Improved Triester Approach for the Synthesis of Pentadecathymidylic Acid[1]

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Abstract: The synthesis of a pentadecanucleotide of thymidine has been achieved by a modified phosphotriester method which produces significantly improved yields and allows for potentially large scale manipulation. The key step is the use of a fully protected mononucleotide such as 5′-*O*-monomethoxytritylthymidine-3′-*p*-chlorophenyl β-cyanoethyl phosphate to eliminate phosphorylation and thus simplify purification of the product. Extension of the chain length from the 3′ toward the 5′ end produces improved yield.

Despite the fact that the synthesis of polynucleotides of reasonable size (12–20 units) has been achieved by the classical diester method,[1,3] there remain some definite practical limitations in this approach, such as low yields using stoichiometric amounts of each reactant, especially with longer chain length substrates and laborious and time-consuming isolation procedures. In this paper, we report an improved triester approach for an efficient synthesis of thymidine pentadecamer. The following paper[4] will describe the development of various condensing and phosphorylating reagents during the present studies and their application to the synthesis of polynucleotides of defined sequence.

The triester method as originally reported[5,6] and subsequently employed[7-10] involves the phosphorylation of the 3′-hydroxyl group of a 5′-protected mononucleoside (**1**) followed by condensation with the 5′-primary hydroxyl group of an incoming 3′-protected nucleoside. Triisopropylbenzenesulfonyl chloride (TPS)[11] has been used exclusively as the condensing reagent. Since each internucleotidic bond in the desired product is present as a triester function, purification by the more conventional technique of organic solvent extraction and simple column chromatography on silica gel becomes possible. However, we observed that the initial phosphorylation of the 3′-hydroxyl group of 5′-protected mononucleoside **1** did not give a pure 3′-phosphodiester component in quantitative yield, thus the addition of the 3′-protected mononucleoside led to a complicated reaction mixture. These mixtures could not be resolved quantitatively using ordinary silica gel chromatography to yield the desired product. To overcome this problem, we modified the accepted triester approach "two step" reaction, i.e., phosphorylation and subsequent coupling. The basic feature of our approach is to start the synthesis of an oligonucleotide from the fully protected mononucleotide **4** containing a fully masked 3′-phosphate group. Since each intermediate oligonucleotide synthesized thus contained the fully masked 3′-phosphate group, the necessity for a phosphorylation step at each condensation stage was eliminated, thus simplifying the approach. A preliminary communication of this work has already appeared.[12] A similar modification has also been employed by Cramer et al.[13]

Our initial choices for protecting groups included monomethoxytrityl[14] for the 5′-hydroxyl function of thymidine as an acid labile group, phenyl[5,7] for the internucleotidic phosphate bond as an alkaline labile group, and finally β-cyanoethyl also for terminal phosphate because of its known extremely labile nature with alkali.[15] Thus phosphorylation of the 3′-hydroxyl of **1** was accomplished with phenyl phosphate in the presence of triisopropylbenzenesulfonyl chloride (TPS). After a reaction period of 8 hr, excess β-cyanoethanol was added along with additional TPS. The fully protected mononucleotide **4** was isolated by chromatography on silica gel with chloroform–methanol as eluting solvent in 75–85% yield. Treatment of **4** with 80% acetic acid afforded the 5′-hydroxy, 3′ fully protected intermediate **5**, whereas a mild alkaline treatment (0.05 *N* sodium hydroxide, dioxane–water 4:1 v/v) selectively removed the β-cyanoethyl group to give a 5′-protected nucleoside 3′-phenyl phosphate **3**. Condensation of these two components in stoichiometric amounts in the presence of TPS produced dinucleotide **6**. The reaction mixture from this and each condensation was analyzed by silica gel TLC using chloroform with 1–10% (v/v) methanol as developing solvent. It was always observed that the required product, i.e., **6**, appeared as an elongated spot completely separated from and with higher R_f value than those of the other components of the reaction mixture **3** and **5**. Unreacted **5** was strongly adsorbed because of the free hydroxyl group, but **3** was even more strongly held because of its negative charge. After the usual work-up, the reaction mixture was submitted to silica gel column chromatography, and the desired product was separated cleanly and completely from the starting as well as the side components (for yields see Table I). Fractions from each column chromatography was easily monitored by silica gel TLC, and it was usually found that the elongated spot was partially resolved into a couple of overlapping spots, each giving a positive test for the trityl group. Since these spots gave the same product on complete deblocking, one might surmise they represented simply diastereoisomeric phosphate esters. A similar observation has also been reported by the earlier workers.[16]

The dinucleotide **6** on mild alkali treatment and further condensation with **5** gave the fully protected trinucleotide **8** (see Figure 1). By extending the chain from the 5′ end toward the 3′ end as outlined in Figure 2, the synthesis of fully protected nonanucleotide **12** was achieved in reasonable yield using approximately stoichiometric amounts of each component. The results are given in Table I. For the synthesis of chain lengths larger than hexanucleotide, two problems became apparent: (i) removal of 3′-terminal β-cyanoethyl group required increasingly stronger alkaline conditions which caused partial removal of the phenyl groups from the internucleotide phosphate functions; (ii) complete removal of the phenyl groups from the triester bonds became increasingly difficult with the increase in the chain length.

