A convenient approach to the synthesis of medium size oligodeoxyribonucleotides by improved new phosphite method

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ABSTRACT
Improvement of the new phosphite method for the synthesis of oligodeoxyribonucleotides using the deoxyribonucleoside 3'-bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite unit has been carried out via the hydrolysis and capping steps, without any side reaction products. The new phosphite unit and capping agent, bis(1,1,1,3,3,3-hexafluoro-2-propyl)-2-propyl phosphite, is readily activated by N-methylimidazole under very mild condition on a solid support. This operation involves a one pot reaction, which is an advantage over both the phosphite and H-phosphonate approaches. The mechanism of internucleotidic bond formation of the new phosphite method is also discussed.

INTRODUCTION
Recently, the developments of phosphite [1], phosphoramidite [2], and H-phosphonate [3] approaches have enabled the rapid chemical synthesis of oligo- and polydeoxyribonucleotides on solid supports. The phosphoramidite approach has been accessible and more successful in application to molecular biology. However, the phosphoramidite approach requires a phosphate protecting group and capping and oxidation reaction (an oxidation reaction is carried out at the end of each coupling reaction) during the course of synthesis compared with the H-phosphonate approach. On the other hand, the H-phosphonate approach also has some disadvantages: instability of the coupling agent (pivaloyl chloride) [4—6] and necessity for a large excess of the phosphorylating agent to prepare the H-phosphonate units [5,6].

In a current study, we have reported [7] a simple method for the synthesis of deoxyribonucleoside 3'-H-phosphonates using the transesterification of a new agent, bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphonate. This phosphorylating agent was easily activated by pyridine to give the reactive N-phosphonylpyridine intermediates.

Based upon the above facts, we considered whether deoxyribonucleoside 3'-bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphites (3) could be used as new monomer building blocks that had high reactivity as a trivalent phosphorus compound, like trialkyl phosphites [8]. Therefore, we have studied the synthesis of oligodeoxyribonucleotides by use of the phosphite units (3) on solid support [9]. However, we now found that the oligodeoxyribonucleotides prepared by our phosphite method is contaminated by small amounts of the side product, long size oligomer than desired oligomer. In an attempt to improve our original approach, our syntheses were carried out to evaluate the relative effectiveness of the two different synthetic cycles and the effect of capping during oligonucleotide synthesis. Further, the mechanism of internucleotidic bond formation of the new phosphite method is also discussed.

GENERAL MATERIALS AND METHODS
1H-NMR spectra were recorded on a JEOL JNMP 100 spectrometer with TMS as an internal standard. 31P-NMR spectra were recorded in CH3CN or CDC13 on a Bruker AM-400 spectrometer using 85% H3PO4 as an internal standard. Ultraviolet spectra were recorded on a Shimazu UV-160 spectrometer. Thin layer chromatography (TLC) was carried out on Merck Kieselgel 6OF254 plates which were developed in system A (CH2Cl2-MeOH, 9:1, v/v), system B (CH2Cl2-MeOH, 95:5, v/v). Reverse phase TLC was carried out on Merck silanized silica gel;[RP-8F 60F254] plates with a mixture of acetone and 0.02 M triethylammonium acetate (TEAA) (6:4, v/v) as the eluting agent. Column chromatography was carried out on silica gel (BW-300; Fuji Davison Co.Ltd.) and alkylated silica gel (C-18, Waters Associates Inc.). THF was continuously refluxed over sodium/benzophenone and distilled prior to use. CH2Cl2 was distilled from P2O5 and stored over activated 4-A molecular sieves. CH3CN was distilled twice from P2O5 and from CaH2 and then stored over activated 4-A molecular sieves. N,N-Diisopropylethylamine, DMF, and lutidine were freshly distilled from CaH2.

