Nucleoside S-Alkyl Phosphorothioates. III.[1] Application to Oligonucleotide Synthesis

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Abstract: The S-ethyl phosphorothioate group has been used as a protecting group in oligonucleotide synthesis. Nucleoside 5′-S-ethyl phosphorothioates were condensed with several partially protected mononucleotides to give a series of dinucleotides containing 5′-S-ethyl phosphorothioate groups. These were partially unblocked and converted into the corresponding trinucleotide 5′-S-ethyl phosphorothioates by further condensation. The acyl groups of the fully protected di- and trinucleotide S-ethyl phosphorothioates could be removed with aqueous ammonia without destruction of the S-ethyl moiety. S-Ethyl di- and trinucleotides were used as intermediates in the preparation of a variety of derivatives; (a) treatment with aqueous iodine removed the S-ethyl group and gave the corresponding unprotected oligomers, (b) treatment with iodine in methanol produced the corresponding 5′-methyl esters, (c) treatment with iodine and inorganic pyrophosphate gave the corresponding oligomer 5′-triphosphates, and (d) treatment of an S-ethyl trinucleotide with acetylated adenosine 5′-phosphate gave, after subsequent deacetylation, a 5′,5′-pyrophosphate derivative of the trimer with adenosine 5′-phosphate. Thymidine 5′-S-ethyl phosphorothioate (**1**) has been self-condensed using iodine in pyridine, and a series of linear and cyclic oligonucleotides was isolated.

Problems encountered in the chemical synthesis of oligonucleotides are threefold: (a) the search for efficient condensation methods leading to the phospho diester linkage, (b) the use of specific, reversible masking procedures for the protection of those functional groups not to be involved in internucleotide bond formation, and (c) the development of efficient methods for the separation of the products. In the deoxyribonucleotide series, considerable progress has been made in recent years, especially by Khorana and his colleagues, as shown by their current endeavors to synthesize an entire gene,[2] that of yeast alanine transfer RNA. Although at the present time no oligonucleotide longer than 20 units has yet been chemically synthesized, larger oligomers have been prepared by joining together fragments by means of the recently discovered enzyme polynucleotide ligase.[3] A limitation of the use of this enzyme lies in the fact that phosphodiester bond synthesis will only occur when the fragments are held in double-stranded conformation by base pairing.

The protection of the 5′-terminal phosphate is a basic problem in oligonucleotide synthesis, since without protection self-condensation will occur, and a complex mixture of oligonucleotides is produced. One of the most widely used protecting groups which have been used for this purpose is the base-labile β-cyanoethyl ester.[4] It suffers from the disadvantage that it is not inert to the alkaline treatment required to remove esters elsewhere in the molecule. The β,β,β-trichloroethyl group, a group susceptible to reductive removal,[5] has been successfully used for oligonucleotide synthesis, although our own work[6] has shown that the conditions necessary for its removal (a zinc–copper couple) are not compatible with the N-benzoylcytosine moiety. A masked ribonucleoside subject to eventual removal by periodate oxidation followed by β elimination has also been used as a protecting group during oligonucleotide synthesis.[7]

Our own work with nucleoside S-ethyl phosphorothioates[1] had indicated that these compounds were quite stable to a variety of manipulations, especially at an alkaline pH. It was also observed that the S-ethyl substituent could be activated toward hydrolysis by mild oxidation,[8] so its use in oligonucleotide synthesis was suggested. The present paper describes experiments toward this objective.

The pyridinium salt of thymidine 5′-S-ethyl phosphorothioate (**1**, Scheme I) was condensed with 3′-O-acetylthymidine 5′-phosphate (d-pT-OAc) using dicyclohexylcarbodiimide (DCC) as the condensing agent, and the mixture was purified by DEAE cellulose column chromatography. In this way the protected dinucleotide EtS-dpTpT-OAc (**2**)[9] was readily obtained in a yield of 84%. Treatment of **2** with aqueous iodine cleaved the P–S bond and produced the corresponding dinucleotide acetate d-pTpT-OAc (**3**). The mechanism for this type of reaction presumably involves labilization of the P–S bond by formation of an iodosulfonium intermediate; further discussion has been presented in paper II of this series.[1] Subsequent removal of the acetyl group gave the unprotected dinucleotide d-pTpT. Treatment of EtS-dpTpT-OAc (**2**) with concentrated ammonium hydroxide removed the acetyl group while leaving the S-ethyl group intact, and EtS-dpTpT (**4**) was prepared in quantitative yield. This partially pro-

(1) Paper II in this series: A. F. Cook, M. J. Holman, and A. L. Nussbaum, *J. Am. Chem. Soc.*, **91**, 1522 (1969).

(2) N. K. Gupta, E. Ohtsuka, V. Sgaramella, H. Buchi, A. Kumar, H. Weber, and H. G. Khorana, *Proc. Nat. Acad. Sci. U. S.*, **60**, 1338 (1968).

(3) (a) B. Weiss and C. Richardson, *ibid.*, **57**, 1021 (1967); (b) B. M. Olivera and I. R. Lehman, *ibid.*, **57**, 1426 (1967); (c) S. B. Zimmerman, J. W. Little, C. K. Oshinsky, and M. Gellert, *ibid.*, **57**, 1841 (1967); (d) M. L. Gefter, A. Becker, and J. Hurwitz, *ibid.*, **58**, 240 (1967); (e) N. R. Cozzarelli, N. E. Melechen, T. M. Jovin, and A. Kornberg, *Biochem. Biophys. Res. Commun.*, **28**, 578 (1967).

