Developments in Syntheses of Oligodeoxyribonucleotides and Their Organic Derivatives[1]

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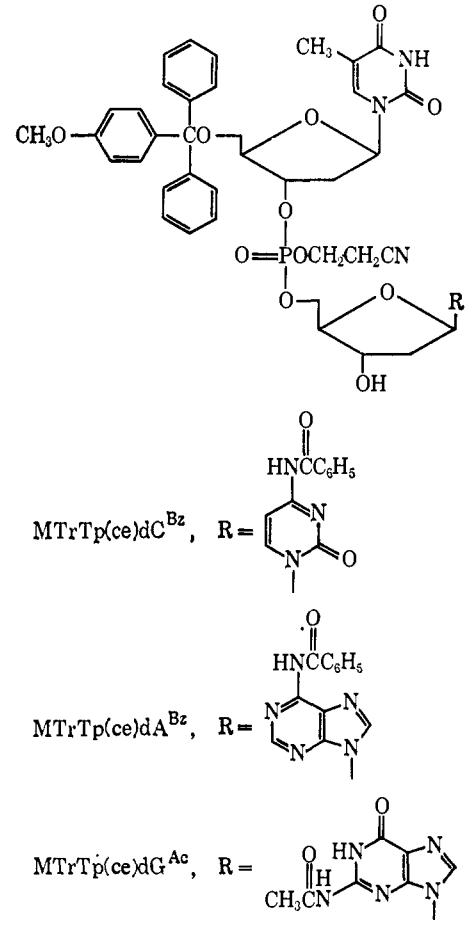
Abstract: The β-cyanoethyl phosphotriester approach to the synthesis of oligonucleotides is shown to be general for deoxyribonucleosides by the synthesis of MTrTp(ce)dC^{Bz}, MTrTp(ce)dA^{Bz}, and MTrTp(ce)dG^{Ac} and the conversion of these derivatives to TpdC, TpdA, and TpdG, respectively. The utility of the synthetic scheme is also extended by the observation that β-benzoylpropionyl serves as a protecting group for the 3′-O of nucleosides employed in the condensation reaction. This group, which prevents the formation of isomers with 3′–3′-internucleotide links, can be removed selectively with hydrazine in pyridine–acetic acid. The chemistry is illustrated with the synthesis of the β-cyanoethyl ester of 5′-O-(*p*-monomethoxytrityl)thymidylyl-(3′–5′)-3′-O-(β-benzoylpropionyl)thymidine and the conversion of this compound to higher molecular weight oligonucleotide derivatives, from which TpTpT and TpTpdC are prepared. With the aid of the β-benzoylpropionyl protecting group, intermediates can be obtained which enable one to use the phosphotriester approach to join preformed blocks containing two or three nucleoside units. The synthesis of TpTpTpT and TpTpTpTpTpT by this method is described.

In this paper the β-cyanoethyl phosphotriester method for synthesis of oligodeoxyribonucleotides, previously demonstrated by the preparation of derivatives of thymidine,[2] is extended in three ways. It is shown that each of the common deoxyribonucleosides will react with a β-cyanoethylphosphoryl nucleoside to form a phosphotriester derivative, that β-benzoylpropionyl[1b] serves as an effective protecting group for the 3′-O- of nucleosides, ensuring the exclusive formation of 3′–5′ internucleotide links, and that chains containing two or three nucleotide units can be joined by the phosphotriester method.

Derivatives of Dinucleoside Monophosphates. To determine whether the β-cyanoethyl phosphotriester approach would be applicable to deoxyribonucleosides other than thymidine, we investigated several condensations involving the other common deoxyribonucleosides. In one series, 50 μmol of 5′-O-(*p*-monomethoxytrityl)-thymidine was phosphorylated with β-cyanoethyl phosphate and mesitylenesulfonyl chloride. One half of the MTrTp(ce)[2] thus prepared was treated (2 hr) with 50 μmol of N-benzoyldeoxycytidine and 75 μmol of 2,4,6-triisopropylbenzenesulfonyl chloride in pyridine. The other half was similarly treated with N-benzoyldeoxyadenosine and 2,4,6-triisopropylbenzenesulfonyl chloride. Removal of the protecting groups with ammonium hydroxide and aqueous acetic acid, gave TpdC and TpdA. The yields, based on *p*-monomethoxytritylthymidine and determined spectrophotometrically from products eluted from paper chromatograms, were 56 and 83%, respectively. As in the case of TpT,[2] a portion of the dinucleoside phosphate (8% for TpdC and 4% for TpdA) was not degraded by snake venom phosphodiesterase, indicative of the formation of a small amount of material with the 3′–3′ phosphodiester link.

For isolation and characterization of the products, the reactions were scaled up and the 3′–3′ contaminates were removed as in the synthesis of MTrTp(ce)T.[2] Thus, 1 mmol of 5′-O-(*p*-monomethoxytrityl)thymidine was phosphorylated with excess β-cyanoethyl phosphate and the resulting product was condensed with 1.5 mmol of N-benzoyldeoxyadenosine or N-benzoyldeoxycytidine. The nucleotidic products isolated from these reactions were then treated with *p*-monomethoxytrityl chloride in order to derivatize the isomers possessing a

Chart I



(1) Part XV in a series on nucleotide chemistry. A preliminary account of a portion of the work has appeared: (a) R. L. Letsinger, M. H. Caruthers, P. S. Miller, and K. K. Ogilvie, *J. Am. Chem. Soc.*, **89**, 7146 (1967); (b) for part XIV see R. L. Letsinger and P. S. Miller, *ibid.*, **91**, 3356 (1969).

