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Chemical Synthesis of Deoxyribonucleotide with a 5′-Phosphoryl Group on a Polystyrene Polymer Support by the Phosphotriester Method

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Highly lipophilic amines [trityloxyethylamine (TEAm) and *N*-trityloxyethylaniline (TEAn)] were introduced onto a 5′-phosphoryl group and the stability of the phosphoramidate linkage to acid was studied at the diester level. It was found that the phosphoramidate linkage using TEAn was cleaved by 80% aqueous acetic acid within 1 h at room temperature. However, it required 2 h to remove TEAm from the phosphate. By using the TEAn group, 5′-phosphorylated pentadecadeoxyribonucleotide pTCCAGGGTCTGGTAC was synthesized on a polystyrene support by the phosphotriester method. After partial deblocking, the pentadecamer with the TEAn group could be easily isolated on a reversed-phase column due to the high lipophilicity of the TEAn group.

The chemically synthesized 5′-phosphorylated pentadecamer was successfully joined by using deoxyribonucleic acid ligase in the presence of a template.

Keywords——oligodeoxyribonucleotide synthesis; phosphotriester method; 5′-phosphoryl group; polymer support; DNA ligase

Oligodeoxyribonucleotides, which can be synthesized rapidly on a polymer support by means of either the phosphotriester or phosphite-triester approach, are indispensable tools for biological studies. Since chemically synthesized oligonucleotide usually has a hydroxyl group at the 5′- end, it must be phosphorylated with T_4-polynucleotide kinase and adenosine triphosphate (ATP). Though several synthetic methods for 5′-phosphorylated oligonucleotide has been reported by other workers,[1] the methods usually involve a solution phase synthetic technique. In order to prepare 5′-phosphoryl oligonucleotide easily and rapidly, polymer-supported oligonucleotide synthesis seems preferable. Though some work on solid-phase synthesis was reported,[2] the need for rapid purification was not considered. By using a lipophilic trityl group for the protection of the 5′-hydroxyl group, the 5′-protected oligonucleotide could be purified easily on a reversed-phase column.[3] In this paper, we wish to describe lipophilic protecting groups for the protection of the 5′-phosphate of nucleosides and the synthesis of 5′-phosphoryl oligodeoxyribonucleotides on a polystyrene support by the phosphotriester approach.

A 5′-phosphorylated oligodeoxyribonucleotide can be used directly for the gene construction[4] of a protein or the synthesis of mutated forms[5] of the deoxyribonucleic acid (DNA). It can be attached to biotin[6] or ethylenediaminetetracetic acid (EDTA)[7] through an aminoethylene linker for use as a hybridization probe or in DNA strand cleavage studies. It should be also possible to prepare 5′-phosphoryl oligonucleotides on a large scale for conformational studies.[8]

Results and Discussion

For the preparation of trityloxyethylamine (TEAm) (**5**),[9] *N*-2-hydroxyethylphthalimide

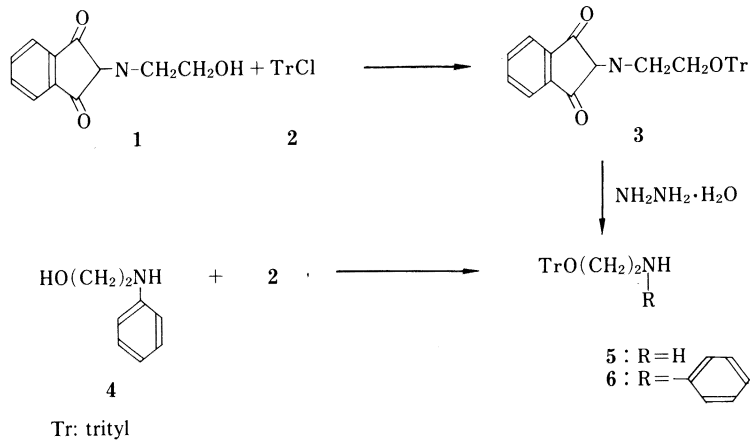
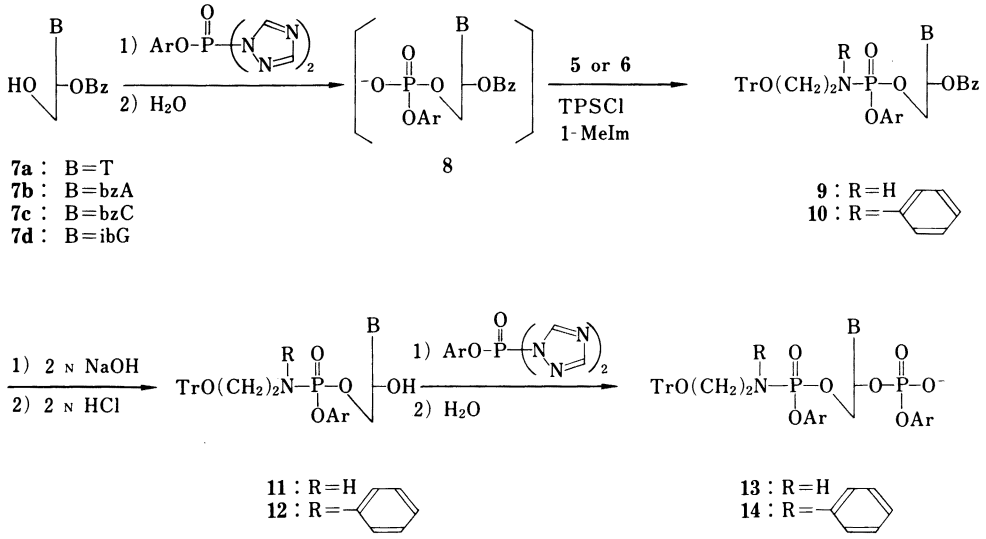