(4) G. M. Tener, *J. Am. Chem. Soc.*, **83**, 159 (1961); S. A. Narang, T. M. Jacob, and H. G. Khorana, *ibid.*, **89**, 2158 (1967).

(5) A. Franke, F. Eckstein, K. H. Scheit, and F. Cramer, *Chem. Ber.*, **101**, 944 (1968).

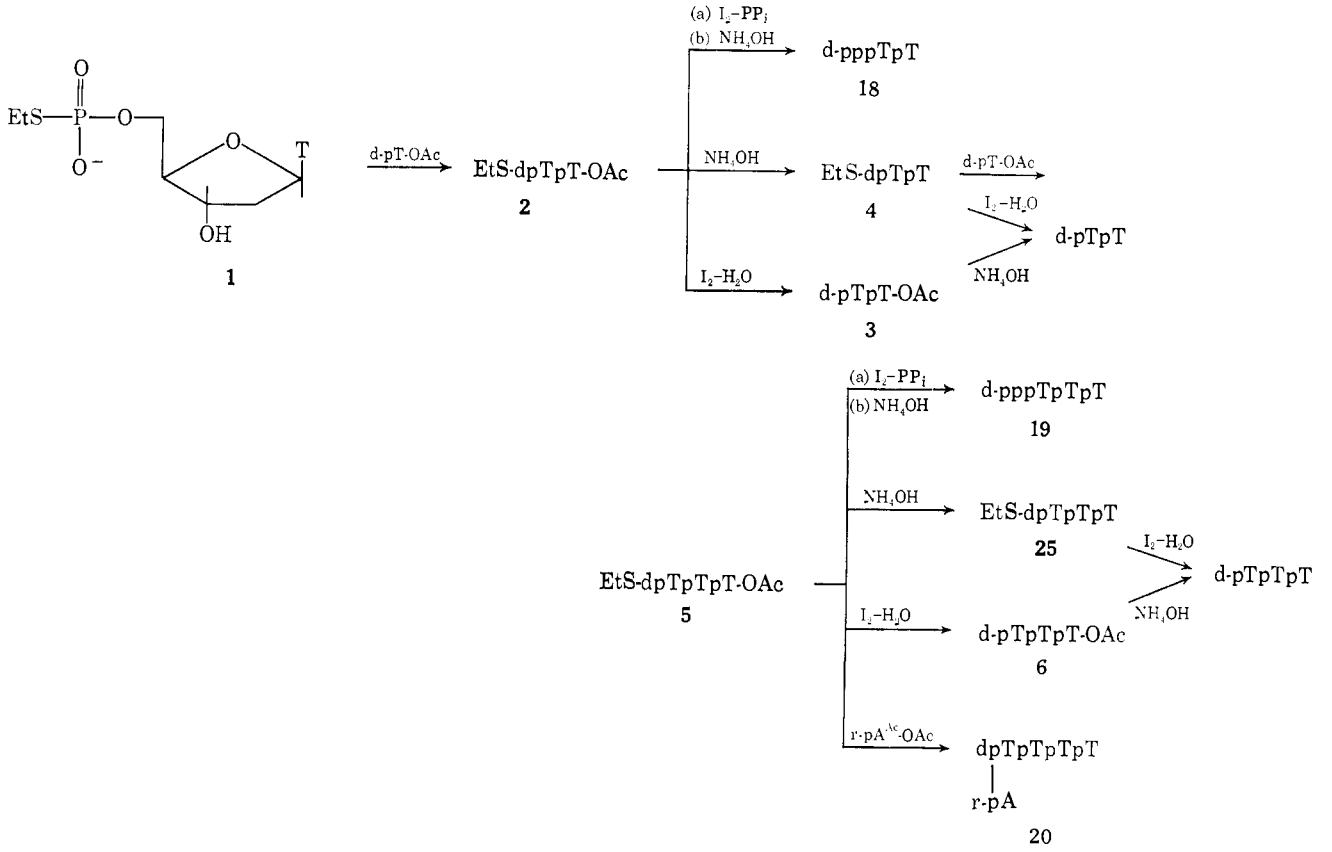
(6) A. F. Cook, *J. Org. Chem.*, **33**, 3589 (1968).

(7) F. Kathawala and F. Cramer, *Ann.*, **709**, 185 (1967).

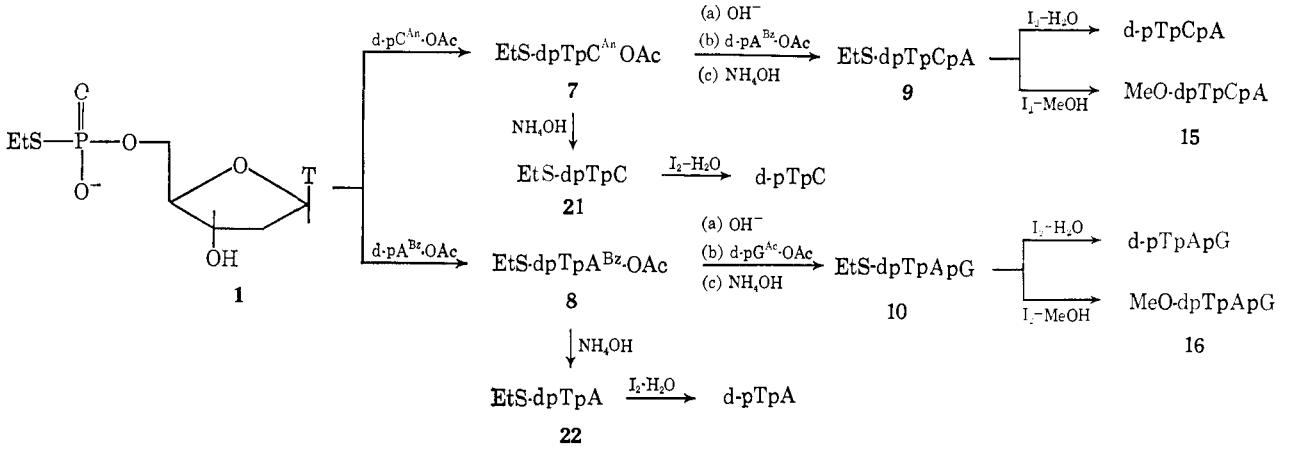
(8) T. Wieland and R. Lambert, *Chem. Ber.*, **89**, 2476 (1956).