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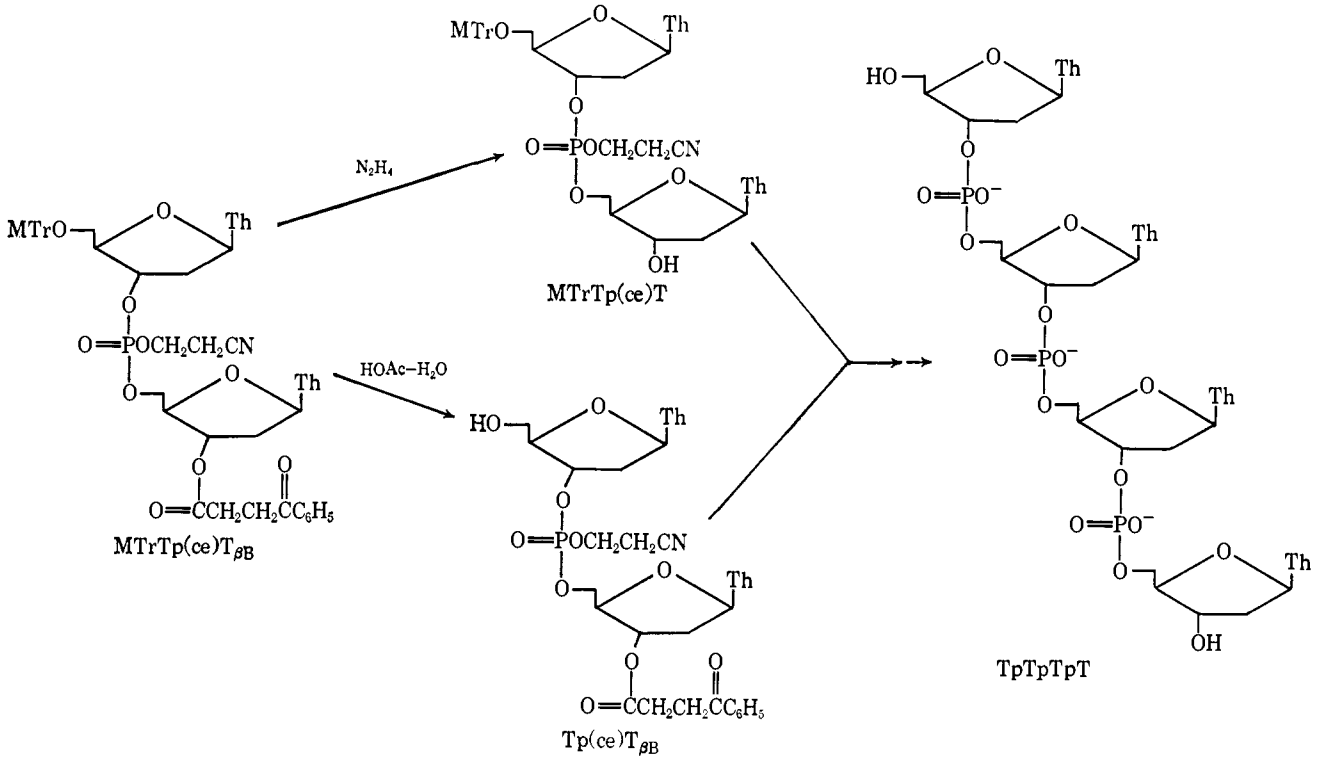
(2) R. L. Letsinger and K. K. Ogilvie, *ibid.*, **89**, 4801 (1967); **91**, 3350 (1969).

Chart II



5′-OH group (*i.e.*, material with a 3′–3′ phosphotriester link). Chromatography on silica gel with ethyl acetate and tetrahydrofuran afforded the pure dinucleoside phosphate derivatives as white powders. They were characterized by elemental analysis and by hydrolysis. In this manner MTrTp(ce)dA^{Bz} and MTrTp(ce)dC^{Bz} were obtained in yields of 55 and 51% (see Chart I). When the blocking groups were removed, the resulting dinucleoside phosphates were found to be completely degradable to nucleoside and nucleoside phosphate by both spleen and snake venom phosphodiesterase; therefore, they must have been free of material with 3′–3′ internucleotide links.

MTrTp(ce)dG^{Ac} was prepared in the same manner with two modifications in the isolation and purification technique. Enzymatic assays on small samples of TpdG prepared and isolated directly by paper chromatography showed that the product was completely degradable by both snake venom and spleen phosphodiesterase. Since this observation indicates that the condensation involving N-acetyldeoxyguanosine occurs selectively at the 5′-O to give only the 3′–5′ isomer of the dinucleoside phosphate derivative,[3] the step involving treatment of the reaction products with *p*-monomethoxytrityl chloride was omitted in the work-up procedure. The other change was necessitated by the fact that MTrTp(ce)dG^{Ac} did not elute from silica gel with tetrahydrofuran. This compound was eluted from the silica gel with methanol and then rechromatographed on Mallinckrodt ChromAR sheets to remove traces of material extracted from the silica gel with methanol. The yield of MTrTp(ce)dG^{Ac} was 51%.

Syntheses with β-Benzoylpropionylnucleosides. While the procedure for purifying the 3′–5′-linked isomers by derivatizing and removing the 3′–3′-linked isomers is satisfactory for the preparation of short-chain oligonucleotides, the alternative of utilizing 3′-O-protected nucleosides in the condensation to form the triester, thereby preventing formation of 3′–3′ phosphotriester links, is more attractive for an extended synthesis. In the case of reactions conducted on an insoluble polymer support use of 3′-O-protected derivatives is essential since the 3′–3′-linked isomers cannot be separated while the material is bound to the support.

