OLIGONUCLEOTIDIC COMPOUNDS. XLIII.*

SYNTHESIS OF SOME DIRIBONUCLEOSIDE PHOSPHATE ANALOGUES CONTAINING THE PHOSPHOROTHIOIC O,O-DIESTER BOND

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Uridine 5'-phosphorothioic acid (*Ib*) was converted on successive treatment with acrylonitrile and acetic anhydride into 2',3'-di-O-acetyluridine 5'-S-(2-cyanoethyl)phosphorothioate (*Ic*). 2',3'-O-Ethoxymethylene-N^2-acetylguanosine 5'-S-(2-cyanoethyl)phosphorothioate (*IIc*) was prepared from 2',3'-O-ethoxymethyleneguanosine on treatment with a mixture of S-(2-cyanoethyl)phosphorothioate and N,N-dicyclohexylcarbodiimide followed by reaction with acetic anhydride. Successive treatment of 5'-O-acetyl-2'-O-tetrahydropyranyluridine 3'-phosphate (*III*) with alkaline phosphatase, ammonia, and dimethoxytrityl chloride afforded 5'-O-dimethoxytrityl-2'-O-tetrahydropyranyluridine (*IVb*). 5'-O-Acetyl-N^6-acetyl-2'-O-tetrahydropyranyladenosine 5'-phosphate (*V*) was converted on treatment with alkaline phosphatase and then ammonia to 2'-O-tetrahydropyranyladenosine (*VIIIc*). Reaction of the pyridinium salt of compound *Ic* with the uridine derivative *IVb* in the presence of 2,3,5- triisopropylbenzenesulfonyl chloride afforded the O,O,S-triester *IXa* which was converted into uridinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-uridine (*IXd*) on successive removal of protecting groups. Analogously, compounds *VI* and *Ic* afforded adenosinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-uridine; uridinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-guanosine (*XId*) was prepared from compounds *IVb* and *IIc*.

In the preceding paper[1], a novel approach to the O,O-diesters of phosphorothioic acid via the O,O,S-triesters has been exemplified on the synthesis of thymidine-phosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-thymidinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-thymidine. In the present paper, we wish to demonstrate the use of this approach in the ribo series on the synthesis of phosphorothioate O,O-diester analogues of uridylyl-uridine, adenylyl-uridine, and uridylyl-guanosine. Compounds carrying the phosphorothioate group on the $C_{(5')}$-hydroxylic function have been used as the starting material in the synthesis of the thio-internucleotidic bond.

In the synthesis of uridinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-uridine (*IXd*) and adenosinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-uridine (*Xc*), 2',3'-di-O-acetyluridine 5'-S-(2-cy-

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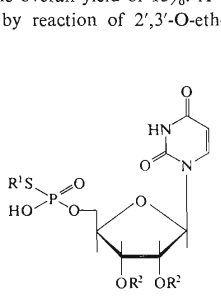
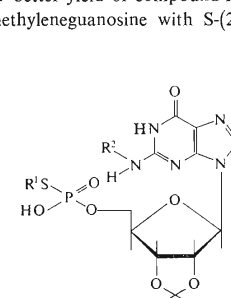
anoethyl)phosphorothioate (*Ic*) has been used as the clue intermediate. Compound *Ic* was prepared from 2′,3′-di-O-benzoyluridine which was converted into 2′,3′-di-O-benzoyluridine 5′-S-(2-carbomylethyl)phosphorothioate (*Ia*) by reaction with S-(2-carbamoylethyl)phosphorothioate pyridinium salt[2] and N,N-dicyclohexylcarbodiimide. The 2-carbamoylethyl group was split off by refluxing in 0·2M-NaOH. The benzoyl groups were quantitatively removed by the action of a mixture of concd. aqueous ammonia and methanol. The thus-obtained uridine 5′-phosphorothioic acid (*Ib*) was isolated in the form of the barium salt. The S-(2-cyanoethyl) ester of compound *Ib* was prepared by the action of acrylonitrile on the triethylammonium salt of compound *Ib* at pH 8–9, analogously to the procedure of the preceding paper[1]. On treatment with acetic anhydride in pyridine, the 2-cyanoethylation product was converted into the thioate *Ic* which was isolated as the pyridinium salt. By the action of a solution of iodine in aqueous acetone, the 2-cyanoethyl group is quantitatively removed under the formation of phosphorothioic O-monoester with the electrophoretical mobility of 0·88 in respect to uridylic acid (at pH 7·5). The thioate *Ic* does not react with potassium ferricyanide; in the presence of concd. aqueous ammonia, the β-elimination of the 2-cyanoethyl group takes place under the formation of a bis(nucleosidephosphoryl)disulfide of the $[\text{uridine-5'-PO(OH)}]_2S_2$ type, electrophoretical mobility $0{\cdot}92_{Up}$ (at pH 7·5).

