JOURNAL
OF THE AMERICAN CHEMICAL SOCIETY

VOLUME 98, NUMBER 5 MARCH 3, 1976

Studies on Polynucleotides. CXLIII.[1] A Rapid and Convenient Method for the Synthesis of Deoxyribooligonucleotides Carrying 5′-Phosphate End Groups Using a New Protecting Group[2]

K. L. Agarwal,[3a] Y. A. Berlin,[3b] H.-J. Fritz,[3e] M. J. Gait, D. G. Kleid,[3c] R. G. Lees,[3f] K. E. Norris,[3d] B. Ramamoorthy, and H. G. Khorana*

Contribution from the Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received July 10, 1975*

Abstract: A new and rapid method for the synthesis of protected dinucleotides of defined sequence carrying 5′-phosphomonoester groups is described. The key step is the use of a highly lipophilic protecting group for the 5′-phosphate group of the terminal nucleotide. This allows the isolation of the products by convenient solvent extraction procedures and thus obviates time-consuming anion exchange or gel filtration methods. The synthetic steps are: (1) N-protected deoxyribonucleotide 5′-monophosphates are converted to the corresponding 5′-diesters by reaction with the lipophilic 2-(*p*-tritylphenyl)thioethanol (TPTE) or 2-(*p*-tritylphenyl)sulfonylethanol (TPSE) in the presence of triisopropylbenzenesulfonyl chloride or dicyclohexylcarbodiimide (the yields are 65–70%); (2) the diesters are further condensed with suitably protected mononucleotides, and the resulting protected dinucleotides are isolated by organic solvent extraction procedures in yields of 50–65%. The group TPSE is then removed simply by a mild alkaline treatment. For removal of TPTE, the sulfide group is first oxidized to the sulfone. Extension of the above method to the synthesis of a number of tri- and tetranucleotides is described.

Chemical synthesis of polynucleotides of defined nucleotide sequence has been useful in the study of various aspects of the chemistry, physical chemistry, enzymology, and molecular biology of nucleic acids. Thus, for example, precise elucidation of the mode of action of a number of nucleolytic enzymes[4] and of the genetic code[5] was greatly aided by the availability of defined polynucleotides. In more recent years, attention has been focused on the development of methods for the synthesis of macromolecular bihelical DNA of defined sequence and, in particular, of genes for certain transfer RNA's,[6,7] including the adjacent DNA regions which signify the initiation and termination of their transcription.[1] Further, synthetic deoxyribopolynucleotides have formed the basis of a general and useful approach for the determination of the nucleotide sequence of the above control regions in DNA.[8-11]

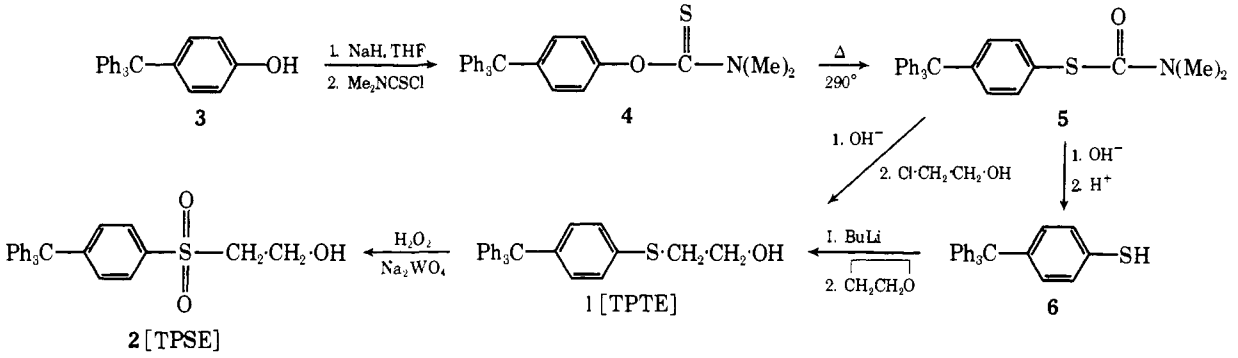
In future studies of the biological functions of the nucleic acids, for example, gene structure and function relationships, mechanisms of the protein-nucleic acid interactions, and the controlled expression of genetic information, the synthesis of defined bihelical DNA's clearly will continue to be highly useful and necessary.

In the combined chemical-enzymatic approach to the synthesis of macromolecular DNA, chemical synthesis of short deoxypolynucleotide segments continues to determine the progress in the synthesis of the required defined DNA molecules. Thus, despite intensive efforts made to date,[12,13] chemical synthesis has continued to be a time-consuming process. Therefore, efforts continue to be made in this laboratory so as to enhance the rapidity and efficiency of the chemical procedures. A significant improvement in efficiency would result from the ready availability of large amounts of protected mono- and oligonucleotide blocks carrying 5′-phosphate groups. While protected mononucleotides are readily prepared on a satisfactory (20–60 mmol) scale, the large-scale preparation of di-, tri-, and tetranucleotide blocks continues to involve considerable effort, and there is room for improving rapidity and efficiency.

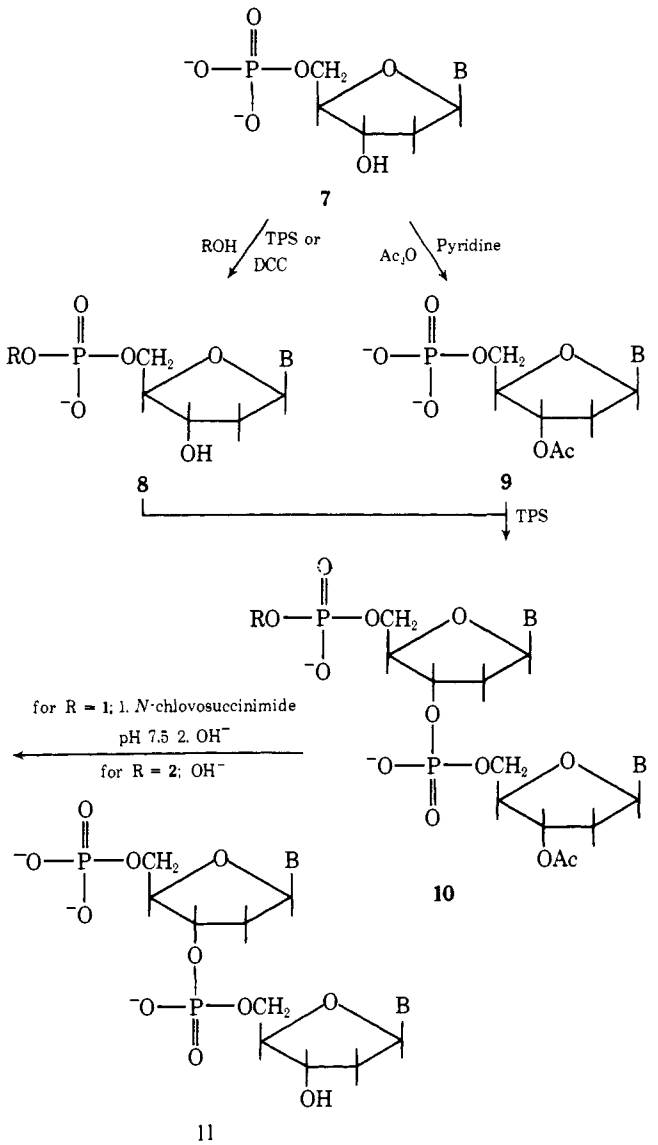
The present paper reports on a new method for the synthesis of oligonucleotide blocks which represents a substantial improvement over the current procedures.

