NUCLEIC ACID COMPONENTS AND THEIR ANALOGUES. CXLVI.*

PREPARATION OF SOME NUCLEOTIDE DERIVATIVES OF 6-METHYLURIDINE, 3-(β-D-RIBOFURANOSYL)URACIL, AND 3-(β-D-RIBOFURANOSYL)-6-METHYLURACIL. INVESTIGATIONS OF THEIR TEMPLATE ACTIVITY AND BEHAVIOUR TOWARDS SOME NUCLEOLYTIC ENZYMES

A. HOLÝ, R. W. BALD** and F. ŠORM

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

Received January 13th, 1971

The ribonucleoside 5′-phosphates *III* derived from the title nucleosides *I* were prepared *via* the 2′,3′-O-ethoxymethylene derivatives *II* by phosphorylation with β-cyanoethyl phosphate and removal of the protecting groups. The 5′-diphosphates *IVa,b* were isolated as by-products. The 5′-nucleotide *IIIc* was obtained by phosphorylation of the nucleoside *Ic* with phosphorus oxychloride in triethyl phosphate. Condensation of protected nucleosides *II* with 5′-O-acetyl-2′-O-tetrahydropyranyluridine 3′-phosphate (*V*) in the presence of N,N′-dicyclohexylcarbodiimide afforded the protected dinucleoside phosphates *VIa,b*, the deblocking of which led to the free dinucleoside phosphates *VIIa,b*. Compound *VIIc* was prepared by the enzymatically catalyzed reaction of uridine 2′,3′-cyclic phosphate with the nucleoside *Ic* in the presence of pancreatic ribonuclease. The 5′-nucleotides *III* are substrates for alkaline phosphatase *Escherichia coli* and intestinal alkaline phosphates; the snake venom 5′-nucleotidase degradation proceeds slowly. The dinucleoside phosphates *VII* which contain the nucleosides *I*, are resistant to the snake venom phosphodiesterase.

The oligonucleotides of the type pGpN, GpUpN, and pGpUpN (N = the nucleoside *I*) were prepared by the enzymatically catalysed reaction of the corresponding guanosine 2′,3′-cyclic phosphates with nucleosides *I* or dinucleoside phosphates *VII*. Replacement of uridine in the second or third position of the valine codon by the nucleoside *I* resulted in a loss of the template activity of the oligonucleotide for the binding of Val-tRNA to ribosomes.

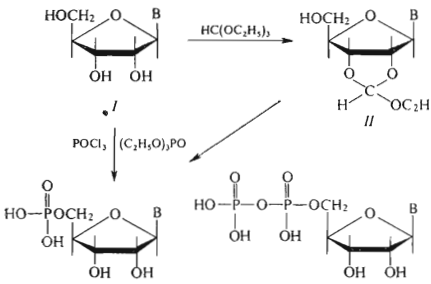
In earlier papers[1,2] of this Series, we have reported the anomalous behaviour of 1- and 3-ribofuranosyl-6-methyluracil 2′,3′-cyclic phosphates towards some ribonucleases. The results were interpreted in terms of the influence of a change in conformation of the nucleoside portion of the molecule on the interaction between the substrate and the enzyme. With the 1-ribofuranosyl derivative, this change might be caused by the steric influence of the substituent at position 6. In the case of uracil

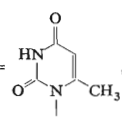
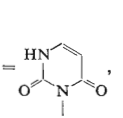
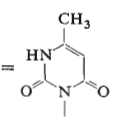
* Part CXLV: This Journal *36*, 4066 (1971).

** Present address: Max-Planck-Institut für Molekulare Genetik, Berlin-West.

3-ribofuranosyl derivatives, the different character of the heterocyclic system might lead to a different interaction with the sugar moiety. In this connection, we have been interested in the preparation of some other nucleotide derivatives both of the 1- and 3-series and their behaviour towards other nucleolytic enzymes.

