Nucleophilic Reactions of Some Nucleoside Phosphorothioates[1]

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Abstract: The synthetic intermediates 5′-*O*-tosylthymidine 3′-cyanoethyl phosphorothioate, 5′-*O*-tosyl-N^4-benzoyldeoxycytidine 3′-cyanoethyl phosphorothioate, and 5′-*O*-tosyl-N^6-benzoyldeoxyadenosine 3′-cyanoethyl phosphorothioate were prepared by the reaction of the corresponding 5′-*O*-tosylnucleosides with $PSCl_3$, followed by a treatment with hydracrylonitrile. After the alkaline hydrolysis of the cyanoethyl group the labile 5′-*O*-tosyldeoxyribonucleoside 3′-phosphorothioates underwent an intramolecular nucleophilic displacement reaction under the formation of a novel six-membered cyclic phosphorothioate ring system. The rate of the formation of 5′-*O*-deoxy-5′-thiothymidine 3′,5′-cyclic phosphorothioate was followed in water and dimethylformamide. Several intermolecular nucleophilic reactions of thymidine 3′-phosphorothioate were studied, including a Michael addition and the displacement of 5′-*O*-tosylates. As a result of displacement, di- and trinucleotides containing unnatural P–S–C (5′) linkages were formed.

Work on nucleotide analogs has been gratifying due to the dual aspects of novel chemistry and, sometimes, interesting biological activity. Our particular interest in oligo-5′-deoxy-5′-thionucleotides stems from mechanistic considerations concerning oligonucleotide synthesis, namely, these compounds can be obtained *via* displacement reactions. The problem of condensing nucleotides gains a new dimension with the participation of sulfur in the esterification process. The consequence of the displacement with thiophosphate is that it leads to the formation of a P–S–C linkage, *i.e.*, the originally "natural" nucleoside portion also becomes an analog in addition to the already unnatural thiophosphate. Yet, it is hoped that this twofold derivatization in oligo-5′-deoxy-5′-thionucleotides would not affect their information content and they could substitute for the natural oligonucleotides in many respects.

The first analog of this kind, a poly-5′-deoxy-5′-thiouridylate of the random linkage type, was obtained *via* phosphate activation by Michelson.[2] In general, this class of polynucleotide analog is more accessible through displacement reactions with a thiophosphate ester. The method of *S*-alkyl ester synthesis of Åkerfeldt[3] had already been applied in the nucleotide field for the preparation of 5′-*S*-nucleosidyl monoesters.[4] A successful thiophosphorylation of the 3′ hydroxyl group was first accomplished by Cook[5] using *S*-2-carbamoylethyl phosphorothioate and DCC. Cook also found that nucleoside 5′- and 3′-phosphorothioates can react efficiently with 5′-deoxy-5′-iodothymidine at 70°. In our laboratory, we have carried out polymerization experiments leading to oligo-2′,5′-dideoxy-5′-thionucleotides using 5′-*O*-tosyldeoxyribonucleoside 3′-cyanoethyl phosphorothioates and the corresponding monoesters as the starting materials.[6] In this paper we describe the preparation of some synthetic intermediates which can be useful in stepwise syntheses and in polymerization. We have also studied the efficiency of the displacement reactions on a few simple models.

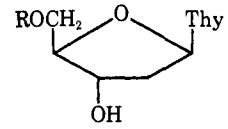
Results

All the nucleotides which were used as starting materials and synthetic intermediates in this project were 3′-phosphorothioates. The thiophosphorylation of the 3′ hydroxyl group was carried out by the versatile reagent, $PSCl_3$, which could be equally used in the preparation of phosphorothioate monoesters, diesters, and pyrophosphates. In this simple procedure the nucleoside components, 5′-*O*-dimethoxytritylthymidine (**1**), 5′-*O*-tosylthymidine (**2**), 5′-*O*-tosyl-N^6-benzoyldeoxyadenosine (**3**), and 5′-*O*-tosyl-N^4-benzoyldeoxycytidine (**4**), were treated with a threefold excess of $PSCl_3$ in pyridine at 5° for 5–6 hr. The products were then treated successively with hydracrylonitrile and triethylammonium bicarbonate. The main reaction products were the nucleoside 3′-cyanoethyl phosphorothioates (**5**, **6**, **7**, and **8**) which were separated either by ion exchange or Sephadex chromatography from the varying amounts of pyrophosphates (types **10** and **11**). A pyrophosphate, presumably **11**, was the main product (69%) when the hydracrylonitrile step was omitted. Thymidine 3′-phosphorothioate[5] (**12**) could be best obtained (90% yield) through the acidic and alkaline hydrolysis of the pyrophosphates. The alternative route *via* cyanoethyl esters **5** and **9** was less efficient.

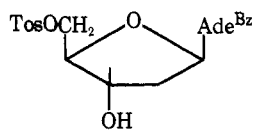
In the course of preparation of these compounds, care must be exercised to avoid oxidation. While molecular oxygen may be of concern only for monoesters at high pH, *N*-oxides present in triethylamine may cause the degradation of diesters. The treatment of the diester **9** with ferricyanide and subsequent alkaline hydrolysis led to the formation of a disulfide isomeric to that obtained by Eckstein[7] from thymidine 5′-phosphorothioate.

Intramolecular Displacement Reactions. In order to conveniently study nucleophilic displacement reactions at equimolar concentrations of the reactants, we chose the intramolecular displacement occurring in 5′-*O*-tosylnucleoside 3′-phosphorothioates (**13–15**). The half-life of the reaction in water at pH 9, 20°, is ~5 hr (Table I). In dimethylformamide the displacement is too rapid to be measured accurately at 20°. It has a half-life of the order of 10 min (Table II).[8]

(1) This research was supported by grants from the National Institute of Health (GM 16213–04) and the Robert A. Welch Foundation (A-401).
(2) A. M. Michelson, *J. Chem. Soc.*, 979 (1962).
(3) S. Åkerfeldt, *Acta Chem. Scand.*, **16**, 1897 (1962).
(4) A. Hampton, L. W. Brox, and M. Bayer, *Biochemistry*, **8**, 2303 (1969).
(5) A. F. Cook, *J. Amer. Chem. Soc.*, **92**, 190 (1970).
(6) J. Nagyvary, S. Chladek, and J. Roe, *Biochem. Biophys. Res. Commun.*, **39**, 878 (1970).
(7) F. Eckstein, *J. Amer. Chem. Soc.*, **88**, 4292 (1966).
(8) In the hope of obtaining some more stable 5′ activated nucleoside 3′-phosphorothioates, 5′-chlorothymidine and 5′-bromothymidine were



