PHOSPHONYLMETHYL ANALOGUES OF RIBONUCLEOSIDE 2',3'-CYCLIC PHOSPHATES AND 2'(3')-NUCLEOTIDE METHYL ESTERS: SYNTHESIS AND PROPERTIES

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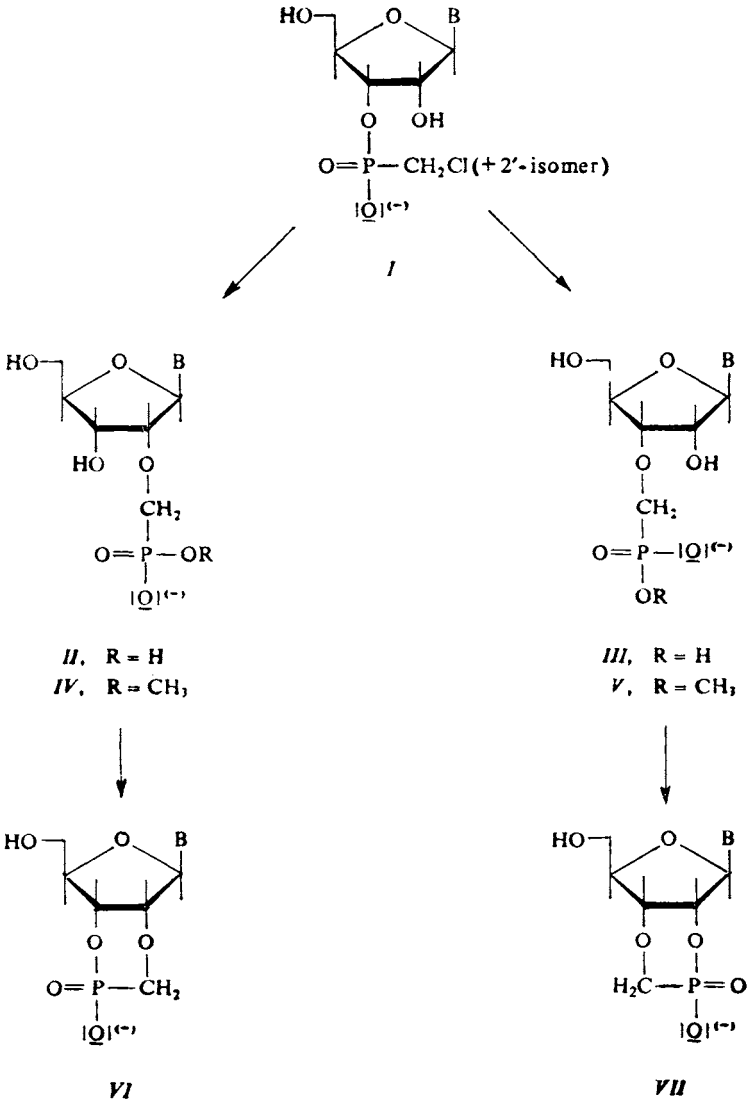
2'(3')-O-Phosphonylmethylribonucleosides *II*, *III* were prepared by intramolecular cyclisation of ribonucleoside 2'(3')-chloromethanephosphonates *I* in alkali. Treatment of the phosphonylmethyl derivatives with dicyclohexylcarbodiimide afforded cyclic six-membered esters *VI*, *VII* which are stable in acid and easily hydrolyze in alkali. Ribonucleases A, T1, T2, U2 and spleen cPDase do not split the cyclic phosphonate ester linkage of these analogues of ribonucleoside 2',3'-cyclic phosphates.

Recently, we have described synthesis and principal properties of a novel class of nucleotide analogues, O-phosphonylmethyl derivatives of nucleosides. These compounds which are supposed to be close analogues of natural metabolites bear a chemically stable linkage of the O—C—P type which substantiates their resistence against enzymatic dephosphorylation[1,2]. We have since proved that some of these analogues can replace natural substrates and act as inhibitors of some enzymatic reactions[3-5].

In order to further examine the response of these analogues toward enzymes involved in nucleic acid metabolism we now describe the synthesis of the analogues of ribonucleoside 2',3'-cyclic phosphates and 2'- or 3'-nucleotide methyl esters and their behaviour toward ribonucleases.