Protection of the 5′-Phosphate Group. Sometime ago we reported on a method for the synthesis of dinucleotides bearing 5′-phosphate groups which too aimed at organic solvent extraction of the products.[14] In this procedure the 5′-phosphate of the terminal nucleotide was protected by the formation of a phosphoramidate linkage with the lipophilic amine, *p*-aminophenyltriphenylmethane. Although a number of dinucleotides was prepared by this method and the latter used in oligonucleotide syntheses, the procedure was not completely satisfactory due to N-sulfonylation of the phosphoramidate group by triisopropylbenzenesulfonyl

Scheme I. Synthesis of TPTE and TPSE



Scheme II. General method for the synthesis of dinucleotides



1, R = $Ph_3CC_6H_4$-*p*-SCH_2CH_2–; 2, R = $Ph_3CC_6H_4$-*p*-$SO_2CH_2CH_2$–; B = thymine, *N*-anisoylcyfosine, *N*-isobutyrylguanine, or *N*-benzoyladenine

chloride (TPS).[19] This competing reaction caused losses in the yields of the di- and the higher oligonucleotides. In the present work, we have developed the use of S-substituted mercaptoethanols as a new class of phosphate protecting group. A preliminary report[15] described the use of 2-(phenyl- or 2-naphthylthioethanol in protection of 5′-phosphate groups. These were found to be superior to the previously described phosphoramidate protecting group, since the group was stable and free of side reactions during subsequent synthetic steps. The group could be removed by oxidation with *N*-chlorosuccinimide to the sulfone followed by a mild alkaline treatment to give the free phosphomonoester. However, such protected nucleotides could not be rapidly isolated by simple organic solvent extraction, although purification could be achieved by using trityl-cellulose column chromatography.[13] Clearly, use of a more lipophilic S-substituted mercaptoethanol as the protecting group was desirable. Therefore, the use of 2-(*p*-tritylphenyl)thioethanol (TPTE) and its oxidized form, 2-(*p*-tritylphenyl)sulfonylethanol (TPSE), was studied.

A number of routes for the synthesis of TPTE (**1**) and TPSE (**2**) was tried. Generally, reactions involving electrophilic substitution of the trityl cation on thiophenol or its derivatives met with little or no success. However, the method of Newman and Karnes[16] for the conversion of phenols to thiophenols via dialkylthiocarbamates proved satisfactory. Thus, as shown in Scheme I, the sodium salt of *p*-tritylphenol (**3**) was treated with *N,N*-dimethylthiocarbamoyl chloride in tetrahydrofuran under reflux to give *O*-*p*-tritylphenyl *N,N*-dimethylthiocarbamate (**4**) in 94% yield. Thermal rearrangement of **4** at 290° for 30 min under an inert atmosphere afforded *S*-(*p*-tritylphenyl) *N,N*-dimethylthiocarbamate (**5**) in similarly high yield. Subsequent treatment with methanolic potassium hydroxide led to formation of *p*-tritylthiophenol (**6**) in 98% yield. Treatment of **6** with *n*-butyllithium followed by reaction with ethylene oxide gave TPTE (**1**) in 82% yield. However, **1** was most conveniently obtained directly from the thiocarbamate **5**. After alkaline treatment of **5** as before, the anion of **6** was treated immediately, without prior isolation, with 2-chloroethanol. TPTE (**1**) was obtained in 99% yield. TPTE (**1**) was converted to TPSE (**2**) in 91% yield by oxidation with hydrogen peroxide, the reaction being catalyzed by tungstate ions.[17]

Dinucleotide Blocks. Scheme II outlines the general procedure for the use of TPTE and TPSE in the preparation of dinucleotide blocks. N-protected mononucleotides (**7**) were condensed with an excess of **1** in the presence of TPS in pyridine. Although sometimes the desired TPTE mononucleotide (**8**) could be isolated merely by organic solvent extraction, it was usually preferable to purify these derivatives by column chromatography on silica gel using aqueous acetonitrile solvent systems to remove, in particular, traces of nucleotidic side products. Yields of the required products were 65–75%, depending on the nucleotide. Unreacted TPTE could be recovered from the column and reused subsequently. TPTE derivatives of the mononucleotides were characterized by their mobility on TLC and by their reconversion to the starting mononucleotide under the conditions used to remove the blocking group from dinucleotide derivatives (see below). Moreover, the TPTE derivatives could be

treated with ammonia at 50° for 24 hr to remove the N-protecting groups without loss of TPTE.

The TPTE derivatives (**8**) were then condensed with 3′-O-acetylated mononucleotides (**9**) in the presence of TPS. Unreacted **8** was removed by extraction from an aqueous triethylammonium bicarbonate (TEAB) solution into ethyl acetate by continuous solvent-solvent extraction. In certain cases, complete removal of the TPTE mononucleotides required further extraction with ethyl acetate containing small amounts of 1-butanol. TPTE dinucleotides (**10**) could then be extracted into ethyl acetate-butanol (7:3, v/v) or methylene chloride-butanol (7:3, v/v). There was some variability in ease of extraction of TPTE derivatives of mono- and dinucleotides depending on base composition, but in all cases suitable manipulation of extraction conditions yielded pure TPTE-dinucleotides in 50-60% yield based on the TPTE mononucleotides used.

Removal of the TPTE group was effected in two stages. Treatment with *N*-chlorosuccinimide in dioxane-water mixtures containing Tris buffer (pH 7.5) afforded the corresponding sulfone. The oxidation was highly pH dependent, but at the above pH the reaction was generally complete in 10 min. Since the sulfide and sulfone both have similar R_f values on TLC, an alternative method was used to ascertain that oxidation was complete. An aliquot was treated with sodium hydroxide solution at 0° for 5 min under the standard conditions for removal of 3′-*O*-acetyl groups from the nucleotides.[6] Only the sulfone underwent β elimination to give the free dinucleotide which could easily be distinguished from its TPTE derivative on TLC. Oxidation by *N*-chlorosuccinimide is specific for the sulfide moiety, and no side reaction of the nucleotides was observed.

After completion of the oxidation, the reaction was terminated by addition of pyridine. Subsequent alkaline treatment as described above liberated the free dinucleotide (**11**). Either of two procedures could be used for removal of nonnucleotidic by-products resulting from the oxidation reaction. Passage of the final solution through a short, fat DEAE-cellulose column at low ionic strength (0.05 M TEAB) allowed selective adsorption of the dinucleotide, which was subsequently eluted with 0.2 M TEAB. Alternatively, before treatment with alkali the mixture could be "diafiltered" using an Amicon ultrafiltration apparatus fitted with a UMO5 membrane. In this process, small molecules such as succinimide and its derivatives passed through the membrane, whereas protected dinucleotides (and higher oligonucleotides) were almost quantitatively retained.

Table I summarizes the yields and characteristics of seven dinucleotides prepared by the TPTE method. Yields are based in each case on the TPTE mononucleotide components. Some of the alternative techniques described above are illustrated in four examples given in the Experimental Section.

While the use of the protecting group, TPTE, for the above-described syntheses of dinucleotide blocks is highly satisfactory, the use of TPSE protecting group offers some advantages over the TPTE method. This blocking group can be removed from the dinucleotide merely by treatment with alkali. Moreover, since the oxidation of the sulfide to the sulfone is performed at an earlier stage, the need for separation of nonnucleotidic by-products resulting from the oxidation reaction is obviated.

The steps in the preparation of the dinucleotides using TPSE are similar to those shown above in Scheme II. Thus, TPSE mononucleotides prepared as described for the TPTE derivatives can be purified by solvent-solvent extraction in yields of 70-80%. Silica gel column chromatography was no longer necessary when DCC was used as the condensing agent rather than TPS. The preparation of TPSE dinucleotides followed an analogous procedure to that described for TPTE dinucleotides. By organic solvent extraction, the TPSE dinucleotides were extracted into ethyl acetate-1-butanol (7:3) or methylene chloride-1-butanol using varying proportions. Removal of the TPSE group was effected using the same general procedure which is used for the removal of the 3′-*O*-acetyl groups.

Four examples of dinucleotides prepared by the TPSE method are given in Table II. The yields ranged from 78% for dpT-T to 45% for dpibG-ibG.