1, R = DMTr
2, R = Tos



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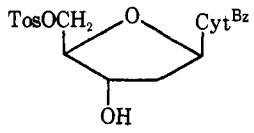

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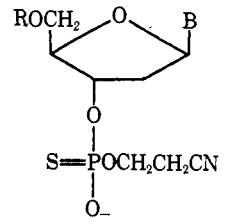
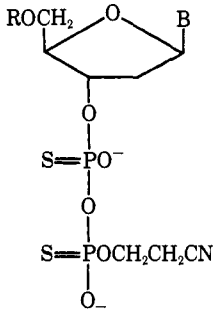


5, R = DMTr; B = Thy
6, R = Tos; B = Thy
7, R = Tos; B = Ade^{Bz}
8, R = Tos; B = Cyt^{Bz}
9, R = H; B = Thy



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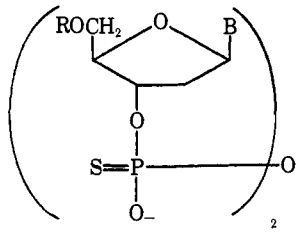

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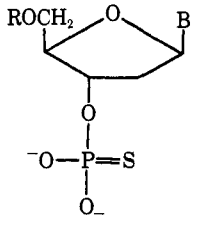
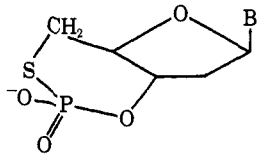
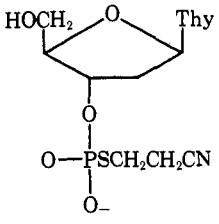


12, R = H; B = Thy
13, R = Tos; B = Thy
14, R = Tos; B = Ade^{Bz}
15, R = Tos; B = Cyt^{Bz}



16, B = Thy
17, B = Ade
18, B = Cyt



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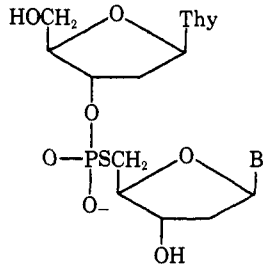
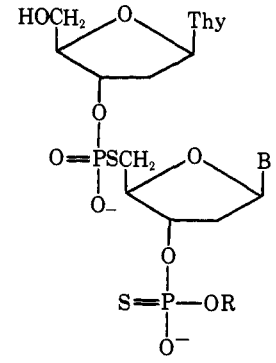
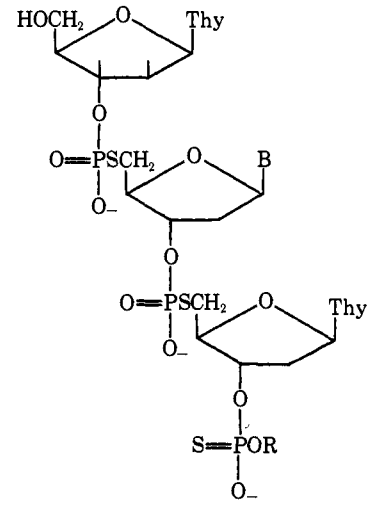


20, B = Thy
21, B = Ade



22, B = Thy; R = CH_2CH_2CN
23, B = Ade^{Bz}; R = CH_2CH_2CN
24, B = Thy; R = H
25, B = Ade^{Bz}; R = H



26, B = Thy; R = CH_2CH_2CN
27, B = Ade^{Bz}; R = CH_2CH_2CN

The products of these intramolecular displacement reactions are the 2′,5′-dideoxy-5′-thionucleoside 3′,5′-cyclic phosphorothioates **16**, **17**, and **18**. Some loss of

Table I

Time, min	% **16**
20	11
150	37
270	51
1200	98

Table II

Time, min	% **13**	% **16**	% Oligo
30	45	55	
180	15	85[a]	
330	7	78	15
1220	0	81	19

[a] The oligonucleotides and **16** were not separated.

thiophosphorylated by the general procedure, and the cyanoethyl esters were isolated by DEAE–cellulose chromatography. When the alkaline elimination of the protecting group was conducted in aqueous solution, the cyclization was complete in less than 24 hr, the only by-product being about 10% desulfurized monoester. In dimethylformamide, the displacement was complete and almost quantitative, within 6 hr.

sulfur prevents the reaction from being quantitative. If the concentration is above 0.01 *M*, a corresponding degree of polymerization also takes place, but in 0.01 *M* solution only traces of cyclic di- and trinucleotides

were formed. Compounds **16** and **17** were purified by passing through ion exchange and Sephadex G-10 columns and by precipitation to remove the contaminating tosylate ions. The deoxycytidylate **18** was isolated in small quantities by paper chromatography. Effort was made to crystallize only 5′-deoxy-5′-thiothymidine 3′,5′-cyclic phosphorothioate (**16**), which was fully analyzed.[9] The pyrimidine nucleotides **16** and **18** were resistant to snake venom phosphodiesterase, but **17** hydrolyzed readily. This behavior parallels that of the natural nucleoside 3′,5′-cyclic phosphates.[10] The thio compounds were also stable to 0.1 *N* KOH at 20° for 24 hr in the absence of oxygen.

The cyclic nucleotides **16**, **17**, and **18** are also formed from cyanoethyl esters **6**, **7**, and **8** on storage at room temperature. Keeping a dilute dimethylformamide solution of **6** at 37° for 2 days produced **16** in better than 90% yield.

Intermolecular Nucleophilic Reactions. (1) The addition of a diesterified phosphorodithioate to an aliphatic double bond has been described.[11] We have found that thymidine 3′-phosphorothioate reacts with acrylonitrile at room temperature with formation of both the *S*-cyanoethyl ester **19** and the *O*-cyanoethyl ester **9**, the former being the main product. The ratio of **19** to **9** could be determined by ferricyanide oxidation of the diester products which were eluted as a mixture from the DEAE column. Only **9** undergoes oxidation by this reagent, and the product is easily separated from **19** by electrophoresis. When the cyanoethylation was carried out in dimethylformamide in the presence of some triethylammonium bicarbonate (pH 7.5), 87% of **19** was found, while in the presence of some ammonium acetate (pH 5.5) a lesser quantity (73%) of **19** was found. The *S*-cyanoethyl ester is the sole product of the reaction of **12** with β-bromopropionitrile, in analogy to the similar reactivity of the 5′-phosphorothioate.[5]

The alkaline β elimination of the *O*-cyanoethyl group in the phosphorothioate **9** proceeded smoothly giving **12** without the decrease of rate that characterized the elimination of the *S*-cyanoethyl group in thymidine 5′-*S*-cyanoethyl phosphorothioate.[5] However, if the acrylonitrile formed in the hydrolysis was not evaporated prior to neutralization of the reaction mixture, a varying degree of diester formation was observed.