Our first attempt to alleviate these deblocking problems was to investigate new protecting groups which could be removed more selectively. The trichloroethyl[17] group, which requires specific deblocking conditions, has been used to re-

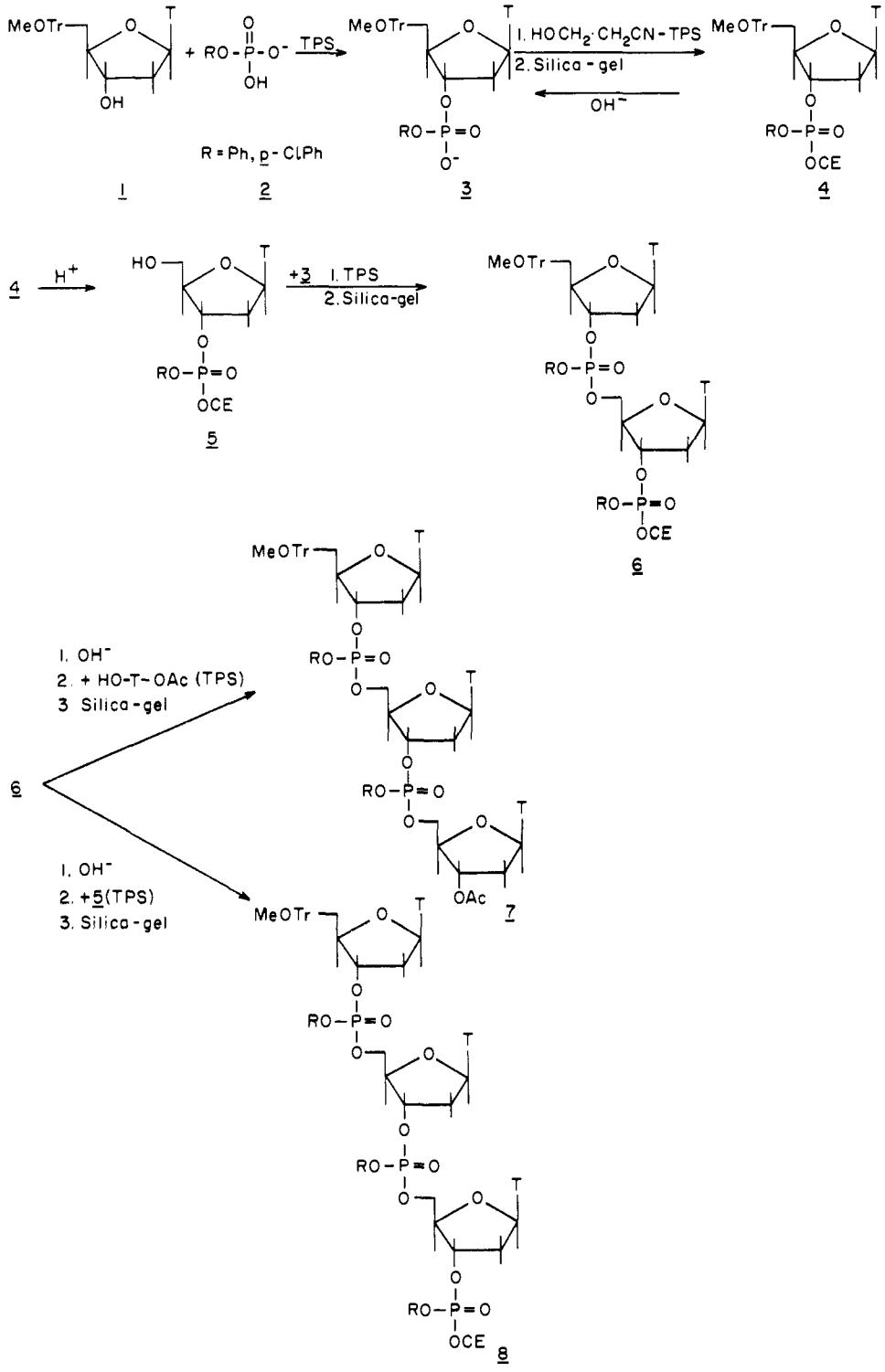

Figure 1. Synthesis of fully protected trinucleotides.