The Synthesis of Tri- and Tetranucleotide Blocks. While only a limited number of tri- and tetranucleotides has been prepared, the method is clearly promising for further work. Both the 5′-TPTE and TPSE mononucleotides could be used in the synthesis of the trinucleotides provided conden-

Table I. Synthesis and Characterization of Protected and Unprotected Oligonucleotides Using TPTE as the Protecting Group

Compound	% Yield[c]	Chromatography (R_f) A[a]	B[a]	C[a]	D[a]	Q[b]	Uv spectra $\epsilon_{260}/\epsilon_{280}$ calcd	found
d-TPTE-pT-T			2.01					
dpT-T	85	0.74		0.90	0.74		1.37	1.34
d-TPTE-pibG-ibG		2.39	2.49			1.8		
dpibG-ibG	68	1.11		1.35		0.6	1.45	1.47
d-TPTE-pbzA-T			2.36					
d-pbzA-T	58	1.32		1.38			0.79	0.82
dpA-T		0.75		0.82				
d-TPTE-pibG-anC						1.8		
dpibG-anC	28	1.37		1.40		0.7	1.10	1.11
dpG-C				0.88	0.30		1.24	1.31
d-TPTE-panC-T			2.22				1.19	1.19
dpanC-T	49	1.09	0.92		1.48		0.88	0.84
dpC-T		0.44			0.95		1.31	1.18
d-TPTE-pT-ibG		3.7	1.93				1.72	1.65
dpT-ibG	45		1.15	1.04	1.38		1.43	1.54
dpT-G				0.75	0.66		1.42	1.56
d-TPTE-pbzA-bzA		3.2	2.4				0.85	0.88
dpbzA -bzA	45	1.52	1.24				0.59	0.64
dpA-A				0.74	1.53		7.0	4.2
dpibG-ibG-anC	15		0.95			0.7	1.13	1.19
dpG-G-C				0.53	0.44		1.32	1.38
dpT-bzA-bzA-ibG	22[d]			0.54			0.86	0.91
dpT-A-A-G				0.31	0.39			
dpT-ibG-bzA-bzA	13[d]			1.20	1.46		1.02	1.11
dpT-G-A-A				0.43	0.55		0.86	1.02

[a] Paper chromatography R_f values with respect to dpT. [b] TLC R_f values with respect to xylene cyanol FF blue. [c] Yields with respect to TPTE mononucleotides. [d] Yields with respect to TPTE dinucleotides.

Table II. Synthesis and Characterization of Protected and Unprotected Oligonucleotides Using TPSE as the Protecting Group

Compound	% Yield[c]	Chromatography (R_f) A[a]	B[a]	C[a]	D[a]	Q[b]	Uv spectra $\epsilon_{260}/\epsilon_{280}$ calcd	found
d-TPSE-pT-T-OAc		1.16	2.21			1.5		
dpT-T	78	0.87	0.88	0.46			1.38	1.39
d-TPSE-pibG-ibG-OAc						1.7		
d-pibG-ibG	45	1.11		1.35		0.6	1.45	1.47
d-TPSE-pT-anC-OAc		1.70	2.30			2.0		
dpT-anC	79	1.39	1.16	1.45			0.90	0.88
dpT-C		0.81	0.71	0.26			1.18	1.18
dpanC-anC	51		0.96				0.72	0.69
dpC-C				0.68	1.06		1.00	1.04
dpibG-ibG-anC	40[d]		0.95	1.42		0.5		
dpG-G-C				0.44			1.32	1.36

[a] Paper chromatography R_f values with respect to dpT. [b] TLC R_f values with respect to xylene cyanol FF blue. [c] Yield calculated with respect to TPSE mononucleotides. [d] Yield calculated with respect to dinucleoside diphosphate.

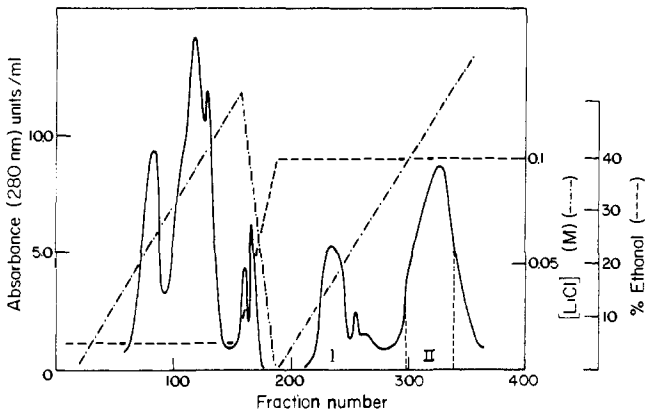


Figure 1. Separation of the reaction products in the condensation of d-TPTE-pibG with dpibG-anC-OAc. A DEAE-cellulose column (75 × 2.5 cm) preequilibrated with 0.02 M Tris hydrochloride (pH 7.5) containing 5% ethanol was used. The gradient elution is as shown in the figure. Peak I contained unreacted d-TPTE-pibG, peak II contained the trinucleotide d-TPTE-pibG-ibG-anC-OAc. Fractions were pooled as shown by the broken vertical lines. Concentration of LiCl (·····); concentration of ethanol (- - - -).

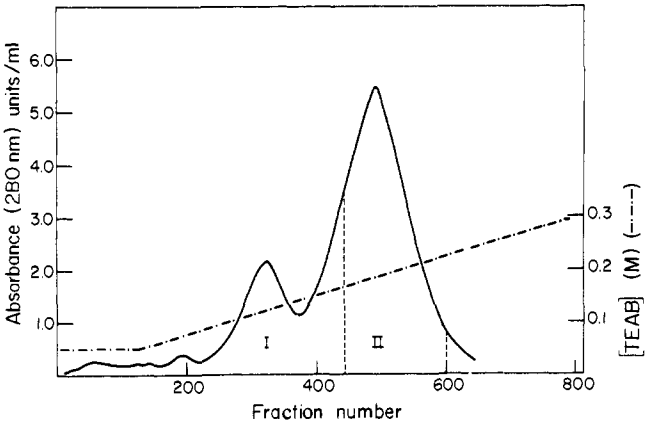


Figure 2. Rechromatography of the reaction products formed in the synthesis of dpT-bzA-bzA-ibG on a DEAE-cellulose column (80 × 1.2 cm) preequilibrated with 0.05 M TEAB containing 10% ethanol. The column was eluted with a TEAB gradient as shown in the figure. Peak II contained the tetranucleotide and was pooled as shown by the broken vertical lines. Concentration of TEAB (·····).

sations were effected with preformed dinucleotide blocks carrying 5′-phosphate group. Thus, the trinucleotide dpibG-ibG-anC was synthesized by condensation of TPSE-pibG with pibG-anC-OAc. The fully protected trinucleotide was isolated by extraction with methylene chloride–1-butanol, and the yield was 40% as based on the starting dinucleotide. The synthesis of the same trinucleotide was also accomplished by condensation of d-TPTE-pibG with the above dinucleotide block. In the work-up, as an alternative to the extraction procedure, it was found that TPTE-containing oligonucleotides are retarded on DEAE-cellulose chromatography (Figure 1) in the same way that monomethoxytrityl-containing oligonucleotides are,[6] such that here d-TPTE-pibG-ibG-anC-OAc (peak II) could be eluted free from other contaminants by linear salt gradient elution in the presence of 40% ethanol.

In the preparation of the tetranucleotide, dpT-bzA-bzA-ibG, the TPTE dinucleotide d-TPTE-pT-bzA was condensed with the dinucleotide dpbzA-ibG-OAc. Organic solvent extraction was satisfactory in separating TPTE dinucleotide from TPTE tetranucleotide, but contamination of the latter with the symmetrical pyrophosphate, O-(pbzA-ibG-OAc)$_2$, necessitated DEAE-cellulose column chroma-

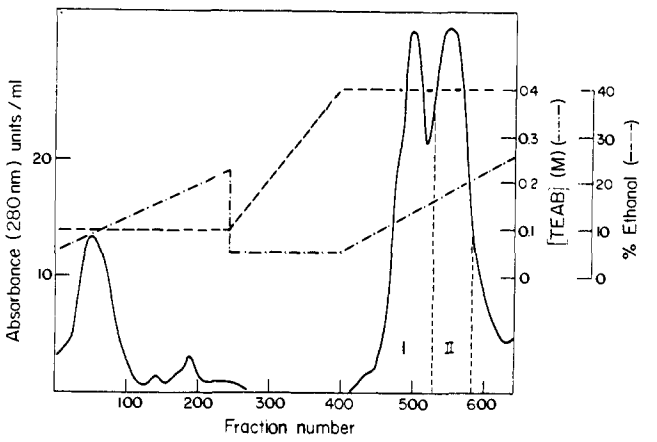


Figure 3. Separation of the reaction products in the condensation of d-TPTE-pT-ibG with d-pbzA-bzA-OAc on a DEAE-cellulose column (95 × 2.8 cm) preequilibrated with 0.05 M TEAB containing 10% ethanol. The gradient elution is as shown in the figure. Peak I contained unreacted d-TPTE-pT-ibG. Peak II contained the tetranucleotide d-TPTE-pT-ibG-bzA-bzA-OAc. Fractions were pooled as shown by the broken vertical lines. Concentration of TEAB (·····); concentration of ethanol (- - -).