Dicyclohexylcarbodiimide (DCC) and CS2 were redistilled before use. 1,1,1,3,3,3-Hexafluoro-2-propanol was purchased from Sentral Glass Co. Ltd. and alkylated silica gel (C-18, Waters Associates Inc.). Snake

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venom phosphodiesterase and alkaline phosphatase were purchased from Boehringer Mannheim.

The chain elongation steps were carried out in an Applied Biosystems Model 381A DNA synthesizer using CPG column containing 0.2 μmol of partially-protected dT and dA.

Electrophoretic gels were either 20% polyacrylamide/7% urea and run at 400V.

Reverse-phase HPLC was performed on a Shimazdu LC-6A system using a TSKgel oligo-DNA RP for analysis and Inersil ODS for purification with a linear gradient of CH₂CN in 0.1 M triethylammonium acetate (pH 7.0). For anion exchange HPLC, the TSKgel DEA-2SW, DEA-DEAE, and DEA-5PW columns were used with a linear gradient of ammonium formate in 20% CH₃CN.

The dT-CPG (39 μmol/g) and dA-CPG (28 μmol/g) were prepared as described previously [10].

Preparation of Deoxyribonucleoside 3'-Bis(1,1,1,3,3,3-2-Propyl) Phosphites(3)

After co-evaporation with dry pyridine, N-acetyl-5'-O-dimethoxytrityl-nucleosides (I) (1 mmol) was dissolved in CH₂Cl₂ (10 ml) and tris(1,1,1,3,3,3-hexafluoro-2-propyl) phosphate (2) (0.34 ml, 1.2 mmol) was added. After 5 min, the mixture solution was poured into n-hexane and the supernatant solution was taken off from the reaction mixture. The residue was co-evaporated with n-hexane to give a white foam, which could be used as the phosphate unit directly in the coupling reaction, or could be purified, if necessary, by silica gel column chromatography.

5'-O-DMTr-deoxythymidine 3'-O-bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite (3a): Yield: 88%. 31P-NMR (CDCl₃, 85% H₃PO₄) δ 140.2 ppm.

N°-Benzoyl-5'-O-DMTr-deoxyadenosine 3'-O-bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite (3b): Yield: 85%. 31P-NMR (CDCl₃, 85% H₃PO₄) δ 141.2 ppm.

N°-Anisoyl-5'-O-DMTr-deoxycytidine 3'-O-bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite (3c): Yield: 83%. 31P-NMR (CDCl₃, 85% H₃PO₄) δ 140.2 ppm.

N°-Isobutyryl-5'-O-DMTr-deoxyguanosine 3'-O-bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite (3d): Yield: 81%. 31P-NMR (CDCl₃, 85% H₃PO₄) δ 141.1 ppm.

5'-O-(Dimethoxytrityl)-3'-thymidine 3'-O-(Benzoyl)-5'-thymidine Phosphonate (4).

The phosphate unit (3a) (976 mg, 1 mmol) was treated with 3'-O-benzoylthymidine (380 mg, 1.1 mmol) in the presence of Melm (0.24 ml, 3 mmol) in CH₃CN (1 ml). After 10 min, the mixture was quenched with 0.1 M Melm/2%H₂O-THF and the solution was extracted with CH₂Cl₂ (5 ml x 2). The organic layer was dried over Na₂SO₄, evaporated and chromatographed on silica gel to give 4 (836 mg, 89%). 31P-NMR (CDCl₃, 85% H₃PO₄) δ 8.63, 7.33 ppm.

Synthesis of a Capping Agent (6)

To a solution of 2-propyl phosphorodichloridite (20.1 g, 125 mmol) and triethylamine (48.8 ml, 350 mmol) in dry ether (150 ml) at -20°C, 1,1,1,3,3,3-hexafluoro-2-propanol (52.8 ml, 500 mmol) in dry ether (50 ml) was added. The mixture was allowed to warm up to room temperature, and was stirred for an additional 12 h. Petroleum ether (100 ml) was then added. The products were kept overnight at 4°C and were filtered. The filtrate was concentrated, and the residue was distilled under reduce pressure. The main fraction (43.3 g, 82%) was obtained as a colorless liquid: b.p. 48°C/18 mmHg; 31P-NMR (CDCl₃, 85% H₃PO₄) δ139.9 ppm.