The 2'- and 3'-O-phosphonylmethylribonucleosides *II*, *III* can be prepared by an intramolecular cyclisation reaction of the 2'(3')-chloromethanephosphonylribonucleosides *I* in aqueous alkali. The starting materials *I* are available on treatment of suitably protected ribonucleosides with chloromethanephosphonyl chloride in the presence of pyridine[2]. The accessibility of these key-compounds has been now significantly improved by the use of "hydrolyzed chloromethanephosphonyl chloride" in the analogy to Efimov's phosphorylation reaction[6]. The reaction now proceeds with a nearly quantitative yield of compound *I*. A simultaneous use of alkali-labile groups at one of the 2',3'-*cis*-diol hydroxyls enables to prepare isomerically pure phosphonyl derivative *II*, *III* in an efficient "one-flask" synthesis. As a suitable alkali-labile group one can use not only the obvious acyl functions, but also the

tetraisopropyldisiloxanyl moiety[7] which protects simultaneously the 3'- and 5'--hydroxyl groups[8]. Although the latter group is usually cleaved by fluoride anion, it is split completely under the alkaline conditions used for the preparation of compounds *II*, *III*. Thus, the application of this strategy opens a direct route to 3'-O-phosphonylmethylribonucleosides *III* and their derivatives.



In formulae *I*—*VII*, B stands for a uracil-1-yl, b cytosin-1-yl, c adenin-9-yl, d guanin-9-yl residue.

Contrary to the cyclisation of 2'(3')-ribonucleosides which easily proceeds in aqueous ammonia–tert-butanol solution by the action of DDC, the analogues *II* and *III* produce a more complex reaction mixture which contains, in addition to the expected cyclic esters, significant amounts of by-products, most probably phosphonamidates; these compounds are less polar, alkali-stable and generate the starting phosphonate *II* or *III* upon acidic treatment. However, the cyclisation to the compounds *VI* and *VII* can be easily achieved when the reaction with DCC is performed in the absence of excess ammonia.

Similarly to the phosphonates *II* and *III*, the isomeric cyclic esters *VI*, *VII* are not interconvertible and, though they can be easily separated by HPLC, their preparative separation from a mixture of isomers is difficult. The cyclic phosphonates are very stable in acidic media resembling in this respect six-membered cyclic phosphodiesters. However, they differ substantially from the latter and from the five-membered 2',3'-cyclic nucleotides by an easy hydrolysis in alkaline solutions which results in the starting phosphonates *II* or *III* as the only products. This sensitivity to alkaline hydrolysis explains the formation of compounds *II* and *III* from chloromethanephosphonates *I* which evidently proceeds *via* cyclic intermediates.

This unique susceptibility of cyclic phosphonates to a nucleophilic attack is the principle of a simple preparation of the methyl esters *IV* and *V* which are formed in a reaction of the above chloromethanephosphonyl derivatives *I* with sodium methoxide in anhydrous methanol solution. The cyclic intermediate which is formed in a thermodynamically controlled reaction is subsequently opened by the methoxide anion to produce the methyl ester. Methyl esters *IV* and *V* are also stable in acid solutions but undergo a smooth cleavage to the phosphonylmethyl derivatives *II*, *III* in alkali.

The ribonucleases catalyze the cleavage of the internucleotidic (or nucleotide ester) linkages in two steps: *a*) the intramolecular nucleotidyl group transfer to the 2'-hydroxyl group which results in the 2',3'-cyclic phosphate formation and *b*) hydrolysis of the latter to the 3'-nucleotide. The reactions can be both base-specific and non-specific[9]. In the investigation of the behaviour of the above nucleotide analogues as the models for more complicated structures of nucleic acid analogues we have chosen the methyl esters *IV* and *V* for the study of the transfer reaction and the cyclic phosphonates *VI*, *VII* for the study of the hydrolytic reaction catalyzed by ribonucleases and other hydrolases.

The results show that none of the enzymes used, either specific (purine-specific RNAse U2, pyrimidine-specific RNAse A, guanine-specific RNAse T1) or non-specific (RNAse T2, spleen cPDase) is capable either of transfer of the phosphonylmethyl residue in compounds *IV* and *V*, or of hydrolysis of the cyclic esters *VI* and *VII*. Under the conditions used, the control experiments with ribonucleoside 2',3'-cyclic phosphates invariably resulted in a complete cleavage of the substrate by the assayed enzyme.

