A combined use of triphenyl phosphite and 2,2'-dipyridyl diselenide as a coupling reagent in oligonucleotide synthesis

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ABSTRACT

Oligonucleotides and nucleoside phosphoramidates can be synthesized in high yields by a combined use of triphenyl phosphite and 2,2'-dipyridyl diselenide as a coupling reagent.

Until recently, there were only two useful methods for the synthesis of natural internucleotidic bonds, namely, dicyclohexylcarbodiimide method¹ and arylsulfonyl chloride method^{2,3} developed by Khorana and his coworkers. Other approaches often generate a mixture of undesirable symmetrical pyrophosphate formed from the nucleotide component, and don't give practically sufficient yield of coupling reaction for the formation of internucleotidic bond.

Recently, Mukaiyama has described a new coupling method by use of triphenylphosphine and 2,2'-dipyridyl disulfide.⁴ According to this method, several oligonucleotides were synthesized in high yields⁵⁻⁷. In the course of our experiment, we found that the coupling reaction accompanied a side reaction in which the hydroxy group of sugar moiety of nucleosides reacted with coupling reagents to form the corresponding 5'-S-pyridylthio-5'-deoxyribonucleosides.⁸ In order to avoid the side reaction, screenings were performed by use of several tervalent phosphorus compounds and disulfides or diselenides. Finally, we found that a combined use of triphenyl phosphite and 2,2'-dipyridyl diselenide is the most suitable combination for the synthesis of internucleotidic bonds.

In this paper, we describe the synthesis of oligonucleotides and nucleoside phosphoramidates by use of triphenyl phos-

phite $[(\text{PhO})_3\text{P}]$ and 2,2'-dipyridyl diselenide $[(\text{PySe})_2]$.

When 5'-O-tritylthymidine (TrT) (0.12 mmol) was treated with 3'-O-acetylthymidine 5'-phosphate (pTOAc) (0.1 mmol) in the presence of $(\text{PhO})_3\text{P}$ (0.5 mmol) and $(\text{PySe})_2$ (0.5 mmol) at room temperature for 1 day, 5'-O-tritylthymidylyl(3'→5')-thymidine 3'-O-acetate (TrTpTOAc) was obtained along with pyridine-2-selenone and triphenyl phosphate. The protected dithymidine derivative (TrTpTOAc) was treated first with 80% aqueous acetic acid at 100°C for 10 min and then with 0.5N sodium hydroxide at room temperature for 1 hr to give thymidylyl(3'→5')thymidine (TpT)⁹ in 75% yield based on pTOAc.

In the above experiment, pyridine is the most suitable solvent than the others such as hexamethylphosphortriamide (HMPA) or dimethylformamide (DMF). Screenings for the synthesis of TpT by use of several trivalent phosphorus compounds and solvents are listed in Table 1.

Table 1. Synthesis of Thymidylyl(3'→5')Thymidine (TpT)

R_3P	Solvent	TpT Yield(%)
Ph_3P	pyridine	7
Ph_3P	HMPA	10
Ph_3P	DMF	5
$(\text{p-MeOPh})_3\text{P}$	pyridine	14
$\text{n-Bu}_3\text{P}$	pyridine	35
$(\text{PhS})_3\text{P}$	pyridine	25
$(\text{PhO})_3\text{P}$	pyridine	75
$(\text{PhO})_3\text{P}$	DMF	59

In order to synthesize oligonucleotides containing adenosine, guanosine, and cytidine residues, 5'-O-monomethoxy-tritylthymidine (MMTrT) was used in place of TrT. Dinucleoside monophosphates⁹⁻¹¹ were synthesized as shown in Table 2.

B:thymine, N⁶-benzoyladenine, N⁴-anisoyl-cytosine, or N²-isobutyrylguanine.

R:acetyl or isobutyryl.

