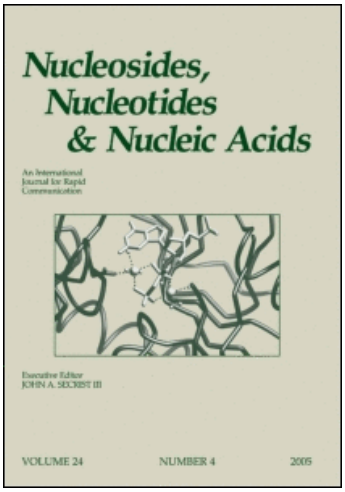

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Online publication date: 05 December 2003

To cite this Article Karpenko, Inna L. , Jasko, Maxim V. , Andronova, Valeriya L. , Ivanov, Alexander V. , Kukhanova, Marina K. , Galegov, Georgy A. and Skoblov, Yury S.(2003) 'Synthesis and Antiherpetic Activity of Acyclovir Phosphonates', Nucleosides, Nucleotides and Nucleic Acids, 22: 3, 319 — 328
To link to this Article: DOI: 10.1081/NCN-120021431
URL: http://dx.doi.org/10.1081/NCN-120021431

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 22, No. 3, pp. 319–328, 2003

Synthesis and Antiherpetic Activity of Acyclovir Phosphonates

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ABSTRACT

Phosphonate derivatives of acyclovir containing phosphorous acid and ethoxycarbonylphosphonic acid residues as well as their isopropyl esters were prepared. They selectively inhibited the herpes simplex virus 1 reproduction in Vero cell culture, the efficacy of esters being 3-4 times higher than that of **ACV**. The hydrolysis of the synthesized compounds was studied in the PBS buffer and human blood serum.

Key Words: Acyclovir; Herpes simplex virus type 1; Phosphonate.

INTRODUCTION

Acyclovir (9-[(2-hydroxyethyloxy)methyl]guanine) (**ACV**) is one of the widely used drugs for treatment of herpes infection. The mechanism of its action involves

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DOI: 10.1081/NCN-120021431
1525-7770 (Print); 1532-2335 (Online)

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a series of successive stages, namely, phosphorylation catalyzed by viral and cellular kinases, the HSV DNA polymerase-catalyzed incorporation of the resulting triphosphate into the growing DNA chain, and termination of the proviral DNA synthesis. However, the bioavailability of acyclovir is low. Many attempts were made to increase it by the introduction of chemical groups facilitating the cell penetration, affecting the metabolism pathway and rate, etc.[1] This approach was successfully realized in antiherpetic drugs Valaciclovir® and Famciclovir®.

In the case of nucleotide analogues displaying antiviral activity this "pronucleotide" approach was also rather promising. For example, when 3′-azido-3′-deoxythymidine and 2′,3′-didehydro-2′,3′-dideoxythymidine were modified with phosphonic acid derivatives, the resulting phosphonates occasionally demonstrated an enhanced anti-HIV activity and lower toxicity than those of parent nucleosides.[2,3] Moreover, we showed earlier that acyclovir hydrogenphosphonate **I** was active in Vero cell cultures infected with several HSV-1 strains including the **ACV**-resistant strain.[4] It is worth noting that the modification of this type was not always successful. In the case of 5-substituted 2′-deoxyuridines, the phosphonates of this type did not offer advantages over the parent nucleosides in respect to antiviral properties.[5–7]

The aim of our work was the synthesis of **ACV** ethoxycarbonylphosphonate **II**, isopropyl esters **III** and **IV**, and carboxyphosphonate **V** and evaluation of their properties towards various HSV-1 strains including **ACV**-resistant ones.

