NUCLEIC ACID COMPONENTS AND THEIR ANALOGUES. CXXXVIII.*

SYNTHESIS OF 2',3'-CYCLIC PHOSPHATES DERIVED FROM SOME PYRIMIDINE RIBONUCLEOSIDES AND THEIR BEHAVIOUR TOWARDS PANCREATIC RIBONUCLEASE AND RIBONUCLEASE T2

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Starting from unprotected ribonucleosides *I*, the 2',3'-cyclic phosphates *III* of 5-chloro- (*IIIa*), 5-fluoro- (*IIIb*), 5-amino- (*IIIc*), 5-dimethylamino- (*IIId*), 5-hydroxy- (*IIIe*), 5-ethyl- (*IIIf*), 6-methyl- (*IIIg*), and 5,6-dimethyluridine (*IIIh*) have been prepared *via* the nucleoside 2'(3')-phosphites *II*. Orotidine 2',3'-cyclic phosphate (*IIIi*) and its $N_{(3)}$-methyl derivative, *IIIj*, the isocytidine derivative *V*, the 5-methyl-6-azauridine derivative *VI*, and the $C_{(1')}$-methylthymidine derivative *VII* have been obtained analogously. The 2',3'-cyclic phosphates of 2-thiouridine (*VIII*), of 2-thio-6-azauridine (*IX*), and of 4-thio-6-azauridine (*X*) have been prepared by reaction of the appropriate ribonucleoside with phosphoric acid in the presence of trichloroacetonitrile. $N_{(3)}$-Methyl-6-azauridine 2',3'-cyclic phosphate (*XII*) has been synthesized from the 6-azauridine derivative *XI* on alkylation with dimethylformamide dimethylacetal. Tritylation of 5-nitrouridine (*XIII*) afforded the 5'-trityl derivative *XIV* the reaction of which with 2-cyanoethyl phosphate and N,N'-dicyclohexylcarbodiimide and the subsequent alkaline and acidic work-up led to the 2'(3')-phosphate *XV*. Treatment of the latter compound with ethyl chloroformate afforded 5-nitrouridine 2',3'-cyclic phosphate (*XVI*). The specificity of the above 2',3'-cyclic phosphates to pancreatic ribonuclease and ribonuclease T2 has been investigated.

In previous papers from this Laboratory[1-5], syntheses of various cyclic five-membered phosphoric acid diesters derived from base- or sugar-modified nucleosides have been reported along with a study on the sensitivity of these diesters towards the enzymatically catalysed hydrolysis. In order to get additional information on the substrate specificity and mechanism of action of pancreatic ribonuclease as a base-specific enzyme[6,7] and ribonuclease T2 as a relatively non-specific one[8,9], we have synthesized several 2',3'-cyclic phosphates of base-substituted pyrimidine ribonucleosides and other related compounds, and studied their behaviour towards

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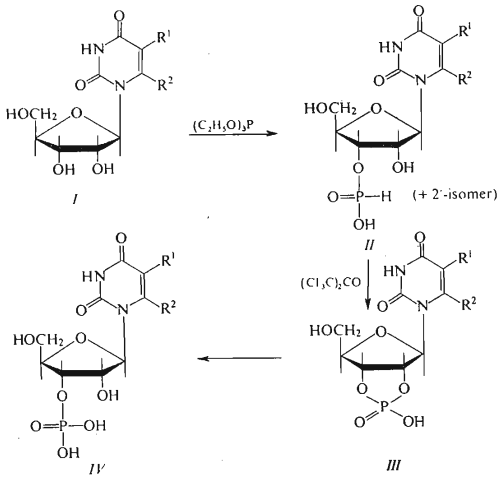
both enzymes mentioned. In the present paper, we wish to report these syntheses along with conclusions on the qualitative substrate requirements of the enzymes examined.

As a general method for the synthesis of most 2',3'-cyclic phosphates, a two-step procedure has been used consisting in treatment of unprotected nucleosides *I* with triethyl phosphite and the subsequent oxidative cyclisation of the resulting 2'(3')-phosphites *II* with hexachloroacetone[10] (Scheme 1). In this series of compounds, some derivatives have been already reported, namely, 5-methyl- (*cf.* ref.[11]), 5-bromo-, and 5-iodouridine 2',3'-cyclic phosphate[12]. In connection with investigations on the influence of 5-substituents in enzymatic reactions, the 5-ethyl derivative *IIIf*, the halo derivatives *IIIa* and *IIIb*, the 5-amino derivative *IIIc*, the 5-dimethylamino



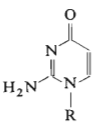
SCHEME 1

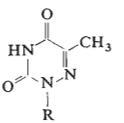
In formulae *I*—*IV*

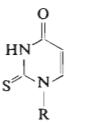
a, $R^1 = Cl$, $R^2 = H$
b, $R^1 = F$, $R^2 = H$
c, $R^1 = NH_2$, $R^2 = H$
d, $R^1 = (CH_3)_2N$, $R^2 = H$
e, $R^1 = OH$, $R^2 = H$
f, $R^1 = C_2H_5$, $R^2 = H$
g, $R^1 = H$ $R^2 = CH_3$
h, $R^1 = R^2 = CH_3$
i, $R^1 = H$, $R^2 = COOH$
j, $N_{(3)}-CH_3$ $R^1 = H$, $R^2 = COOH$

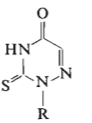
derivative *IIId*, and the 5-hydroxy derivative *IIIe* have been now synthesized. Furthermore, the 2′,3′-cyclic phosphates of orotidine (*IIIi*) and its $N_{(3)}$-methyl derivative *IIIj*, of 6-methyluridine (*IIIg*), and 5,6-dimethyluridine (*IIIh*) have been prepared with the use of the same synthetic procedure.

