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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-

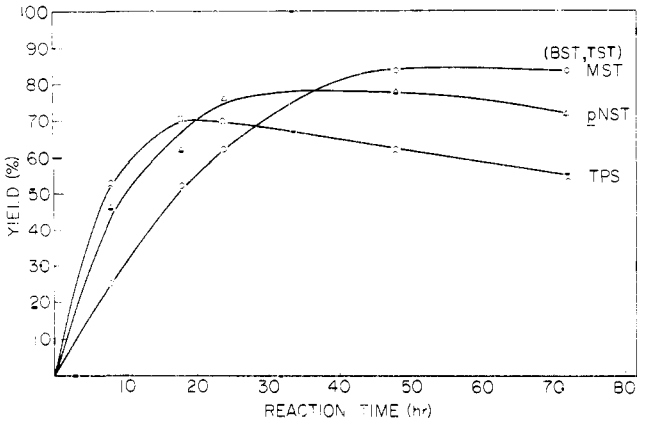


Figure 1. Relative rate of dinucleotide synthesis. For details, see text.

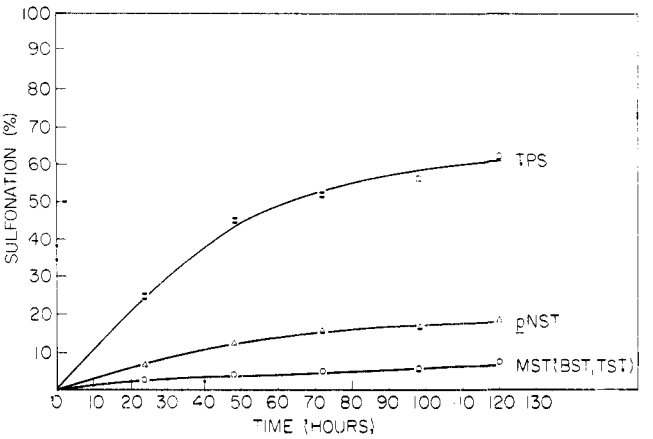


Figure 2. Rate of Sulfonation of 3′-*O*-acetylthymidine. For details, see text.

ous triazoles (**3a–g**) were easily prepared in almost quantitative yields by condensing the corresponding arylsulfonyl chloride with (1*H*)-1,2,4-triazole in the presence of triethylamine (equimolar ratio) in chloroform solution. Each of the compounds was characterized by elemental and spectra analysis.

Kinetic Studies Using Arylsulfonyltriazoles. The kinetics of the reaction in anhydrous pyridine at room temperature between 5′-monomethoxytritylthymidine-3′-*p*-chlorophenyl phosphate (1 mol equiv) and 3′-acetylthymidine (1.2 mol equiv) were determined, and the results are shown in Figure 1. Thus benzenesulfonyltriazole (BST) and *p*-toluenesulfonyltriazole (TST) were similar in reactivity to mesitylenesulfonyltriazole (MST) which took 36–48 hr to produce a maximum yield (78–85%) of the dinucleotide, while *p*-nitrobenzenesulfonyltriazole (*p*-NBST) was found to be the most reactive yielding 75% of product in 24 hr. Compared with TPS, the rate of reaction using MST or *p*-NBST was rather slow, *but the reaction mixture was much cleaner and the yield of condensed product was much higher.* We also felt it was quite pertinent to compare these compounds in their rate of sulfonation of the primary 5′-hydroxyl group of a nucleotide, and the results using 3′-acetylthymidine (1 mol equiv) and various sulfonyltriazole or TPS (5 mol equiv) in anhydrous pyridine at room temperature are shown in Figure 2. Thus in 5 days, BST, TST, and MST only caused sulfonation to the extent of 7% in each case, 18% for *p*-NBST whereas, in the case of TPS, the sulfonation reaction of 5′-hydroxyl group was 60%. These results clearly indicated distinct advantages for the arylsulfonyltriazoles over TPS in building the triester phosphate linkage between the nucleotides.

Triazole as Phosphate Activator. Phosphorylation is one of the most crucial steps in the synthesis of polynucleotides by the triester approach. With the above encouraging results, we investigated the ability of (1*H*)-1,2,4-triazole to catalyze the phosphorylation of a 5′-protected mononucleoside. This was accomplished by stirring *p*-chlorophenyl phosphorodichloridate (1.2 mol equiv) with (1*H*)-1,2,4-triazole (2.4 mol equiv) and triethylamine (2.4 mol equiv) in anhydrous dioxane for 1 hr at room temperature, filtering off the precipitated triethylammonium chloride, and using the resultant filtrate directly for the phosphorylation of 5′-dimethoxytrityl N-protected nucleoside (1 mol equiv). The preceding sequence must be performed without interruption due to the labile nature of the phosphorylating reagent. After keeping the solution at room temperature for 6 hr, it was treated with β-cyanoethanol (4 mol/equiv) for 20 hr to obtain the fully protected derivative in yields of 70–85%.