Thus, the results of our present investigation suggest: *a*) Though the formation of a six-membered cyclic phosphonate ring by chemical activation is as easy as the ring closure of the five-membered 2′,3′-cyclic nucleotides, the ribonucleases are uncapable of catalysis of such a transfer under the *in vitro* conditions. *b*) Though the cyclic phosphonates are very much susceptible to a nucleophilic attack, such a reaction with an activated water molecule cannot be mediated by ribonucleases. This failure may be due to the presence of the CH_2-grouping in the immediate vicinity of the phosphorus atom or rather[14] by a distorted conformation of the analogue which would disturb the complex formation. Alternatively, such a hypothetical complex, if arisen, might not be able to form the activated (pentacovalent) state or to undergo consecutive transformations. The sensitivity of ribonucleases to conformational changes in the substrate molecule is supported *e.g.* by the stability of the five-membered 2′,3′-cyclic phosphates derived from β-D-ribopyranosyl analogues of nucleosides[10–12]. *c*) The phosphonylmethyl analogues of oligonucleotides would most probably be resistant towards the transfer or hydrolytic reactions catalyzed by ribonucleases. However, since the above negative data may also be due to the very low probability of the complex formation (or an extremely short life of such a species) with the monomers, the co-operative effect of the neighbouring base binding to the enzyme might significantly enhance the activity towards the analogues of dinucleotides. To ensure the stability of the analogous internucleotide linkage it will be necessary to study the latter type of compounds.

EXPERIMENTAL

If not stated otherwise, the solutions were evaporated at 40°C/2 kPa and the compounds dried at 13 Pa over phosphorus pentoxide. Ultraviolet absorption spectra were recorded on a Specord UV/VIS instrument (Carl Zeiss, Jena, G.D.R.) in aqueous solutions. The absorption maxima corresponded closely to the values given for the corresponding nucleotides. HPLC Analysis was performed on a high pressure glass column (3·3 × 150 mm) of Separon SIX 18 (5 μ) with UV-detector LCD-254 and recorder EZ 11 (Laboratory Equipment, Prague, Czechoslovakia). Samples were applied with a sample valve injector (7 μl sample loop) and Constametric I high pressure pump (LDC, U.S.A.) was used for elution (flow rate, 0·5 ml/min). Analysis was run at room temperature, detection at 254 nm (0·32 AUFS). DEAE-Sephadex A-25 column chromatography was performed on columns (15 × 250 mm) of the title material with the linear gradient (2 × × 1 000 ml) of triethylammonium hydrogen carbonate pH 7·5 (end concentration, 0·2 mol l^{-1}); continuous detection with Uvicord apparatus (LKB, Uppsala, Sweden), elution rate 3 ml/min. Combined fractions were taken down *in vacuo*, codistilled twice with methanol and the residues in water (10 ml) run through a column (20 × 1 cm) of Dowex 50X8 (Li^+). The UV-absorbing fractions eluted with water were evaporated *in vacuo*, dried with ethanol and precipitated from methanol solutions with ether. Chromatography on Dowex 1 columns was made with the use of 15 × 250 mm columns of Dowex 1X2 (acetate) resin with a linear gradient (2 × 2 000 ml) of acetic acid (end concentration, 2 mol l^{-1}). Elution rate, 3 ml/min. Fractions were evaporated, codistilled twice with water and the compounds isolated by ethanol–ether precipitation.

Materials. The following O-phosphonylmethyl derivatives have been prepared by the formerly described[2] procedure (numbers in parentheses refer to the isomers): uridine (2′ + 3′), cytidine (2′ + 3′), adenosine (2′ + 3′), guanosine (2′ + 3′), uridine (3′). The following enzymes were used: pancreactic ribonuclease A (Lachema, Czechoslovakia), RNase T1, RNase T2 and RNase U2 (Sankyo, Japan), spleen cPDase (Koch-Light, England).

Preparation of Phosphonylating Reagent

To a solution of 501 mg (3 mmol) chloromethanephosphonyl chloride in pyridine (5 ml) there was added water (54 µl, 3 mmol) under stirring and cooling with ice. After 15 min stirring under exclusion of external moisture, pyridine hydrochloride was centrifuged and the supernatant added to the corresponding nucleoside derivative (1 mmol).