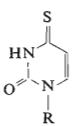
The synthesis of other nucleoside 2′,3′-cyclic phosphates related to uridine derivatives has been performed analogously, namely, of the isocytidine derivative *V*, the 5-methyl-6-azauridine derivative *VI* (the 6-azauridine derivative *XI* has been reported earlier[13]), and the $C_{(1')}$-methylthymidine derivative *VII*. The attempted application of the above method for the phosphorylation of 2-thiopyrimidine ribonucleosides failed, probably because of the reaction of phosphorous acid triester with the reactive 2-thio group (on the other hand, with the less reactive thio group in 6-thiopurine nucleosides, the reaction proceeds as usual[1]). Consequently, the

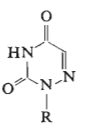

V

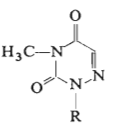

VI


VIII


IX

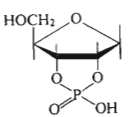

X

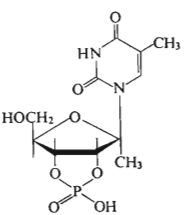

XI


XII

In formulae *V*, *VI*, *VIII*–*XII*

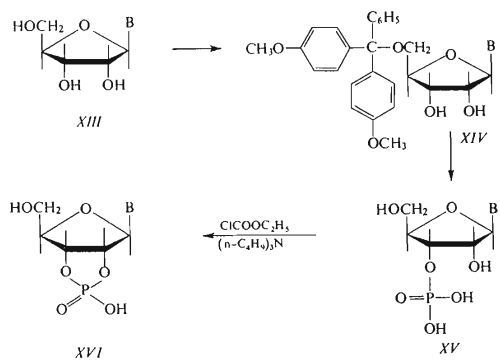
R =




VII

2',3'-cyclic phosphates of 2-thiouridine (*VIII*), 2-thio-6-azauridine (*IX*), and 4-thio-6-azauridine (*X*) have been synthesized from unprotected nucleosides and triethylammonium phosphate in the presence of trichloroacetonitrile[14].

5-Nitrouridine 2',3'-cyclic phosphate (*XVI*) has been prepared from 5-nitrouridine (*XIII*) by the route shown in Scheme 2 since the above general procedure failed. Thus, compound *XIII* was transformed by reaction with substituted trityl chloride to the 5'-tritylated derivative *XIV* the treatment of which with 2-cyanoethyl phosphate and N,N'-dicyclohexylcarbodiimide followed by removal of protecting groups first in weakly alkaline and then weakly acidic media afforded the 2'(3')-phosphate *XV*. The latter was converted to compound *XVI* by the action of ethyl chloroformate[15,16].



SCHEME 2

In formulae *XIII*—*XVI* B = 5-nitrouracil

With the use of modified substrates, an inhibitory effect of the $N_{(3)}$- (or next-to-2-carbonyl)- substituent has already been proved[6]. In this connection, the $N_{(3)}$-methyl-6-azauridine derivative *XII* has been prepared by methylation of compound *XI* with dimethylformamide dimethylacetal[17].

The above 2',3'-cyclic phosphates have been identified by their chromatographic and electrophoretic properties as well as by ultraviolet absorption spectra (small shifts of absorption maxima observed in some cases, with respect to the spectra of starting compounds are due to the phosphate substitution effect[18]). Furthermore, the structure of a five-membered 2',3-cyclic phosphate was confirmed by the acetic acid hydrolysis resulting in a mixture of 2'- and 3'-isomers of nucleotides *IV*. Another

confirmation of the structure consists in the enzymatical degradation with pancreatic ribonuclease and ribonuclease T2 leading in most cases specifically to 3′-isomers of nucleotides *IV*. Finally, dephosphorylation of *IV* with alkaline phosphatase *E. coli* affords the starting nucleosides identical on comparison with authentic specimens.

As shown by pancreatic ribonuclease degradation of 5-substituted uridine derivatives *IIIa–IIIf*, *XVI*, and of the 5-methyl-6-azauridine derivative *VI*, the substituent at position 5 does not exert any significant effect on the enzymatically catalysed hydrolysis of uridine 2′,3′-cyclic phosphate. This observation is consistent with earlier results[6] on the substrate specificity of pancreatic ribonuclease. Within the series of compounds examined, the substitution with either electron-withdrawing or electron-donating groups does not exert any qualitative effect upon the enzymatic reaction. The heterocyclic base of some substrates is assumed as completely or partially deprotonated under the experimental conditions (pH 8·0): *IIIb*, pK_a 7·48; *IIIe*, pK_a 7·55; *VI*, *XVI*, pK_a 6·35. Consequently, any participation of $N_{(3)}$-hydrogen atom of the pyrimidine moiety in mechanism of the enzymatical reaction (as postulated earlier[19]) can be hardly expected. Our findings are in agreement with those obtained in the case of compounds the heterocyclic bases of which lack any hydrogen atom at position corresponding to $N_{(3)}$ of uracil, namely, the 2-pyridone derivatives[2,6] and the 2-pyrimidinone derivatives[14].