place the β-cyanoethyl group. We tried tribromoethyl[18] as the 3′-phosphate protecting group and found that extremely mild conditions, i.e., Zn-Cu alloy in DMF for 10 min at room temperature, were required to liberate the 3′-terminal phosphodiester group. These conditions were much milder than any reported to date. Again beyond the hexamer stage, quantitative deblocking was difficult, and furthermore various products were observed in the case of heterosequences. Finally we overcame this problem by extending the oligonucleotide chain in the opposite direction instead of the conventional way, i.e., from the 3′ end toward the 5′ end as outlined in Figure 3. This approach required the deblocking of an acid labile protecting group, i.e., monomethoxytrityl group from the 5′ end instead of a β-cyanoethyl group from the 3′ end before each condensation step which was achieved quantitatively. As alternative aryl groups for protection of the internucleotide phosphate, we investigated various substituted phenyl groups such as: (a) a *p*-nitrophenyl group, which was found to be too labile to remove the β-cyanoethyl group selectively; (b) a *p*-methylthiophenyl group, which was found to be quite satisfactory during the coupling reaction, but whose complete removal with alkali after oxidation to sulfoxide was found to be difficult. Finally, we found the *p*-chlorophenyl group to be quite suitable

Table I. Reaction Conditions and the Yields by Triester Approach

3′-Phosphodiester component[a]	Amount, mmol	5′-Hydroxyl component	Amount, mmol	TPS, mmol	Products	Product yields, %	Solvent for column chloroform–methanol	Completely deblocked[b] compd[c] Identity	Yield, %
[MeOTr] T-ClPh	22	T∓CE	20	45	[MeOTr] T∓T∓CE	75	40:1		
[MeOTr] T-ClPh	4.5	T(OAc)	4	9	[MeOTr] T∓T(OAc)	85	40:1	T_2	90
[MeOTr] T-ClPh	11	T∓T∓CE	10	22	[MeOTr] T∓T∓T∓CE	71	30:1		
[MeOTr] T-ClPh	2.2	T∓T(OAc)	2	4.5	[MeOTr] T∓T∓T(OAc)	74	30:1	T_3	86
[MeOTr] T∓T∓T-ClPh	5	T∓T∓T∓CE	4	10	[MeOTr] T∓T_4∓T∓CE	61	25:1		
[MeOTr] T∓T_4∓T-ClPh	3	T∓T∓T∓CE	2.5	6	[MeOTr] T∓T_7∓T∓CE	39	20:1		
[MeOTr] T∓T∓T-ClPh	5	T∓T∓T(OAc)	4	10	[MeOTr] T∓T_4∓T(OAc)	67	25:1	T_6	71
[MeOTr] T∓T∓T-ClPh	3	T_5∓T(OAc)	2.5	6	[MeOTr] T∓T_7∓T(OAc)	49	20:1	T_9	65
[MeOTr] T∓T∓T-ClPh	1.2	T_8∓T(OAc)	1	2.5	[MeOTr] T∓T_{10}∓T(OAc)	35	15:1	T_{12}	60
[MeOTr] T∓T∓T-ClPh	0.7	T_{11}∓T(OAc)	0.5	1.5	[MeOTr] T∓T_{13}∓T(OAc)	29	14:1	T_{15}	50

[a] Abbreviations are as suggested by the IUPAC-IUB, *Biochemistry*, 9, 4022 (1970). A phosphodiester linkage is represented by *hyphen* and phosphotriester linkage is represented by (∓) symbol. Each internal internucleotidic phosphate is protected with *p*-chlorophenyl group (ClPh). [b] Deblocking was carried out with 0.1 *N* NaOH in aqueous dioxane followed by 80% acetic acid treatment at room temperature. [c] Diester.

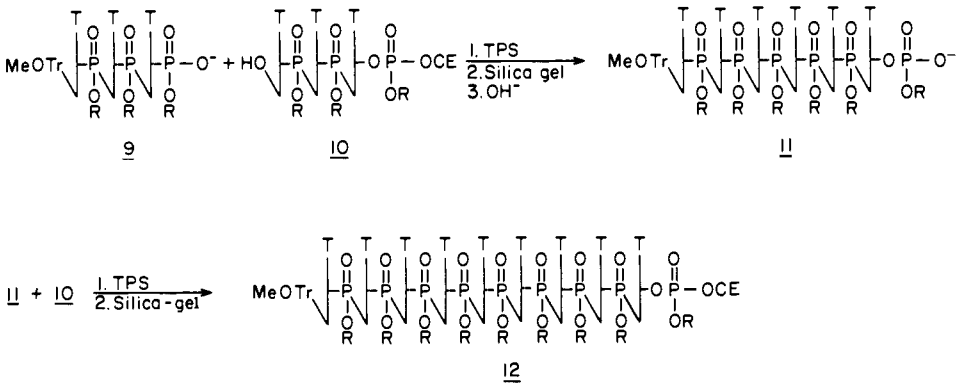

Figure 2. Building the oligonucleotide chain from 5′ toward 3′ end.

since it could be removed almost quantitatively with mild alkali treatment even beyond dodecanucleotide. This group has been extensively used in the present studies.