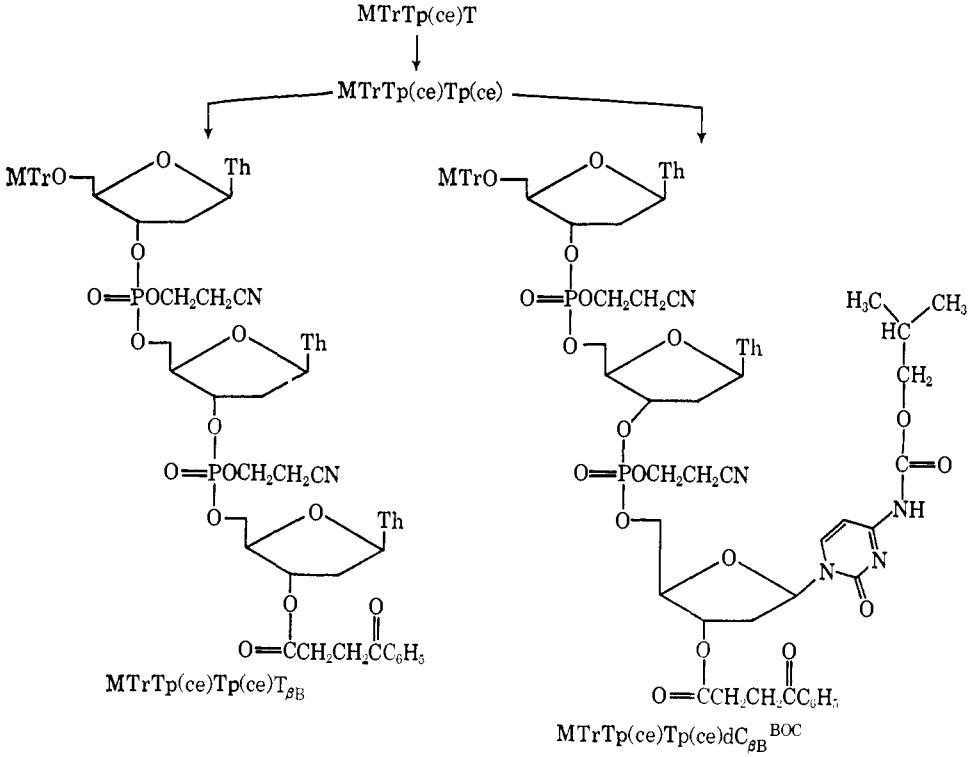
An investigation of the chemistry of esters of β-benzoylpropionic acid indicated that the β-benzoyl propionyl group should serve well as a masking group for the 3′-O of nucleosides in these syntheses. To test the utility of this group we accordingly prepared 3′-O-β-benzoylpropionylthymidine[1] ($T_{\beta B}$), 3′-O-β-benzoylpropionyl-N-isobutyloxycarbonyldeoxycytidine[1] (dC$_{\beta B}^{BOC}$), and 3′-O-β-benzoylpropionyl-N-benzoyldeoxyadenosine[4] (dA$_{\beta B}^{Bz}$) and subjected these compounds to reaction with 3′-O-(β-cyanoethylphosphoryl)-thymidine derivatives.

Phosphorylation of 5′-O-(*p*-monomethoxytrityl)-thymidine with β-cyanoethyl phosphate followed by reaction with 3′-O-β-benzoylpropionylthymidine gave MTrTp(ce)T$_{\beta B}$ (Chart II). This compound was isolated in 63% yield, which is comparable to the yield of MTrTp(ce)T obtainable from the corresponding reaction of thymidine.[2] In addition, the TpT obtained on

(3) The possibility that the 3′–3′ isomer of TpdG is attacked by both snake venom and spleen phosphodiesterase is highly unlikely in view of the enzymatic selectivity found for the other dinucleoside monophosphates.

(4) G. W. Grams and R. L. Letsinger, *J. Org. Chem.*, 33, 2589 (1968). This compound was prepared before it was found that hydrazine would remove an N-benzoyl group on the adenine ring as well as O-β-benzoylpropionyl groups (R. L. Letsinger, P. S. Miller, and G. W. Grams, *Tetrahedron Letters*, 2621 (1968)). For multipstep syntheses it would be necessary to use a group other than benzoyl at the N position (*e.g.*, isobutyloxycarbonyl); however the benzoyl derivative serves adequately to test the reactivity of a 3′-O-β-benzoylpropionyldeoxyadenosine derivative in a condensation to give a phosphotriester.

Chart III



removal of the protecting groups was all the 3′–5′ isomer as judged by the enzymatic assay.

MTrTp(ce)$T_{\beta B}$ prepared in this manner is a useful intermediate for the synthesis of higher molecular weight compounds since the 3′- and the 5′-O-protecting groups can be removed independently without disturbing the β-cyanoethyl group. Aqueous acetic acid reacts selectively at the 5′-ether function to give Tp(ce)$T_{\beta B}$ and tritanol whereas hydrazine hydrate in pyridine–acetic acid attacks the 3′-O-β-benzoylpropionyl group, yielding MTrTp(ce)T and 4,5-dihydro-6-phenylpyridazone. Although of less synthetic value, it is of interest that the β-cyanoethyl group can be eliminated selectively by brief exposure to aqueous ammonium hydroxide at room temperature. Under these conditions neither the methoxytrityl ether nor the β-benzoylpropionic ester functions are attacked.

That the sequence can be extended further was demonstrated by the synthesis of MTrTp(ce)Tp(ce)$T_{\beta B}$ and MTrTp(ce)Tp(ce)$dC_{\beta B}^{BOC}$ (Chart III). These compounds were obtained in yields of 71 and 40%, respectively, by cyanoethylphosphorylation of MTrTp(ce)T, obtained from the hydrazinolysis of MTrTp(ce)$T_{\beta B}$, and condensation of the resulting phosphodiester with the appropriate 3′-O-β-benzoylpropionylnucleoside. Each compound was converted cleanly to the trinucleoside diphosphate (TpTpT or TpTpdC) by successive treatment with ammonium hydroxide and aqueous acetic acid. Since these compounds were completely degradable enzymatically, the experiments show that the β-benzoylpropionyl group satisfactorily protects the 3′-O of both thymidine and deoxycytidine during formation of the phosphotriester. That it will also serve satisfactorily for the deoxyadenosine analog was shown by preparation of TpdA (58% yield) from 5′-O-(*p*-monomethoxytrityl)thymidine and 3′-O-β-benzoylpropionyl-N-benzoyldeoxyadenosine.