On the contrary, substitution of uracil at position 6 by the methyl group (compounds *IIIg* and *IIIh*) results in a complete resistance of the phosphodiester linkage towards the enzymatic hydrolysis. Comparison of circular dichroism (or optical rotation) data of both starting nucleosides[20,21] indicated conversion of the nucleoside conformation into the *syn*-form. The lack of enzyme activity can be easily understood with the assumption of the 2-carbonyl group taking part in the mechanism of hydrolysis[6]. This participation would be lower with a predominant *syn*-conformation of the pyrimidine ring.

Curiously enough, the orotidine derivative *IIIi* represents a satisfactory substrate for the enzyme. The positive sign of Cotton effect[22] should not necessarily reflect the *anti*-conformation of the nucleoside (the change of the ultraviolet-absorbing system due to the 6-carboxylic substituent has not been taken into account). Nevertheless, substitution of orotidine (in *IIIi*) by the $N_{(3)}$-methyl group leads to a compound (*IIIj*) resistant to the action of the enzyme, similarly to the pairs of uridine and $N_{(3)}$-methyluridine derivatives or the corresponding analogues *XI* and *XII*; this observation represents a good evidence of the *anti*-conformation of the base in compound *IIIi*. The decrease of enzyme activity in compound *IIIi* might be due to the presence of an ionized carboxylic group ($pK_a < 1·5$). Consequently, the substrate might be attached non-specifically to another strongly basic side chain of the enzyme, not necessarily in the vicinity of active sites. An analogous lack of enzyme activities to orotidine nucleotides has been observed also with 5′-nucleotidase and snake venom phosphodiesterase. Unfortunately, the attempted synthesis of the corres-

ponding orotidine methyl ester derivative which might throw some light on these problems, failed.

4-Thiouridine 2',3'-cyclic phosphate has been reported[6] as a substrate for pancreatic ribonuclease. The 6-aza analogue *X* is also cleaved by this enzyme. On the other hand, the 2-thio derivatives *VIII* and *IX* are poor substrates in this respect. This fact might be explained by an increased nucleophilicity of the 2-thiocarbonyl group in comparison with the carbonyl group and its influence on the conformation of the nucleoside. Only an indirect evidence on 2-thiouridylic acid esters as substrates for pancreatic ribonuclease has been supplied, arising from the presence of 2-thiouridine 3'-phosphate in the RNase-hydrolysate of RNA[23]. Replacement of 2-carbonyl group of uridine 2',3'-cyclic phosphate by an amino function (resulting in the isocytidine derivative *V*) is accompanied by a complete loss of the enzyme activity, again in accordance with the assumed participation of the 2-carbonyl group in the hydrolytic step. Finally, the $C_{(1')}$-methyl derivative *VII* has been investigated to get some information on the effect of a $C_{(1')}$-substituent on the enzymatic reaction. Under the experimental conditions used, compound *VII* was resistant to the enzyme action. Interpretation of this finding is rather difficult because of a scarce knowledge on the relation of the conformation and the optical properties of the nucleoside which exhibits a positive sign of optical rotation but a remarkably decreased intensity[24]. This behaviour might reflex a more or less fixed conformation(s) unsuitable for the enzyme action.

Ribonuclease T2 (EC 2.7.7.24) represents a rather non-specific ribonuclease which splits the phosphodiester linkage of pyrimidine and purine nucleotides with a preference for 3'-adenylic acid esters[8,9]. The data summarised in Table I and concerning degradation of 2',3'-cyclic phosphate examined, indicate that there is *a*) no remarkable effect of the 5-substituent at the pyrimidine moiety upon the hydrolysis and, *b*) no qualitative effect of the 6-methyl or $N_{(3)}$-methyl group in the same respect (*cf.*[9]). This observation is considerably different from the requirements of pancreatic ribonuclease and indicates that neither a bulky substituent at position $N_{(3)}$, nor the conformation of the nucleoside moiety plays any role of importance in the enzyme action. Degradation of the orotidine derivative *IIIi* is decreased in comparison with other pyrimidine nucleotides, probably due to a non-specific interaction of an electronegatively charged base residue with another enzyme region. The lack of activity of the enzyme towards the compound *IIIj* might be due to accumulation of two lowering effects, namely, the carboxylic group and the $N_{(3)}$-methyl group (*cf.* ref.[9]). Both the 2-thio derivatives *VIII* and *IX* as well as the 4-thio derivative *X* are split by ribonuclease T2, although to a different extent. This difference might also be due to an inhibitory effect of the product, but exact conclusions could be drawn from kinetic data only.

A very significant decrease of the affinity to the enzyme has been found with the $C_{(1')}$-methyl derivative *VII* the splitting extent of which is within the range of experi-

mental errors. Interpretation of this effect would require a more detailed investigation with the use of compounds bearing various substituents at position $C_{(1')}$.

We should like to emphasize that the data on the affinity of all compounds discussed in the present paper towards both enzymes examined can be taken only as qualitative. An exact interpretation would require evaluation of kinetic data; experiments in this direction are in progress and will be published elsewhere.