Synthesis of Di-, Tri-, and Hexanucleotides of Defined Sequence. In order to thoroughly test the generality of these reagents, we have carried out large scale synthesis of di-, tri-, and hexanucleotides according to the scheme outlined in Figure 5. Thus 5′-dimethoxytritylnucleoside-3′-*p*-chlorophenyl phosphate (**8**) (1 mol equiv) was coupled with 3′-acetyl N-protected nucleoside **9** (1 mol equiv) in the presence of each condensing reagent (MST, *p*-NBST, or TPS, 2–3 mol equiv) in anhydrous pyridine at room temperature. The reaction went to completion in 48 hr with MST, 24 hr with *p*-NBST, and 18 hr with TPS. The reaction mixture was then decomposed with aqueous pyridine, followed by extraction with chloroform, and the chloroform solution washed with 0.1 *M* triethylammonium bicarbonate buffer, pH 7.5. The chloroform solution was coevaporated in the presence of toluene and the residue submitted to silica gel column chromatography to isolate the desired compound using chloroform with methanol (2–5%). The reaction conditions and yields of each synthesized oligomer are given in Tables I and II. As shown in Table I, the use of triazoles, i.e., MST and *p*-NBST, as condensing reagents remarkably increased yields as compared with TPS *especially in the sequences containing purine bases*, although TPS was confirmed to be a reasonably good reagent in the synthesis of oligonucleotides provided only pyrimidine bases were present.[1] Further, it is noteworthy to mention that the *N*-benzoyl group of the cytosine moiety was quite stable in the presence of MST or *p*-NBST as condensing reagents. The deprotection of fully protected di-, tri-, and hexanucleotides was carried out with 0.1 *N* sodium hydroxide–dioxane water at room temperature for 3–6 hr. After neutralization with Dowex 50 (pyridinium form), the concentrated residue was treated with concentrated ammonia for 3 hr at 50° to remove any N-protecting group and finally with acetic acid (80%) for 20 min at room temperature. The deprotected compounds were isolated by gel filtration on Sephadex G-25 (superfine). The R_f's of the unprotected components are given in Table III and yields in Tables I and II.

Finally all the unprotected oligonucleotides prepared by the triester approach using MST or *p*-NBST were checked for their purity by TLC on Avicel-Cellulose plates using various solvents (R_f's in Table III); spleen phosphodiesterase digestion resulted in their expected ratio of nucleoside to nucleotide components (results in Table IV). The sequence of each hexanucleotide was confirmed by a two-dimensional chromatographic pattern of 5′-^{32}P-labeled hexanucleotide after partial digestion with snake venom phosphodiesterase according to the procedures of Sanger[16] and Wu.[17] A typical patter for [5′-P^{32}]-G-A-G-C-G-G is given in Figure 3.

Table I. Comparison of the Yields of the Product Using New Condensing Reagents with Respect To TPS

5'-Protected[a] component (1 mol equiv)	3'-Protected component (1.2 mol equiv)	Product	Product (using 2 mol equiv) based on 5'-protected component: TPS	MST	p-NBST	Solvent for column chloroform:methanol	Deblocked diester compd: Identity	Yield, %
		Fully Protected Dinucleotides						
[(MeO)$_2$Tr]dbzA-ClPh	dbzA∓CE	[(MeO)$_2$Tr]dbzA∓bzA∓CE	30	75	78	45:1	dA-A-ClPh	70
[(MeO)$_2$Tr]T-ClPh	dacG∓CE	[(MeO)$_2$Tr]dT∓acG∓CE	34	65	68	25:1	dT-G-ClPh	85
[(MeO)$_2$Tr]dacG-ClPh	dbzA∓CE	[(MeO)$_2$Tr]dacG∓bzA∓CE	22	66	52	30:1	dG-A-ClPh	70
[(MeO)$_2$Tr]dbzC-ClPh	dacG∓CE	[(MeO)$_2$Tr]dbzC∓acG∓CE	38	70	80	28:1	dC-G-ClPh	77
[(MeO)$_2$Tr]dbzA-ClPh	dT∓CE	[(MeO)$_2$Tr]dbzA∓T∓CE	52	73	81	40:1	dA-T-ClPh	83
[(MeO)$_2$Tr]dbzA-ClPh	dbzC∓CE	[(MeO)$_2$Tr]dbzA∓bzC∓CE	63	74	70	45:1	dA-C-ClPh	78
[(MeO)$_2$Tr]T-ClPh	dbzA∓CE	[(MeO)$_2$Tr]dT∓bzA∓CE	62	72	78	40:1	dT-A-ClPh	72
[(MeO)$_2$Tr]dbzC-ClPh	dbzC∓CE	[(MeO)$_2$Tr]dbzC∓bzC∓CE	71	83	80	45:1	dC-C-ClPh	80
[(MeO)$_2$Tr]dbzC-ClPh	T∓CE	[(MeO)$_2$Tr]dbzC∓T∓CE	63	79	82	40:1	dC-T-ClPh	85
		Fully Protected Trinucleotides						
[(MeO)$_2$Tr]dbzA∓bzA-ClPh	T∓CE	[(MeO)$_2$Tr]dbzA∓bzA∓T∓CE	45	74	71	38:1	dA-A-T-ClPh	75
[(MeO)$_2$Tr]dbzA∓T-ClPh	dbzA∓CE	[(MeO)$_2$Tr]dbzA∓T∓bzA∓CE	51	76	78	35:1	dA-T-A-ClPh	76
[(MeO)$_2$Tr]dbzA∓T-ClPh	T(oAc)	[(MeO)$_2$Tr]dbzA∓T∓T(oAc)	59	82	78	30:1	dA-T-T	82
[(MeO)$_2$Tr]dbzA∓bzC-ClPh	dbzA∓CE	[(MeO)$_2$Tr]dbzA∓bzC∓bzA∓CE	52	70	72	35:1	dA-C-A-ClPh	72
[(MeO)$_2$Tr]dT∓acG-ClPh	T∓CE	[(MeO)$_2$Tr]dT∓acG∓T∓CE	32	64	52	20:1	dT-G-T-ClPh	82
[(MeO)$_2$Tr]T∓bzA-ClPh	T∓CE	[(MeO)$_2$Tr]dT∓bzA∓T∓CE	55	72	70	30:1	dT-A-T-ClPh	88
[(MeO)$_2$Tr]dacG∓bzA-ClPh	dacG∓CE	[(MeO)$_2$Tr]dacG∓bzA∓acG∓CE	18	58	40	22:1	dG-A-G-ClPh	72
[(MeO)$_2$Tr]dbzC-ClPh	dacG∓acG(oAc)	[(MeO)$_2$Tr]dbzC∓acG∓acG(oAc)	20	68	59	20:1	dC-G-G	75
[(MeO)$_2$Tr]dbzC∓bzC-ClPh	dacG∓CE	[(MeO)$_2$Tr]dbzC∓bzC∓acG∓CE	32	61	58	25:1	dC-C-G-ClPh	75
[(MeO)$_2$Tr]dbzC∓T-ClPh	dbzC∓CE	[(MeO)$_2$Tr]dbzC∓T∓bzC∓CE	65	76	80	32:1	dC-T-C-ClPh	78

