A Simple Protecting Group Protection-Purification "Handle" for Polynucleotide Synthesis. II.

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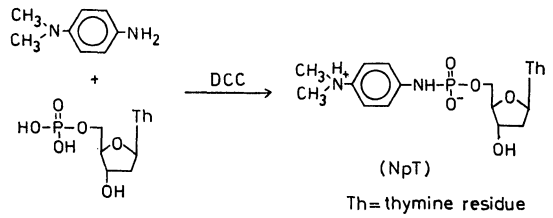
A new method for the synthesis of oligonucleotides by employing a protecting group protection-purification "handle" was investigated. It was found that 3′-*O*-acetylthymidylyl(5′→3′)thymidine 5′-phosphor[*p*-*N*,*N*-dimethylamino]anilidate (NpTpTOAc) was prepared in 65% yield from thymidine 5′-phosphor[*p*-*N*,*N*-dimethylamino]anilidate (NpT) and 3′-*O*-acetylthymidine 5′-phosphate (pTOAc) in the presence of triisopropylbenzenesulfonyl chloride (TPS). The NpTpTOAc was selectively isolated by the use of Dowex 50W-X2 and Amberlite IR-45 resins. After treatment of NpTpTOAc with 80% acetic acid, pTpTOAc was obtained in 60% yield based on pTOAc. Similarly, trinucleotide derivative, pTpTpTOAc, was isolated in 31% yield based on pTpTOAc.

Application of the solid phase method to oligonucleotide synthesis has recently been studied.[1] However, the desired sequence of oligonucleotides obtained is accompanied with several truncated and failure sequences as the purity of the product depends on the yield of coupling reactions on polymer supports, and only 100% yield in every coupling can prevent the formation of truncated and failure sequences.[2] Separation of oligonucleotides prepared by the present method seems to be more difficult than the usual method because the contents of truncated and failure sequences are uniformly much more than those of a true sequence. Especially, higher yields of coupling reactions except in the case of 100% yield would cause much difficulty. In order to remove the restriction of solid phase synthesis, a new synthetic method of oligonucleotides, in which true sequence of oligonucleotide may be selectively prepared without using column chromatography, has been established.[3]

The results of experiments for the preparation of oligothymidylates designed by a new method are described in this paper. The coupling reactions of nucleotide were carried out in solution in the absence of polymer. In the next stage, the desired oligonucleotide was seaprated from the other by-products using an ion exchange resin utilizing salt formation or molecular adsorption between the protecting group and the ion exchange resin by taking advantage of the separation on solid phase.

The protecting group (N) on nucleoside 5′-phosphate end is of importance, since during the course of the experiment the group acts as a protecting group of phosphate in the coupling reactions and also functions as a "handle" for separation. A basic *N*,*N*-dimethyl-*p*-phenylenediamino group was chosen as the protecting group on the nucleoside 5′-phosphate end. This was introduced to form a phosphoroanilidate linkage which could be cleaved easily by treatment with 80% acetic acid at 80°C for 3 hr.

Thymidine 5′-phosphoroanilidate (NpT) was prepared in 90% yield when thymidine 5′-phosphate was treated with *N*,*N*-dimethyl-*p*-phenylenediamine in the presence of dicyclohexylcarbodiimide (DCC) according to a modification of the procedure of Moffatt.[4] The NpT was purified and isolated from the reaction mixture by treatment with Dowex 50W-X2 (H^+ form) since the resin adsorbs NpT by a salt formation between sulfonic acid group of Dowex 50W-X2 and *N*,*N*-dimethyl anilino group of NpT, which excluded the formation of the inner salt of NpT.



NpT is stable pink oil and can be stored in dry pyridine at 0°C for more than one month. The possible self-condensation reaction[5] into the corresponding pyrophosphate, P^1,P^2-dithymidine 5′-pyrophosphate, was not observed even when it was treated with Dowex 50W-X2 (H^+ form).

When a mixture of 1.3 equivalent of NpT and 1 equivalent of 3′-*O*-acetylthymidine 5′-phosphate (pTOAc) was treated with 3 equivalent of triisopropylbenzenesulfonyl chloride (TPS) in dry pyridine at room temperature for 13 hr, the corresponding dinucleotide derivative, NpTpTOAc, was formed in 65% yield along with P^1,P^2-di-3′-*O*-acetylthymidine 5′-pyrophosphate $(pTOAc)_2$, the starting materials pTOAc and NpT being recovered. These compounds were detected by paper chromatography and the yield was determined spectrophotometrically.

