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Synthesis of Antisense Oligodeoxyribonucleotide Analogues by Use of Deoxyribonucleoside 3′-Bis(1,1,1,3,3,3-hexafluoro-2-propyl) Phosphites as New Key Intermediates

Hideo Hosaka, Testuo Watanabe, Yoshikazu Suzuki, and Hiroshi Takaku*

Department of Industrial Chemistry, Chiba Institute of Technology, Narashino, Chiba 275, Japan

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ABSTRACT

The deoxyribonucleoside 3′-bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite units (**3**) *are used in the chemical synthesis of oligodeoxyribonucleotides on solid supports. The new phosphite units* (**3**) *were prepared easily by reaction of nucleosides and tris-(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite* (**2**). *They are readily activated by* N*-methylimidazole under very mild conditions on a solid support. This operation involves a one step reaction, which is an advantage over both the phosphite and* H*-phosphonate approaches. The use of deoxyribonucleoside phosphite intermediates in the synthesis of antisense oligodeoxyribonucleotides is also described.*

INTRODUCTION

Recently, the developments of phosphite, phosphoramidite, and *H*-phosphonate approaches by Letsinger [1], Caruthers [2], and Matteucci [3] 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 [4]. However, the phosphoramidite approach requires a phosphate protecting group and a 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) [5] and a necessity for a large excess of the phosphitylating agent to prepare the *H*-phosphonate units [6].

In this paper, we wish to report a more efficient approach to the synthesis of oligodeoxyribonucleotides (DNA) containing internucleotide phosphate analogues by deoxyribonucleoside 3′-bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphites as new key intermediates.

RESULTS AND DISCUSSION

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 phosphonylating agent was easily activated by pyridine to give the reactive *N*-phosphonylpyridine intermediates.

Based on 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 a high reactivity as a trivalent phosphorus compound like trialkyl phosphites [8]. Therefore, we studied the synthesis of deoxyribonucleoside 3′-bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite units (**3**). First, we examined the

*To whom correspondence should be addressed.

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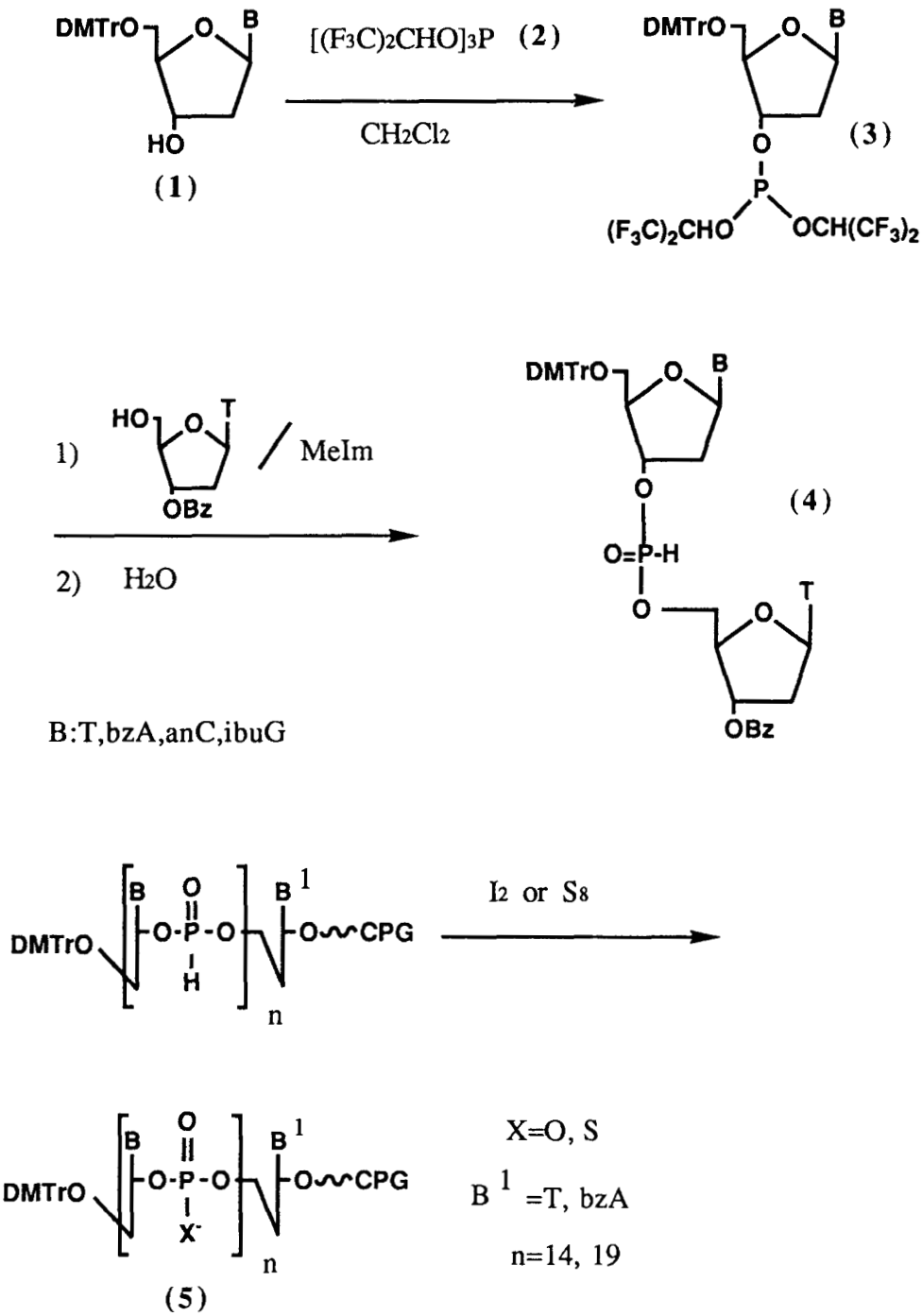


