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Nucleoside 3'-*N*,*N*-Dialkylphosphonamidates as Novel Nucleotide Units for the Solution-Phase Oligonucleotide Synthesis

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Abstract: *Nucleoside 3'-N,N-diisopropylphosphonamidate building blocks for oligonucleotide synthesis were prepared in high yields from appropriately protected nucleosides by use of chlorobis(N,N-diisopropylamino)phosphine as a phosphitylating reagent. The 5'-dimethoxytrityl group of the nucleoside 3'-N,N-diisopropylphosphonamidates was removed selectively without cleavage of the P-N bond under mild acidic conditions. It was found that the nucleoside 3'-N,N-diisopropylphosphonamidates were resistant to oxidation with tert-butyl hydroperoxide. Chain elongation using the phosphonamidate building block was attempted both toward 5'- and 3'-directions and trithymidylate was synthesized. Nucleoside 3'-N,N-diisopropylphosphonamidates were found to be potentially useful as 3'-terminal nucleotide units for the solution-phase oligonucleotide synthesis.*

Introduction

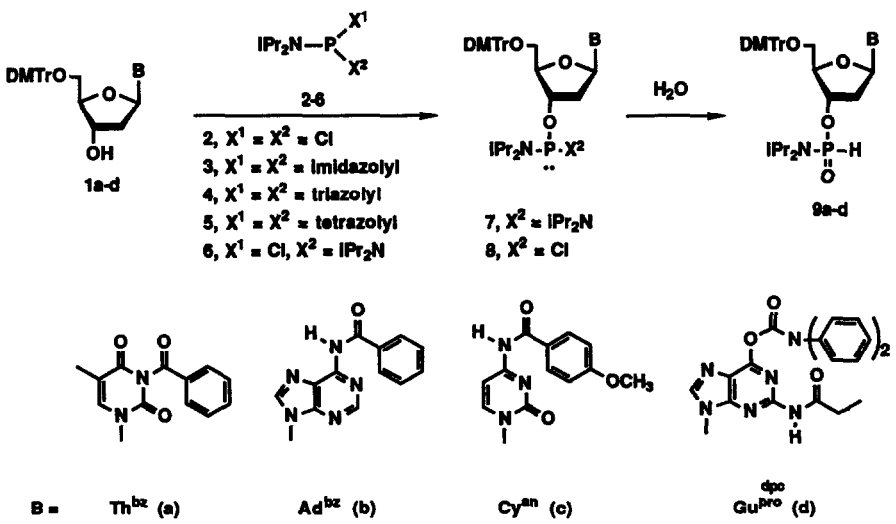
Current techniques of the solid-phase DNA synthesis[1] have provided great contributions to the study on molecular biology, especially, in the field of genetic engineering. However, the routine synthesis of DNA fragments by use of the phosphoramidite method[2,3] on a solid support yields only few μmol of the products, and consumes a large excess of the expensive nucleoside 3'-phosphoramidites and 1*H*-tetrazole. Compared with the automated solid-phase phosphoramidite method, the solution-phase phosphotriester method has remarkable advantageous points. For example, the reaction proceeds almost stoichiometrically and is suitable for relatively large scale synthesis of short oligonucleotides. An additional attractive feature of the phosphotriester method makes the chain elongation possible both toward 5'- and 3'-directions.[4] The nucleotide unit in the standard phosphotriester method[4,5] has a trityl-type protective group for the 5'-hydroxyl and a properly protected phosphotriester at the 3'-position. The 3'-phosphotriester is stable under mild acidic conditions for deprotection of the 5'-trityl group and also stable during the condensation reaction for chain elongation toward the 5'-direction. On the contrary, currentry used nucleoside 3'-phosphonic acid derivatives such as nucleoside 3'-phosphoramidites or nucleoside 3'-*H*-phosphonates[6,7] cannot be applied to the 3'-terminal nucleotide units in the solution-phase synthesis because the nucleoside 3'-phosphoramidites are unstable during chain elongation process toward 5'-direction; acidic conditions for detritylation, 1*H*-tetrazole promoted condensation, and oxidation. The nucleoside 3'-*H*-phosphonates are relatively inert to acid[8] and oxidant,[9] but react with condensing reagents.

Recently, we have reported a novel method for internucleotidic bond formation using nucleoside 3'-*N,N*-dialkylphosphonamidates (**9**) as starting nucleotide units.[10] Compound **9** can be converted to the corresponding phosphorochloridite quantitatively by using tris(2,4,6-tribromophenoxy)dichlorophosphorane (BDCP).[11,12] The 3'-*N,N*-dialkylphosphonamidate is stable under mild acidic conditions and much more resistant to oxidation compared with the other phosphonic acid derivatives such as trialkyl phosphites or dialkyl phosphonates.[13]

In this paper, we wish to describe a novel strategy for the solution-phase synthesis of oligodeoxyribonucleotides by use of the nucleoside 3'-*N,N*-diisopropylphosphonamidates (**9**) as building blocks.

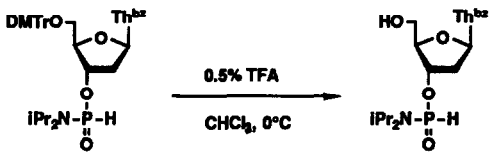
Results and Discussion

Synthesis of Nucleoside 3'-*N,N*-Diisopropylphosphonamidates (9). Several phosphitylating reagents were tested for the synthesis of nucleoside 3'-*N,N*-diisopropylphosphonamidates (**9**). For example, dichloro-*N,N*-diisopropylaminophosphine[14,15] (**2**), *N,N*-diisopropylaminobis(imidazolyl)phosphine (**3**), and *N,N*-diisopropylaminobis(triazolyl)phosphine (**4**) did not react with 5'-*O*-dimethoxytrityl-N^3-benzoylthymidine (**1a**) in the presence of pyridine in THF at -78°C for 2 h. *N,N*-Diisopropylaminobis(tetrazolyl)phosphine (**5**) was allowed to react with **1a** in dichloromethane at -8°C in the presence of diisopropylethylamine and the reaction was complete within 1 h. In this case, bis(3'-*O*-nucleoside) *N,N*-diisopropylphosphoramidite (ca. 20%) was formed as a side product. After usual work up and silica gel column chromatography, 5'-*O*-dimethoxytrityl-N^3-benzoylthymidin-3'-yl *N,N*-diisopropylphosphonamidate (**9a**) was obtained in 48% yield. Chlorobis(*N,N*-diisopropylamino)-phosphine (**6**)[16,17] was also applied to the synthesis of **9**. van Boom reported that nucleoside 3'-bis(*N,N*-diisopropyl)phosphorodiamidites **7** were synthesized using **6** as a phosphitylating reagent in the presence of triethylamine in dioxane. When the reaction was carried out by using *N,N*-dimethylaniline in place of triethylamine in dichloromethane, the reaction was complete within 15 min and one of the amino group of **7** was automatically activated by *N,N*-dimethylanilinium chloride, formed in the reaction of **1a** with **6**, to give the corresponding phosphorochloridite (**8a**).[10]