(9) The abbreviated nomenclature in this paper follows that described in *J. Biol. Chem.*, **241**, 527 (1966); and H. Schaller and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3841 (1963). Abbreviations not described therein include: EtSP, S-ethyl phosphorothioate ($EtSPO_3^-$); MeOP, methyl phosphate.

Scheme I. Thymidine Oligonucleotide Derivatives



Scheme II. Oligomers Derived from Thymidine 5′-S-Ethyl Phosphorothioate (**1**)

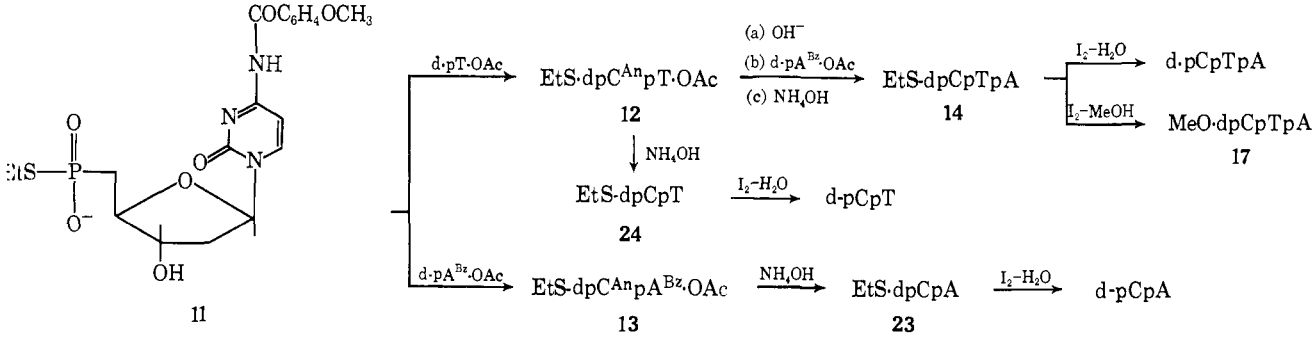


tected dinucleotide was condensed with an excess of d-pT-OAc, using mesitylenesulfonyl chloride as the condensing agent, and the protected derivative **5** was obtained in reasonable yield. Treatment of **5** with aqueous iodine removed the S-ethyl protecting group and gave the trinucleotide acetate **6** from which the trinucleotide d-pTpTpT was obtained by treatment with ammonium hydroxide. Both this material and the dinucleotide d-pTpT were characterized by chromatographic comparison with authentic samples prepared by a conventional method,[10] and by their complete degradation to d-pT by venom diesterase. This sequence of reactions demonstrated that the S-ethyl phosphorothioate moiety was resistant to the action of condensing agents and the basic conditions required to remove the other protecting groups, and thus was a suitable protecting group for use in oligonucleotide synthesis.

The reactions of **1** with other mononucleotides were next investigated. Condensation of **1** with d-pC^{An}-OAc using DCC in pyridine for 4 days gave the protected dinucleotide EtS-dpTpC^{An}-OAc (**7**, Scheme II) in 65% yield and, similarly, the reaction of **1** with d-pA^{Bz}-OAc gave EtS-dpTpA^{Bz}-OAc (**8**) in 32% yield. The protecting groups in both of these dinucleotides **7** and **8** could be removed as previously described, and the corresponding unprotected dinucleotides d-pTpA and d-pTpC were readily obtained. These materials were characterized by their uv absorption and susceptibility to venom diesterase, the expected mononucleotides being produced in essentially equimolar proportions. Both compounds **7** and **8** could be partially deprotected by treatment with 1 *N* sodium hydroxide at 0° for 20

(10) G. M. Tener, H. G. Khorana, R. Markham, and E. H. Pol, *J. Am. Chem. Soc.*, **80**, 6223 (1958).

Scheme III. Oligomers Derived from N-Anisoyldeoxycytidine 5′-S-Ethyl Phosphorothioate



min,[11] and their de-O-acetylated counterparts were obtained without destruction of the S-ethyl moiety. Condensation of EtS-dpTpA^{Bz} with the protected deoxyguanosine nucleotide d-pG^{Ac}-OAc gave the trinucleotide EtS-dpTpA^{Bz}pG^{Ac}-OAc, and similarly, a condensation of EtS-dpTpC^{An} with d-pA^{Bz}-OAc readily gave EtS-dpTpC^{An}pA^{Bz}-OAc. The protected trinucleotides were deacylated by treatment with concentrated ammonium hydroxide to give the S-ethyl derivatives **9** and **10** from which the trinucleotides d-pTpApG and d-pTpCpA were obtained by treatment with aqueous iodine.