The final deblocking of the internucleotide phosphate protecting group is the most important step in the triester approach as the chain length of the oligonucleotides grows. For example, the fully protected trinucleotide **7** on treatment with 0.1 *N* sodium hydroxide in aqueous dioxane at room temperature for 1 hr yielded 75–80% of the expected trinucleotide MeOTr-T-T-T(OH)(diester), 15% of a component containing a 3′–3′ bond, and 5% of cyclic dinucleotide. These products can be rationalized by facile deblocking of the 3′-hydroxyl group which then undergoes an intramolecular displacement at one of the internucleotide (triester) phosphates. Depending on which triester bond is displaced, one will obtain the three types of products. Similar observations have been reported by Khorana[19] and Smith[20] but presumably overcome by Reese[7] by tetrahydropyranylating the free hydroxyl group before further alkali treatment. At the hexamer stage, we obtained a higher yield (85%) of the expected product because of the increased stability of 3′-acetyl group. We overcame this deblocking problem by using fluoride anion, a strong nucleophile[21] to phosphate, in the form of tetraethylammonium fluoride in a mixture of tetrahydrofuran and aqueous pyridine to afford 3′,5′-diester product in reasonable yield (ca. 95%). Presumably fluoride anion functions as a very small nucleophile, able to attack the highly hindered internucleotide triester phosphate and bring about deblocking. The phosphofluoridate is then subsequently easily hydrolyzed to the phosphodiester due to the high P–F bond energy. However, deblocking of oligonucleotides longer than nona was best accomplished with 0.1 *N* sodium hydroxide in aqueous dioxane because of the increased stability of the 3′-acetyl group, although the recovery of the completely deblocked product decreased with increasing chain length (see Table I). The

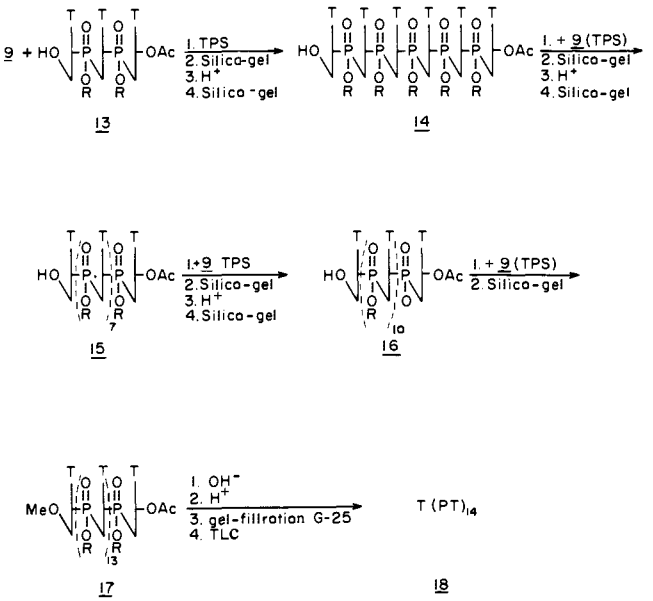

Figure 3. Synthesis of thymidine pentadecamer by growing chain from 3′ toward 5′ end.

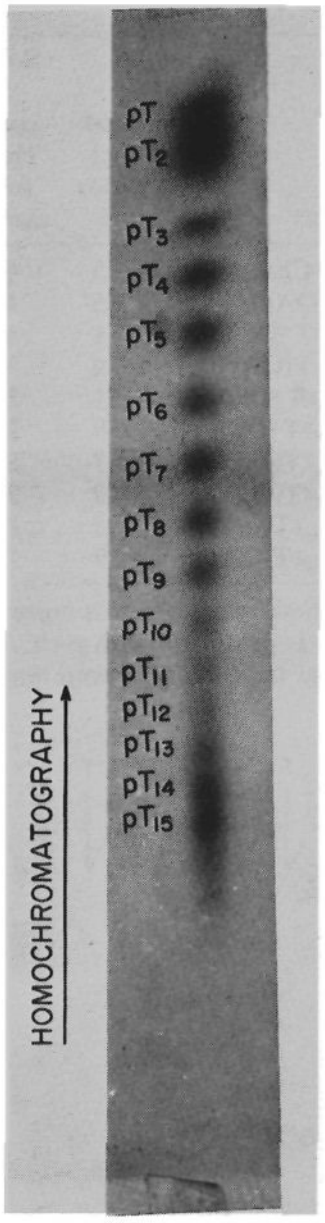

Figure 4. Homochromatography of a partial snake venom phosphodiesterase digest of the p*T_{15} labeled at the 5′ end with [γ-^{32}P]ATP by the T_4 polynucleotide kinase reaction.