Chart 1

(**1**) was first treated with 1.3 eq of trityl chloride (TrCl) in CH_2Cl_2 and diisopropylethylamine was added as a catalyst. Next, 33 eq of hydrazine hydrate was added. After the reaction, the product was separated on a silica gel column, to give **5** in 82% yield. To prepare *N*-trityloxyethylaniline (TEAn) (**6**),[10] *N*-hydroxylethylaniline (**4**) was reacted with 1.6 eq of TrCl in CH_2Cl_2 and diisopropylethylamine was again used as the catalyst. Following the usual reaction, **6** was obtained in 94% yield after separation on a silica gel column.

The preparation of nucleotide-5′-phosphoramidate derivatives by using **5** and **6** was done as follows. The 3′-*O*-benzoylthymidine-5′-*o*-chlorophenylphosphate (**8a**) (which was prepared from 3′-*O*-benzoylthymidine by the phosphorylation with *o*-chlorophenylphosphoroditriazolide followed by hydrolysis with H_2O), was first activated with 1,3,5-triisopropylbenzenesulfonyl chloride (TPSCl) and 1-methylimidazole (1-MeIm)[11] for 10 min at room temperature. Then 2 eq of lipophilic amine **5** or **6** was added to the mixture. The condensation reaction was very rapid (within 10 min) at room temperature. After separation on a silica gel column, the phosphoramidate derivative (**9a**) or (**10a**) was obtained as a solid in a yield of 58% or 45%, respectively, after precipitation into *n*-hexane from a solution in CH_2Cl_2. To find suitable deblocking conditions, **9a** or **10a** was treated with conc. NH_4OH at 50 °C for 5 h. It was found that the 3′-benzoyl and *o*-chlorophenyl protecting groups were removed from **9a**. In the case of **10a** only the benzoyl group was removed and the *o*-chlorophenyl group was completely stable. Though the *o*-chlorophenyl group was stable on treatment with 0.5 M N^1,N^1,N^3,N^3-tetramethylammonium *syn*-pyridine-2-aldoximate (TMG-PAO) in dioxane–pyridine–H_2O (4 : 2 : 1, v/v) at room temperature overnight, it was removed by the treatment with 0.5 M TMG-PAO at 55 °C overnight. This treatment did not cleave internucleotidic linkages.[12] After purification on a reversed-phase column, phosphoramidate derivatives which had only the TEAm or TEAn protecting group were treated with 80% aqueous acetic acid. It was observed that the TEAn group was smoothly removed with 80% acetic acid at room temperature within 1 h to give pT. However, the TEAm group was only partially removed by the same treatment. It required more than 2 h for complete deprotection.

Compound **9a** or **10a** was then treated with 2 N NaOH for 10 min at 0 °C for selective removal of the 3′-benzoyl group. After this treatment **11a** or **12a** was obtained in a yield of 75% or 72%, respectively, after isolation on a silica gel column. The free 3′-hydroxyl group of **11a** or **12a** was then phosphorylated with *o*-chlorophenylphosphoroditriazolide followed by hydrolysis with H_2O in quantitative yield. By the same procedure, phosphoroamidate derivatives of other nucleosides **13b—d** and **14b—d** were prepared from **7b—d**.