Table 2. Synthesis of Dinucleoside Monophosphate

Mononucleotide (mmol)	(PhO) ₃ P-(PySe) ₂ (mmol)	Product	Yield (%)
d-pTOAc (0.2)	1.0	TpT	89
d-pA ^{Bz} OAc (0.2)	1.0	TpA	78
d-pC ^{An} OAc (0.2)	1.0	TpC	75
d-pG ^{iBu} iBu (0.2)	1.0	TpG	75

MMTrT (0.1 mmol) was used in the above reactions.

Next, trinucleoside diphosphates were synthesized by the reactions of dinucleoside monophosphates prepared in the above experiments with 3'-O-acetylnucleoside 5'-phosphates.

When 5'-O-tritylthymidylyl(3'→5')thymidine (TrTpT) (0.05 mmol) was treated with pTOAc (0.10 mmol) in the presence of (PhO)₃P (0.50 mmol) and (PySe)₂ (0.50 mmol) in dry pyridine (0.5 ml) at room temperature for 2 days, 5'-O-tritylthymidylyl-

(3'→5')thymidylyl(3'→5')thymidine (TrTpTpT)¹² was obtained in 73% yield after removal of acetyl group by usual work-up.

B_1, B_2, B_3 : thymine, N^6 -benzoyladenine, N^4 -anisoyl-cytosine, or N^2 -isobutyrylguanine.
 R : trityl or monomethoxytrityl.
 R_1 : acetyl.

In a similar manner, some trinucleoside diphosphates^{10,12,13} were obtained as shown in Table 3.

Table 3. Synthesis of Trinucleoside Diphosphates

Dinucleoside phosphate	Mononucleotide	Product	Yield (%)
TrTpT	d-pTOAc	TrTpTpT	73
MMTrTpT	d-pA ^{Bz} OAc	MMTrTpTpA	64
MMTrTpT	d-pC ^{An} OAc	MMTrTpTpC	62
MMTrTpA ^{Bz}	d-pA ^{Bz} OAc	MMTrTpApA	63
MMTrTpA ^{Bz}	d-pTOAc	MMTrTpApT	68

In each case, dinucleoside phosphate (0.05 mmol), mononucleotide (0.1 mmol), triphenyl phosphite (0.5 mmol), and 2,2'-dipyridyl diselenide (0.5 mmol) were used.

There is an approach to the synthesis of inter-nucleotidic bond in which nucleoside 3'-phosphate is coupled with 5'-hydroxy group of nucleoside components. We attempted to synthesize deoxyribooligonucleotides by use of nucleoside 3'-phosphates.

First, phosphorylation reaction of thymidine 3'-hydroxy group was tried by means of β -cyanoethyl phosphate¹⁴ in the presence of $(\text{PhO})_3\text{P}$ and $(\text{PySe})_2$.

When 5'-O-tritylthymidine (2 mmol) was treated with β -cyanoethyl phosphate (4 mmol) in the presence of $(\text{PhO})_3\text{P}$ (20 mmol) and $(\text{PySe})_2$ (20 mmol) in dry pyridine (20 ml) at room temperature for 1 day, 5'-O-tritylthymidine 3'-phosphate (TrTp)^{15,16} was obtained in 80% yield after removal of β -cyanoethyl group by alkaline hydrolysis.

Next, 5'-O-tritylthymidine 3'-phosphate (TrTp) (0.05 mmol) prepared in the above experiment was treated with 3'-O-acetylthymidine (TOAc) (0.05-0.2 mmol) in the presence of $(\text{PhO})_3\text{P}$ (0.25 mmol) and $(\text{PySe})_2$ (0.25 mmol) in dry pyridine (0.5 mmol) to form dithymidine phosphate (TpT)^{16,17}, as shown in Table 4.

When the reaction was carried out in a mixed solvent of pyridine and triphenyl phosphite (1:1 v/v) in the presence of 5 equiv. of $(\text{PySe})_2$, TpT was obtained in almost quantitative yield after removal of the protecting groups.