[a] Abbreviations are as suggested by the IUPAC–IUB, *Biochemistry*, 9, 4022 (1970). A phosphodiester linkage is represented by *hypen* and phosphotriester linkage is represented by (∓) symbol. Each internal internucleotidic phosphate is protected with *p*-chlorophenyl group (ClPh).

Table II. The Reaction Conditions and Yields of Various Hexanucleotides Using *p*-Nitrobenzenesulfonyltriazole as the Condensing Reagent

5'-Protected[a] component	Amount, mmol (g)	5'-Hydroxyl component	Amount, mmol (g)	p-NBST, mmol	Product	Yield, % (g)	Solvents for column chloroform:methanol	Deblocked diester compd: Product	Yield, %
[(MeO)$_2$Tr]dbzA∓bzA∓T-ClPh	2.5 (4.6)	dbzT∓acG∓T∓CE	2.5 (3.5)	7.5	[(MeO)$_2$Tr]dbzA∓bzA∓T∓T∓acG∓T∓CE	55 (4.3)	20:1	dA-A-T-T-C-G-ClPh	72
[(MeO)$_2$Tr]dacG∓bzA∓acG-ClPh	1.0 (1.8)	dbzC∓acG∓acG(oAc)	1.0 (1.5)	3.0	[(MeO)$_2$Tr]dacG∓bzA∓acG∓bzC∓acG∓acG(oAc)	48 (1.6)	15:1	dG-A-G-C-G-G	70
[(MeO)$_2$Tr]dbzC∓bzC∓acG-ClPh	2.0 (3.7)	dbzC∓T∓bzC∓CE	2.0 (3.0)	6.0	[(MeO)$_2$Tr]dbzC∓bzC∓acG∓bzC∓T∓bzC∓CE	50 (3.35)	25:1	dC-C-G-C-T-C-ClPh	75
[(MeO)$_2$Tr]dbzA∓bzC∓bzA-ClPh	1.0 (2.1)	dbzA∓T∓T(oAc)	1.0 (1.3)	3.0	[(MeO)$_2$Tr]dbzA∓bzC∓bzA∓bzA∓T∓T(oAc)	62 (2)	25:1	dA-C-A-A-T-T	71

[a] Phosphodiester bond is represented by (-) and phosphotriester bond by (∓).

In conclusion, the present studies have clearly demonstrated the usefulness of new condensing reagents such as mesitylenesulfonyl- and *p*-nitrobenzenesulfonyltriazoles which are highly efficient for the oligonucleotide synthesis of defined sequence by the triester approach.

Experimental Section

General Methods and Materials. Thymidine and deoxyadenosine (Calbiochem), deoxyguanosine (Nutritional Biochem. Corp.), deoxycytidine (PWA, Mannheim, West Germany), (1*H*)-1,2,4-triazole, dimethoxytrityl chloride, benzenesulfonyl chloride, *p*-toluenesulfonyl chloride, mesitylenesulfonyl chloride, 2,4,6-triisopropylbenzenesulfonyl chloride, *o*-nitro- and 2,4-dinitrobenzenesulfonyl chlorides (Aldrich), *p*-nitrobenzenesulfonyl chloride (Eastman Kodak), silica gel F_{254}, Avicel-Cellulose TLC plates, and silica gel H grade for column chromatography (Brinkman), DEAE-cellulose (1:9) plates (20 × 40 cm) (Analtec), polynucleotide kinase (Bioenergetics), [γ-^{32}P]-ATP (ICN, Irvine), and Cellogram strip (Kalex Scientific Co., N.Y.) were purchased commercially.