Ar: *o*-chlorophenyl TPSCl: 1,3,5-triisopropylbenzenesulfonyl chloride
1-MeIm: 1-methylimidazole Bz: benzoyl

Chart 2

By using **13a** and **14a**, a 5′-*O*-phosphorylated dimer, pTpC, was prepared: 5 eq of **13a** or **14a** was condensed with the 5′-hydroxyl group of *N*-4-benzoyldeoxycytidine bound to a polystyrene support with 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT) at room temperature for 40 min. After treatment with 0.5 M TMG-PAO at either room temperature or 55 °C overnight followed by conc. NH_4OH at 55 °C for 6 h, the dimer which still retained the TEAm or TEAn group was separated on a reversed phase (C_{18}) column with a linear gradient of CH_3CN (10%→50%) in 50 mM triethylammonium acetate (TEAA) (pH 7). The product was eluted with 30% CH_3CN as a single peak, then treated with 80% acetic acid at room temperature for 2 h or 1 h, respectively. This reaction gave unprotected dimer pTpC which was hydrolyzed to give pT and pC in equal amounts by venom phosphodiesterase. However, as it is known that deoxyadenosine is sensitive to acid treatment, rather drastic acid treatment should be avoided. In fact, when (TEAm) pTpA, prepared by the same procedure, was treated with 80% aqueous acetic acid for 3 h, some depurination (7%) was observed. It seems that the TEAn group is superior for the synthesis of 5′-phosphorylated oligonucleotide. However, the TEAm group can be used for the synthesis of nucleoside 5′-di or triphosphate.[5]

The usefulness of this method was demonstrated by applying it to the synthesis of a longer chain oligonucleotide, the pentadecamer pTCCAGGGTCTGGTAC, using **14a**. Starting with 3 μmol of *N*-benzoyldeoxycytidine bound to polystyrene, six protected dimers TA, GG, CT, GT, GG, CA, one monomer C[3,13] and **14a** were condensed successively with MSNT according to the reaction cycle shown in Table I. After the reaction, the resin was treated successively with 0.5 M TMG-PAO at 60 °C overnight and conc. NH_4OH at 60 °C overnight to remove the base and phosphate protecting groups and to release the nucleotidic compounds from the resin. The pentadecamer which still possessed a lipophilic TEAm group was separated on a reversed-phase (C_{18}) silica gel column with a linear gradient of CH_3CN in 50 mM TEAA, pH 7. As shown in Fig. 1a, it was eluted slowly (with 25% CH_3CN) and could be separated easily from truncated short oligonucleotides and the other reagents, which were eluted at the front. To remove the triethylammonium acetate salt used as a buffer in the column eluant from the pentadecamer, the residue, after evaporation of the volatile organic

TABLE I. One Cycle of Operation

Operation	Solution	Volume
1) Wash	CH_2Cl_2	1 ml (3 times)
2) Detritylation	3% Cl_3CCOOH in CH_2Cl_2	1 ml for 30 s (4 times)
3) Wash	CH_2Cl_2–pyridine (9 : 1, v/v)	1 ml (3 times)
4) Wash	Pyridine	1 ml (3 times)
5) Coevaporation	Nucleotide + pyridine	0.2 ml
6) Condensation	MSNT + pyridine	0.4 ml (40 min)
7) Wash	Pyridine	1 ml
8) Capping	1 M Ac_2O, 50 mM DMAP in pyridine	1 ml (1 min)
9) Wash	Pyridine	1 ml

MSNT = 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole. DMAP = 4-dimethylaminopyridine.

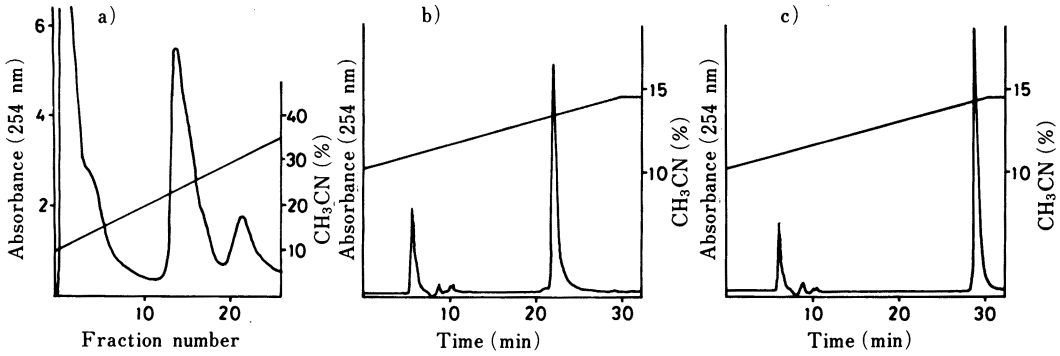


Fig. 1a. Isolation of d-(TEAn)-pTCCAGGGTCTGGTAC on a Reversed-Phase (C-18) Silica Gel Column (0.7 × 8 cm) Using a Linear Gradient of Acetonitrile (10—35%) in 50 mM Triethylammonium Acetate (Total, 100 ml)

