# A New General Method for the Synthesis of Phosphate-Protected Deoxyribooligonucleotides. IV[1]

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**Abstract:** The attachment of aromatic protecting groups to the terminal phosphate of deoxyribonucleotides greatly increases their affinity for benzoylated DEAE-Sephadex. Columns of the absorbent are capable of resolving condensation mixtures containing fragments thus protected from incoming mononucleotide, together with derived side products not bearing such aromatic protecting groups. Various protected di- and trinucleotides were obtained, in excellent purity, in yields of 40–70 and 30–60%, respectively. A novel phosphate-protecting group developed for this purpose, 2-phenylmercaptoethanol, is very stable to all manipulations encountered in the synthesis; however, when oxidized to the sulfoxide derivative with sodium metaperiodate, it is easily removed with 2 *N* sodium hydroxide at room temperature in 30 min.

Although synthetic polynucleotides have played an important role in our understanding of various biological processes at the molecular level,[3a] their syntheses still represent a major problem. This is mainly due to the very tedious and time consuming nature of current synthetic methods. It has become, therefore, of paramount importance to develop new and simpler methods for the synthesis of polynucleotides. In this paper we wish to report (i) a new synthetic approach which offers an easy procedure for the separation of the growing chains of intermediate oligonucleotides from the incoming nucleotidic units; (ii) various new phosphate protecting groups containing one aromatic ring; and finally (iii) quick and easy characterization of oligonucleotides (using amounts equivalent to less than 1.0 absorbance unit at 260 mμ) by Avicel-Cellulose tlc plate and double scanning technique developed in our laboratory.

**New Synthetic Approach.** The basic principle of our new approach is that the attachment of an aromatic protecting group to the 5′-phosphate of a nucleotidic unit **2** markedly increases its binding to benzoylated DEAE-Sephadex.[3b] On condensation of **3** with a second compound, a 3′-*O*-acetyl N-protected nucleoside 5′-phosphate **4** containing nonaromatic protecting groups, the aromatic group will be found only in the unreacted starting material **3** and the product **5** (see Chart I). Thus, when the reaction mixture is passed through a benzoylated DEAE-Sephadex column, **3** and **5** will be the only components of the reaction mixture retained by the BD-DEAE-Sephadex due to the affinity between their aromatic rings. All the other components which lack the aromatic group such as unused **4**, pyrophosphate of **4** (a serious side product), will be eluted quickly. The components **3** and **5** can then be easily eluted by washing the column with stronger buffer solutions containing ethyl alcohol (see Figure 1). The desired product is then separated from the starting material by either gel filtration[4a] or preparative tlc.[4b] The preliminary reports of this work have already appeared.[5] A similar approach has been reported by Japanese workers.[6]

**New Aromatic Phosphate Protecting Groups.** In order to develop this approach, it was considered essential to search for suitable phosphate protecting groups containing a benzene ring. Various substituted phenols were investigated as potential protecting groups. Although aryl esters of mononucleoside 5′-phosphate could easily be prepared by the usual dicyclohexylcarbodiimide reaction, more drastic conditions (2 *N* sodium hydroxide, 50–100° 15–60 min) were generally required for the complete hydrolysis. This treatment resulted in the formation of small amounts of deamination and other side products. However, 3,5-dinitrophenol, 2-nitro-4-chlorophenol, and pentachlorophenol were found to be promising, since these groups only required the action of 2 *N* sodium hydroxide (100°, 15 min) for the complete hydrolysis of their nucleotidic esters.

Next, various aromatic hydracrylamide derivatives, **1a**, **1b**, **1c**, prepared by condensing β-propiolactone with the appropriate aromatic amines,[7] were studied. Their esters, known to cleave under mild alkali condition *via* a β elimination mechanism,[8] were prepared by reaction with mononucleoside 5′-phosphate in the presence of dicyclohexylcarbodiimide or triisopropylbenzenesulfonyl chloride. As expected, treatment with 2 *N* sodium hydroxide at room temperature smoothly hydrolyzed the phenylhydracrylamide ester (**3a**) in 45 min, *p*-methoxyphenylhydracrylamide ester (**3b**) in 90 min, and the benzylhydracrylamide ester (**3c**) in 8 hr.

(1) Part III: S. A. Narang and J. J. Michniewicz, *Anal. Biochem.*, in press.

(2) (a) National Research Council Postdoctoral Fellows, 1968–1970; (b) guest workers, Chemistry Department, Carleton University, Ottawa, Canada; (c) NRCC No. 12577.

(3) (a) H. G. Khorana, H. Büchi, H. Ghosh, N. Gupta, T. M. Jacob, H. Kössel, R. Morgan, S. A. Narang, E. Ohtsuka, R. D. Wells, *Cold Spring Harbor Symp. Quant. Biol.*, **31**, 39 (1966); (b) I. Gillam, S. Milward, D. Blew, M. von Tigerstrom, E. Wimmer, and G. M. Tener, *Biochemistry*, **6**, 3042 (1967).

(4) (a) S. A. Narang, J. J. Michniewicz, and S. K. Dheer, *J. Amer. Chem. Soc.*, **91**, 936 (1969); (b) S. A. Narang, O. S. Bhanot, S. K. Dheer, J. Goodchild, and J. J. Michniewicz, *Biochem. Biophys. Res. Commun.*, **41**, 1248 (1970).

(5) (a) S. A. Narang, O. S. Bhanot, J. Goodchild, and R. H. Wightman, *Chem. Commun.*, 91 (1970); (b) S. A. Narang, O. S. Bhanot, J. Goodchild, J. J. Michniewicz, R. H. Wightman, and S. K. Dheer, *ibid.*, 516 (1970).

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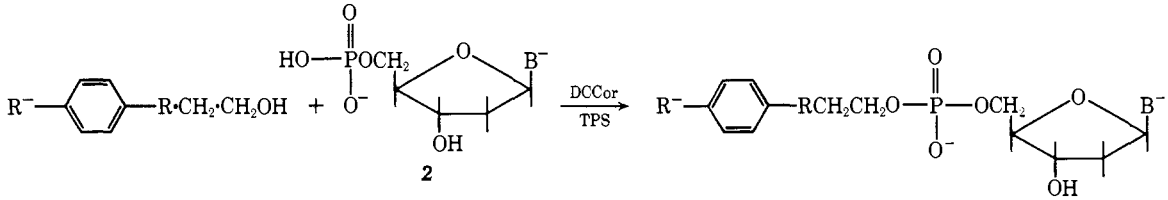
(8) E. Cherbuliez, G. Cordahi, and J. Rabinowitz, *Helv. Chim. Acta*, **43**, 863 (1960).