3′-*O*-Acetylthymidine, 3′-*O*-*N*-diacetyldeoxyguanosine, *N*-acetyl-5′-*O*-dimethoxytrityldeoxyguanosine, *N*-benzoyl-5′-*O*-dimethoxytrityldeoxyadenosine, *N*-benzoyl-5′-*O*-dimethoxytrityldeoxycytidine, *N*-benzoyl-3′-acetyldeoxyadenosine, and *N*-benzoyl-3′-acetyldeoxycytidine were prepared according to published procedures.[18] Compounds containing dimethoxytrityl group were detected by spraying the thin layer plates with 10% perchloric acid and drying on hot plate. Samples of fully protected mononucleotides for the elemental analysis were prepared by precipitation from tetrahydrofuran with hexane.

Solvent Systems. The same solvent systems were used as described in the previous paper.[1]

General Method for the Preparation of Arylsulfonyltriazoles (3a–g). A chloroform (20 ml) solution of triethylamine (10.1 g, 0.1 mol) was added to a suspension of arylsulfonyl chloride (0.1 mol) and (1*H*)-1,2,4-triazole (6.9 g, 0.1 mol) in chloroform (200 ml) with ice cooling. The reaction mixture was stirred for 1 hr at 20° and then washed with water (3 × 100 ml) to remove triethylammonium chloride. The chloroform solution was dried over anhydrous sodium sulfate and, after evaporation of solvent, the residue

Table III. R_f Values[a] of Deoxyoligonucleotide on *TLC Plates*

Oligonucleotide containing phosphodiester groups	Solvent systems B	C	D
Dinucleotides			
dA-Ap(ClPh)	4.6	1.8	2.5
dT-Gp(ClPh)	2.4	1.6	1.4
dG-Ap(ClPh)	3.4	1.4	1.9
dC-Gp(ClPh)	3.0	1.8	1.5
dA-Tp(ClPh)	4.6	1.7	1.7
dA-Cp(ClPh)	4.7	1.8	2.1
dT-Ap(ClPh)	4.8	1.7	1.8
dC-Cp(ClPh)	4.5	2.0	1.5
dC-Tp(ClPh)	4.1	2.1	1.4
Trinucleotides			
dA-A-Tp(ClPh)	2.5	1.6	2.0
dT-G-Tp(ClPh)	1.4	1.5	1.1
dG-A-Gp(ClPh)	0.9	1.1	1.6
dC-G-Gp(ClPh)	0.8	1.2	1.3
dA-T-Ap(ClPh)	2.7	1.6	1.7
dA-C-Ap(ClPh)	2.4	1.3	2.0
dT-A-Tp(ClPh)	1.7	1.7	1.6
dC-C-Gp(ClPh)	1.8	1.2	1.3
dC-T-Cp(ClPh)	2.3	1.7	1.2
dA-T-T	4.4	3.8	1.5
Hexanucleotides			
dA-A-T-T-G-Tp(ClPh)		0.71	0.6
dA-C-A-A-T-T		0.58	0.5
dG-A-G-C-G-G		0.30	0.40
dC-C-G-C-T-Cp(ClPh)		0.65	0.40

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

Table IV. Spleen Phosphodiesterase Digestion of Hexanucleotides

Compd	*Nucleotide:* nucleoside	Molar ratio of products Found	Theor
dA-C-A-A-T-T	dAp:dCp:Tp:T	2.9:1:0.9:1	3:1:1:1
dG-A-G-C-G-G	dGp:dAp:dCp:G	2.7:1:0.8:0.9	3:1:1:1
dA-A-T-T-G-Tp(ClPh)[a]	dAp:T:dGp	1.8:1.9:1	2:2:1
dC-C-G-C-T-Cp(ClPh)[a]	dCp:cGp:Tp	2.8:1.1:0.9	3:1:1

[a] Spleen phosphodiesterase failed to cleave the phosphodiester bond containing *p*-chlorophenyl group at the 3′-terminal quantitatively.

was recrystallized from the appropriate organic solvent giving the arylsulfonyltriazole in almost quantitative yields. Each of the compounds was characterized by its elemental analysis and NMR spectra as detailed below. (See Figure 4.)

3a [1-(Benzenesulfonyl)-(1*H*)-1,2,4-triazole (BST)]: mp 104° (recrystallized from benzene); NMR[19] ($CDCl_3$, ppm from Me_4Si) δ 8.82 (H_5), 8.08 (H_3). Anal. Calcd for $C_8H_7N_3O_2S$: C, 45.94; H, 3.37; N, 20.09; S, 15.34. Found: C, 45.79; H, 3.41; N, 19.86; S, 15.36.

3b [1-(*p*-Toluenesulfonyl)-(1*H*)-1,2,4-triazole (TST)]: mp 107° (from benzene); NMR ($CDCl_3$, ppm from Me_4Si) δ 8.81 (H_5), 8.08 (H_3). Anal. Calcd for $C_9H_9N_3O_2S$: C, 48.43; H, 4.06; N, 18.83; S, 14.34. Found: C, 48.54; H, 4.03; N, 18.59; S, 14.18.

3c [1-(Mesitylenesulfonyl)-(1*H*)-1,2,4-triazole (MST)]: mp 135° (from benzene); NMR ($CDCl_3$, ppm from Me_4Si) δ 8.85 (H_5), 8.02 (H_3). Anal. Calcd for $C_{10}H_{13}N_3O_2S$: C, 52.58; H, 5.22; N, 16.73; S, 12.74. Found: C, 52.76; H, 5.24; N, 16.57; S, 12.55.