(2) Synthesis of Dinucleoside Monophosphate Analogs d(NpsN).[12] The synthesis of thymidylyl-(3′-*O*→5′-S)-5′-deoxy-5′-thiothymidine (**20**) *via* displacement of the 5′ iodide in pyridine–water at 70° was reported.[5] The use of dry dimethylformamide in our hands obviated the need for an elevated temperature. Compound **20** was isolated in over 65% yield when **12** was allowed to react 20 hr with 3–5 equiv of 5′-*O*-tosylthymidine. An almost quantitative reaction of **12** resulted after a prolonged reaction time. Under the same conditions, 5′-*O*-tosyl-N^6-benzoyldeoxyadenosine proved to be less reactive, and d(TpsA) (**21**) was obtained in 50% yield after deblocking and purification on a DEAE–cellulose column. For the complete separation of the tosylate ion, further chromatography on a Sephadex G-10 column was necessary. Compounds **20** and **21** purified in this manner gave satisfactory uv and nmr spectra and were degraded by purified snake venom phosphodiesterase. However, the ratio of nucleoside to nucleotide was higher than the calculated value. This was due to the chemical instability of the thioester pdsN in the incubation mixture. In the case of d(TpsA), two nucleosides were clearly separated and T/(pdsA + dsA) = 1.03 was found.

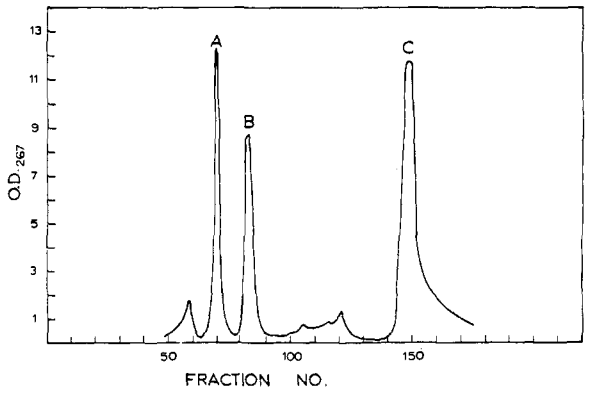


Figure 1. Isolation of the trinucleotide **26** by gel permeation chromatography on Sephadex G-25. For details see text.

(3) Synthesis of the Dinucleotide d(TpsTps) (24) and d(TpsA^{Bz}ps) (25). The condensation of thymidine 3′-phosphorothioate with 2.5 and 3.5 equiv of **6** and **7**, respectively, gave the protected dinucleotides **22** and **23** in 55 and 27% yields. Some polymerization of **6** and **7** took place even at 20°, thus limiting the applicability of this type of protection. The dinucleotides **24** and **25** were obtained following the alkaline removal of the cyanoethyl groups. They gave the expected chromatographic, electrophoretic, and uv values.

(4) Synthesis of Trinucleotides. In order to achieve a chain elongation, the cyanoethyl groups in **22** and **23** were removed with alkali, and the dinucleotides **24** and **25** (triethylammonium or ammonium salts) were treated with 3–5 equiv of **6** for 24–48 hr. In addition to the main products, **26** and **27**, substantial amounts of desulfurized starting material, unreacted **24** and **25**, and some polymeric material were obtained. A typical Sephadex G-25 pattern is given in Figure 1. In two experiments, yields of 50% for **26** and 10% for **27** were obtained. A further improvement is precluded owing to the reactivity of the phosphorothionate diester linkage. The trinucleotides **26** and **27** were not studied in any detail, but an increase in molecular weight was evident from the Sephadex elution pattern. They contained 3′-cyanoethyl groups as judged from the increased electrophoretic mobility after treatment with alkali.

Discussion

In the course of the characterization of the various compounds the only difficulty was the interpretation of the nmr spectrum of the cyclic phosphorothioate **16**. The same irregular quartet-like feature (Figure 2) was observed in the spectra of three different preparations, in both the NH_4^+ and Ba^{2+} salts. As in all 5′-deoxy-5′-thio compounds, the 5′ protons exhibited a characteristic upfield shift. The C-1′–H in **17** and **18** appeared as the usual triplet; its coupling constant (J =

(9) ORD and CD spectra will be separately reported.
(10) G. I. Drummond, M. W. Gilgan, E. J. Reiner, and M. Smith, *J. Amer. Chem. Soc.*, **86**, 1626 (1964).
(11) W. E. Bacon and W. M. LeSuer, *ibid.*, **76**, 670 (1954).
(12) The abbreviation dsN stands for any 2′,5′-dideoxy-5′-thionucleoside; d(NpsNps) is the corresponding dinucleotide with a 3′-phosphorothioate end group.

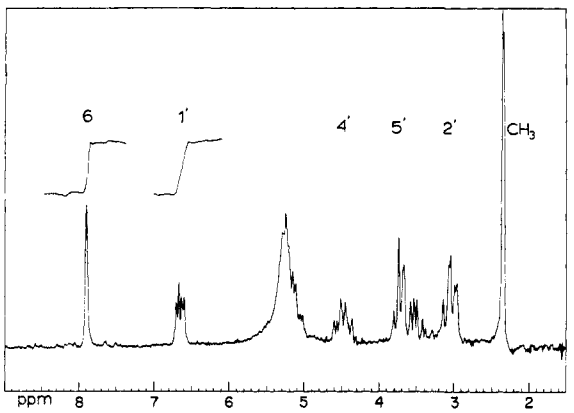


Figure 2. The 100-MHz nmr spectrum of 5′-deoxy-5′-thiothymidine 3′,5′-cyclic phosphorothioate (**16**) in D_2O relative to TMS (external).

8 Hz) is quite large as compared to the corresponding value of 3′,5′-cyclic AMP (J = 1 Hz).[13] This tends to confirm the conclusion gained from the inspection of molecular models that there is no particular strain in these cyclic phosphorothioates. The possibility that **16** also contained a P=S isomer could be ruled out by the lack of reactivity toward C_2H_5Br. The interpretation of Figure 2 will have to await the nmr study of all 3′,5′-cyclic nucleotides of the deoxyribo series which has not yet been published. The appearance of the C-1′ protons in **20** and **21** is normal.

