OLIGONUCLEOTIDIC COMPOUNDS. XLII.* SYNTHESIS OF THYMIDINEPHOSPHOROTHIOYL-($O^{3'} \rightarrow O^{5'}$)-THYMIDINEPHOSPHOROTHIOYL-($O^{3'} \rightarrow O^{5'}$)-THYMIDINE**

A. MALKIEVICZ*** and J. SMRT

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

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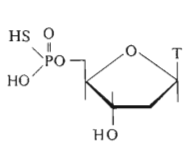
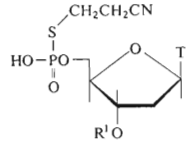
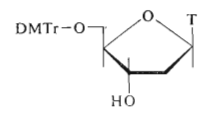
Reaction of thymidine 5'-phosphorothioate (*I*) with acrylonitrile at pH 8—9 and the subsequent treatment with acetic anhydride in pyridine affords 3'-O-acetylthymidine 5'-S-(2-cyanoethyl)-phosphorothioate (*IIb*). By the action of pyridinium S-(2-cyanoethyl)phosphorothioate and 2,3,5-triisopropylbenzenesulfonyl chloride, 5'-O-dimethoxytritylthymidine (*III*) is converted to 5'-O-dimethoxytritylthymidine 3'-S-(2-cyanoethyl)phosphorothioate (*IV*). Reaction of compounds *IIb* and *III* in the presence of 2,3,5-triisopropylbenzenesulfonyl chloride and the subsequent treatment with 90% aqueous acetic acid affords thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-3'-O-acetylthymidine [P-S-(2-cyanoethyl) ester] (*VI*). Reaction of compounds *IV* and *VI* accomplished by the action of 2,3,5-triisopropylbenzenesulfonyl chloride and the subsequent removal of protecting groups affords thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-thymidine (*X*).



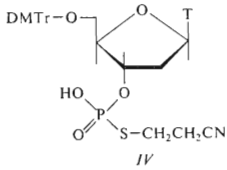
I



IIa, $R^1 = H$
IIb, $R^1 = COCH_3$

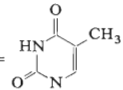


III



IV

In all formulae T =



DMTr = dimethoxytrityl

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*** Present address: Institute of Technology, Department of Chemistry, Lodz, Poland.

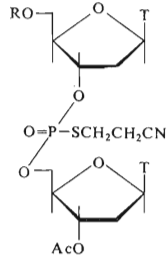
Polynucleotidic chains are used by the living matter for the storage of genetic informations as well as for the transport of these informations into the protein-synthesising systems. Processes involving informations stored in the sequence of bases in a polynucleotidic chain, may be investigated or controlled by means of synthetic chains of known sequences. The synthetic oligo- or polynucleotides may be successfully used only in simple biochemical systems lacking enzymes which cleave the internucleotidic bond. The more complex systems and the untouched living matter would probably require the use of such synthetic analogues that would be resistant towards enzymes or at least relatively less susceptible than the naturally occurring polynucleotides.

Such a requirement could be realised by synthesis of oligonucleotide analogues, the internucleotidic bond of which is formed by phosphorothioic acid O,O-diester[2]. The enzymatically prepared polyribonucleotide analogues of this type maintain the ability to form double-stranded polymers and the messenger ability, being however more resistant towards nucleases than the parent substances.

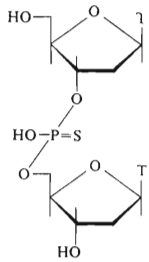
The O,O-dinucleoside esters of phosphorothioic acid have been synthesised by condensation of a nucleoside phosphorothioate with a nucleoside bearing a free hydroxylic function[3,4]; this method is not suitable for the preparation of longer chains since a mixture of O,O- and O,S-diester is obtained in each step. The unequivocal synthesis of phosphorothioic acid O,O-diester would to our opinion involve the O,O,S-triester; the sulfur atom of this triester would be protected by such a group which could be easily removed in the final step of the synthesis. This O,O,S-triester could be obtained by condensation of a O,S-diester with the hydroxylic function of the other component by the action of an aromatic sulfonyl chloride, analogously to the triester synthesis of the internucleotidic bond[5].