6-Methyluridine 5'-phosphate (*IIIa*) was prepared from the free nucleoside[3] *Ia* by reaction with ethyl orthoformate[4] and phosphorylation of the resulting 2',3'-O-ethoxymethylene derivative *IIa* with β-cyanoethyl phosphate according to Tener[5]. Work-up of the reaction mixture with triethylamine in anhydrous media afforded the 5'-nucleotide *IIIa* along with about 10% of the corresponding 5'-diphosphate[6] *IVa*. Analogously, 3-(β-D-ribofuranosyl)-6-methyluracil[3] (*Ib*) furnished the 5'-nucleotide *IIIb* and 5'-diphosphate *IVb* (Scheme 1).



In formulae *I*—*IV a*: B =  , *b*: B =  , *c*: B = 

SCHEME 1

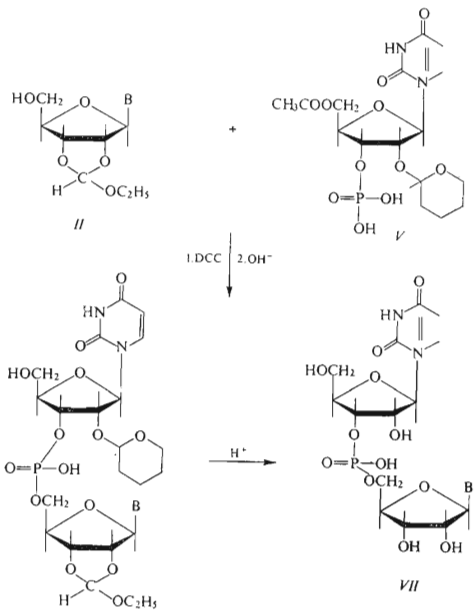
The 5'-phosphate *IIIc* was obtained as the main product by a simpler route, namely, by phosphorylation of 3-(β-D-ribofuranosyl)uracil[2,7] (*Ic*) with phosphorus oxychloride in triethyl phosphate[8].

The 5'-nucleotides *III* were isolated as ammonium salts, homogeneous on paper chromatography and electrophoresis, and afforded satisfactory data on elemental analysis. Their ultraviolet absorption spectra did not practically differ from those of the corresponding nucleosides.

Degradations of 5′-nucleotides *III* with phosphomonoesterases were performed under standard conditions (under these conditions, uridine 5′-phosphate is dephosphorylated quantitatively after 2 hours). It has been found that the alkaline phosphatase *E. coli* degradation as well as the intestinal alkaline phosphatase degradation of compounds *IIIa–IIIc* is quantitative while only 40–50% of the material is degraded by the action of *Crotalus adamanteus* snake venom 5′-nucleotidase. All three enzymes afforded the starting nucleoside *I* as the degradation product the characterisation of which was performed on comparison with an authentic specimen. These results are in accordance with our earlier findings on the nature of the interaction between the substrate and the above enzymes, shown in investigations concerning the action of enzymes on L-nucleotides[9]. Thus, both the alkaline phosphatase *E. coli* and the intestinal phosphatase split the L-nucleotides; consequently, they interact with the substrate without any participation of the appropriate heterocyclic base. For this reason, the change of the character or conformation of the nucleoside moiety does not exert qualitatively any influence on the action of the enzyme. On the other hand, the snake venom 5′-nucleotidase does not split the L-nucleotides[9,10]: it obviously interacts with the substrate at three centers, including the interaction with the heterocyclic base itself or with the whole nucleoside portion of the molecule. The decreased activity of this enzyme with nucleotides *III* might be due to the above mentioned changes of the character of the heterocyclic base or, conformation of the nucleoside moiety[1,2].