A comparison of some practical value can be made between phosphate esters and phosphorothioate esters with respect to the facility with which they form diester linkages by various mechanisms. While phosphates are not known to undergo Michael addition, we found that nucleoside phosphorothioates add rapidly to the double bond of acrylonitrile. The reaction proceeds rapidly in aqueous dimethylformamide over the pH range of 5–9. It is quite surprising that, in addition to the expected *S*-cyanoethyl phosphate **19**, some *O*-cyanoethyl compound was also found, and their ratio depended on the pH. Whether or not *O*-cyanoethylation took place directly at the monoester level or through an intermediary triester formation or *via* a more complex four-center mechanism deserves further study. The only known participation of the oxygen in thiophosphates in nucleophilic reactions is the attack on phosphoryl and acyl halides.[14] Accordingly, bicarbonate hydrolysis of thiophosphoryl chloride esters leads to the exclusive formation of normal (P–O–P) pyrophosphates.

In contrast to ordinary phosphates, monoesterified phosphorothioates are good nucleophiles toward 5′-*O*-tosyl nucleosides at room temperature. The use of elevated temperature is of no advantage because the hydrolysis of the thiophosphate to orthophosphate is also accelerated. A strong solvent effect could be seen clearly in the intramolecular displacement reaction **6** → **16**, in which the change from water to dimethylformamide enhanced the rate some 30-fold. Steric hindrance of the 5′ carbon as a rate-determining factor can be invoked to explain the differences in displacement on the purine *vs.* pyrimidine nucleosides **2** and **3**.

(13) C. D. Jardetzky, *J. Amer. Chem. Soc.*, **84**, 62 (1962).
(14) A. J. Kirby and S. G. Warren, "The Organic Chemistry of Phosphorus," Elsevier, New York, N.Y., 1967, p 227.

It is interesting to make at least a qualitative comparison between the classical oligonucleotide synthesis *via* phosphate activation and thiophosphorylation *via* displacement. The ease of forming the 3′,5′-cyclic esters is very much favored if the sulfur atom is incorporated into the ring. This is probably due to the size of the sulfur atom which can accommodate itself with less strain in the trans-fused ring system. In the intermolecular reaction, the rate and yield of the displacement have remained to this date under all conditions below the phosphorylation by dicyclohexylcarbodiimide.[15] For the present, only the synthesis of the simple dinucleoside monophosphates is satisfactory. In the absence of suitable protection for the sulfur in the diesters **6**, **7**, and **8**, these compounds are reactive in organic solvents and cannot be efficiently used in stepwise syntheses.

In spite of the preliminary state of the art, there are some very attractive features in the displacement reaction involving phosphorothioate, suggesting that a stepwise synthesis could be efficiently performed. (1) The diester types **19**, **20**, and **21**, *i.e.*, the end products, are not reactive below 80°. (2) The yield of the individual steps is limited only by a slow loss of sulfur, and the use of a large excess of activated monomer would be beneficial. (3) The only by-product is the phosphomonoester which is completely unreactive at room temperature and would not interfere with the subsequent chain elongation steps. (4) The esterification step itself does not require the protection of OH and NH_2 groups, although such protection might have been necessary for the preparation of synthetic intermediates; it is also unaffected by the presence of moisture.

Further interest in oligo-5′-thionucleotides will depend on the degree to which these compounds may substitute in the functions of natural oligonucleotides. Physical–chemical studies[9] and molecular models indicate that the thio analogs are capable of forming secondary structures.

Experimental Section

General. Chromatography was carried out by the descending technique on Whatman no. 1 paper with the solvent mixtures: A, 2-propanol–concentrated NH_4OH–H_2O (7:1:2) and B, 1-butanol–ethanol–0.5 M NH_4OOCCH_3 (5:3:2). Eastman Chromagram sheets were used for thin-layer chromatography (tlc) on silica gel in solvent mixtures C, 1-butanol–acetone–H_2O (4:2:1), and D, $CHCl_3$–CH_3OH (95:5). Paper electrophoresis was performed in a Savant flat-plate apparatus on Whatman no. 3 MM paper in 0.05 M sodium phosphate, pH 7.0, and 0.05 M sodium acetate, pH 5.5. R_f values and electrophoretic mobilities are listed in Table III.

The uv spectra were taken on a Beckman DU spectrophotometer. The nmr spectra were measured on a Varian HA-100 apparatus in D_2O with TMS as an external standard at ambient temperature. The yields were determined spectrophotometrically without regard to hypochromicity.

For enzymic degradation, the samples (1 μmol) were incubated with purified snake venom diesterase (Worthington; 0.05 mg of protein) in 0.05 M Tris–chloride, pH 8.8. The ratios of the degradation products were determined spectrometrically after eluting them from the chromatograms.

Microanalyses were performed by M-H-W Laboratories.

N^6-Benzoyl-5′-*O*-tosyl-2′-deoxyadenosine (3). N^6-Benzoyl-2′-deoxyadenosine[16] (4.0 g, 11.3 mmol) was dissolved in pyridine (50 ml) and a solution of *p*-toluenesulfonyl chloride (3.2 g, 17.3 mmol)

(15) T. M. Jacob and H. G. Khorana, *J. Amer. Chem. Soc.*, **87**, 368 (1965).
(16) H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *ibid.*, **85**, 3821 (1963).

