 9 mmole) in pyridine (15 ml) was taken in a 250 ml three-necked flask with argon inlet and magnetic stirring. The bis-phosphorylating reagent prepared above (54 ml) was added slowly at room temperature. After stirring the reaction mixture for 3 h, thymidine (2.83 g; 11.7 mmole) was added and stirred for 15 min. Then, N-methylimidazole (3.69 g; 45 mmole) was added at room temperature and the reaction was stirred for 8 h. Triethylammonium bicarbonate (1 M solution, 40 ml) was added to quench the reaction. The reaction mixture was diluted with dichloromethane (200 ml) and washed with water (100 ml), brine (100 ml) and dried ($MgSO_4$). The dried extract

was filtered, concentrated and purified by flash chromatography using silica gel eluting first with dichloromethane, followed by dichloromethane:methanol (9:1, v/v). The fractions corresponding to the desired product were combined and concentrated.. Yield 5.35 g (61%). ^{31}P NMR ($CDCl_3$) δ 61.9, 63.2.

Typical Experimental Procedure: To a stirred solution of anhydrous 1-hydroxy-6-(trifluoromethyl)benzotriazole (7.31 g; 36 mmole) in anhydrous dioxane (12 ml) in a 250 ml flask under argon was added anhydrous pyridine (6.4 ml; 79.2 mmole) at room temperature. Then a solution of 2-chlorophenylphosphorodichloridothioate[25,26] (3.93 g; 15 mmole) in anhydrous dioxane (60 ml) was added over a period of 15 min. at room temperature. Stirring was continued for 5 h. The pyridinium hydrochloride salt was filtered under argon and the clear solution of bis-phosphorylating reagent was used as a stock solution. 5'-DMT Thymidine (4.9 g; 9 mmole) in pyridine (15 ml) was taken in a 250 ml three-necked flask with argon inlet and magnetic stirring. The bis-phosphorylating reagent prepared above (54 ml) was added slowly at room temperature. After stirring the reaction mixture for 3 h, 3'-O-acetylthymidine (3.33 g; 11.7 mmole) was added and stirred for 15 min. Then, N-methylimidazole (3.69 g; 45 mmole) was added at room temperature and the reaction was stirred for 8 h. Triethylammonium bicarbonate (1 M solution, 40 ml) was added to quench the reaction. The reaction mixture was diluted with dichloromethane (200 ml) and washed with water (100 ml), brine (100 ml) and dried ($MgSO_4$). The dried extract was filtered, concentrated and purified by flash chromatography using silica gel eluting first with dichloromethane, followed by dichloromethane:methanol (9:1, v/v). The fractions corresponding to the desired product were combined and concentrated.. Yield 7.6 g (86%). ^{31}P NMR ($CDCl_3$) δ 61.8, 63.1.

Synthesis of homothymidine tetramer: To a stirred solution of anhydrous 1-hydroxy-6-(trifluoromethyl)benzotriazole (0.731 g; 3.6 mmole) in anhydrous dioxane (1.2 ml) in a 50 ml flask under argon was added anhydrous pyridine (0.64 ml; 7.92 mmole) at room temperature. Then a solution of 2-chlorophenylphosphorodichloridothioate[25,26] (0.393 g; 1.5 mmole) in anhydrous dioxane (6 ml) was added over a period of 15 min. at room temperature. Stirring was continued for 5 h. The pyridinium hydrochloride salt was filtered under argon and the clear solution of bis-phosphorylating reagent was used as a stock solution. 5'-DMT-TpsT-OH (0.88 g; 0.9 mmole) in pyridine (1.5 ml) was taken in a 50 ml three-necked flask with argon inlet and magnetic stirring. The bis-phosphorylating reagent prepared above (5.4 ml) was added slowly at room temperature. After stirring the reaction mixture for 3 h, 5'-HO-TpsT-OAc (0.71 g; 1.0 mmole) was added and stirred for 15 min. Then, N-methylimidazole (0.369 g; 4.5 mmole) was added at room temperature and the reaction was stirred for 8 h. Triethylammonium bicarbonate (1 M solution, 10 ml) was added to quench the reaction. The reaction mixture was diluted with dichloromethane (20 ml) and washed with water (10 ml), brine (10 ml) and dried ($MgSO_4$). The dried extract was filtered, concentrated and purified by flash chromatography using silica gel eluting first with dichloromethane, followed by

dichloromethane:methanol (9:1, v/v). The fractions corresponding to the desired product were combined and concentrated.. Yield 1.69 g (88%). ^{31}P NMR ($CDCl_3$) δ 62.85-62.37 (b, m); m/z 1875.45.

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