Fig. 1b, c. Reversed-Phase HPLC Analysis of b) d-pTCCAGGGTCTGGTAC and c) d-TCCAGGGTCTGGTAC Using a Linear Gradient of Acetonitrile in 0.1 M Triethylammonium Acetate at a Flow Rate of 0.7 ml/min

solvent, was applied to a Sep-Pak C_{18}. After washing of the column with H_2O to remove the salt, the product was eluted with 80% acetic acid and the TEAn group was removed by keeping the eluate at room temperature for 1 h. Completely unprotected pentadecamer was purified by reversed-phase high pressure liquid chromatography) (HPLC) (Fig. 1b). To compare the retention times of the pentadecamer with and without phosphate at the 5′-end, the pentadecamer with the same sequence but no phosphate at the 5′-end was prepared by the same procedure. It is apparent that the 5′-phosphorylated pentadecamer was eluted faster than the non-phosphorylated one (Fig. 1b, c). The existence of the 5′-phosphate on the pentadecamer was analyzed as follows. At first, the purified pentadecamer was found to be unphosphorylated by [γ-^{32}P]ATP and polynucleotide kinase (Fig. 2, lane 1). However, after treatment with bacterial alkaline phosphatase to remove the 5′-phosphate, it could be phosphorylated as in the case of the non-phosphorylated pentadecamer (Fig. 2, lane 2, 3). The labeled pentadecamer was next subjected to mobility shift analysis to confirm its nucleotide sequence.[14]

In order to determine whether synthetic 5′-phosphorylated pentadecamer could be a substrate for the T_4-DNA ligase reaction, [γ-^{32}P]CCTCCTACCGTTGAAC (Fig. 3, I) and CAGACCCTGGAGTTCAACGG (Fig. 3, III) were prepared as an acceptor and the template, respectively. They were mixed with pTCCAGGGTCTGGTAC, annealed and then

Fig. 2. Analysis by Homochromatography

Lane 1, 5′-*O*-phosphorylated pentadecamer was labeled with [γ-^{32}P]ATP and polynucleotide kinase; lane 2, 5′-*O*-phosphorylated pentadecamer was treated with bacterial alkaline phosphatase then labeled; lane 3, non-phosphorylated pentadecamer was labeled.

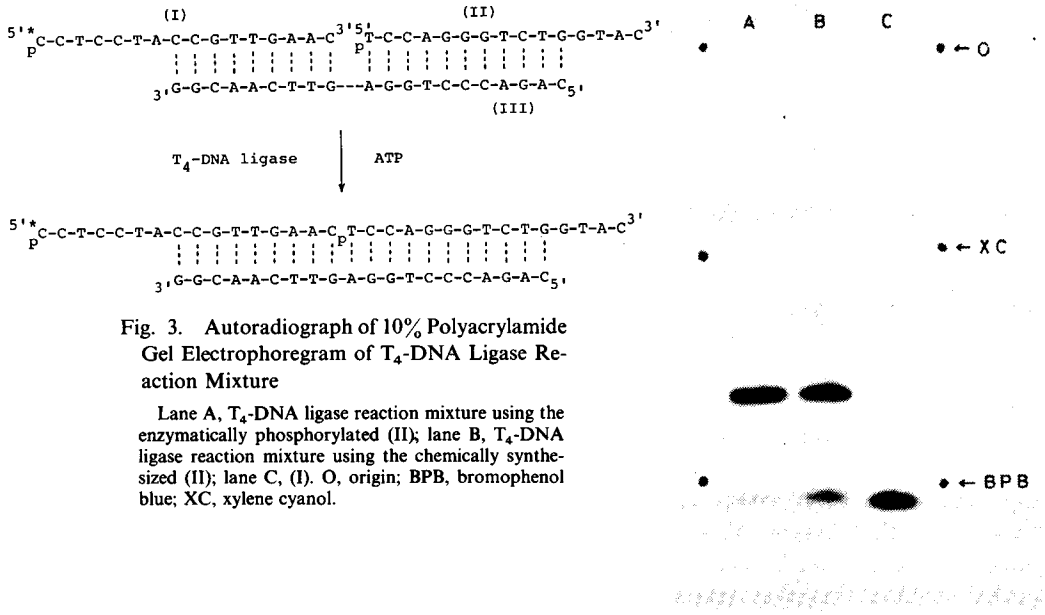


Fig. 3. Autoradiograph of 10% Polyacrylamide Gel Electrophoregram of T_4-DNA Ligase Reaction Mixture