Table III

Compd	R_f values A	B	C	Electrophoresis pH 7.0	pH 5.5
dT	0.63				
pdT	0.14	0.05		1.0	1.0
dA	0.56				
pdA	0.10	0.02			
3	0.87				
12	0.16	0.05	0.10	1.14	1.35
9	0.66		0.59	0.46	0.56
19	0.66		0.54	0.46	0.56
dsT	0.60				
pdsT	0.15				1.14
13				0.91	1.16
6	0.75		0.65	0.47	
16	0.56	0.29		0.62	
$d(Tps)_2O$	0.35		0.27	0.86	
$d(TosTps)_2O$	0.78		0.72	0.41	
$d(Tps\text{-})_2$		0.15		0.85	1.05
dsA	0.55				
pdsA	0.11				0.95
14			0.25	0.73	
7	0.72	0.83	0.71	0.35	
17	0.35		0.27	0.53	0.57
Cyclic $dA^{Bz}ps$	0.53	0.46	0.55	0.40	
15				0.86	1.10
8	0.78			0.42	
18	0.38	0.25		0.70	
20	0.47	0.26	0.40	0.39	0.57
21	0.40		0.27	0.35	0.52
22		0.12		0.75	
24				1.25	
23			0.34	0.65	
25			0.05	0.97	
26		0.10		0.96	
27			0.36	0.84	

in 50 ml of pyridine was added slowly under cooling and stirring. The reaction mixture was kept 1 day at 5° and then concentrated to a small volume, and 100 ml of ethyl acetate and ice were added. The upper layer was separated, washed with water, dried by $MgSO_4$, and evaporated to dryness. The ditosyl derivative was extracted with an ethyl acetate–ether mixture, and the residue crystallized from ethyl acetate. The crystals were filtered, washed with ethyl acetate, and dried *in vacuo* (4.1 g, 72%). The compound was uniform on paper chromatography and tlc: mp 126–129°; uv max (95% C_2H_5OH) 280 mμ (ε 20,600), 224 (24,000), min 248 (10,600).

Anal. Calcd for $C_{24}H_{23}O_6N_5S$ (503.57): C, 56.58; H, 4.55; N, 13.75; S, 6.29. Found: C, 56.25; H, 4.66; N, 13.88; S, 6.57.

General Method for the Preparation of Various Nucleoside 3′-Cyanoethyl Phosphorothioates. One millimole of the nucleoside derivatives **1, 2, 3,** and **4** was dissolved in 3 ml of pyridine, 0.3 ml of thiophosphoryl chloride was added at 0°, and the solution was kept at 5° for 6 hr. After the addition of 2 ml of hydracrylonitrile under cooling on ice, the phosphorylation was terminated within 30–60 min. The solution was evaporated *in vacuo* to a gum and extracted twice with a 1:1 mixture of ether and petroleum ether (50 ml); the residue was dissolved in 5 ml of pyridine and poured into a mixture of 2 *M* $Et_3NH \cdot HCO_3$, pH 8, and acetone (1:1, 50 ml) under cooling with ice and stirring for 1.5 hr. Two-thirds of the volume was evaporated, and the residual syrup was extracted two times with 50-ml portions of chloroform. The chloroform solution was dried over $MgSO_4$, filtered, and evaporated to dryness. Then the residue was dissolved in 50% ethanol and the solution applied to a DEAE-cellulose column (3 × 64 cm, HCO_3^- form). The elution was performed with a linear gradient made of 50% ethanol (1 l.) and 0.1 *M* $Et_3NH \cdot HCO_3$, pH 8 (1 l., in 50% ethanol). Three well-separated uv-absorbing peaks were found: the first comprised the unreacted nucleosides; the second peak at *ca.* 0.04 *M* represented the main product (**5, 6, 7,** and **8**). The last to elute were the pyrophosphates, types **10** and/or **11**.

5′-*O*-Tosylthymidine 3′-Cyanoethyl Phosphorothioate (6). Compound **6** obtained by the above procedure (4750 OD_{266} units, 44%) was homogeneous on paper chromatography and tlc. For analysis a sample was freeze dried from dioxane–water and dried over P_2O_5 *in vacuo* at room temperature for 24 hr. *Anal.* Calcd for $C_{26}H_{36}N_4O_9PS_2$ (643.71): P, 4.80; S, 9.93. Found: P, 4.41; S, 9.87. The nmr spectrum was complex but the H-1′ (t, δ 6.66 ppm, J = 7 Hz) and CH_3 (s, δ 2.25) of the thymidine moiety, the two triplets of the cyanoethyl group (δ 3.34 and 4.30, J = 6.2 Hz), and all aromatic protons could be identified, uv max (95% C_2H_5-OH) 266 mμ (ε 10,900); min 244 (4800).

N^6-Benzoyl-5′-*O*-tosyl-2′-deoxyadenosine 3′-Cyanoethyl Phosphorothioate (7). The nucleoside **3** (0.5 g, 1 mmol) was thiophosphorylated and purified as described above in the general procedure. The yield was 9900 OD_{280} units, 0.48 mmol (48%). After repeated freeze drying from dioxane, the amorphous powder exhibited a P/S ratio of 0.53; calcd, 0.5. It was homogeneous by paper chromatography, tlc, and electrophoresis. The uv spectrum was similar to that of **3**.

5′-Deoxy-5′-thiothymidine 3′,5′-Cyclic Phosphorothioate (16). **A.** 5′-*O*-Tosylthymidine[17] (200 mg, 0.5 mmol) was dissolved in 1 ml of anhydrous pyridine and treated with 0.1 ml of $PSCl_3$ at +5° for 6 hr. To this solution was added 5 ml of 2 *N* KOH and 5 ml of pyridine with continuous cooling and stirring at 5° for 1 day. After that, the pH was adjusted to 11 with Dowex 50 (H^+ form), and the solution was diluted and filtered. The nucleotides were absorbed on a Dowex 1-X2 ion-exchange column (1 × 30 cm, CO_3^{2-} form) at 5°, and the column was washed successively with 50 ml of 0.001 *N* KOH and 2 l. of 0.03 *M* $Et_3NH \cdot HCO_3$, pH 8. The cyclic phosphate **16** was eluted as a distinct peak at 800–960 ml. After repeated freeze drying, one obtained 103 mg (2300 OD_{267} units) of chromatographically pure product, which was crystallized from methanol (mp 186–190°) and dried at 55° *in vacuo* over P_2O_5. *Anal.* Calcd for $C_{16}H_{28}N_5O_6PS$ (421.42): C, 45.63; H, 6.70; P, 7.35; S, 7.60. Found: C, 45.15; H, 6.85; P, 6.78; S, 7.75. The nmr spectrum of the NH_4^+ salt (Figure 2) contained the following signals: 7.90 (d, 1, J = 1 Hz, C_6H), 6.65 (m, 1, $C_{1'}$ H), 4.50 (m, 1, $C_{4'}$ H), 3.7 (m, 2, $C_{5'}$ H), 3.01 (m, 2, $C_{2'}$ H), 2.36 ppm (d, 3, J = 1 Hz, CH_3); uv max (H_2O, pH 7) 267 mμ (ε 9600), min 235 (3080)

B. Compound **6** (1200 OD units) was dissolved in 10 ml of of dimethylformamide and 0.5 ml of 40% Et_4NOH was added at 0°. After 10 min a sample was concentrated and analyzed by electrophoresis (2–5°, 2 hr, 40 V/cm). No starting material was found.