RESULTS AND DISCUSSION

Several methods of synthesis of nucleoside 5′-phosphonates have been described. Ethoxycarbonylphosphonic acid was coupled with guanine-containing nucleosides in the presence of 1,3-dicyclohexylcarbodiimide; the yields of the products did not thereby exceed 50%.[8] To phosphonylate protected guanosine nucleosides, ethoxycarbonylphosphonic dichloride was used.[9] Although the yields of target products were 72–85%, the extra stage of the preparation of phosphonylating agent can be referred to disadvantages of the method. In the reaction of ethoxycarbonylmethylphosphonic acid with **ACV** in the presence of TPSCl, the acid residue was added both to the side chain and the guanine base. When N-acetyl derivative of **ACV** was used in this reaction, the target product was obtained, but its yield achieved only 9%.[6]

We used the coupling of **ACV** with ethoxycarbonylphosphonic acid (prepared *in situ* by the treatment of triethyl phosphonoformate with Me_3SiBr) in the presence of PivCl as a condensing agent (Sch. 1). The yield of isolated phosphonate **II** was 74%. Isopropyl esters **III IV** were prepared by the coupling of isopropanol with phosphite **I** in the presence of PivCl or with ethoxycarbonylphosphonate **II** in the presence of TPSCl. Carboxyphosphonate **V** was obtained by treatment of phosphonate **II** with aqueous alkali.

The homogeneity of the compounds was controlled by HPLC; their structure was confirmed by ^1H and ^{31}P NMR spectral data. The ^{31}P NMR spectrum of ester **III** in CD_3OD represented a resonance group interpreted as ddt with the coupling constants $^1J_{P,H} = 712$ Hz and $^3J_{P,H} \sim 9$ Hz (see Fig. 1A); it was transformed into a

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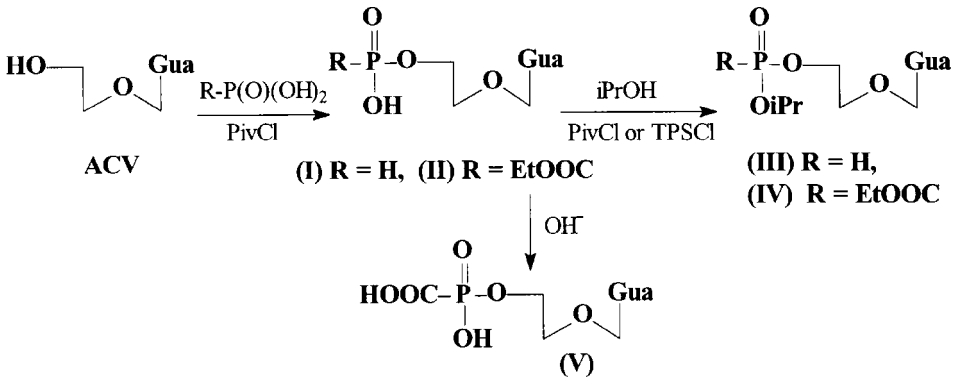


Scheme 1.

broad singlet when the proton-phosphorus interaction was decoupled (Fig. 1B). It is worth noting that in the case of D_2O as a solvent, the hydrogen atom in the P-H bond was substituted by a deuterium one. It was clearly observed in the pattern of the proton-decoupled ^{31}P-NMR spectrum, in which a singlet resonance at 8.6 ppm disappeared and a triplet at 8.3 ppm with $^{1}J_{P,D} = 109$ Hz was observed