Chart I. Synthesis of Phosphate-Protected Deoxyribooligonucleotides



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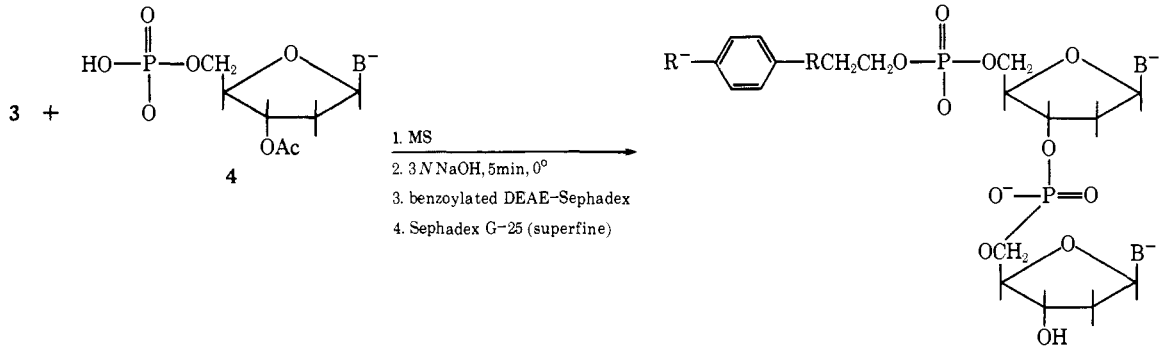
3

1a, $R^- = H$; R = NHCO (phenylhydracrylamide)
1b, $R^- = CH_3O$; R = NHCO (methoxyphenylhydracrylamide)
1c, $R^- = H$; R = $CH_2NHCO$ (benzylhydracrylamide)
1d, $R^- = H$; R = *S* (2-phenylmercaptoethanol)



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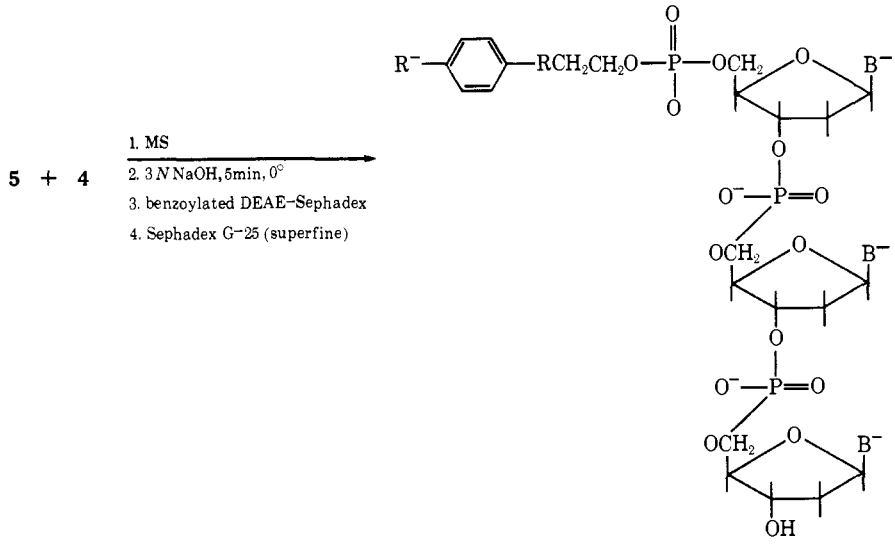
5



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$B^-$ = thymine, *N*-acetyladenine, *N*-acetylguanine, and *N*-isobutyloxycarbonylcytosine.

Finally, we investigated 2-phenylmercaptoethanol[9] **1d**, a new phosphate protecting group, which has many novel features. It can easily be introduced under the usual conditions and the corresponding sulfide ester is very stable to conditions commonly encountered in oligonucleotide synthesis, such as (a) 2 *N* sodium hydroxide at room temperature for 4 hr; (b) concentrated ammonium hydroxide at 50° for 2 hr; (c) aqueous pyridine at room temperature for 1 week; (d) mesitylenesulfonyl chloride in anhydrous pyridine at room temperature for 3 hr; (e) aqueous hydrochloric acid (pH 2) at room temperature for 3 days. However, when "activated" this group is readily removed. Thus, the 2-phenylmercaptoethyl group was removed by treating the protected nucleotide in aqueous solution or in 0.1 *M* triethylammonium bicarbonate buffer with sodium metaperiodate (approximately 5 *M* excess—as a 0.1 *M* aqueous solution) at room temperature for 1 hr. It was apparently oxidized to the corresponding sulfoxide derivative[10] 7. After decomposition of the excess periodate with ethylene glycol the solution was concentrated to dryness *in vacuo*. The nucleotidic component was isolated by extracting the white residues with 90% aqueous pyridine. The free nucleotide was then liberated by treatment with 2 *N* sodium hydroxide at room temperature for 30 min. Each of the four monodeoxynucleotides (*i.e.*, d-pA,

(9) H. H. Ford-Moore, R. A. Peters, and R. H. Wakelin, *J. Chem. Soc.*, 1754 (1949).

(10) (a) N. J. Leonard and C. R. Johnson, *J. Org. Chem.*, 27, 282 (1962); (b) M. Joaquina and S. A. Amaral, *J. Chem. Soc. C*, 2495 (1969).

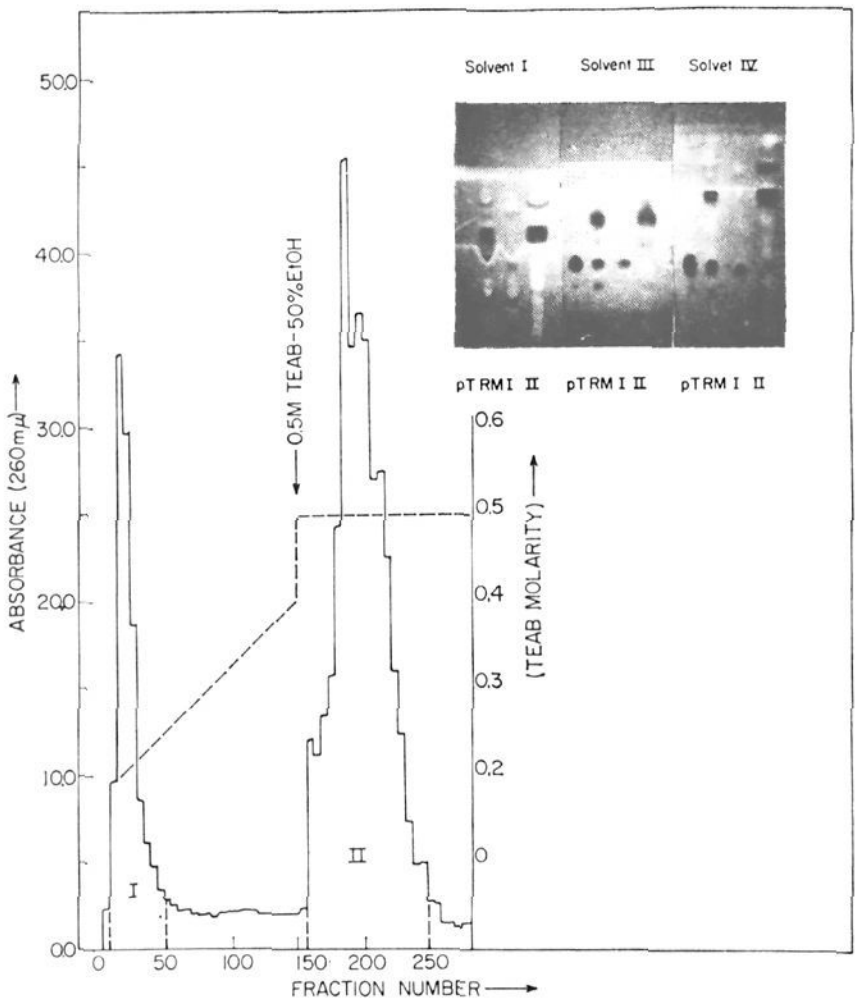



Figure 1. Column chromatography of the reaction mixture on a benzoylated DEAE-Sephadex column (1.5 × 40 cm) in the preparation of PME-pTpT. A linear gradient of 0.2–0.4 *M* triethylammonium bicarbonate buffer, pH 7.1, 1-l. each, was applied and fractions of about 20 ml were collected every 2 min. It was followed by elution with 0.5 *M* triethylammonium bicarbonate (pH 7.1)–50% ethyl alcohol, 1 l., and fractions of 10 ml were collected every 10 min. Peak II mainly contained a mixture of PME-pT and PME-pTpT as shown by the photograph of the plates in three solvent systems. (RM stands for reaction mixture.)