In this reaction, bis(3'-*O*-nucleoside) phosphonate was slightly formed. Crude **8a** was hydrolyzed to **9a** and purified by silica gel column chromatography. However, bis(*N,N*-diisopropylamino)phosphonodiamidate formed by hydrolysis of excess **6** could not be removed from **9a** by silica gel column chromatography, but it could be completely removed by precipitation of the crude **9a** from *n*-hexane. After filtration, pure **9a** was obtained in 67% yield. When pyridine was employed instead of *N,N*-dimethylaniline in dichloromethane, the reaction was complete in 10 min and the formation of bis(3'-*O*-nucleoside) phosphonate was negligible. Since **9a** was found to be slightly soluble in *n*-hexane at room temperature, precipitation was performed under cooling at -30°C to give pure **9a** in 80% yield. In a similar manner, the other phosphonamidate units (**9b-d**) were obtained in 84-88% yields. The phosphonamidates are stable in chloroform at room temperature for several weeks and at -20°C for several years as solid.

Stability of Nucleoside 3'-*N,N*-Diisopropylphosphonamidates (9) To Acid and Oxidizing Reagent. Compared with tervalent alkyl nucleoside 3'-*N,N*-dialkylphosphoramidites, nucleoside 3'-*N,N*-dialkylphosphonamidates **9** were much more stable for acid hydrolysis. The P-N bond of **9** was completely hydrolyzed with 80% acetic acid at 20°C for 8 h.[13] The stability of **9** to acid enable us to deprotect the 5'-dimethoxytrityl group of **9** without cleavage of the P-N bond. It was found that the 5'-dimethoxytrityl group of **9** was removed selectively with 0.5% trifluoroacetic acid in chloroform at 0°C. For instance, **9a** was treated with 0.5% trifluoroacetic acid in chloroform at 0°C for 45 min and after usual work up, N^3-benzoylthymidin-3'-yl *N,N*-diisopropylphosphonamidate (**12**) was obtained in 89% yield. **12** was further treated with 0.5% trifluoroacetic acid in chloroform at 0°C for 24 h, ca. 20% of N^3-benzoylthymidin-3'-yl phosphonate was detected by TLC.

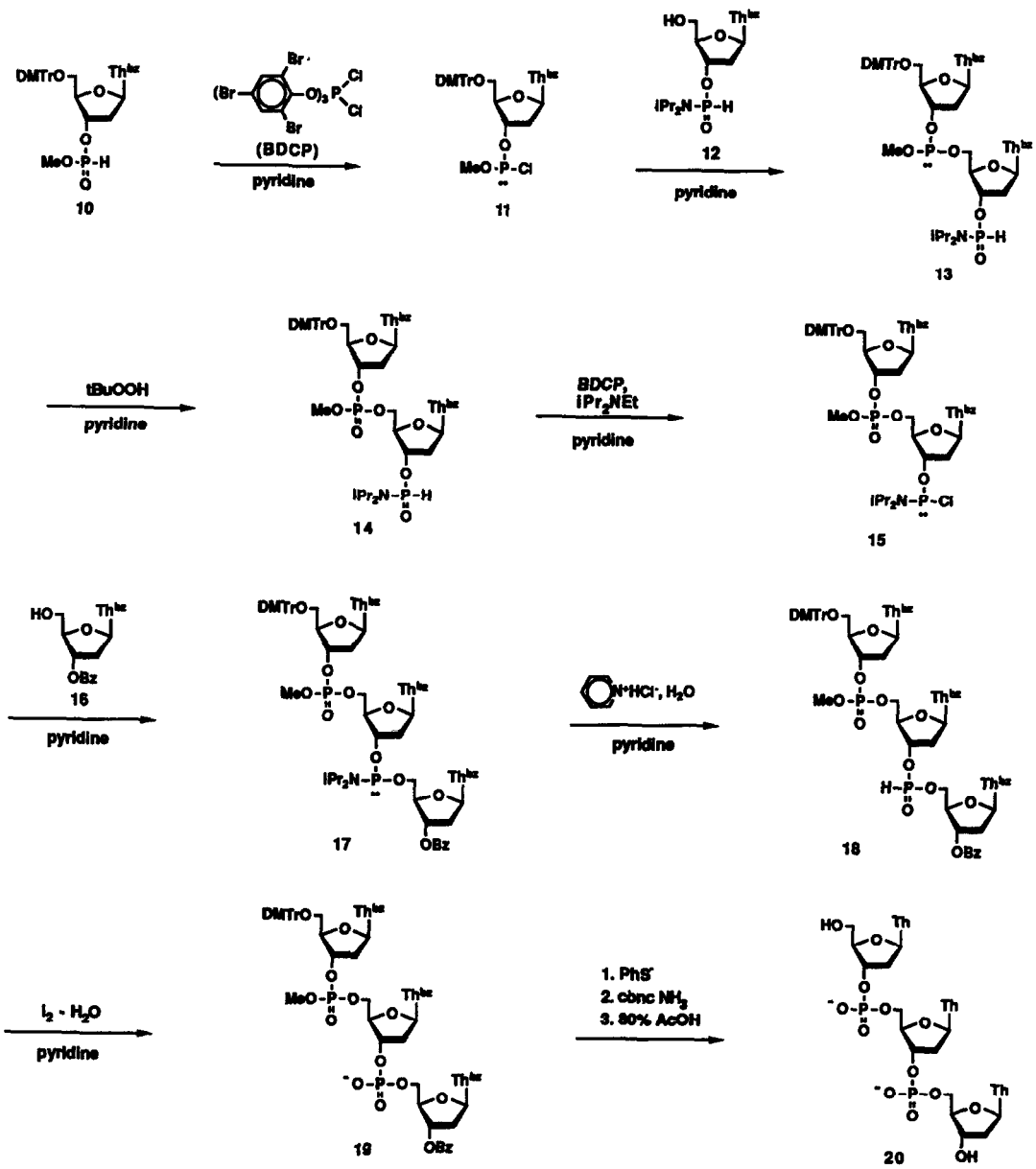


9a **12**

Next the stability of **9** to oxidizing reagents was tested: **9** was allowed to react with 3 equiv of iodine in pyridine-water (98:2, v/v)[9] to afford the corresponding phosphoramidate. TLC monitoring indicated that a half life time of **9** was 2.5 h and the reaction was complete in 12 h. In contrast, **9** was hardly oxidized with *tert*-butyl hydroperoxide (3 equiv) in dry pyridine for 12 h.