tography (Figure 2). This contaminant could be avoided in the analogous preparation of dpT-ibG-bzA-bzA by performing DEAE-cellulose column chromatography *before* removal of the TPTE group (Figure 3). However, in this particular case, TPTE-containing di- and tetranucleotides were not fully separated and further DEAE-cellulose chromatography was performed after removal of the TPTE group.[20]

Comments. From the above, it can be seen that the use of a highly lipophilic, easily removable protecting group offers important advantages over the cyanoethyl and other alternative phosphate protecting groups which have been proposed.[21] The rapid solvent extraction procedure facilitates the large-scale preparation of the dinucleotide blocks which are in demand in increasingly large amounts for the synthesis of large numbers of polynucleotide segments. The present approach can be used for the synthesis of tri- and tetranucleotide blocks although further work will clearly be necessary to simplify the procedures. While trinucleotides were prepared rapidly and in reasonably satisfactory yields, the synthesis of the tetranucleotide blocks requires improvement. An important direction for further work is indicated by the inherent difference between the TPTE and TPSE groups. While the former, because of its alkaline stability, can be used in stepwise condensations in conjunction with the acetyl group at the 3′ end, the TPSE group can only be used by condensing TPSE mononucleotides with preformed oligonucleotide blocks. Since TPSE does offer the advantage of ready removal by a mild alkaline treatment, methods for its use in stepwise condensation clearly necessitate the substitution of the usual acetyl group by a group removable under nonalkaline conditions. Work along this line is in progress.

Experimental Section

Materials and General Methods. TPS, *p*-tritylphenol, and *N,N*-dimethylaminothiocarbamoyl chloride were obtained from Aldrich Chemical Co. (Milwaukee) and 2-chloroethanol from Eastman Chemicals. TPS was freshly crystallized from dry pentane or petroleum ether, and *N,N*-dimethylaminothiocarbamoyl chloride was distilled in vacuo before use. Pyridine was treated with a small amount of chlorosulfonic acid before distillation, redistilled from sodium hydroxide, and stored over predried molecular sieves (Linde 4A).

Condensation reactions were carried out as described previously.[14] DEAE-cellulose columns were run at 4° using triethylammo-

nium bicarbonate (TEAB, pH 7.5) gradients or at room temperature using Tris hydrochloride buffer (pH 7.5) and lithium chloride gradients. Melting points were determined on a capilliary melting point apparatus and are uncorrected. Membrane ultrafiltrations were performed using Amicon Models 2000 or 400S for large scale desalting and Model 8MC for analytical-scale desalting of column fractions. UMO5 membranes were used throughout. Nuclear magnetic resonance spectra were determined on a Varian T-60 spectrophotometer with tetramethylsilane as an internal standard. Infrared spectra were recorded on a Perkin-Elmer Model 567 spectrophotometer.

Paper chromatography was performed by the descending technique on Whatman 1 or 40, and thin-layer chromatography (TLC) was carried out on precoated silica gel plates containing a fluorescent indicator (Eastman 6062). Solvent systems were as described earlier[18] with one addition: solvent Q—2-butanol-water-pyridine (70:50:0.1, v/v). When this system was used, TLC plates were generally prerinsed briefly in 30% aqueous acetone and the acetone allowed to evaporate in an air stream. Plates were run for about 16 hr in an unlined tank and the R_f of compounds compared to markers of xylene cyanol FF blue dye. Despite some irreproducibility of R_f's, the system has excellent resolving power for fully protected oligonucleotides up to 10–11 in chain length.

***O*-*p*-Tritylphenyl *N,N*-Dimethylthiocarbamate (4).** To a stirred solution of *p*-tritylphenol (25.2 g, 75 mmol) in anhydrous tetrahydrofuran (THF, 500 ml) was added sodium hydride (2.4 g, 0.1 mol) in small portions at room temperature. After being refluxed for 4 h, the reaction mixture was cooled to room temperature and treated with a solution of *N,N*-dimethylaminothiocarbamoyl chloride (3.72 g, 60 mmol) in tetrahydrofuran (50 ml). The mixture was stirred at room temperature for 30 min and then heated under reflux overnight. The resulting yellow solution, while still warm (~30°), was added slowly to ice-cold water (1 l.) with stirring. The flocculent white precipitate was filtered off, washed thoroughly with water (1 l.), and dried. The crude product was crystallized from chloroform–petroleum ether (1:2) to give 119 g (94%) of **4**: mp 266–267°; ir ($CHCl_3$) 3060, 2980, 2940, 1590, 1530, 1490, 1440, 1400, 1290, 1180, 1150, 1140 cm^{-1}.

Anal. Calcd for $C_{28}H_{25}SO$: C, 82.15; H, 6.11. Found: C, 82.28; H, 6.04.

***S*-*p*-Tritylphenyl *N,N*-Dimethylthiocarbamate (5).** The above product (**4**) (112 g) was heated slowly on an oil bath under an inert atmosphere to 290° and kept at this temperature for a further 30 min. The amber melt was dissolved in chloroform (600 ml), the solution treated with activated charcoal and concentrated, and the product crystallized from chloroform–petroleum ether (400:1200, v/v) to give 105.9 g (94%) of **5**: mp 185–186°; ir ($CHCl_3$) 3060, 3000, 2940, 1660, 1590, 1490, 1440, 1370, 1260, 1100, 1090 cm^{-1}.

Anal. Calcd for $C_{28}H_{25}SO$: C, 82.15; H, 6.11. Found: C, 83.31; H, 6.19.

***p*-Tritylthiophenol (6).** The above product (**5**) (105 g) was dissolved in tetrahydrofuran (400 ml) and the solution treated with 1 M potassium hydroxide solution (800 ml) prepared in pyridine–methanol–water (65:30:5, v/v). The reaction mixture was heated under reflux overnight and poured into ice-cold glacial acetic acid (2 M; 1200 ml). This was then heated on a steam bath until the solution temperature reached 60°, and the white precipitate which formed was collected by filtration and washed thoroughly with water. The crude product (104 g) was crystallized from 90% aqueous ethanol to give **6** (86 g, 98%): mp 230–232°; ir ($CHCl_3$) 3600, 3060, 3000, 1595, 1490, 1445, 1400, 1200, 1180, 1040, 1020 cm^{-1}.

Anal. Calcd for $C_{25}H_{20}S$: C, 85.23; H, 5.68; S, 9.09. Found: C, 85.16; H, 5.49; S, 8.91.

2-(*p*-Tritylphenyl)thioethanol (TPTE, 1). Route A. *S*-*p*-Tritylphenyl *N,N*-dimethylthiocarbamate (**5**) (34.6 g, 0.077 mol) was dissolved in tetrahydrofuran (180 ml) by heating to reflux. The solution was cooled, then treated with 1 N methanolic potassium hydroxide solution (180 ml; 0.18 mol) with stirring, and the mixture heated under reflux for 12 h under an inert atmosphere. At this point a sample was removed and acidified with 10% acetic acid in tetrahydrofuran. TLC of the sample in ether–petroleum ether (3:7, v/v) showed complete conversion to a new product with R_f 0.8, identical to a marker of **6**. 2-Chloroethanol (18.6 g, 0.23 mol) was then added dropwise over a period of 10 min to the above reaction mixture, when precipitation of potassium chloride was observed. The mixture was further heated under reflux for 1 h and then poured into a conical flask containing 1 l. of chloroform, 1 l. of water, 7 g of ammonium chloride, and 1 kg of crushed ice. Rapid stirring caused partitioning of the reaction products, and after separation of the phases, the aqueous layer was reextracted once with 500 ml of chloroform. The combined organic phase was evaporated to dryness, and the crude product was crystallized from ethanol. The yield of **1** was 29.7 g (71 mmol; 92%; mp 167–168°). An additional crop of crystals (2.25 g, 5.6 mmol, 7%; mp 160–65°) was obtained from the mother liquor. NMR ($CDCl_3$) δ 7.23 (m, 19), 2.77 (m, 2), 3.10 (t, 2); 2.35 (m, 1); ($CDCl_3$ + D_2O) δ 7.23 (m, 19), 3.77 (t, 2), 3.10 (t, 2); ir ($CHCl_3$) 3600, 3080, 3060, 3000, 2940, 1690, 1595, 1480, 1445, 1390, 1060, 1035, 1015 cm^{-1}; uv λ_{max} 263 nm (ϵ 13900), ϵ_{260} 13.150, ϵ_{280} 4650.

