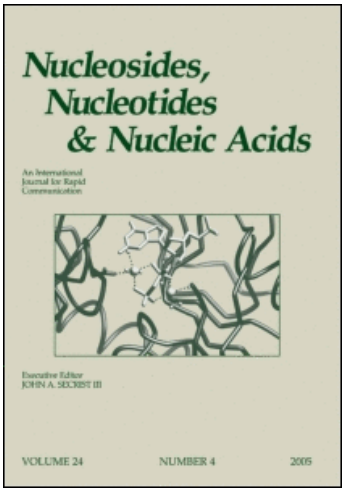

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INVESTIGATION OF A FACILE SYNTHETIC METHOD FOR PHOSPHOROTHIOATE DIMER SYNTHONS IN OLIGONUCLEOTIDE PHOSPHOROTHIOATES SYNTHESIS

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ABSTRACT: A facile synthetic method of a phosphorothioate dimer block was investigated. Dinucleoside phosphite triester intermediates were obtained in one-pot synthesis by the coupling of a protected nucleoside bearing free 5'-OH and a protected nucleoside bearing free 3'-OH in the presence of phosphorous trichloride (PCl_3) and 1,2,4-triazole. The intermediates were easily sulfurized to afford the desired phosphorothioate dimer blocks in 33-64% overall yields.

INTRODUCTION

Recently, the importance of antisense oligonucleotides has been recognized in medical treatment, so that a number of clinical trials are under way.[1] Phosphorothioate oligonucleotides are the most intensively investigated nuclease-resistant antisense agents. For clinical evaluation, large amounts of highly pure phosphorothioate antisense oligonucleotides are needed. Synthesis of phosphorothioate oligonucleotides is usually achieved by solid-phase phosphoramidite-monomer coupling methodology followed by stepwise sulfurization of the phosphite triester intermediate (**Scheme 1, B**).[2] A problem, however, commonly experienced in this procedure is the generation of shorter deletion sequences[3] and natural phosphodiester linkages. These impurities would come from the inefficiency of the coupling and sulfurization reactions required in each elongation step. The reduction of such impurities is certainly an important issue.

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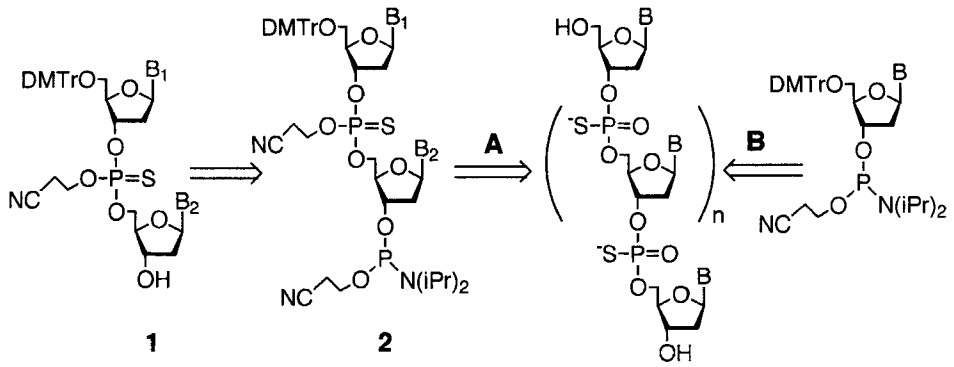
The "block condensation approach"[4-14] is considered to be an effective solution of this problem (**Scheme 1, A**).



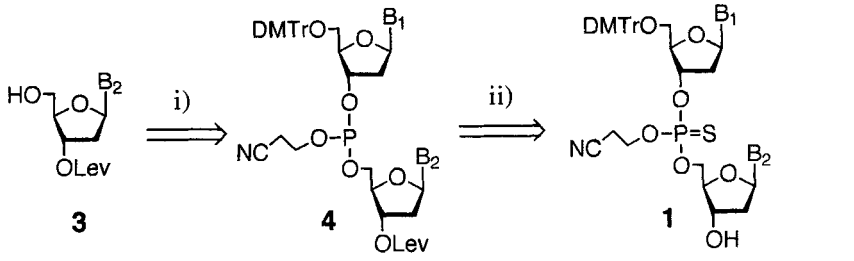
SCHEME 1

A recent report of Krotz *et al.*[4] indicated that the utilization of phosphorothioate dimer building blocks capable of reduction of the total reaction steps in the solid-phase phosphoramidite methodology led to a significant reduction of such impurities without decreasing the yield of the phosphorothioate oligonucleotide. Papers dealing with a convenient method to prepare phosphorothioate dimer building blocks, however, are very few[4] compared to those for "normal DNA" counterparts.[5-12] In this paper, we would like to describe the result of our investigation to develop a new practical phosphorothioate dimer synthesis utilizing a cheap and simple phosphitylating agent, phosphorous trichloride (PCl_3).