monomethoxytrityl group was removed by treatment with 80% acetic acid at room temperature for 40 min. The completely deblocked product was isolated by gel filtration on Sephadex G-25 (superfine).[22]

The purity of each diester product was checked by TLC on Avicel-Cellulose,[23] using solvent systems B, C, and D, and the data are documented in Table II. It was further checked by homochromatography[24,25] of each 5′-^{32}P labeled oligonucleotide prepared by the enzymatic phosphorylation with polynucleotide kinase[26] and [γ-^{32}P]-ATP. The chain lengths of hexa-, nona-, dodeca-, and pentadecamer were analyzed by homochromatography of partially digested ^{32}P oligomer according to the procedure of Wu.[25] Each compound gave the expected number of radioactive spots, i.e., 6 for hexamer, 9 for nona-, and 12 for dodecamer. The results for [^{32}P]pentadecamer are shown in Figure 4. Similar procedure has also been employed successfully to determine the defined sequence of pentadecamer prepared by the phosphotriester approach.[27]

In conclusion, by the use of a protected mononucleotide containing a fully masked 3′-phosphate group, followed by silica gel chromatography, large scale synthesis of polynucleotides has become possible in fairly high yields using approximately stoichiometric amounts of each component. Furthermore, this approach offers two distinct advantages in the field of polynucleotide synthesis: i.e., (i) absence of pyrophosphate due to its instability in the presence of aqueous pyridine; and (ii) silica gel chromatography provides a rapid method of large scale purification procedure which generally takes less than 24 hr.

Table II. R_f Values[a] of Thymidine-Containing Deoxyribopolynucleotides on Avicel-Cellulose TLC Plates

	Solvent system		
Oligonucleotides	B	C	D
T_2	0.85	1.09	
T_3	0.65	0.90	
T_6		0.31	1.00
T_9		0.12	0.71
T_{12}		0.09	0.43
T_{15}		0.05	0.21

[a] R_f values with respect to pT.

Experimental Section

General Methods and Materials. Thymidine (Calbiochem), phenyl phosphodichloridate, β-cyanoethanol, trichloroethanol, tribromoethanol, triisopropylbenzenesulfonyl chloride (TPS), monomethoxytrityl chloride (Aldrich), silica gel F_{254} TLC plates, Avicel-Cellulose TLC plate containing fluorescence indicator, silica gel H grade (Brinkman), DEAE-cellulose (1:9) plates (20 × 40 cm) (Analtec), polynucleotide kinase (Bioenergetic), [γ-^{32}P]-ATP (ICN, Irvine), and cellogram strip (Kalex Scientific Co., N.Y.) were purchased commercially. *p*-Chlorophenyl phosphorodichloridate[28] and zinc–copper dust[29] were prepared according to the published procedure. Compounds containing mono-*p*-methoxytrityl groups were detected by spraying the thin layer plates with 10% perchloric acid and drying them on hot plate. Samples for the elemental analysis were prepared by precipitation from tetrahydrofuran with hexane.

Solvent Systems. For the oligonucleotides containing phosphotriester groups, silica gel F_{254} TLC plates were developed in solvent A, chloroform–methanol (1–10% v/v); for oligonucleotides containing phosphodiester groups, silica gel F_{254} or Avicel-Cellulose plates were developed in solvents B [isopropyl alcohol–concentrated ammonium hydroxide–water (7:1:2 v/v)], C [isobutyric acid–1 *M* ammonium hydroxide–0.1 *M* EDTA (100:60:1.6)], and D [*n*-propyl alcohol–concentrated ammonium hydroxide–water (55:10:35)]. DEAE-cellulose plates were developed using homomix III or V prepared according to the published procedure.[25]