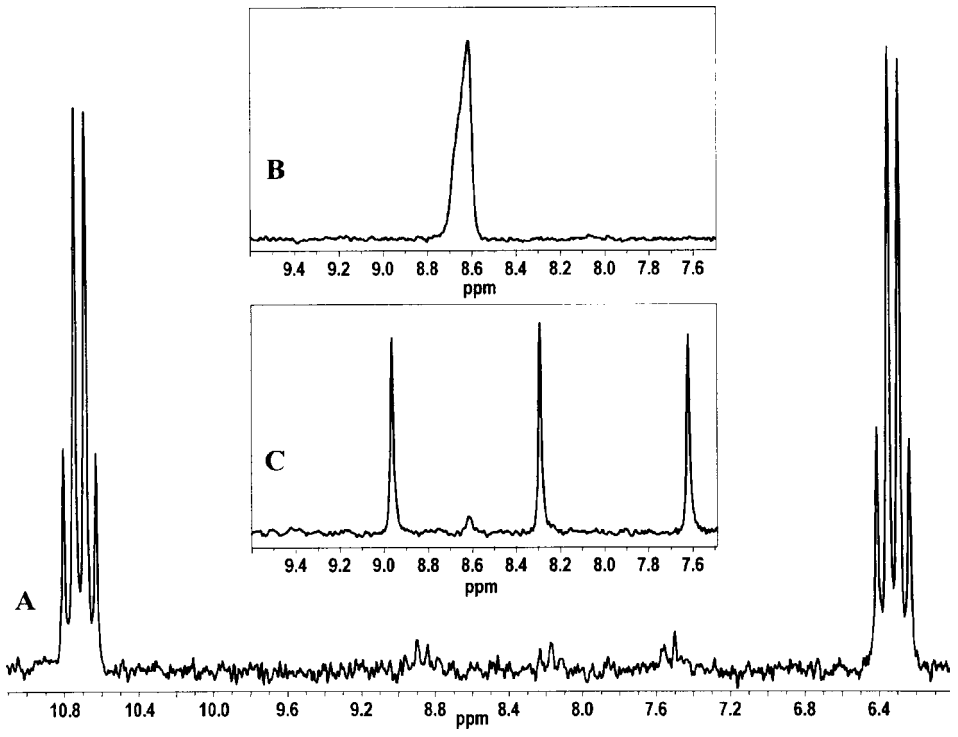


Figure 1. ^{31}P-NMR patterns of ester **III**.

(Fig. 1C). When the spectrum of ester **III** was registered in DMSO-d_6, the coupling constant $^1J_{P,H}$ was decreased to the value of 698 Hz.

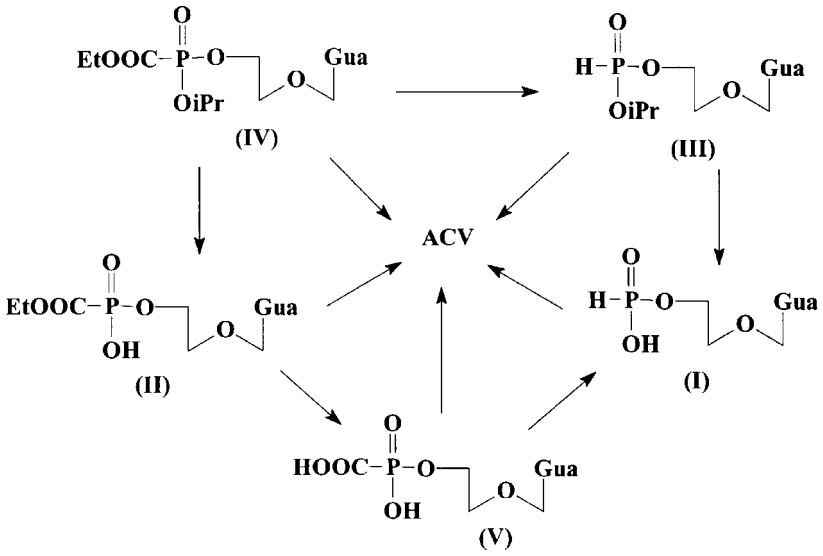
When planning the synthesis of hydrogenphosphonate and phosphonoformate **ACV** derivatives **II**–**V**, we presumed that these compounds are actual **ACV** precursors. When penetrated into the cell, they will be converted in **ACV** with different efficacy. Scheme 2 demonstrates possible hydrolysis pathways for the synthesized compounds independently of the reaction mechanism.

We studied the hydrolysis of compounds **I**–**V** in PBS and in human blood serum (Table 1); the aliquots were analyzed by HPLC analysis. Anionic phosphonates **I**, **II** and **V** displayed high stability both in PBS and in human blood serum. Carboxyphosphonate **V** was extraordinarily stable: only **ACV** was found after 3 days of incubation in trace quantities.