After passing the mixture through a column of Dowex 50W-X2 (H^+ form), NpT was selectively adsorbed on the resin as described in the preparation

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2) E. Bayer, H. Eckstein, K. Hägele, W. A. König, W. Brüning, H. Hagenmaier, and W. Parr, *J. Amer. Chem. Soc.*, **92**, 1735(1970).

3) T. Hata, K. Tajima, and T. Mukaiyama, *ibid.*, **93**, 4928 (1971).

4) J. G. Moffatt and H. G. Khorana, *ibid.*, **83**, 649 (1961).

5) V. M. Clark, G. W. Kirby, and A. R. Todd, *J. Chem. Soc.*, **1957**, 1497.

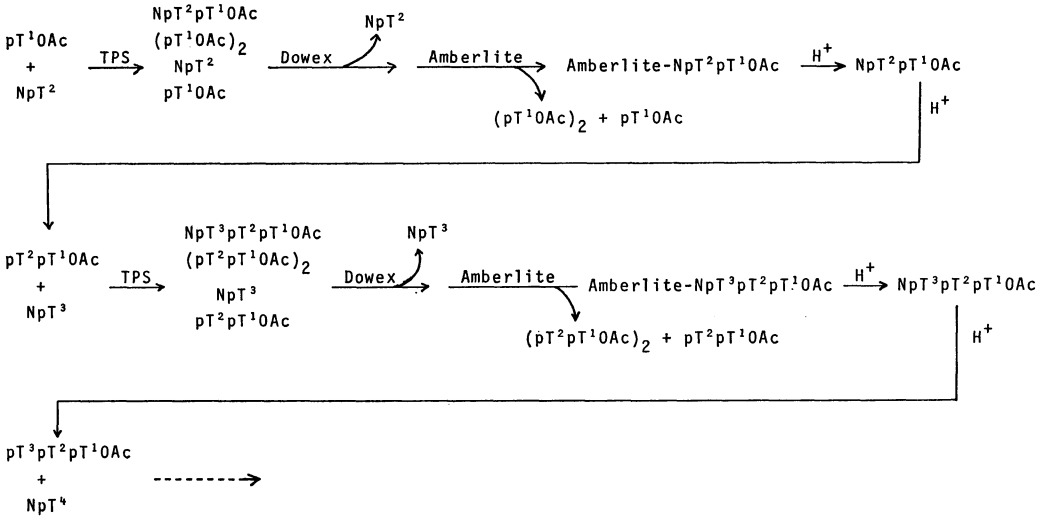
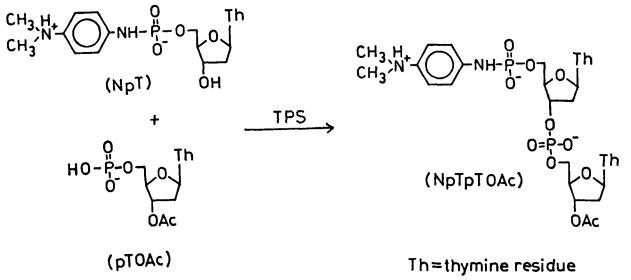


Chart 1. General synthetic scheme.



of NpT. The eluate containing NpTpTOAc, $(pTOAc)_2$ and pTOAc was neutralized with 0.1N sodium hydroxide. When the solution was passed through a column of Amberlite IR-45 (free base), NpTpTOAc was selectively adsorbed on the resin excluding adsorption of the other compounds, pTOAc and $(pTOAc)_2$. After washing the resin with neutral water, the resin was treated with 80% acetic acid and the eluate containing NpTpTOAc was heated at 80°C for 3 hr. The solution was concentrated under reduced pressure for removal of acetic acid, and the residue was dissolved in water. *N,N*-Dimethyl-*p*-phenylenediamine was removed by treatment of the aqueous solution with Dowex 50W-X2 (H^+ form). The eluate was lyophilized to remove a trace of moisture and stored in dry pyridine. pTpTOAc was isolated in 60% yield based on pTOAc.[6]

It is most probable that Amberlite IR-45 having anilino group includes *N,N*-dimethyl-*p*-phenylenediamino group of NpTpTOAc by a force of molecular adsorption. This was supported by the adsorption of the base *N,N*-dimethyl-*p*-phenylenediamine on the IR-45 resin. Further, when a mixture of 1 equivalent of pTpTOAc and 3 equivalent of NpT was treated with 3 equivalent of TPS in dry pyridine at room temperature for 14 hr, the corresponding trinucleotide derivative, NpTpTpTOAc, was obtained in 34% yield along with NpT and $(pTpTOAc)_2$.[7] pTpTpTOAc was isolated by means of a similar procedure (see Experimental) to that described for pTpTOAc.