For the synthesis of oligonucleotides containing a deoxycytidylic acid residue in the 5′-terminal position, the protected monomer **11** (Scheme III) was required. This material was synthesized from 3′-O-acetyl-N-anisoyldeoxycytidine by condensation with pyridinium S-ethyl phosphorothioate, followed by deacetylation of the product with 1 *N* sodium hydroxide. Although several by-products were formed during the sodium hydroxide treatment, the desired material was obtained in reasonable yield. Condensations of **11** with d-pT-OAc and d-pA^{Bz}-OAc readily gave the protected dinucleotides **12** and **13**, respectively, from which the unprotected dinucleotides d-pCpT and d-pCpA could be obtained in the usual way. Compound **12** was also deacetylated and condensed with d-pA^{Bz}-OAc to give EtS-dpCpTpA^{Bz}-OAc in good yield, from which the unprotected trinucleotide d-pCpTpA was prepared *via* the S-ethyl derivative **14**. Paper-chromatographic properties of the various nucleoside S-ethyl phosphorothioates are listed in Table V.

An important asset of the S-ethyl phosphorothioate group as a protecting group in oligonucleotide synthesis lies in its versatility. We have previously shown that at the monomer level, diphosphate, triphosphate, phosphoromorpholidate, pyrophosphate, and a wide variety of diester functions can be prepared *via* nucleoside S-ethyl phosphorothioate intermediates. By the same procedure, a wide variety of functional groups can be introduced at the 5′-terminal position of oligonucleotides by treatment of the S-ethyl derivative with the appropriate nucleophile in the presence of iodine. In this connection, a series of oligonucleotides with a methyl phosphate at the 5′-terminal position were required in these laboratories as model compounds for a sequence determination method as recently described.[12]

These methyl phosphate derivatives **15**, **16**, and **17** (Schemes II and III) were prepared by treatment of the individual S-ethyl trinucleotides **9**, **10**, and **14** with iodine in anhydrous methanol. Similarly, the triphosphates d-pppTpT (**18**) and d-pppTpTpT (**19**, Scheme I) have been prepared by treatment of **2** and **5**, respectively, with tributylammonium pyrophosphate in 2-picoline, this solvent being employed to reduce the dismutation of the triphosphates.[13] These oligomer 5′-triphosphates were conveniently characterized by their susceptibility to a diesterase from carrot,[14] since this enzyme degrades nucleoside 5′-triphosphates at slower rates than internucleotide linkages; thus d-pppTpT (**18**) was cleaved to give thymidine 5′-monophosphate and thymidine 5′-triphosphate in equimolar amounts. Similarly, d-pppTpTpT (**19**) upon incubation gave the monophosphate and the triphosphate in the ratio 2:1. Oligomers containing 5′-terminal triphosphates have previously been synthesized from phosphoromorpholidate[15] and phosphorimidazolidate[16] intermediates.

A trinucleotide containing a 5′-terminal pyrophosphate linkage to adenylate (**20**) has been prepared.[17] Although a reaction of **5** with adenosine 5′-phosphate in the presence of iodine gave, after deacetylation, a very low yield of **20**, the yield was greatly increased when adenosine 5′-phosphate was acetylated before use. In the former case the amino group of adenosine 5′-phosphate may be a sufficiently good nucleophile to compete with phosphate for attack on the activated species.

Although **20** was resistant to the action of bacterial alkaline phosphatase, degradation by venom diesterase produced adenosine 5′-phosphate and thymidine 5′-phosphate in the ratio of 1:3. The presence of a pyrophosphate bond in **20** was demonstrated by treatment with acetic anhydride in pyridine,[18] followed by concentrated aqueous ammonia; adenosine 5′-phosphate and d-pTpTpT were produced.

In the syntheses of the oligomers so far described, the phospho diester bonds have been formed by activation of the appropriate monoester with DCC or a sulfonyl chloride, the S-ethyl group serving as a protecting group until the desired chain length had been obtained. The S-ethyl group has also been used as an activating group in the preparation of oligomers of thymidine 5′-phos-

(11) T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, **87**, 2971 (1965).

(12) T. Gabriel, W. Y. Chen, and A. L. Nussbaum, *ibid.*, **90**, 6833 (1968). This degradation sequence, from the 3′ end of the molecule, included the removal of 3′-phosphates with bacterial alkaline phosphatase. Protection of the 5′-phosphate was therefore needed to prevent its cleavage by the monoesterase.

(13) W. E. Wehrli and J. G. Moffatt, *J. Am. Chem. Soc.*, **87**, 3760 (1965).

(14) C. Harvey, L. Malsman, and A. L. Nussbaum, *Biochemistry*, **6**, 3689 (1967).

(15) A. M. Duffield and A. L. Nussbaum, *J. Am. Chem. Soc.*, **86**, 111 (1964).

(16) D. E. Hoard and D. G. Ott, *ibid.*, **87**, 1785 (1965).

(17) This compound has also been prepared by the morpholidate procedure, and used during a study of the enzyme polynucleotide ligase: Z. W. Hall and I. R. Lehman, *J. Biol. Chem.*, **244**, 43 (1969).