In principle, MTrTp(ce)$T_{\beta B}$ could also be prepared by phosphorylating 3′-O-β-benzoylpropionylthymidine with β-cyanoethyl phosphate and condensing the resulting phosphodiester (p(ce)$T_{\beta B}$) with 5′-O-(*p*-monomethoxytrityl)thymidine. This approach, however, has not proved satisfactory in our hands. The reaction with β-cyanoethyl phosphate proceeded well; however, the subsequent attempt to form the phosphotriester by reaction at the 3′-OH was unsuccessful. Most of the 5′-O-(*p*-monomethoxytrityl)thymidine was recovered unchanged.

Syntheses with Preformed Oligonucleotide Blocks. In the syntheses thus far described in this series, oligonucleotides were constructed in a stepwise fashion by addition of one nucleoside unit at a time. For syntheses of higher molecular weight materials it would be desirable to build with preformed oligonucleotide segments containing two or more nucleoside units. That the phosphotriester method can be adapted to syntheses of this type was demonstrated by the preparation of TpTpTpT and TpTpTpTpTpT from segments containing two and three nucleoside units, respectively.

The tetrathymidine derivative was obtained by condensation of MTrTp(ce)Tp(ce) with Tp(ce)$T_{\beta B}$ in pyridine in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (Chart II). Removal of the protecting groups afforded TpTpTpT that was completely degradable enzymatically. The yield was influenced by the concentration of the reactants in the condensation reaction. Under the standard conditions used for the reactions involving addition of single nucleoside units, the yield of TpTpTpT was only 27%. The low yield in this case probably reflects the greater steric hindrance to approach at the 5′-OH in the dinucleoside derivative. When the pyridine solution was concentrated so that the reaction occurred in a viscous gum, the yield rose to 52%. The hexathymidine derivative, TpTpTpTpTpT,

Table I. Properties of Dinucleoside Phosphate Derivatives

	Yield, %	Mp,[a] °C	λ_{max},[b] mμ (ε)	λ_{min},[b] mμ (ε)	R_f in solvent EtOAc[c]	R_f in solvent THF[c]	R_f in solvent C[d]
MTrTp(ce)dA^{Bz}	55	130–133	275 (26,900)	247 (17,900)	0.10	0.70	0.85
MTrTp(ce)dC^{Bz}	51	130–136	262 (31,800)	243 (22,300)	0.10	0.70	0.87
MTrTp(ce)dG^{Ac}	51	146–148	261 (22,100)	244 (15,900)	0.0	0.15	0.78
MTrTp(ce)T$_{\beta B}$	63	~125	245 (23,310)	227 (25,400)	0.21	0.76	

[a] The melting points are generally not sharp and vary somewhat from one preparation to another. [b] In 95% C_2H_5OH. [c] On Eastman Chromagram sheets 6060 with ethyl acetate (EtOAc) and tetrahydrofuran (THF). [d] Paper chromatography on Whatman 3 MM paper in solvent C.

Table II. Analytical Data

Compound	Formula	Calcd, % C	Calcd, % H	Calcd, % N	Found, % C	Found, % H	Found, % N
MTrTp(ce)dA^{Bz}	$C_{50}H_{49}N_8O_{12}P\cdot H_2O$	59.88	5.13	11.17	59.83	5.25	10.38
MTrTp(ce)dC^{Bz}	$C_{49}H_{49}N_6O_{13}P\cdot H_2O$	60.12	5.25	8.59	60.18	5.22	8.14
MTrTp(ce)dG^{Ac}	$C_{45}H_{47}N_8O_{13}P\cdot H_2O$	56.48	5.16	11.71	56.40	5.01	11.45
MTrTp(ce)T$_{\beta B}$	$C_{53}N_{54}N_5O_{15}P$	61.68	5.27	6.79	61.13	5.20	6.74

was similarly prepared in 41% yield from MTrTp(ce)Tp(ce)T and Tp(ce)Tp(ce)T$_{\beta B}$, these two reagents having been obtained from MTrTp(ce)Tp(ce)T$_{\beta B}$ by selective removal of the *p*-monomethoxytrityl on one hand and the β-benzoylpropionyl groups on the other.

Experimental Section

For the equipment and procedures used in carrying out the reactions and characterizing the products by electrophoresis, chromatography, and spectral analysis see ref 2. The solvents used in paper chromatography were: A, isopropyl alcohol–concentrated ammonium hydroxide–water (7:1:2); C, 1 *M* ammonium acetate–ethanol (3:7); E, 0.5 *M* ammonium acetate–ethanol (3:7, adjusted to pH 3.5 with acetic acid); F, *n*-propyl alcohol–concentrated ammonium hydroxide–water (55:10:35).

Elemental analyses were performed by the Micro Tech Laboratories, Skokie, Ill. and by Miss H. Beck, Chemistry Department, Northwestern University.

General Procedure for Synthesis of β-Cyanoethyl Derivatives of Dinucleoside Phosphates. 5′-O-(*p*-Monomethoxytrityl)thymidine (0.515 g, 1 mmol) and 2.0 mmol of pyridinium mono-β-cyanoethyl phosphate were mixed and dried by dissolving in pyridine and removing the pyridine under vacuum. Mesitylenesulfonyl chloride (0.654 g, 3.0 mmol) and pyridine (2 ml) were added and the solution was stirred for 7 hr, whereupon 2 ml of water was added. After 14 hr of additional stirring, the solution was diluted with 2 ml of water and extracted with chloroform (three 10-ml portions). The chloroform extracts were combined, washed with water, concentrated to a gum, dried by evaporation of pyridine (four 5-ml portions), and finally taken up in 2 ml of dry pyridine.

For the coupling reaction 1.5 mmol of nucleoside and 2.0 mmol (0.604 g) of 2,4,6-triisopropylbenzenesulfonyl chloride were added to the solution of MTrTp(ce). The mixture was stirred for 24 hr, diluted with 2 ml of water, stirred 10 hr, again diluted with 2 ml of water, and finally extracted with chloroform (two 15-ml portions). All material possessing the methoxytrityl group was present in the chloroform solution, as determined by spraying thin layer chromatograms of samples taken from the aqueous and the chloroform layers.