A general synthetic scheme developed for oligothymidylate derivatives is outlined in Chart 1.

In conclusion, it should be noted that the use of the basic protecting group in oligonucleotide synthesis resulted in the selective preparation of oligonucleotides. The chain of oligomer propagates from the head, whereas in the solid phase method it propagates from the tail, *i.e.*, in the former, the oligonucleotide can be purified at each step where the protecting group N enacts the role of handle for separation. This is why the oligomer is selectively prepared. The principle of this method could be applied for the synthesis of polypeptides and other complex molecules.

Experimental

General Procedure. Paper chromatography was performed by a descending technique using Toyo Roshi No. 51 paper. Solvent systems used were: 2-propanol, concentrated ammonium hydroxide, water (7:1:2 v/v) (Solvent 712); ethanol, 1M ammonium acetate (7:3 v/v, pH=7.5) (Solvent 73). Pyridine was purified as follows; Commercial pyridine was distilled over *p*-toluenesulfonyl chloride, phosphorus pentoxide and over potassium hydroxide and stored over calcium hydride. 2,4,6-Triisopropylbenzenesulfonyl chloride (TPS) was prepared by a procedure in literature.[8] *N,N*-Dimethyl-*p*-phenylenediamine was purified by distillation (bp 79—82°C/0.7 mmHg), before use and stored in dry pyridine.

Preparation of NpT. A mixture of pyridinium salt of thymidine 5′-phosphate (0.5 mmol) and *N,N*-dimethyl-*p*-phenylenediamine (3 mmol) was combined with a mixture of water (1 ml) and *t*-butyl alcohol (5 ml). DCC (2.5 mmol) was then added. The mixture was heated under gentle reflux on a glycerin bath at 93—94°C for 3 hr and concentrated to dryness under reduced pressure. The residue was suspended in water (10 ml) and the suspension was washed with three portions of ether (3×10 ml). The aqueous layer was then extracted with three portions of chloroform and concentrated *in vacuo* (1 ml). This was applied to a column

6) Hydrolysis of the acetyl group from pTpTOAc was observed under acidic conditions. Acetylation with acetic anhydride afforded pure pTpTOAc in quantitative yield.

7) $(pTpTOAc)_2$ refers to P^1,P^2-di-3′-*O*-acetylthymidylyl(3′→5′)-thymidine 5′-pyrophosphate. No pTpTOAc was detected from the reaction mixture.

8) R. Lohrmann and H. G. Khorana, *J. Amer. Chem. Soc.*, **88**, 8296 (1966).

TABLE 1. CHROMATOGRAPHIC AND SPECTRAL PROPERTIES OF THE REPORTED COMPOUNDS

Compound	Pc[a] solvent		Spectral data[c] (in H_2O, pH=9)	
	712	73	λ_{max} ($10^{-3}\varepsilon$)	λ_{min} (mμ)
pT	0.20	0.22		
NpT	0.58		251 (17.2)	230
pTOAc	0.22	0.33		
NpTpTOAc	0.45		255 (25.0)	235
pTpTOAc	0.12	0.29		
pTpT[b]	0.09	0.19		
NpTpTpTOAc	0.24		266 (33.0)	236
pTpTpTOAc	0.05	0.12		
pTpTpT[b]	0.04	0.10		

a) Pc=paper chromatography.
b) The compound has been reported by Khorana and co-workers.[9] The R_f values of the compounds agree well with those given in literature. The oligothymidylates prepared by the present method were characterized by degradation to pT with snake *venom* phosphodiesterase and by mobility of the oligothymidylates relative to pT on paper electrophoresis (phosphate buffer, pH 8).
c) The ε_{max} values were obtained and calculated from those of the corresponding free thymidylates.

(1.5×15 cm) of Dowex 50W-X2 (H^+ form). The column was washed first with water (150 m*l*) and then eluted with 0.2N aqueous triethylamine. The eluate was concentrated to dryness under reduced pressure. NpT was obtained in 90% (7740 O.D.$_{251}$ units at pH 9) yield as pink oil which was chromatographically pure. Spectral data are given in Table 1.