It is interesting to note that phosphonate **II** was hydrolyzed by two alternative ways to give **ACV** or carboxyphosphonate **V**. The hydrolysis in PBS gave **ACV** and **V** in a ratio of about 1 : 1, whereas carboxyphosphonate **V** prevailed in human blood serum. When comparing the **ACV** accumulation rates from **II** in the aforementioned media, one can imply that carboxyphosphonate **V** is mainly resulted from enzymatic hydrolysis in human blood serum, whereas **ACV** is a product of chemical hydrolysis.

Chemical transformations of phosphonates **IV** and **V** could involve the formation of the corresponding hydrogenphosphonates **III** and **I** as was observed for some phosphonoformates.[10] However, according to the data presented in Table 1, the products of such transformation were not found.

The table demonstrates the averaged data of three independent series; the experimental error for the compounds **I**–**IV** did not exceed 10%.



Scheme 2.

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Table 1. Stability of compounds **I–V** in PBS and human blood serum.

	PBS			Human blood serum		
Comp.	Incubation time	Relative amount of the components*		Incubation time	Relative amount of the components*	
I	72 h	**I:ACV**	98:2	72 h	**I:ACV**	82:18
II	72 h	**II:ACV:V**	84:7:9	72 h	**II:ACV:V**	38:8:54
III	90 min	**III:ACV**	53:47	35 min	**III:ACV**	48:52
IV	45 min	**IV:ACV:II**	54:36:10	10 min	**IV:ACV:II**	35:47:18
V	72 h	**V**	~100**	72 h	**V**	~100**

*The amount of starting compound at t = 0 min is taken as 100%.
Traces of **ACV were observed.

The stability of esters **III** and **IV** is substantially lower both in the buffer and in the blood serum if compared with phosphonates **I** and **II**, respectively. It is essential that **ACV** was the only detected hydrolysis product of ester **III** in all the conditions under study. Since phosphonate **I** is hydrolytically stable, we can imply that ester **III** is hydrolyzed directly to **ACV** (Sch. 2).

ACV and phosphonate **II** were identified as hydrolysis products of ester **IV** both in the buffer and blood serum. Ester **III** was not found. The major product was also **ACV**, although the rate of accumulation of phosphonate **II** was higher in the blood serum (Fig. 2).

We studied antiherpetic activity of the synthesized phosphonates **I–V** in Vero cell culture infected with various HSV-1 strains. According to the data presented in Table 2, compounds **I–V** are low toxic. They completely inhibited virus reproduction (ID_{95}) in Vero cells infected with HSV-$1/L_2$ at nontoxic concentrations. Uncharged esters **III** and **IV** were more effective than **ACV**. Compounds **II–V** were inactive towards laboratory strain of **ACV**-resistant herpes simplex virus

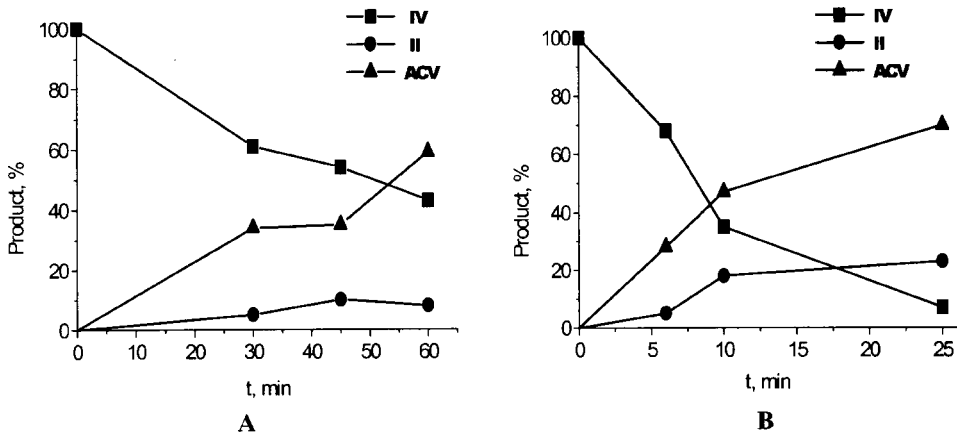

Figure 2. Hydrolysis of ester **IV** in PBS (A) and human blood serum (B).

Table 2. Inhibition of HSV-1 reproduction in Vero cell culture with compounds **I**–**V**.