Anal. Calcd for $C_{27}H_{24}OS$: C, 81.78; H, 6.10. Found: C, 81.30; H, 6.06.

Route B. *p*-Tritylthiophenol (**6**) (10.56 g, 30 mmol) was treated with *n*-butyllithium (19.2 ml, 36 mmol) in dry tetrahydrofuran (500 ml) for 10 min at room temperature. The solution was cooled to 0° and cold ethylene oxide (12 ml) added. The flask was sealed and allowed to stand for 2 h at room temperature. The solution was concentrated in vacuo to 250 ml and then added to ice-cold water containing acetic acid (10 ml). The precipitate which formed was collected by filtration, washed with water, and crystallized from aqueous ethanol (90%) to give 9.8 g of **1** (82.3%) with mp 167–169°.

2-(*p*-Tritylphenyl)sulfonylethanol (TPSE) (2). TPTE (**1**) (39.6 g, 0.10 mol) was dissolved in dioxane (500 ml) and mixed with an aqueous solution (125 ml) of sodium tungstate dihydrate[18] (105 mg, 0.5 mmol) adjusted to pH 5.5 with acetic acid. The mixture was heated to 70° and 7.3 M hydrogen peroxide (20 ml, 0.146 mol) was added in 5-ml portions. Uptake of peroxide was followed by the starch–iodide test performed on aliquots. After 2.5 h, more hydrogen peroxide (10 ml, 0.073 mol) was added and the reaction allowed to proceed for a further 14 h at 70°. TLC of the reaction mixture in chloroform showed the absence of starting material. Excess peroxide was destroyed by addition of 1 M sodium hydrogen sulfite (20 ml, 0.02 mol) and the solution added to iced water (6 l.) with stirring. The product, which formed a white suspension, was stirred further for 30 min and the resulting precipitate collected by filtration and washed well with water. The crude material (41 g) was crystallized from methanol (2.5 l.) to give **2** (30.5 g, 71%; mp 182–184°). Further crystals were obtained from the mother liquor (8.4 g, 20%): mp 180–183°; NMR ($CDCl_3$) δ 7.47 and 7.80 (d and d, AB quartet, J_{AB} = 8 Hz), 7.23 (m, 15), 4.00 (t, 2), 3.33 (t, 2); ir ($CHCl_3$) 3550, 3080, 3060, 2930, 1590, 1490, 1440, 1310, 1140, 1130 cm^{-1}.

Anal. Calcd for $C_{27}H_{24}O_3S$: C, 75.67; H, 5.64; O, 11.20. Found: C, 75.51; H, 5.60; O, 11.44.

General Procedure for the Preparation of Deoxynucleoside 5′-(2-(*p*-Tritylphenyl)thioethyl) Phosphates. To a dry solution of the deoxynucleoside 5′-phosphate (1 mmol) in pyridine (15 ml) was added TPS (2 mmol) and the solution shaken for 3 min at room temperature. A solution of TPTE (3 mmol) in pyridine (15 ml) was added and the mixture concentrated to 20 ml. After 30 min at room temperature the mixture was checked by TLC in solvent H for the absence of unreacted nucleotide, and then the mixture was concentrated to a thick oil. Chromatography on a neutral silica gel column (Merck 7734) was performed and the column monitored by TLC in solvent H. Washing the column with acetonitrile eluted TPS and TPTE[22] and elution with acetonitrile–water (95:5, v/v) eluted small amounts of side products. The required product was generally eluted with acetonitrile–water (90:10, v/v), pure fractions were pooled and concentrated to an anhydrous pyridine solution, and the product was precipitated by dropwise addition of this solution to an excess of ether–petroleum ether (1:1, v/v). The precipitate was collected by centrifugation, washed 3 times with ether, and dried in vacuo to give the nucleotide TPTE derivative.

Thymidine 5′-(2-*p*-Tritylphenyl)thioethyl) Phosphate (d-TPTE-pT). To a dry solution of pyridinium thymidine 5′-phosphate (800 mg, 2 mmol) in pyridine (30 ml) was added TPS (848 mg, 2.8 mmol) and the solution shaken for 3 min at room temperature. A solution of TPTE (2.4 g, 6 mmol) in pyridine (30 ml) was added, the mixture concentrated to 25 ml, and reaction allowed to proceed for 30 min at room temperature. The product (d-TPTE-pT) was isolated by the procedure described above. The yield was 1.36 g (75%).

N-Anisoyldeoxycytidine 5'-(2-(p-Tritylphenyl)thioethyl) Phosphate d-TPTE-panC). An anhydrous pyridine solution (75 ml) of pyridinium dpanC (4.0 mmol) was treated with TPS (12.4 g, 8.0 mmol) for 3 min at room temperature. A dry solution of TPTE (4.6 g, 12 mmol) in pyridine (75 ml) was added, the reaction mixture concentrated to 30 ml, and reaction allowed to proceed for 2 h at room temperature. The product (d-TPTE-pbzA) was isolated by the standard procedure described above. The yield was 2.9 g (70%).

N-Benzoyldeoxyadenosine 5'-(2-(p-Tritylphenyl)thioethyl) Phosphate (d-TPTE-pbzA). To a dry solution of pyridinium dpibG (1.0 mmol) in pyridine (16 ml) was added TPS (606 mg, 2 mmol) and the solution shaken for 3 min at room temperature. A solution of TPTE (2.4 g, 6 mmol) in pyridine (35 ml) was added, the reaction mixture concentrated to 20 ml, and the reaction allowed to proceed for 45 min at room temperature. The product (d-TPTE-pbzA) was isolated by the standard procedure described above. The yield was 2.65 g (65%).

N-Isobutyryldeoxyguanosine 5'-(2-(p-Tritylphenyl)thioethyl) Phosphate (d-TPTE-pibG). To a dry solution of pyridinium dpibG (1 0 mmol) in pyridine (16 ml) was added TPS (606 mg, 2 mmol) and the solution shaken for 3 min at room temperature. A solution of TPTE (885 mg, 3 mmol) in pyridine (16 ml) was added, the reaction mixture concentrated to 10 ml, and reaction allowed to proceed for 30 min at room temperature. The product (d-TPTE-pibG) was isolated by the standard procedure described above. The yield was 1.4 g (70%).

Synthesis of Dinucleotides Using TPTE as the Protecting Group. Dinucleotide dpT-T. An anhydrous pyridine solution of d-TPTE-dpT (744 mg, 1 mmol) and pyridinium dpT-OAc (528 mg, 1.2 mmol) was kept for 3 h at room temperature in the presence of TPS (726 mg). A 1 N solution of diisopropylethylamine in pyridine (6.5 ml) and water (6.5 ml) was added with cooling and the solution kept at room temperature overnight. After concentration and addition of TEAB (0.05 M; 25 ml) the solution was extracted with ethyl acetate in a continuous solvent–solvent extractor. Traces of d-TPTE-pT present in the aqueous solution were removed by further extraction with ethyl acetate–1-butanol (9:1, 2 × 50 ml). The aqueous TEAB solution was then extracted with ethyl acetate–1-butanol (70:30, 3 × 40 ml), the combined organic extracts were concentrated to an anhydrous pyridine solution, and the product (d-TPTE-pT-T-OAc) was precipitated by dropwise addition to an excess of dry ether (yield: 903 mg).