The realisation of this proposal has been first attempted in the deoxyribo series because of the easier accessibility of the starting compounds. The sulfur atom was protected by the 2-cyanoethyl group which has been some time ago proposed by Letsinger[5] for triester synthesis of the internucleotidic bond. The clue compound of the synthesis, namely, thymidine 5'-S-(2-cyanoethyl)phosphorothioate (*IIa*) has been prepared by Cook[6] by reaction of thymidine 5'-phosphorothioate (*I*) with 3-bromopropionitrile. Alternatively, the S-(2-cyanoethyl) ester of nucleoside thiophosphates may be prepared by a direct cyanoethylation of nucleoside phosphorothioates with acrylonitrile[7]. Thymidine 3'-phosphorothioate is claimed[7] to react with acrylonitrile under buffered conditions to afford S-(2-cyanoethyl) ester along with 27% of the O-(2-cyanoethyl) ester (at pH 5·5) or with 13% of the O-ester (at pH 7·5). The amount of the O-ester in the crude product was however determined[7] by a method which, to our opinion, was not suitable for this purpose. Thus, the crude cyanoethylation product was treated with potassium ferricyanide and then conc. aqueous ammonia was added after a certain period of time. The thus-obtained product was subjected after an inaccurately stated period of time to electrophoresis: the presence of the O-ester was deduced[7] from the formation of a bis(nucleoside-

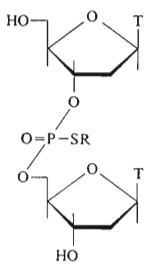
phosphoryl)disulfide with reference to the paper of Eckstein[8]; this author, however, performed the ferricyanide oxidation of phosphorothioic acid O-monoester, not of the



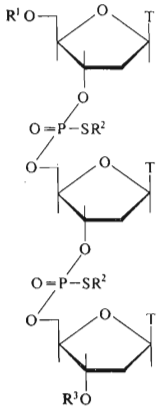
V, R = dimethoxytrityl
VI, R = H



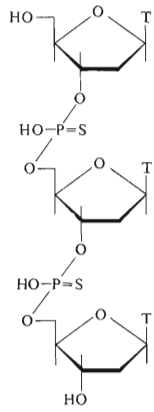
VIII



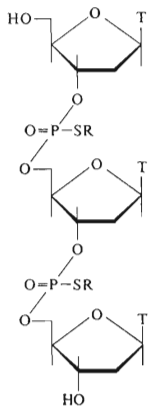
IXa, R = CH_2CH_3
IXb, R = $CH_2C_6H_4NO_2$



VIIa, R^1 = dimethoxytrityl
$R^2 = CH_2CH_2CN$, $R^3 = COCH_3$
VIIb, R^1 = H, $R^2 = CH_2CH_2CN$
$R^3 = COCH_3$



X



XIa, R = CH_3CH_3
XIb, R = $CH_2C_6H_4NO_2$

corresponding O,O-diester. The phosphorothioic acid O,O-diester can be hardly assumed to react with potassium ferricyanide since, *e.g.*, the bis(dialkoxyphosphoryl)-disulfides exhibit a higher oxidation-reduction potential than the ferricyanide[9]. In this laboratory, we did not observe any reaction of thymidinephosphorothioyl-$(O^{3'} \rightarrow O^{5})'$-thymidine (*VIII*) with the ferricyanide. Addition of ammonia[7] to the ferricyanide-containing reaction mixture was accompanied by β-elimination of the 2-cyanoethyl group both with the O-(2-cyanoethyl) and the S-(2-cyanoethyl), ester, though at a somewhat slower rate in the latter case[6]. Consequently, the results depend on the time of action of the alkaline medium. We have shown that also the treatment of thymidine 5'-S-(2-cyanoethyl)phosphorothioate (*IIa*) with potassium ferricyanide and conc. aqueous ammonia led to the formation of the corresponding bis(nucleosidephosphoryl)disulfide (about 50% after 48 h).

In the cyanoethylation of phosphorothioic acid O-esters, the yields of the diesters were found to increase with the increasing pH value of the solution. At pH 8 – 9, the reaction is quantitative and affords exclusively the S-(2-cyanoethyl) derivatives under the conditions stated, as shown by iodine tests. When the O-(2-cyanoethyl) esters are formed at a lower pH value, they can be isomerised to the more stable S-isomers by raising the pH value[10].