RESULTS AND DISCUSSION

We investigated a new general synthetic method of phosphorothioate blockmers for the synthesis of phosphorothioate oligonucleotides. The aim of this work was to develop a practical synthesis of the dimer units of the general structure **1**, which is the precursor of **2**. We tried the dimer synthesis using PCl_3, which is one of the simplest phosphitylating reagents. Thus, 3'-*O*-Levulinyl (Lev) thymidine (**3-a**, 1.0 mmol) was allowed to react with PCl_3 (1.1 mmol) in the presence of pyridine (1.1 mmol). The reaction mixture was subsequently treated with a pyridine solution of 1,2,4-triazole (4.0 mmol) and 2,4,6-collidine (2.0 mmol). To the mixture, 5'-*O*-dimethoxytritylthymidine (DMTr-T, 1.0 mmol) (**5-a**) was added at –20 °C followed by ethylene cyanohydrin (2.0 mmol) at room temperature (Procedure A). The whole reaction mixture was diluted with ethyl acetate and the organic layer was washed with sat. $NaHCO_3$. After drying of the obtained organic layer, the crude phosphite triester compound **4-a** was used for the next

sulfurization reaction, which was achieved by treatment of **4-a** with elemental sulfur in pyridine (50 °C), without further purification. The resulting dinucleoside phosphorothioate triester was treated with hydrazine hydrate to remove the levulinyl group. The desired T-T dimer (**1-a**) was obtained in a satisfactory overall yield (50 %) after silica gel column chromatography (**Scheme 2**).



a: B_2=T
b: B_2=C^{Bz}
c: B_2=A^{Bz}
d: B_2=G^{iBu}

a: B_1=T, B_2=T
b: B_1=C^{Bz}, B_2=T
c: B_1=A^{Bz}, B_2=T
d: B_1=G^{iBu}, B_2=T
e: B_1=T, B_2=C^{Bz}
f: B_1=T, B_2=A^{Bz}
g: B_1=T, B_2=G^{iBu}
h: B_1=A^{Bz}, B_2=C^{Bz}

a: B_1=T, B_2=T
b: B_1=C^{Bz}, B_2=T
c: B_1=A^{Bz}, B_2=T
d: B_1=G^{iBu}, B_2=T
e: B_1=T, B_2=C^{Bz}
f: B_1=T, B_2=A^{Bz}
g: B_1=T, B_2=G^{iBu}
h: B_1=A^{Bz}, B_2=C^{Bz}

T=thymine, C^{Bz}=N^4-benzoylcytosine, A^{Bz}=N^6-benzoyladenine, G^{iBU}=N^2-isobutyrylguanine

i). a) PCl_3 (1.1 eq.), pyridine (1.1 eq.), THF, 0 °C to r.t., 3h; b) 1,2,4-triazole (4.0 eq.), pyridine (20 eq.), 2,4,6-collidine (2.0 eq.), -20 °C, 20 min; c) compound **5** in THF, -20 °C; d) $NCCH_2CH_2OH$, 0 °C to r.t., 2h; ii). a) S_8, pyridine, 50 °C, 30 min; b) AcOH, $NH_2NH_2•H_2O$, 0 °C, 10 min.

SCHEME 2: Preparation of the dimers by procedure A.

We found that the amount of pyridine used in the above phosphitylation step affected the yield of **1-a**. The use of an excess of pyridine (20 equiv.) or removal of pyridine from the first step led to significant reduction of the yield of the dimer (19 and 43%, respectively). Interestingly, the removal of 1,2,4-triazole from the reaction also lowered the yield of **1-a** significantly (28%).

i). a) PCl_3 (1.1 eq.), 1,2,4-triazole (4.0 eq.), *N*-methylmorpholine (9 eq.), THF, 0 °C, 30 min; b) compound **3**, 0 °C; c) $NCCH_2CH_2OH$, 0 °C to r.t., 2h; ii). a) S_8, pyridine, 50 °C, 30 min; b) AcOH, $NH_2NH_2 \cdot H_2O$, 0 °C, 10 min.