(18) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **81**, 4660 (1959).

phate by the addition of iodine. Treatment of thymidine 5′-S-ethyl phosphorothioate (**1**) with iodine in pyridine in the absence of any external nucleophile produced extensive self-condensation by attack of the 3′-hydroxyl group on the iodine-activated phosphorothioate. After a reaction period of 65 hr, water was added to convert the remaining S-ethyl phosphorothioate groups to phosphate groups. The mixture was then treated with acetic anhydride in pyridine followed by concentrated ammonium hydroxide in order to reduce the level of pyrophosphates in the mixture. The product was applied to a DEAE cellulose column, and upon elution linear oligonucleotides up to the nonanucleotide and cyclic oligonucleotides up to the hexamer were obtained and identified by their susceptibility or resistance to snake venom phosphodiesterase, and by chromatographic comparison with authentic materials.[19] The yields and the distribution of products obtained from this reaction (Table I) are very similar to those

Table I. Products Identified from the Self-Condensation of Thymidine 5′-S-Ethyl Phosphorothioate

Product	Linear nucleotides yield, %[a]	Cyclic nucleotides yield, %[a]
Mononucleotide	18.1	
Dinucleotide	7.6	15.7
Trinucleotide	7.9	4.4
Tetranucleotide	5.8	1.9
Pentanucleotide	3.2	1.1
Hexanucleotide	1.9	1.1
Heptanucleotide	1.1	
Octanucleotide	0.9	
Nonanucleotide	0.5	

[a] Calculated without regard to hypochromic effects.

reported for the polymerization of thymidine 5′-phosphate using DCC as the condensing agent,[10] the cyclic dinucleotide being the main product in both reactions.

Enzymatic Experiments. For characterization, the fully protected di- and trinucleotide S-ethyl phosphorothioates were treated with aqueous ammonia to remove the acyl-protecting groups, and the products were then treated with snake venom phosphodiesterase. The mononucleotides were quantitatively released, together with ethanethiol which was recognized by its characteristic odor. The relative proportions of each mononucleotide were determined by elution and spectrophotometric assay of the appropriate spots obtained from paper chromatography of the mixture. In those cases where adequate resolution was not achieved, the material for analysis was hydrolyzed to its constituent bases which were then separated by paper chromatography and assayed as before (see Table IV).

Experimental Section

General Procedures. Paper chromatography was carried out by the descending technique using Whatman No. 1 paper. The following solvent systems were routinely used: solvent A, ethanol–0.5 *M* ammonium acetate, pH 3.8 (7:3, v/v); solvent B, acetonitrile–0.1 *M* ammonium acetate, pH 7 (6:4, v/v); solvent C, ethanol–1 *M* ammonium acetate, pH 7.5 (7:3, v/v). Visualization of sulfur-containing spots on paper chromatograms was carried out as described by Wieland and Lambert.[8] Pyridine was dried by distillation over potassium hydroxide and stored over Linde Molecular Sieve Type 4A. Ultraviolet measurements were performed on a Zeiss PMQ II instrument. Whatman DE 23 cellulose was used for ion exchange column chromatography. The enzymatic experiments were carried out as previously described.[1]

Preparation of EtS-dpTpT-OAc (2). A mixture of EtS-dpT (**1**, 0.83 mmol, pyridinium salt) and d-pT-OAc (2.5 mmol, pyridinium salt) was dried by evaporation of pyridine (three 2-ml portions), dissolved in dry pyridine (5 ml), and treated with DCC (971 mg) for 72 hr with shaking. Water (10 ml) was added and, after 18 hr at 0°, the precipitate was filtered and washed with 50% aqueous pyridine. The filtrate and washings were evaporated to dryness, and the residue was dissolved in water (50 ml). The solution was applied to a DEAE cellulose column (50 × 4 cm, acetate form) and eluted with a linear gradient of 4 l. of 0.005 *M* triethylammonium acetate, pH 6.0, in the mixing vessel and 4 l. of 0.25 *M* triethylammonium acetate, pH 6.0, in the reservoir. The chromatographic profile is shown in Figure 1. Fractions 282–325 were combined and evaporated to give EtS-dpTpT-OAc (**2**), 6940 OD_{267} units (43%)[20] (see Table II).

Table II. Identification of the Products Formed in the Synthesis of EtS-dpTpT-OAc

Peak	Fraction	OD_{267} units	Identification
I	13–80	2980	Pyridine and unidentified components
II	131–145	1750	Unidentified component
III	146–170	2350	EtS-dpT and unidentified component[a]
IV	218–272	7580	d-pT-OAc
V	273–281	523	Mainly P^1,P^2-di(3′-O-acetylthymidine-5′) pyrophosphate
VI	282–325	6940	EtS-dpTpT-OAc
VII	330–355	380	Unidentified
VIII	371–400	360	Unidentified

[a] This material gave EtS-dpT upon treatment overnight with concentrated aqueous ammonia.

EtS-dpTpC^{An}-OAc (7). Compound **1** (0.094 mmol) and d-pC^{An}-OAc (0.4 mmol) in pyridine (3 ml) were treated with DCC (124 mg) with shaking for 72 hr. The required product **7** was isolated by the method described in the previous experiment, and eluted from the column (60 × 2.3 cm) using a gradient of 2 l. of 0.005 *M* buffer in the mixing vessel and 2 l. of 0.2 *M* buffer in the reservoir. **7**, 1360 OD_{303} units (65%), was eluted from the column at a buffer strength of 0.14 *M*.