Condensation of the 2′,3′-O-ethoxymethylene derivatives *IIa,b* with 2′-O-tetrahydropyranyl-5′-O-acetyluridine 3′-phosphate[11] (*V*) in the presence of N,N′-dicyclohexylcarbodiimide and the subsequent removal of protecting groups first in alkaline and then in acidic media led to the (3′→5′)-diribonucleoside phosphates *VII* (Scheme 2). Compound *VIIc* was obtained by reaction of uridine 2′,3′-cyclic phosphate with the nucleoside *Ic* in the presence of pancreatic ribonuclease[12]. The complete pancreatic ribonuclease degradation of compounds *VII* to uridine 3′-phosphate and the nucleoside *I* in equimolar ratio simultaneously represents a proof of the structure *VII*. The complete degradation was observed also with the ribonuclease T2. On the other hand, compounds *VII* are completely resistant to the action of snake venom phosphodiesterase under standard conditions. As shown by investigations on L-nucleotides[10], the snake venom phosphodiesterase interacts similarly to 5′-nucleotidase (*vide supra*) with the substrate under participation of the heterocyclic base or the whole nucleoside unit. It may be assumed from the complete resistance of compounds *VII* towards the action of the snake venom phosphodiesterase that the sensitivity to the changed conformation of the corresponding portion of the molecule is higher with the snake venom phosphodiesterase than with 5′-nucleotidase. It is noteworthy in this connection that the p*K* value of nucleosides *I* does not considerably differ from that of uridine. Consequently, the effect observed with both snake venom enzymes must be due to the decreased interaction of the base

with the peptidic chain of the enzyme. With compounds *IIIa* and *VIIa*, the changed conformation follows from circular dichroism spectra of the nucleoside[1,13] *Ia* while with other compounds this change is assumed on the basis of earlier investigations[2].



For B in formulae *II*, *VI*, *VII*, Scheme 1

SCHEME 2

None of the nucleosides *I* shows any bacteriostatic activity on the growth of *E. coli* B in synthetic media up to the concentration of 1000 μg per 1 ml.* Incubation of nucleosides *I* with the S 100 fraction from *E. coli* B under standard conditions (50% of uridine was split to uracil under these conditions) resulted in an 8·5% degradation of the nucleoside linkage with the compound *Ia* while compounds *Ib* and *Ic* were practically resistant.

* This measurement was performed by Dr I. Votruba of this Institute.

The changed conformation of the nucleoside *Ia* in respect to uridine as well as the changed heterocyclic system of uracil 3-ribosyl derivatives *Ib,c* should assert itself also in the protein-synthesizing system *in vitro* and to influence the interaction of ribosomes, aminoacyl-tRNA, and messenger RNA (containing the nucleoside of type *I* as the codon component). This effect was studied by measurement of the [^{14}C]-Val-tRNA binding to ribosomes[14] in the presence of oligonucleotides which contain the nucleosides *I*. For this purpose, we have prepared three types of compounds, analogous to valine codons, namely (N = the nucleoside *I*), pGpN, GpUpN, and pGpUpN (*cf.*[15]). All these types of compounds were prepared by enzymatically catalysed reactions in the presence of ribonuclease[16] T1:

$$\text{pGcp} + \text{N} \rightleftarrows \text{pGpN}$$

$$\text{Gcp} + \text{UpN} \rightleftarrows \text{GpUpN}$$

$$\text{pGcp} + \text{UpN} \rightleftarrows \text{pGpUpN}$$

Abbreviations: G, guanosine; pG, guanosine 5′-phosphate, cp, 2′,3′-cyclic phosphate; N, nucleoside *I*; UpN, dinucleoside phosphate *VI*.

None of the present oligonucleotides exhibited any appreciable template activity both under standard conditions and at a three-fold concentration. With 6-methyluridine (*Ia*) derivatives, this failure might be ascribed to the changed conformation of the nucleoside since other portions of the heterocyclic base which are involved in the formation of complementary base pairs (N^3—H and the carbonyl function at position 4) remain untouched. In view of the changed nucleoside conformation with compounds of the pGpN and GpUpN type, one base pair cannot take part in the complex formation because of a changed distance between the interacting groups of the codon and anticodon. Moreover, the changed conformation of the nucleoside component may influence the character of the neighbouring base and thus change conformation of the whole template molecule. This idea is in accordance with investigations on the template activity of compounds of the pGpUpN (N = *Ia*) type (Table I). In this case, the lower activity due to the loss of one base pair formation (with 6-methyluridine) should be somewhat compensated by the substitution effect of the 5′-phosphate group[15].