SCHEME 1

synthesis of tris(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite (**2**) as a new phosphitylating agent for the preparation of the monomer building blocks (**3**). However, we have now found that the agent **2** prepared by the procedure of Denny et al. [9] is contaminated by small amounts of the side product, bis(1,1,1,3,3,3-hexafluoro-2-propyl) phosphonate. We then found that the following procedure gave a rel-

TABLE 1 Isolated Yields and ^{31}P-NMR Spectra Analysis of Compounds **3a–d**

Compound	*Yields (%)*	*^{31}P-NMR (chemical shift (ppm))*
3a	88	140.2
3b	85	141.2
3c	83	140.2
3d	81	141.1

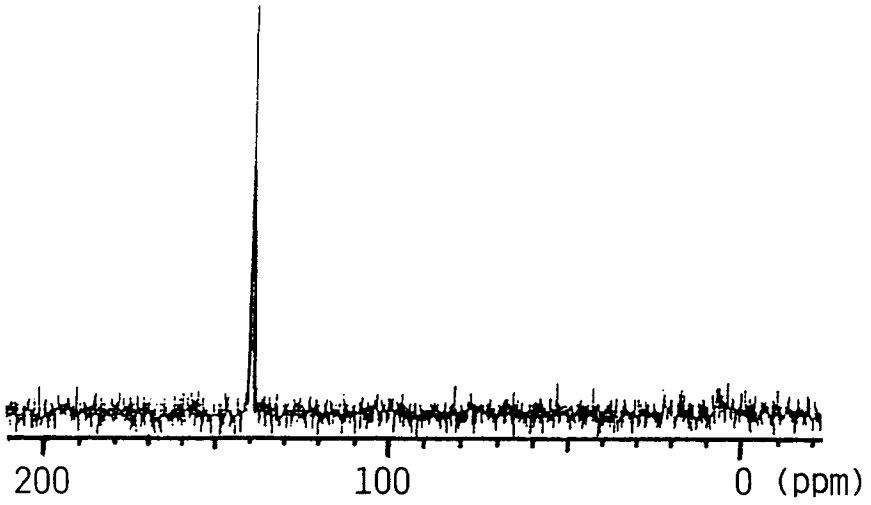


FIGURE 1 ^{31}P-NMR spectrum of DMTrTp(HFP)$_2$ (**3a**) obtained after separation by only a washing procedure from the reaction mixture.

atively pure sample of **2**. The reaction of phosphorus trichloride with the lithium salt of 1,1,1,3,3,3-hexafluoro-2-propoxide gave **2** in 85% yield. The required building blocks (**3**) were readily prepared by allowing the deoxyribonucleosides (**1**) to react with a slight excess of **2** (1.2 mol equiv) in CH_2Cl_2 for 10 min, followed by washing, workup, and chromatography of the products. Isolated yields and ^{31}P-NMR spectroscopic data are listed in Table 1. On the other hand, the building blocks (**3**) used in the coupling reaction were purified by washing with dry *n*-hexane and each yielded on coevaporation a colorless foam that was ^{31}P-NMR spectroscopically pure (Figure 1).