SCHEME 3: Preparation of the dimers by procedure B.

Other dimer units (**1-b** to **f**) prepared by this procedure are listed in **TABLE 1**. As shown in **TABLE 1**, the overall yields of the dimer building blocks were in a satisfactory range (30-50%). When 3'-*O*-Lev-N^2-isobutyryldeoxyguanosine (**3-d**) was used as the starting material, a complex mixture was obtained without formation of the desired T-G dimer (**1-g**). This result could be explained in terms of some side-reaction involving the unprotected 6-position of the guanine base with PCl_3.

Assuming that the condensation between the two nucleoside components to form the desired products proceeded through the formation of 3'-*O*-Lev nucleoside 5'-phosphorous bis(1,2,4-triazolide),[15] we investigated another approach for the preparation of the above T-G dimer unit. In this method, phosphorous tris(1,2,4-triazolide), *in situ* generated as a milder phosphitylation reagent, was allowed to react with 5'-*O*-DMTr nucleoside (Procedure B). Thus, 1,2,4-triazole (4.0 mmol) was treated with PCl_3 (1.1 mmol) in the presence of *N*-methylmorpholine (9.0 mmol) in anhydrous THF at 0 °C. To this mixture were added successively 5'-*O*-DMTr-thymidine (**5-a**, 1.0 mmol), 3'-*O*-Lev-guanosine (**3-a**, 1.0 mmol) and ethylene cyanohydrin (2.0 mmol). The reaction mixture was worked up and the crude phosphite triester was sulfurized in the same manner as described above. After silica gel column chromatography, the desired T-G dimer was obtained in an overall yield of 33% (**Scheme 3**). This successful result could be due to the suppression of the side-reaction mentioned above by using a phosphitylation reagent milder than PCl_3. The method was found to be applicable to the preparation of other dimers such as T-T, T-A, T-C and A-C and gave better results as summarized in TABLE 2. HPLC analysis[16] indicated that the purity of the dimer units (**1-a** to **1-h**) thus obtained was more than 96% (data not shown).

CONCLUSION

We have successfully developed a new, convenient synthetic method to prepare phosphorothioate dimer building blocks. The current method may have several advantages over previous methods. For example, we utilized a quite cheap

TABLE 1. Synthesis of dimers 1 (Procedure A)

B_1	B_2	Compound	Yield (%) from **3**	^{31}P-NMR($CDCl_3$)
T	T	**1a**	50	68.53, 68.01
C^{Bz}	T	**1b**	40	68.52, 67.78
A^{Bz}	T	**1c**	32	68.21, 68.15
G^{iBu}	T	**1d**	40	69.27, 68.97
T	C^{Bz}	**1e**	31	68.55, 68.16
T	A^{Bz}	**1f**	34	68.92, 68.16
T	G^{iBu}	**1g**	0	

TABLE 2. Synthesis of dimers 1 (Procedure B)

B_1	B_2	Compound	Yield (%) from **5**	^{31}P-NMR($CDCl_3$)
T	T	**1a**	64	68.40, 67.88
T	C^{Bz}	**1e**	45	68.30, 68.08
T	A^{Bz}	**1f**	44	68.55, 68.08
T	G^{iBu}	**1g**	33	68.00, 67.74
A^{Bz}	C^{Bz}	**1h**	36	68.89, 68.60

phosphitylating agent, PCl_3, instead of the rather expensive phosphoramidite reagents or tetrazole. This would provide a cost-effective route to preparation of dimer blocks, particularly in the case of large-scale synthesis. Also, the phosphitylation of the protected nucleosides and the formation of the dimer blocks were achieved in a one-pot method. Thus, the necessary purification steps, such as silica gel column chromatography, are minimal.

These dimer blocks can be utilized for the preparation of phosphorothioate antisense agents retaining sufficient purity for clinical use.

EXPERIMENTAL

Thin-layer chromatography (TLC) analysis was carried out on Merck silica gel 60 F254-precoated plates. Column chromatography was performed with silica gel 60 (MERCK 7734 or 9385). ^{31}P-NMR spectra were recorded at 270 MHz with JEOL GSX-270 spectrometer in $CDCl_3$. Fast-atom bombardment mass spectra were recorded in positive ion mode with JEOL JMS-AX500 using a magic bullet.