2′,3′-O-Ethoxymethylene-N^2-acetylguanosine 5′-S-(2-cyanoethyl)phosphorothioate (*IIc*), the nucleotidic component in the synthesis of uridinephosphorothioyl-$(O^{3'} \rightarrow O^{5'})$-guanosine (*XId*), was prepared analogously from 2′,3′-O-ethoxymethyleneguanosine *via* 2′,3′-O-ethoxymethyleneguanosine 5′-S-(2-carbamoylethyl)-phosphorothioate (*IIa*) and 2′,3′-O-ethoxymethyleneguanosine 5′-phosphorothioate (*IIb*) in the overall yield of 15%. A much better yield of compound *IIc* (55%) was obtained by reaction of 2′,3′-O-ethoxymethyleneguanosine with S-(2-cyanoethyl)-





Ia, $R^1 = CH_2CH_2CONH_2$, $R^2 = COC_6H_5$
Ib, $R^1 = R^2 = H$
Ic, $R^1 = CH_2CH_2CN$, $R^2 = COCH_3$

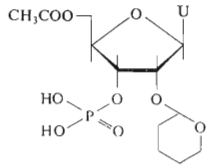
IIa, $R^1 = CH_2CH_2CONH_2$, $R^2 = H$
IIb, $R^1 = R^2 = H$
IIc, $R^1 = CH_2CH_2CN$, $R^2 = COCH_3$

-phosphorothioate pyridinium salt[3] and N,N'-dicyclohexylcarbodiimide followed by acetylation. The specimens of compound *IIc* obtained by both routes were identical on chromatography and electrophoresis and were quantitatively converted into the corresponding phosphorothioic O-monoester ($E_{7.5}$, $0{\cdot}87_{Up}$) by the action of a solution of iodine in aqueous acetone. On treatment with a mixture of concd. aqueous ammonia and potassium ferricyanide, the bis(nucleosidephosphoryl)disulfide ($E_{7.5}$, $0{\cdot}74_{Up}$) was obtained.

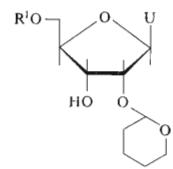
The nucleosidic components for the $C_{(5')}$-phosphorothioyl derivatives *Ic* and *IIc* must carry a free hydroxylic function at position $C_{(3')}$ while the remaining hydroxyls are protected. An uridine derivative of this type, namely, 5'-O-dimethoxytrityl-2'-O-tetrahydropyranyluridine (*IVb*) was prepared from 2'-O-tetrahydropyranyluridine (*IVa*) by the action of dimethoxytrityl chloride. Compound *IVa* was in turn obtained by deacetylation of the enzymatically prepared 5'-O-acetyl-2'-O-tetrahydropyranyluridine. The enzymatic method[4] consists in the action of alkaline phosphatase on a salt of 5'-O-acetyl-2'-O-tetrahydropyranyluridine 3'-phosphate at pH 9·5. It has been now found that the presence of excess calcium or barium ions is required for the quantitative course of the enzymatic dephosphorylation in the relatively concentrated solution. The reaction (accompanied by precipitation of barium phosphate) is accomplished within a few hours when barium chloride is added to the incubation mixture. After work-up with ammonia, compound *IVa* is extracted from the residue with an organic solvent in a practically quantitative yield. When chromatographed on a thin layer of silica gel in the solvent system 9 : 1 chloroform-methanol, compound *IVa* exhibits two spots of diastereoisomers in accordance with the reported properties of 2'-O-tetrahydropyranyluridine prepared by another route[5]. The predominating isomer of a lower R_F value and of a higher m.p. value was isolated by crystallisation.

The adenosine derivative with a free $C_{(3')}$-hydroxylic function, namely, 5'-O--acetyl-N^6-acetyl-2'-O-tetrahydropyranyladenosine (*VI*) was prepared analogously by enzymatical dephosphorylation of the corresponding 3'-phosphate *V*. In this case, the incubation was performed at pH 7·5 to prevent the cleavage of the N^6-acetyl group. In the presence of excess barium ions, the reaction was not slower in spite of a lower pH value. The thus-obtained mixture of diastereoisomers can be separated by thin-layer chromatography. As shown by the ultraviolet spectrum of the product, the N^6-acetyl group prevents the deamination of adenosine with adenylate deaminase which is present in the crude alkaline phosphatase.

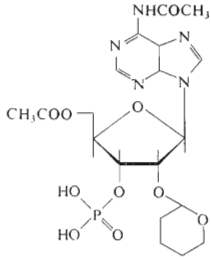
Another adenosine derivative with a free $C_{(3')}$-hydroxylic function, namely, 5'-O-benzoyl-N^6-benzoyl-2'-O-tetrahydropyranyladenosine (*VIIIb*), was prepared by a reported[6] chemical procedure. On treatment with 98% formic acid, 5'-O-benzoyl--N^6-benzoyl-2',3'-O-ethoxymethyleneadenosine (*VII*) afforded the crystalline 3'-O-formyl derivative *VIIIa*, the NMR spectrum of which was in good agreement with the reported[5] data of an adenosine derivative carrying a free $C_{(2')}$-hydroxylic function.



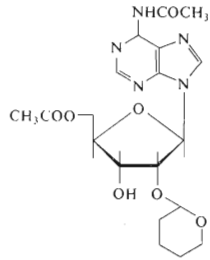
III



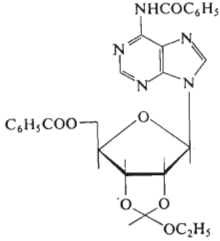
IVa, $R^1 = H$
IVb, R^1 = dimethoxytrityl



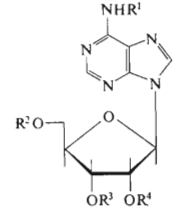
V

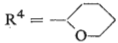


VI

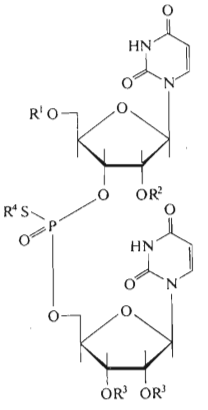


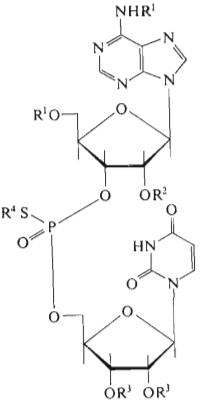
VII



VIIIa, $R^1 = R^2 = COC_6H_5$, $R^3 = CHO$, $R^4 = H$
VIIIb, $R^1 = R^2 = COC_6H_5$, $R^3 = H$, $R^4 =$ 
VIIIc, $R^1 = R^2 = R^3 = H$, $R^4 =$ 