The presence of nucleosides *Ib,c* in the corresponding oligonucleotides changes the character of the whole heterocyclic base[2]. There are changes in the base pair formation with the complementary base, in the conformation of the nucleoside, and in the conformation of the whole nucleotide due to the mutual interaction of vicinal nucleosides. Conclusively, the 3-ribosyl derivatives of uracil are inactive in the second or third position of the valine codon and, probably, in the triplet codon at all.

TABLE I

Stimulation of [^{14}C]Val-tRNA Binding (in μμmol) to Ribosomes (Conditions, see Experimental)

Template[a] $0{\cdot}1A_{260}$[b]	[^{14}C]Val-tRNA bound	Template[a] $0{\cdot}1A_{260}$[b]	[^{14}C]Val-tRNA bound
None	0·70	pGp 6-MeU	0·73
GpUpU	2·46 (2·96)	pGp 3-RfU	0·56
GpUp6-MeU	0·68 (0·80)	pGp 6-Me-3-RfU	0·73
GpUp3-RfU	0·72 (0·70)	pGpUpU	4·20 (5·95)
GpUp6-Me-3-RfU	0·75 (0·72)	pGpUp6-MeU	1·01 (1·32)
pGpU	1·05	pGpUp6-Me-3-RfU	0·75 (0·82)

[a] Abbreviations, see above; 6-MeU *Ia*, 3-RfU *Ib*, 6-Me-3-RfU *Ic*; [b] in parentheses, values for $0{\cdot}3A_{260}$ of the template.

EXPERIMENTAL

Methods

Paper chromatography was performed by the descending technique 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), and S_2, ethanol–2M ammonium acetate (5 : 2). For R_F values see Table II.

Paper electrophoresis was performed by the technique of Markham and Smith[17] on paper Whatman No 3 MM in 0·1M triethylammonium hydrogen carbonate (pH 7·5) at 20 V/cm for 1 hour. For electrophoretical mobilities see Table II.

Detection of spots was performed under ultraviolet light (Chromatolite). The phosphorus-containing compounds were detected with the use of the reagent according to Isherwood[18].

Ultraviolet absorption spectra were taken in 0·01M-HCl on a Beckman DU apparatus. The following molar extinction coefficient values at 260 nm were used for calculation of quantitative measurements: uridine, 10000; guanosine, 11800; compound *Ia*, 11200 (*cf.*[3]); compound *Ib*, 8250; compound *Ic*, 8500. The hyperchromicity was not considered in calculations of molar extinction coefficients of oligonucleotides. One optical density unit at 260 nm is that amount of the test substance which shows in 1 cm cell in a solution of pH 2 at 260 nm the absorbancy equal to one ($1A_{260}^{pH}$).

Enzymatical degradations were performed with 2 μmol of compounds *III* or 1 μmol of compounds *VII* in 100 μl of 0·05M Tris-HCl buffer solution (pH 9·0) with the use of the following enzymes (incubation at 37°C for 4 hours): *a*) alkaline phosphatase *E. coli* (Worthington, A grade), 20 μl of the enzyme suspension in ammonium sulfate; *b*) alkaline intestinal phosphatase (Boehringer), 20 μl of the enzyme solution in 50% aqueous glycerine; *c*) *Crotalus adamanteus* 5′-nucleotidase (Worthington), 40 μg of the protein; *d*) *Crotalus terr. terr.* snake venom phosphodiesterase (Boehringer), 20 μg of the enzyme. With enzymes *a*) to *c*), uridine 5′-phosphate (quantitative degradation within less than 2 hours) was used as standard while with the enzyme *d*), uridylyl-(3′→5′)-uridine was used (quantitative degradation after 1 hour). With compounds *VI*, the phos-

phodiesterase degradation was performed also at 37°C for 24 hours. The mixtures after incubation were analysed by chromatography in the solvent system S_1. The percentage of degradation was determined spectrophotometrically.