According to Cook[6], thymidine S-(2-cyanoethyl)phosphorothioate (*IIa*) does not afford on self-condensation any oligomeric products; it could be inferred from this finding that the formation of an O,O,S-triester does not occur or that a 3',5'-cyclic triester is obtained. The reaction of 3'-O-acetylthymidine 5'-S-(2-cyanoethyl)phosphorothioate (*IIb*) with 5'-O-dimethoxytritylthymidine (*III*) by the action of 2,3,5-triisopropylbenzenesulfonyl chloride has been now observed to afford the O,O,S-triester *V* in 80% yield. By the action of aqueous ammonia, the triester *V* is converted to a dimethoxytrityl-containing substance, the immobility of which on thin-layer chromatography in 9 : 1 chloroform–methanol solvent system points to the occurrence of an ionic substance (O,O-diester). The O,O,S-triester *V* was also treated with 90% aqueous acetic acid and the course of detritylation was checked by thin-layer chromatography. The reaction was quantitative after 2 h. The product, namely, thymidinephosphorothioyl-$(O^{3'} \rightarrow O^{5'})$-3'-O-acetylthymidine [P-S-(2-cyanoethyl) ester] (*VI*), was isolated by chromatography on a loose layer of silica gel. Deblocking of the ester *VI* with 1 : 1 methanol-conc. aqueous ammonia afforded thymidine-phosphorothioyl-$(O^{3'} \rightarrow O^{5'})$-thymidine[1]. The above results have shown the realizability of the triester synthesis of phosphorothioic acid O,O-diester *via* O,O-dialkyl--S-(2-cyanoethyl) esters.

The above discussed synthesis was performed with the use of a nucleoside 5'-S--(2-cyanoethyl) ester. The other approach consists in the reaction of a nucleoside 3'-(2-cyanoethyl) ester with the $C_{(5')}$-hydroxylic function of the second component. In the latter approach, 5'-O-dimethoxytritylthymidine-3'-S-(2-cyanoethyl)phosphorothioate (*IV*) served as the active component. Compound *IV* was prepared in a high

yield by condensation of 5'-O-dimethoxytritylthymidine (*III*) with the pyridinium salt of S-(2-cyanoethyl)phosphorothioate[11] by the action of 2,3,5-triisopropylbenzenesulfonyl chloride. This procedure represents the most advantageous method for the preparation of S-(2-cyanoethyl) derivatives of phosphorothioic acid O-esters as well as of phosphorothioic acid O-esters alone (because of the ready removability of the 2-cyanoethyl group), *cf.* ref.[10]

The phosphorothioate grouping of compound *IV* is resistant to potassium ferricyanide. In the presence of potassium ferricyanide, conc. aqueous ammonia splits off the 2-cyanoethyl group and the resulting phosphorothioic acid O-ester is oxidized with the ferricyanide unter the formation of the corresponding bis(nucleosidephosphoryl)disulfide. The 2-cyanoethyl and the dimethoxytrityl groups are quantitatively removed by the action of an acidic 1% solution of iodine in 50% aqueous acetone (checked by electrophoresis).

Condensation of the triester *VI* with the pyridinium salt of the diester *IV* by the action of triisopropylbenzenesulfonyl chloride afforded the protected trinucleotide *VIIa* in 38% yield. The dimethoxytrityl group was removed on treatment with 90% aqueous acetic acid under the formation of compound *VIIb*. Removal of the acetyl and the 2-cyanoethyl group from compound *VIIb* with the methanol-aqueous ammonia solvent mixture afforded the phosphorothioate analogue *X* of thymidylyl-thymidylyl-thymidine. The structure of compound *X* (as inferred from the synthesis) was confirmed on comparison with the thionucleotide *VIII* by chromatography (slower mobility of *X*) and electrophoresis (faster mobility of *X*). Another proof of structure of compounds *VIII* and *X* consists in S-alkylation with alkyl halides; this reaction is characteristic of salts of phosphorothioic acid O,O-diester[12,13]. Thus, treatment of compounds *VIII* and *X* with ethyl bromide in methanol and with *p*-nitrobenzyl bromide in dimethylformamide afforded products, the chromatographic and electrophoretic behaviour of which was similar to that of the O,O,S-triesters *IXab* and *XIab*.