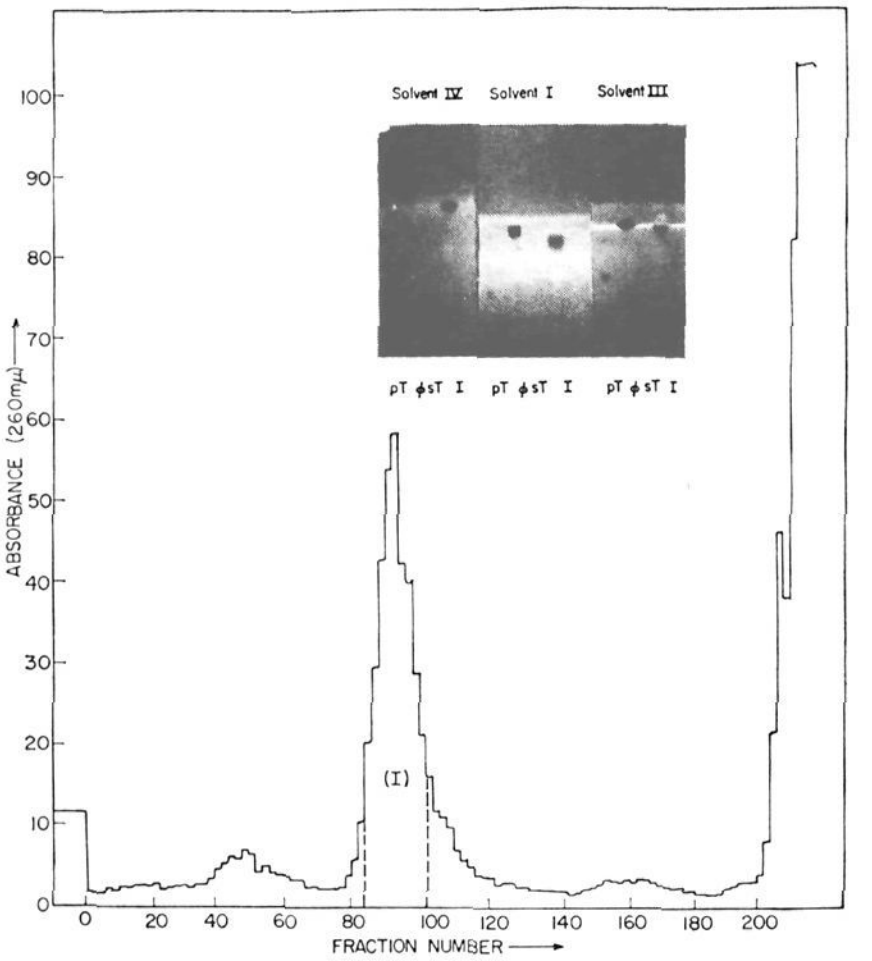



Figure 2. Chromatography of peak II (containing mainly PME-pT and PME-pTpA$^{Ac}$) from the benzoylated DEAE-Sephadex column chromatography on a Sephadex G-25 (superfine) K 50–100 column for the isolation of PME-pTpA$^{Ac}$. Peak I contained the desired product as shown by tlc photography.

d-pC, d-pG, and pT) thus obtained from protected mononucleotides *via* a complete deblocking sequence (see Chart II) was fully characterized by thin-layer chromatography in three solvent systems and ultraviolet spectroscopy to ensure that the periodate treatment in no way affected the heterocyclic bases. It was also surprising to find that the sulfide esters (**3d**, **5d**, or **6d**) bind more strongly to Sephadex than their corresponding oxidized derivatives[11] sulfoxide **7**. This novel property also offers a potentially new separation technique.

**Synthesis of Protected Di- and Trinucleotides.** The main strategy in the present synthetic approach was to protect the 5′-phosphate of the starting monodeoxynucleotide with an aromatic protecting group, whereas the amino and 3′-hydroxyl functions of the incoming monodeoxynucleotide should be blocked with nonaromatic protecting groups. Thus, the 3′-hydroxyl and amino functions of adenosine and guanosine were conveniently protected with the acetyl group[12] whereas the isobutyloxycarbonyl group was used for cytosine.[13,14]

The 2-phenylmercaptoethanol **1d** protecting group has been found to be more suitable than phenylhydracrylamide[15] due to its greater stability toward mesitylenesulfonyl chloride. The protected dinucleotide **5d** was prepared by condensing the 2-phenylmercaptoethyl 5′-phosphate protected monodeoxynucleotide **3c** with N-protected 2′-*O*-acetyl monodeoxynucleoside 5′-phosphate (**4**) (1.2 molar equiv) in the presence of mesitylenesulfonyl chloride (2.4 molar equiv) for 3 hr. After terminating the reaction with aqueous pyridine (25%), the reaction mixture was kept at room temperature for 1 hr. The 3′-*O*-acetyl group was then removed by the usual treatment with 3 *N* sodium hydroxide (equal volume) at 0° for 5 min followed by neutralization with an excess of Dowex-50 resin (pyridinium form). The filtrate was evaporated *in vacuo* to remove pyridine and applied to a benzoylated DEAE-Sephadex[16] column. Elution with a linear gradient of the triethylammonium bicarbonate buffer, pH 7.5, removed the incoming nucleotide **4**, lacking aromatic protecting group, along with pyrophosphate. Subsequent washing with 0.5 *M* triethylammonium bicarbonate–50% ethyl alcohol, eluted strongly, retained nucleotide components **3**

(11) This binding property may be due to the interactions of the unshared electrons of the Sephadex ether oxygen with the d orbital of the sulfur.

(12) (a) R. K. Ralph and H. G. Khorana, *J. Amer. Chem. Soc.*, **83**, 2926 (1961); (b) R. K. Ralph, W. J. Connors, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 1983 (1963).