In cases where 3′–3′-linked isomer was to be removed, the chloroform layer was concentrated at reduced pressure and the residue, after drying by evaporation of pyridine, was stirred for 15 hr with 370 mg (1.2 mmol) of *p*-monomethoxytrityl chloride in 2 ml of pyridine. The solvent was then removed at reduced pressure and the residual gum taken up in a small volume of chloroform.

Separation and purification was achieved by chromatography in two portions on a silica gel column (45 × 2 cm) made up with ethyl acetate. In a typical case impurities were removed by eluting the column with 3–4 l. of ethyl acetate. The β-cyanoethyl phosphotriester derivative was then eluted with tetrahydrofuran (300–900 ml), the separation being followed by thin layer chromatography. Intermediate fractions which contained both the cyanoethyl phosphotriester and impurities were purified by rechromatography. The products (>0.5 g in each case) were recovered by precipitation from the tetrahydrofuran solutions by addition of hexane. In the preparation of MTrTp(ce)dG^{Ac} the treatment with *p*-monomethoxytrityl chloride was omitted and the product, which moved very slowly on silica gel, was eluted with methanol. The material recovered from the methanol was somewhat contaminated, as indicated by a low value for the nitrogen analysis. It was further purified by chromatography on Mallinckrodt ChromAR Sheet 500. Chromatography and elution of 15 mg of the crude product from the ChromAR sheet (6.5 × 8 in.) with tetrahydrofuran followed by precipitation with hexane gave 13.5 mg of good quality MTrTp(ce)dG^{Ac}.

The procedure for MTrTp(ce)T$_{\beta B}$ differed from the "general method" in that 2.5 mmol of β-cyanoethyl phosphate and 4 mmol of mesitylenesulfonyl chloride were used in the initial phosphorylation and, of course, the purification step involving *p*-monomethoxytrityl chloride was omitted.

Data on yields and properties of the products are given in Table I and analytical data are presented in Table II.

Thymidylyl-(3′–5′)-deoxyadenosine was prepared on a small scale by a similar procedure from 13 mg (0.025 mmol) of 5′-O-(*p*-monomethoxytrityl)thymidine, 0.05 mmol of β-cyanoethyl phosphate, and 26 mg (0.05 mmol) of N-benzoyl-3′-O-(β-benzoylpropionyl)deoxyadenosine.[4] The volume of pyridine was 0.3 ml. Protecting groups were removed by stirring the products with 80% aqueous acetic acid and then with ammonium hydroxide in pyridine. Chromatography in solvent F showed Tp (23%, R_f 0.48), TpdA (58%, R_f 0.63), dA (R_f 0.75), and spots at 0.87 and 0.92. The yields are based on MTrT. Assay of TpdA by hydrolysis with snake venom phosphodiesterase showed thymidine (R_f^F 0.78, 1.60 OD units) and pdA (R_f^F 0.42, 2.57 OD units). Assay with the spleen enzyme yielded Tp (R_f^F 0.44, 1.75 OD units) and deoxyinosine[5] (R_f^F 0.63, 2.45 OD units).

Dinucleoside Monophosphates. The procedure used to prepare the dinucleoside monophosphates is represented by the conversion of dMTrTp(ce)dA^{Bz} to TpdA. A solution of 10 mg of dMTrTp(ce)-dA^{Bz} in 0.5 ml of pyridine and 1 ml of ammonium hydroxide was stirred for 3 days. The solvent was taken off under reduced pressure and the residue was dissolved in 1 ml of 80% aqueous acetic acid. After 90 min, the solution was applied as a strip to Whatman 3 MM paper and eluted with solvent F. A single nucleotide product was found; R_f 0.65 in solvent F; electrophoretic mobility relative to Tp at pH 8.0, 0.32. It was characterized by hydrolysis with snake venom phosphodiesterase to thymidine (R_f^F 0.74) and deoxyadenosine 5′-phosphate (R_f^F 0.43) and with spleen phosphodiesterase to deoxyinosine[5] (R_f^F 0.65) and thymidine 3′-phosphate (R_f^F 0.42).

All of the dinucleoside monophosphates were completely degraded by both spleen and snake venom phosphodiesterases as judged by paper chromatography and paper electrophoresis. The

(5) Deoxyadenosine is converted to deoxyinosine by the enzyme preparation used in this work: K. K. Ogilvie and R. L. Letsinger, *Biochem. Biophys. Res. Commun.*, **30**, 273 (1968).

ratio of nucleotide to nucleoside measured spectrophotometrically for the products of each of the hydrolyses was 1.03 ± 0.05 for reactions catalyzed by snake venom phosphodiesterase and 0.95 ± 0.01 for reactions catalyzed by spleen phosphodiesterase.

β-Cyanoethyl Ester of 5′-O-(*p*-Monomethoxytrityl)thymidylyl-(3′–5′)-thymidine (MTrTp(ce)T). To 100 mg (0.10 mmol) of MTrTp(ce)$T_{\beta B}$ in 2.4 ml of pyridine was added 0.3 ml of hydrazine hydrate in 0.6 ml of acetic acid. After 3.5 hr the solution was concentrated to a small volume and applied to a silica gel column (25 × 1.5 cm) made up in ethyl acetate. Elution with 300 ml of ethyl acetate and then 250 ml of tetrahydrofuran yielded from the tetrahydrofuran solution 60 mg (71%) of MTrTp(ce)T, mp 122–130°. For characterization approximately 1 mg was hydrolyzed with 0.5 ml of 80% aqueous acetic acid (20 min on steam bath). Chromatography of the solution on Whatman 3 MM paper with solvent F gave a single nucleotidic band (TpT, R_f^F 0.63) which was eluted with water and lyophilized to a powder. This material was completely degraded by snake venom phosphodiesterase (pT/T = 0.92) and by spleen phosphodiesterase (Tp/T = 0.96).