EtS-dpTpA^{Bz}-OAc (8). A mixture of **1** (0.094 mmol) and d-pA^{Bz}-OAc (0.1 mmol) was treated with DCC (200 mg) in pyridine (1 ml) with shaking for 72 hr. After the usual purification procedure **8**, 840 OD_{280} units (32%) was eluted from the column at 0.13 *M* buffer strength.

3′-O-Acetyl-N-anisoyldeoxycytidine. N-Anisoyldeoxycytidine was treated with *p*-methoxytrityl chloride in the usual way,[21] and the trityl compound was converted into 3′-O-acetyl-N-anisoyldeoxycytidine by the method described for the corresponding N-benzoyl compound,[6] yield 57%, mp 186–187°, uv max (CH_3OH) 287 mμ (ε 25,000).

Anal. Calcd for $C_{19}H_{21}N_3O_7$: C, 56.58; H, 5.21; N, 10.42. Found: C, 56.60; H, 5.06; N, 10.54.

N-Anisoyldeoxycytidine 5′-S-Ethyl Phosphorothioate (11). The 3′-O-acetyl derivative of **11** was prepared by condensation of 3′-O-acetyl-N-anisoyldeoxycytidine with S-ethyl phosphorothioate in the same way as described for the N-benzoyl compound,[1] yield 27%.

Anal. Calcd for $C_{21}H_{25}N_3NaO_9PS \cdot 1.5H_2O$: C, 43.74; H, 4.89; N, 7.29; P, 5.37. Found: C, 43.53; H, 4.56; N, 7.29; P, 4.94.

This material (640 mg, 1.11 mmol of sodium salt) was deacetylated by treatment with 1 *N* aqueous sodium hydroxide for 20 min. After neutralization with Dowex 50 resin (pyridinium form), the

(19) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 675 (1961).

(20) All yields are calculated without regard to the hypochromicity of the products.

(21) H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3821 (1963).

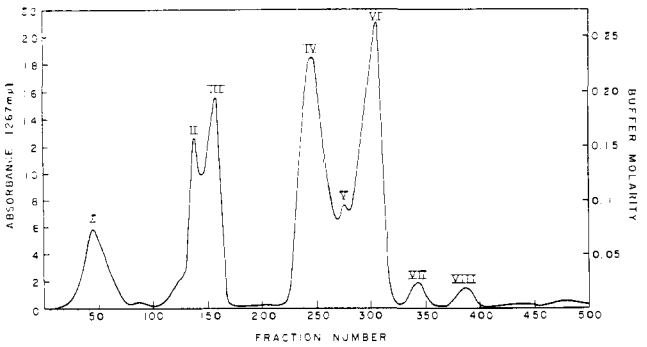


Figure 1. Chromatographic analysis of the reaction mixture from the condensation of EtS-dpT and dpT-OAc. For identification of the products, see Table II.

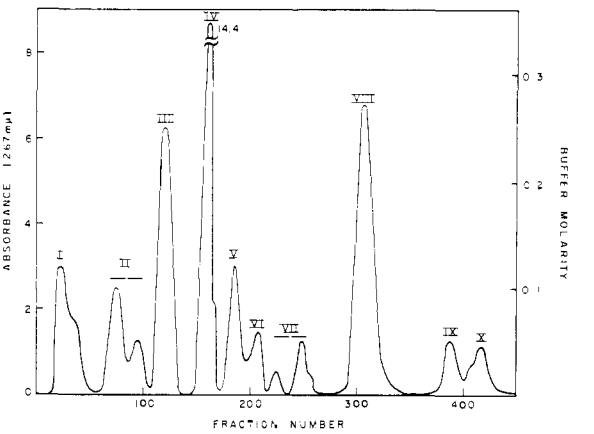


Figure 2. Chromatographic analysis of the reaction mixture in the preparation of the trinucleotide EtS-dpTpTpT-OAc. For identification of the products, see Table III.

solution was evaporated to dryness, dissolved in water and applied to a DEAE column (44 × 3.5 cm, acetate form), and eluted with a linear gradient of triethylammonium acetate, pH 6 (0.005–0.1 *M*). N-Anisoyldeoxycytidine 5′-S-ethyl phosphorothioate (**11**) was eluted from the column at a buffer strength of 0.05–0.07 *M*, 13,500 OD_{303} units (54%). This material was converted into the pyridinium form for use in subsequent experiments.

EtS-dpC^{An}pT-OAc (12). A solution of **11** (67 μmol), d-pT-OAc (213 μmol), and DCC (84 mg) in pyridine (1 ml) was shaken at room temperature for 64 hr. After the usual purification procedure **12**, 665 OD_{303} unit, (44%) was eluted from the column (66 × 2 cm) at a gradient concentration of 0.18 *M*.

EtS-dpC^{An}pA^{Bz}-OAc (13). A mixture of **11** (45 μmol) and d-pA^{Bz}-OAc (145 μmol) in pyridine (1 ml) was treated with DCC (200 mg) for 72 hr. After the normal purification procedure, **13** was eluted at a buffer strength of 0.18 *M*, 546 OD_{303} units (47%).

EtS-dpTpTpT-OAc (5). A sample of **2** (260 μmol) was treated overnight with concentrated aqueous ammonia (2 ml) and then evaporated to dryness. The product was converted into the pyridinium form and condensed with d-pT-OAc (1.3 mmol) using mesitylenesulfonyl chloride (590 mg) in pyridine (4 ml) for 4 hr. Water (1 ml) was added, and after storage overnight the mixture was evaporated, dissolved in water, and applied to a DEAE column (65 × 3.2 cm, acetate form). The chromatographic profile is shown in Figure 2. The protected nucleotide **5** was eluted from the column in tubes 288–325, 2480 OD_{267} units (33%) (Table III).