TABLE II
Paper Chromatography and Electrophoresis

Compound	R_F		E_{Up}[a]
	S_1	S_2	
Uridine	0·50	0·76	—
Ia	0·55	0·77	—
Ib	0·64	0·80	—
Ic	0·54	0·76	—
IIa	0·85	—	—
IIb	0·87	—	—
Uridine 3′-Phosphate	0·12	0·27	1·00
IIIa	0·12	0·31	0·98
IIIb	0·20	0·35	0·90
IIIc	0·18	0·25	1·00
IVa	0·08	0·15	0·98
IVb	0·12	—	0·80
VIa	0·66	—	0·45
VIb	0·73	—	0·45
VIIa	0·31	0·48	0·48
VIIb	0·37	0·63	0·47
VIIc	0·24	0·49	0·45
Uridylyl-(3′→5′)-uridine	0·22	0·49	0·45
Uridine 2′,3′-Cyclic Phosphate	0·39	0·67	0·72

[a] The mobility refers to uridine 3′-phosphate in 0·1M triethylammonium hydrogen carbonate under standard conditions.

*Assay of aminoacyl-*tRNA *binding to ribosomes.* The incubation mixture contained 0·05M Tris acetate (pH 7·2), 0·1M ammonium chloride, 0·03M magnesium acetate, $2·5A_{260}$ units of ribosomes, $0·6A_{260}$ unit of tRNA acylated with 22 μmol of [^{14}C]valine (specific activity, 146 μC : : μmol), and $0·1A_{260}$ unit of the oligonucleotide (if not stated otherwise). Total volume: 0·05 ml. After incubation (at 24°C for 20 min), the mixture was diluted with 3 ml of the same buffer, filtered on Millipore filters, washed with four 5-ml portions of cold buffer, the filter mounted on a planchet, dried, and the radioactivity determined in a proportional methane-flow counter (Frieseke and Hoepfner). For the results see Table I.

6-Methyluridine 5'-Phosphate (*IIIa*)

A mixture of 6-methyluridine[3] (*Ia*; 516 mg; 2 mmol), dimethylformamide (5 ml), ethyl orthoformate (2 ml), and 6M hydrogen chloride in dimethylformamide (0·2 ml) was stirred until a solution was obtained. The solution was kept at room temperature overnight, then treated with triethylamine (1 ml), and the mixture evaporated to dryness at 35°C/0·1 Torr. The residue was coevaporated with toluene (10 ml) and then dissolved in 50% aqueous pyridine (10 ml). To this solution of compound *IIa* there was added 5 mmol of β-cyanoethyl phosphate pyridinium salt in pyridine[4] (20 ml) and the mixture was coevaporated with six 20 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 (6 g), the whole shaken at room temperature for two hours and then set aside for 6 days. Triethylamine (2 ml) was added, the mixture kept at room temperature for 10 min, diluted with water (100 ml), and washed with three 50 ml portions of ether. The aqueous phase was concentrated at 30°C/15 Torr to the volume of about 5 ml, the concentrate diluted with water (20 ml), concentrated aqueous ammonia (20 ml), and triethylamine (5 ml), the whole heated at 60°C for 2 hours, and concentrated as above to the volume of about 5 ml. The concentrate was diluted with water (50 ml), filtered through Celite, and the filtrate applied to a column (80 × 4 cm) of DEAE cellulose (Cellex D, Calbiochem, standard capacity). The column was eluted with water (rate, 6 ml per min) to the drop of the nucleoside absorption (the elution was followed continuously with the use of the Uvicord apparatus) and then with the 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); fractions were taken in 10 min intervals at the rate of 3 ml per min. The corresponding fractions were combined, evaporated at 35°C/15 Torr to dryness, and the residues coevaporated with methanol (50 ml each) under similar conditions: portion 1, 0·10—0·15M buffer solution; portion 2, 0·16—0·20M buffer solution. The residues were dissolved in 50% aqueous acetic acid (5 ml each), the solutions heated at 50°C for 40 min, and each mixture chromatographed for 3 days on 4 sheets of paper Whatman No 3 MM in the solvent mixture S_1. Bands corresponding to products *IIIa* and *IVa* were eluted with dilute (1 : 100) aqueous ammonia (20 ml) and the eluates freeze-dried. Yield (from portion 1), 250 mg (33·2%) of the ammonium salt of compound *IIIa*, homogeneous on chromatography and electrophoresis. Molecular weight: 370 (determined spectrophotometrically), 355 (calculated for the monoammonium salt); N/P, 3·1. Degradations with alkaline phosphatase *E. coli* and intestinal alkaline phosphatase were quantitative while only 45% of the material was split with 5'-nucleotidase (standard conditions). Yield (from portion 2), 135 mg (15%) of the diammonium salt of compound *IVa*; molecular weight, 460 (determined spectrophotometrically; ratio phosphorus to 6-methyluridine, 2·02 : 1. Compound *IVa* is identical with an authentic specimen prepared from compound *IIIa* (*vide infra*) and is split quantitatively with alkaline phosphatase *E. coli* to compound *Ia*.