Stepwise Removal of β-Cyanoethyl and β-Benzoylpropionyl Groups from MTrTp(ce)$T_{\beta B}$. To 5 mg of MTrTp(ce)$T_{\beta B}$ in 0.1 ml of pyridine was added 0.1 ml of ammonium hydroxide. Cleavage of the β-cyanoethyl group was complete within 1 min as indicated by chromatography of the mixture on silica slides with tetrahydrofuran; the R_f of the starting material was 0.76 and after 1 min of reaction in ammonium hydroxide the R_f of the nucleotidic product was 0.10.

The course of the reaction was followed by paper chromatography with a solvent consisting of 1 *M* aqueous ammonium acetate and isopropyl alcohol (2:8 v/v) adjusted to pH 6.5 with acetic acid. This solvent was selected since MTrTp(ce)$T_{\beta B}$ and MTrTpT were stable in it and gave distinguishable R_f values (0.91 for MTrTp(ce)$T_{\beta B}$ and 0.60 for MTrTpT). Chromatography of the product from a reaction in ammonium hydroxide that had proceeded for ~2 min showed only one spot, at R_f 0.74 as expected for MTrTp$T_{\beta B}$. As the reaction progressed, two new substances appeared at R_f 0.62 (MTrTpT) and at 0.86 (cleavage fragment derived from the benzoylpropionyl group) and the relative intensity of the material at R_f 0.74 decreased. After 2 hr about 50% of the benzoylpropionyl groups had been cleaved off and within 24 hr MTrTp$T_{\beta B}$ had been converted completely to MTrTpT.

β-Cyanoethyl Ester of Thymidylyl-(3′–5′)-3′-O-(β-benzoylpropionyl)thymidine (Tp(ce)$T_{\beta B}$). A solution of 100 mg (0.10 mmol) of MTrTp(ce)$T_{\beta B}$ in 20 ml of 80% aqueous acetic acid was heated on a steam bath for 10 min. The solvent was removed at reduced pressure and the residue was taken up in a small amount of tetrahydrofuran and chromatographed on a silica gel column (25 × 2 cm) by elution first with 200 ml of ethyl acetate and then with 500 ml of ethyl acetate–tetrahydrofuran (1:1 v/v), 50-ml fractions being collected. The first three fractions contained tritanol and fractions 6–13 contained the nucleotidic product. Fractions 6–13 were combined and concentrated. On addition of hexane 67 mg (90%) of Tp(ce)$T_{\beta B}$ was obtained as a white precipitate. It softened at 105° and melted principally 113–116°; R_f (tlc) in tetrahydrofuran 0.62, in 1:1 tetrahydrofuran–ethyl acetate 0.29; uv (95% ethanol) λ_{max} 249.5 mμ (ε 22,300), λ_{min} 228 mμ (ε 11,900), shoulder λ 263 mμ (ε 20,500).

Anal. Calcd for $C_{33}H_{38}N_5O_{14}P$: C, 51.50; H, 4.98; N, 9.10. Found: C, 51.63; H, 5.00; N, 8.78.

Bis-β-cyanoethyl Ester of Thymidylyl-(3′–5′)-thymidylyl-(3′–5′)-3′-O-(β-benzoylpropionyl)thymidine (MTrTp(ce)Tp(ce)$T_{\beta B}$). MTrTp(ce)T (0.872 g, 1 mmol) was mixed with 2 mmol of pyridinium β-cyanoethyl phosphate and 0.654 g (3 mmol) of mesitylenesulfonyl chloride in 5 ml of pyridine. The solution was concentrated to 2 ml (very viscous) and stirred at room temperature for 6.5 hr, whereupon 1 ml of pyridine and 2 ml of water were added. After 12 hr of stirring the solvent was evaporated and the residue was taken up in 30 ml of chloroform. This solution was washed twice with 5 ml of water saturated with sodium chloride (sodium chloride was added to reduce the solubility of the nucleotide derivatives in water); then the chloroform was stripped off and the residue was dried by evaporation of pyridine (three 5-ml portions). To MTrTp(ce)Tp(ce) thus prepared was added 0.604 g (2 mmol) of 2,4,6-triisopropylbenzenesulfonyl chloride and 0.810 g (2 mmol) of 3′-O-(β-benzoylpropionyl)thymidine in 5 ml of pyridine. The solution was successively concentrated to 2 ml, stirred for 32 hr, diluted with 2 ml of water and 1 ml of pyridine, stirred for 18 hr, and extracted twice with 10-ml portions of chloroform. The chloroform extracts were washed with water (10 ml) and evaporated, leaving a fluffy white solid which was dissolved in 3 ml of tetrahydrofuran and applied to a column (50 × 3.5 cm) of silica gel in ethyl acetate. Elution was carried out with 1 l. each of ethyl acetate, ethyl acetate–tetrahydrofuran mixtures in volumetric ratios of 9:1, 8:2, 7:3, 6:4, and 5:5, tetrahydrofuran, and tetrahydrofuran–methanol (9:1). Fractions of 200 ml were collected. Fractions 17–37, which contained the MTrTp(ce)Tp(ce)$T_{\beta B}$ were combined and evaporated. The residue was dissolved in tetrahydrofuran, filtered to remove some insoluble material (probably extracted from the silica gel) and reprecipitated with hexane. The MTrTp(ce)Tp(ce)$T_{\beta B}$, collected by centrifugation, amounted to 0.991 g (71%); mp 129–132° (softening from 121°); R_f (tlc, tetrahydrofuran) 0.70; ultraviolet spectrum: λ_{max} (95% ethanol) 263 mμ (ε 29,100), 250 mμ (ε 29,500), 238 mμ (ε 30,300); λ_{min} 257 mμ (ε 28,700), 244 mμ (ε 29,300), 229 mμ (ε 28,200).

Anal. Calcd for $C_{66}H_{70}N_8O_{22}P_2 \cdot H_2O$: C, 56.33; H, 5.16; N, 7.96. Found: C, 56.27; H, 5.16; N, 7.77.