In order to investigate the utility of monomer building blocks (**3**) as the starting materials for the oligodeoxyribonucleotide synthesis, several experiments have been carried out. The coupling properties of building block (**3a**) were first studied with the 3′-*O*-benzoylthymidine (1.1 mol equiv) in the presence of *N*-methylimidazole (MeIm) at room temperature. After 10 min, the reaction was monitored by ^{31}P-NMR spectroscopy (Figure 2). The spectrum of the reaction mixture showed that a signal of the building block unit (**3a**) completely disappeared and new signals were observed at δ 8.68 and 7.33. The chemical shift suggested that **3a** was readily converted into the corresponding *H*-phosphonate diester (**4**). The result clearly indicates that the protected dithymidine (3′–5′) phosphonate (**4**) was isolated in high yield (89%) without any side reaction products. In the above reaction, when 4-(dimethylamino) pyridine (DMAP), triethylamine, imidazole, and pyridine were used in place of MeIm, the rate of the coupling reaction was considerably slower.

To ascertain the coupling efficiency of a new phosphite approach, we tried to effect a solid phase synthesis of d(Tp)$_{14}$T. The reaction was carried out on controlled pore glass (14 mg, 17.5 μmol/g) [10] with an Applied Biosystems Model 381A DNA synthesizer. Our first syntheses were carried out to

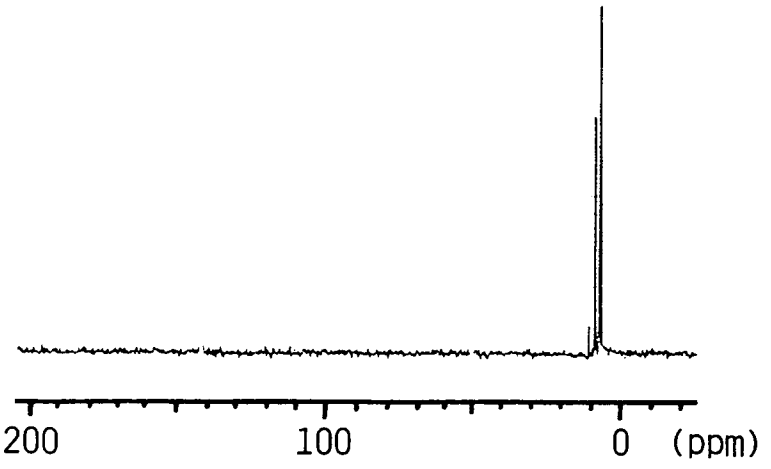


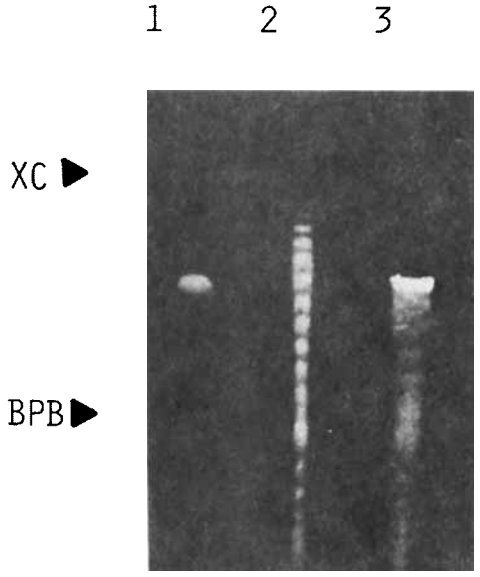
FIGURE 2 ^{31}P-NMR spectrum of the mixture obtained by the reaction of **3a** with 3′-*O*-benzoylthymidine in the presence of MeIm in CH_3CN within 10 min. Chemical shift values are given in ppm relative to H_3PO_4.