3-(β-D-Ribofuranosyl)-6-methyluracil 5'-Phosphate (*IIIb*)

The title compound was prepared analogously to the phosphate *IIIa* from 2 mmol of the nucleoside[3] *Ib*. Yield, 30%. The product was homogeneous on paper chromatography and electrophoresis. Degradations with alkaline phosphatase *E. coli* and intestinal phosphatase were quantitative while only 60% of the material was split with 5'-nucleotidase (standard condition). In addition to the 5'-phosphate *IIIb*, there was obtained 6% of the 5'-diphosphate *IVb* which was characterised analogously to compound *IVa*.

3-(β-D-Ribofuranosyl)uracil 5′-Phosphate (*IIIc*)

A mixture of the nucleoside *Ic* (40 mg; 0·16 mmol), triethyl phosphate (1·5 ml), and phosphorus oxychloride (0·1 ml) was stirred at 0°C for 2 hours, treated with 5 ml of water and 0·3 ml of triethylamine, the whole heated at 70°C for 20 min, and evaporated to dryness at 40°C/15 Torr. The residue was chromatographed for 3 days on 2 sheets of paper Whatman No 3 MM in the solvent system S_1. The band corresponding to the product *IIIc* was processed as above to afford 32 mg (60%) of the ammonium salt of compound *IIIc*, homogeneous on paper chromatography and electrophoresis. Molecular weight: 345 (determined spectrophotometrically); 337 (calculated for the monoammonium salt). Degradations with alkaline phosphatase *E. coli* and intestinal alkaline phosphatase to compound *Ic* are quantitative while only 40% of the material is split with 5′-nucleotidase (standard conditions).

6-Methyluridine 5′-Diphosphate[19] (*IVa*)

The ammonium salt of compound *IIIa* (0·5 mmol) was converted into the free acid on a column (1 × 5 cm) of Dowex 50 X 8 (H^+) ion exchange resin. The column was eluted with 50 ml of water, the eluate evaporated almost to dryness at 30°C/15 Torr, the residue diluted with methanol (10 ml) and tri-n-butylamine (0·5 ml), the whole evaporated to dryness under similar conditions, the residue coevaporated with four 20 ml portions of ethanol, and dried over phosphorus pentoxide at 0·1 Torr overnight to afford the crude tri-n-butylammonium salt of compound *IIIa*. A mixture of this salt, dioxane (3 ml), tri-n-butylamine (0·45 ml), and diphenylphosphoryl chloride (0·30 ml) was shaken at room temperature for 3 hours. The dioxane was evaporated at 30°C/0·1 Torr, the residue washed by decantation with two 10 ml portions of ether, briefly dried at 0·1 Torr, and dissolved in a mixture of phosphoric acid tri-n-butylammonium salt (2 mmol) and pyridine (3 ml). The solution was kept at room temperature overnight, evaporated at 30°C : : 15 Torr, the residue dissolved in water (10 ml), the solution acidified to pH 3 with hydrochloric acid, kept 30 min, made alkaline to pH 9 with aqueous ammonia, and evaporated to dryness at 30°C/15 Torr. The residue was chromatographed for 3 days on 3 sheets of paper Whatman No 3 MM in the solvent system S_1. The corresponding bands were eluted and the eluates processed as above to afford 100 mg (44%) of the ammonium salt of compound *IVa*, identical with that obtained by phosphorylation of compound *IIa* (*vide supra*). Molecular weight: 460 (determined spectrophotometrically); 452·3 (calculated for the bis-ammonium salt); N/P, 2·05; the ratio P to 6-methyluridine, 2·12.