To allow comparison of NMR spectra, compound *VIIIa* was converted on refluxing in methanol into a derivative with free hydroxylic functions at positions $C_{(2')}$ and $C_{(3')}$. Under catalysis of trifluoroacetic acid, the tetrahydropyranyl group was introduced into the position $C_{(2')}$ and the 3'-O-formyl group was selectively removed by the action of 95 : 5 ethanol–concd. aqueous ammonia mixture. The product *VIIIb* was characterised by conversion into the known 2'-O-tetrahydropyranyladenosine[5] (*VIIIc*) which was isolated in the form of two crystalline diastereoisomers. An iden-





IXa, R^1 = dimethoxytrityl, R^2 =  , $R^3 = COCH_3$, $R^4 = CH_2CH_2CN$

IXb, R^1 = H, R =  , $R^3 = COCH_3$, $R^4 = CH_2CH_2CN$

IXc, $R^1 = R^3 = R^4$ = H, R^2 = 

IXd, $R^1 = R^2 = R^3 = R^4$ = H

IXe, $R^1 = R^3$ = H, R^2 =  , $R^4 = CH_2CH_3$

IXf, $R^1 = R^2 = R^3$ = H, $R^4 = CH_2CH_3$

Xa, $R^1 = R^3 = COCH_3$, R^2 =  , $R^4 = CH_2CH_2CN$

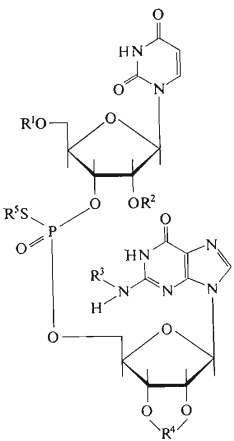
Xb, $R^1 = R^3 = R^4$ = H, R^2 = 

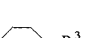
Xc, $R^1 = R^2 = R^3 = R^4$ = H

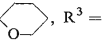
Xd, $R^1 = R^3$ = H, R^2 =  , $R^4 = CH_2CH_3$

tical (on chromatography) mixture of diastereoisomers *VIIIc* was obtained from the enzymatically prepared compound *VI* by the action of methanolic ammonia.

Condensations of the pairs *IVb*–*Ic*, *VI*–*Ic*, and *IVb*–*IIc* were accomplished by the action of 2,3,5-triisopropylbenzenesulfonyl chloride[1]. The completely blocked O,O,S-triester was isolated in the first case only (*IXa*, 52%). With compound *X*, it was possible to isolate the product resulting after cleavage of the alkalilabile protecting groups (*Xb*, 62%); with compound *XI*, the product resulting after removal of the dimethoxytrityl group was isolated (*XIb*, 6%). The low yield in the condensation of the guanosine derivative *IIc* might be due to the decreased reactivity of tetra-

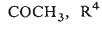


XIa, R^1 = dimethoxytrityl, R^2 =  , $R^3 = COCH_3$, R^4 =  , $R^5 = CH_2CH_2CN$

XIb, R^1 = H, R^2 =  , $R^3 = COCH_3$, R^4 =  , $R^5 = CH_2CH_2CN$

XIc, $R^1 = R^3 = R^5 = H$, R^2 = , R^4 =

XId, $R^1 = R^2 = R^3 = R^4 = R^5 = H$

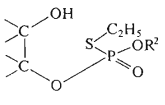
XIe, $R^1 = R^3 = H$, R^2 =  , R^4 =  , $R^5 = CH_2CH_3$

alkyl thiopyrophosphate which is formed in the first step. The partially protected intermediates *IXc*, *Xb*, and *XIc* containing the 2′-O-tetrahydropyranyl group, were examined as to their reaction with ethyl bromide. Analogously to the deoxyribo series[1], the corresponding O,O,S-triesters *IXe*, *Xd*, and *XIc* were obtained, though at a lower rate. The resulting O,O,S-triesters may be readily distinguished from the starting O,O-diesters by a different mobility on thin-layer chromatography and the loss of mobility on electrophoresis.

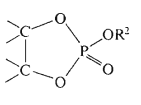
As shown by preliminary attempts to remove the 2′-O-tetrahydropyranyl group of compound *IXc* by the action of aqueous acetic acid, a simultaneous desulfurisation occurred to a considerable extent under the formation of uridylyl-uridine. Thus, when compound *IXc* was kept in 50% aqueous acetic acid at 50°C for 20 min, the extent of desulfurisation was 32%. On the other hand, a similar treatment with 0·5M-HCl was not accompanied by any desulfurisation.

On the basis of this observation, the final deblocking was performed on treatment with 0·5M-HCl (compound *IXc*) and 0·2M-HCl (compounds *Xb* and *XIc*). The electrophoretical mobility of the final products is the same as that of the oxygen counter parts UpU, ApU, and UpG. When chromatographed in the solvent system 2-propanol–conc. aqueous ammonia–water, compound *Xc* migrates similarly to ApU but compounds *IXd* and *XId* are markedly more mobile than UpU and UpG. Furthermore, the stability of the *ribo*-internucleotidic bond in uridinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-uridine (*IXd*) in acidic and alkaline media was compared with that of the parent UpU. Thus, by the action of 0·5M-HCl at 50°C for 1 h, the phosphoric acid diester is cleaved by 85% and the phosphorothioic acid diester by 32%. On the other hand in alkaline medium (0·5M-NaOH, 20°C, 30 min), the phosphorothioyl analogue is cleaved somewhat faster (35%) than the parent phosphate (23%).