Synthesis of Trithymidylate (TpTpT). The unique characters of the *N,N*-diisopropylphosphonamidates, stability against acid and oxidizing reagents, enable us to examine the elongation of oligonucleotide chain both toward 5'- and 3'-directions. For example, methyl 5'-*O*-dimethoxytrityl-N^3-benzoylthymidin-3'-yl phosphonate (**10**) was treated with 1.2 equiv of BDCP in pyridine for 5 min to give the corresponding phosphorochloridite (**11**).[18,19] It was allowed to react with N^3-benzoylthymidin-3'-yl *N,N*-diisopropylphosphonamidate (**12**) and the reaction was monitored by ^{31}P NMR. After 5 min, formation of the dimer (**13**) having an internucleotidic phosphite triester (139.98, 140.57, 140.86 ppm) and a *N,N*-diisopropylphosphonamidate (13.56, 14.24 ppm) at the 3'-terminus was observed (Figure 1A). In this reaction, BDCP (-64.62 ppm) was completely consumed and

the 3'-phosphonamidate group was not activated at all. To the reaction mixture was added 6 equiv of *tert*-butyl hydroperoxide and ^{31}P NMR was further recorded for 10 min. It was observed that the internucleotidic phosphite was selectively oxidized to the phosphotriester (-0.19, -0.39 ppm) and the 3'-phosphonamidate (14.05, 14.92 ppm, J_{PH} = 638.7 Hz, pyridine-d_5) was not oxidized (Figure 1B). The resulting dimer **14** was isolated in 90% yield (based on **12**) by silica gel column chromatography. The ^{31}P NMR spectra of purified **14** were shown in Figure 2.



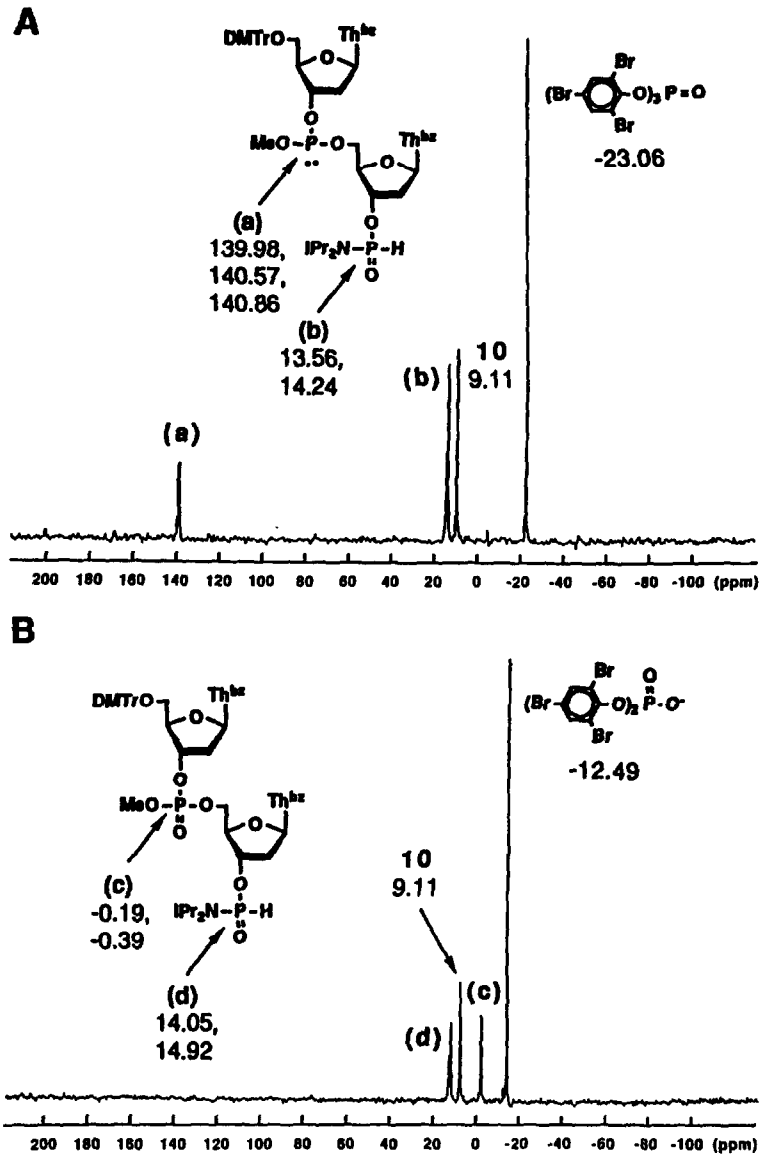


Figure 1. ^{31}P NMR spectra: A, reaction mixture obtained by the reaction of **12** with **11** in pyridine for 5 min; B, reaction mixture after oxidation with *t*-butyl hydroperoxide in pyridine for 10 min and aqueous work up.

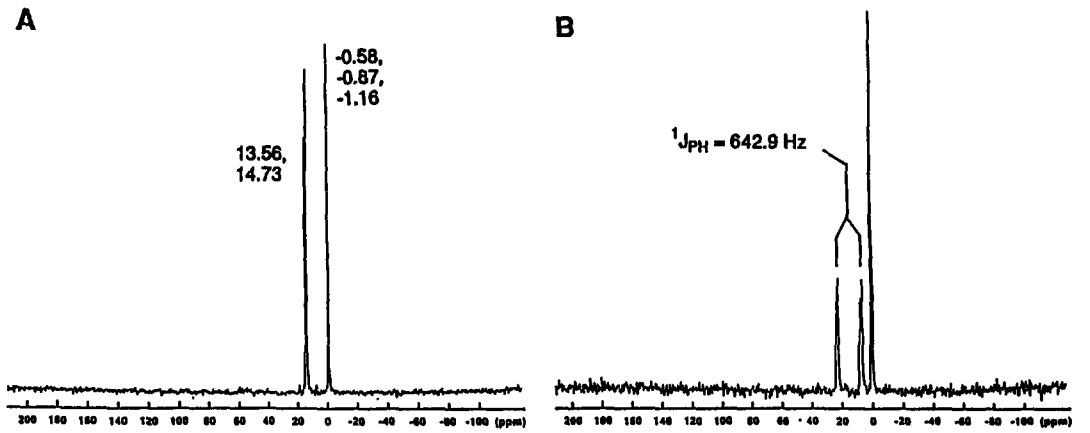


Figure 2. ^{31}P NMR spectra of **14** in $CDCl_3$: A, ^1H-decoupled spectrum; B, ^1H-coupled spectrum.