Preparation of NpTpTOAc. Triethylammonium salt of NpT (0.13 mmol) and 3′-*O*-acetylthymidine 5′-phosphate (pTOAc) (0.1 mmol) were dissolved in pyridine (1 m*l*) and the mixture was concentrated to dryness. This was repeated five times for complete removal of moisture. The residue was dissolved in pyridine (0.3 m*l*) and TPS (0.3 mmol)was added. The mixture was allowed to stand at room temperature for 13 hr and water (0.3 m*l*) was added. After 3 hr, the solvent was removed *in vacuo* and the residue was suspended in water (3 m*l*). The aqueous solution was washed with ether and then with chloroform and concentrated to *ca.* 0.5 m*l*. The solution was applied to a column (1.5×5 cm) of Dowex 50W-X2 (H^+ form). The column was washed with water (300 m*l*). The eluate contains NpTpTOAc, $(pTOAc)_2$ and pTOAc. It was concentrated to *ca.* 2 m*l* and neutralized with 0.1N sodium hydroxide and then Amberlite IR-45 (free base) (3 m*l*) was added. After 30 min, the mixture was applied to a column (1.5×6 cm) of Amberlite IR-45 (free base). The column was washed with water (100 m*l*) for removal of $(pTOAc)_2$ and pTOAc and then 50% acetic acid (10 m*l*) was added. It was allowed to stand for 30 min. The column was eluted and washed with water. This was repeated once more. Eluate and washings were combined and concentrated *in vacuo*. NpTpTOAc was obtained in 65% (1625 O.D.$_{255}$ units at pH 9) yield as colorless oil which was chromatographically pure. The spectral data are given in Table 1.

Preparation of pTpTOAc. NpTpTOAc (0.06 mmol) was dissolved in 80% acetic acid (5 m*l*). The solution was allowed to stand at 80°C for 3 hr and concentrated to dryness. The residue was dissolved in water (1 m*l*) and applied to a column (1.5×2 cm) of Dowex 50W-X2 (H^+ form). The column was eluted with water and the eluate was concentrated. pTpTOAc was obtained in 92% yield based on NpTpTOAc and stored in dry pyridine. When hydrolysis of the acetyl group from pTpTOAc was observed, acetylation with acetic anhydride afforded pure pTpTOAc in quantitative yield.

Preparation of NpTpTpTOAc. pTpTOAc (0.015 mmol) and triethylammonium salt of NpT (0.045 mmol) were dissolved in pyridine (1 m*l*) and the mixture was concentrated to dryness. This was repeated five times for complete removal of moisture. The residue was then dissolved in pyridine (0.15 m*l*) and TPS (0.045 mmol) was added. The mixture was allowed to stand at room temperature for 14 hr and water (0.5 m*l*) was then added under cooling. It was allowed to stand at room temperature for 3 hr and then concentrated *in vacuo*. The residue was suspended in water (3 m*l*). The aqueous solution was washed with ether and with chloroform and then concentrated to *ca.* 0.5 m*l*. It was applied to a column (1.5×5 cm) of Dowex 50W-X2 (H^+ form). The column was eluted with water (150 m*l*). The eluate contains NpTpTpTOAc, $(pTpTOAc)_2$ and pTpTOAc. It was concentrated to *ca.* 1 m*l* and neutralized with 0.1N sodium hydroxide. Amberlite IR-45 (free base) was added to the solution. After being left to stand at room temperature for 30 min, it was applied to a column (1.5×5 cm) of Amberlite IR-45 (free base). The column was washed with water for removal of $(pTpTOAc)_2$ and pTpTOAc and then 50% acetic acid (10 m*l*) was added. The column was allowed to stand for 30 min, then eluted and washed with water. This was repeated once more. Eluate and washings were combined and concentrated under reduced pressure. NpTpTpTOAc was obtained in 31% (153 O.D.$_{266}$ units at pH 9) yield as colorless oil which was chromatographically pure. The spectral data are given in Table 1.

Preparation of pTpTpTOAc. NpTpTpTOAc (0.047 mmol) was dissolved in 80% acetic acid (2 m*l*). The solution was allowed to stand at 80°C for 3 hr and concentrated to dryness. The residue was dissolved in water (1 m*l*) and 1 m*l* of Dowex 50W-X2 (H^+ form) was added and allowed to stand at room temperature for 10 min. Dowex 50W-X2 was filtered and washed with water. Filtrate and washings were combined and concentrated *in vacuo*. pTpTpTOAc was obtained in 91% yield based on NpTpTpTOAc and stored in dry pyridine.

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9) a) P. T. Gilham and H. G. Khorana, *J. Amer. Chem.* Soc., **80**, 6212 (1958). b) H. G. Khorana and J. P. Vizsolyi, *ibid.*, **83**, 675 (1961).