Table III. Identification of the Products Formed in the Synthesis of the Trinucleotide EtS-dpTpTpT-OAc

Peak	Fraction	OD_{267} units	Identification
I	7–45	1000	Pyridine and unidentified components
II	61–105	875	Unidentified
III	108–135	1485	Mesitylenesulfonic acid
IV	148–170	2660	d-pT-OAc
V	173–195	700	P^1,P^2-Di(3′-O-acetylthymidine-5′) pyrophosphate and EtS-dpTpT
VI	199–214	249	EtS-dpTpT
VII	218–260	408	Unidentified
VIII	288–325	2480	EtS-dpTpTpT-OAc
IX	375–400	347	Unidentified
X	401–430	346	Unidentified

EtS-dpTpCpA (9). Compound **7** (47 μmol) was deacetylated by treatment with 1 *N* sodium hydroxide (10 ml) for 20 min at 0°. The product was neutralized, converted into the pyridinium form, and treated with d-pA^{Bz}-OAc (250 μmol) and mesitylenesulfonyl chloride (545 mg) in pyridine (3 ml) for 4 hr. The protected trinucleotide EtS-dpTpC^{An}pA^{Bz}-OAc was obtained by the usual purification procedure and eluted from the column (50 × 2 cm) at 0.28 *M* buffer strength, 432 OD_{303} units (35%). **9** was obtained by treatment of the protected trinucleotide with concentrated aqueous ammonia for 16 hr.

EtS-dpTpApG (10). Compound **8** (26 μmol) was deacetylated as described in the previous experiment, and treated with d-pG^{Ac}-OAc (83 μmol) and DCC (35 mg) in pyridine (1 ml) with shaking for 5 days. After the normal isolation procedure, EtS-dpTpA^{Bz}-pG^{Ac}-OAc, 130 OD_{280} units (13%), was eluted at a buffer strength of 0.24 *M*. Deacylation in the usual way gave **10**.

EtS-dpCpTpA (14). A solution of **12** (39 μmol) was deacetylated as described for **7**, and the product was condensed with d-pA^{Bz}-OAc (174 μmol) using mesitylenesulfonyl chloride (768 mg) in pyridine (6 ml) for 3.5 hr. After addition of water (6 ml) and column purification, the protected trimer EtS-dpC^{An}pTpA^{Bz}-OAc, 365 OD_{303} units (35%), was obtained at a buffer strength of 0.29 *M*. **14** was produced by subsequent treatment with concentrated aqueous ammonia.

Removal of the S-Ethyl Group with Aqueous Iodine. Samples of the S-ethyl dinucleotides **4, 21, 22, 23,** and **24,** and the S-ethyl trinucleotides **9, 10, 14,** and **25** (approximately 1 μmol) were each treated with iodine (10 mg) in acetone/water (1:1, 2 ml) for 16 hr. The product was extracted with ether (three 2-ml portions), and the aqueous layer was evaporated to dryness to give the unprotected di- or trinucleotide.

Preparation of d-pppTpT (18). A mixture of tributylammonium pyrophosphate (250 μmol) and EtS-dpTpT-OAc (**2**, 53 μmol) was dried by repeated evaporation of 2-picoline, dissolved in 2-picoline (3 ml), and treated with iodine (73 mg) for 16 hr. The product was evaporated to a syrup which was partitioned between ether and water. The aqueous layer was washed with ether (three 5-ml portions), evaporated to dryness, and treated with concentrated aqueous ammonia. After 3 hr the precipitate was removed by filtration and washed with aqueous ammonia. The filtrate and washings were evaporated to dryness, dissolved in water, and applied to a DEAE cellulose column (50 × 2 cm, bicarbonate form) which was eluted with a linear gradient of triethylammonium bicarbonate, pH 7.5 (4 l., 0.005–0.5 *M*). **18** was eluted at a buffer strength of 0.26 *M*, 696 OD_{267} units (69%).

Preparation of d-pppTpTpT (19). This procedure was the same as described for **18.** The following quantities were used: tributylammonium pyrophosphate (174 μmol), EtS-dpTpT-OAc (33 μmol), 2-picoline (3 ml), iodine (53 mg). Compound **19** was eluted at a buffer strength of 0.35 *M*, 453 OD_{267} units (48%).

Synthesis of Trinucleotide 5′-Methyl Esters 15, 16, and 17. The appropriate S-ethyl derivative **9, 10,** or **14** (7 μmol) in dry methanol (1.5 ml) and pyridine (0.5 ml) was treated with iodine (40 mg) overnight at room temperature. Water (2 ml) was added, and after 1 hr the solution was evaporated to dryness, the residue was dissolved in water (2 ml) and extracted with ether (three 2-ml portions). The aqueous layer was evaporated to low bulk, and purified by paper chromatography using Whatman No. 3MM paper and acetonitrile/water (6:4, v/v) as the developing solvent. The main uv-absorbing band (approximately R_f 0.5) was cut out and extracted with water, and the extracts were evaporated to dryness to give the required methyl ester.