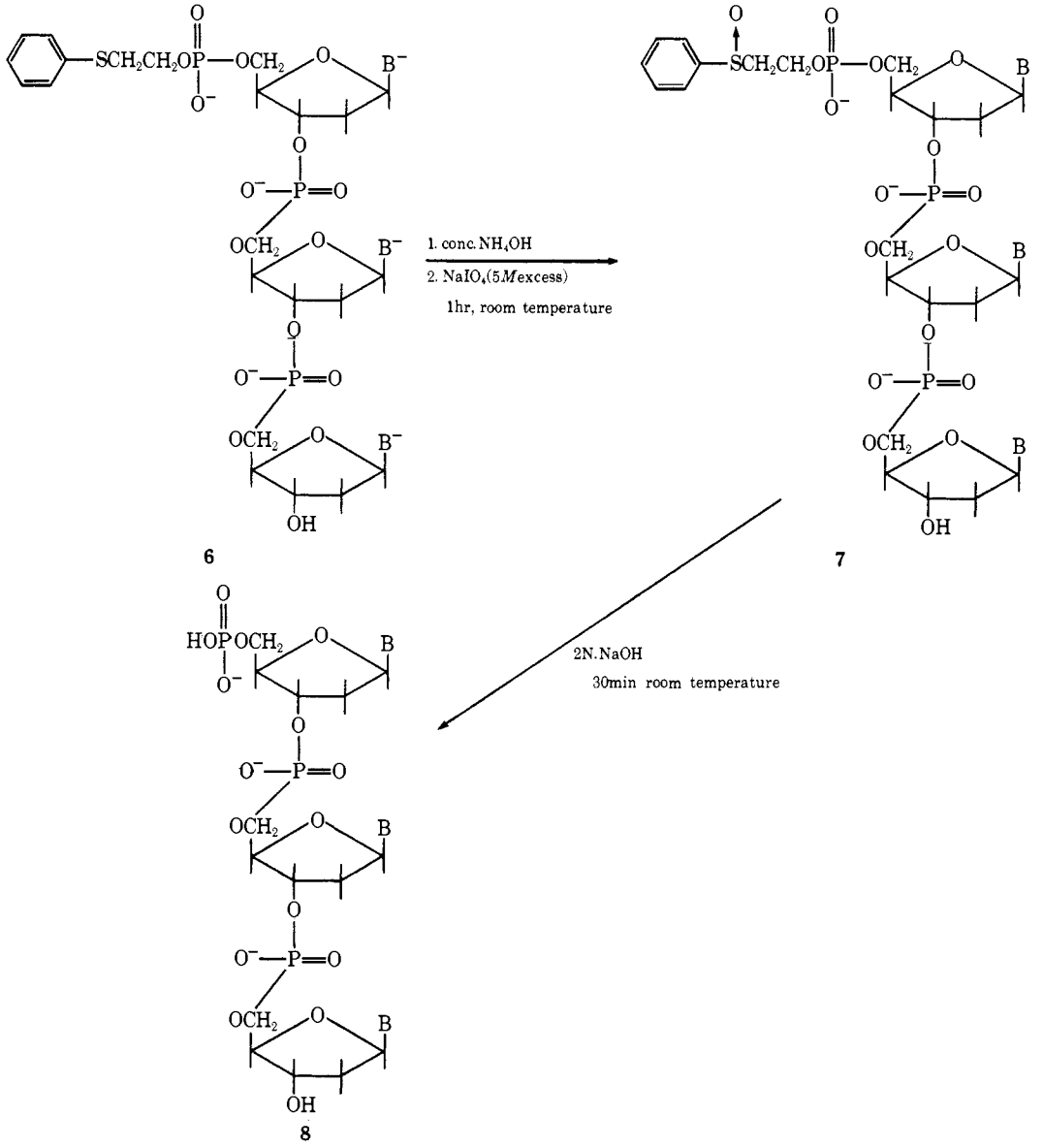
(13) R. L. Letsinger and P. S. Miller, *ibid.*, **91**, 3356 (1969).

(14) The benzoylated DEAE-Sephadex has also been found to retain partially *N*-isobutyloxycarbonyldeoxycytidine 5′-phosphate. This retention may be due to the polarization of the cytosine ring due to the presence of the isobutyloxycarbonyl group.

(15) An excess of mesitylenesulfonyl chloride (more than 5 molar equiv) was found to cause some deblocking of the *p*-methoxyphenyl hydracrylamide groups from their nucleotidic esters, followed by polymerization.

(16) Benzoylated DEAE-Sephadex has been found to have six–seven times greater capacity than benzoylated DEAE-cellulose. It can be easily reused after storing with 0.1 *M* triethylammonium bicarbonate buffer pH 7.1 for 4–6 months without losing appreciable retention capacity for the aromatic protected oligonucleotides: J. J. Michniewicz, O. S. Bhanot, J. Goodchild, S. K. Dheer, R. H. Wightman, and S. A. Narang, *Biochim. Biophys. Acta*, **224**, 626 (1970).

Chart II. Deblocking of 2-Phenylmercaptoethyl Protecting Group




B = thymine, adenine, guanine, and cytosine

and **5** which essentially contained aromatic protecting groups. A typical profile of the benzoylated DEAE-Sephadex column chromatography along with its tlc pattern is given in Figure 1. Finally, the triethylammonium bicarbonate–ethyl alcohol eluted fractions were pooled, concentrated *in vacuo* in the presence of added pyridine and further fractionated on Sephadex G-25 (superfine) column. A typical elution pattern in the isolation of protected dinulceotide and its tlc pattern is given in Figure 2. Similarly, the syntheses of protected trinucleotides were carried out by condensing phosphate-protecting dinucleotide **5d** with 3′-*O*-acetyl N-protected mononucleoside 5′-phosphate **4**. The product was isolated by fractionation on benzoylated DEAE-Sephadex followed by Sephadex (G-25 superfine) gel filtration (see Figure 3 for a typical profile on Sephadex G-25 superfine). The list of all the protected di- and trinucleotides prepared by the present method and their yields and reaction conditions are given in Tables I and II, respectively. Using the 2-phenylmercaptoethanol protecting group, the protected dinucleotides were prepared in 65–70% and protected trinucleotides in 50–65% yields.

**Characterization of Oligonucleotides.** The homogeneity of fully unprotected oligonucleotides was checked by thin-layer chromatography on Avicel-Cellulose plates (0.1 mm thickness). The mobilities of each compound in three solvent systems are given in Table III. This technique offers the following advantages over the conventional paper chromatography: (i) a very small amount (0.5–1.0 absorbance unit at 260 mμ) of the oligonucleotides is required to give well-resolved spots; (ii) a much shorter time (30 min to 4 hr) is required to develop the tlc plate; and (iii) characterization and quantitative estimation of the spots can be obtained directly from the tlc plates using the double scanning technique. Thus, the time-consuming manual operation of isolating the compounds from the chromatograms has been eliminated.[17]

Final characterization of each unprotected trinucleotide listed in Table II was achieved by their

(17) S. A. Narang and J. J. Michniewicz, *Anal. Biochem.*, in press.