2′-O-Phosphonylmethyluridine (*IIa*)

The reaction was performed with 2′,5′-di-O-benzoyluridine (1 mmol) and the above reagent (3 mmol). After stirring for 4 h at room temperature 10 ml 1 mol l^{-1} triethylammonium hydrogen carbonate pH 7·5 precooled to 0°C was added. The mixture was stirred for 15 min and extracted with chloroform (100 ml). The extract was washed with the same buffer (0·1 mol l^{-1}, 2 × 50 ml) and the solvent evaporated *in vacuo*. The residue was dissolved in 50 ml of 1 mol l^{-1} lithium hydroxide in 40% aqueous dioxane and incubated at 40°C for 20 h. Dioxane was then evaporated *in vacuo*, the residue neutralized with hydrochloric acid and evaporated *in vacuo*. After codistillation with ethanol (3 × 50 ml) the residue was taken up in aceton–methanol mixture (1 : 1, 200 ml), stirred for 2 h and centrifuged. The sediment was dissolved in water, residual organic solvents removed *in vacuo* and the compound was purified by Sephadex A-25 chromatography. Yield 86% of the lithium salt of *IIa*.

3′-O-Phosphonylmethyladenosine (*IIIc*)

a) 3′,5′-O-Tetraisopropyldisiloxane-1,3-diyladenosine[7] (1 mmol) was stirred overnight with a mixture of dimethylformamide (10 ml) and dimethoxymethyldimethylamine (3 ml) and the solvents were taken down at 40°C/13 Pa. 50% Aqueous pyridine (20 ml) and a few pieces of dry ice were added to the residue and after 30 min the solution was taken down and dried with pyridine (3 × 20 ml) under the same conditions. The phosphonylation agent was added and the reaction and workup were performed as described for the uridine derivative. Alkaline treatment was prolonged for 15 h at 60°C. The compound was finally purified by Dowex 1 chromatography (*cf.* above) to yield 60% of *IIIc*.

b) 3′,5′-O-Tetraisopropyldisiloxane-1,3-diyl-N^6-benzoyladenosine[13] (1 mmol) was treated with 5 mmol of the phosphonylation agent in 10 ml pyridine essentially as described for the uridine derivative. The chloroform extract was evaporated and the residue in tetrahydrofuran (10 ml) treated with 1 mol l^{-1} tetra-n-butylammonium fluoride solution in the same solvent (5 ml). After 30 min at room temperature, the solution was taken down *in vacuo* and the residue incubated for 20 h at 40°C with 1 mol l^{-1} sodium hydroxide solution. The mixture was neutralized by addition of Dowex 50X8 (pyridinium form), the slurry filtered by suction, washed with 10% aqueous pyridine and the filtrate concentrated *in vacuo*. The resulting solution was alkalified by ammonia to pH 8–9 and applied on the Dowex 1 column. Yield 65% of *IIIc*.

Preparation of the 2′,3′-Cyclic Phosphonate Esters *VI*, *VII*

A refluxing solution of 2′- or 3′-O-phosphonylmethylnucleoside (*II*, *III*, ammonium salt, 1 mmol) in 50% aqueous dimethylformamide (20 ml) was treated dropwise during 1 h with a solution

of N,N′-dicyclohexylcarbodiimide (2 g) in tert-butanol (10 ml). After additional reflux for 7 h the mixture was cooled, filtered and the filtrate evaporated *in vacuo*. The residue was treated with water (100 ml), filtered and the filtrate reevaporated *in vacuo*, dissolved again in water (100 ml) and the solution extracted with ether (3 × 20 ml). The aqueous phase was concentrated *in vacuo* and purified on DEAE-Sephadex A-25. Homogeneous cyclic esters *VI, VII* were isolated as lithium salts in 95—98% preparative yields. Compounds derived from uridine, cytidine, adenosine, and guanosine were prepared by this procedure. The cyclic esters derived from all four nucleosides mentioned are stable in 0·1 mol l^{-1} HCl for 72 h at room temperature whereas 0·1 mol l^{-1} LiOH cleaves these compounds quantitatively to the derivatives *II* or *III* under the same conditions.