The results of the present paper represent a starting point for investigations on the stepwise synthesis of analogues of oligonucleotidic chains carrying phosphorothioyl O,O-diester bonds.

EXPERIMENTAL

Thin-layer chromatography was performed on ready-for-use Silufol UV_{254} plates (Kavalier Glassworks, Votice, Czechoslovakia) in the following solvent systems: T_1, 2-propanol–conc. aqueous ammonia–water (7 : 1 : 2); T_2, chloroform–methanol–pyridine (90 : 5 : 5); T_3, chloroform–methanol (9 : 1); T_4, chloroform–methanol–pyridine (8 : 1 : 1). The preparative runs were performed in the same systems on a 6 mm thick layer of loose silica gel (particle size, 10—60 micron) containing a fluorescent indicator (produced by Service Laboratories of this Institute in Prague - Suchdol). The dimethoxytrityl derivatives were detected by pressing a strip of paper to the moist chromatographic layer of loose silica gel and spraying the paper with a 10% solution

of perchloric acid in 30% aqueous acetic acid. The bands were eluted with 1 : 1 chloroform–methanol solvent mixture (T_e). Electrophoresis was performed on paper Whatman No 1 (immersed in tetrachloromethane) in E_1, 0·05M triethylammonium hydrogen carbonate (pH 7·5).

Thin-layer chromatographical mobilities in systems T_1 and T_3, and the electrophoretical mobility in the buffer solution *E*: uridine 2′(3′)-phosphate (0·1, 0, 1·0); *I* (0·46, 0, 0·92); *II* (0·84, —, 0·50); *IV* (0·84, —, 0·05); *V* (—, 0·60, —); *VI* (—, 0·24, —); *VIIa* (—, 0·27, —); *VIIb* (—, 0·07, —); *VIII* (0·58, 0, 0·59); *XIa* (—, 0·16, —); *IXb* (—, 0·25, —); *X* (0·41, 0, 0·89); *XIa* (—, 0·15, —); *XIb* (—, 0·20, —).

Thymidine 5′-Phosphorothioate (*I*)

In the preparation of the title compound (*I*), the reported[6] procedure was used with some modifications in the isolation. The reaction mixture consisting of 3′-O-acetylthymidine (2 mmol), S-(2-carbamoylethyl)phosphorothioate pyridinium salt (5 mmol), N,N′-dicyclohexylcarbodiimide (2 g), pyridine (5 ml), and hexamethylphosphoric triamide is allowed to stand for 4 days, diluted with water (5 ml), kept for additional 2 h, and evaporated under diminished pressure. The hexamethylphosphoric-triamide-containing residue is treated with 0·2M-NaOH (100 ml), the resulting mixture refluxed for 15 min, and allowed to cool. Pyridinium Dowex 50 ion exchange resin is then added to obtain pH 7. The resin is filtered off and the filtrate evaporated under diminished pressure. The residue is chromatographed on a 40 × 16 × 0·6 cm layer of loose silica gel in the solvent system T_1. The ultraviolet-absorbing band (R_F 0·55) is eluted with water and the eluate passed through Dowex 50 (H^+) ion exchange resin. The effluent is adjusted to pH 7·5 by the addition of barium hydroxide and concentrated under diminished pressure to the volume of 20 ml. The precipitate is removed by centrifugation and the supernatant is diluted with ethanol (40 ml). The solid is isolated by centrifugation, washed successively with 66% aqueous ethanol, 99% ethanol, and finally with ether, and air-dried. Yield, 454 mg of the barium salt of *I*.

3′-O-Acetylthymidine 5′-S-(2-Cyanoethyl)phosphorothioate (*IIb*)

The ammonium salt of compound *I* (obtained by the preparative thin-layer chromatography as stated above) is dissolved in 50% aqueous dimethylformamide (8 ml) and the solution is adjusted to pH 8—9 with triethylamine. Acrylonitrile (2 ml) is then added, the whole mixture stirred at toom temperature for 20 h, and finally passed through a column of pyridinium Dowex 50 ion exchange resin (50 ml). The eluate was evaporated to dryness under diminished pressure and the residue coevaporated with three 10 ml portions of 9 : 1 ethanol–triethylamine. The ethanol is removed by coevaporation with pyridine and the final residue is dissolved in pyridine. As shown by spectrophotometry after chromatography of an aliquot on paper Whatman No 1 in the solvent system T_1, the solution contained 0·5 mmol of the triethylamonium salt of thymidine 5′-S-(2-cyanoethyl)phosphorothioate, identical on electrophoresis and chromatography with a specimen prepared according to ref.[6] As shown by quantitative decyanoethylation with iodine, the corresponding O-(2-cyanoethyl) derivative is absent.