TABLE 2 Automated Synthetic Cycles

Step	Reagent	(Step) Time/min Cycle A	Cycle B	Cycle C
1	3% Cl_3CCOOH/CH_2Cl_2	(1) 0.5	(1) 0.5	(1) 0.5
2	CH_2Cl_2	(2) 0.5	(2) 0.5	(2) 0.5
3	CH_3CN	(3) 0.5	(3) 0.5	(3) 0.5
4	0.25 M phosphite unit/0.5 M MeIm/CH_3CN	(4) 15.0	(4) 15.0	(4) 15.0
5	CH3CN	(2) 1.0	(5) 1.0	(5) 1.0
6	0.1 M MeIm/2% H_2O/THF		(6) 2.0	(6) 2.0
7	CH_3CN		(7) 1.0	(7) 1.0
8	Capping (0.5 M HFPP/1.5 M MeIm/CH_3CN		(2)	(8) 5.0
				(6) 1.0
				(7) 1.0
				(2) (0.5)
	End Cycles			
1	Oxidation (0.1 M I_2/THF:Py:H_2O = 44:3:3)	(1) 20.0	(1) 20.0	(1) 20.0
2	CH_2Cl_2	(2) 1.0	(2) 1.0	(2) 1.0
3	3% Cl_3CCOOH/CH_2Cl_2	(3) 0.5	(3) 0.5	(3) 0.5
4	The DNA was removed from polymer, deprotected (conc. NH_4OH, 55°C, 6 h) and evaporated.			

evaluate the relative effectiveness of the two different synthetic cycles and the effect of building block concentration on coupling yield. For these syntheses the synthetic cycles used are shown in Table 2 and are referred to as cycle A and cycle B.

FIGURE 3 20% polyacrylamide gel electrophoresis in 7 M urea of the authentic sample, $d(Tp)_{14}T$ (lane 1) and the crude mixtures, $d(Tp)_{14}T$ (Lane 2: cycle A; Lane 3: cycle B).



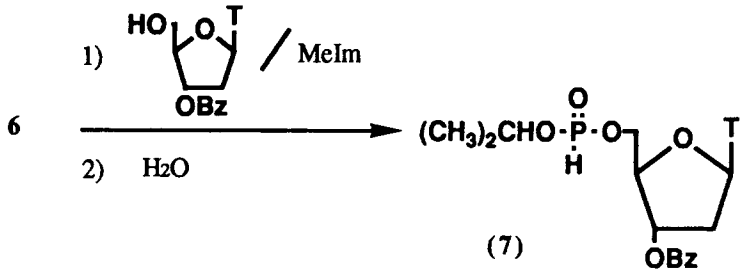
However, cycle A was not satisfactory for the synthesis of $d(Tp)_{14}T$, with the formation of a long oligomer rather than the desired oligomer occurring, and we found that it was difficult to effect the synthesis of oligodeoxyribonucleotides using the synthetic cycle A (Figure 3, lane 2). In these cases, it was necessary to add a hydrolytic step to give cycle B, which is shown in Table 2. In this case, the desired oligomers were obtained without any by-products (Figure 3, lane 3).

Further, $d(Tp)_{14}T$ was synthesized under the following conditions: 0.05 M, 0.1 M, and 0.25 M, all 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. [5] that a capping reaction for the unreacted 5′-hydroxyl group on a solid support is necessary to be able to effect the chemical synthesis of oligodeoxyribonucleotides by the *H*-phosphonate approach. We have found evidence by HPLC that the coupling reaction is approximately 96% efficient in converting 3′-*O*-benzoylthymidine to **4**. Of the 4% remaining 3′-*O*-benzoylthymidine, the unreacted 5′-hydroxyl group is involved 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 2).

We first tested the utility of the agent used with

$[(F_3C)_2CHO]_2POCH(CH_3)_2$ **(6)**



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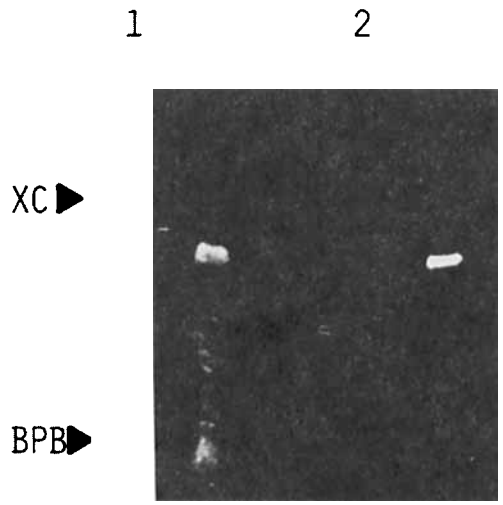