In order to elongate the chain toward 3'-terminus, the dimer **14** was further treated with 1.5 equiv of BDCP in the presence of diisopropylethylamine in pyridine. After 5 min, ^{31}P NMR spectrum (Figure 3) showed that the 3'-*N,N*-diisopropylphosphonamidate was completely converted into the corresponding phosphorochloridite **15** (179.51 ppm). To the reaction mixture was added 3'-*O*-benzoyl-N^3-benzoylthymidine **16**. The trimer **17** having the phosphoramidite (149.19, 149.18 ppm) and the phosphotriester (-0.10 ppm) was observed after 5 min by ^{31}P NMR. It was treated with aqueous pyridine in the presence of pyridinium chloride for 5 min to give the trimer **18** having the phosphonate diester (8.61, 9.98 ppm) and the phosphotriester (-0.10 ppm) bonds. To the mixture iodine was added and the mixture was stirred for 10 min to give the trimer **19** having the phosphodiester (-1.07 ppm) and the phosphotriester (-0.10 ppm) bonds. **19** was isolated by silica gel column chlomatography in 79% yield on the basis of **16**.

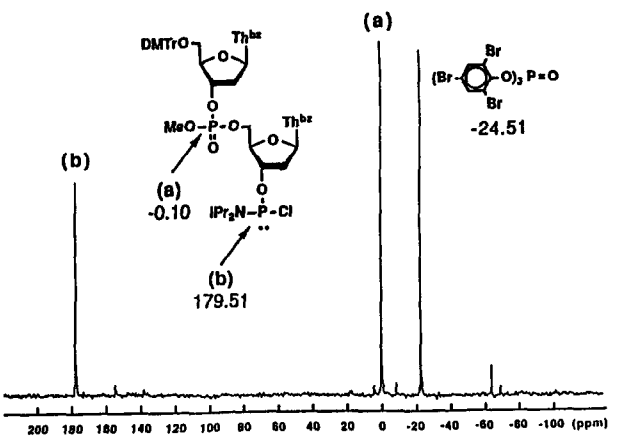


Figure 3. ^{31}P NMR spectrm of the reaction mixture obtained by the reaction of **14** with BDCP in pyridine in the presence of diisopropylethylamine for 5 min.

Deprotection of the trimer was attempted as follows: **19** was treated with benzenethiol-triethylamine-dioxane (1:1:2, v/v/v)[20] at room temperature for 1 h and with concentrated ammonia at room temperature for 12 h. Finally, dimethoxytrityl group was removed by treatment of 80% acetic acid for 30 min. After washing with ether, pure trithymidylate (**20**) was obtained in 91% without further purification (Figure 4 and Figure 5A). The resulting trithymidylate was completely digested with snake venom phosphodiesterase to give thymidine and thymidine 5'-phosphate in the rational ratio (Figure 5B).

In conclusion, nucleoside 3'-*N,N*-diisopropylphosphonamidates are the first example of the nucleoside 3'-phosphonic acid derivatives as 3'-terminal nucleotide units which can perform the chain elongation of oligonucleotides both toward 5'- and 3'-directions.

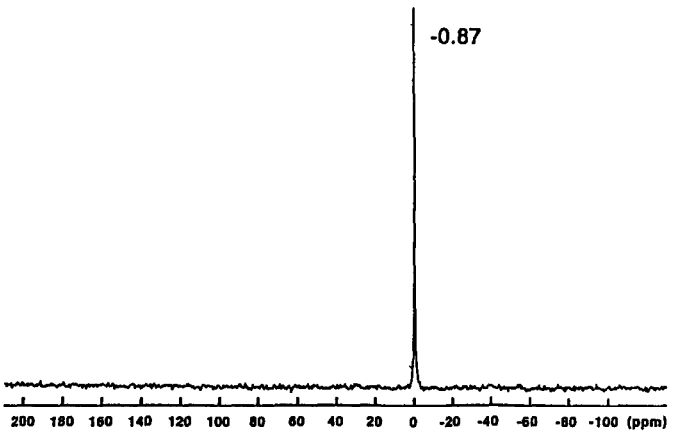


Figure 4. ^{31}P NMR spectrum of crude trithymidylate in D_2O.

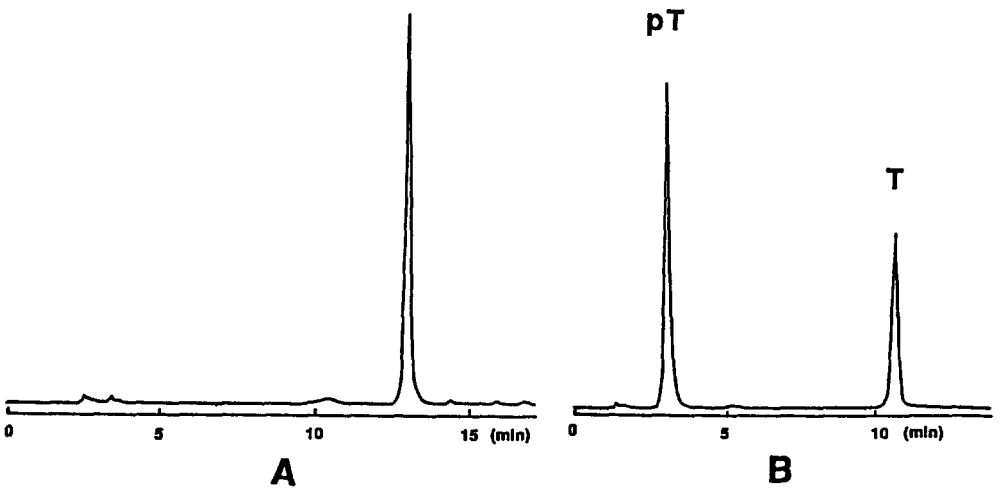


Figure 5. Reversed phase HPLC profiles: **A**, crude trithymidylate; **B**, after digestion with snake venom phosphodiesterase.