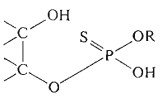
In connection with investigations on the stability of the *ribo*-thiointernucleotidic bond in alkaline and acidic media, it was of interest to examine the behaviour of the O,O,S-triester system in the neighbourhood of a free *cis*-hydroxylic function. It has



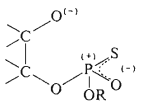
XII



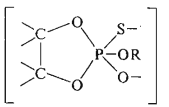
XIII



XIV



XV



XVI

been known from the field of derivatives of phosphoric acid that the diesters of uridine 3'-phosphate are unstable over a wide pH range and decompose into uridine 2'--phosphate and uridine 3'-phosphate monoesters and uridine 2',3'-cyclic phosphate[7]. When heated with ethyl bromide, compound *IXd* afforded a product, the chromatographical and electrophoretical behaviour of which points to an O,O,S-triester. The product is stable in water as well as in the ammonia-containing chromatographic system. The conversion of the structure *XII* into the cyclic triester *XIII* is obviously highly improbable.

The above observations permit to draw some conclusions on the mechanism. Thus, it may be assumed in accordance with the generally accepted explanation of the cleavage of the *ribo*-internucleotidic bond[8,9] that a pentacovalent transition complex *XVI* is formed by the cleavage of phosphorothioyl analogues. In 0·5M-HCl, a completely protonated structure *XIV* may be assumed in which the positive charge on phosphorus (in contrast to the parent phosphate) is lowered to such an extent that the formation of a pentacovalent structure is highly improbable. On the other hand in alkaline medium, the nucleophilic attack of the hydroxylic function in the completely ionised structure *XV* is easier than with the parent phosphate.

In the desulfurisation of the O,O-diester with aqueous acetic acid, the participation of the *cis*-hydroxylic function can be hardly assumed in view of the lack of any perceivable cleavage of the internucleotidic bond. The reaction might proceed through a S-acetyl phosphate intermediate.

EXPERIMENTAL

Thin-layer chromatography was performed on ready-for-use Silufol UV_{254} plates (Kavalier Glassworks, Votice, Czechoslovakia) in the solvent systems T_1, 2-propanol-concd. aqueous ammonia–water (7 : 1 : 2), and T_2, chloroform-methanol (9 : 1). Preparative runs were carried out on loose layers of silica gel (particle size, 10—60 micron) containing a fluorescent indicator (produced in Service Laboratories of this Institute in Prague - Suchdol) in the solvent systems T_1; T_2; T_3, chloroform–methanol (98 : 2); T_4, chloroform–methanol (95 : 5); T_5, chloroform–pyridine–methanol (90 : 5 : 5); T_6, chloroform–pyridine–methanol (70 : 15 : 15); and T_7, chloroform–methanol (7 : 3). The solvent system T_6, chloroform–methanol (1 : 1) was used as eluant. Spots were detected by viewing in UV light. Esters of phosphorothioic acid were specifically detected by iodine vapours (white spots on a dark background). The NMR data were measured in a mixture of hexadeuteriodimethyl sulfoxide and deuteriochloroform in the δ scale. 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_2, and the electrophoretical mobility in the buffer solution E_1: uridine 2'(3')-phosphate (0·1, 0, 1·0); *Ia* (0·85, —, —); *Ib* (0·30, —, —); *Ic* (0·43, —, 0·45); *IIa* (0·64, —, —); *IIb* (0·29, —, —); *IIc* (0·37, —, 0·45); *IVa* (—, 0·21 + 0·28, —); *IVb* (—, 0·73, —); *VI* (—, 0·46 + 0·57, —); *VIIIa* (—, 0·71, —); *VIIIb* (—, 0·92, —); *VIIIc* (—, 0·23 + 0·34, —); *IXa* (—, 0·55, —); *IXb* (—, 0·30, —); *IXc* (0·41, —, 0·46); *IXd* (0·35, —, 0·41); UpU (0·26, —, 0·41); *Xb* (0·33, —, 0·35); *Xc* (0·45, —, 0·34); ApU (0·43, —, 0·34); *XIa* (—, 0·60, —); *XIb* (—, 0·43, —); *XIc* (0·65, —, —); *XId* (0·36, —, 0·37); UpG (0·23, —, 0·37)

Uridine 5'-Phosphorothioate (*Ib*)

A mixture of S-(2-carbamoyl)phosphorothioate pyridinium salt (5 mmol) and 2',3'-di-O-benzoyluridine (2 mmol) was repeatedly coevaporated with pyridine, the final residue dissolved in pyridine (5 ml), and the solution kept in a closed vessel with hexamethylphosphoric triamide (5 ml) and N,N-dicyclohexylcarbodiimide (2 g) for 4 days at room temperature. Water is then added (5 ml), the mixture allowed to stand for 3 h, filtered, and the material on the filter washed with 50%