FIGURE 4 20% polyacrylamide gel electrophoresis in 7 M urea of the crude mixture, $d(Tp)_{19}T$ (Lane 1: without capping, Lane 2: capping).

the phosphoramidite approach for a capping (Ac_2O/DMAP) of the unreacted 5′-hydroxyl group [11]. However, the coupling reaction did not proceed smoothly, and the product contained some impurities that could not be separated by HPLC. In order to overcome this problem, we have attempted to find a more effective capping agent for the unreacted 5′-hydroxyl group on a solid support in the oligonucleotide synthesis by the *H*-phosphonate approach. Finally, we found bis(1,1,1,3,3,3-hexafluoro-2-propyl)-2-propyl phosphite (HFPP) (**6**), which could be prepared easily 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 readily activated by MeIm under very mild conditions. The capping properties of **6** have first been studied with 3′-*O*-benzoylthymidine (1.2 mol equiv) and **6** (1.0 mol equiv) in the presence of MeIm in CH_3CN at room temperature. After 10 min, the reaction mixture was treated with 0.1 M MeIm in THF:H_2O (98:2, v/v) and the reaction was monitored by ^{31}P-NMR spectroscopy (Figure 5). The spectrum of the reaction mixture showed that a signal of the capping agent **6** completely disappeared and new signals were observed at δ 8.07 and 7.22. The chemical shift suggested that **6** was readily converted into the corresponding *H*-phosphonate diester (**7**).

On the basis of this finding, the synthetic cycle used for the synthesis of $d(Tp)_{19}T$ is shown in Table 2 and is referred to as 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 2, with capping). It is clear from the gel results that the crude sequences were already pure, indicating the effectiveness of a new type capping agent, **6**.

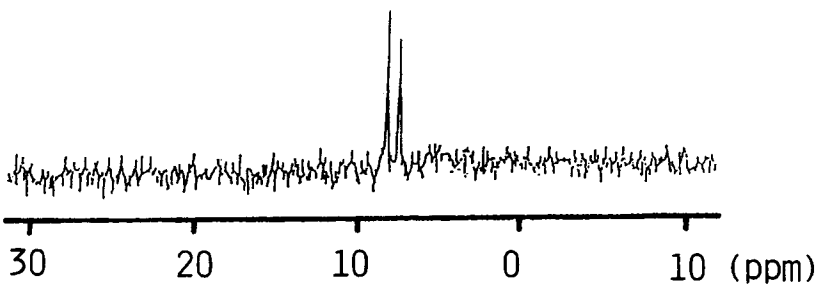
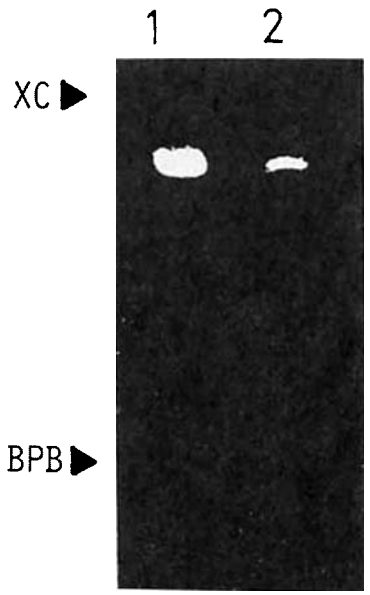


FIGURE 5 ^{31}P-NMR spectrum of the mixture obtained by the reaction of 6 with 3′-*O*-benzoylthymidine in the presence of MeIm in CH_3CN within 10 min. Chemical shift values are given in ppm relative to H_3PO_4.

Recently, oligonucleotides complementary to viral RNA have been shown to inhibit viral replication in cell cultures with Rous sarcoma virus [12, 13], human immunodeficiency virus (HIV) [14, 15], vesicular stomatitis virus [16–18], herpes simplex virus [16, 19], and influenza virus [20]. 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 the antisense oligonucleotides have been modified on the backbone as methylphosphonates [16, 19] or phosphorothioates [15].