EXPERIMENTAL

Paper Chromatography and Electrophoresis

Descending paper chromatography was performed on paper Whatman No 1 (preparative runs on paper Whatman No 3 MM) in the solvent systems S_1, 2-propanol–concentrated aqueous ammonia–water (7 : 1 : 2), S_2, ethanol–1M ammonium acetate (5 : 2); and S_3, 1-butanol–acetic acid–water (5 : 2 : 3). Paper electrophoresis was carried out on 16 cm wide strips of paper Whatman No 3 MM in 0·1M triethylammonium hydrogen carbonate (E_1) at 20 V/cm for 1 hour. Preparative runs were performed in 0·2M buffer solution at 10 V/cm for 1 1/2—2 hours. For R_F values and electrophoretical mobilities see Table II.

Ultraviolet Absorption Spectra

The measurements were performed on a Beckman DM spectrophotometer in 0·01M-HCl. One optical density unit (1 A) is defined as that amount of the ultraviolet-absorbing material which dissolved in 1 ml of 0·01M-HCl causes at a given wavelength (mostly at 260 nm) the absorbancy

TABLE I

Enzymatic Degradation of Nucleoside 2',3'-Cyclic Phosphates with Pancreatic Ribonuclease and Ribonuclease T2

For test conditions see the Experimental Part; ++ good substrate; + poor substrate; (+) only traces of enzymatic hydrolysis; — no splitting.

Substrate	Pancreatic ribonuclease	Ribonuclease T2	Substrate	Pancreatic ribonuclease	Ribonuclease T2
IIIa	++	++	*IIIj*	—	—
IIIb	++	++	*V*	—	+
IIIc	++	++	*VI*	++	+
IIId	++	++	*VII*	(+)	(+)
IIIe	++	++	*VIII*	+	+
IIIf	++	++	*IX*	+	+
IIIg	—	++	*X*	++	+
IIIh	—	++	*XII*	—	+
IIIi	+	+	*XVI*	++	+

TABLE II

Paper Chromatography and Electrophoresis

Compound	R_F			E_{Up}[a]
	S_1	S_2	S_3	
Nucleosides				
Uridine	0·45	0·70	0·42	—
Ia	0·45	0·74	—	—
Ib	0·45	0·70	—	0·45
Ic	0·35	0·58	—	—
Id	0·60	0·82	—	—
Ie	0·19	0·62	—	0·47
If	0·67	—	—	—
Ig	0·51	—	—	—
Ih	0·63	—	—	—
Ii	0·28	0·51	0·16	0·66
Ij	0·54	0·70	0·27	0·60
Isocytidine	0·45	—	—	—
6-Azauridine	0·44	—	—	0·58
5-Methyl-6-azauridine	0·56	—	—	0·56
$C_{(1')}$-Methylthymidine	0·68	0·81	—	—
2-Thiouridine	0·40	0·73	0·50	0·42
2-Thio-6-azauridine	0·42	0·61	—	0·59
4-Thio-6-azauridine	0·50	—	—	0·59
5-Nitrouridine (*XIII*)	0·49	—	—	0·50
2′,3′-Cyclic Phosphates[b]				
Uridine 2′,3′-cyclic phosphate	0·36	—	—	0·67
IIIa	0·31	0·60	—	0·97
IIIb	0·38	—	—	0·95
IIIc	0·36	0·50	—	0·50
IIId	0·50	—	—	0·50
IIIe	0·18	0·55	—	0·91
IIIf	0·55	0·67	—	0·54
IIIg	0·40	—	—	0·55
IIIh	0·50	—	—	0·57
IIIi	0·18	0·42	—	1·08
IIIj	0·38	0·60	0·34	1·05
V	0·39	—	—	0·52
VI	0·40	0·58	—	0·82
VII	0·56	0·72	—	0·50
VIII	0·25	—	—	0·89
IX	0·35	—	—	1·10
X	0·38	—	—	1·14
XI	0·36	—	—	1·05
XII	0·56	—	—	0·60
XVI	0·18	—	—	1·06

TABLE II
(*Continued*)

Compound	R_F			E_{Up} [a]
	S_1	S_2	S_3	
2′(3′)-Nucleotides				
Uridine 2′(3′)-phosphate	0·12	0·25	—	1·00
IVa	0·07	—	—	1·10
IVb	0·10	0·21	—	1·25
IVc	0·16	—	—	0·95
IVd	0·20	—	—	0·90
IVe	0·03	0·18	—	1·30
IVf	0·22	—	—	0·99
IVg	0·18	—	—	1·00
IVh	0·23	—	—	1·00
IVi	0·06	0·15	—	1·36
IVj	0·14	0·24	—	1·31
Isocytidine 2′(3′)-phosphate	0·12	—	—	0·95
5-Methyl-6-azauridine 2′(3′)-phosphate	0·12	0·58	—	1·13
$C_{(1')}$-Methylthymidine 2′(3′)-phosphate	0·20	—	—	0·95
2-Thiouridine 2′(3′)-phosphate	0·05	—	—	1·17
2-Thio-6-azauridine 2′(3′)-phosphate	0·08	—	—	1·40
4-Thio-6-azauridine 2′(3′)-phosphate	0·10	—	—	1·40
$N_{(3)}$-Methyl-6-azauridine 2′(3′)-phosphate *XV*	0·08	—	—	1·25

[a] Electrophoretical mobility referred to uridine 2′(3′)-phosphate in E_1; [b] R_F and E_{Up} values of nucleoside 2′(3′)-phosphites *II* are indistinguishable from those of 2′,3-cyclic phosphates *III*.