Experimental

General Remarks. CH_2Cl_2 was distilled from CaH_2 after being refluxed for several hours, and stored over molecular sieves 4A. Pyridine was distilled after being refluxed over *p*-toluenesulfonyl chloride for several hours, redistilled from CaH_2 and stored over molecular sieves 4A. Tris(2,4,6-tribromophenoxy)dichlorophosphorane (BDCP) was prepared by the procedure as reported previously.[12] Chlorobis(*N,N*-diisopropylamino)phosphine (**6**) was prepared by literature procedure.[16] *tert*-Butyl hydroperoxide (3.0 M solution in 2,2,4-trimethylpentane) was purchased from Aldrich Chemical Co., Inc. ^1H NMR spectra were obtained at 270 MHz on a JEOL-EX-270 spectrometer with tetramethylsilane as an internal standard in $CDCl_3$ and with sodium 3-(trimethylsilyl)propane sulfonate as an external standard in D_2O. ^{13}C NMR spectra were obtained at 67.8 MHz on a JEOL-EX-270 spectrometer with tetramethylsilane as an internal standard. The signals of the ^1H and ^{13}C NMR spectra were assigned by analysis of the ^1H-^1H COSY and ^1H-^{13}C COSY spectra obtained on a JEOL-EX-270 spectrometer. ^{31}P NMR spectra were obtained at 40.5 MHz on a JEOL-FX-100 spectrometer using 85% H_3PO_4 as an external standard. FAB mass spectra were obtained on a JEOL-JMS-DX-303 mass spectrometer. UV spectra were recorded on a Hitachi 220A spectrophotometer. Thin layer chromatography were performed on precoated glass plates of Kieselgel 60 F_{254} (Merck, No. 5715) and developed by CH_2Cl_2-MeOH (20:1, v/v). Silica gel column chromatography was carried out using Wakogel C-200. Reversed phase HPLC was performed on a column of μBondasphere 5μ C18 100 Å, 3.9 mm x 15 cm (Nihon Waters Ltd., No. 10066) with a linear gradient of 0-60% MeCN in 0.1 M ammonium acetate buffer (pH 7.0) at 50°C for 40 min at a rate of 1.0 mL/min.

5'-*O*-Dimethoxytrityl-N^3-benzoylthymidin-3'-yl *N,N*-diisopropylphosphonamidate (9a). *Typical procedure* : **6** (0.320 g, 1.2 mmol) was added to a mixture of **1a**[21] (0.648 g, 1.0 mmol, dried by repeated coevaporation with dry pyridine) and pyridine (0.12 mL, 1.5 mmol) in dry CH_2Cl_2 (10 mL) at room temperature. After being stirred for 10 min, 0.5 M pyridinium chloride in pyridine-H_2O (98:2, v/v, 0.1 ml) was added. The mixture was concentrated and the resulting gum was dissolved in ether-pyridine (4:1, v/v, 50 mL) and washed three times with 5% $NaHCO_3$ (50 mL). The aqueous layer was back extracted three times with ether. The organic layer and washings were combined and dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give a colorless foam. It was dissolved in CH_2Cl_2 (2 mL) and precipitated with *n*-hexane (100 mL) at -30°C. The precipitate was filtered and washed with cold *n*-hexane. The filtrate was concentrated and dissolved in CH_2Cl_2 (1 mL) and precipitated with *n*-hexane (25 mL) at -30°C. The second crop was filtered and washed with cold *n*-hexane. The precipitates were combined and dried in vacuo to give **9a** (0.634 g, 80%) as a white solid: ^{31}P NMR ($CDCl_3$) δ 13.18, 13.47 (J_{PH} = 631.2 Hz); ^1H NMR ($CDCl_3$) δ 1.20, 1.21 (12H, 2d, J = 7.3 Hz and 6.9 Hz, CH_3 of isopropyl), 1.37, 1.41 (3H, 2d, $J_{5\text{-Me},6\text{-H}}$ = 1.0 Hz and 0.7 Hz, 5-CH_3), 2.49 (1H, m, 2'-H), 2.63 (1H, m, 2"-H), 3.35-3.49 (4H, m, 5',5"-H and CH of isopropyl), 3.80 (6H, s, OCH_3 of DMTr), 4.28, 4.32 (1H, 2m, 4'-H), 5.14 (1H, m, 3'-H), 6.45, 6.48 (1H, 2dd, $J_{1',2'}/J_{1',2''}$ = 5.8 Hz and 8.1Hz, $J_{1',2'}/J_{1',2''}$ = 5.6 Hz and 7.1 Hz, 1'-H), 6.85 (4H, 2d, J = 8.6 Hz and 7.7 Hz, 3,3',5,5'-H of DMTr), 6.85, 6.92 (1H, 2d, J_{PH} = 636.5 Hz and 635.4 Hz, PH), 7.16-7.41 (9H, m, ArH of DMTr except for 3,3',5,5'-H), 7.50 (2H, m, 3,5-H of benzoyl), 7.65 (1H, tt, J = 7.5 Hz and 1.3 Hz, 4-H of benzoyl), 7.74 (1H, bs, 6-H), 7.94 (2H, m, 2,6-H of benzoyl); ^{13}C NMR ($CDCl_3$) δ 11.63, 11.72 (5-CH_3), 22.68, 22.92 (J_{PNCC} = 8.6 Hz and 6.1 Hz, CH_3 of isopropyl), 39.73 (2'-C), 45.22, 45.26, (J_{PNC} = 7.0 Hz and 6.1 Hz, CH of isopropyl), 55.29 (OCH_3 of DMTr), 62.96, 63.36 (5'-C), 74.37, 75.03 (J_{POC} = 6.1 Hz and 6.1 Hz, 3'-C), 84.69 (1'-C), 85.14, 85.43 (J_{POCC} = 4.8 Hz and 4.9 Hz, 4'-C), 87.26,

87.33 (*tert*-C of DMTr), 111.53, 111.66 (5-C), 113.37 (3,3',5,5'-C of DMTr), 128.08, 128.19, 130.13, 130.51, 131.52 (ArC of DMTr except for 3,3',5,5'-C), 129.04, 129.13 (3,5-C of benzoyl), 130.51 (2,6-C of benzoyl), 131.62 (1-C of benzoyl), 135.00, 135.09 (4-C of benzoyl and 1,1'-C of DMTr), 135.25 (6-C), 144.02 (1"-C of DMTr), 149.36, 149.43 (2-C), 158.83 (4,4'-C of DMTr), 162.78 (4-C), 169.00 (C=O of benzoyl); FAB MS m/z 794 (M^+); Anal. Calcd for $C_{44}H_{50}N_3O_9P$: C, 66.40; H, 6.33; N, 5.28. Found: C, 66.70, H, 6.46; N, 4.94.