Lane A, T_4-DNA ligase reaction mixture using the enzymatically phosphorylated (II); lane B, T_4-DNA ligase reaction mixture using the chemically synthesized (II); lane C, (I). O, origin; BPB, bromophenol blue; XC, xylene cyanol.

treated with DNA ligase at 20 °C for 9 h. To compare the regular DNA-ligase reaction, pentadecamer with the same sequence but no phosphate at the 5′-end was phosphorylated with ATP and polynucleotide kinase. It was also annealed with the acceptor and template,

and then treated with DNA ligase. The mixture was analyzed by 10% polyacrylamide gel electrophoresis. As shown in Fig. 3, very little difference was observed in the two autoradiograms (lanes A and B). A new product which migrated in the position of 30-mer was seen in both cases.

In this work, we prepared two lipophilic amines, TEAn and TEAm, for the protection of the phosphoryl group. Though both groups were successfully introduced onto the 5′-phosphoryl group of nucleoside by TPSCl and 1-MeIm, it was found that the TEAn group was more suitable for this purpose owing to its instability to acid and higher lipophilicity. We also achieved the synthesis and purification of a 5′-phosphorylated pentadecamer by the phosphotriester method on a polystyrene support.

Materials and Methods

Thin-layer chromatography (TLC) was performed on plates of Kieselgel $60F_{254}$ (Merck). Column chromatography was performed on Kieselgel 60 (Merck). Reversed-phase chromatography was performed on alkylated silica gel (C_{18}, 55—105 μ, Waters). Sep-Pak C-18 was purchased from Waters. HPLC was performed on an Altex 322MP chromatography system. Reversed-phase HPLC was carried out on Nucleosil C-18 (5 μ) packed into a stainless steel column (i.d. 0.6 × 20 cm) under a pressure of 500 kg/cm^2.
500 kg/cm^2.

Venom phosphodiesterase was purchased from Boehringer Mannheim. T_4-polynucleotide kinase and T_4-DNA ligase were purchased from Takara Shuzo Co. Ltd.

Synthesis of Trityloxyethylamine (TEAm) (5)——Trityl chloride (1.8 g, 6.5 mmol) was added to a solution of *N*-hydroxyethylphthalimide (955 mg, 5 mmol) in dry CH_2Cl_2 (20 ml) and diisopropylethylamine (1.4 ml, 8 mmol). After 2 h, the completion of the reaction being confirmed by TLC (CH_2Cl_2 : MeOH = 10 : 1, *Rf* 0.35→0.81), hydrazine hydrate (8 ml, 165 mmol) was added to the mixture. Then methanol was added until the mixture became a homogeneous solution. After 1 h, TLC showed that the reaction was completed (CH_2Cl_2 : MeOH = 10 : 1, *Rf* 0.81→0.24). The mixture was diluted with CH_2Cl_2 (30 ml), washed with 0.1 M triethylammonium bicarbonate (TEAB) (pH 7.5) (30 ml × 4) and evaporated. The residue was applied to a silica gel column (i.d. 3.5 × 7 cm) and eluted with an increasing MeOH concentration in CH_2Cl_2. The fractions containing **5** were pooled and evaporated. The residue was dried *in vacuo* to give a white precipitate. Yield, 1.25 g (4.12 mmol, 82%).

Synthesis of *N*-Trityloxyethylaniline (TEAn) (6)——Trityl chloride (2.2 g, 8 mmol) was added to a solution of β-anilinoethanol (0.63 ml, 5 mmol) in dry CH_2Cl_2 (20 ml) and diisopropylethylamine (1.7 ml, 10 mmol). The mixture was stirred for 2 h at room temperature, and the completion of the reaction was confirmed by TLC (CH_2Cl_2, *Rf* 0→0.81). The mixture was diluted with CH_2Cl_2 (20 ml), washed with 0.1 M TEAB (20 ml × 3) and evaporated. The residue was separated on a silica gel column (i.d. 3.5 × 4 cm). The fractions containing **6** were pooled and evaporated. Yield, 1.78 g (4.7 mmol, 94%).