The above product was dissolved in dioxane–1M Tris hydrochloride, pH 7.5 (9:1, v/v; 60 ml), and *N*-chlorosuccinimide (1.32 g) was added. The solution was kept at room temperature for 10 min and the reaction followed by TLC in solvent G. Pyridine (10 ml) was added and the mixture allowed to stand for 30 min before being concentrated and extracted continuously with ethyl acetate. The aqueous TEAB solution (30 ml) was evaporated to a pyridine solution (15 ml), ethanol (15 ml) added and then the solution treated with 2 N sodium hydroxide solution (30 ml) for 5 min at 0°. After neutralization with Dowex-50 (pyridinium form) and removal of the resin by filtration, the resulting aqueous solution was concentrated to an anhydrous pyridine solution[23] and the product was precipitated by dropwise addition to an excess of dry ether. The yield of dpT-T was 85%.

The Dinucleotide d-pibG-ibG. An anhydrous pyridine solution (15 ml) of d-TPTE-pibG (910 mg, 1 mmol), pyridinium d-pibG-OAc (1.08 g, 2 mmol), and TPS (910 mg, 3 mmol) was kept for 3 h at room temperature. The reaction was terminated by the standard procedure and kept at room temperature overnight. The solution was concentrated and dissolved in TEAB (0.05 M; 50 ml), and the solution extracted with ethyl acetate in a continuous solvent–solvent extractor. The aqueous TEAB solution was further extracted with ethyl acetate–1-butanol (70:30, 3 × 50 ml), the combined organic extracts were concentrated to an anhydrous pyridine solution, and the product was precipitated by dropwise addition to an excess of dry ether. The yield of d-TPTE-pibG-ibG-oAc was 1.1 g. The TPTE group was removed as described for d-pT-T to yield the dinucleotide dpibG-ibG (68%).

The Dinucleotide d-pbzA-T. An anhydrous pyridine solution (10 ml) of d-TPTE-pbzA (810 mg, 0.8 mmol) and pyridinium d-pT-oAc (400 mg, 1 mmol) was allowed to react for 3 h at room temperature in the presence of TPS (606 mg, 2 mmol). The reaction was terminated by the standard procedure and the mixture kept at room temperature overnight. The solution was concentrated and dissolved in TEAB (0.05 M; 30 ml) and the solution extracted continuously with ethyl acetate. The aqueous TEAB solution was extracted with ethyl acetate–1-butanol (7:3, 2 × 75 ml), the combined organic extracts were concentrated to an anhydrous pyridine solution, and the product was precipitated by dropwise addition to an excess of anhydrous ether to give d-TPTE-pbzA-T-oAc (903 mg). The TPTE group was removed as described for dpT-T. The yield of the dinucleotide, dpbzA-T, was 58%.

The Dinucleotide dpibG-anC. An anhydrous pyridine solution (50 ml) of d-TPTE-pibG (2.29 g, 2.6 mmol) and d-panC-oAc (2.86 g, 5.4 mmol) was kept for 5 h at room temperature in the presence of TPS (3.84 g, 12.7 mmol). The reaction was terminated by the standard procedure and the mixture allowed to stand at room temperature for 16 hr. The solution was concentrated and dissolved in Tris hydrochloride (0.02 M; pH 7.5; 250 ml) and the solution extracted continuously with ethyl acetate. A trace of d-TPTE-pibG present in the aqueous solution was removed by further extraction with ethyl acetate containing 3, 5, and 8% 1-butanol successively (100 ml each). The dinucleotide was extracted from the aqueous solution with ethyl acetate–1-butanol (7:3, v/v), the combined organic extracts were concentrated by repeated addition of pyridine to give an anhydrous pyridine solution, and the product was precipitated by dropwise addition to an excess of dry ether. The yield of d-TPte-pibG-anC-oAc was 1.4 g.

The TPTE group was removed from the dinucleotide by the following procedure. The dinucleotide was dissolved in dioxane–0.5 M Tris hydrochloride (pH 7.5, 8:2 v/v; 300 ml) and the solution treated with *N*-chlorosuccinimide (2.5 g, 18.7 mmol). After 15 min at room temperature TLC showed the presence of a small amount of starting material. A further amount (1 g) of *N*-chlorosuccinimide was added and the oxidation allowed to proceed for a further 10 min.

At this point TLC of the reaction mixture showed the absence of starting material. Pyridine (20 ml) was added and the mixture allowed to stand for 30 min before concentration to 50 ml to remove most of the dioxane. The solution was "diafiltered" in an Amicon ultrafiltration system fitted with a UMO5 membrane, using pyridine–dioxane–water (2:20:78) as the wash solution. Complete removal of succinimide was followed by TLC in solvent H. The TLC plate was exposed to iodine vapors, and, among other spots, a white spot at the origin showed the presence of succinimide. The retentate was evaporated to 30 ml in the presence of pyridine and the solution treated with 2 N sodium hydroxide solution in the usual way at 0° for 10 min. After standard work-up the aqueous solution (250 ml) was extracted with ethyl acetate–1-butanol (8:2, v/v; 3 × 250 ml) and the aqueous layer concentrated to an anhydrous pyridine solution. Product was precipitated by dropwise addition to an excess of dry ether. The yield of d-pibG-anC was 28%.

A General Procedure for Preparation of Deoxynucleoside 5'-(2-(p-Tritylphenyl)sulfonylethyl) Phosphate. To an anhydrous pyridine solution (5 ml) of the N-protected mononucleotide (1 mmol) was added TPSE (2 mmol), pyridinium Dowex 50 (1 g), and DCC (10 mmol) and the reaction mixture shaken in the dark at room temperature for 16–24 h. The reaction was followed by TLC in solvent G. Water (5 ml) was added, the reaction mixture kept at room temperature overnight before being filtered, and the precipitate washed with 50% aqueous pyridine. The filtrate and washings were combined and extracted with diisopropyl ether (3 × 100 ml) to remove TPSE. The aqueous solution was concentrated, dissolved in 0.2 M TEAB, and extracted twice with ethyl acetate–1-butanol (8:20, v/v). The combined organic extracts were back-washed once with 0.2 M TEAB and the organic layer was concentrated to an anhydrous pyridine solution and product precipitated by dropwise addition to an excess of ether–petroleum ether (1:1, v/v). Yields ranged from 70 to 80%.

Thymidine 5'-(2-(p-Tritylphenyl)sulfonylethyl) Phosphate (d-TPSE-pT). An anhydrous pyridine solution (3 ml) of pyridinium thymidine 5'-phosphate (0.5 mmol), TPSE (1 mmol), pyridinium Dowex-50 (1 g), and DCC (5 mmol) was shaken in the dark at room temperature overnight. After the usual work-up (see general procedure), the product was isolated by precipitation into anhydrous ether. The yield of d-TPSE-pT was 79%.

N-Benzoyldeoxyadenosine 5'-(2-(p-Tritylphenyl)sulfonylethyl) Phosphate (d-TPSE-pbzA). Pyridinium d-pbzA (0.5 mmol), TPSE (1 mmol), pyridinium Dowex-50 (1 g), and DCC (5 mmol) was

dissolved in dry pyridine (3 ml) and the solution shaken in the dark for 20 h at room temperature. The reaction was terminated and worked up by standard procedure to give d-TPSE-pbzA (74%).

***N*-Isobutyryldeoxyguanosine 5'-(2-(*p*-Tritylphenyl)sulfonylethyl) Phosphate (d-TPSE-pibG).** An anhydrous pyridine solution (3 ml) of pyridinium d-pibG (0.5 mmol), TPSE (1 mmol), and pyridinium Dowex-50 (0.8 g) was shaken in the presence of DCC (5 mmol) for 22 h at room temperature in the dark. After the usual work-up, product was isolated by standard procedure to give d-TPSE-pibG (75%).

***N*-Anisoyldeoxycytidine 5'-(2-(*p*-Tritylphenyl)sulfonylethyl) Phosphate (d-TPSE-panC).** An anhydrous pyridine solution (250 ml) of pyridinium d-panC (10 mmol) and TPSE (20 mmol) was allowed to react for 5 h at room temperature in the presence of TPS (20 mmol). After the usual work-up, product was purified by silica gel column chromatography as described for d-TPTE-pT. The desired product was precipitated by dropwise addition of its pyridine solution into an excess of dry ether. The yield of d-TPSE-panC was 50%.