**Table I.** Synthesis of Protected Dinucleotides[a]

| Phosphate protected *component* | Amount, *mmol* | 3′-*O*-Acetyl *component* | Amount, *mmol* | *MS* | *Product* | Isolated *yield*, % |
|---|---|---|---|---|---|---|
| MPH-pT | 5.0 | pT-oAc | 6.0 | 12.0 | MPH-pTpT | 60 |
| MPH-pT | 2.0 | d-pA$^{Ac}$-oAc | 3 | 4.5 | MPH-pTpA$^{Ac}$ | 40 |
| MPH-pT | 1.4 | d-pG$^{Ac}$-oAc | 1.5 | 2.0 | MPH-pTpG$^{Ac}$ | 45 |
| MPH-pC$^{Boc}$ | 2.0 | pT-oAc | 2.5 | 5.0 | MPH-pC$^{Boc}$pT | 53 |
| MPH-pC$^{Boc}$ | 2.0 | d-pA$^{Ac}$-oAc | 2.5 | 5.0 | MPH-pC$^{Boc}$pA$^{Ac}$ | 40 |
| MPH-pC$^{Boc}$ | 0.6 | d-pC$^{Boc}$-oAc | 0.9 | 2.0 | MPH-pC$^{Boc}$pC$^{Boc}$ | 45 |
| MPH-pA$^{Ac}$ | 1.5 | d-pC$^{Boc}$-oAc | 2.0 | 4.0 | MPH-pA$^{Ac}$pC$^{Boc}$ | 30 |
| MPH-pA$^{Ac}$ | 2.5 | pT-oAc | 3.0 | 6.0 | MPH-pA$^{Ac}$pT | 40 |
| MPH-pG$^{Ac}$ | 0.5 | pT-oAc | 0.75 | 1.5 | MPH-pG$^{Ac}$pT | 35 |
| PME-pG$^{Ac}$ | 1.2 | pT-oAc | 2.4 | 4.8 | PME-pG$^{Ac}$pT | 70 |
| PME-pA$^{Ac}$ | 1.0 | pT-oAc | 2.0 | 4.0 | PME-pA$^{Ac}$pT | 65 |
| PME-pC$^{Boc}$ | 0.9 | pT-oAc | 1.8 | 3.6 | PME-pC$^{Boc}$pT | 68 |
| PME-pT | 2.0 | pT-oAc | 4.0 | 8.0 | PME-pTpT | 72 |

[a] The following abbreviations are used: MPH, *p*-methoxyphenylhydracrylamide; PME, 2-phenylmercaptoethanol; Boc, isobutyloxycarbonyl group; MS, mesitylenesulfonyl chloride; TPS, triisopropylbenzenesulfonyl chloride.

**Table II.** Synthesis of Protected Trinucleotides

| Phosphate protected component | Amount, mmol | 3′-*O*-Acetyl component | Amount, mmol | MS | Product | Isolated yield, % |
|---|---|---|---|---|---|---|
| MPH-pTpT | 1.5 | d-pA$^{Ac}$-oAc | 2.0 | 4.0 | MPH-pTpTpA$^{Ac}$ | 50 |
| MPH-pTpT | 0.2 | d-pC$^{Boc}$-oAc | 0.3 | 0.6 | MPH-pTpTpC$^{Boc}$ | 32 |
| MPH-pTpT | 2.0 | pT-oAc | 3.0 | 6.0 | MPH-pTpTpT | 45 |
| MPH-pTpT | 1.0 | d-pG$^{Ac}$-oAc | 1.5 | 3.0 | MPH-pTpTpG$^{Ac}$ | 40 |
| MPH-pA$^{Ac}$pC$^{Boc}$ | 0.4 | d-pA$^{Ac}$-oAc | 0.6 | 1.2 | MPH-pA$^{Ac}$pC$^{Boc}$pA$^{Ac}$ | 42 |
| MPH-pC$^{Boc}$pT | 1.0 | d-pC$^{Boc}$-oAc | 1.2 | 2.4 | MPH-pC$^{Boc}$pTpC$^{Boc}$ | 41 |
| MPH-pC$^{Boc}$pA$^{Ac}$ | 0.30 | d-pT-oAc | 0.40 | 0.8 | MPH-pC$^{Boc}$pA$^{Ac}$pT | 38 |
| MPH-pC$^{Boc}$pC$^{Boc}$ | 0.25 | d-pC$^{Boc}$-oAc | 0.3 | 0.6 | MPH-pC$^{Boc}$pC$^{Boc}$pC$^{Boc}$ | 40 |
| MPH-pTpA$^{Ac}$ | 0.2 | d-pG$^{Ac}$-oAc | 0.3 | 0.6 | MPH-pTpA$^{Ac}$pG$^{Ac}$ | 41 |
| MPH-pTpA$^{Ac}$ | 1.2 | d-pC$^{Boc}$-oAc | 1.5 | 3.0 | MPH-pTpA$^{Ac}$pC$^{Boc}$ | 42 |
| MPH-pG$^{Ac}$pT | 0.5 | d-pA$^{Ac}$-oAc | 0.75 | 1.5 | MPH-pG$^{Ac}$pTpA$^{Ac}$ | 38 |
| MPH-pA$^{Ac}$pT | 0.8 | d-pC$^{Boc}$-oAc | 1.0 | 2.0 | MPH-pA$^{Ac}$pTpC$^{Boc}$ | 43 |
| PME-pTpT | 1.1 | d-pG$^{Ac}$-oAc | 2.0 | 6.0 | PME-pTpTpG$^{Ac}$ | 60 |
| PME-pTpA$^{Ac}$ | 0.9 | d-pG$^{Ac}$-oAc | 1.5 | 3.0 | PME-pTpA$^{Ac}$pG$^{Ac}$ | 59 |
| PME-pA$^{Ac}$pC$^{Boc}$ | 0.5 | d-pA$^{Ac}$-oAc | 1.0 | 4.0 | PME-pA$^{Ac}$-pC$^{Boc}$pA$^{Ac}$ | 58 |
| PME-pA$^{Ac}$pT | 0.7 | d-pC$^{Boc}$-oAc | 1.4 | 6.0 | PME-pA$^{Ac}$pTpC$^{Boc}$ | 50 |
| PME-pC$^{Boc}$pT | 1.2 | d-pC$^{Boc}$-oAc | 2.4 | 10.0 | PME-pC$^{Boc}$pTpC$^{Boc}$ | 55 |
| PME-pG$^{Ac}$pT | 0.3 | d-pA$^{Ac}$-oAc | 0.6 | 2.0 | PME-pG$^{Ac}$pTpA$^{Ac}$ | 65 |

digestion (0.5–1.0 absorbance unit at 260 m$\mu$) with snake venom and spleen phosphodiesterase enzymes followed by tlc on Avicel-Cellulose plate (see results in Table IV). The *in situ* identification of each spot was carried out by scanning each spot at 260 and 280 m$\mu$. All the oligonucleotides reported in this paper have been found to be completely digested with the spleen and snake venom phosphodiesterase enzymes to their expected molar ratio of mononucleotide to mononucleoside.

In conclusion, the new synthetic method reported in this paper has greatly simplified many of the major practical problems associated with oligonucleotide synthesis, such as (i) development of a new and stable phosphate-protecting group, (ii) a new and efficient isolation procedure to improve the purity of the synthetic oligonucleotides and finally (iii) a more precise characterization technique on tlc plates with a microscale amount. The synthesis of longer oligonucleotides will be the subject of forthcoming papers.