Table 4. Synthesis of Thymidylyl(3'→5')Thymidine (TpT)

TOAc (mmol)	Solvent	$(\text{PhO})_3\text{P}-(\text{PySe})_2$ (mmol)	TpT Yield(%)
0.05	pyridine	0.25	64
0.1	pyridine	0.25	92
0.2	pyridine	0.25	92
0.05	$(\text{PhO})_3\text{P}$	0.25	57
0.1	$(\text{PhO})_3\text{P}$	0.25	75
0.1	pyridine $(\text{PhO})_3\text{P}$ (1:1)	0.25	quant.

The above reactions were carried out by use of 0.05 mmol of TrTp at room temperature for 1 day.

Further, 5'-O-tritylated dithymidine derivative, TrTpT, was allowed to react with β -cyanoethyl phosphate in the presence of $(\text{PhO})_3\text{P}$ and $(\text{PySe})_2$ in dry pyridine at room temperature for 1 day to afford 5'-O-tritylthymidylyl(3'→5')thymidine 3'- β -cyanoethyl phosphate (TrTpTpce). After removal of β -cyanoethyl group, TrTpTp^{1,16} was obtained in 69% yield.

The TrTpTp (0.05 mmol) was further treated with TOAc (0.1 mmol) in the presence of $(\text{PhO})_3\text{P}$ (0.5 mmol) and $(\text{PySe})_2$ (0.5 mmol) in dry pyridine (0.5 ml) at room temperature for 2 days to give the corresponding trithymidine diphosphate derivative (TrTpTpTOAc). After removal of the protecting groups, thymidylyl(3'→5')thymidylyl(3'→5')thymidine (TpTpT)¹⁶ was obtained in 95% yield based on TrTpTp.

It is noted that no side reaction was observed during the coupling reaction and only unchanged starting materials were detected.

From these facts, it is concluded that the coupling reagents, $(\text{PhO})_3\text{P}$ and $(\text{PySe})_2$ were widely utilized to the

stepwise synthesis of oligonucleotide in which both of nucleoside 5'-phosphate and nucleoside 3'-phosphate could be used as the unit of nucleotides.

Finally, it has been tried to the synthesis of nucleoside 5'-phosphoramidates by use of $(\text{PhO})_3\text{P}$ and $(\text{PySe})_2$. Mukaiyama and Hashimoto⁵ reported that the reaction of thymidine 5'-phosphate reacted with aniline (N) in the presence of 10 equiv. each of triphenylphosphine and 2,2'-dipyridyl disulfide to form thymidine 5'-phosphoranilidate (NpT) and thymidine 5'-phosphorodianilidate (NNpT).

On the other hand, when 2 equiv. each of triphenylphosphine and 2,2'-dipyridyl disulfide was employed in the above reaction, NpT was obtained in 80% yield.

In the present experiment, $(\text{PhO})_3\text{P}$ and $(\text{PySe})_2$ were used in place of triphenylphosphine and 2,2'-dipyridyl disulfide and it was found that monoanilidate, thymidine 5'-phosphoranilidate (NpT) was obtained exclusively in 94% yield even when 5 equiv. each of $(\text{PhO})_3\text{P}$ and $(\text{PySe})_2$ were used.

RNH_2 : aniline, 3-(N,N-diethylaminomethyl)aniline, or morpholine.

B: thymine, N⁶-benzoyladenine, N⁴-anisoyl-cytosine, or N²-isobutyrylguanine.

In a similar manner, several nucleoside 5'-phosphoramidates were synthesized in high yields as shown in Table 5.

Table 5. Synthesis of Nucleoside 5'-Phosphoramidates

Mononucleotide (0.05 mmol)	Amine (0.5 mmol)	Yield (%)	max, nm
d-pT	aniline	94	267,226
d-pA ^{Bz}	aniline	90	280,230
d-pC ^{An}	aniline	85	302,(283)
d-pG ^{iBu}	aniline	87	257,211
d-pT	3-(N,N-diethylamino- methyl)aniline	84	267,236
d-pT	morpholine	96	267

The above reactions were carried out by use of 0.25 mmol of $(\text{PhO})_3\text{P}$ and 0.25 mmol of $(\text{PySe})_2$ in dry pyridine.