3d [1-(2,4,6-Triisopropylbenzenesulfonyl)-(1*H*)-1,2,4-triazole (TPST)]: mp 112° (benzene); NMR ($CDCl_3$, ppm from Me_4Si) δ 8.80 (H_5), 8.05 (H_3). Anal. Calcd for $C_{18}H_{25}N_3O_2S$: C, 60.88; H, 7.51; N, 12.53; S, 9.47. Found: C, 61.22; H, 7.69; N, 12.30; S, 9.71.

3e [1-(*o*-Nitrobenzenesulfonyl)-(1*H*)-1,2,4-triazole (*o*-NBST)]: mp 128–130° (benzene); NMR ($CDCl_3$, ppm from Me_4Si) δ 8.90 (H_5), 8.10 (H_3). Anal. Calcd for $C_8H_6N_4O_4S$: C, 37.80; H, 2.38; N, 22.05; S, 12.60. Found: C, 37.58; H, 2.40; N, 21.66; S, 12.41.

3f [1-(*p*-Nitrobenzenesulfonyl)-(1*H*)-1,2,4-triazole (*p*-NBST)]: mp 147–149° (ethyl acetate); NMR ($CDCl_3$, ppm from Me_4Si) δ 8.82 (H_5), 8.12 (H_3). Anal. Calcd for $C_8H_6N_4O_4S$: C, 37.80; H, 2.38; N, 22.05; S, 12.60. Found: C, 37.66; H, 2.32; N, 21.98; S, 12.44.

3g [1-(2,4-Dinitrobenzenesulfonyl)-(1*H*)-1,2,4-triazole (DNBST)]: mp 170–172° (acetone); NMR ($CDCl_3$, ppm from Me_4Si) δ 8.93 (H_5), 8.16 (H_3). Anal. Calcd for $C_8H_5O_6S$: C, 32.12; H, 1.68; N, 23.41; S, 10.73. Found: C, 31.90; H, 1.61; N, 23.02; S, 10.90.

Relative Rates of Synthesis of Dinucleotide. An anhydrous pyridine solution (25 ml) of 5′-*p*-methoxytritylthymidine-3′-*p*-chlorophenyl phosphate (5 mmol) and 3′-*O*-acetylthymidine (6.0 mmol) was prepared by repeated evaporation of pyridine. The solution was then divided into five equal portions (5 ml each) which were treated with mesitylenesulfonyltriazole (MST), benzenesulfonyltriazole (BST), *p*-toluenesulfonyltriazole (TST), 2,4,6-triisopropylbenzenesulfonyl chloride (TPS), and *p*-nitrobenzenesulfonyltriazole (*p*-NBST) (using 2 mmol in each case). Aliquots (0.1 ml) were removed at intervals and treated with 0.1 *M* triethylammonium bicarbonate buffer, pH 7.1 (0.1 ml). After 1 hr, toluene (1 ml) was added, and the resultant solution was evaporated to dryness in vacuo; the residue was then dissolved in chloroform (0.5 ml) and applied to silica gel TLC plates (0.25 mm thickness). The plates were developed with chloroform:methanol (10:1), and the band corresponding to the desired fully protected dinucleotide (R_f 1.5 with respect to 3′-*O*-acetylthymidine) was scraped from the TLC plates and eluted with chloroform:methanol (5:1). The yield of the nucleotide component was determined spectrophotometrically using an extinction value of 19400 at 267 nm for the *p*-chlorophe-

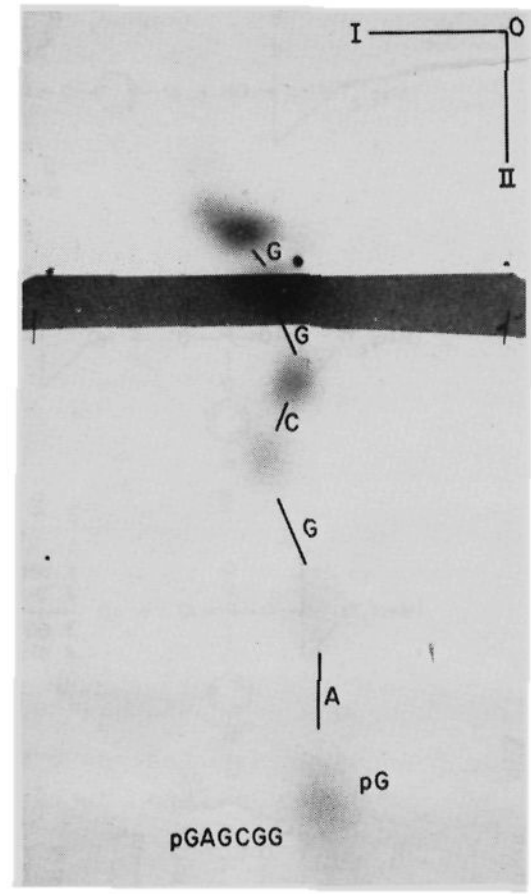
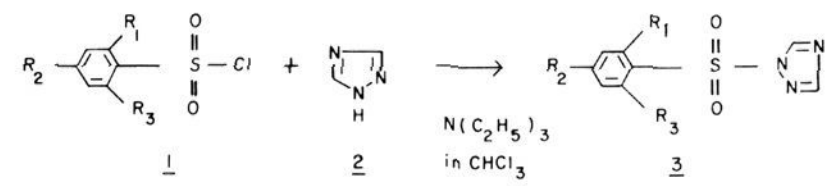