		HSV-1/L_2			HSV-1/L_2/R			HSV-1 (Avd)		
Comp.	CTD_{50} μM	ID_{50} μM	ID_{95} μM	SI	ID_{50} μM	ID_{95} μM	SI	ID_{50} μM	ID_{95} μM	SI
I	3922	20.4	81.7	192	40.8	327	96	20.4	81.7	192
II	> 2646	330.7	661	> 8	331	661	> 8	–	–	–
III	> 3021	0.45	1.89	> 6713	181	> 363	> 16.7	1.9	3.8	> 1590
IV	> 2481	0.37	0.77	> 6705	149	> 298	> 16.7	0.77	3.1	> 3222
V	> 2857	35.7	71.4	> 80	286	> 286	> 10	143	286	> 20
ACV	> 2226	1.82	2.27	> 1223	486	> 486	> 4.6	6.3	101	353

CTD_{50} – 50% cytotoxic dose for uninfected Vero cells; ID_{50} and ID_{95} – doses inhibiting the viral cytopathogenic effect by 50 and 95%, respectively; SI – selectivity index (CTD_{50}/ID_{50} ratio).

(HSV-1/L_2/R). This fact indirectly supports our hypothesis about the transformation of compounds **II**–**V** into **ACV**. At the same time esters **III** **IV** were substantially active towards the clinical isolate of HSV-1 (Avd) strain weakly resistant to **ACV**.

Their potency may be accounted for by more effective penetration through cell membranes and, as a consequence, by higher intracellular **ACV** concentration. More thorough studies of cellular metabolism of compounds **III** and **IV** will allow the evaluation of the correctness of this assumption.

The analysis of the data on hydrolysis of compounds **I**–**V** in PBS and blood serum shows that only some of the possible transformations presented on Scheme 2 are realized. If to compare these data with the ones on antiherpetic activity, one can assume that the rate and mechanism of hydrolysis of compounds **I** and **V** in Vero cells differed from those in blood serum. This hypothesis is based on the observed discrepancy between high hydrolytic stability and pronounced antiviral activity of phosphonates **I** and **V** (Tables 1 and 2).

EXPERIMENTAL

Triethyl phosphonoformate, PivCl, and Dowex 50 W X 8 were from Fluka, TPSCl was from Aldrich. Hydrogenphosphonate **I** was obtained as described in Ref.[4].

UV spectra were recorded on a Shimadzu UV-2401PC spectrophotometer (Japan). ^{1}H NMR spectra (400 MHz) and ^{31}P NMR spectra (162 MHz) were registered on a Bruker AMX III-400 spectrometer (Germany). Column chromatography was performed on DEAE-Toyopearl 650 M (Toyo Soda, Japan), Kieselgel 60 (63–100 μm), LiChroprep RP-8, and RP-18 (25–40 μm) (Merck, Germany). HPLC was performed on a Gilson chromatograph (France) using a Lichrosorb RP-18 (7 μm, Merck) column (4 × 150 mm) in a gradient of MeOH in 5 mM sodium phosphate buffer (pH 4.8); gradient A: 0% for 5 min, 0 → 7% for 5 min, 7 → 17% for 25 min, and 17 → 66% for 5 min; gradient B: 0% for 5 min; 0 → 7% for 5 min,

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$7 \rightarrow 17\%$ for 25 min, $17 \rightarrow 53\%$ for 5 min, $53 \rightarrow 66\%$ for 5 min, and 66% for 5 min; flow rate 0.4 mL/min; detection at 254 nm.