Experimental

General Methods and Materials. Reagent grade pyridine was distilled after treatment with p-toluenesulfonyl chloride and dried over calcium hydride for 2 weeks. DMF and HMPA were distilled and dried over molecular sieves (linde molecular sieves, 4A). Paper chromatography was preformed by the technique using Toyo Roshi No. 51 paper. The solvent systems used were: solvent A, isopropyl alcohol:concentrated ammonia:water (7:1:2 v/v); solvent B, ethyl alcohol:1M ammonium acetate (pH 7.5) (7:3 v/v). Paper electrophoresis was carried out in a commercially available apparatus capable of giving a potential of 1200V using potassium phosphate (0.05M, pH 8.0) buffer. The R_f value and paper electrophoretic mobility of different compounds are listed in Table 6.

The compounds containing trityl group were made visible on paper chromatograms by spraying the chromatograms with dilute perchloric acid and warming in a oven. The compounds containing trityl group appeared yellow; those containing monomethoxytrityl group appeared yellowish orange. Phosphorus-containing compounds were located on chromatograms with Hanes-Isherwood spray¹⁸ followed by ultraviolet irradiation.¹⁹

5'-O-Tritylthymidine (TrT) and 5'-O-monomethoxytrityl-thymidine (MMTrT) were prepared by the method of Khorana.^{15,20}

Table 6. Paper Chromatography and Electrophoresis of Different Compounds.

Compound	Rf in solvent		Paper electrophoresis relative mobility (pH 8.0)
	A	B	
TrT	0.85		
TrTpT	0.71	0.89	0.28
TrTpTpT	0.49	0.72	0.51
TrTp	0.51		0.69
TrTpTp	0.27		0.89
TOAc	0.78	0.85	
TpT	0.43	0.59	0.52
TpC	0.34	0.53	0.45
TpG	0.39	0.58	0.47
TpA	0.40	0.66	0.36
TpTpT	0.23		0.63
TppT	0.22		0.78
TpTp	0.05	0.35	
Tp	0.13		
pT	0.18	0.38	1.00
pA	0.13	0.23	
pC	0.08	0.15	
pG	0.07	0.16	
pTOAc		0.45	0.95
pC ^{An} OAc		0.56	
pG ^{iBu} iBu		0.60	
pA ^{Bz} OAc		0.45	
pC ^{An}		0.42	
pG ^{iBu}		0.50	
pA ^{Bz}		0.33	
T	0.65		
MMTrT			
MMTrTpT	0.80		
MMTrTpTpA	0.66		
MMTrTpTpC	0.57		
MMTrTpA ^{Bz}	0.78	0.88	
MMTrTpApT	0.68		
MMTrTpApA	0.63		
PhNHpT	0.65	0.75	0.65
PhNHpA ^{Bz}	0.62	0.74	0.77
PhNHpG ^{iBu}	0.59	0.70	0.63
PhNHpC ^{An}	0.55	0.71	0.70
MpT ^a	0.50	0.67	0.76
DEAMP ^b	0.67	0.81	

a, M=morpholine. b, DEAM=3-(N,N-diethylaminomethyl)aniline.

3-O-Acetylthymidine 5'-phosphate (pTOAc)²¹, 3'-O-acetyl-N⁴-anisoyldeoxycytidine 5'-phosphate (d-pC^{An}OAc)²², 3'-O-isobutyl-N²-isobutyldeoxyguanosine 5'-phosphate (d-pG^{iBu}OiBu)²³, and 3'-O-acetyl-N⁶-benzoyldeoxyadenosine (d-pA^{Bz}OAc)²⁴ were prepared by literature procedures and used as the pyridinium salts.