Figure 3. Two-dimensional fingerprinting of a partial snake venom phosphodiesterase digest of the hexanucleotide p*G-A-G-C-G-G.



a BST ($R_1 = R_2 = R_3 = H$)
b TPST ($R_1 = R_2 = R_3 =$ iso-propyl)
c TST ($R_1 = R_3 = H$ $R_2 = CH_3$)
d O-NBST ($R_1 = NO_2$ $R_2 = R_3 = H$)
e DNBST ($R_1 = R_2 = NO_2$ $R_3 = H$)
f P-NBST ($R_1 = R_3 = H$ $R_2 = NO_2$)
g MST ($R_1 = R_2 = R_3 = CH_3$)

Figure 4. Synthesis of various arylsulfonyltriazoles.

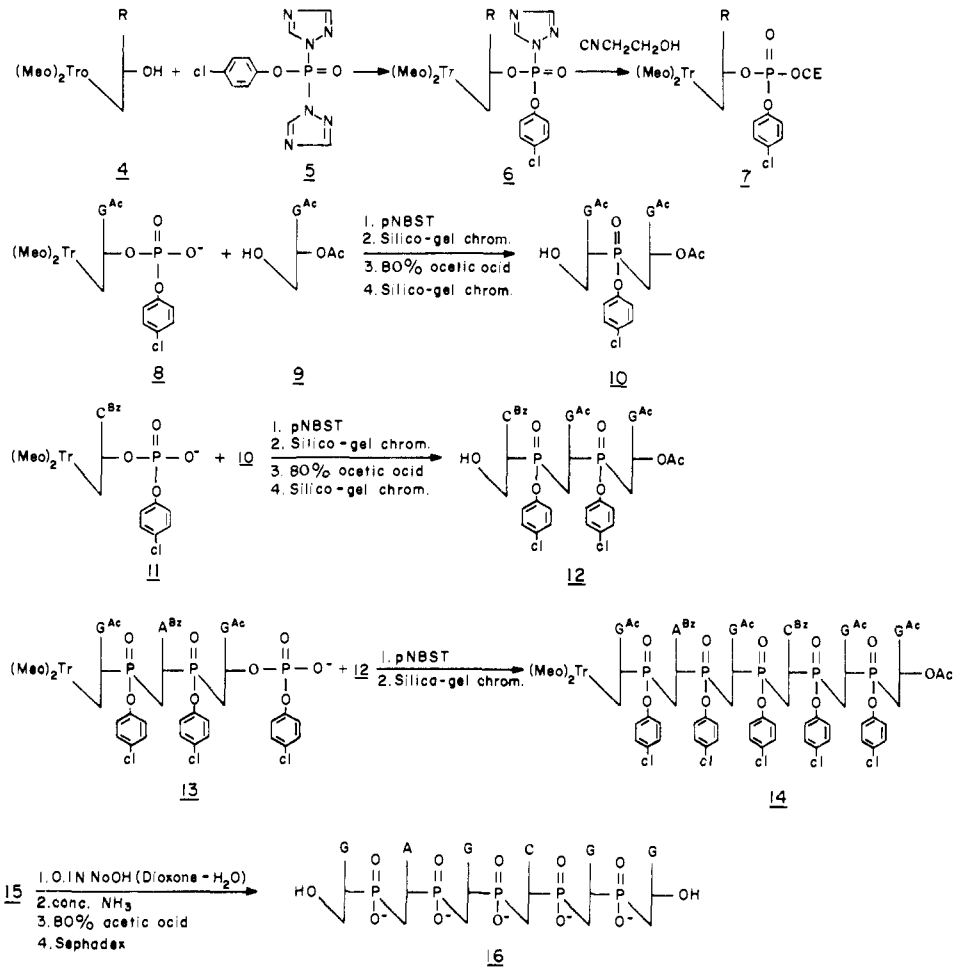


Figure 5. Synthesis of fully protected hexanucleotide of the defined sequence by the improved triester approach.

nyl phosphate ester of 5′-*O*-(*p*-monomethoxytrityl)thymidylthymidine. The results are shown in Figure 1.

Rate of Sulfonation of 3′-*O*-Acetylthymidine Using Arylsulfonyltriazoles. Anhydrous pyridine solutions (2 ml) of 3′-*O*-acetylthymidine (28.4 mg, 0.1 mmol) were each treated with either MST, BST, TST, *p*-NBST, or TPS (0.5 mmol, 5 mol equiv in each case). Aliquots were taken at various time intervals and evaporated in vacuo with the addition of toluene to remove pyridine completely. The residues were dissolved in chloroform:methanol (5:1) and applied to silica gel TLC plates which were developed with chloroform:methanol (10:1). Examination under uv light of the developed plates showed two bands which corresponded to 3′-*O*-acetylthymidine and its sulfonated product. The mobilities of 5′-*O*-mesitylsulfonyl, 5′-*O*-benzenesulfonyl, 5′-*O*-toluenesulfonyl, 5′-*O*-nitrobenzenesulfonyl, or 5′-*O*-2,4,6-triisopropylbenzenesulfonyl derivatives of 3′-*O*-acetylthymidine with respect to T(oAc) are 1.6, 1.7, 1.8, 1.7, and 1.9, respectively. These compounds were isolated from the TLC plates in the usual manner using chloroform–methanol (5:1), the yields were determined spectrophotometrically, and the results are indicated in Figure 2.