Preparation of 5′-*O*-Monomethoxytritylthymidine-3′-*p*-chlorophenyl β-Cyanoethyl Phosphate (4). A mixture of 5′-*O*-monomethoxytritylthymidine (**1**) (20 mmol) and *p*-chlorophenyl phosphate (**2**) (25 mmol) was condensed in the presence of triisopropylbenzenesulfonyl chloride (TPS) (50 mmol) in anhydrous pyridine (100 ml) at room temperature. After 8 hr, an excess of β-cyanoethanol (100 mmol) was added to this reaction mixture together with additional TPS (20 mmol). The reaction was stopped after 20 hr by the addition of aqueous pyridine (50%, 200 ml) and kept further at room temperature for 1 hr. The product was taken up in chloroform (500 ml) which was backwashed with 0.1 *M* triethylammonium bicarbonate buffer pH 7.5 (3 × 100 ml) followed by water (1 × 100 ml). The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure; the residue was coevaporated with toluene (3 × 50 ml) to remove traces of pyridine. The clear residue was dissolved in chloroform (20 ml) and applied to silica gel (250 g) packed in a glass column of diameter 7 cm. The column was eluted with chloroform–methanol (2%, 2 l.), and fractions (15 ml) were collected every 10 min. Every second fraction was checked by silica gel TLC using chloroform–methanol (9:1) solvent. The desired product **4** was isolated at 75–85% yield: mp 75–78°; uv spectrum (95% ethanol); λ_{max} 267 mμ (ϵ 11700); λ_{max} 235 mμ (ϵ 23000); λ_{min} 254 mμ (ϵ 8500); NMR ($CDCl_3$, ppm from Me_4Si) δ 10.1 (1 H, s, NH), 7.5 (1 H, broad s, H-6), 6.5 (1 H, t, $H_{1'}$), 4.3 (3 H, m, $H_{4'}$ and OCH_2), 3.75 (3 H, s, OCH_3), 2.7 (4 H, m, $H_{2'}$ and CH_2CN), 1.4 (3 H, s, CH_3). Anal. Calcd for $C_{39}H_{37}N_3O_9PCl$: C, 61.47; H, 4.88; N, 5.54. Found: C, 61.27; H, 4.92; N, 5.41.

Deblocking of β-Cyanoethyl Group from the Fully Protected Nucleotide. The fully protected thymidine **4** (10 mmol) was treated with 0.1 *N* sodium hydroxide (500 ml) in dioxane–water (4:1) for 30 sec at room temperature. The reaction mixture was neutralized with Dowex-50w (pyridinium form). After filtering the resin, the filtrate was made anhydrous by coevaporation with added pyri-

dine. The product was precipitated by the addition of this pyridine solution (200 ml) to anhydrous ether (1 l.). The pure product **3** was isolated in quantitative yield by centrifugation followed by ether washing (3 × 100 ml).

Deblocking of Monomethoxytrityl Group. The fully protected mononucleotide **4** (10 mmol) was treated with acetic acid (80%, 100 ml) at room temperature for 4 hr. The solvent was evaporated, and the residue was coevaporated with excess toluene below 30° under reduced pressure. The residue was dissolved in chloroform (50 ml) and applied to silica gel (250 g) (column size 7 × 20 cm) and then eluted with chloroform–methanol (3%, 1 l.) at room temperature. Fractions of 15 ml/10 min were collected. The product **5** was isolated in 90% yield after monitoring the fractions by silica gel TLC in chloroform–methanol (9:1 v/v) solvent. An analytical sample which softened at 115° (precipitated from tetrahydrofuran and hexane) and became clear melted at 122°: uv spectrum (95% ethanol) λ_{max} 267 mμ (ε 9700); λ_{min} 234 mμ (ε 3500). Anal. Calcd for $C_{19}H_{21}N_3O_8PCl$: C, 46.90; H, 4.32; N, 8.64. Found: C, 46.85; H, 4.41; N, 8.53.

Preparation of Fully Protected Dinucleotide Containing 3′-*p*-Chlorophenyl β-Cyanoethyl Phosphate (6). A solution of anhydrous pyridine (50 ml) containing 5′-monomethoxytritylthymidine-3′-*p*-chlorophenyl phosphate (**3**) (10 mmol) and thymidine 3′-*p*-chlorophenyl β-cyanoethyl phosphate (**5**) (12 mmol) was treated with triisopropylbenzenesulfonyl chloride (TPS) (20 mmol) for 20 hr. The reaction mixture was then decomposed by adding aqueous pyridine (50%, 50 ml). After 1 hr at room temperature, the reaction mixture was taken up in chloroform (300 ml), washed with 0.1 *N* triethylammonium bicarbonate buffer, pH 7.5 (3 × 100 ml), and water (1 × 100 ml). After drying (Na_2SO_4) and filtering, the chloroform solution was evaporated to dryness and the residue coevaporated with toluene (500 ml). This residue was then dissolved in chloroform (20 ml) and chromatographed on silica gel (300 g) in a column of diameter 7 cm by eluting with chloroform–methanol (3%, 2 l.). The product **6** was isolated in 75–80% yield. An analytical sample softened at 130° (precipitated from tetrahydrofuran and hexane) and completely melted at 138–140°: uv spectrum (95% ethanol) λ_{max} 267 mμ (ε 19400); λ_{min} 250 mμ (ε 12000). Anal. Calcd for $C_{55}H_{53}N_5O_{16}P_2Cl_2$: C, 56.27; H, 4.51; N, 5.97. Found: C, 56. 17; H, 4.45; N, 5.85.

Preparation of Fully Protected Trinucleotide Containing 3′-*p*-Chlorophenyl β-Cyanoethyl Phosphate Group 8. The β-cyanoethyl group from fully protected dinucleotide **6** was removed by treatment with 0.1 *N* sodium hydroxide dioxane as described above.