2-Selenopyridine. To a solution of selenium (6.0g, 76 mmol) in absolute ethanol (150 ml) sodium borohydride (3.2g, 84 mmol) was added with vigorous stirring under cooling in an ice bath. After the initial vigorous forming had subsided (about 15 min), the ice bath was removed and the mixture was kept standing at room temperature for 12 hr. To the mixture containing sodium hydrogen selenide, 2-chloropyridine (8.59g, 76 mmol) was added. It was refluxed for 19 hr. Precipitate mainly sodium chloride was filtered off and the filtrate was concentrated to dryness

under reduced pressure at 30°C. The residue was washed with cold water and dried over phosphorus pentoxide in vacuum desiccator. 2-Selenopyridine (7.7g, 60%) was obtained and recrystallized from benzene to give yellow needles; mp. 136-138°C (lit.^{25,26} 132-137°C).

2,2'-Dipyridyl diselenide. To a solution of 2-selenopyridine (1.58g, 10 mmol) in water (100 ml) air was bubbled for 12 hr. 2,2'-dipyridyl diselenide separated and filtered. It was washed with a small amount of water and dried. The product was recrystallized from petroleum ether to give yellow needles; 1.1g (74%), mp. 48°C (lit.²⁵ 47.5-48°C).

Condensation of 5'-O-Tritylthymidine with 3'-O-Acetylthymidine 5'-Phosphate. General Procedure. A mixture of 5'-O-tritylthymidine (TrT) (0.12 mmol) and 3'-O-acetylthymidine 5'-phosphate (pTOAc) (0.1 mmol) was rendered anhydrous by addition of dry pyridine and finally dissolved in 0.5 ml of a solvent (pyridine, DMF, or HMPA). Then, (PySe)₂ (0.5 mmol) and a tervalent phosphorus compound (R₃P) (0.5 mmol) were added under exclusion of moisture. The reaction mixture was kept at room temperature for 24 hr, whereupon, water (1 ml) was added. After continuous stirring for 12 hr, the solution was concentrated to a gum. The residue was treated with 0.5 ml of 80% acetic acid at 100°C for 10 min. It was concentrated under reduced pressure and the residue was treated with 1 ml of 0.5N sodium hydroxide. The alkaline solution was kept at room temperature for 1 hr and neutralized with Dowex 50W-X2 (pyridinium form). After the solution was concentrated in vacuo, the residue was dissolved in water (5 ml) and the insoluble pasty material containing tritylcarbinol, (PySe)₂, and phosphorus compounds such as triphenyl phosphate and triphenylphosphine oxide were removed by extraction with ether. The yield of thymidylyl(3'→5')-thymidine (TpT)⁹ was determined spectrophotometrically after separation of the products either by paper chromatography of aliquots in solvent A or paper electrophoresis. The results are listed in Table 1.

Synthesis of Dinucleoside Phosphates.⁹⁻¹¹ General Procedure. A mixture of 5'-O-monomethoxytritylthymidine (MMTrT) (0.1 mmol) and 3'-O-acyl protected nucleoside 5'-phosphate (0.2 mmol) was

rendered anhydrous by addition of dry pyridine. Finally, the residue was dissolved in the required volume of dry pyridine. Then, $(\text{PhO})_3\text{P}$ (1.0 mmol) and $(\text{PySe})_2$ (1.0 mmol) were added and it was kept at room temperature for 1 day. Work-up of the reaction mixture as described above in the case of TpT, gave dinucleoside phosphate. It was estimated spectrophotometrically after separation of the products by paper chromatography in solvent A. The results are summarized in Table 2.