Phosphorylation of a 5′-N-Protected Nucleoside (Preparation of Fully Protected Nucleotides). (i) Using Mesitylenesulfonyltriazole (MST). The 5′-*O*-dimethoxytrityl acyl deoxynucleosides (10 mmol) in anhydrous pyridine (40 ml) were treated with *p*-chlorophenyl phosphate (12 mmol) in the presence of MST (30 mmol) for 36 hr at 20°. Next, β-cyanoethanol (40 mmol) along with additional MST (10 mmol) was added to the reaction mixture and kept a further 24 hr at 20°. The reaction mixture was decomposed by adding water (10 ml) under cooling and then chloroform (100 ml) and finally washing with triethylammonium bicarbonate buffer (3 × 50 ml). The chloroform layer was dried over anhydrous sodium sulfate and evaporated in the presence of excess toluene to a gum. The gum was dissolved in chloroform (50 ml) and chromatographed on a silica gel column (500 g) by eluting with chloroform–methanol (50:1; 2 l.). In the case of fully protected guanosine, chloroform–methanol (30:1) was necessary for eluting the column. The yields of fully protected mononucleotides were from 80 to 90%.

(ii) **Using (1*H*)-1,2,4-Triazole.** A mixture of *p*-chlorophenyl phosphodichloridate (12 mmol), (1*H*)-1,2,4-triazole (25 mmol), and triethylamine (24 mmol) was stirred in anhydrous dioxane (100 ml) for 1 hr at room temperature. The precipitated triethylammonium chloride was removed by filtration, and the filtrate was concentrated (to 50 ml) under reduced pressure, temperature not exceeding 20°. To this solution was added the N,5′-protected nucleoside (10 mmol), and the reaction mixture was kept at 20° for 8 hr; subsequently β-cyanoethanol (40 mmol) was added and the reaction maintained overnight. The fully protected mononucleotide was isolated by the usual procedure as described above in 70–85% yields.

(a) 5′-*O*-(Di-*p*-methoxytrityl)-*N*-benzoyldeoxyadenosine-3′-*p*-Chlorophenyl β-Cyanoethyl Phosphate: mp 68–70°; uv spectrum (95% ethanol) λ_{max} 280 mμ (ε 20500); λ_{min} 255 mμ (ε 12700); ir bands (in $CHCl_3$) 3400, 3000, 2920, 2843, 2250, 1710, 1610, 1490, 1250 cm^{-1}; NMR ($CDCl_3$, ppm from Me_4Si) δ 8.7 (1 H, s, NH), 8.3 (1 H, s, H-8), 6.5 (1 H, t, $H_{1'}$), 4.37 (3 H, m, $H_{4'}$ and OCH_2), 3.80 (3 H, s, OCH_3), 2.7 (4 H, m, $H_{2'}$ and CH_2CN). Anal. Calcd for $C_{47}H_{42}O_9N_6PCl$: C, 62.66; H, 4.70; N, 9.33. Found: C, 62.62; H, 4.92; N, 9.29.

(b) 5′-*O*-(Di-*p*-methoxytrityl)-*N*-benzoyldeoxycytidine-3′-*p*-chlorophenyl β-Cyanoethyl Phosphate: mp 77–80°; uv spectrum (95% ethanol) λ_{max} 305 mμ (ε 9600); λ_{max} 261 mμ (ε 20700); λ_{min} 285 mμ (ε 7000); and λ_{min} 242 mμ (ε 13000); ir bands (in $CHCl_3$) 3400, 3000, 2250, 1700, 1663, 1480, 1250 cm^{-1}; NMR ($CDCl_3$, ppm from Me_4Si) δ 9.3 (1 H, b s, NH), 6.33 (3 H, t, $H_{1'}$), 4.33 (3 H, m, $H_{4'}$ and OCH_2), 3.79 (3 H, s, OCH_3), 2.66 (4 H, m, $H_{2'}$ and CH_2CN). Anal. Calcd for $C_{46}H_{42}O_{10}N_4PCl$: C, 62.96; H, 4.83; N, 6.39. Found: C, 63.19; H, 5.01; N, 6.33.

(c) **5′-*O*-(Di-*p*-methoxytrityl)-*N*-acetyldeoxyguanosine-3′-*p*-chlorophenyl β-Cyanoethyl Phosphate:** mp 82–84°; uv spectrum (95% ethanol) λ_{max} 278 mμ (ε 17100); λ_{max} 262 mμ (ε 18500); λ_{min} 272 mμ (ε 16400); and λ_{min} 250 mμ (ε 17200); ir bands (in $CHCl_3$) 3200, 2920, 2250, 1695, 1608, 1488, 1250 cm^{-1}; NMR ($CDCl_3$, ppm from Me_4Si) δ 9.75 (1 H, s, NH), 7.85 (1 H, s, H-8), 6.2 (1 H, t, $H_{1'}$), 4.4 (3 H, m, $H_{4'}$ and OCH_2), 3.80 (3 H, s, OCH_3), 2.71 (4 H, m, $H_{2'}$ and CH_2CN), 2.1 (3 H, s, CH_3CO). Anal. Calcd for $C_{42}H_{40}O_{10}N_6PCl$: C, 58.94; H, 4.67; N, 9.82. Found: C, 58.81; H, 4.72; N, 9.75.