Synthesis of Oligodeoxyribonucleotides

The LCAA-CPG support loaded with first nucleoside (0.2 μmol) was packed in a small ABI column which is part of an Applied Biosystems 381A DNA Synthesizer. The reaction cycle of chain elongation was carried out by a control programmed series of reagent and solvent washes based on a program of the DNA synthesis with the following modifications:

1) coupling: 0.25 M phosphate unit (3) and 0.5 M methylimidazole in dry CH₂CN in delivered in 4 alternating burst of 4 sec (Melm) followed by 10 sec (phosphate + Melm) with wait time 5 min.

2) unblocking: 3% trichloracetic acid in CH₂Cl₂ delivered in 5×10 sec bursts with intermediate 1 sec reverse flushes.

3) hydrolysis: 0.1 M Melm in 2% aqueous THF solution delivered in two 10 sec bursts with total intermediate wait time of 120 sec.

4) capping: 0.5 M HFPP and 1.5 M Melm in CH₂CN in delivered one two 10 sec bursts with total intermediate wait time 5 min.

Deprotection and Isolation of Oligodeoxyribonucleotides

After oxidation, the column was washed with CH₂CN and ether. Further the column was treated with concentrated ammonia for 1 h at room temperature. The solution was eluted from column and heated in a sealed vial at 55°C for 5–8 h, except oligonucleotides. The solution was concentrated and the residue was dissolved in water. The solution was passed through a membrane filter (EKICRODISC 13, Gelman Sciences Japan) [11]. The deprotected oligonucleotide was analyzed and purified by the anion exchange HPLC or reverse phase HPLC. The appropriate fractions were collected and lyophilized from sterile water. The purify and chain length were analyzed by anion exchange HPLC and PAEG.

Enzymatic Digestions

The oligonucleotide (0.2 A₆₀₀ units) was dissolved in 0.01 M TRIS/HCl buffer (pH 8.8) (500 μl) and digested with snake venom phosphodiesterase (5 μg) at 37°C for 2 h. The mixture was further incubated with alkaline phosphatase (5 μg) at 37°C for 1 h. Degradation products were analyzed by the reverse phase HPLC using a TSKgel oligo-DNA RP with a nonlinear gradient of CH₂CN (5% during 60 min) in 0.1 M TEAA (pH 7.0).

RESULTS AND DISCUSSION

First, the required building blocks (3) were readily prepared by allowing the deoxyribonucleosides (1) to react with a slight excess of tris(1,1,1,3,3,3-hexafluoro-2-propyl) phosphate 2 [6,12] (1.2 molar equiv.) in CH₂Cl₂ for 10 min, followed by washing work-up and chromatography of the products. Isolated yields and 31P-NMR spectroscopic data are listed in the experimental section. On the other hand, the building blocks (3) used in the coupling reaction were purified by washing with dry n-hexane to yield on co-evaporation colorless foams, which were 31P-NMR spectroscopically pure (Scheme 1).
In order to investigate the utility of these phosphite units (3) as starting materials for oligodeoxyribonucleotide synthesis, several experiments have been made. The coupling properties of the phosphite units (3a) were first studied with 3'-O-benzoylthymidine (1.1 molar equiv.) in the presence of N-methylimidazole (MeIm) at room temperature. After 10 min, the reaction was monitored by $^{31}$P-NMR spectroscopy. The spectrum of the reaction mixture showed that the signal of the phosphite unit (3a) completely disappeared. New signals were observed at 8.68 and 7.33 ppm. The chemical shift suggested that 3a was readily converted to the corresponding H-phosphonate diester (4). The protected dithymidine (3'-5') phosphonate (4) was isolated in high yield (89%) without any side reaction products.