## Experimental Section

**General Methods and Materials.** All deoxymononucleoside 5′-phosphates were obtained from Calbiochem. Mesitylenesulfonyl chloride (MS), triisopropylbenzenesulfonyl chloride (TPS), substituted phenols, and isobutyl chloroformate were commercially available. Phenylhydracrylamide, *p*-methoxyphenylhydracrylamide, benzylhydracrylamide,[7] and 2-phenylmercaptoethanol[9] were obtained by the previously reported procedures. Avicel-Cellulose

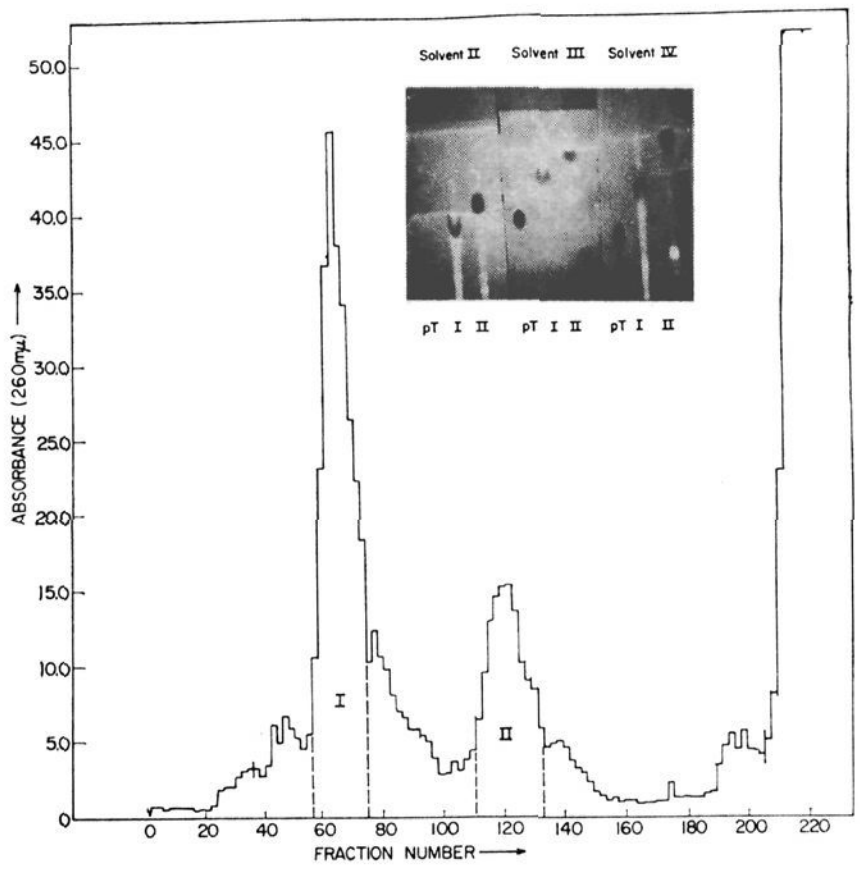



Figure 3. Chromatography of peak II (containing mainly PME-pTpA$^{Ac}$ and PME-pTpA$^{Ac}$pG$^{Ac}$) from the benzoylated DEAE-Sephadex column chromatography on a Sephadex G-25 (superfine) K 50–100 column for the isolation of PME-pTpA$^{Ac}$pG$^{Ac}$. Peaks I and II contained PME-pTpA$^{Ac}$pG$^{Ac}$ and PME-pTpA$^{Ac}$, respectively, as shown by tlc photography.

Table III. $R_f$ Values of Protected Deoxyribooligonucleotides

| Compd | Tlc in solvent system[a] I | III | IV | Compd | Tlc in solvent system[a] I | III | IV |
|---|---|---|---|---|---|---|---|
| Protected Mononucleotides | | | | Protected Trinucleotides | | | |
| MPH-pT | 4.0 | 1.5 | 2.5 | MPH-pTpTpT | 2.5 | 1.2 | 1.8 |
| MPH-pA$^{Ac}$ | 4.2 | 2.5 | 2.8 | MPH-pC$^{Boc}$pTpC$^{Boc}$ | 3.5 | 1.4 | 1.5 |
| MPH-pA | 3.9 | 2.4 | 2.4 | MPH-pCpTpC | 3.0 | 1.2 | 1.3 |
| MPH-pC$^{Boc}$ | 4.3 | 2.1 | 2.9 | MPH-pC$^{Boc}$pA$^{Ac}$pT | 3.1 | 1.5 | 1.4 |
| MPH-pC | 4.0 | 1.9 | 2.3 | MPH-pCpApT | 2.7 | 1.2 | 1.3 |
| MPH-pG$^{Ac}$ | 3.9 | 1.8 | 2.4 | MPH-pC$^{Boc}$pC$^{Boc}$pC$^{Boc}$ | 3.5 | 1.7 | 1.5 |
| MPH-pG | 3.6 | 1.5 | 1.9 | MPH-pCpCpC | 3.0 | 1.3 | 1.2 |
| PME-pT | 4.01 | 1.8 | 2.6 | MPH-pTpA$^{Ac}$pG$^{Ac}$ | 3.5 | 1.4 | 1.6 |
| PME-pA$^{Ac}$ | 6.0 | 2.8 | 4.9 | MPH-pTpApG | 2.8 | 1.2 | 1.3 |
| PME-pA | 5.4 | 2.2 | 1.9 | MPH-pTpTpC$^{Boc}$ | 3.6 | 1.3 | 2.0 |
| PME-pC$^{Boc}$ | 6.1 | 2.9 | 5.1 | MPH-pTpTpC | 3.5 | 1.3 | 1.8 |
| PME-pC | 5.8 | 2.0 | 2.8 | MPH-pTpTpA$^{Ac}$ | 2.5 | 1.3 | 1.5 |
| PME-pG$^{Ac}$ | 5.4 | 1.8 | 5.0 | MPH-pTpTpA | 2.1 | 1.2 | 1.3 |
| PME-pG | 3.0 | 2.0 | 1.9 | MPH-pTpG$^{Ac}$pT | 3.2 | 1.4 | 1.4 |
| Protected Dinucleotides | | | | MPH-pTpGpT | 2.8 | 1.2 | 1.2 |
| MPH-pTpT | 3.8 | 1.4 | 2.3 | MPH-pTpTpG$^{Ac}$ | 3.1 | 1.4 | 1.3 |
| MPH-pC$^{Boc}$pT | 4.0 | 1.6 | 1.76 | MPH-pTpTpG | 2.75 | 1.2 | 1.2 |
| MPH-pCpT | 3.5 | 1.4 | 1.5 | MPH-pA$^{Ac}$pC$^{Boc}$pA$^{Ac}$ | 3.8 | 1.9 | 1.5 |
| MPH-pC$^{Boc}$pA$^{Ac}$ | 3.9 | 2.0 | 1.7 | MPH-pApCpA | 2.6 | 1.7 | 1.4 |
| MPH-pCpA | 3.1 | 1.8 | 1.5 | MPH-pG$^{Ac}$pTpA$^{Ac}$ | 3.1 | 1.5 | 1.3 |
| MPH-pC$^{Boc}$pC$^{Boc}$ | 4.1 | 1.9 | 1.8 | MPH-pGpTpA | 2.8 | 1.3 | 1.1 |
| MPH-pCpC | 3.5 | 1.5 | 1.4 | MPH-pA$^{Ac}$pTpC$^{Boc}$ | 3.0 | 1.5 | 1.3 |
| MPH-pTpA$^{Ac}$ | 3.7 | 1.6 | 1.8 | MPH-pApTpC | 2.7 | 1.2 | 1.3 |
| MPH-pTpA | 3.2 | 1.6 | 1.5 | MPH-pTpApC | 3.1 | 1.5 | 1.4 |
| MPH-pTpG$^{Ac}$ | 3.6 | 1.5 | 1.6 | MPH-pTpApC | 2.8 | 1.3 | 1.2 |
| MPH-pTpG | 3.1 | 1.4 | 1.3 | PME-pToToG$^{Ac}$ | 3.4 | 1.3 | 1.8 |
| MPH-pA$^{Ac}$pC$^{Boc}$ | 4.1 | 2.1 | 1.8 | PME-pTpTpG | 2.8 | 1.1 | 1.3 |
| MPH-pApC | 3.0 | 1.8 | 1.5 | PME-pTpA$^{Ac}$pG$^{Ac}$ | 3.1 | 1.5 | 1.4 |
| MPH-pA$^{Ac}$pT | 3.8 | 1.65 | 1.81 | PME-pTpApG | 2.8 | 1.3 | 1.2 |
| MPH-pApT | 3.15 | 1.6 | 1.45 | PME-pA$^{Ac}$pC$^{Boc}$pA$^{Ac}$ | 3.9 | 2.5 | 1.5 |
| MPH-pG$^{Ac}$pT | 3.6 | 1.45 | 1.7 | PME-pApCpA | 2.9 | 1.9 | 1.4 |
| MPH-pGpT | 3.5 | 1.5 | 1.6 | PME-pA$^{Ac}$pTpC$^{Boc}$ | 4.5 | 2.0 | 1.6 |
| PME-pTpT | 3.9 | 1.3 | 2.2 | PME-pApTpC | 3.5 | 1.8 | 1.3 |
| PME-pG$^{Ac}$pT | 3.7 | 1.6 | 2.2 | PME-pC$^{Boc}$pTpC$^{Boc}$ | 4.9 | 2.3 | 1.9 |
| PME-pGpT | 3.5 | 1.4 | 1.9 | PME-pCpTpC | 3.1 | 1.9 | 1.7 |
| PMe-pA$^{Ac}$pT | 5.1 | 2.2 | 1.7 | PME-pG$^{Ac}$pTpA$^{Ac}$ | 3.2 | 1.3 | 1.7 |
| PME-pApT | 4.2 | 2.1 | 1.5 | PME-pGpTpA | 2.9 | 1.2 | 1.5 |
| PME-pC$^{Boc}$pT | 5.3 | 2.5 | 2.1 | | | | |
| PME-pCpT | 4.1 | 1.9 | 1.8 | | | | |
| PME-pA$^{Ac}$pC$^{Boc}$ | 4.8 | 2.6 | 1.9 | | | | |
| PME-pApC | 4.0 | 2.0 | 1.7 | | | | |