aqueous pyridine. The filtrate and washing are combined, concentrated to a small volume under diminished pressure, and the hexamethylphosphoric-triamide-containing concentrate is diluted with 0·2M-NaOH (100 ml). The reaction mixture is refluxed for 20 min, allowed to cool, adjusted to pH 7 with pyridinium Dowex 50 ion exchange resin, filtered, and the filtrate evaporated under diminished pressure. The residue is dissolved in a mixture of methanol (20 ml) and concd. aqueous ammonia (20 ml), the solution kept at room temperature for 20 h, evaporated, and the residue chromatographed on two 16 × 40 × 0·6 cm layers of silica gel in the solvent system T_1. The ultraviolet-absorbing band (R_F, about 0·23) is eluted with water, the eluate passed through a small column of Dowex 50 (H^+) ion exchange resin, the effluent neutralised with aqueous barium hydroxide (pH 7·5), and concentrated under diminished pressure to the volume of 50 ml. The precipitate is removed by centrifugation and the supernatant is diluted with ethanol (150 ml) to deposit the barium salt of compound *Ib*. The salt is collected by centrifugation, washed twice with 66% aqueous ethanol, once with 99% ethanol, and once with ether, and air-dried. Yield, 530 mg of the barium salt of the phosphorothioate *Ib*, the chromatographical and electrophoretical properties of which are almost identical with those of uridine 5′-phosphate.

2′,3′-Di-O-acetyluridine 5′-S-(2-Cyanoethyl)phosphorothioate (*Ic*)

The barium salt of compound *Ib* (500 mg) is converted to the triethylammonium salt with the use of triethylammonium Dowex 50 ion exchange resin. The effluent is evaporated to dryness under diminished pressure, the residue dissolved in 80% aqueous dimethylformamide (10 ml), and the solution is kept with acrylonitrile (1 ml) at room temperature overnight. The reaction mixture is evaporated to dryness under diminished pressure, the residue is repeatedly coevaporated with pyridine, and the final residue is dissolved in a mixture of acetic anhydride (5 ml) and pyridine (15 ml). The solution is kept at room temperature for 20 h, evaporated under diminished pressure, the residue coevaporated with two portions of pyridine and the final residue dissolved in 50% aqueous pyridine (10 ml). After 3 h at room temperature, the solution is passed through a column of pyridinium Dowex 50 ion exchange resin, the effluent evaporated to dryness under diminished pressure, and the residue coevaporated with five portions of pyridine. The final residue is dissolved in pyridine (10 ml) and the solution is added dropwise with stirring into ether (250 ml). The precipitate is collected with suction, washed with ether, and dried under diminished pressure. Yield, 378 mg of the pyridinium salt of the thioate *Ic*. For $C_{16}H_{20}N_3O_{10}PS.C_5H_5N$ (556.4) calculated: 10.07% N, 5.49% P, 5.58% S; found: 9.96% N, 6.04% P, 5.36% S.

N^2-Acetyl-2′,3′-O-ethoxymethyleneguanosine 5′-S-(2-Cyanoethyl)phosphorothioate (*IIc*)

A suspension of guanosine (previously dried for several hours at 50°C/0.1 Torr; 2·54 g; 9 mmol) in a mixture of dimethylformamide (20 ml), triethyl orthoformate (8 ml), and 6M-HCl in dimethylformamide (2 ml) is stirred at room temperature overnight[10]. The resulting solution is adjusted to pH 8 with triethylamine and kept at 0°C for several hours. The precipitate of triethylammonium chloride is filtered off and washed with dimethylformamide. The combined filtrates are evaporated under diminished pressure and the residue is repeatedly coevaporated with pyridine. To the final residue, there is added a solution of S-(2-cyanoethyl)phosphorothioate pyridinium salt[3] (20 mmol) in pyridine (25 ml), hexamethylphosphoric triamide (25 ml), and N,N-dicyclohexylcarbodiimide (15.5 g). The reaction mixture is kept at room temperature for 4 days and treated with water (20 ml) and cyclohexane (40 ml). After 3 h at room temperature, the mixture is filtered and the precipitate washed with 30% aqueous pyridine. The lower layer of the filtrate is concentrated under diminished pressure to the volume of 30 ml and the concentrate is chromatographed on five 16 × 40 × 0·6 cm layers of loose silica gel in the solvent system

T_1. The ultraviolet-absorbing bands (R_F value of about 0.65) are eluted with 60% aqueous methanol. Triethylamine (2 ml) is added to the eluates, the whole is evaporated under diminished pressure, and the residue is coevaporated with three 11 ml portions of 10 : 1 ethanol–triethylamine mixture and then with several portions of pyridine. The final residue is dissolved in a mixture of acetic anhydride (10 ml) and pyridine (25 ml), the whole kept at room temperature for 3 days, and evaporated to dryness under diminished pressure. The residue is coevaporated with three portions of pyridine and the final residue is dissolved in 50% aqueous pyridine (25 ml). After 3 h at room temperature, the solution is passed through a small column of pyridinium Dowex 50 ion exchange resin, the effluent evaporated to dryness under diminished pressure, and the residue repeatedly coevaporated with pyridine. The final residue is dissolved in pyridine (20 ml) and the solution is added dropwise with stirring into ether (540 ml). The precipitate is collected with suction, washed with ether, and dried under diminished pressure. Yield, 3·15 g (53%) of the pyridinium salt of the thioate *IIc*. For $C_{18}H_{23}N_6O_9PS.C_5H_5N$ (609·5) calculated: 16.09% N, 5·09% P, 5·26% S; found: 16·54% N, 4·97% P, 5·95% S. When the preparation is performed analogously to that of the compound *Ic*, there is obtained only 15% of the thioate *IIc*.