Acetic anhydride (5 ml) is added to the above pyridine solution, the reaction mixture kept at room temperature for 20 h, and evaporated at 20°C/1 Torr. The residue is kept in 50% aqueous pyridine (10 ml) for 3 h and then passed through a column of pyridinium Dowex 50 (20 ml). The column is eluted with additional 50% aqueous pyridine and the eluate is evaporated at 20°C/1 Torr. The water is removed by repeated coevaporations with pyridine. The final residue is dissolved in pyridine (10 ml) and the solution is added dropwise with stirring into ether (300 ml). The precipitate is collected with suction, washed with ether, and dried under diminished pressure. Yield,

279 mg of the pyridinium salt of compound *IIb*. For $C_{15}H_{20}N_3O_8PS.C_5H_5N$ (512·4) calculated: 10·94% N, 6·05% P, 6·25% S; found: 10·27% N, 6·00% P, 6·76% S.

5′-O-Dimethoxytritylthymidine 3′-S-(2-Cyanoethyl)phosphorothioate (*IV*)

A mixture of S-(2-cyanoethyl)phosphorothioate pyridinium salt[12] (2 mmol) and 5′-O-dimethoxytritylthymidine (1 mmol) is repeatedly coevaporated with pyridine at 20°C/1 Torr and the final residue is dissolved in pyridine (10 ml). The solution is shaken with 2,3,5-triisopropylbenzenesulfonyl chloride (600 mg) for 10 min, concentrated to a half of the original volume under diminished pressure, and the concentrate kept at 20°C for 20 h. Water is then added (5 ml) and the mixture is extracted with chloroform (30 ml). The extract is washed, dried over magnesium sulfate, concentrated to the volume of 10 ml, and the concentrate added dropwise with stirring into ether (200 ml). The precipitate is collected with suction, washed with ether, and dried under diminished pressure. Yield, 600 mg (78%) of the pyridinium salt of compound *IV*. For $C_{34}H_{36}N_3O_9PS.C_5H_5N$ (772·7) calculated: 7·25% N, 4·03% P, 4·19% S; found: 7·14% N, 3·85% P, 3·99% S.

5′-O-Dimethoxytritylthymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-3′-O-acetylthymidine [P-S-(2-Cyanoethyl)Ester] (*V*)

A mixture of the pyridinium salt of compound *IIb* (137 mg; 0·2 mmol) and 5′-O-dimethoxytritylthymidine (217 mg; 0·4 mmol) is coevaporated with three portions of pyridine and the final residue is shaken with 2,3,5-triisopropylbenzenesulfonyl chloride (180 mg) and pyridine (5 ml) for 10 min. The reaction mixture is concentrated just to crystallisation, kept at room temperature for 20 h, diluted with chloroform (3 ml), and chromatographed on one 20 × 20 × 0·6 cm layer of loose silica gel in the solvent system T_2. The dimethoxytrityl-group-positive band (R_F 0·50) is eluted with the solvent system T_e, the eluate evaporated, and the residue dried under diminished pressure. Yield, 154 mg (80%) of the triester *V*. For $C_{46}H_{50}N_5O_{14}PS$ (959·9) calculated: 7·29% N, 3·23% P, 3·33% S; found: 6·98% N, 2·83% P, 3·17% S.

Thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-3′-O-acetylthymidine [P-S-(2-Cyanoethyl) Ester] (*VI*)

A solution of the triester *V* (130 mg) in 90% acetic acid (5 ml) is kept at 20°C for 2 h and evaporated at 20°C/1 Torr. The acetic acid is removed by repeated coevaporations with 1-butanol. The final residue is dissolved in chloroform and chromatographed on one 20 × 20 × 0·6 cm layer of loose silica gel in the solvent system T_3. The ultraviolet-absorbing band (R_F 0·25) is eluted with the solvent system T_e, the eluate evaporated, and dried under diminished pressure. Yield, 80 mg of compound *VI*. For $C_{25}H_{32}N_5O_{12}PS$ (657·6) calculated: 10·66% N, 4·64% P, 4·87% S; found: 10·45% N, 4·39% P, 4·72% S.