The rate of formation of the internucleotidic bond depended on the base agent. It was found that when N-ethylmorpholine, 4-(dimethylamino)pyridine (DMAP), and tri-n-buthylamine were used as base agents, the internucleotidic bond was not completely formed on a practical time scale (Figure 1). When N-methylimidazole (MeIm) or N-ethylimidazole (EtIm) was used, the internucleotidic bond was nearly formed within 2 min. The yield, however, slightly increased with MeIm.

Next, we decided to investigate the activation process and the coupling reaction using $^{31}$P-NMR spectroscopy (Figure 2). The phosphite unit (3a) (1 molar equiv.) was dissolved in dry CH$_3$CN and the solution was monitored by $^{31}$P-NMR (Figure 2a). A peak at 140.2 ppm in the low field region was assigned to the phosphite unit (3a). Moreover, when MeIm (5 molar equiv.) was added to the above mixture, the corresponding intermediate N-methylimidazolide (5) was not observed as a $^{31}$P-NMR resonance peak. The intermediate 5 that must be present in equilibrium with 3, most be so reactive intermediate that the equilibrium lies predominantly towards 3 (Scheme 2). Finally, to the resulting solution was added 3'-O-benzoylthymidine (1.5 molar equiv.). The spectrum of the reaction mixture then showed that the double new peak was observed at high field region (8.55 and 7.61ppm) corresponded to the H-phosphonate diester (4) (Figure 2b).

To ascertain the coupling efficiency of a new phosphite approach, we tried to effect solid phase synthesis of d-(Tp)$_{14}$T

![Scheme 1](image1.png)

![Scheme 2](image2.png)

**Fig. 1.** Rate of the formation of H-phosphonate internucleotidic bond by estimated by $^{31}$P-NMR. The phosphite unit (3a) was mixed with 3'-O-benzoylthymidine in the presence of bases in CH$_3$CN.

**Fig. 2.** $^{31}$P-NMR spectra of the reaction mixture for the H-phosphonate internucleotidic bond formation. a) 3a was dissolved in CH$_3$CD. b) 3'-O-Benzoylthymidine was added to the mixture of 3a and MeIm.
coupling yield. For these syntheses the synthetic cycles used are evaluated to determine the effectiveness of the two different synthetic approaches. We have found that bis(1,1,1,3,3,3-hexafluoro-2-propyl)-2-propyl phosphite (HFPP) is effective. It can be easily prepared in 82% yield by treatment of 2-propyl phosphorodichloridite with 1,1,1,3,3,3-hexafluoro-2-propanol in the presence of triethylamine and can be readily activated by MeIm under very mild conditions. The capping properties of HFPP were first studied with 3′-O-benzoylthymidine (1.2 molar equiv.) and HFPP (1.0 molar equiv.) in the presence of MeIm in CH₂CN at room temperature. After 10 min, the reaction mixture was treated with 0.1 M MeIm in THF:H₂O (98:2, v/v) and the reaction was monitored by 31P-NMR spectroscopy. The spectrum of the reaction mixture showed that a signal of the capping agent completely disappeared; new signals were observed at 8.07 and 7.22 ppm. The chemical shift suggested that HFPP was readily converted into the corresponding H-phosphonate diester.

Further, d-(Tp)₄T was synthesized under the following conditions: 0.05 M; 0.1 M; and 0.25 M; each in the presence of 0.5 M MeIm as activator. The results indicated that the most effective procedure employed the phosphite unit (3a) at a concentration of 0.25 M.

It has been clearly shown by Andrus et al. [4] that a capping reaction for the unreacted 5′-hydroxyl group is necessary in the chemical synthesis of oligodeoxyribonucleotides by the H-phosphonate approach on a solid support. We have found evidence by HPLC that the coupling reaction is approximately 96% efficient in converting 3′-O-benzoylthymidine to 3′-O-benzoylthymidine remains, and the 5′-hydroxyl groups are available for reaction in the next cycle. Consequently, without an effective capping reaction, oligodeoxyribonucleotides cannot be purified free from the deletion sequences (Figure 4, compare lanes 1 and 3).