Synthesis of Dinucleotides Using TPSE as the Protecting Group. Dinucleotide d-pT-T. An anhydrous pyridine solution (2.0 ml) of pyridinium d-pT-oAc (0.2 mmol) and d-TPSE-pT (0.1 mmol) was kept in the presence of TPS (0.4 mmol) for 5.5 h at room temperature. After the usual work-up, the aqueous pyridine solution was concentrated, dissolved in 0.2 M TEAB, and extracted with ethyl acetate in a continuous solvent-solvent extractor to remove unreacted d-TPSE-pT. The aqueous phase was extracted with methylene chloride–1-butanol (7:3; 3 × 5 ml). The combined organic extracts were washed with TEAB (0.2 M, 2 × 3 ml) and concentrated to an anhydrous pyridine solution and product precipitated by dropwise addition to an excess of dry ether. The yield of d-TPSE-pT-T-oAc was 78%.

The TPSE group was removed by treatment of a solution of the dinucleotide in pyridine–ethanol (1:1, v/v; 1.6 ml) with 2 N sodium hydroxide solution (1.6 ml) at 0° for 5 min. The reaction mixture was then neutralized with excess pyridinium Dowex-50 and extracted with methylene chloride–1-butanol (7:3) to remove TPSE, and after concentration of the aqueous phase to an anhydrous pyridine solution, product was precipitated by dropwise addition to an excess of dry ether. Yield of dpT-T was 78%.

The Dinucleotide d-pT-anC. An anhydrous pyridine solution (10 ml) of d-TPSE-pT (1.16 mmol) and pyridinium d-panC-oAc (2.32 mmol) was kept in the presence of TPS (4.64 mmol) for 5.5 h at room temperature. After the usual work-up, the desired dinucleotide was isolated by the same solvent extraction and precipitation procedure as described for d-TPSE-pT-T-oAc. The yield of the dinucleotide d-TPSE-pT-anC-oAc was 79%.

The TPSE group was removed from the dinucleotide by treatment with 2 N sodium hydroxide solution and product isolated as described above. The yield of d-pT-anC was 79%.

The Dinucleotide dpanC-anC. An anhydrous pyridine solution (45 ml) of d-TPSE-panC (4.4 mmol) and pyridinium d-panC-oAc (8.8 mmol) was kept in the presence of TPS (22 mmol) for 5.5 h at room temperature. After the usual work-up and continuous extraction procedure with ethyl acetate as described above, the dinucleotide was extracted from the aqueous solution with methylene chloride–1-butanol (8.5:1.5, v/v; 3 × 100 ml) and the combined organic extracts were back-washed with TEAB (0.2 M; 2 × 50 ml). The organic phase was concentrated in the presence of pyridine, taken up into pyridine–ethanol (1:1, v/v; 70 ml), and treated with 2 N sodium hydroxide solution as described for d-TPSE-pT-T-OAc. Product was precipitated by dropwise addition of its anhydrous pyridine solution into an excess of dry ether. Yield of d-panC-anC was 51%.

The Dinucleotide dpibG-ibG. TPSE-dpibG (0.2 g, 0.3 mmol) and d-pibG-oAc (0.3 g, 0.62 mmol) were treated with TPS (440 mg, 1.46 mmol) in anhydrous pyridine (5 ml) for 5.75 h at room temperature. After the usual work-up, the dinucleotide was isolated by the same solvent extraction, alkali treatment, and precipitation procedure as described for d-pT-T. The yield of the dinucleotide dpibG-ibG was 45%.

Synthesis of a Trinucleotide using TPSE as the Phosphate Protecting Group. The Trinucleotide dpibG-ibG-anC. d-TPSE-pibG (2.25 g, 2.5 mmol) and dpibG-anC-oAc (0.942 g, 0.85 mmol) were treated with TPS (1.46 g, 4.8 mmol) in pyridine (20 ml) for 5 h at room temperature. The reaction was quenched and the mixture worked up in the usual way. Excess TPSE mononucleotide was removed by continuous extraction with ethyl acetate overnight followed by one manual extraction with ethyl acetate–1-butanol (80:20). The TPSE trinucleotide was then extracted from the remaining aqueous solution into dichloromethane–1-butanol (9:1). The organic phase was evaporated to an anhydrous pyridine solution (20 ml) and product precipitated by dropwise addition to ether (1500 ml). The yield of d-TPSE-pibG-ibG-anC-oAc was 0.73 g (44%).

The TPSE and acetyl groups were removed by alkaline treatment under standard conditions and product precipitated as described above. The yield of dpibG-ibG-anC was 0.53 g (40%, based on dinucleoside diphosphate).

Synthesis of Tri- and Tetranucleotides Using TPTE as the Phosphate Protecting Group. The Trinucleotide dpibG-ibG-anC. d-TPTE-ibG (409 mg, 0.45 mmol) and d-pibG-anC-oAc (825 mg, 0.65 mmol) were kept in the presence of TPS (730 mg, 2.3 mmol) in anhydrous pyridine (5 ml) for 6 h at room temperature. The reaction was terminated and the mixture allowed to stand overnight. This was concentrated to an oil, diluted with Tris hydrochloride (0.02 M; 100 ml; pH 7.5), and extracted continuously with diisopropyl ether. The aqueous solution was chromatographed on DEAE-cellulose (Cl^- form; 2.4 × 75 cm) using a linear gradient of lithium chloride as shown in Figure 1. Peak II contained the desired trinucleotide and pure fractions were pooled and desalted by membrane filtration. Product was precipitated from its anhydrous pyridine solution by dropwise addition to an excess of dry ether. The yield of d-TPTE-pibG-ibG-anC-oAc was 27%.

A solution of this product in dioxane–0.4 M Tris hydrochloride, pH 7.5 (20 ml; 15:5, v/v), was treated with *N*-chlorosuccinimide (170 mg, 1.2 mmol) for 25 min at room temperature. Pyridine (2 ml) was added and after a further 20 min the solution was "diafiltered" as described previously. The retentate contained the trinucleotide and was concentrated in the presence of pyridine, made up to pyridine–ethanol (1:1, 10 ml), and treated with 2 N sodium hydroxide solution (10 ml) at 0° for 5 min. After usual work-up, the aqueous pyridine solution was extracted with ethyl acetate–1-butanol (3:1, v/v; 3 × 100 ml) and product precipitated form its anhydrous pyridine solution by dropwise addition to an excess of dry ether. The yield of dpibG-ibG-anC was 15% overall.

The Tetranucleotide d-pT-bzA-bzA-ibG. d-TPTE-pT-bzA (700 mg, 0.5 mmol) and d-pbzA-ibG-oAc (784 mg, 0.7 mmol) were kept in the presence of TPS (1.1 g, 3.5 mmol) in anhydrous pyridine (10 ml) for 5 h at room temperature. The reaction was terminated, and after the usual work-up and continuous ethyl acetate extraction, the aqueous TEAB solution was concentrated to an oil in the presence of pyridine, dissolved in TEAB (0.05 M; 30 ml), and extracted with ethyl acetate–1-butanol (3:2, v/v; 4 × 50 ml). The aqueous phase was then extracted with methylene chloride–1-butanol (7:3, v/v; 3 × 75 ml). Although the desired tetranucleotide was extracted into the organic phase, it still contained a trace of d-TPTE-pT-bzA. Attempts to purify this mixture further by organic solvent extraction failed, and the product was precipitated from its anhydrous pyridine solution by dropwise addition to an excess of dry ether. The precipitate was dissolved in dioxane–0.2 M Tris hydrochloride (40 ml; 1:1, v/v) and the solution treated with *N*-chlorosuccinimide (600 mg, 5 mmol) for 10 min at 0°. Pyridine (20 ml) was added, and after 20 min at room temperature the solution was concentrated and chromatographed twice on a DEAE-cellulose column (bicarbonate form; 1.6 × 85 cm) using TEAB as the eluting buffer. Peak II of the second column (Figure 2) contained the desired tetranucleotide dpT-bzA-bzA-ibG (22%).