TABLE III
Synthesis of Nucleoside 2′,3′-Cyclic Phosphates

Compound	Starting nucleoside mmol	Yield, %	Method	Compound	Starting nucleoside mmol	Yield, %	Method
IIIa	3·0	50	*A*	*IIIi*	0·25	25	*B*
IIIb	0·2	47	*B*	*IIIj*	1·00	17	*A*
IIIc	2·0	65	*A*	*V*	2·0	68	*A*
IIId	3·5	54	*A*	*VI*	2·0	71	*A*
IIIe	2·0	48	*A*	*VII*	0·06	56	*B*
IIIf	0·08	61	*B*	*VIII*	0·25	1	*C*
IIIg	0·25	48	*B*	*IX*	0·17	10	*C*
IIIh	0·5	63	*B*	*X*	0·17	7	*C*

equal to one. The following molar extinction coefficients and spectral characteristics were used (at pH 2, if not stated otherwise): *Ia* (pH 7), λ_{max} 277 nm, λ_{min} 240 nm, ε_{max} 9000, $A_{250/260}$ 0·49, $A_{280/260}$ 1·61; *Ib*, λ_{max} 269 nm, λ_{min} 234 nm, ε_{max} 8900, ε_{260} 7850, $A_{250/260}$ 0·65, $A_{280/260}$ 0·82, $A_{290/260}$ 0·35; *Ic* (pH 7), λ_{max} 294 nm, ε_{294} 7400, $A_{280/260}$ 1·88, $A_{290/260}$ 2·20; at pH 2, λ_{max} 265 nm; *Id*, λ_{max} 267 nm, ε_{max} 9500, ε_{260} 8700, $A_{250/260}$ 0·66, $A_{280/260}$ 0·65, $A_{290/260}$ 0·16; *Ie*, λ_{max} 280 nm, ε_{280} 8000, $A_{250/260}$ 0·70, $A_{280/260}$ 1·37, $A_{290/260}$ 1·17; *If*, λ_{max} 267 nm, ε_{260} 8750, $A_{250/260}$ 0·65, $A_{280/260}$ 0·72; *Ig*, λ_{max} 261 nm, ε_{260} 11 200, $A_{250/260}$ 0·80, $A_{280/260}$ 0·05; *Ih*, λ_{max} 268 nm, ε_{268} 10500, $A_{250/260}$ 0·65, $A_{280/260}$ 0·69, $A_{290/260}$ 0·22; orotidine (*Ii*), λ_{max} 267 nm, ε_{max} 9600, $A_{250/260}$ 0·65, $A_{280/260}$ 0·80; *XIII*, λ_{max} 238 nm, 305 nm, ε_{305} 10000; isocytidine (pH 7), λ_{max} 258 nm, ε_{260} 6000, $A_{250/260}$ 1·03, $A_{280/260}$ 0·15; 5-methyl-6-azauridine, λ_{max} 261 nm, ε_{260}, $A_{250/260}$ 0·80, $A_{280/260}$ 0·50, $A_{290/260}$ 0·17; 2-thio-6-azauridine (pH 7), λ_{max} 269 nm, ε_{269} 18000, $C_{(1')}$,5-dimethyluridine, λ_{max} 267 nm, ε_{260} 8750; $N_{(3)}$-methyl-6-azauridine, λ_{max} 260 nm, ε_{260} 6000; 2-thiouridine (pH 7), λ_{max} 275 nm, λ_{max} 13600; 4-thio-6-azauridine (pH 7), λ_{max} 243 nm, 327 nm, ε_{327} 17000.

Materials

5-Chlorouridine, 5-hydroxyuridine, and orotidine were purchased from Calbiochem Ltd., Los Angeles, California, U.S.A. 5-Fluorouridine was a gift of Dr J. Weil, University Strasbourg, France. 5-Ethyluridine was obtained from Dr P. Swierkowski, Institute of Biochemistry and Biophysics, Warsaw, Poland. 2-Thiouridine and 2-thio-6-azauridine were kindly offered by Dr H. Vorbrüggen, Schering AG, West-Berlin. 6-Methyluridine, 5,6-dimethyluridine, 5-methyl-6-azauridine (Dr M. Prystaš), $C_{(1')}$,5-dimethyluridine (Dr J. Farkaš), and 4-thio-6-azauridine (Dr J. Beránek) were synthesized in our Institute. 6-Azauridine 2′,3′-cyclic phosphate has been reported elsewhere[13]. $N_{(3)}$-Methylorotidine was prepared by a reported procedure[25]. Dimethylformamide was dried over phosphorus pentoxide, distilled under diminished pressure, and stored over molecular sieves. The solution of hydrogen chloride in dimethylformamide was prepared by saturation of dimethylformamide with dry gaseous hydrogen chloride at 0°C until the mixture solidified. Afterwards, the crystals were dissolved with the use of such an amount of dimethylformamide to bring the concentration to the value of 6M-HCl.