5'-*O*-Dimethoxytrityl-*N*^{6}-benzoyldeoxyadenosin-3'-yl *N,N*-diisopropylphosphonamidate (9b). The above procedure with **1b**[22] (0.460 g, 0.70 mmol) gave **9b** (0.479 g, 85%) as a white solid: ^{31}P NMR ($CDCl_3$) δ 12.88, 13.76 (J_{PH}= 634.8 Hz); ^{1}H NMR ($CDCl_3$) δ 1.25, 1.26 (12H, 2d, J = 6.9 Hz and 6.3 Hz, CH_3 of isopropyl), 2.85 (1H, m, 2'-H), 3.07 (1H, m, 2"-H), 3.37-3.57 (4H, 5',5"-H and CH of isopropyl), 3.77 (6H, s, OCH_3 of DMTr), 4.39, 4.44 (1H, 2m, 4'-H), 5.22 (1H, m, 3'-H), 6.53, 6.55 (1H, 2dd, $J_{1',2'}$/$J_{1',2"}$ = 5.9 Hz and 6.4Hz, $J_{1',2'}$/$J_{1',2"}$ = 5.9 Hz and 7.1 Hz, 1'-H), 6.78, 6.79 (4H, 2d, J = 8.6 Hz and 8.3 Hz, 3,3',5,5'-H of DMTr), 6.93, 6.97 (1H, 2d, J_{PH} = 632.1 Hz and 639.7 Hz, PH), 7.16-7.32 (5H, m, 2",3",4",5",6"-H of DMTr), 7.35, 7.37 (4H, 2d, J = 8.6 Hz and 8.3 Hz, 2,2',6,6'-H of DMTr), 7.51 (2H, m, 3,5-H of benzoyl), 7.60 (1H, tt, J = 7.3 Hz and 1.3 Hz, 4-H of benzoyl), 8.03 (2H, m, 2,6-H of benzoyl), 8.16, 8.18 (1H, 2s, 2-H), 8.73, 8.74 (1H, 2s, 8-H), 9.13 (1H, bs, 6-NH); ^{13}C NMR ($CDCl_3$) δ 22.95, 22.96 (J_{PNCC} = 7.3 Hz and 4.9 Hz, CH_3 of isopropyl), 39.19, 39.41 (J_{POCC} = 2.4 Hz, 2'-C), 45.27, 45.35 (J_{PNC} = 6.1 Hz and 4.9 Hz, CH of isopropyl), 55.22 (OCH_3 of DMTr), 63.00, 63.36 (5'-C), 74.63, 74.88 (J_{POC} = 4.9 Hz, 3'-C), 84.58, 84.65 (1'-C), 85.47, 85.67 (J_{POCC} = 6.8 Hz, 4'-C), 86.72, 86.77 (*tert*-C of DMTr), 113.21 (3,3',5,5'-C of DMTr), 123.38 (5-C), 127.01, 130.03 (2",3",4",5",6"-C of DMTr), 127.87 (2,6-C of benzoyl), 128.10 (2,2',6,6'-C of DMTr), 128.86 (3,5-C of benzoyl), 132.76 (4-C of benzoyl), 133.69 (1-C of benzoyl), 135.40, 135.45 (1,1'-C of DMTr), 141.33, 141.42 (2-C), 144.27 (1"-C of DMTr), 149.56 (4-C), 151.50 (6-C), 152.68 (8-C), 158.58 (4,4'-C of DMTr), 164.58 (C=O of benzoyl); FAB MS m/z 805 (M^+).

5'-*O*-Dimethoxytrityl-*N*^{4}-anisoyldeoxycytidin-3'-yl *N,N*-diisopropylphosphonamidate (9c). The same procedure with **1c**[22] (0.664 g, 1.0 mmol) gave **9c** (0.711 g, 88%) as a white solid: ^{31}P NMR ($CDCl_3$) δ 13.37 (J_{PH}= 632.8 Hz); ^{1}H NMR ($CDCl_3$) δ 1.23, 1.24 (12H, 2d, J = 6.6 Hz and 6.6 Hz, CH_3 of isopropyl), 2.39 (1H, m, 2'-H), 2.89 (1H, m, 2"-H), 3.38-3.52 (4H, 5',5"-H and CH of isopropyl), 3.79, 3.80 (6H, 2s, OCH_3 of DMTr), 3.88 (3H, s, OCH_3 of anisoyl), 4.37, 4.42 (1H, 2m, 4'-H), 5.09 (1H, m, 3'-H), 6.34, 6.36 (1H, 2t, $J_{1',2'}$ = $J_{1',2"}$ = 5.9 Hz and $J_{1',2'}$ = $J_{1',2"}$ = 5.3 Hz, 1'-H), 6.85, 6.87 (4H, 2d, J = 8.6 Hz and 8.6 Hz, 3,3',5,5'-H of DMTr), 6.84, 6.95 (1H, 2d, J_{PH} = 637.4 Hz and 633.5 Hz, PH), 6.98 (2H, d, J = 8.8 Hz, 3,3',5,5'-H of anisoyl), 7.21-7.40 (12H, m, 5-H and ArH), 7.86 (2H, d, 8.8 Hz, 2,6-H of anisoyl), 8.14, 8.19 (1H, 2d, J = 7.3 and 7.6 Hz, 6-H), 8.65 (1H bs, 4-NH); ^{13}C NMR ($CDCl_3$) δ 22.96, 22.98 (J_{PNCC} = 9.7 Hz and 7.3 Hz, CH_3 of isopropyl), 40.86 (2'-C), 45.24 (J_{PNC} = 6.1 Hz, CH of isopropyl), 55.24 (OCH_3 of DMTr), 55.56 (OCH_3 of anisoyl), 62.30, 62.84 (5'-C), 73.12, 74.25 (J_{POC} = 4.9 Hz and 4.9 Hz, 3'-C), 85.50, 85.91 (J_{POCC} = 4.8 Hz and 4.9 Hz, 4'-C), 86.97 (*tert*-C of DMTr), 87.10, 87.13 (1'-C), 96.46 (5-C), 113.33 (3,3',5,5'-C of DMTr), 114.27 (3,5-C of anisoyl), 125.28 (1-C of anisoyl), 127.17, 128.05, 128.12, 130.03, 130.10 (ArC of DMTr), 129.63 (2,6-C of anisoyl), 135.04, 135.07, 135.22, 135.25 (1,1'-C of DMTr), 143.95, 143.99 (1"-C of DMTr), 144.26 (6-C), 158.70 (4-C of anisoyl and 4,4'-C of DMTr), 162.17 (2-C), 163.54 (4-C and C=O of anisoyl); FAB MS m/z 811 (M^+).