Synthesis of 9 and 10——*o*-Chlorophenylphosphorodichloridate (0.11 ml, 0.65 mmol) was added dropwise to a solution 1,2,4-triazole (114 mg, 1.65 mmol) in dry CH_2Cl_2 (5 ml) and pyridine (0.5 ml) at 0 °C, and the mixture was stirred for 15 min, 3′-*O*-benzoylthymidine (**7a**) which had previously been co-evaporated with pyridine and then dissolved in CH_2Cl_2–pyridine (5 ml–0.5 ml) was added. After 15 min, a 0.1 M TEAB solution was added to the mixture and the phosphorylated product (**8**) was extracted with CH_2Cl_2 (20 ml). The organic layer was washed with 0.1 M TEAB (5 ml × 3) and evaporated. The residue was co-evaporated with pyridine to remove a trace of water and treated with TPSCl (606 mg, 2 mmol) and 1-methylimidazole (0.28 ml, 3.5 mmol) in CH_2Cl_2 (5 ml) for 10 min. Then TEAm (303 mg, 1 mmol), previously co-evaporated with pyridine and dissolved in CH_2Cl_2 (5 ml), was added. The mixture was stirred for another 30 min. After confirmation of completion of the reaction by TLC (CH_2Cl_2 : MeOH = 10 : 1, *Rf* 0→0.6), the mixture was dropped into *n*-hexane (100 ml) with stirring and kept in a freezer overnight. The supernatant was removed. The oily residue was applied to a silica gel column (i.d. 3 × 4.5 cm) and eluted with an increasing MeOH concentration in CH_2Cl_2. The fractions containing **9** were pooled and evaporated. The residue was taken up in CH_2Cl_2 (3 ml) and dropped into *n*-hexane (50 ml) with stirring. The precipitate was collected and dried *in vacuo*. Yield, 235 mg (0.29 mmol, 58%).

By the same procedure, **9b—d** were obtained in yields of 70%, 83% and 61%, respectively. For the synthesis of compound **10a—d**, TEAn instead of TEAm was used for the condensation reaction. The yields of **10a—d** were 45%, 75%, 45% and 47%, respectively.

Synthesis of 11a, b and 12a——Compound **9** (B = T, 164 mg, 0.2 mmol) was dissolved in MeOH (6 ml). Then 2 N NaOH solution (1 ml) was added with stirring at 0 °C. After 10 min, the completion of the reaction was confirmed by TLC (CH_2Cl_2 : MeOH = 10 : 1, *Rf* 0.65→0.52). The mixture was neutralized by adding 2 N HCl solution, then diluted with CH_2Cl_2 (10 ml), and the organic layer was washed with 0.1 M TEAB (10 ml × 3). The organic layer was evaporated, and the residue was applied to a silica gel column (i.d. 3 × 4 cm). Elution was carried out with an

increasing MeOH concentration in CH_2Cl_2. The fractions containing **11** were pooled and evaporated. The residue was taken up in CH_2Cl_2 (2 ml) and dropped into *n*-hexane (40 ml) with stirring. The precipitate was collected and dried *in vacuo*. Yield, 108 mg (0.15 mmol, 75%). By the same procedure, **11b** and **12a** were obtained in 70% and 88% yields, respectively, from **9b** or **10a**.

Synthesis of 13a, b and 14a——*o*-Chlorophenylphosphorodichloridate (0.05 ml, 0.30 mmol) was added dropwise to a solution of 1, 2, 4-triazole (23 mg, 0.33 mmol) in a mixture of CH_2Cl_2 (5 ml) and pyridine (0.5 ml) with stirring at 0 °C. After 15 min, **11a** (72 mg, 0.1 mmol), which had previously been co-evaporated with pyridine and then dissolved in CH_2Cl_2 (5 ml), was added. After another 20 min, 0.1 M TEAB (10 ml) was added and the product was extracted with CH_2Cl_2 (10 ml). The organic layer was washed with 0.1 M TEAB (10 ml × 4) and evaporated. The residue was co-evaporated with pyridine, and dissolved in CH_2Cl_2 (1 ml), and this solution was dropped into *n*-hexane (50 ml) with stirring. The precipitate was collected and dried *in vacuo*. Yield, 87 mg, 86%. By the same procedure, **13b** and **14a** were obtained in 92% yield in each case.