We first tested the agent (Ac₂O/DMAP) used for capping in the phosphoramidite approach [13]. However, the coupling reaction did not proceed smoothly and the product contained some impurities which could not be separated by HPLC. In order to overcome this problem, we have looked for a more effective capping agent for the unreacted 5′-hydroxyl group in the H-phosphonate approach. We found that bis(1,1,1,3,3,3-hexafluoro-2-propyl)-2-propyl phosphite (HFPP) (6) is effective. It can be easily prepared in 82% yield by treatment of 2-propyl phosphorodichloridite with 1,1,1,3,3,3-hexafluoro-2-propanol in the presence of triethylamine and can be readily activated by MeIm under very mild conditions (Scheme 4). The capping properties of HFPP were first studied with 3′-O-benzoylthymidine (1.2 molar equiv.) and 6 (1.0 molar equiv.) in the presence of MeIm in CH₂CN at room temperature. After 10 min, the reaction mixture was treated with 0.1 M MeIm in THF:H₂O (98:2, v/v) and the reaction was monitored by 31P-NMR spectroscopy. The spectrum of the reaction mixture showed that a signal of the capping agent completely disappeared; new signals were observed at 8.07 and 7.22 ppm. The chemical shift suggested that HFPP was readily converted into the corresponding H-phosphonate diester (7).

**Table 1. Automated Synthetic Cycles.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Cycle A</th>
<th>Cycle B</th>
<th>Cycle C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5% Cl₂CHCOOH/CH₂Cl₂</td>
<td>(1) 0.5</td>
<td>(1) 0.5</td>
<td>(1) 0.5</td>
</tr>
<tr>
<td>2</td>
<td>CH₂Cl₂</td>
<td>(2) 0.5</td>
<td>(2) 0.5</td>
<td>(2) 0.5</td>
</tr>
<tr>
<td>3</td>
<td>CH₂CN</td>
<td>(3) 0.5</td>
<td>(3) 0.5</td>
<td>(3) 0.5</td>
</tr>
<tr>
<td>4</td>
<td>0.25 M phosphate unit/0.5 M MeIm/CH₂CN</td>
<td>(4) 5.0</td>
<td>(4) 5.0</td>
<td>(4) 5.0</td>
</tr>
<tr>
<td>5</td>
<td>CH₂CN</td>
<td>(5) 1.0</td>
<td>(5) 1.0</td>
<td>(5) 1.0</td>
</tr>
<tr>
<td>6</td>
<td>0.1 M MeIm/2% H₂O/THF</td>
<td>(2)</td>
<td>(2) 2.0</td>
<td>(6) 2.0</td>
</tr>
<tr>
<td>7</td>
<td>CH₂CN</td>
<td>(7) 1.0</td>
<td>(7) 1.0</td>
<td>(7) 1.0</td>
</tr>
<tr>
<td>8</td>
<td>Capping (0.5 M HFPP/1.5 M MeIm/CH₂CN)</td>
<td>(2)</td>
<td>(8) 2.0</td>
<td>(2)</td>
</tr>
</tbody>
</table>

**End Cycles**

1. Oxidation (0.1 M I₂/THF:Py·H₂O=44:3:3) (1) 20.0 (1) 20.0 (1) 20.0
2. CH₂Cl₂ (2) 1.0 (2) 1.0 (2) 1.0
3. 1.5% Cl₂CHCOOH/CH₂Cl₂ (3) 0.5 (3) 0.5 (3) 0.5
4. The DNA was removed from polymer, deprotected (conc. NH₄OH, 55°C, 6 h) and evaporated.

**Fig. 3.** 20% Polyacrylamide gel electrophoresis in 7 M urea of the authentic sample, d(Tp)₄T (lane 1) and the crude mixtures, d(Tp)₄T (Lane 2: cycle A; Lane 3: cycle B).