Synthesis of Trinucleoside Diphosphates.^{10,12,13} General

Procedure. A mixture of monomethoxytritylated dinucleoside phosphate (0.05 mmol) and an appropriate 3'-O-acylnucleoside 5'-phosphate (0.1 mmol) was rendered anhydrous by addition of dry pyridine and finally the residue was dissolved in the required volume of dry pyridine. Then, $(\text{PhO})_3\text{P}$ (0.5 mmol) and $(\text{PySe})_2$ (0.5 mmol) were added under exclusion of moisture. The reaction mixture was kept at room temperature for 2 days. After treatment with water (1 ml) it was further kept at room temperature for 12 hr. The solution was concentrated to dryness under reduced pressure and the residue was treated with methanolic ammonia (1 ml). After removal of the solvent, the residue was dissolved in water (10 ml). The resulted suspension was shaken with ether (20 ml) in order to remove $(\text{PySe})_2$ and triphenyl phosphate. The yields of trinucleoside diphosphates were determined spectrophotometrically after separation of the products either by paper chromatography in solvent A or paper electrophoresis. The results are shown in Table 3.

5'-O-Tritylthymidine 3'-Phosphate (TrTp).^{15,16} A mixture of 5'-O-tritylthymidine (TrT) (2 mmol) and β -cyanoethyl phosphate (4 mmol) was rendered anhydrous by addition of dry pyridine. The residue was finally dissolved in dry pyridine (20 ml) and $(\text{PhO})_3\text{P}$ (20 mmol) and $(\text{PySe})_2$ (20 mmol) were added. It was kept at room temperature for 1 day. After removal of the β -cyanoethyl group by treatment with 2N sodium hydroxide at room temperature for 10 min and the solution was neutralized by addition of Dowex 50W-X2 (pyridinium form). The neutral solution was concentrated to small volume. It was chromatographed on a DEAE cellulose column (carbonate form, 90 x 3 cm). Elution was performed first by using a linear gradient of tri-

ethylammonium bicarbonate solution from 0 to 0.25 M (8 l), then, with triethylammonium bicarbonate solution from 0.25 M to 0.5 M (8 l). TrTp appeared in fractions from 110 to 185. The yield was 1.6 mmol (80% based on TrT), $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ 267 nm (ϵ = 9,600, pH 7). It was homogeneous on paper chromatogram developed by solvent A.

Coupling Reaction of 5'-O-Tritylthymidine 3'-Phosphate with 3'-O-Acetylthymidine.^{16,17} A Typical Procedure.

A mixture of pyridinium salt of 5'-O-tritylthymidine 3'-phosphate (TrTp) (0.05 mmol) and 3'-O-acetylthymidine (TOAc) (0.05-0.2 mmol) (see Table 4) was rendered anhydrous by addition of dry pyridine. A gummy residue was in a solvent (0.5 ml) such as pyridine, $(\text{PhO})_3\text{P}$, or pyridine- $(\text{PhO})_3\text{P}$ (1:1 v/v). To the solution $(\text{PhO})_3\text{P}$ (0.25 mmol) and $(\text{PySe})_2$ (0.25 mmol) were added and it was shaken mechanically at room temperature for 1 day. The mixture was treated with water (1 ml) and further kept at room temperature for 12 hr. The solution was concentrated to dryness and the residue was treated with 1 ml of 80% acetic acid at 100°C for 10 min. Evaporation was repeated several times to remove acetic acid completely by addition of water. Then, the residue was treated with 1 ml of 0.5 N sodium hydroxide at room temperature for 1 hr and the solution was neutralized with Dowex 50W-X2 (pyridinium form). After removal of the resin, the solution was concentrated under reduced pressure. The residue was dissolved in water (5 ml) and insoluble tritylcarbinol, $(\text{PySe})_2$, and triphenyl phosphate were removed by extraction with two portions of ether (2 x 5 ml). Estimation of the yield of thymidylyl(3'→5')thymidine (TpT) most frequently was performed spectrophotometrically after elution of the spot from paper chromatogram run in solvent A. The results are listed in Table 4.