The bis-β-cyanoethyl ester of thymidylyl-(3′–5′)-thymidylyl-(3′–5′)-3′-O-(β-benzoylpropionyl)thymidine, (Tp(ce)Tp(ce)$T_{\beta B}$), 73 mg (91%), mp 120–123° (softening from 112°), R_f (tlc) (tetrahydrofuran) 0.56, λ_{max} (95% ethanol) 263.5 mμ (ε 28,000), 253 (ε 27,000), λ_{min} 230 mμ (ε 15,900), was obtained by heating 0.100 g of MTrTp(ce)Tp(ce)$T_{\beta B}$ in 2 ml of 80% aqueous acetic acid on a steam bath, evaporating the solvent, and chromatographing the residue on a silica gel column.

Anal. Calcd for $C_{46}H_{54}N_8O_{21}P_2 \cdot H_2O$: C, 48.68; H, 4.97; N, 9.87. Found: C, 48.41; H, 4.86; N, 9.67.

The bis-β-cyanoethyl ester of 5′-O-(*p*-monomethoxytrityl)-thymidylyl-(3′–5′)-thymidylyl-(3′–5′)-thymidine, MTrTp(ce)Tp(ce)T, was prepared by removing the β-benzoylpropionyl group from 0.175 g of MTrTp(ce)Tp(ce)$T_{\beta B}$ by reaction (5 hr) with 0.04 ml of hydrazine hydrate in 0.9 ml of pyridine and 0.2 ml of acetic acid. Purification by chromatography on silica gel in the usual way gave a total of 0.124 g (80%) of MTrTp(ce)Tp(ce)T. The physical properties were the same as those for MTrTp(ce)Tp(ce)T prepared without use of the 3′-O-blocking group.[2] For further characterization a sample of the compound was converted to TpTpT by treatment with 80% aqueous acetic acid and ammonia under the usual conditions. The TpTpT (R_f^F 0.53; R_m^{Tp} 0.47 at pH 8.0) was completely degraded to pT (R_f^F 0.42, 19.5 OD units) and T (R_f^F 0.75, 10.40 OD units) in a ratio pT/T = 1.87 by snake venom phosphodiesterase and to Tp (R_f^F 0.46, 6.36 OD units) and T (R_f^F 0.82, 3.21 OD units) with a ratio Tp/T = 1.98 by spleen phosphodiesterase.

Bis-β-cyanoethyl Ester of 5′-O-(*p*-Monomethoxytrityl)-thymidylyl-(3′–5′)-thymidylyl-(3′–5′)-N-isobutyloxycarbonyl-3′-O-(β-benzoylpropionyl)deoxycytidine (MTrTp(ce)Tp(ce)$dC_{\beta B}^{BOC}$). a. This compound was prepared by essentially the same method used for MTrTp(ce)Tp(ce)$T_{\beta B}$, with 3′-O-(β-benzoylpropionyl)-N-isobutyloxycarbonyl deoxycytidine[1b] ($dC_{\beta B}^{BOC}$) (0.730 g, 1.5 mmol) replacing 3′-O-(β-benzoylpropionyl)ethymidine. Since the crude product isolated by chromatography on silica gel contained a salt of 2,4,6-triisopropylbenzenesulfonic acid (R_f on tlc with tetrahydrofuran 0.25) as well as MTrTp(ce)Tp(ce)$dC_{\beta B}^{BOC}$ (R_f on tlc with tetrahydrofuran 0.67), it was rechromatographed with 50% tetrahydrofuran–ethyl acetate and with tetrahydrofuran as eluents. The contaminant was eluted with the tetrahydrofuran–ethyl acetate mixture and the nucleotide derivate was obtained in the tetrahydrofuran solution. A total of 0.423 g (29%) of MTrTp(ce)Tp(ce)$dC_{\beta B}^{BOC}$ was obtained. For preparation of the analytical sample, the product was rechromatographed again on silica gel H (Merck) plates (1 mm thick). The compound melted at 112–115°.

Anal. Calcd for $C_{70}H_{77}N_9O_{23}P_2 \cdot 2H_2O$: C, 55.67; H, 5.37; N, 8.35. Found: C, 55.44; H, 5.24; N, 8.27.

Removal of the blocking groups by treatment successively with ammonium hydroxide (72 hr) and 80% acetic acid yielded TpTpdC (R_f 0.49 in solvent F and 0.14 in solvent A) which was separated from neutral material by electrophoresis (pH 7) and degraded to Tp and dC (2.24:1) by spleen phosphodiesterase and to T, pT, and pdC (1.0:1.2:0.7) by snake venom phosphodiesterase.[6]

b. In experiment a, a considerable quantity of material (R_f 0.48 on tlc in ethyl acetate) was obtained which appeared to be a product of reaction of 2,4,6-triisopropylbenzenesulfonyl chloride at the 5′-OH of $dC_{\beta B}^{BOC}$. The conditions of the reaction were accordingly modified to reduce the amount of sulfonylation of the nucleoside derivative. MTrTp(ce)T (1 mmol) was phosphorylated as before; then the MTrTp(ce)Tp(ce) was stirred with 333 mg (1.1

(6) The extinction coefficients used in calculating these values, 9.6 × 10^3 for thymidine and the thymidine phosphate and 7.4 × 10^3 for deoxycytidine and its phosphate at 260 mμ, were taken from Table V, H. Kössel, H. Büchi, and H. G. Khorana, *J. Am. Chem. Soc.*, **89**, 2185 (1967).

mmol) of 2,4,6-triisopropylbenzenesulfonyl chloride in 2 ml of pyridine for 3.5 hr, following which 0.504 g (1.2 mmol) of 3′-O-(β-benzoylpropionyl)-N-isobutyloxycarbonyldeoxycytidine was added. The mixture was stirred to dissolve the nucleoside, approximately 1 ml of the pyridine was stripped off, and the remaining thick syrup was stirred for 72 hr. Ethanol (1 ml) was then added, the solvent was taken off, and the residue was dissolved in chloroform. The products were partially separated by chromatography on silica gel (4 × 42 cm) by elution in succession with ethyl acetate, 50% ethyl acetate–tetrahydrofuran, and 1% methanol in tetrahydrofuran. Fractions containing MTrTp(ce)Tp(ce)dC$_{\beta B}^{BOC}$ were collected and rechromatographed. At this stage the product still contained a small amount of the triisopropylbenzenesulfonic acid salt as a contaminant. Elution with tetrahydrofuran through a short column (6.5 × 3 cm) of silica gel PF$_{254}$ (Merck) yielded 0.582 g (40% based on MTrTp(ce)T) (of MTrTp(ce)pT(ce)dC$_{\beta B}^{BOC}$, mp 125–130° with softening from 120°, which was homogeneous on tlc in ethyl acetate (R_f 0.03), tetrahydrofuran (R_f 0.65), and methanol (R_f 0.65). The infrared spectrum was identical with that for the product isolated from experiment a.