The fully protected trinucleotide **8** was synthesized by condensing 5′-monomethoxyltrityl dinucleotide (5 mmol) with **5** (7.5 mmol) in anhydrous pyridine (25 ml) in the presence of TPS (10 mmol) for 20 hr at room temperature. After the usual work-up, the protected trinucleotide **8** was obtained by silica gel chromatography (250 g) in a column of diameter 7 cm by eluting with chloroform–methanol (4%, 2 l.). The yield was 60–65%. The fully protected trinucleotide **7** was prepared by condensing the dinucleotide with 3′-*O*-acetylthymidine as described above. The product was isolated in 75–80% yield: uv spectrum (95% ethanol) λ_{max} 267 mμ (ε 28900); λ_{min} 249 mμ (ε 17500).

General Method for the Synthesis of Hexa and Higher Oligonucleotides. An anhydrous pyridine solution (5 ml per mmol of the nucleotidic component) containing 5′-hydroxyl oligonucleotide 3′-*p*-chlorophenyl β-cyanoethyl phosphate and 5′-monomethoxytrityl oligonucleotide 3′-*p*-chlorophenyl phosphate (1.2 mol equiv relative to the hydroxyl component) was treated with TPS (2 mol equiv relative to the 3′-phosphodiester component) for 2 days at room temperature. After the usual work-up as described above, the desired fully protected oligonucleotide was isolated by silica gel chromatography. The reaction conditions, chromatographic details, and isolated yields are given in Table I.

Complete Removal of Protecting Groups. Removal of 3′-Acetyl and *p*-Chlorophenyl Group from the Internucleotidic Phosphate Function of Oligonucleotides up to Hexanucleotides. (i) Using Tetraethylammonium Fluoride. The fully protected hexanucleotide (50 mg) compound in a solution of tetrahydrofuran–pyridine–water (8:1:1) (1.5 ml) containing tetraethylammonium fluoride (0.1 *M*, 2–3 equiv per phosphate group) was kept overnight at room temperature. After evaporation of the solvent, the residue was coevaporated with ammonium hydroxide (5%). The monomethoxytrityl group was removed by treatment with acetic acid (80%, 10 ml) at room temperature for 4 hr, excess acetic acid was removed under reduced pressure, and the residue was dissolved in water (5 ml). This solution was washed with ether (2 × 2 ml), and the aqueous solution was evaporated. The completely unprotected compound was isolated first by gel filtration on G-25 SF (K25-100) column followed by further purification on Avicel-Cellulose TLC plate in solvent systems B and C.

(ii) Using Sodium Hydroxide. A sample of oligonucleotide (0.01 mmol) was shaken with 0.5 *N* sodium hydroxide (10 ml) and dioxane (40 ml) at room temperature for various lengths of time depending upon the chain lengths, i.e., 1 hr for di-, 3 hr for tri-, 9 hr for hexa-, 15 hr for nona-, and 24 hr for dodeca- and pentadecanucleotides. Dowex-50w resin (pyridinium form) was added to neutralize the alkali and, after filtration, the pyridine solution was concentrated in vacuo to dryness. The monomethoxytrityl group was removed as described above, and isolated yields are given in Table I.

Characterization of Completely Unprotected Oligonucleotides Containing 3′,5′-Phosphodiester Group. I. Avicel-Cellulose TLC. The homogeneity of each intermediate compound (1–5 OD_{260nm}) was checked using Avicel-Cellulose plates containing fluorescent indicator with solvent B, C,[30] and D. These plates not only quench any fluorescence but permit detection of traces of impurities using ≤1.0 OD_{260nm} of the product. The mobility of each compound is given in Table II.

II. Homochromatography. The homogeneity of higher oligothymidine such as hexa-, nona-, dodeca-, and pentadecamer was further confirmed by labeling each one at the 5′ terminal with polynucleotide kinase and [γ-^{32}P]-ATP according to Richardson's procedure,[26] followed by isolation on Sephadex G-25 (SF K10-100) column. A sample of 2 μl was applied to homochromatographic TLC plates DEAE-cellulose (1:9) (20 × 40 cm). The plate was developed according to Wu's procedure[25] in homo-mix III. The autoradiogram showed a single spot for hexa-, nona-, dodeca-, and pentadecamer with R_f values with respect to the blue dye of 0.66, 0.47, 0.28, and 0.12, respectively.

III. Snake Venom Phosphodiesterase Degradations. The oligonucleotide (0.5–1.0 absorbance unit at 260 nm in 0.1 *M* ammonium carbonate buffer, pH 8.5) (50 μl) was treated with snake venom phosphodiesterase (Worthington) (20 μl) (1 mg/5 ml) for 3–5 hr at 37°. The solution was then concentrated to about 10 μl in vacuo and chromatographed on Avicel-Cellulose TLC plates in solvent B. The nucleotide material was extracted from the appropriate spot with 0.01 *N* hydrochloric acid. The absorbance of the solution was recorded on a Gilford spectrophotometer Model 2400 using the extract from an equivalent area of cellulose as a blank. The results of enzymatic degradations are given in Table III.