5′-O-Dimethoxytritylthymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-3′-O-acetylthymidine [Bis-P_1-S,P_2-S-(2-cyanoethyl) Ester] (*VIIa*)

A mixture of the triester *VI* (60 mg) and the pyridinium salt of compound *IV* (155 mg) is coevaporated with three portions of pyridine and the residue is shaken with 2,3,5-triisopropylbenzenesulfonyl chloride (120 mg) in pyridine (5 ml) for 5 min. The reaction mixture is evaporated under diminished pressure just to crystallisation, kept at room temperature for 20 h, diluted with chloroform, and chromatographed on one 20 × 20 × 0·6 cm layer of loose silica gel in the solvent system T_4. The dimethoxytrityl-group-positive band (9—15 cm) is eluted with the solvent system T_e, the eluate evaporated under diminished pressure, and the residue coevaporated re-

peatedly with toluene to remove pyridine. The residue is then rechromatographed as above except for the solvent system T_3. The ultraviolet-absorbing band (R_F 0·43) is eluted with the eluant T_e, the eluate evaporated, and the residue dried under diminished pressure. Yield, 44 mg (38%) of compound *VIIa*.

Thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-thymidine (*X*)

A solution of compound *VIIa* (40 mg) in 90% aqueous acetic acid (5 ml) is kept at 20°C for 2 h, evaporated at 20°C/1 Torr, and the residue coevaporated repeatedly with 1-butanol to remove acetic acid. The thus-obtained detritylated derivative *VIIb* is dissolved in a mixture of methanol (1 ml) and conc. aqueous ammonia (1 ml), the solution kept at 50°C for 1 h, cooled down, and chromatographed on one 20 × 20 × 0·6 cm layer of loose silica gel in the solvent system T_1. The ultraviolet-absorbing band (R_F 0·50) is eluted with water, the eluate evaporated to dryness under diminished pressure, the silicic-acid-containing residue taken up into a little water, filtered, and freeze-dried. Yield, 22 mg of the ammonium salt of compound *X*.

Reaction of O,O-Diesters *VIII* and *X* with Ethyl Bromide and *p*-Nitrobenzyl Bromide

Compounds *VIII* and *X* (about 1 mg each) were dissolved in 0·03 ml of methanol (reaction with ethyl bromide) or 0·03 ml of dimethylformamide (reaction with *p*-nitrobenzyl bromide), the solutions treated with the corresponding halide (about 10 mg each), the whole kept at 50°C for 6 h, cooled down, and chromatographed on a thin layer of silica gel (Silufol UV_{254}) in the solvent system T_3. On treatment with ethyl bromide, the O,O-diesters *VIII* and *X* were converted to the corresponding triesters *IXa* and *XIa* in c. 50% yield; the triesters *IXb* and *XIb* were obtained in an almost quantitative yield on treatment with *p*-nitrobenzyl bromide.

Reaction of Thiophosphoric Acid Diesters with Potassium Ferricyanide

A. Compounds *IIb* and *IV* (about 5 mg each) were kept with finely ground potassium ferricyanide (10 mg) in 50% aqueous acetone (0·05 ml) at 20°C for 20 h. As shown by chromatography in T_1 and electrophoresis in E_1, compound *II* did not react at all and compound *IV* split off the dimethoxytrityl group. The reaction mixtures were then treated with 0·05 ml of concd. aqueous ammonia each and subjected to electrophoresis in the buffer solution E_1. As shown by withdrawal of samples in intervals of 12 h, there are gradually formed electrophoretically more mobile compounds ($0{\cdot}84_{Up}$), namely, bis(nucleosidephosphoryl)disulfides. After 48 h, the yield is about 50%.

B. Powdered potassium ferricyanide (5 mg) was added to a solution of compound *VIII* (2 mg) in 50% aqueous acetone (0·05 ml). The samples were withdrawn in intervals of 24 h and analysed by chromatography in T_1 and electrophoresis in E_1. Even after 5 days, the starting compound *VIII* did not show any change.

Elemental analyses were carried out in the Analytical Department (Dr J. Horáček, Head) of this Institute.

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