General Method for the Synthesis of Di-, Tri-, or Hexanucleotide of Defined Sequences. A 5′-*O*-dimethoxytrityl-N-protected nucleotide (or oligonucleotide)-3′-*p*-chlorophenyl phosphate (1 mol equiv) and an appropriate nucleotide containing free 5′-hydroxyl and fully protected 3′-*O* phosphate group (1.2 mol equiv) were mixed in anhydrous pyridine (5 ml per g of the nucleotidic components) and treated with TPS, MST, or *p*-NBST (2 mol equiv based on 5′-protected component) for 36 hr at 20°. The reaction mixture was then decomposed by addition of water (10 ml per g) with cooling, followed by extraction with chloroform (100 ml per g). The chloroform layer was washed with 0.1 *N* triethylammonium bicarbonate buffer (three times) and then with water, dried over anhydrous sodium sulfate, and under reduced pressure evaporated to a gum in the presence of excess toluene. The gum was dissolved in chloroform and purified by column chromatography on silica gel providing isolated yields of various oligonucleotides as documented in Tables I and II. (See Figure 5.)

Complete Deblocking of the Fully Protected Oligonucleotides. A fully protected oligonucleotide (100 mg) was treated with 0.1 *N* sodium hydroxide in dioxane–water (4:1) (100 ml) for 3–6 hr at 20° to remove the cyanoethyl and the *p*-chlorophenyl group from the internal phosphotriester functions. The solution was neutralized with Dowex-50 resin (pyridinium form) and filtered and the filtrate evaporated in vacuo to a gum. The gum was dissolved in concentrated ammonia and sealed in a flask which was heated at 50° for 3 hr to deblock the N-protecting groups. This solution was concentrated to dryness in vacuo, the residue was dissolved in water (50 ml) and washed with ether (2 × 10 ml), and the aqueous layer was evaporated to dryness. The residue then was treated with 80% acetic acid (50 ml) for 20 min at room temperature to remove the dimethoxytrityl group. The excess acetic acid was removed by evaporation in the presence of pyridine, the resulting material dissolved in 0.1 *M* triethylammonium bicarbonate buffer, pH 7.5, and the desired unprotected oligonucleotide was finally isolated in pure form by gel filtration on Sephadex G-25 (SF) K25-100 columns at 4°.

Characterization of Oligonucleotides. (a) Silica Gel TLC. All of the phosphotriester compounds were checked for purity on silica gel TLC plates in two solvent systems. Before a sample was applied to silica gel TLC plate, pyridine was removed completely by coevaporation with toluene. The compound was dissolved in chloroform for application to the plate. Some components showed elongated (multiple) spots due to the presence of diastereoisomeric phosphate triesters;[1] however, after complete deblocking, such compounds produced a single sharp spot on TLC in the usual aqueous solvent systems.

(b) Avicel-Cellulose TLC. The homogeneity of each oligonucleotide (1–5 $O.D._{260\text{-}nm}$) purified by gel filtration on a Sephadex column was checked by TLC on Avicel-Cellulose plates 0.1 mm in thickness in various aqueous solvent systems. About 0.5–1 absorbance unit at 260 nm of each oligonucleotide showed only one spot on the plates in *two* solvent systems. The R_f values of each compound are recorded in Table III.

(c) Spleen Phosphodiesterase Digestion. The 5′-hydroxyl oligonucleotide (1–2 absorbance units at 260 nm) was digested with spleen phosphodiesterase (1 mg/ml) in 0.1 *M* ammonium acetate, pH 6.5 (20 μl), for 30 min. The digested solution was chromatographed on Avicel-Cellulose plates (0.1-mm thick) in solvent C. 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 IV.

(d) Sequence Analysis of dG-A-G-C-G-G. The 5′-labeled [^{32}P]-G-A-G-C-G-G was prepared with polynucleotide kinase and [γ-^{32}P]-ATP according to Richardson procedure[20] followed by its isolation on Sephadex G-25 (SK10-100) column. The labeled compound was then digested at 37°C in 15 μl containing 30 μg of RNA carrier (partially hydrolyzed yeast RNA), 50 m*M* Tris-HCl (pH 8), 5 m*M* $MgCl_2$ and 0.2 μg of venom phosphodiesterase. Seven 2-μl samples were removed at increasing time intervals and blown into 5 μl of 1 *M* ammonium hydroxide containing 1 m*M* EDTA. Samples were taken after 10, 20, 40, 60, 90, 120, and 180 min. The combined digest was dried in a desiccator, dissolved in 2–5 μl of water and applied to a cellulose acetate strip (2.5 × 52 cm) with 0.2 μl of the standard mixture of three dyes on each side of the sample. Electrophoresis was carried out at pH 3.5 (pyridine–acetate buffer) for 40 min at 2500 V.[16] After transfer of the oligomers from the cellulose acetate strip onto a DEAE-cellulose (1:9) glass plate (20 × 40 cm), the plate was allowed to air-dry at room temperature for 2–4 hr. Chromatography was then carried out in homo-mix V[17] until the blue dye marker was 1 in. from the top of the plate. Its x-ray fingerprinting pattern is shown in Figure 3.

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