IV. Chain Length Analysis of Pentadecathymidylic Acid. The 5′-labeled pentadecamers moving as a single spot with R_f (with respect to blue dye) 0.12 were digested at 37° in 15 μl containing 30 μg of RNA carrier (partially hydrolyzed yeast RNA), 50 n*M* Tris-HCl (pH 8), 5 m*M* $MgCl_2$, and 0.2 μg of venom phosphodiesterase. One-microliter samples were removed at increasing time intervals 0, 2, 5, 10, 20, 40, 60, 90, 120, and 180 min, and each sample was blown into 5 μl of 1 *M* ammonium hydroxide containing 1 m*M* EDTA. The combined digest was dried in a desiccator, dissolved in 10 μl of water. One-microliter of sample was applied on a DEAE-cellulose plate (5 × 20 cm), and then the plate was developed with homo-mix IV till the blue dye was 2 cm from the top. The radioautogram of the plate is shown in Figure 4.

Table III. Snake Venom Phosphodiesterase Digestion of Thymidine Oligonucleotides

Compd	Molar ratio of T:pT	
	Found	Theoretical
T_2	1:0.9	1:1
T_3	1:1.7	1:2
T_6	1:4.5	1:5
T_9	1:7.5	1:8

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The Use of Arylsulfonyltriazoles for the Synthesis of Oligonucleotides by the Triester Approach[1]

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Abstract: Various arylsulfonyltriazoles have been investigated for inducing the formation of a phosphotriester bond in heterosequence of nucleotides. Mesitylenesulfonyl- and p-nitrobenzenesulfonyltriazoles are found to be most effective. Application of these reagents for the large scale synthesis using nearly stoichiometric amounts of each component, of defined sequence of various di-, tri-, and hexanucleotides, is reported.

The development by Khorana and his co-workers of dicyclohexylcarbodiimide[3] (DCC), mesitylenesulfonyl chloride[4] (MS), and triisopropylbenzenesulfonyl chloride[5] (TPS) as reasonably effective condensing reagents has played a significant role in the synthesis of polynucleotides by the diester method. In the case of the triester synthetic approach,[6] triisopropylbenzenesulfonyl chloride (TPS) has been used almost exclusively as the condensing reagent because dicyclohexylcarbodiimide (DCC) will not activate phosphodiester functions, and mesitylenesulfonyl chloride (MS) causes extensive sulfonation of the primary 5′-hydroxyl group of the nucleotide component, thus blocking possible condensation. In this paper, we describe arylsulfonyltriazoles as a new class of condensing reagents and bis(triazolyl)-*p*-chlorophenyl phosphate as a phosphorylating reagent and demonstrate their effectiveness in the synthesis of oligonucleotides of defined sequence by the triester approach. A preliminary report of this work has already appeared.[7]

The search for new condensing reagents was initiated because of our continued realization of low yields (ca. 20%) when attempting condensation with TPS of products containing purine bases, especially guanine. Cramer et al.[8] have also reported very low yields (~10%) for the synthesis of oligonucleotides containing the guanine unit. These low yields might be attributed to the liberation of hydrogen chloride from the triisopropylbenzenesulfonyl chloride (TPS) during the condensation reaction. We speculated on overcoming this problem by using another arylsulfonyl derivative with a better or less innocuous leaving group such as azide or cyanide, instead of chloride. We investigated the *p*-toluenesulfonyl and *p*-nitrobenzenesulfonyl azides;[9] however, these compounds failed to effect linkage between a phosphodiester group and the 5′-primary hydroxyl group of a nucleotide component or even to cause sulfonation of the 5′-hydroxyl group. The *p*-toluenesulfonyl cyanide[10] readily sulfonated the 5′-hydroxyl group but consequently failed to achieve any formation of the phosphotriester bond, whereas the imidoyl sulfonate[11] would either bring about formation of pyrophosphate or was inert. Recently two new nucleotide condensing reagents have been reported—a polymeric arylsulfonyl chloride[12] which resembles TPS in reactivity and the mixture of triphenylphosphine and 2,2′-dipyridyl disulfide[13]—but both appear to be useful only for the diester approach.

Russian workers[14] have recently reported *p*-toluenesulfonylimidazole as a useful condensing reagent for the chemical synthesis of oligonucleotides, but we found its rate of reaction was very slow. However *N*-acetyltriazole has been reported[15] to be hydrolyzed six times faster at room temperature than *N*-acetylimidazole. This observation prompted us to investigate the reactivity of various arylsulfonyltriazoles and their potential as condensing reagents. The vari-