Synthesis of Dinucleotide (pTpC)——Compound **13a** (15 mg, 15 μmol) or **14a** (16 mg, 15 μmol) was mixed with *N*-4-benzoyldeoxycytidine bound to a polystyrene resin (17 mg, 3 μmol) and co-evaporated with pyridine (0.5 ml). The mixture was treated with MSNT (20 mg, 68 μmol) in pyridine (0.4 ml) at room temperature. After 40 min, the resin was washed successively with pyridine (2 ml × 2), CH_2Cl_2 (2 ml × 3) and ether (2 ml × 2), and dried. The resin was treated with 0.5 M TMG-PAO in dioxane–pyridine–water (5 : 4 : 1 v/v, 1 ml) at room temperature for 12 h (when **13a** was used) or at 60 °C for 12 h (when **14a** was used). After evaporation of the volatile materials, the product was treated with a mixture of conc. NH_4OH (2 ml) and pyridine (0.5 ml) at 60 °C for 5 h. The mixture was centrifuged and the resin was washed with 50% aqueous pyridine (1 ml). The combined solution was washed with ethyl acetate (6 ml × 3) and evaporated. The residue was dissolved in 0.1 M TEAA (pH 7, 0.2 ml) and applied to a reversed-phase (C_{18}) silica gel column (0.7 × 5 cm). Elution was carried out with a linear gradient formed from 50 ml of 10% acetonitrile in 0.1 M TEAA (mixing vessel) and 50 ml of 50% acetonitrile in 0.1 M TEAA (reservoir). The fractions containing the desired product (31 A_{267}) were concentrated to a small volume. The residue was dissolved in H_2O (2 ml) and applied to a Sep-Pak C-18 column previously washed successively with H_2O (10 ml), 60% aqueous acetonitrile (10 ml) and H_2O (20 ml). The column was washed with H_2O (20 ml), then the product was eluted with 80% aqueous acetic acid (6 ml), and the eluate was kept at room temperature for 1 h. The mixture was diluted with H_2O (3 ml), washed with ether (6 ml × 3) and evaporated. An aliquot of the sample (2.5 A_{267} units), after isolation by the paper electrophoresis, was treated with a bacterial alkaline phosphatase (69 units/ml) (3 μl) in 5 mM TEAB (100 μl) at 37 °C for 4 h. The mixture was separated by paper electrophoresis after heating at 100 °C for 5 min in the presence of 40 mM nitrilotriacetic acid (NTA) (13 μl). The de-phosphorylated dimer (TpC) was then treated with snake venom phosphorodiesterase [(1 mg/ml) (3 μl)] in 0.2 M TEAB at 37 °C for 4 h. The mixture was analyzed by paper electrophoresis to separate T (0.96 A_{267} at pH 7.5) and pC (1.30 A_{280} at pH 2). The ratio found was 1.03 : 1.

Synthesis of Oligonucleotides——The target 5′-*O*-phosphorylated oligonucleotide was synthesized according to the reaction cycle shown in Table I. Starting from *N*-4-benzoyldeoxycytidine bound to a polystyrene support (17 mg, 3 μmol), the protected dimer (20 mg, 15 μmol) was condensed with MSNT (20 mg, 67 μmol) in pyridine (400 μl). At the last coupling, **14a** (16 mg, 15 μmol) was used for condensation. After the reaction, the procedures used for deprotection and isolation were similar to those described for the synthesis of the dimer. The loaded polystyrene support was treated with 0.5 M TMG-PAO (1 ml) at 60 °C overnight and with conc. NH_4OH (2.5 ml). Then the mixture was separated on a reversed-phase (C_{18}) silica gel column to obtain the pentadecamer with a TEAn group at the 5′-end (65 A_{267}) (Fig. 1a). After the removal of the salt and treatment with 80% AcOH, the unprotected pentadecamer was further purified by reversed-phase HPLC (Fig. 1b). Other oligonucleotides with hydroxyl group at the 5′-end (CCTCCTACCGTTGAAC, TCCAGGGTCTGGTAC, CAGACCCTGGAGTTCAACGG) were prepared by the reported procedure.[3)]

Kinase Reaction of Oligonucleotide——The synthetic 5′-*O*-phosphorylpentadecamer pTCCAGGGTCTGGTAC (0.02 A_{260} unit, 150 pmol), the de-phosphorylated pentadecamer (0.02 A_{260} unit, 150 μmol) obtained by treatment with bacterial alkaline phosphatase (0.04 unit) in 0.1 M TEAB (5 μl) at 55 °C for 1.5 h, followed by heating at 100 °C for 5 min in the presence of NTA, and the pentadecamer with the same sequence but no phosphoryl group at the 5′-end (0.02 A_{260} unit, 150 pmol) were treated with [γ-^{32}P]ATP (4000 cpm/pmol, 83 μM, 0.5 μl) and T_4-polynucleotide kinase (1 unit/μl, 1 μl) in 10 μl of 50 mM Tris–HCl (pH 9.6), 1 mM $MgCl_2$, 2 mM spermine, 10 mM dithiothreitol and 0.1 M KCl at 37 °C for 1 h. An aliquot (about one-tenth) was analyzed by homochromatography in Homomix I.[9a)]