Preparation of 20. Adenosine 5′-phosphate monohydrate (61 mg) was acetylated by treatment with pyridine (1 ml) and acetic anhydride (0.5 ml) overnight with shaking at room temperature. Water (0.5 ml) was added to the clear solution, and after 4 hr the product was evaporated to dryness. The S-ethyl derivative **5** (17 μmol) was added, and the mixture was dried by evaporation of pyridine (three 2-ml portions). Iodine (63 mg) was then added,

Table IV. Characterization of S-Ethyl Derivatives of Oligonucleotides

Compound	Base T	C	A	G
EtS-dpTpT	2			
EtS-dpTpC	1	1.03		
EtS-dpCpT	1	1.06		
EtS-dpTpA	1		1.05	
EtS-dpCpA		1	1.11	
EtS-dpTpTpT	3			
EtS-dpTpApG	1		1.09	1.09
EtS-dpTpCpA	1.11	1	1.14	
EtS-dpCpTpA	1	1.39	0.78	

and after storage overnight the solution was evaporated, dissolved in water (5 ml), and extracted with ether (three 5-ml portions). The aqueous layer was treated overnight with an equal volume of concentrated aqueous ammonia, and evaporated to dryness. The residue was dissolved in water (25 ml), and purified by passage through a DEAE cellulose column (60 × 2.3 cm, bicarbonate form), which was eluted with triethylammonium bicarbonate, pH 7.5. A linear gradient of 0.005–0.35 *M* was used, and the required product was obtained at a buffer molarity of 0.25 *M*, 501 OD_{260} units (69%).

Self-Condensation of 1. The pyridinium salt of **1** (0.97 mmol) was dried by evaporation of pyridine (three 10-ml portions), and treated with iodine (1.29 g) in dry pyridine (5 ml) for 70 hr at room temperature. Water (5 ml) was added, and after 2 hr the solution was evaporated to dryness, dissolved in water (10 ml), and extracted with ether (three 10-ml portions). The aqueous layer was evaporated to dryness, and treated with pyridine (10 ml) and acetic anhydride (5 ml) overnight. The solution was cooled to 0°, and water was added with cooling. After 5 hr at 0° the solution was evaporated to dryness and treated with concentrated aqueous ammonia (30 ml) for 16 hr. The product was evaporated to dryness, dissolved in water, and applied to a DEAE cellulose column (55 × 3.4 cm, bicarbonate form). The column was eluted with a linear gradient of 4 l. of triethylammonium bicarbonate buffer, pH 7.5 (0.005 *M*) in the mixing vessel and 4 l. of the same buffer (0.5 *M*) in the reservoir. The appropriate fractions were combined, evaporated to dryness, and the products were identified by paper chromatographic comparison with authentic samples,[19] and by their susceptibility or resistance to snake venom diesterase. The products are listed in Table I.

Table V. Paper Chromatographic Properties of Oligonucleotide Derivatives

Compound	R_f (solvent A)	R_f (solvent B)
EtS-dpT	0.68	0.64
EtS-dpC	0.60	0.61
EtS-dpTpT	0.45	0.54
EtS-dpTpC	0.38	0.51
EtS-dpTpA	0.34	0.50
EtS-dpCpT	0.41	0.51
EtS-dpCpA	0.34	0.37
EtS-dpTpTpT	0.33	0.43
EtS-dpTpCpA	0.19	0.29
EtS-dpTpApG	0.19	0.15
EtS-dpCpTpA	0.15	0.31
MeO-dpTpCpA	0.20	0.29
MeO-dpTpApG	0.16	0.25
MeO-dpCpTpA	0.14	0.31
d-pT	0.46	0.39
d-pTpCpA	0.18[a]	0.11
d-pCpTpA	0.18	0.12
d-pTpApG	0.08	0.11
d-pppTpT	0.17	0.26
d-pppTpTpT	0.10	0.20
d-pTpTpT	0.28	0.23
|		
r-pA		

[a] This, and the remaining R_f values in this column, are calculated with respect to d-pT.

Characterization of Oligonucleotides. Samples of each oligomer were digested with snake venom diesterase, and the products were separated by paper chromatography in system A or C. The appropriate spots were cut out, eluted with water, and measured spectrophotometrically. Samples containing both d-pA and d-pC were not well separated by these systems. In these cases, the original sample was hydrolyzed to its constituent bases by treatment with 80% formic acid for 1 hr at 175°, and the products were separated by paper chromatography in acetonitrile/0.1 *M* ammonium acetate/concentrated ammonium hydroxide/*n*-butyl alcohol 6:2:1:1 (v/v). The appropriate spots were cut out and assayed as before. The results are summarized in Table IV. In Table V are given paper chromatographic properties of oligonucleotide derivatives.

Secondary Isotope Effects in Reactions Catalyzed by Yeast and Muscle Aldolase[1]

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Abstract: The aldolase-catalyzed condensation of (1*R*)-[1-^{3}H]dihydroxyacetone-P with D-glyceraldehyde-3-P to give [3-^{3}H]fructose-1,6-diP was found to go more slowly than normal under conditions which previously indicated that the proton abstraction step is rate determining. Furthermore, slower reaction of fructose-diP containing ^{3}H at C-3 or C-4 is characteristic of both the yeast and muscle enzymes, indicating that the C–C cleavage step (kinetic effect) or glyceraldehyde-P release (equilibrium effect) is the rate-limiting step for the formation of the first product, glyceraldehyde-3-P.

The enzyme-catalyzed aldol cleavage of fructose-1,6-diP (FDP)[4] is known to occur in an ordered sequence in which C–C bond cleavage and release of D-glyceraldehyde-3-P (G3P) precede the protonation of the

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(4) I. A. Rose, E. L. O'Connell, and A. H. Mehler, *J. Biol. Chem.*, 240, 1758 (1965).