2′- and 3′-O-Phosphonylmethylnucleoside Methyl Esters *IV, V*

Nucleoside 2′(3′)-chloromethanephosphonate (0·5 mmol, ammonium or lithium salt[2]) was dried by codistillation with dioxane *in vacuo*. This material was dissolved in 1 mol l^{-1} sodium methoxide in methanol (10 ml) and the solution left to stand overnight at 40°C under exclusion of moisture. The mixture was added to a suspension of Dowex 50X8 (pyridinium) (50 ml) in 50% aqueous pyridine (50 ml) precooled with ice. After 30 min stirring the suspension was filtered and

TABLE I
HPLC Data

Compound[a,b]	k[c]	System[d]	Compound[a,b]	k[c]	System[d]
IIa	3·90	A	*IIc*	1·94	B
IIIa	3·48	A	*IIIc*	1·13	B
IVa, Va	6·75; 7·65	A	*IVc*	4·11	B
VIa	4·80	A	*Vc*	5·17	B
VIIa	4·05	A	*VIc*	3·62	B
2′,3′-U > p	2·03	A	*VIIc*	5·72	B
3′-Up	4·53	A	2′,3′-A > p	2·74	B
IIb + IIIb	1·83; 2·50	A	2′-Ap	1·79	B
IVb + Vb[e]	4·28; 4·85	A	3′-Ap	1·15	B
VIb + VIIb[f]	2·58; 2·95	A	*IId + IIId*	1·20; 2·03	C
2′,3′-C > p	1·48	A	*IVd + Vd*[g]	3·28; 4·05	C
2′-Cp	2·05	A	*VId + VIId*	2·45; 4·40	C
3′-Cp	2·63	A	2′,3′-G > p	0·88	A
			2′-Gp	0·69	A
			3′-Gp	0·41	A

[a] Paper chromatography of compounds *II*—*VII* in the system 2-propanol–conc. ammonia–water (7 : 1 : 2) and paper electrophoresis (20 V/cm) at pH 7·5 were identical with the data of their nucleotide counterparts; [b] abbreviations: one-letter symbolics, >p cyclic nucleotide; [c] capacity factor $k = (t_R - t_0)/t_0$, t_R retention time, t_0 hold-up time; [d] 0·1 mol l^{-1} triethylammoniumborate pH 8·76 (A), containing 10% methanol (B) or 5% methanol (C); [e] ratio 46 : 54; [f] ratio 57 : 43; [g] ratio 62 : 38.

washed with 50% aqueous pyridine, the combined filtrates were evaporated *in vacuo*, codistilled with water to remove pyridine and finally with 0·05 mol l^{-1} triethylammonium hydrogen carbonate (pH 7·5). The purification by DEAE-Sephadex A-25 chromatography afforded the title compounds (as lithium salts) in 90—95% yields. Mixed isomers *IV*, *V* derived from uridine, cytidine, adenosine, and guanosine were prepared by this procedure.

The compounds *IV* and *V* are stable in 0·1 mol l^{-1} HCl after 72 h at room temperature and decompose quantitatively to compounds *II*, *III* by treatment with 0·1 mol l^{-1} LiOH after 10 h at room temperature.

Enzyme Assays

The incubation mixture contained 125 nmol compounds *IV*— *VII* in 100 μl 0·05 mol l^{-1} sodium citrate buffer pH 7·5 (RNase A and T1), pH 6·3 (RNase T2 and spleen cPDase) or pH 4·5 (RNase U2) and the following enzyme amounts: 7·5 μg RNase A, 12·5 e.u. RNase T1 or T2, 0·25 e.u. spleen cPDase, and 25 e.u. RNase U2. The assays with ribonucleoside 2',3'-cyclic phosphates were performed under identical conditions. Control experiments of non-enzymatic hydrolysis were run under the same conditions with both types of compounds except for the enzymes which were omitted. The mixtures were incubated at 40°C for 15 h and frozen before analysis at —60°C. Samples (1·5—2 μl) were analyzed by HPLC technique (Table I).

Under the given conditions, control experiments of non-enzymatic hydrolysis with compounds *IV*— *VII* were essentially negative; in the presence of enzymes, compounds *IV*— *VII* were intact whereas the ribonucleoside 2',3'-cyclic phosphates were in all cases quantitatively transformed into the corresponding nucleotides.

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