DNA Ligase Reaction——By means of the procedure mentioned above, the hexadecamer CCTCCTACCG-TTGAAC was labeled with [γ-^{32}P]ATP and the pentadecamer TCCAGGGTCTGGTAC was phosphorylated at the 5′-end with cold ATP by using T_4-polynucleotide kinase. The labeled hexadecamer (0.05 A_{260} unit, 0.33 nmol), d-CAGACCCTGGAGTTCAACGG (0.05 A_{260} unit, 0.33 nmol) as a template were mixed with either the synthetic 5′-*O*-phosphorylated pentadecamer (0.05 A_{260} unit, 0.33 nmol) or enzymatically phosphorylated pentadecamer (0.05 A_{260} unit, 0.33 nmol) in 27 μl of 66 mM Tris–HCl pH 7.6, 6.6 mM $MgCl_2$, and 500 μM ATP. The mixture was heated to 75 °C for 10 min and then cooled slowly to 20 °C over a period of 1 h. Then 1.5 μl of 0.2 M β-mercaptoethanol and 1.5 μl of T_4-DNA ligase (350 units/μl, 1.5 μl) were added to the mixture. This mixture was then incubated at 20 °C for 9 h. An aliquot (5 μl) of the mixture was dissolved in 8 M urea, 10 mM EDTA and 40 mM Tris–borate

(pH 8.4) containing 0.04% (w/v) marker dye (3 μl) and loaded onto a 10% polyacrylamide gel for electrophoresis at 400 V. The result is shown in Fig. 3.

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References and Notes

1) *a*) E. Ohtsuka, T. Tanaka and M. Ikehara, *Nucleic Acids Res.*, **7**, 1283 (1979); *b*) G. van der Marel, G. Veenemar and J. H. van Boom, *Tetrahedron Lett.*, **22**, 1463 (1981); *c*) G. van der Marel, C. A. A. van Boechel, G. Wille and J. H. van Boom, *Nucleic Acids Res.*, **10**, 2337 (1982).
2) *a*) E. Uhlman and J. Engels, *Tetrahedron Lett.*, **27**, 1023 (1986); *b*) T. Horn and M. S. Urdea, *ibid.*, **27**, 4705 (1986).
3) T. Tanaka and T. Oishi, *Chem. Pharm. Bull.*, **33**, 5178 (1985).
4) M. Ikehara, E. Ohtsuka, T. Tokunaga, S. Nishikawa, S. Uesugi, T. Tanaka, Y. Aoyama, S. Kikyodani, K. Fujimoto, K. Yanase, K. Fuchimura and H. Morioka, *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 4695 (1986).
5) M. J. Zoller and M. Smith, *Methods Enzymol.*, **100**, 468 (1983).
6) A. Chollet and E. H. Kawashima, *Nucleic Acids Res.*, **13**, 1529 (1985).
7) B. C. F. Chu and L. E. Orgel, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 963 (1985).
8) K. Majumder, S. K. Brahmachari and V. Sasisekharan, *FEBS Lett.*, **198**, 240 (1986).
9) T. Tanaka, Y. Yamada and M. Ikehara, *Tetrahedron Lett.*, **27**, 3269 (1986).
10) T. Tanaka, Y. Yamada and M. Ikehara, *Tetrahedron Lett.*, **27**, 5641 (1986).
11) V. A. Efimov, S. V. Reverdatto and O. G. Chakhmakhcheva, *Tetrahedron Lett.*, **23**, 961 (1982).
12) T. P. Patel, M. A. Chauncey, T. A. Millican, C. C. Bose and M. A. W. Zaton, *Nucleic Acids Res.*, **12**, 6853 (1984).
13) 5′-Hydroxyl, heterocyclic amino and phosphate groups were protected by dimethoxytrityl, benzoyl or isobutyryl and *o*-chlorophenyl groups, respectively.
14) *a*) E. Jay, R. Padmanabhan and R. Wu, *Nucleic Acids Res.*, **1**, 331 (1974); *b*) E. Jay, C. P. Bahl and R. Wu, *Anal. Biochem.*, **74**, 73 (1976).