The 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′-dCACCCAATTCTG-AAAATGGA-3′, a complementary sequence to the splice acceptor site in HTLV-III [14, 15], was carried out smoothly. The solid support was treated with conc ammonia at 55°C for 6 h. The tritylated 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 (Figure 6, lane 1). The properties of four nucleosides were analyzed by reverse phase C-18 HPLC after hydrolysis with snake venom phosphodiesterase and alkaline phosphatase (Figure 7).

FIGURE 6 20% Polyacrylamide gel electrophoresis in 7 M urea of the oligodeoxyribonucleotides. Lane 1: dCACCCAATTCTGAAAATGGA; Lane 2: dCsAsCsCsCs-AsAsTsTsCsTsGsAsAsAsAsTsGsGsA.



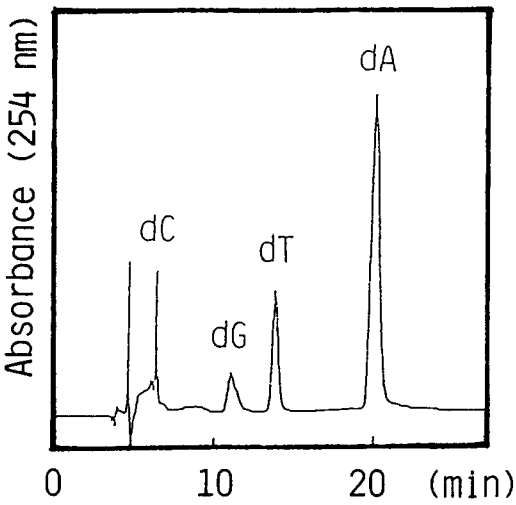


FIGURE 7 Analysis of the products obtained after hydrolysis of dCACCCAATTCTGAAAATGGA with snake venom phosphodiesterase and alkaline phosphatase on a TSKgel oligo-DNA RP column. Elution was performed with a 5% CH_3CN in 0.1 M triethylammonium acetate (pH 7.0). The flow rate was 0.7 mL/min.

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′-dCsAsCsCsCsAsAsTsTsCsTsGsAsAsAsAsTsGsGsA-3′ was effected by TSKgel DEAE 2SW HPLC. The main peak was found to be homogeneous by reverse phase C-18 HPLC and by gel electrophoresis (Figure 6, lane 2). In this case, small amounts of oligomer attached to CPG were 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.

SUMMARY

These results and those shown previously clearly demonstrate that transesterification of a new type of phosphite unit can be very effective for the synthesis of medium size DNA fragments on a solid support. They are readily activated by *N*-methylimidazole under very mild conditions. It is noted that this operation involves a one-step reaction, which is an advantage over both the phosphite and *H*-phosphonate approaches. Further, the antisense oligomers synthesized here were tested in an inhibition assay that will be published very soon [21].

EXPERIMENTAL SECTION

General Materials and Methods

^{1}H-NMR spectra were recorded on a JEOL JNMPS 100 spectrometer with TMS as an internal standard. ^{31}P-NMR spectra were recorded in CH_3CN or $CDCl_3$ on a Bruker AM-400 spectrometer using 85% H_3PO_4 as an internal standard. Ultraviolet spectra were recorded on a Shimazu UV-160 spectrometer. Thin layer chromatography (TLC) was carried out on Merck Kieselgel $60F_{254}$ plates, which were developed in system A (CH_2Cl_2–MeOH, 9:1, v/v), system B (CH_2Cl_2–MeOH, 95:5, v/v). Reverse phase TLC was carried out on Merck silanized silica gel; [RP-8F $60F_{254}$] 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 from sodium/benzophenone and distilled prior to use. CH_2Cl_2 was distilled from P_2O_5 and stored over activated 4-A° molecular sieves. CH_2CN was distilled twice from P_2O_5 and from CaH_2 and then stored over activated 4-A° molecular sieves. Pyridine was distilled twice from *p*-toluenesufonyl chloride and from CaH_2 and then stored over activated 4-A° molecular sieves. *N,N,*-Diisopropylethylamine, DMF, and lutidine were freshly distilled from CaH_2. Dicylcohexylcarbodiimide (DCC) and CS_2 were redistilled before use. 1,1,1,3,3,3-Hexafluoro-2-propanol was purchased from Sentral Glass Co. Ltd. Long-chain alkylamino controlled pore glass was purchased from Electro Nucleonics Inc. Snake venom phosphodiesterase and alkaline phosphatase were purchased from Beohringer Mannhein.