Dinucleoside Phosphates *VIa,b*

The calcium salt of compound[11] *V* (1·5 mmol) was applied in 30% aqueous pyridine (10 ml) to a column (10 × 1 cm) of pyridinium Dowex 50 X 8 ion exchange resin, the column eluted with 70 ml of 30% aqueous pyridine, the eluate evaporated at 30°C/15 Torr to the volume of about 10 ml, and treated with a solution of 2 mmol of the 2′,3′-O-ethoxymethylene derivative *IIa,b* (*cf.* the above preparation of compound *IIIa*). The whole mixture was dried by repeated coevaporations with pyridine (six 20 ml portions) at 30°C/0·1 Torr, the residue dissolved in pyridine (10 ml), the solution shaken with N,N′-dicyclohexylcarbodiimide (1·6 g) for 2 hours, and the mixture kept then at room temperature for 5 days. Water (5 ml) and triethylamine (0·5 ml) were then added, the mixture kept for 30 min, diluted with water (50 ml), extracted with two 20 ml portions of ether, and the aqueous phase concentrated at 30°C/15 Torr to the volume of about 5 ml. The concentrate was heated at 50°C with 10 ml of water and 10 ml of concentrated aqueous ammonia for one hour, the mixture concentrated to a small volume, and the concentrate filtered through Celite. The filtrate was applied to a column of DEAE-cellulose (*vide supra*) and the co-

lumn eluted with water until the absorption of the nucleoside *II* ceased. The elution was then continued 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 0·2M buffer solution in the reservoir) at the rate of 3 ml per min, the fractions being taken in 10 min intervals. The corresponding fractions (0·08—0·12M) of the dinucleoside phosphate *VI* were combined, evaporated at 35°C/15 Torr, the residue coevaporated with three 20 ml portions of ethanol under similar conditions, dissolved in ethanol (5 ml), and the solution added dropwise under stirring into ether (100 ml). The precipitate was collected by centrifugation, washed with ether, and dried over phosphorus pentoxide at 0·1 Torr to afford 45% of the triethylammonium salt of compound *VIa* (the yield is based on the nucleoside *Ia*) or 43% of the triethylammonium salt of compound *VIb* (the yield refers to the nucleoside *Ib*). The product is homogeneous on paper chromatography in the solvent system S_1 and electrophoresis. The spectrophotometrical determination of the molecular weight indicates the content of about 90%.

Dinucleoside Phosphates *VIIa,b*

The triethylammonium salt of compounds *VIa,b* (100 μmol) in 50% aqueous acetic acid (2 ml) was heated at 50°C for 30 min and chromatographed for 2 days on 3 sheets of paper Whatman No 3 MM in the solvent system S_1. The bands corresponding to phosphates *VII* were eluted with dilute (1 : 100) aqueous ammonia (20 ml), the eluates freeze-dried, and the yield determined spectrophotometrically. The ammonium salt of the dinucleoside phosphate *VIIa* was obtained in 70% yield. Ultraviolet spectrum (pH 2): λ_{max} 262 nm, λ_{min} 230 nm, $A_{250/260}$ 0·77, $A_{280/290}$ 0·30, $A_{290/260}$ 0·05. The ammonium salt of the dinucleoside phosphate *VIIb* was obtained in 83% yield. Ultraviolet spectrum (pH 2): λ_{max} 263 nm, λ_{min} 232 nm, $A_{250/260}$ 0·72, $A_{280/260}$ 0·42, $A_{290/260}$ 0·08. The pancreatic ribonuclease degradation (1 μmol of *VII* in 100 μl of 0·05M Tris-HCl buffer, pH 8, contg. 0·5 μg of the enzyme purchased from Calbiochem, incubation at 37°C for 3 hours) was higher than 98% in both cases. On the other hand, the *Crotallus terr. terr.* snake venom phosphodiesterase degradation (standard conditions) did not occur (even after incubation for 24 hours).