2′-O-Tetrahydropyranyluridine (*IVa*)

A solution of the calcium salt of 2′-O-tetrahydropyranyl-5′-O-acetyluridine 3′-phosphate[11] (5 g) and barium chloride (2 g) in water (200 ml) is adjusted to pH 9·5 with aqueous ammonia. A solution of alkaline calf intestine phosphatase (Calbiochem) (100 mg) in water (10 ml) is then added and the whole is incubated at 37°C under occasional additions of aqueous ammonia to keep the pH value at 9—9.5. When the precipitation of the inorganic phosphate is finished (after about 4 h), concd. aqueous ammonia is added (100 ml), and the whole is kept at room temperature for 20 h. The mixture is then evaporated in the presence of lauryl alcohol (0.1 ml) and the residue is extracted with two 100 ml portions of boiling ethanol. The extract is evaporated, the residue dissolved in methanol (20 ml), and the solution diluted with ether (100 ml) to deposit on standing crystals which are collected with suction, washed with ether, and dried. Yield, 1.8 g of compound *IVa*, m.p. 162—163°C, R_F value in the solvent system T_1, 0.21. In addition to this substance, the mother liquor contains the other diastereoisomer of the R_F value 0.28. The analytical sample is obtained on recrystallisation from methanol–ether; m.p. of *IVa*, 198 to 199°C. For $C_{14}H_{20}N_2O_7$ (328·3) calculated: 51.21% C, 6.10% H, 8.52% N; found: 50·93% C, 6.16% H, 8·54% N.

5′-O-Dimethoxytrityl-2′-O-tetrahydropyranyluridine (*IVb*)

A solution of compound *IVa* (740 mg) and dimethoxytrityl chloride (800 mg) in pyridine (5 ml) is kept at room temperature overnight and poured with stirring into a mixture (100 g) of ice and water. The precipitate is collected with suction, washed with water, and dried under diminished pressure over phosphorus pentoxide. The crude product is dissolved in chloroform (5 ml) and then cyclohexane (50 ml) is added. The solid is collected with suction, washed with cyclohexane, and dried under diminished pressure. Yield, 1.3 g of compound *IVb*, m.p. 104—106°C. The analytical sample (m.p. 108°C) is obtained on recrystallisation from the same solvent mixture. For $C_{35}H_{38}N_2O_9$ (630.7) calculated: 66·60% C, 6·02% H, 4·44% N; found: 66·53% C, 6·27% H, 4·47% N.

N^6-Acetyl-5′-O-acetyl-2′-O-tetrahydropyranyladenosine (*VI*)

A solution of the calcium salt[12] of compound *V* (5 g), barium chloride (5 g), and alkaline phosphatase (Calbiochem) (100 mg) in water (150 ml) was incubated at 37°C and the course was

checked by thin-layer chromatography in the solvent system T_1. When the spot of the phosphate *V* disappeared (after about 4 h), the mixture was evaporated under diminished pressure in the presence of lauryl alcohol (0·05 ml). The residue was extracted with a boiling mixture (100 ml) of chloroform–acetone (1 : 1). The extract was filtered and the filtrate evaporated to afford 2·786 g of compound *VI* in the form of a foam consisting of two diastereoisomers (R_F 0·46 and 0·53), as shown by thin-layer chromatography in the solvent system T_2. For $C_{19}H_{25}N_5O_7$ (435·4) calculated: 52·50% C, 5·75% H, 16·11% N; found: 52·15% C, 5·83% H, 15·92% N. UV spectrum (methanol): λ_{max} 271—271 nm, λ_{min} 236 nm; $A_{250/260}$ 0·80, $A_{280/260}$ 0·45.

5′-O-Benzoyl-N^6-benzoyl-3′-O-formyladenosine (*VIIIa*)

2′,3′-O-Ethoxymethyleneadenosine (8·3 g) was converted into the dibenzoate *VII* as reported[13]. The chloroform extract after the benzoylation was dried, evaporated, the residue coevaporated with toluene, and the final residue dissolved in 98% formic acid (100 ml). After 20 min at room temperature, the solution was evaporated at 20°C/1 Torr and the residue chromatographed on six 16 × 40 × 0·6 cm layers of loose silica gel in the solvent system T_3. The ultraviolet-absorbing bands (R_F value of about 0·31) were eluted with the solvent system T_e and the eluates were evaporated under diminished pressure. The residual foam was dissolved at 40°C in chloroform (5 ml) and the solution was diluted with ethanol (60 ml) containing 0·1% of formic acid. The mixture was kept at room temperature for 20 h to deposit crystals which were collected and washed with ethanol. Yield, 4·8 g of compound *VIIIa*, m.p. 104—7°C. For $C_{25}H_{21}N_5O_7$ (503·4) calculated: 59·65% C, 4·17% H, 13·94% N; found: 59·83% C, 4·34% H, 13·28% N. NMR spectrum: H(1′) doublet, 6·12 p.p.m. (J = 5·7 Hz). The recrystallised 2′-O-formyl isomer (mother liquor): H(′) doublet, 6·34 p.p.m. (J = 3·8 Hz).

Methanolysis. Compound *VIIIa* (300 mg) was refluxed in methanol (8 ml) for 3 h, evaporated, and the residue chromatographed or one 16 × 40 × 0·6 cm layer of loose silica gel in the solvent system T_4. The ultraviolet-absorbing band (R_F value 0·41) was eluted with the solvent system T_e and the eluate evaporated to afford 220 mg of N^6-benzoyl-5′-O-benzoyladenosine. NMR spectrum: H(1′) doublet, 6·05 p.p.m. J= 4·9 Hz.