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

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

Reverse-phase HPLC was performed on a Shimazu LC-6A system using a TSKgel oligo-DNA RP for analysis and Inertsil ODS for purification with a linear gradient of CH_3CN in 0.1 M triethylammonium acetate (pH 7.0). For anion exchange HPLC, the TSKgel DEAE-2SW, DEAE-NPR, and DEAE 5PW columns were used with a linear gradient of ammonium formate in 20% CH_3CN.

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

Tris-(1,1,1,3,3,3-hexafluoro-2-propyl) Phosphite (**1**)

To a solution of *n*-BuLi (40 mmol, 25 mL of a 1.6 M solution) in hexane at −80°C, 1,1,1,3,3,3-hexafluoro-2-propanol (4.4 mL, 42 mmol) was added under a nitrogen atmosphere. The mixture was allowed to warm slowly to room temperature, and was stirred for an additional 1 h. The mixture was evaporated in vacuo to leave a white solid. The solid was dissolved in dry ether (20 mL) and PCl_3 (1.05 mL, 13 mmol) was then added at −20°C under a nitrogen atmosphere. The mixture was kept at room temperature for 6 h and then filtered. The filtrate was concentrated, and the residue was distilled under reduced pressure. The main fraction (5.81 g, 85%) was obtained as a colorless liquid: bp 60°/25 mmHg (lit. [9] 87°C/47 mmHg); ^{31}P-NMR ($CDCl_3$, 85% H_3PO_4) δ 41.09. ^{1}H-NMR ($CDCl_3$) δ 4.80 (dh, $J_{H,P}$ = 9.5 Hz, $J_{H,F}$ = 5.1 Hz).

Preparation of Deoxyribonucleoside 3'-Bis(1,1,1,3,3,3-hexafluoro-2-propyl) Phosphites (**3**)

After coevaporation with dry pyridine, each *N*-acyl-5'-*O*-dimethoxytritylnucleoside (**1**) (1 mmol) was dissolved in CH_2Cl_2 (10 mL) and tris-(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite (**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 coevaporated with *n*-hexane to give a white foam. The white foam could be used as the phosphite unit directly in the coupling reaction or was purified, if necessary, by silica gel column chromatography. The isolated yields and the ^{31}P-NMR analysis data are shown in Table 1.

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, a solution of 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 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 reduced pressure. The main fraction (43.3 g, 82%) was obtained as a colorless liquid: bp 48°C/18 mmHg; ^{31}P-NMR ($CDCl_3$, 85% H_3PO_4) δ 139.9.

Synthesis of Oligodeoxyribonucleotides

The LCAA-CPG support loaded first with 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 phosphite unit (**3**) and 0.5 M methylimidazole in dry CH_3CN is delivered in 4 alternating bursts of 4 sec (MeIm) followed by 10 sec (phosphite + MeIm) with a wait time of 15 min.
2. Unblocking: 3% trichloroacetic acid in CH_2Cl_2 delivered in 5 × 10 sec bursts with intermediate 1 sec reverse flushes.
3. Hydrolysis: 0.1 M MeIm 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 MeIm in CH_3CN is delivered in two 10 sec bursts with a total intermediate wait time of 5 min.

Deprotection and Isolation of Oligodeoxyribonucleotides

After oxidation, the column was washed with CH_3CN and ether. Further, the column was treated with concentrated ammonia for 1 h at room temperature. The solution was eluted from the column and heated in a sealed vial at 55°C for 5–8 h, except for oligonucleotides. The solution was concentrated and the residue was dissolved in water. The solution was passed through a membrane filter (EKICROD-ISC 13, Gelman Sciences Japan) [22]. The deprotected oligonucleotide was analyzed and purified by anion exchange HPLC or reverse phase HPLC. The appropriate fractions were collected and lyophilized from sterile water. The purity and chain length were analyzed by anion exchange HPLC and PAEG (Figure 6).

Enzymatic Digestions

The oligonucleotide (0.5 A_{260} 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 reverse phase HPLC using a TSKgel oligo-DNA RP with a nonlinear gradient of CH_3CN (5% during 60 min) in 0.1 M TEAA (pH 7.0) (Figure 7).

ACKNOWLEDGMENT

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