**Fig. 4.** 20% Polyacrylamide gel electrophoresis in 7 M urea of the crude mixture, d(TT(ATTT)) (Lane 1: without capping, Lane 2: phosphoramidite method, Lane 3: with capping).
On the basis of this finding, a capping step was added to the synthetic cycle used for the synthesis of the 30 mer dTT(ATTT)$_2$ (Table 1, cycle C). The effect of capping during oligomer synthesis was assessed by electrophoresis on a 20% PAG (Figure 4, lane 1, without capping and lane 3, with capping). It is clear from the gel results that the crude sequence by cycle C was already pure, indicating the effectiveness of the new capping agent, 6. We have also compared the use of the improved new phosphite and phosphoramidite methods in terms of the deprotection of dTT(ATTT)$_2$. Although there are not a significant difference between the two quantitatively (Figure 4, lane 2, phosphoramidite method; lane 3 improved new phosphite method), the improved new phosphite method was easier to prepare the phosphite units (3) and unnecessary of the protection at the phosphorus center.

Recently, oligonucleotides complementary to viral RNA have been shown to inhibit viral replication in cell cultures with Rous sarcoma virus [14,15], human immunodeficiency virus (HIV) [16,17], vesicular stomatitis virus [18—20], herpes simplex virus [18,21], and influenza virus [22]. Experiments with normal (phosphodiester-bonds) antisense oligodeoxyribonucleotides as inhibitors have indicated that these comparatively short oligonucleotides can be taken up by cells in culture and may also be effective in producing modulation of gene expression. The relatively short half-lives of normal oligonucleotides in serum and in cells due to the presence of nucleases, and low permeability of these charged molecules into normal cells, limit their potential usefulness in vivo. To overcome these problems, some of antisense oligonucleotides have been modified on backbone as methylphosphonates, [18,21] or phosphorothioates [17].

To availability of the new phosphite approach can be demonstrated in the synthesis of antisense oligonucleotide analogues having phosphate and phosphorothioate bonds. The synthesis of antisense oligodeoxyribonucleotide, 5'-dCACC-AATTCTGAAAATGGA-3', complementary sequence to the splice acceptor site in HTLV-HI [16,17] was performed smoothly. The solid support was treated with conc. ammonia at 55°C for 6 h. The triethylated product was separated by reverse phase C-18 silica gel and unblocked with 80% AcOH. The unblocked oligomer was further purified by reverse phase C-18 HPLC. The main peak was found to be homogeneous by TSKgel DEAE 2SW and gel electrophoresis. It was hydrolyzed with snake venom phosphodiesterase and alkaline phosphatase and the four nucleosides were analyzed by reverse phase C-18 HPLC.

For the phosphorothioate, the oxidation step was replaced by treatment with 5% sulfur in CS$_2$/pyridine/triethylamine (45:45:10) for up to 2 h, depending on the chain length. After the usual deprotection, isolation of the desired oligomer, 5'-dCAsAsCsCsCsAsAsTsTsTsGsAsAsAsAsTsGsGSA-3' was performed by TSKgel DEAE 2SW HPLC. The main peak was found to be homogeneous by reverse phase C-18 HPLC and by gel electrophoresis. In this case, a small amount of oligomer attached to CPG was taken before the final treatment with sulfur and oxidized with 0.1 M I$_2$ solution to phosphodiesters. The product was used for determination of the base composition by enzymatic degradation to nucleosides followed by HPLC.

These results and those shown above clearly demonstrate that the improved new phosphite unit method could prove to be very effective for the synthesis of medium size DNA fragments on a solid support. The phosphite unit (3) and capping agent (6) are readily activated by N-methylimidazole under very mild conditions. It is noted that this operation involves a one-pot reaction, which is an advantage over both the phosphite and H-phosphonate approaches. Further, this method can be considered as an easy and efficient route a variety of internucleotide phosphate analogues of DNA containing the naturally occurring deoxynucleotides.

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