5'-*O*-Dimethoxytrityl-N^2-propionyl-O^6-diphenylcarbamoyldeoxyguanosin-3'-yl *N,N*-diisopropylphosphonamidate (9d). The same procedure with **1d**[23] (0.821 g, 1.0 mmol) gave **9d** (0.816 g, 84%) as a white solid: ^{31}P NMR ($CDCl_3$) δ 12.98, 14.05 (J_{PH}= 635.9 Hz); ^{1}H NMR ($CDCl_3$) δ 1.19, 1.23 (3H, 2t, J = 7.3 Hz and 6.9 Hz, CH_3 of propionyl), 1.24 (12H, 2d, J = 6.9 Hz, CH_3 of isopropyl), 2.70 (2H, q, J = 7.3 Hz, CH_2 of propionyl), 2.75 (1H, m, 2'-H), 2.96 (1H, m, 2"-H), 3.36-3.56 (4H, m, 5',5"-H and CH of isopropyl), 3.74 (6H, s, OCH_3 of DMTr), 4.32, 4.39 (1H, 2m, 4'-H), 5.22 (1H, m, 3'-H), 6.39, 6.43 (1H, 2dd, $J_{1',2'}$/$J_{1',2"}$ = 5.6 Hz and 8.3Hz, $J_{1',2'}$/$J_{1',2"}$ = 5.9 Hz and 6.6 Hz, 1'-H), 6.76, 6.77 (4H, 2d, J = 9.2 Hz and 8.9 Hz, 3,3',5,5'-H of DMTr), 6.91, 6.96 (1H, 2d, J_{PH}= 631.8 Hz and 639.1 Hz, PH), 7.14-7.45 (19H, m, ArH), 7.95, 8.00 (1H, 2bs, 2-NH), 8.08 (1H, s, 8-H); ^{13}C NMR ($CDCl_3$) δ 8.91 (CH_3 of propionyl), 22.92, 22.94 (J_{PNCC} = 8.6 Hz and 6.1 Hz, CH_3 of isopropyl), 39.21, 39.50 (2'-C), 45.26, 45.31 (J_{PNC} = 6.1 Hz and 6.1 Hz, CH of isopropyl), 55.20 (OCH_3 of DMTr), 63.18, 63.47 (5'-C), 74.37, 74.64 (J_{POC} = 4.9 Hz and 7.4 Hz, 3'-C), 84.46 (1'-C), 85.29, 85.62 (J_{POCC} = 6.1 Hz and 4.9 Hz, 4'-C), 86.63, 86.72 (*tert*-C of DMTr), 113.21 (3,3',5,5'-C of DMTr), 121.20, 121.26 (5-C), 126.99, 127.22, 128.07, 129.18, 129.97, 130.01 (ArC), 135.34, 135.42 (1,1'-C, DMTr), 141.78 (1-C of diphenylcarbamoyl), 142.17, 142.28 (8-C), 144.29 (1"-C of DMTr), 150.37 (4-C), 152.07 (2-C), 154.45, 154.59 (6-C), 156.19 (C=O of diphenylcarbamoyl), 158.60 (C=O of propionyl and 4,4'-C of DMTr); FAB MS m/z 968 (M^+).

N^3-Benzoylthymidin-3'-yl *N,N*-diisopropylphosphonamidate (12). To a solution of **9a** (0.159 g, 0.20 mmol) in $CHCl_3$ (20 mL) at 0°C was added 1% trifluoroacetic acid in $CHCl_3$ (20 mL). The reaction mixture was stirred at 0°C for 45 min. To the above mixture was added pyridine (1 mL) and washed three times with 5% $NaHCO_3$, and the aqueous layer was back extracted three times with $CHCl_3$. The organic layer and washings were combined and dried over Na_2SO_4, filtered, and concentrated to dryness. The residue was applied to a silica gel column. Chromatography was performed first with CH_2Cl_2-ether (1:1, v/v) containing 1% of Et_3N followed with CH_2Cl_2 containing 1% of Et_3N, applying a gradient of MeOH (0-2%). The fractions containing **12** were combined and concentrated to give **12** (0.088 g, 89%) as a colorless foam: ^{31}P NMR ($CDCl_3$) δ 13.47, 14.14 (J_{PH}= 642.4 Hz); ^{1}H NMR ($CDCl_3$) δ 1.24, 1.25 (12H, 2d, J = 6.9 Hz and 6.9 Hz, CH_3 of isopropyl), 1.96 (3H, s, 5-CH_3), 2.38 (1H, m, 2'-H), 2.49 (1H, m, 2"-H), 3.45, 3.51 (2H, m, J = 6.9 Hz, CH of isopropyl), 3.90 (2H, m, 5',5"-H), 4.62 (1H, m, 4'-H), 5.17 (1H, m, 3'-H), 6.31 (1H, t, $J_{1',2'}$=$J_{1',2"}$ = 6.3 Hz, 1'-H), 6.94, 6.96 (1H, 2d, J_{PH}= 644.3 Hz and 633.8 Hz, PH), 7.49 (2H, t, J = 7.6 Hz, 3,5-H of benzoyl), 7.65 (1H, tt, J = 7.6 Hz and 1.3 Hz, 4-H of benzoyl), 7.80, 7.82 (1H, 2d, $J_{6\text{-H},5\text{-Me}}$ = 1.3 Hz and 1.0 Hz, 6-H), 7.93 (2H, d, J = 7.6 Hz, 2,6-H of benzoyl); ^{13}C NMR ($CDCl_3$) δ 12.67 (5-CH_3), 22.87 (J_{PNCC} = 8.5 Hz, CH_3 of isopropyl), 39.35 (2'-C), 45.41, 45.44 (J_{PNC} = 7.4 Hz and 6.1 Hz, CH of isopropyl), 61.65, 61.74 (5'-C), 73.45, 74.30 (J_{POC} = 6.1 Hz and 4.8 Hz, 3'-C), 85.28, 85.35 (1'-C), 86.17, 86.39 (J_{POCC} = 3.6 Hz and 3.7 Hz, 4'-C), 111.21 (5-C), 129.16 (3,5-C of benzoyl), 130.46 (2,6-C of benzoyl), 131.55 (1-C of benzoyl), 135.07 (4-C of benzoyl), 135.92 (6-C), 149.40, 149.43 (2-C), 162.84 (4-C), 168.98 (C=O of benzoyl).