[a] All $R_f$ values are with respect to pT.

Table IV. Snake Venom Phosphodiesterase Digestion of Trinucleotides

| Compd | Nucleoside:nucleotides | Molar ratio of products Found | Theor |
|---|---|---|---|
| d-TpTpG | T:pT:d-pG | 1:0.9:1.1 | 1:1:1 |
| d-TpTpC | T:pT:d-pC | 1:1.2:1.1 | 1:1:1 |
| d-TpTpA | T:pT:d-pA | 1:0.8:1.1 | 1:1:1 |
| d-TpApG | T:d-pA:d-pG | 1:1.2:1.1 | 1:1:1 |
| d-TpApC | T:d-pA:d-pC | 1:0.9:1.1 | 1:1:1 |
| d-ApCpA | d-A:d-pC:d-pA | 1:1.2:1.1 | 1:1:1 |
| d-ApTpC | d-A:pT:d-pC | 1:1.0:1.1 | 1:1:1 |
| d-GpTpA | d-G:pT:d-pA | 1:1.1:0.9 | 1:1:1 |
| d-CpTpC | d-C:pT:d-pC | 1:1.2:0.8 | 1:1:1 |

plates (0.1 mm thickness and 1.0 mm thickness) were purchased from Brinkman Inc. and Analtech, respectively.

**Solvent Systems.** Avicel-Cellulose plates were chromatographed with the following solvent systems by the ascending technique: I, isopropyl alcohol–concentrated ammonia–water (7:1:3, v/v); II, isopropyl alcohol–5% ammonium hydroxide (2:1, v/v); III, isobutyric acid–1 *M* ammonium hydroxide–0.1 *M* EDTA (100:60:1.6, v/v); and IV, Lelvoir pH 7.5, 1 *M* ammonium hydroxide—ethyl alcohol (7:3, v/v).

**Photographic Records of Tlc Plates.** A Polaroid Land camera U-5, equipped with filters (No. 54 and 4A) and loaded with Polaroid film type 104, was used. The photograph of the tlc plate was taken by illuminating it with three ultraviolet lamps (Fischer Scientific UVS-12) in a darkroom with the exposure time from 15 to 20 sec.

**Scanning of the Tlc Plates.** A Zeiss chromatogram scanner (by Stahl) attached with a digital integrator (Vidar 6300 autolab) was employed with the following settings: slit width, 0.5 mm; chart and motor drive speeds, 40 and 50 mm/min, respectively.

**Uv Monitoring of the Column Chromatographic Fractions.** In the present studies, the automatic uv absorbance mesaurements of fractions were made by using Gilford spectrophotometer Model 2400 in conjunction with Sampler Model 530 and Data Lister Model 4008. This device automatically feeds, prints out absorbance readings, and plots the profile of the elution pattern on the chart of the column chromatography simultaneously (see Figures 1–3).

***p*-Methoxyphenylhydracrylamide.** Our typical procedure was modified from that of Gresham, *et al.*[7] β-Propiolactone (37.5 g, 0.55 mol) was added to a stirred solution of *p*-anisidine (61.5 g, 0.5 mol) in ethyl acetate (300 ml) with sufficient cooling to keep the temperature below 30°. The solution was then allowed to stand at room temperature overnight followed by treatment with aqueous sodium hydroxide (2.5 *N*, 200 ml) to remove any acidic component. After removing and drying the solvent, the solid was recrystallized from ethyl acetate–ether after treatment with charcoal. *p*-Methoxyphenylhydracrylamide was isolated as white crystals, mp 103° (40% yield); uv ($H_2O$) $\lambda_{max}$ 247 mμ (ε 10,000). *Anal.* Calcd for $C_{10}H_{13}NO_3$: C, 61.59; H, 6.71. Found: C, 61.23; H, 6.54.

**$N^4$-Isobutyloxycarbonyldeoxycytidine 5′-Phosphate.** The pyridinium salt of deoxycytidine 5′-phosphate (1.0 mmol) was treated with isobutyl chloroformate (10 mmol) under anhydrous conditions with stirring for 4 hr. It was next treated with aqueous pyridine

(25%) and left overnight. The solution was concentrated and then treated with 0.8 *N* sodium hydroxide (30 ml) in dioxane (30 ml) with stirring for 30 min at room temperature to remove the isobutyloxycarbonyl group at the 3'-position followed by neutralization with Dowex-50 resin (pyridinium form). The filtrate was concentrated and the desired compound was isolated by gel filtration on Sephadex G-25 (superfine) in 70% yield. Its mobilities in three solvent systems are given in Table III.

**General Method for the Protection of the 5'-Phosphate of Deoxymononucleosides with Aromatic Protecting Groups.** An anhydrous solution of the pyridinium salt of N-protected deoxymononucleoside 5'-phosphate **2** (5 mmol) and appropriate aromatic alcohol **1a**, **1b**, **1c**, or **1d** (50 mmol) in anhydrous pyridine (100 ml) was treated at room temperature with dicyclohexylcarbodiimide (50 mmol) for 24 hr. The reaction mixture was next decomposed with aqueous pyridine (25%, 200 ml) and kept at room temperature overnight. The insoluble material was removed by filtration and the filtrate was concentrated *in vacuo* at 25° to ensure removal of pyridine. The resulting suspension was extracted with ethyl acetate (four 150-ml portions) in order to remove excess aromatic alcohol and reagent. The aqueous layer was concentrated *in vacuo* to about 10–20 ml volume in the presence of added pyridine. The pure desired ester was conveniently isolated (75% yield) by a gel filtration on Sephadex G-25 (superfine) column. A triester derivative (10% yield) was also recovered from the pyridine peak.