The main portion was kept at 20° for 6 hr, neutralized with acetic acid, concentrated *in vacuo*, and precipitated with ether. The precipitate was centrifuged off and dissolved in 0.5 ml of 10% barium acetate, and the nucleotides were again precipitated with 10 ml of acetone. This preparation was further purified by passing it through a Sephadex G-10 column (bed volume 1.250 ml, V_e = 350 ml) in distilled water; yield 950 OD_{267} units, 90% of barium salt; it contains some barium tosylate according to elementary analysis and nmr.

In contrast to **6** and other phosphorothioates, **16** did not react with C_2H_5Br at 37° with formation of a triester.

2′,5′-Dideoxy-5′-thioadenosine 3′,5′-Cyclic Phosphorothioate (17). The nucleoside **3** (0.3 g, 0.6 mmol) was dissolved in 2 ml of dry pyridine, and 0.18 ml of $PSCl_3$ was added slowly at 0°. After standing for 4 hr at 5°, the reaction mixture was evaporated *in vacuo* and the residual gum was extracted by two 15-ml portions of ether. The residue was dissolved in pyridine and poured under cooling and stirring into a 1:1 mixture (25 ml) of 2 *M* $Et_3NH \cdot HCO_3$, pH 8, and acetone. After 1 day the solution was concentrated *in vacuo* to a small volume and repeatedly freeze dried from water. The residue was applied to a DEAE-cellulose column (3 × 64 cm, HCO_3^-). The elution by a linear gradient (50% ethanol, 1 l.; 0.1 *M* $Et_3NH \cdot HCO_3$, pH 8, in 50% ethanol, 1 l.) produced, in addition to a large neutral fraction, one major nucleotide peak at 0.05 *M*, which corresponded to the N^6-benzoyl derivative of **17**. The yield was 1600 OD_{285} units, 12%. The uv spectrum was similar to N^6-benzoyl-2′-deoxyadenosine (H_2O, pH 2, λ_{max} 285 mμ (ε 22,500); λ_{min} 260 mμ). On further elution of the column with 2 *M* buffer, a second peak of 2150 OD_{285} units appeared, which consisted, at least partially, of pyrophosphates.

The freeze-dried sample (32 mg) was dissolved in a 1:1 mixture of CH_3OH and concentrated NH_4OH and set aside for 48 hr. The solution was evaporated, and the crude product subjected to ion exchange chromatography on a DEAE-cellulose column under the previously used conditions. Further purification was carried out on a Sephadex G-10 column (2.3 × 80 cm) in 0.1 *M* aqueous $Et_3NH \cdot HCO_3$; 5.5-ml fractions were collected at 15-min intervals. The fractions 51–61 were freeze dried, and the dry powder extracted with methanol–ether to remove traces of benzoate.

(17) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 816 (1955).

The K^+ salt of **17** (1080 OD_{260} units, 0.07 mmol) was obtained by passage through Dowex 50 (K^+).

A similar quantity of cyclic nucleotide can be obtained from the pyrophosphate fraction by hydrolysis at pH 13. The overall yield of **17** runs around 20%; nmr 8.25 (s, 1, C_8H), 8.18 (s, 1, C_2H), 6.40 ppm (t, 1, J = 8 Hz, C_1,H).

17 was rapidly and quantitatively hydrolyzed by purified snake venom phosphodiesterase with the formation of a monoester that possessed mobilities identical with 5′-dAMP on electrophoresis and paper chromatography in solvent B.

5′-Deoxy-5′-thiocytidine 3′,5′-Cyclic Phosphorothioate (18). The cyanoethyl ester **8** was prepared from 1 mmol of 5′-*O*-tosyl-N^6-benzoyldeoxycytidine[18] according to the general procedure in 27% yield. The crude product was kept in concentrated NH_4OH for 2 days. One-half of the solution was applied to a small Dowex 1-X2 column ($HCOO^-$ form) but no compound with the expected properties of **18** could be eluted with 0.1 *N* HCOOH. The rest of the reaction mixture was then separated on Whatman no. 3 MM paper in solvent A. The NH_4^+ salt of **18** (15 mg) was isolated among other unidentified polymeric and neutral fractions. The sample was insufficient for a detailed analysis, but the spectral and the chromatographic properties were in accordance with the structure: uv max (H_2O, pH 7) 270 mμ (ε 9200); nmr 8.16 (d, 1, J = 7.5 Hz, C_6H), 6.71 (d, 1, J = 7.5 Hz, C_5H), 6.62 ppm (t, 1, J = 8–9 Hz, C_1′H).

Compound **18** remained unchanged following the treatment with snake venom phosphodiesterase.

Kinetics of Intramolecular Displacement 13 → 16 in 10% Ethanol. In two experiments, 5–10-mg quantities of **6** were dissolved in 0.5 ml of ethanol and deblocked with 4.5 ml of 0.1 *N* KOH at 0° during 15 min. After standing at 20° for various times, the aliquots were first concentrated *in vacuo*, treated with an excess of Dowex 50 (NH_4^+ form) and analyzed by electrophoresis. The uv absorbing spots and appropriate blanks were eluted with water, and the number of OD_{267} units determined. The percentage of **16** was calculated, taking into consideration a small amount of hydrolysis of **13** (Table I).

Kinetics of Intramolecular Displacement 13 → 16 in Dimethylformamide. A solution of 50 OD_{267} units of **6** in 0.5 ml of ethanol–H_2O (1:1) was adjusted to pH 13 with 40% Et_4NOH. After 20 min at 0°, 2 ml of dimethylformamide was added, and the volume quickly reduced *in vacuo* to 1 ml. Aliquots were analyzed as described above. The reaction times include a 20-min correction for the time of evaporation. The results shown below in Table II are the average of two measurements. No correction was made for the insignificant desulfurization of **13**.

Thymidine 3′-Cyanoethyl Phosphorothioate (9). 5′-*O*-Dimethoxytritylthymidine[16] (1) (1.2 g, 2 mmol) was converted to the cyanoethyl phosphorothioate according to the general procedure described above. After evaporation of the $CHCl_3$ solution, the residue was dissolved in 80% CH_3COOH (50 ml) and left at room temperature for 4 hr. After freeze drying it was extracted twice with a mixture of ether and petroleum–ether (100 ml, 1:1), dissolved in 50% ethanol and subjected to ion exchange chromatography on DEAE–cellulose as described in the general method. The title compound **9** was eluted as the first nucleotidic material, followed by an equal peak of pyrophosphates of type **10** and **11**. Compound **9** was found homogeneous on electrophoresis and paper chromatography. The yield was determined spectrophotometrically (0.595 mmol, 30%): uv max (H_2O) 266 mμ (ε 9600), min 235 (3200).