Ribonucleoside 2′,3′-Cyclic Phosphates; General Procedure (*cf.* Table III)

A. *Macroscale preparations.* The corresponding ribonucleoside (1—10 mmol) was stirred with a dimethylformamide–triethyl phosphite mixture (2 : 1; 7 ml per 1 mmol of the nucleoside) and a small excess of 6M-HCl in dimethylformamide (with basic nucleosides, more than 1 equivalent was necessary) until a clear solution was obtained. The pH value of the reaction mixture was checked with the use of a moistened reagent paper and, if necessary, additional drops of 6M-HCl in dimethylformamide were added to keep the pH in the range of 1—2. The resulting solution was allowed to stand at room temperature overnight, treated with an excess (10—20 ml per 1 mmol of the nucleoside) of 0·4M triethylammonium hydrogen carbonate (pH 7·5), and the whole immediately evaporated at 35°C/15 Torr. The residual oil was applied to a column (25 × × 3·5 cm) of DEAE-cellulose (Cellex D, standard capacity, Calbiochem) equipped with an Uvicord apparatus (Uppsala, Sweden) for continuous measurements of ultraviolet absorption, elution rate 3 ml per min. The column was eluted first with water until the nucleoside absorption ceased and then with 0·4M triethylammonium hydrogen carbonate. The corresponding ultraviolet-absorbing fraction of compound *II* was evaporated at 35°C/15 Torr to dryness preferably under the addition of lauryl alcohol to prevent foaming. The residue was coevaporated with three 50 ml portions of ethanol and finally dried over phosphorus pentoxide at 0·1 Torr/60°C

for 2 hours. In the case of acidic nucleosides (*Ia,b,e,i*), the above chromatography had to be modified as follows. The column (80 × 4 cm) of the above DEAE-cellulose was eluted with the use of a linear gradient of triethylammonium hydrogen carbonate, pH 7·5 (2 l of water in the mixing chamber, 2 l of 0·3M buffer solution in the reservoir), the fractions being taken in 10 min intervals (elution rate, 3 ml per min). The second fraction, containing the corresponding nucleoside 2′(3′)-phosphite *II*, was processed (evaporation and drying) as above. Compound *II* was shaken with a fresh mixture of dimethylformamide and hexachloroacetone (2 : 1; 6 ml per 1 mmol) the resulting solution allowed to stand at room temperature overnight under exclusion of atmospheric moisture, and taken to dryness at 40°C/0·1 Torr. The residue was coevaporated with two 10 ml portions of dimethylformamide and finally dissolved in 0·4M triethylammonium hydrogen carbonate, pH 7·5 (20 ml per 1 mmol), the solution shaken with ether (0·3 vol.), and the whole allowed to stand at room temperature for one hour. The aqueous phase was then washed once more with the same volume of ether, and concentrated at 35°C/15 Torr to a half of the original volume. The concentrate was applied to a column (80 × 4 cm) of DEAE-cellulose and the elution performed as above. The product *III* was isolated from the second (main) fraction by evaporating to dryness at 35°C/15 Torr, coevaporating with three 50 ml portions of ethanol, and drying over phosphorus pentoxide at 20°C/0·1 Torr. The foamy residue was dissolved in a small amount (as small as possible) of ethanol and the solution added dropwise under stirring into 300—500 ml of ether. The precipitate was collected by centrifugation (if oily, the ether layer is discarded, the oily layer dissolved again in 10—20 ml of ethanol, and the ethanolic solution is added as above into 200 ml of ether), washed with ether, and dried over phosphorus pentoxide at 20°C/0·1 Torr. The purity of products was determined by paper chromatography and electrophoresis as well as by spectrophotometry in 0·01M-HCl (content determination). When necessary, the desalting can be achieved either by passage through a Sephadex G-10 column and elution with water (20 ml per hour), or, by adsorption on deactivated charcoal at pH 4—5, washing with water, and desorption which is performed by elution with a 5 : 3 : 2 water–methanol–pyridine mixture adjusted with ammonia to the pH value of 8—9.

B. *Microscale preparations.* The corresponding ribonucleoside (10—500 μmol) was treated with dimethylformamide (2 ml), triethyl phosphite (1 ml), and a few drops of 6M-HCl in dimethylformamide. The resulting solution was adjusted, if necessary, to pH 1—2 (moistened pH paper), allowed to stand at room temperature overnight, treated with 10 ml of 0·4M triethylammonium hydrogen carbonate (pH 7·5), and the whole evaporated to dryness at 35°C/15 Torr. The residue was chromatographed in the solvent system S_1 on several sheets of paper Whatman No 3 MM (up to 50 mg of the starting nucleoside per one sheet) overnight. Both nucleoside and nucleoside 2′(3′)-phosphite (*II*) bands were eluted with dilute (1 : 100) aqueous ammonia (25—50 ml). The eluate containing the product *II* was evaporated to dryness at 35°C/15 Torr, repeatedly coevaporated with ethanol, and finally dried over phosphorus pentoxide at 50°C/0·1 Torr. The glassy residue was shaken with a mixture of dimethylformamide (2 ml) and hexachloroacetone (1 ml), preferably in the presence of glass beads, until a solution was obtained. After standing overnight, the solution was treated directly with 0·4M triethylammonium hydrogen carbonate, pH 7·5 (20 ml), kept at room temperature for one hour, diluted with an equal volume of water, and the whole washed with one 25 ml portion of ether. The aqueous phase was concentrated at 35°C/15 Torr to the volume of about 10 ml and the concentrate applied to column (20 × 1 cm) of Biogel P-2. The column was eluted with water (rate, 2—3 ml per min) with the use of a Uvicord apparatus.* The eluate was concentrated under diminished pressure and the concentrate