Doubling Experiments. The standard reaction sequence was used to convert two blocks containing two thymidine units to TpTpTpT and two blocks containing three thymidine units to TpTpTpTpTpT on a small scale. The technique is represented by the preparation of the hexanucleoside pentaphosphate.

a. TpTpTpTpTpT. MTrTp(ce)Tp(ce)T·H$_2$O (15 mg, 0.012 mmol) and pyridinium β-cyanoethyl phosphate, after drying by distillation of pyridine *in vacuo*, were mixed with 11 mg (0.05 mmol) of mesitylenesulfonyl chloride in 1 ml of pyridine and the volume was reduced to ~0.1 ml. After 7 hr all of the nucleotide material had been phosphorylated as indicated by the fact that none of the methoxytrityl-containing material moved on tlc in tetrahydrofuran. The solution was diluted with 1 ml of pyridine and 1 ml of water, stirred for 15 hr, and evaporated. The residue was taken up in chloroform (2 ml) and washed with saturated aqueous sodium chloride. After evaporation of the chloroform, the residue was dried by distillation of pyridine (four 1-ml portions) and then dissolved in 1 ml of pyridine along with 27.5 mg (0.024 mmol) of Tp(ce)Tp(ce)T$_{\beta B}$ and 10 mg (0.024 mmol) of 2,4,6-triisopropylbenzenesulfonyl chloride. The solution was concentrated and stirred for 48 hr at room temperature; then the alkaline labile blocking groups were taken off by stirring with 2 ml of ammonium hydroxide and 1 ml of pyridine for 14 hr. The solvent was evaporated and the residue was warmed with 1 ml of 80% aqueous acetic acid on a steam bath for 10 min.

For characterization of the products an aliquot (0.1 ml from 1.0 ml total) was spotted on Whatman 3 MM paper and developed with solvent E. Four spots were found, corresponding to TpTpTpTpTpT (R_f^E 0.084), TpTpTp (R_f^E 0.26), TpTpT (R_f^E 0.38), and a substance at R_f^E 0.85. The products were eluted and the yields determined spectrophotometrically at 267 mμ: TpTpTpTpTpT 41% (2.62 OD units) and TpTpTp 56% (1.74 OD units); both yields were based on MTrTp(ce)Tp(ce)T. The TpTpT, which was derived from the excess Tp(ce)Tp(ce)T$_{\beta B}$, amounted to 3.61 OD units. These materials account for 90% of the total amount of MTrTp(ce)Tp(ce)T and Tp(ce)Tp(ce)T$_{\beta B}$ used in the reaction. Electrophoresis at pH 7.0 also showed four spots: R_m (relative to Tp) 0.75 for TpTpTpTpTpT, 0.97 for TpTpTp, 0.55 for TpTpT and 0.05 (unidentified).

The remainder of the reaction solution was chromatographed on paper with solvent E and the TpTpTpTpTpT fraction was eluted and rechromatographed in solvent F. The TpTpTpTpTpT was eluted and isolated by concentration and lyophilization of the extract. On enzymatic hydrolysis with snake venom phosphodiesterase a sample was completely degraded to pT (R_f^F 0.41, 7.28 OD units) and T (R_f^F 0.76, 1.55 OD units) with pT/T = 4.7.

b. TpTpTpT. This preparation was carried out in essentially the same manner used for TpTpTpTpTpT, starting with 10 mg (0.011 mmol) of MTrTp(ce)T and 34 mg (0.04 mmol) of Tp(ce)T$_{\beta B}$. When the phosphotriester condensation step was carried out for 72 hr in a concentrated solution (~0.1 ml), a 47% yield of TpTpTpT and a 51% yield of TpTp (based on MTrTp(ce)T were obtained. When the phosphotriester condensation step was carried out in 0.2 ml of pyridine for 48 hr, the yields of TpTpTpT and TpTp were 22 and 59%, respectively.

The Radiation Chemistry of Biochemical Disulfides. II. Lipoic Acid[1]

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Abstract: α-Lipoic acid (6-thioctic acid) (I) is destroyed in high yield upon exposure to X-rays in aerated or deaerated aqueous solution. Small amounts, only, of thiol and of oxidation products (sulfonic and sulfinic acid, etc.) and hydrogen peroxide are produced. The major product appears to be a cyclic dimer. A mechanism is presented which accommodates all of the experimental observations. Kinetic treatment of the mechanism affords it support and requires a linear increase in radiation-chemical yield with concentration such that $G_0(-\text{lipoate}) = 2(G_{OH} + G_H) + K[\text{lipoate}]$ at lipoate concentrations above 5–10 mM. The radiobiological significance of the work is briefly discussed.

Exposure of dilute, air-saturated solutions of the amino and peptide disulfides, cystamine,[2] cystine,[3] and oxidized glutathione,[4] to moderate doses of X-rays gives the corresponding sulfonic acids almost exclusively.

A mechanism and a kinetic treatment have been proposed.[3] These account for sulfonic acid production and for the dependence of sulfonic acid yield upon disulfide concentration and radiation dose rate among other variables. Key steps in the proposed mechanism are the formation and heterolysis of a disulfide radical cation.

It seemed likely that either the thiyl radical or the radical cation might cause disproportionation or poly-

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(4) Paper to be published.