The Tetranucleotide d-pT-ibG-bzA-bzA. To an anhydrous solution of d-TPTE-pT (4.7 g, 6.7 mmol) and d-pibG-oAc (5.8 g, 12.0 mmol) was added TPS (7.7 g, 25.4 mmol) and the solution concentrated to 100 ml and kept at room temperature for 5.33 h. The reaction was terminated by the standard procedure, and after the usual work-up and continuous extraction with ethyl acetate, the aqueous solution was further extracted with ethyl acetate–1-butanol (93:7, v/v). The aqueous TEAB solution, which still contained a trace of d-TPTE-pT, was extracted with ethyl acetate–1-butanol (75:25, v/v) and the organic phase concentrated in the presence of pyridine, dissolved in pyridine–ethanol (1:1), and treated with 2 N sodium hydroxide solution at 0° for 5 min. After standard work-up, the solution was concentrated to an oil and dissolved in TEAB (0.1 M; 200 ml). The solution was extracted with several portions

of ethyl acetate containing increasing amounts of 1-butanol (5–10%) which removed final traces of d-TPTE-pT. The desired dinucleotide was then extracted into ethyl acetate–1-butanol (7:3, v/v), the organic phase concentrated to an anhydrous pyridine solution, and the product precipitated by dropwise addition in an excess of dry ether. The yield of d-TPTE-pT-ibG was 45% (4.7 g).

To an anhydrous pyridine solution (30 ml) of this dinucleotide (2.7 g, 1.7 mmol) and d-pbzA-bzA-oAc (2.04 g, 2.0 mmol) was added TPS (2.7 g, 8.9 mmol) and the solution concentrated to 25 ml. After 6 h at room temperature, the reaction was terminated, and after usual work-up the product was chromatographed on a DEAE-cellulose column (bicarbonate form; 2.8 × 95 cm) eluted with gradients of TEAB. Peak II (Figure 3) contained the tetranucleotide which was concentrated to an anhydrous pyridine solution and product precipitated by dropwise addition to an excess of dry ether. The yield of d-TPTE-pT-ibG-bzA-bzA-oAc was 28% (0.47 mmol).

The above tetranucleotide (1.22 g, 0.46 mmol) was dissolved in dioxane–0.2 M Tris hydrochloride (pH 7.5; 1:1, v/v; 60 ml) and treated with *N*-chorosuccinimide (710 mg, 5.3 mmol) for 18 min. Further addition of *N*-chlorosuccinimide (240 mg, 1.8 mmol) was necessary in order to obtain complete oxidation after a further 10 min. Pyridine (18 ml) was added and the mixture left at room temperature for 15 min. After treatment with sodium hydroxide solution in the usual way, the neutralized solution was concentrated to an anhydrous pyridine solution (30 ml) containing diisopropylethylamine (3 ml). This was added dropwise to an excess of dry ether to precipitate the tetranucleotide (800 mg), which was further chromatographed on a DEAE-cellulose column (95 × 2.8 cm; bicarbonate form). The product (column pattern not shown) was eluted with an increasing linear gradient of TEAB (3 l. each of 0.05 and 0.30 M TEAB in 10% ethanol). Fractions corresponding to the tetranucleotide were pooled and concentrated to an anhydrous pyridine solution (25 ml) and the mixture acetylated with acetic anhydride (8 ml) in the presence of diisopropylethylamine (1 ml) under standard conditions. Following normal work-up and isolation, d-pT-ibG-bzA-bzA-oAc was obtained in 13% yield based on d-TPTE-pT-ibG.

References and Notes

(1) The preceding paper in this series (CXLII) is B. Ramamoorthy, R. G. Lees, D. G. Kleid, and H. G. Khorana, *J. Biol. Chem.*, in press.

(2) This work has been supported by grants from the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service (Grant No. CA11981), the National Science Foundation, Washington, D.C. (Grant No. GB-21053X, GB-36881X, and BMX73-06757), the American Cancer Society (Grant No. NP-140), and by funds made available to the Massachusetts Institute of Technology by the Sloan Foundation.

(3) (a) Department of Biochemistry, University of Chicago, Chicago, Ill. 60637; (b) Shemyakin Institute of Bioorganic Chemistry, U.I. Vavilova 32, Moscow, 117312, U.S.S.R.; (c) Department of Biology, Harvard University, Cambridge, Mass. 02138, NIH Postdoctoral Fellow 1972–1974; (d) The Danish Institute of Protein Chemistry, Copenhagen, Denmark; (e) recipient of a postdoctoral fellowship from N.A.T.O; (f) recipient of a postdoctoral fellowship from the Damon Runyon Memorial Fund for Cancer Research, Inc.

(4) H. G. Khorana, *Enzymes* **5**, 79–94 (1961).

(5) H. G. Khorana, *Proc. Int. Congr. Biochem., 7th, 1967*, 17 (1968).

(6) H. G. Khorana, K. L. Agarwal, H. Büchi, M. H. Caruthers, N. K. Gupta, K. Kleppe, A. Kumar, E. Ohtsuka, U. L. RajBhandary J. H. van de Sande, V. Sgaramella, T. Terao, H. Weber, and T. Yamada, *J. Mol. Biol.*, **72**, 209 (1972).

(7) H. G. Khorana, K. L. Agarwal, P. Besmer, H. Büchi, M. H. Caruthers, P. J. Cashion, M. Fridkin, E. Jay, K. Kleepe, R. Kleepe, A. Kumar, P. C. Loewen, R. C. Miller, K. Minamoto, A. Panet, U. L. RajBhandary, B. Ramamoorthy, T. Sekiya, T. Takeya, and J. H. van de Sande, *J. Biol. Chem.*, in press.

(8) P. C. Loewen, T. Sekiya, and H. G. Khorana, *J. Biol. Chem.*, **249**, 217 (1974).

(9) T. Sekiya and H. G. Khorana, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 2978 (1974).

(10) K. L. Agarwal, Y. A. Berlin, D. G. Kleid, V. D. Smirnov, and H. G. Khorana, *J. Biol. Chem.*, in press.

(11) D. G. Kleid, K. L. Agarwal, and H. G. Khorana, *J. Biol. Chem.*, in press.

(12) K. L. Agarwal, A. Yamazaki, P. J. Cashion, and H. G. Khorana, *Angew. Chem., Int. Ed. Engl.*, **11**, 451 (1972).

(13) P. J. Cashion, M. Fridkin, K. L. Agarwal, E. Jay, and H. G. Khorana, *Biochemistry*, **12**, 1985 (1973).

(14) K. L. Agarwal, A. Yamazaki, and H. G. Khorana, *J. Am. Chem. Soc.*, **93**, 2754 (1971).

(15) K. L. Agarwal, M. Fridkin, E. Jay, and H. G. Khorana, *J. Am. Chem. Soc.*, **95**, 2020 (1973).

(16) M. S. Newman and H. A. Karnes, *J. Org. Chem.*, **31**, 3980 (1966).

(17) H. S. Schultz, H. G. Freyermuth, and S. K. Buc, *J. Org. Chem.*, **28**, 1140 (1963).

(18) K. L. Agarwal and H. G. Khorana, *J. Am. Chem. Soc.*, **94**, 3578 (1972).

(19) The system of abbreviation for polynucleotides follows recommendations of IUPAC–IUB Commission of Biochemical nomenclature [*Eur. J. Biochem.*, **15**, 203–208 (1970)]. The protected oligonucleotides are abbreviated according to the suggestions made by Waldo E. Cohn, NAS–NRC Office of Biochemical Nomenclature, and have been defined previously by Schaller and Khorana [*J. Am. Chem. Soc.*, **85**, 3841 (1963)]. TEAB is triethylammonium bicarbonate. DIEA is diisopropylethylamine.

(20) The tetranucleotide dpanC-anC-anC-anC has been recently prepared by the TPTE method, and in this case the TPTE tetranucleotide could be isolated by a DEAE-cellulose column free from contaminants.

(21) For example, references quoted in ref. 15.

(22) Unreacted TPTE could be recovered in good yield by evaporation of the acetonitrile solution to dryness followed by dissolution in benzene. After washing twice with 0.1 M NaOH solution, the organic phase was dried over sodium sulfate and product crystallized from benzene–petroleum ether or 90% aqueous ethanol.

(23) If at the stage the product is not entirely soluble in a few milliliters of dry pyridine the mixture should be diluted with 0.05 M TEAB solution (250 ml) and passed on to a DEAE cellulose column (5 × 3 cm) pre-equilibrated in this solvent. Product can be eluted with 0.5 M TEAB and the solution taken to an anhydrous pyridine solution as before.