Synthesis of the Dimer 14. A solution of BDCP (0.246 g, 0.225 mmol) in dry pyridine (1.5 mL) was added to **10**[21] (0.137 g, 0.188 mmol, dried by repeated coevaporation with dry pyridine) and the mixture was allowed to stand at room temperature for 5 min. The mixture was added to **12** (0.062 g, 0.125 mmol, dried by repeated coevaporation with dry pyridine). The mixture was stirred at room temperature for 5 min and a 3.0 M solution of *tert*-butyl hydroperoxide in 2,2,4-trimethylpentane (0.25 mL, 0.75 mmol) was added. After being stirred for 20

min, the mixture was diluted with $CHCl_3$ and washed three times with 5% $NaHCO_3$, and the aqueous layer was back extracted three times with $CHCl_3$. The organic layer and washings were combined and dried over Na_2SO_4, filtered, and concentrated to dryness. The residue was applied to a silica gel column and chromatography was performed with CH_2Cl_2 containing 1% of Et_3N, applying a gradient of MeOH (0-1.5%). The fractions containing **14** were combined and concentrated to give **14** (0.137 g, 90%) as a colorless foam: ^{31}P NMR ($CDCl_3$) δ 13.56, 14.73 (J_{PH}= 642.9 Hz, phosphonamidate), -0.58, -0.87, -1.16 (phosphotriester); ^1H NMR ($CDCl_3$) δ 1.21, 1.22 (12H, 2d, J = 6.9 Hz and 6.6 Hz, CH_3 of isopropyl), 1.43 (3H, s, 5-CH_3 of pTp), 1.93 (3H, m, 5-CH_3 of Tp), 2.22 (1H, m, 2'-H of Tp), 2.42-2.58 (2H, m, 2"-H of Tp and 2'-H of pTp), 2.69 (1H, m, 2"-H of pTp), 3.36-3.60 (4H, m, 5',5"-H of Tp and CH of isopropyl), 3.72, 3.73, 3.78 (3H, 3d, J_{POCH} = 11.5 Hz, 11.2 Hz, and 11.2 Hz, $POCH_3$), 3.80 (6H, s, OCH_3 of DMTr), 4.20-4.34 (4H, m, 5',5"-H of pT, 4'-H of Tp and pTp), 4.98 (1H, m, 3'-H of Tp), 5.21 (1H, m, 3'-H of pTp), 6.31 (1H, m, 1'-H of Tp), 6.44 (1H, dd, $J_{1',2'}/J_{1',2"}$ = 5.6 Hz and 7.8 Hz, 1'-H of pTp), 6.87 (4H, d, J = 8.9 Hz, 3,3',5,5'-H of DMTr), 6.89, 6.89, 6.91, 6.91 (1H, 4d, J_{PH}= 636.1 Hz , 637.7 Hz, 639.9 Hz, and 640.1 Hz, PH), 7.24-7.95 (21H, m, 6-H and ArH).

Synthesis of the Trimer 19. To a solution of **14** (0.100 g, 0.082 mmol, dried by repeated coevaporation with dry pyridine) in dry pyridine (1 mL) was added a mixture of BDCP (0.134 g, 0.123 mmol) and diisopropylethylamine (0.044 mL, 0.25 mmol) in dry pyridine (1 mL). The mixture was allowed to stand at room temperature for 5 min and added to a solution of **16** (0.025 g, 0.055 mmol, dried by repeated coevaporation with dry pyridine) in dry pyridine (0.5 mL). After being stirred for 10 min, 0.5 M solution of pyridinium chloride in pyridine-H_2O (98:2, v/v, 2 mL) was added to the mixture. After being stirred for 10 min, I_2 (0.104 g, 0.41 mmol) was added and the mixture was stirred for 10 min. Then saturated $Na_2S_2O_4$ aq was added to the mixture and diluted with $CHCl_3$. The organic layer was washed three times with 0.5 M triethylammonium hydrogen carbonate and the aqueous layer was back extracted three times with $CHCl_3$. The organic layer and washings were combined and dried over Na_2SO_4, filtered, and concentrated to dryness. The residue was applied to a preparative TLC developed with CH_2Cl_2-MeOH (20:1, v/v) containing 1% of Et_3N to give **19** (triethylammonium salt; 0.070 g, 79%) as a foam: ^{31}P NMR ($CDCl_3$) δ -0.68, -0.97 (phosphotriester), -1.74 (phosphodiester); ^1H NMR ($CDCl_3$) δ 1.23 (9H, t, J = 7.3 Hz, CH_3 of Et_3NH^+), 1.41 (3H, s, 5-CH_3), 1.93 (3H, m, 5-CH_3), 2.01 (3H, s, 5-CH_3), 2.43-2.76 (6H, m, 2',2"-H), 2.97 (6H, q, J = 7.3 Hz, CH_2 of Et_3NH^+), 3.42, 3.55 (2H, 2m, 5',5"-H), 3.69, 3.73 (3H, 2d, J_{POCH} = 11.2 Hz and 15.5 Hz, $POCH_3$), 3.79 (6H, s, OCH_3 of DMTr), 4.08-4.41 (7H, 5',5"-H and 4'-H), 4.90, 5.20, 5.63 (3H, 3m, 3'-H), 6.33, 6.42, 6.48 (3H, 3m, 1'-H), 6.86 (4H, d, J = 8.9 Hz, 3,3',5,5'-H of DMTr), 7.18-7.99 (29H, m, 6-H and ArH).

Synthesis of Trithymidylate. Compound **19** (0.042 g, 25.8 mmol) was treated with PhSH-Et_3N-dioxane (1:1:2, v/v/v, 1 mL) at room temperature for 1 h. The mixture was diluted with pyridine-H_2O (1:1, v/v) and washed five times with ether. The aqueous layer was concentrated to dryness and the residue was treated with concd NH_3-pyridine (9:1, v/v, 20 mL) for 12 h at room temperature. The mixture was concentrated to dryness and the residue was treated with 80% AcOH (20 mL) at room temperature for 30 min. AcOH was removed by repeated coevaporation with water and the aqueous solution was washed five times with ether. The aqueous layer was concentrated and coevaporated with EtOH for several times. The resulting solid was dried over P_4O_{10} under reduced pressure at 40°C for two days to give trithymidylate (ammonium salt; 0.021 g, 91%) as an amorphous

solid. Resulting trithymidylate was completely digested with snake venom phosphodiesterase in 0.1M Tris-HCl buffer (pH 8.0) at 37°C for 3 h to give thymidine and thymidine 5'-phosphate in 1.0:1.9 ratio (analyzed by reversed phase HPLC). ^{31}P NMR (D_2O) δ -0.87; ^{1}H NMR (D_2O) δ 1.84, 1.86, 1.87 (9H, 3s, 5-CH_3), 2.33-2.51 (6H, m, 2',2"-H), 3.78 (2H, m, 5',5"-H), 4.08-4.10 (4H, m, 5',5"-H), 4.15-4.16 (2H, m, 4'-H), 4.55, 4.76, 4.86 (3H, 3m, 3'-H), 6.17 (1H, t, J = 7.0 Hz, 1'-H), 6.20 (2H, t, J = 7.0 Hz, 1'-H), 7.60 (1H, s, 6-H), 7.65 (2H, s, 6-H); UV (H_2O, pH 7.0) λ_{max} = 266 nm, λ_{min} = 236 nm.

This paper is dedicated to Professor Fritz Eckstein on the occasion of his 60th birthday.

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