The homogeneities of protected diester compounds were checked by the tlc in solvents I, III, and IV and their $R_f$ values are given in Table III.

**General Method for the Synthesis of 5'-Phosphate-Protected Di- and Trinucleotides.** An anhydrous pyridine solution (10 ml) of the tri-*n*-hexylammonium salt of aromatic protected 5'-phosphate of mono- or dinucleotide **3d** or **5d** (1.0 mmol) and 3'-*O*-acetyl N-protected deoxymononucleoside 5'-phosphate **4** (1–2 mmol) was treated with mesitylenesulfonyl chloride (2 molar equiv) with respect to **4** for 3 hr at room temperature. The reaction mixture was decomposed with aqueous pyridine (25%, 10 ml) and kept further for 1 hr at room temperature. It was next treated with 3 *N* sodium hydroxide (20 ml) at 0° for 5 min followed by neutralization with an excess of Dowex-50 resin (pyridinium form). After filtration, the filtrate was evaporated *in vacuo* at 20° to free it from pyridine.

**Isolation of the Protected Oligonucleotides. (a) Chromatography on Benzoylated DEAE-Sephadex Column.** The above aqueous solution (free from pyridine) was applied to a benzoylated DEAE-Sephadex column[18] (100-ml bed volume) at 4°. The column was first eluted with a linear gradient of triethylammonium bicarbonate buffer pH 7.1 from 0.2 to 0.4 *M* (1 l. each), followed by the washing with 0.5 *M* triethylammonium bicarbonate buffer containing 50% ethyl alcohol. A typical elution pattern of the column chromatography along with its tlc results is given in Figure 1 (reaction mixture in the preparation of PME-pTpT).

**(b) Gel Filtration on Sephadex G-25 (Superfine).** The pooled fractions of peak II from the benzoylated DEAE-Sephadex column (described above) were concentrated *in vacuo* to 5–10 ml in the presence of added pyridine and applied through a sample applicator to Sephadex G-25 (superfine) column K 50–100 at 4°. The column was eluted with 0.1 *M* triethylammonium bicarbonate buffer, pH 7.1. The typical elution patterns and the results of characterization of different peaks by tlc are documented in Figure 2 (isolation of PME-pTpA$^{Ac}$) and Figure 3 (isolation of PME-pTpA$^{Ac}$pG$^{Ac}$).

**(c) Preparative Thick-Layer Chromatography.** The concentrated peak II (approximately 1000 absorbance units at 260 mμ) from the benzoylated DEAE-Sephadex column was applied to preparative tlc Avicel-Cellulose plate (1 mm thickness). The plate was developed in the appropriate solvent by the ascending technique for 8–12 hr. The desired product was isolated by removing the cellulose containing the desired compound and transferring it to a sintered glass. The cellulose was first washed with isopropyl alcohol–anhydrous methyl alcohol (85:15, v/v) (two 10-ml portions) followed with anhydrous ether (two 10-ml portions). After drying, the nucleotidic component was eluted with aqueous pyridine (50%, three 10-ml portions). The aqueous pyridine solution was concentrated *in vacuo* in the presence of pyridine. The recovery was between 80–90%.

**Removal of Protecting Groups. (a) Deblocking of Amino Protecting Groups.** The N-protecting groups were removed by heating the fully protected oligonucleotides **5** or **6** with an excess of concentrated ammonia at 50° for various times such as 15 min for the *N*-acetyl of adenosine, 30 min for *N*-isobutyloxycarbonyl of cytidine, 90 min for the *N*-acetyl of guanosine.[19] The solution was then concentrated to dryness *in vacuo*.

**(b) Deblocking of the *p*-Methoxyphenylhydracrylamide Group.** The protected oligonucleotide (0.1 mmol) was treated with 2 *N* sodium hydroxide (10 ml) at room temperature for 90 min, followed by neutralization with Dowex-50 (pyridinium) resin. After filtration, the filtrate was concentrated and the unprotected oligonucleotide was isolated by any of three procedures: (a) preparative tlc; (b) gel filtration on a Sephadex G-25 (superfine) K 25–100 column; (c) precipitation of anhydrous pyridine solution (5 ml) from ethyl acetate (50 ml). The recovery was quantitative.

**(c) Deblocking of the 2-Phenylmercaptoethanol Group.** An aqueous solution (2 ml) (or in 0.1 *M* triethylammonium bicarbonate) of protected oligonucleotide (0.01 mmol) was treated with freshly prepared sodium metaperiodate (approximately 5 *M* excess —as a 0.1 *M* aqueous solution) at room temperature for 1 hr. After decomposition of the excess periodate with ethyl glycol, the solution was concentrated *in vacuo* to dryness. The nucleotidic material was isolated by extracting the white residue with 90% aqueous pyridine (four 2-ml portions). The free oligonucleotide then was liberated by treatment with 2 *N* sodium hydroxide (2 ml) for 30 min. After neutralization with Dowex-50 (pyridinium) resin, the unprotected oligonucleotides were isolated in quantitative yield by the usual procedures as described above.

**Characterization of Oligonucleotides. (a) Tlc on the Avicel-Cellulose Plate.** The homogeneity of each oligonucleotide (protected and unprotected) was checked rigorously by their tlc on Avicel-Cellulose (0.1 mm thickness) plate in three solvent systems. About 0.5–1 absorbance unit at 260 mμ of each oligonucleotide was sufficient to give a strong and well-resolved spot on the plates. The percentage purity of each compound was determined by scanning the tlc plate with the Zeiss Chromatogram Scanner. The $R_f$ values of each compound are given in Table III.

**(b) Enzymatic Digest. (i) Treatment with Bacterial Alkaline Phosphomonoesterase.** Each unprotected oligonucleotide containing free 5'-phosphate (1–2 absorbance units at 260 mμ) in 0.1 *M* ammonium bicarbonate pH 8.5 (20 μl) was treated with bacterial alkaline phosphomonoesterase (50 μl, 5 mg/5 ml of solution) for 30 min at 70°. The dephosphorylated product was isolated by tlc.

**(ii) Snake Venom or Spleen Phosphodiesterase Digestion.** The dephosphorylated oligonucleotide (0.5–1.0 absorbance unit at 260 mμ) was digested with snake venom or spleen phosphodiesterase. The digested solution was chromatographed on Avicel-Cellulose plate (0.1 mm thickness) in solvents III and IV. The *in situ* characterization of each nucleotide was achieved by scanning the plate at 260 and 280 mμ.[17] The *in situ* quantitative estimation was carried out by using the standard curve and internal standard (see results in Table IV).

(18) Generally, a 10-ml bed volume of benzoylated DEAE-Sephadex was sufficient for 1000 absorbance units at 260 mμ of aromatic protected oligonucleotides.

(19) Concentrated ammonia treatment at 50° for 90 min also caused about 30% of the deblocking of *p*-methoxyphenylhydracrylamide, whereas the 2-phenylmercaptoethyl group was quite stable.