Enzymatic Synthesis of the Dinucleoside Phosphate *VIIc*

A solution of uridine 2′,3′-cyclic phosphate ammonium salt (50 μmol) and the nucleoside *Ic* (50 μmol) in 100 μl of 0·05M Tris-HCl buffer, pH 8, was treated at 0°C with 60γ of pancreatic ribonuclease (Calbiochem). After incubation at 0°C for 16 hours, the mixture was chromatographed for 2 days on one sheet of paper Whatman No 3 MM in the solvent system S_1. The band of compound *VIIc* was eluted with dilute (1 : 100) aqueous ammonia (10 ml), the yield determined spectrophotometrically, and the eluate freeze-dried. Yield, 12% of compound *VIIc* (referred to the nucleoside *Ic*). Ultraviolet spectrum (pH 2): λ_{max} 262, λ_{min} 230 nm, $A_{250/260}$ 0·73, $A_{280/260}$ 0·35. The pancreatic ribonuclease degradation (*vide supra*) affords quantitatively uridine 3′-phosphate and the nucleoside *Ic*. On the other hand, the phosphate *VIIc* is completely resistant to the snake venom phosphosphodiesterase.

Enzymatic Synthesis of pGpN, GpUpN and pGpUpN

Ribonuclease T1 (Sankyo Ltd., Japan; 10 e.u.) was added at 0°C to a mixture of the triethylammonium salt of guanosine 2′,3′-cyclic phosphate (Gcp) or 5′-O-phosphorylguanosine 2′,3′-cyclic phosphate (pGcp) (*cf.*[20]) (10 μmol) and the nucleotide *I* or the dinucleoside phosphate *VII* (30—50 μmol) in 100 μl of 0·05M Tris-HCl buffer solution (pH 7·0) and the whole incubated

at 0°C for 16 hours. The resulting mixture was then chromatographed for 2 days on one sheet of paper Whatman No 3 MM in the solvent system 1-butanol–acetic acid–water (5 : 2 : 3). The product was eluted with water and purified by preparative electrophoresis on a 16 cm wide strip of paper Whatman No 3 MM in 0·2M triethylammonium hydrogen carbonate (20 V/cm, 2 hours). After elution with water, the yield was determined spectrophotometrically. The structure of products was determined by ribonuclease T1 degradation ($1A_{260}$ unit of the test substance, 50 µl of 0·05M Tris-HCl buffer, pH 7, 10 e.u. of the enzyme, incubation at 37°C for 3 hours) which led to the starting compound *I* or *VII* and the corresponding nucleotide. The yields of oligonucleotides varied from 1 to 4%.

Degradation of Nucleosides *I* by the Supernatant of S-100 *E. coli* B

A mixture of the nucleoside *I* (4 µmol), 40 µl of 0·01M Tris-HCl buffer solution (pH 7·2) containing 0·01M magnesium acetate, and 40 µl of the supernatant S-100 (*E. coli* B) (*cf.*[15]) was incubated at 37°C for one hour and then analysed by electrophoresis in 0·1M triethylammonium borate, pH 7·5 (40 volt per cm, 90 min). The spots were eluted with 0·01M-HCl and the content of the base and the nucleoside determined spectrophotometrically. Degradation: uridine (50%), *Ia* (8·5%), *Ib* (<1%), *Ic* (<1%).

The authors wish to thank Mrs B. Kopecká, Mrs J. Hlaváčková, and Mr K. Oupický for excellent technical assistance.

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Translated by J. Pliml.