5'-O-Tritylthymidylyl(3'→5')thymidine 3'-Phosphate.^{1,16}

A mixture of 5'-O-tritylthymidylyl(3'→5')thymidine (TrTpT) (1 mmol) and δ -cyanoethyl phosphate (2 mmol) was rendered anhydrous by addition of dry pyridine. The gummy residue was finally dissolved in dry pyridine (10 ml) and $(\text{PhO})_3\text{P}$ (10 mmol) and $(\text{PySe})_2$ (10 mmol) were added. The mixture was kept at room temperature for 1 day. After removal of δ -cyanoethyl group in the usual manner, the solution was concentrated

to small volume. It was chromatographed on DEAE cellulose column (carbonate form, 100 x 2.5 cm). Elution was performed by using a linear gradient with triethylammonium bicarbonate solution from 0.03 M to 0.15 M (4 l). Two ultraviolet absorbing materials were obtained. They were a starting material, TrTpT (fractions from 20 to 32) and the desired product, TrTpTp (fractions from 50 to 89). The later was treated with Dowex 50W-X2 (pyridinium form) and the solution was concentrated in the presence of pyridine. It was homogeneous on paper chromatogram developed by solvent A. The yield of 5'-O-tritylthymidylyl(3'→5')thymidine 3'-phosphate (TrTpTp) was 0.69 mmol (69% based on TrTpT) estimated spectrophotometrically; $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ 267 nm (ϵ =18,500, pH 7).

Coupling Reaction of 5'-O-Tritylthymidylyl(3'→5')thymidine 3'-Phosphate with 3'-O-Acetylthymidine.¹⁶ A mixture of pyridinium salt of 5'-O-tritylthymidylyl(3'→5')thymidine 3'-phosphate (TrTpTp) (0.05 mmol) and 3'-O-acetylthymidine (TOAc) (0.1 mmol) was rendered anhydrous by addition of dry pyridine. Finally, gummy residue was dissolved in dry pyridine (0.5 ml). Then, (PhO)₃P (0.25 mmol) and (PySe)₂ (0.25 mmol) were added under exclusion of moisture. The mixture was kept at room temperature for 2 days. The mixture was treated with water (1 ml) and kept at room temperature for 12 hr. The solution was concentrated to dryness and the residue was treated with 80% acetic acid (1 ml) at 100°C for 10 min. Evaporation of the solvent was repeated several times to remove acetic acid by addition of water. Then, the residue was treated with 0.5 N sodium hydroxide (1 ml) at room temperature for 1 hr and the solution was neutralized with Dowex 50W-X2 (pyridinium form). After removal of resin, the filtrate was concentrated under reduced pressure. The residue was dissolved in water (5 ml) and the insoluble tritylcarbinol, (PySe)₂, and triphenyl phosphate were removed by extraction with two portions of ether (2 x 5 ml). The solution was chromatographed on paper by using solvent A. Two bands containing thymidine (Rf 0.65) and thymidylyl(3'→5')thymidylyl(3'→5')thymidine (TpTpT) (Rf 0.23) were detected. After elution of the later spot by use of water,

TpTpT was obtained in 95% yield based on TrTpT; $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ 267 nm ($\epsilon = 25,400$, pH 7).

Synthesis of Nucleoside 5'-Phosphoramidates.^{5,26,28} General Procedure. To a mixture of a nucleoside 5'-phosphate (0.05 mmol) and an amine (0.5 mmol) in dry pyridine (0.5 ml) were added $(\text{PhO})_3\text{P}$ (0.25 mmol) and $(\text{PySe})_2$ (0.25 mmol). The mixture was allowed to stand at room temperature for 12 hr and concentrated to dryness under reduced pressure. The residue was dissolved in water (5 ml) and the aqueous solution was washed with two portions of ether (2 x 10 ml) to remove the amine, $(\text{PySe})_2$, and triphenyl phosphate. The aqueous solution was concentrated to small volume and applied to paper chromatography. Elution was performed by solvent B. The yields of nucleoside 5'-phosphoramidates were determined spectrophotometrically. The results are listed in Table 5.

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