5′-O-Benzoyl-2′-O-tetrahydropyranyl-N^6-benzoyladenosine (*VIIIb*)

Trifluoroacetic acid (0·8 ml) is added to a solution of compound *VIIIa* (1·2 g), dioxane (10 ml), and dihydropyran (2·5 ml), and the mixture is kept at 20°C for 4 h. Triethylamine is then added, the solution (pH 7·5) is evaporated under diminished pressure, and the residue is dissolved in a mixture (10 ml) of ethanol–concd. aqueous ammonia (95 : 5). After 1 h at room temperature, the solution is evaporated, the residue dissolved in chloroform, and chromatographed on two 16 × 40 × 0·6 cm layers of loose silica gel in the solvent system T_4. The ultraviolet-absorbing bands (R_F value 0·46) are eluted and the eluates evaporated to afford 950 mg of compound *VIIIb* in the form of a foam. The characterisation was accomplished by conversion into the known[5] adenosine derivative *VIIIc*.

2′-O-Tetrahydropyranyladenosine (*VIIIc*)

A solution of compound *VIIIb* (900 mg) in 15% methanolic ammonia (20 ml) was kept at room temperature for 16 h, evaporated, and the residue chromatographed on a 16 × 40 × 0·6 cm layer of loose silica gel in the solvent system T_2 to afford two ultraviolet-absorbing bands (R_F values 0·37 and 0·52) of the diastereoisomers (327 mg and 480 mg, resp.), melting after recrystallisation (R_F 0·37) at 194—196°C (ethanol), reported[5] m.p. 198—201°C, and (R_F 0·52) at 181—182°C

(ethyl acetate), reported[5], m.p. 171–172°C. An identical diastereoisomeric mixture was obtained on heating the enzymatically prepared compound *VI* with methanolic ammonia at 50°C for 6 h.

5′-O-Dimethoxytrityl-2′-O-tetrahydropyranyluridinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-2′,3′-di-O-acetyluridine[P-S-(2-Cyanoethyl) Ester] (*IXa*)

A mixture of the pyridinium salt of compound *Ic* (160 mg; 0·2 mmol) and compound *IVb* (156 mg; 0·25 mmol) is coevaporated with three portions of pyridine and the residue is then shaken with with 2,3,5-triisopropylbenzenesulfonyl chloride (120 mg) and pyridine (5 ml) for 5 min. The reaction mixture is concentrated under diminished pressure just to the beginning of crystallisation, kept at room temperature for 40 h, diluted with chloroform (5 ml), and chromatographed on one 20 × 20 × 0·6 cm layer of loose silica gel in the solvent system T_5. The dimethoxytrityl-group-positive band (R_F 0·52) is eluted with the eluant T_e, the eluate evaporated, the residue coevaporated with toluene, and dried under diminished pressure to afford 113 mg of compound *IXa*. The analytical sample is obtained by rechromatography on a preparative layer of loose silica gel in the solvent system T_2. For $C_{51}H_{58}N_5O_{19}PS$ (1090) calculated: 6·42% N, 2·85% P, 2·93% S; found: 6·11% N, 2·92% P, 2·86% S.

2′-O-Tetrahydropyranyluridinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-uridine (*IXc*)

The triester *IXa* (89 mg) is dissolved in 90% aqueous acetic acid (5 ml), the solution kept at 0°C for 15 h, evaporated at 20°C/1 Torr, and the residue repeatedly coevaporated with 1-butanol. The residual crude compound *IXb* (R_F 0·30 in T_2) is dissolved in methanol (1 ml), the solution is treated with conc. aqueous ammonia (1 ml), heated at 50°C for 1 h, allowed to cool, and chromatographed on a 20 × 20 × 0·6 cm layer of loose silica gel in the solvent system T_1. The ultraviolet-absorbing band (R_F 0·40) is eluted with water, the eluate evaporated under diminished pressure, the silicic-acid-containing residue extracted with water, and the extract freeze-dried to afford 42 mg of the ammonium salt of compound *IXc*. For $C_{23}H_{31}N_4O_{14}PS.NH_3$ (667·6) calculated: 4·79% S; found: 4·53% S.

Reaction with dilute acids. Solutions of compound *IXc* (about 1 mg) in 0·5M-HCl or 50% aqueous acetic acid (0·05 ml each) were heated at 50°C for 20 min, chromatographed on paper Whatman No 1 in the solvent system T_1, and the spots of compound *IXd* and uridylyl-uridine eluted with 0·01M-HCl. The content of eluates was determined spectrophotometrically. The 50% aqueous acetic acid was found to cause desulfurisation by 32% while the heating with 0·5M-HCl was not accompanied by any desulfurisation.

Uridinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-uridine (*IXd*)

Compound *IXc* (10 mg) is dissolved in 0·5M-HCl (0·1 ml) and the solution is heated at 50°C for 20 min. Chromatography on a 20 cm wide strip of paper Whatman 3 MM paper in the solvent system T_1 affords an ultraviolet-absorbing band (R_F 0·23) which is eluted with water and the eluate freeze-dried to afford 8 mg of the ammonium salt of compound *IXd*.

Stability of compound IXd *in comparison with that of uridylyl-(3′→5′)-uridine.* Samples of the title compounds (about 1 mg each) were heated in 0·5M-HCl (0·05 ml) at 50°C for 1 h, the reaction mixtures chromatographed on paper Whatman No 1 in the solvent system T_1, the spots eluted, and the eluates determined spectrophotometrically to indicate a 85% cleavage in the case of uridylyl-(3′→5′)-uridine and a 32% cleavage with the phosphorothioyl analogue *IXd*. When kept at room temperature in 0·5M-NaOH for 30 min, uridylyl-(3′→5′)-uridine was split by 23% and compound *IXd* by 35%.

2′-O-Tetrahydropyranyladenosinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-uridine (*Xb*)

A mixture of the pyridinium salt of compound *Ic* (160 mg; 0·2 mmol) and the adenosine derivative *VI* (110 mg; 0·25 mmol) is coevaporated with three portions of pyridine, the residue dissolved in pyridine (5 ml), and the solution shaken with 2,3,5-triisopropylbenzenesulfonyl chloride (120 mg) for 5 min. The reaction mixture is concentrated to $^1/_3$ of the original volume, kept at room temperature for 3 days, diluted with chloroform (5 ml), and chromatographed on one 20 × 20 × 0·6 cm layer of loose silica gel in the solvent system T_5. All the material (4—17 cm) faster than the dark bands is eluted with T_e, the eluate evaporated, the residue coevaporated with toluene, and the final residue chromatographed on two 16 × 40 × 0·6 cm layers of loose silica gel in the solvent system T_2. The ultraviolet-absorbing band (R_F, about 0·40) is eluted with T_e, the eluated evaporated, and the residue (compound *Xa*) dissolved in a mixture of methanol (2 ml) and concentrated aqueous ammonia (2 ml). The reaction mixture is heated at 50°C for 2 h, allowed to cool, 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, about 0·45) is eluted with water, the eluate evaporated under diminished pressure, the residue extracted with a little water, and the extract freeze-dried to afford 86 mg of the ammonium salt of compound *Xb*. The analytical sample was obtained by rechromatography on paper Whatman No 3 MM in the solvent system T_1. For $C_{24}H_{32}N_7O_{13}PS.NH_3$ (690·6) calculated: 5·17% S; found: 4·91% S.

Adenosinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-uridine (*Xc*)

A solution of compound *Xb* (12 mg) in 0·2M-HCl (0·1 ml) is heated at 50°C for 20 min, allowed to cool, and chromatographed on a half-sheet of paper Whatman No 3 MM in the solvent system T_1. The ultraviolet-absorbing band (R_F 0·19) is eluted with water and the eluate freeze-dried to afford 6 mg of the ammonium salt of compound *Xc*.

2′-O-Tetrahydropyranyluridinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-$N^2$-acetyl-2′,3′-O-ethoxymethyleneguanosine[P-S-(2-Cyanoethyl) Ester] (*XIb*)

A mixture of the pyridinium salt of compound *IIc* (810 mg; 1 mmol) and compound *IVb* (950 mg) is coevaporated with three portions of pyridine, the residue shaken with 2,3,5-triisopropylbenzenesulfonyl chloride (900 mg) and pyridine (5 ml) for 5 min, the mixture evaporated to the beginning of crystallisation, kept at room temperature for 4 days, diluted with chloroform (5 ml) and chromatographed on two 20 × 20 × 0·6 cm layers of loose silica gel in the solvent system T_6. The dimethoxytrityl-group-positive bands (12·5—17·0 cm) are eluted with T_e, the eluates evaporated, and the residue coevaporated with toluene. The final residue is dissolved in 90% aqueous acetic acid (20 ml), the solution kept at 0°C for 20 h, evaporated under diminished pressure, the residue coevaporated with two portions of 1-butanol, and chromatographed on two 16 × 40 × × 0·6 cm layers of loose silica gel in the solvent system T_2. The ultraviolet-absorbing band (R_F 0·50) is eluted with T_e, the eluate evaporated, and the residue dried under diminished pressure to afford 72 mg of compound *XIb*.

2′-O-Tetrahydropyranyluridinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-2′,3′-O-ethoxymethyleneguanosine (*XIc*)

The triester *XIb* (55 mg) is heated at 50°C in a mixture of methanol (2 ml) and concd. aqueous ammonia (2 ml) for 5 h, allowed to cool, 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, about 0·5) is

eluted with water, the eluate evaporated, and the residue freeze-dried to afford 40 mg of the ammonium salt of compound *XIc*. The analytical sample was obtained by rechromatography on paper Whatman No 3 MM in the solvent system T_1. For $C_{27}H_{38}N_7O_{14}PS.NH_3$ (782·7) calculated: 4·19% S; found: 4·01% S.

Uridinephosphorothioyl-($O^{3'} \rightarrow O^{5'}$)-guanosine (*XId*)

A solution of compound *XIc* (13 mg) is heated at 50°C in 0·2M-HCl (0·1 ml) for 20 min, allowed to cool, and chromatographed on a half-sheet of paper Whatman No 3 MM in the solvent system T_1. The ultraviolet-absorbing band (R_F 0·11) is eluted with water and the eluate freeze-dried to afford 8 mg of the ammonium salt of compound *XId*.

Reactions with Ethyl Bromide

A. Samples of compounds *IXc*, *Xb*, and *XIc* (about 1 mg each) are heated at 50°C in a mixture of methanol (0·03 ml), dimethylformamide (0·3 ml), and ethyl bromide (0·05 ml) for 6 h to afford a 50—70% conversion (as shown by chromatography on a thin layer of silica gel in T_2) to the corresponding P-S-ethylesters *IXa*, *Xd*, and *XIe* (R_F values: 0·17, 0·12, and 0·12, resp.).

B. Compound *IXd* (about 1 mg) is heated at 50°C in a mixture of methanol (0·03 ml), dimethylformamide (0·03 ml), and ethyl bromide (0·03 ml) for 1 h. As shown by thin-layer chromatography in T_1, the starting compound disappeared and was converted to a mixture of a substance possessing the R_F value of 0·05 and traces of uridine. The mobility (R_F 0·35) in 7 : 3 chloroform–methanol indicates the structure of the O,O,S-triester *IXf*. The product is electrophoretically immobile.

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

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