Thymidine 3′-Phosphorothioate (12). A. *Via* Pyrophosphate. 5′-*O*-Dimethoxytritylthymidine (0.6 g, 1 mmol) was thiophosphorylated as above, but with the omission of the hydracrylonitrile step. The hydrolysis of the protecting group and the extraction were done as in the preparation of **9**. The products were separated by DEAE–cellulose chromatography (3 × 72 cm, HCO_3^-) with a linear gradient (1 l. of 50% ethanol–1 l. of 0.2 *M* $Et_3NH \cdot HCO_3$ in 50% ethanol, pH 8). There was only one nucleotide peak (6500 OD_{266} units, 69%) which, according to electrophoresis, contained mostly the symmetrical pyrophosphate, type **11**, and a small amount of **12**. The freeze-dried powder was dissolved in water and the Ba^{2+} salt was obtained by ion exchange through a Dowex 50-X2 column. The elementary analysis revealed the following ratios: calcd P/S, 1.00; N/P, 2.00; found P/S, 0.91; N/P, 2.1.

Compound **11** (R = H) (2830 OD_{266} units) was dissolved in 5 ml of 1 *N* KOH and kept under N_2 atmosphere for 5 hr. After the addition of an excess of Dowex 50 (NH_4^+), the solution was filtered and freeze dried. The resulting product contained **12** in 96% purity (0.27 mmol, 90%), some thymidine 3′-phosphate, and a disulfide. The uv spectrum of **12** corresponded to that of **16**; mol wt 554, determined spectrophotometrically. *Anal.* Calcd for $C_{10}H_{21}N_4O_7SP \cdot 10H_2O$ (550.46): P, 5.63; S, 5.82. Found: P, 5.48; S, 6.43; ratio P/S, 0.89.

B. Deblocking of 9. The diester **9** (0.162 mmol) was dissolved in a mixture of dioxane (10 ml) and 1 *N* KOH (10 ml) and kept at 0° for 30 min under the exclusion of O_2. The solution was concentrated *in vacuo*, and an excess of Dowex 50 (NH_4^+) resin was added. The resin was filtered off and washed, and the filtrate was freeze dried. The resultant white powder, which was chromatographically and electrophoretically pure, was identical with **12** obtained by the above method. The yield determined spectrophotometrically was virtually quantitative.

Oxidation of 9 by $K_3[Fe(CN)_6]$. Compound **9** (0.012 mmol) was dissolved in 50% acetone (0.5 ml) and stirred with 20 mg of $K_3[Fe(CN)_6]$ overnight at room temperature. Then some concentrated NH_4OH was added and after a few hours the solution was analyzed by paper chromatography and electrophoresis. The main product had similar mobilities as the disulfide described by Eckstein.[7]

The Reaction of 12 with β-Bromopropionitrile. Compound **12**, Et_3^+NH salt (0.02 mmol) and β-bromopropionitrile (0.1 ml) were allowed to react in 50% pyridine (0.8 ml) at 20° for 24 hr. The solution was evaporated and extracted twice with 10 ml of ether, and the residue was applied to a column of DEAE–cellulose (2 × 56 cm, HCO_3^-). The elution was performed with a linear gradient made of 0.5 l. of 50% ethanol and 0.5 l. of 0.1 *M* $Et_3NH \cdot HCO_3$, pH 8, at a rate of 0.6 ml/min. Following a neutral fraction, the first nucleotidic peak was eluted at a concentration of 0.025 *M*. It contained thymidine 3′-*S*-cyanoethyl phosphorothioate (**19**) (0.011 mmol, 52.5%, by uv) which was uniform on paper chromatograms and electrophoresis. Compound **19** was not oxidized by $K_3[Fe(CN)_6]$, but it was oxidized by I_2[5] under the formation of thymidine 3′-phosphate.

The Reaction of 12 with Acrylonitrile. A. At pH 7.5. Compound **12** (0.04 mmol) was dissolved in a mixture of 0.01 *M* $Et_3NH \cdot HCO_3$ (0.5 ml), dimethylformamide (0.5 ml) and acrylonitrile (0.5 ml). After 14 hr at room temperature, the reaction mixture was evaporated to dryness, coevaporated several times with water, and applied to a DEAE–cellulose column (2.2 × 30 cm, HCO_3^-; 0.5 l. of 50% ethanol–0.5 l. of 0.15 *M* $Et_3NH \cdot HCO_3$, pH 8, in 50% ethanol). The peak eluted at 0.04–0.06 *M* was repeatedly freeze dried (205 OD_{267} units, 0.022 mmol, 55%). The resulting powder was similar to **19** on electrophoresis, but on tlc in solvent A a double spot appeared.

B. At pH 5.5. Compound **12** (0.02 mmol) was dissolved in a mixture of 0.02 *N* NH_4OOCCH_3, pH 5.5 (0.5 ml), dimethylformamide (0.5 ml), and acrylonitrile (0.3 ml). The diester fraction was isolated as above (60 OD_{267} units, 0.063 mmol, 34%).

Samples A and B were treated with $K_3[Fe(CN)_6]$ and NH_4OH as described previously, and the products analyzed by electrophoresis. The spots, corresponding to **19** and the disulfide, d(Tps-)$_2$, were eluted and the OD measured at 267 mμ. The fractions of uv absorption found in the disulfide spot in experiments A and B were 13 and 27%, respectively.