General procedure for synthesis of phosphorothioate dimers (1), Procedure A. To a mixture of 3'-*O*-Lev-2'-deoxynucleoside (3, 1.0 mmol) and pyridine (0.09 ml, 1.1 mmol) in anhydrous tetrahydrofuran (THF) (10 ml) at 0 °C was added phosphorous trichloride (0.1 ml, 1.1 mmol), and the resulting mixture was stirred for 3 h at room temperature. The solution was cooled to –20 °C, and 1,2,4-triazole (0.28 g, 4.0 mmol), pyridine (2.0 ml, 25 mmol) and 2,4,6-collidine (0.25 ml, 2.0 mmol) were added. After the mixture was stirred for 30 min, 5'-*O*-DMTr deoxynucleoside (5, 1.0 mmol) in anhydrous THF (5 ml) was added dropwise to this mixture over 15 min. The mixture was stirred at –20 °C for 15 min and at 0 °C for 1 h. Then, ethylene cyanohydrin (0.14 ml, 2.0 mmol) was added at 0 °C, and the mixture was stirred at room temperature for 1 h. The resulting insoluble materials were removed by filtration, and the filtrate was dissolved in ethyl acetate and washed with saturated $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, filtered, and evaporated.

Sulfurization and deprotection of the levulinyl group. The residue was dissolved in pyridine (10 ml), and elementary sulfur (0.10 g) was added. The resulting mixture was stirred at 50 °C for 30 min. After cooling to 0 °C, acetic acid (6 ml) and hydrazine hydrate (0.10 ml) were added and stirring was continued for an additional 10 min. The reaction mixture was dissolved in chloroform. The chloroform solution was washed with water, saturated $NaHCO_3$ and brine. For separation of the organic and water layers, an appropriate amount of methanol was added. The organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by silica gel column chromatography (chloroform : methanol = 100 : 3 to 100 : 4, V/V) to give the corresponding dimers (**1-a** to **f**). ^{31}P-NMR-chemical shifts of the dimers prepared as described above are listed in **TABLE 1**. Data for **1-a**: FAB$^+$MS 918 $(M+H)^+$. Data for **1-b**: FAB$^+$MS 1007 $(M+H)^+$. Data for **1-c**: FAB$^+$MS 1031 $(M+H)^+$. Data for **1-d**: FAB$^+$MS 1013 $(M+H)^+$. Data for **1-e**: FAB$^+$MS 1007 $(M+H)^+$, 1029 $(M+Na)^+$. Data for **1-f**: FAB$^+$MS 1031 $(M+H)^+$.

Procedure B. To a mixture of 1,2,4-triazole (0.28 g, 4.0 mmol) and *N*-methylmorpholine (1.0 ml, 9 mmol) in anhydrous THF (10 ml) at 0 °C was added phosphorous trichloride (0.10 ml, 1.1 mmol), and the resulting mixture was stirred for 30 min. 5'-*O*-DMTr deoxynucleoside (**5**, 1.0 mmol) in THF (5 ml) was added dropwise to the solution over 15 min at 0 °C. After the mixture was stirred for 1 h, 3'-*O*-Lev deoxynucleoside (**3**, 1.0 mmol) was added to the mixture at 0 °C. After being stirred for 1 h, the mixture was treated with ethylenecyanohydrin (0.14 ml, 2.0 mmol) at 0 °C. Stirring was further continued at room temperature for 1 h. The mixture was filtered off, and the filtrate was dissolved in ethyl acetate and washed with saturated $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, filtered, and evaporated. Sulfurization,

deprotection and purification were carried out in the same manner as Procedure A to give the corresponding dimers (**1-a**, **e** to **h**). ^{31}P-NMR-chemical shift of the dimers prepared as described above are listed in **TABLE 2**. Data for **1-a**: FAB$^+$MS 918 $(M+H)^+$. Data for **1-e**: FAB$^+$MS 100 $(M+H)^+$, 1029 $(M+Na)^+$. Data for **1-f**: FAB$^+$MS 1031 $(M+H)^+$. Data for **1-g**: FAB$^+$MS 1013 $(M+H)^+$, 1035 $(M+Na)^+$. Data for **1-h**: FAB$^+$MS 1020 $(M+H)^+$, 1022 $(M+Na)^+$.

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16. HPLC conditions: column; YMC-Pack ODS-A A-312 150x6.0mm I.D., eluent; 70% CH_3CN-50 mM TEAA (triethylammonium acetate) (pH 7.0), Flow rate; 1.0 ml/min, temperature; ambient (25 °C), detection; UV at 270 nm.

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