* This procedure serves for removal of impurities which as precursors of yellow and brown dyes formed on chromatography in the solvent system S_1 complicate the detection of final products under ultraviolet lamp.

chromatographed on sheets of paper Whatman No 3 MM (for the quantity of the substance to be chromatographed see above) in the solvent system S_1 overnight. Bands of the product *III* were eluted with dilute (1 : 100) aqueous ammonia (20—50 ml), the eluate evaporated to dryness under diminished pressure, and the residue rechromatographed in the same solvent system or subjected to preparative paper electrophoresis (*vide supra*) to remove any contaminant dyes. The final eluate was freeze-dried in glass vials and its content determined spectrophotometrically at pH 2.

C. *Reaction of nucleosides with the triethylammonium salt of phosphoric acid and trichloroacetonitrile* (*cf.* ref.[14]). A mixture of the corresponding nucleoside (0·25 mmol), dimethylformamide (0·5 ml), 1M triethylammonium phosphate in dimethylformamide (0·5 ml; 0·5 mmol), and trichloroacetonitrile (0·35 ml) was heated at 40°C for 30 min. Water (20 ml) and 0·4M triethylammonium hydrogen carbonate, pH 7·5 (20 ml) were then added, the whole mixture washed with two 20 ml portions of ether, and the aqueous layer evaporated to dryness at 35°C/15 Torr. The residue was applied to one sheet of paper Whatman No 3 MM and chromatographed in the solvent system S_1. The band of 2′,3′-cyclic phosphate was cut out, eluted with dilute (1 : 100) aqueous ammonia (25 ml), the eluate evaporated to dryness under the above conditions, and the residue purified by paper electrophoresis in the buffer solution E_1. The band of 2′,3′-cyclic phosphate was eluted with water (10 ml), the content estimated spectrophotometrically at pH 2, and the eluate freeze-dried in glass vials.

For the yields of 2′,3′-cyclic phosphates (Procedures *A*, *B*, and *C*) see Table III.

5′-O-Di(*p*-methoxyphenyl)phenylmethyl-5-nitrouridine (*XIV*)

A mixture of 5-nitrouridine (*XIII*; 1·45 g; 5 mmol), di-(*p*-methoxyphenyl)phenylmethyl chloride (2·4 g; 7 mmol), and pyridine (25 ml) was allowed to stand at room temperature over 2 days, poured into iced water (300 ml), and extracted with three 50 ml portions of chloroform. The extract was washed with two 50 ml portions of water, dried over magnesium sulfate, and evaporated at 35°C/15 Torr. The residue was coevaporated with 50 ml of toluene to remove the pyridine, and finally dissolved in ethanol (40 ml). The ethanolic solution was added dropwise under stirring into light petroleum (400 ml). The precipitate of *XIV* was collected with suction, washed thoroughly with additional light petroleum (200 ml), and dried over phosphorus pentoxide at 20°C/0·1 Torr to afford 2·07 g (70%) of yellow amorphous powder, homogeneous on a thin layer of silica gel (R_F value 0·55 in 5 : 95 methanol–chloroform). For $C_{30}H_{29}N_3O_{10}$ (591·5) calculated: 60·91% C, 4·94% H, 7·10% N; found: 60·70% C, 5·05% H, 7·35% N.

5-Nitrouridine 2′,3′-Cyclic Phosphate (*XVI*)

A mixture of compound *XIV* (1·78 mg; 3 mmol) and the pyridine (20 ml) solution of 2-cyanoethyl phosphate pyridinium salt (10 mmol) was dried by coevaporation with five 25 ml portions of pyridine at 30°C/0·1 Torr. The final residue was dissolved in pyridine (20 ml), the solution treated with N,N′-dicyclohexylcarbodiimide (10·5 g; 50 mmol), and the whole allowed to stand at room temperature for 4 days under exclusion of atmospheric moisture. The mixture was decomposed with water (5 ml), kept at room temperature additional 2 hours, evaporated to dryness at 40°C/15 Torr, and the residue shaken with a mixture (50 ml) of 25% of acetic acid in 50% aqueous methanol. The whole was kept at 50°C for 3 hours, evaporated to dryness at 40°C/15 Torr and shaken with a mixture of water (100 ml) and ether (50 ml). The aqueous phase was washed with additional ether (25 ml) and evaporated to dryness under the above conditions. The residue was heated with dilute (1 : 1) aqueous ammonia (50 ml) at 50°C for 4 hours, the mixture evaporated to dryness, the residue dissolved in water (100 ml), the aqueous solution filtered through