Oligonucleotide Synthesis. d(TpsT) (20). Thymidine 3′-phosphorothioate (**12**, 0.01 mmol), Et_3N^+H salt, was dried by repeated evaporation from a mixture of dimethylformamide and tri-*n*-butylamine; the nucleoside **1** (0.02 mmol), dimethylformamide (1 ml), and Et_3N (0.15 ml) were added, and the reaction mixture was kept at room temperature for 24 hr. The solution was then evaporated to dryness *in vacuo;* the residue was dissolved in 50% ethanol and applied to a DEAE–cellulose column (HCO_3^- form, 2.3 × 30 cm). Elution was performed with a linear gradient made of 0.5 l. of 50% ethanol and 0.5 l. of 0.1 *M* $Et_3NH \cdot HCO_3$, pH 7.5, in 50% ethanol at a rate of 0.7 ml/min. The peak of **20** appeared at 0.045 *M* following the nucleoside peak and preceding some unreacted **12** and thymidine 3′-phosphate. After evaporation and freeze drying, compound **20** was chromatographically and electrophoretically homogeneous, but the nmr spectrum revealed the presence of some tosylate. It was further purified by passing through a Sephadex G-10 column (2.3 × 80 cm) in 0.1 *M* $Et_3NH \cdot HCO_3$ collecting fractions of 5.5 ml/15 min. Fractions 31–36 were freeze dried, and the K^+ salt of **20** was obtained by passage through a Dowex 50 (K^+) column. The yield was 125 OD_{267} units, 65%,

(18) E. Benz, N. F. Elmore, and L. Goldman in "Synthetic Procedures in Nucleic Acids Chemistry," Vol. I, W. W. Zorbach and R. S. Tipson, Ed, Interscience, New York, N. Y., 1968, p 288.

assuming ϵ_{max}^{267} 9600 per thymidine residue. The nmr spectrum contained the clearly separated singlets of the C-6 protons at 7.74 and 7.65 ppm, and two overlapping triplets of the C-1′ protons at 6.34 (J = 7 Hz) and 6.30 ppm (J = 7 Hz), respectively.

Compound **20** is degraded by snake venom phosphodiesterase (see general methods) to form a mixture of 5′-deoxy-5′-thiothymidine 5′-phosphorothioate (pdsT), thymidine, and 5′-deoxy-5′-thiothymidine (sdT). The latter nucleoside is the product of chemical hydrolysis of pdsT; it overlaps with thymidine in paper chromatograms (dT + dsT/pdsT = 1.65).

d(TpsA) (21). Phosphorothioate **12** (0.01 mmol), Et_3N^+H salt, and the nucleoside derivative **3** (0.05 mmol) were allowed to react as described in the preparation of **20**. After the reaction mixture was concentrated to a gum, 1 ml of methanol and 1.3 ml of concentrated NH_4OH were added for the hydrolysis of the benzoyl group (48 hr, 20°). The solution was again reduced to dryness *in vacuo* and the residue subjected to the separation on the same DEAE–cellulose column as described above. Compound **21**, Et_3N^+H salt, was obtained in 50% yield based on uv measurement: uv max H_2O, pH 7) 262 mμ, min 237 mμ; ϵ_{260} (calcd) 23,100; A_{250}/A_{260} 0.65, A_{280}/A_{260} 0.35; nmr 8.42 (s, 1, C_8H), 8.21 (s, 1, C_2H), 7.78 (s, 1, C_6H), 6.45 (t, 1, J = 7 Hz, $C_1'H$, dAdo), 6.18 ppm (t, 1, J = 7 Hz, $C_1'H$, dThd).

The action of snake venom phosphodiesterase in **21** produced, in addition to thymidine, a chromatographically different nucleoside that arose from the nucleotide pdsA. The ratio T/(pdsA + dsA) = 1.03 was found.

Compound 22. A preparation of **12** (0.021 mmol of the Et_3NH^+ salt, plus 20% thymidine 3′-phosphate) was condensed with 0.053 mmol of **6** in 0.2 ml of dimethylformamide over a period of 24 hr. The mixture was separated on a Whatman 3 MM paper in solvent **B** and the major new band at R_f 0.3–0.4 was eluted with water (220 OD_{267} units, 55%). It migrated as a single spot on electrophoresis at pH 7 with a mobility of 0.75 relative to 3′-TMP; upon treatment with 0.2 N KOH, the mobility increased to 1.25.

Compound 23. A pure preparation of **12** (0.01 mmol) was allowed to react with 3.5 equiv of 7 under the conditions given for **20**. The separation of the reaction mixture was carried out on a Sephadex G-25 superfine gel (3 × 105 cm), using 0.1 M $Et_3NH \cdot HCO_3$ in 25% ethanol as the eluent, at a flow rate of 0.3 ml/min. The protected dinucleotide **23** was eluted at 390–420 ml (27% spectrophotometrically); it was uniform on electrophoresis and tlc. According to the elution profile, higher oligomers were also present; for **23** uv max (H_2O) 276 mμ, min 237, sh 262; A_{250}/A_{260} 0.80; A_{280}/A_{260} 1.18; ϵ_{260} 20,800 (calcd).

24 and 25. Small aliquots (0.01–0.02 mmol) of **22** and **23** were kept in 1 N KOH (2 ml) at 0° for 30 min, and the flasks briefly evacuated for the removal of acrylonitrile. After the addition of Dowex 50 (3.5 g, NH_4^+), the solutions were filtered, and the filtrates freeze dried. An expected increase of the electrophoretic mobilities was observed (Table III).

Trinucleotide 26. Two hundred OD_{267} units (0.01 mmol) of **22** were treated with alkali as above, but without the concentration of the reaction mixture. The solution was passed through an excess of Dowex 50-X2 resin (Et_3N^+H form) and freeze dried. The resulting Et_3N^+H salt of **24** was allowed to react with 0.03 mmol of **6** in 0.2 ml of dimethylformamide for 3 days at room temperature. After evaporation, the material was passed through a Sephadex G-25 column (95 × 2.5 cm) in 0.1 M $Et_3NH \cdot HCO_3$ collecting fractions of 3.4 ml/10 min (V_0 = 180 ml; V_i = 480 ml). The elution profile is given in Figure 2. Peak A (150 OD_{267} units, 50%) contains **26** in 90% purity according to electrophoresis and paper chromatography. Peak B is a mixture of two compounds which behave like **22** and **24**, but, presumably, one of them is an S-cyanoethyl ester and the other one is d(TpTp). Peak C comprised the excess of **6**. Longer oligomers are also present. On treatment with alkali, as described for **24**, the monoester was formed according to the increase in electrophoretic mobility.

Trinucleotide 27. Compound **25** (0.01 mmol) NH_4^+ salt was allowed to react with 5 equiv of **6** in 1 ml of dimethylformamide at room temperature for 24 hr. The separation of the products was carried out as described for **23**. Fractions 72–76 contained the main product (10%, by uv) that exhibited the expected electrophoretic mobilities before and after treatment with alkali: uv max (H_2O) 271 mμ, min 236, sh 261; A_{250}/A_{260} 0.74, A_{280}/A_{260} 1.01; ϵ_{260} (calcd) 29,200.

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