Cellit, and the filtrate concentrated under diminished pressure to the volume of 50 ml. The concentrate was applied to a column (80 × 4 cm) of DEAE-cellulose (*vide supra*) and the column eluted with the use of 2 l of water in the mixing chamber and 2 l of 0·5M triethylammonium hydrogen carbonate in the reservoir at the rate of 3 ml per min, the fractions being taken in 10 min intervals. The 0·30—0·35M fraction contained the starting 5-nitrouridine. The 0·45—0·50M fraction containing the 2′(3′)-phosphate *XV* was evaporated at 35°C/15 Torr and the residue chromatographed on 4 sheets of paper Whatman No 3 MM in the solvent system S_1 overnight. The main slowly migrating band of compound *XV* was eluted with dilute (1 : 100) aqueous ammonia (50 ml), the eluate evaporated to dryness at 35°C/15 Torr, and the residue (homogeneous on chromatography and electrophoresis) vigorously stirred for 30 min with a mixture of water (3 ml), tri-n-butylamine (1 ml), and ethyl chloroformate (0·5 ml). Afterwards, another portion of trin-butylamine (0·5 ml) and ethyl chloroformate (0·2 ml) was added, the stirring continued for further 30 min, the whole evaporated at 35°C/15 Torr, and the residue chromatographed on 2 sheets of paper Whatman No 3 MM in the solvent system S_1 overnight. Bands of compound *XVI* (the single absorbing material under ultraviolet light) were eluted with dilute (1 : 100) aqueous ammonia (50 ml), the eluate evaporated to dryness at 35°C/15 Torr, the residue coevaporated with three 25 ml portions of ethanol, and finally dissolved in ethanol (10 ml). The ethanolic solution was poured under stirring into ether (200 ml), the precipitate collected by centrifugation, washed with ether, and dried over phosphorus pentoxide at 20°C/0·1 Torr to afford 110 mg (10%) of compound *XVI* in the form of ammonium salt, chromatographically and electrophoretically homogeneous. Content, >95%.

$N_{(3)}$-Methyl-6-azauridine 2′,3′-Cyclic Phosphate (*XII*)

A mixture of 6-azauridine 2′,3′-cyclic phosphate (*XI*) ammonium salt[17] (208 mg; 0·64 mmol), dimethylformamide (2·5 ml), and dimethylformamide dimethyl acetal (1 ml) was heated at 100°C for 6 hours (after this period of time, a high content of the desired reaction product was determined by paper chromatography in the solvent system S_1). The mixture was then chromatographed directly on 4 sheets of paper Whatman No 3 MM in the solvent systems S_1. Bands (R_F 0·50) containing the product were eluted with dilute (1 : 100) ammonia (25 ml), the eluate evaporated to dryness at 30°C/15 Torr, the residue coevaporated with three 25 ml portions of ethanol, and finally precipitated from methanol (10 ml) with ether (100 ml) under stirring. The precipitate was collected by centrifugation, washed with ether, and dried over phosphorus pentoxide at 20°C/0·1 Torr to afford 152 mg (70%) of compound *XII* in the form of ammonium salt, chromatographically and electrophoretically homogeneous. Content, 97%.

Acid Hydrolysis of Nucleoside 2′,3′-Cyclic Phosphates

The title hydrolysis was performed on heating the substance (1—20 μmol) in 0·20 ml of 50% aqueous acetic acid for 3—4 hours at 50°C. The mixture was then chromatographed on a 25 cm wide strip of paper Whatman No 3 MM in the solvent system S_1. The band of the nucleoside 2′(3′)-phosphate was eluted with water (5 ml) and the eluate (its content was determined spectrophotometrically at pH 2) freeze-dried in glass vials. The yield was almost quantitative. The products (ammonium salts) were homogeneous as shown by paper chromatography and electrophoresis.

Enzymatic Degradations

A. *Alkaline phosphatase* E. coli *degradation of nucleoside 2′(3′)-phosphates*: The test substance (1—2 μmol) in 50 μl of 0·01M Tris-HCl (pH 8·0) was incubated at 37°C for 2 hours with 10 μl

of the enzyme suspension in aqueous ammonium sulfate (Worthington, U.S.A.). In all cases, chromatography in the solvent systems S_1 and S_2 and electrophoresis in the buffer solution E_1 revealed the presence of the corresponding nucleoside as the single product (on comparison with an authentic specimen).

B. *Pancreatic ribonuclease degradation of nucleoside 2',3'-cyclic phosphates*: The test substance (1—2 μmol) in 50 μl of 0·01M Tris-HCl (pH 8·0) was incubated at 37°C for 3—4 hours with 50γ of pancreatic ribonuclease (Calbiochem, 5 fold recrystalised) in 25 μl of the same buffer solution. The course of the degradation was followed by paper chromatography in the solvent system S_1 and electrophoresis in the buffer solution E_1. A blank test was always performed under otherwise identical conditions but in the absence of the enzyme. Compounds which were not split under the conditions stated, were incubated for longer incubation periods with the use of 0·5 to 1·0 mg of the solid enzyme.

C. *Ribonuclease T2 degradations of 2',3'-cyclic phosphates.* The test substance (1—2 μmol) in 50 μl of 0·1M sodium γ,γ-dimethylglutarate (pH 6·2) containing 0·1M-NaCl was incubated at 37°C for 4 hours with the enzyme (50γ protein; obtained from Prof. Dr H. Witzel, Marburg, Germany) in 25 μl of 0·1M-NaCl. The course of splitting was followed by paper chromatography in the solvent system S_1 and electrophoresis in the buffer solution E_1.

For the results obtained in pancreatic ribonuclease and ribonuclease T2 degradations see Table I.

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