Acyclovir Ethoxycarbonylphosphonate, Ammonium Salt (II). Trimethylbromosilane (1.8 mL, 13.9 mmol) was dropped to a precooled (0°C) solution of triethyl phosphonophormate (1 mL, 5.28 mmol) in carbon tetrachloride (3 mL), and after 18 h at 20°C the solution was evaporated and coevaporated with toluene (3×5 mL). The residue was dissolved in 2 mL of pyridine and 2 mL of water and after 1 h the solution was evaporated and coevaporated with pyridine (3×5 mL). The residue was dissolved in pyridine (3 mL) and added to the suspension of acyclovir (770 mg, 3.4 mmol) in DMF (3 mL). The mixture was cooled (0°C) and PivCl (1 mL, 8.1 mmol) was added. After stirring at 5°C for 18 h, the reaction mixture was diluted with water (50 mL), and the solution was loaded on a DEAE-Toyopearl column (3×10 cm). The product was eluted in a linear gradient of NH_4HCO_3 ($0 \rightarrow 0.1$ M, 1 l). The target fractions were concentrated and repurified on a LiChroprep RP-8 column (3×25 cm) by eluting with water. The target fractions were freeze-dried to give 948 mg (74%) of compound **II**; retention time 26 min (gradient A). UV (H_2O, pH 7): λ_{max} 254 nm. ^{1}H-NMR (CD_3OD, δ, ppm, *J*, Hz): 7.88s (1H, H-8), 5.50s (2H, CH_2Gua), 4.18q (2H, J_{CH_2,CH_3} 7.2, CH_2-Et), 4.11m (2H, CH_2OP), 3.75dd (2H, *J* 4.4, 5, CH_2OC), 1.28t (3H, CH_3-Et). ^{31}P-NMR (CD_3OD, δ, ppm, *J*, Hz): −4.51t (*J* 7).

Acyclovir P-(isopropyl) Hydrogenphosphonate (III). To a precooled (0°C) solution of hydrogenphosphonat **I** (71 mg, 0.23 mmol) and isopropanol (0.5 mL) in pyridine (5 mL), PivCl (100 μL, 0.8 mmol) was added, and the mixture was kept overnight at 5°C. After dilution with water (10 mL), the reaction mixture was evaporated in a vacuum, coevaporated with water (3×5 mL), and the residue was purified on a LiChroprep RP-8 column (2×18 cm) eluting in a linear gradient of MeOH ($0 \rightarrow 20\%$, 1 l) in water. The target fractions were evaporated, dissolved in water and freeze-dried to yield 43 mg (56%) of compound **III**; retention time 42 min (gradient B). UV (MeOH): λ_{max} 254 nm. ^{1}H-NMR (DMSO-d_6): 10.62s (1H, NH), 7.82s (1H, H-8), 6.79d (1H, $J_{H,P}$ 698, H-P), 6.51s (2H, NH_2), 5.37s (2H, CH_2Gua), 4.55m (1H, CH-iPr), 4.05m (2H, CH_2OP), 3.66t (2H, *J* 4.4, CH_2OC), 1.23d (6H, $J_{CH_3,CH}$ 6.2, CH_3-iPr). ^{31}P-NMR (DMSO-d_6): 8.16ddt ($^1J_{H,P}$ 698, $^3J_{P,CH_2} \sim {}^3J_{P,CH}$ 9.2).

Acyclovir P-(isopropyl) Ethoxycarbonylphosphonate (IV). To a precooled (5°C) solution of phosphonate **II** (76 mg, 0.2 mmol) in pyridine (5 mL) and isopropanol (0.5 mL), TPSCl (240 mg, 0.8 mmol) was added in two portions during 3 h, the reaction mixture was kept overnight at 5°C, evaporated in a vacuum, coevaporated with water (3×5 mL) and the residue was purified by column chromatography on LiChroprep RP-8 (2×18 cm) eluting in a linear gradient of MeOH ($0 \rightarrow 30\%$, 0.5 L) in water. The fractions containing compound **IV** were evaporated, coevaporated with EtOH, dissolved in $CHCl_3$ (1.9 mL) and MeOH (0.1 mL) and chromatographed on a silica gel column (2.5×25 cm) in a mixture of $CHCl_3$-MeOH 9 : 1 (*v*/*v*). The target fractions were evaporated, the residue was dissolved in water and freeze-dried to give 31 mg (38%) of compound **IV**; retention time 43 min (gradient B). UV (MeOH): λ_{max} 254 nm. ^{1}H-NMR (CD_3OD): 7.87s (1H, H-8), 5.49s (2H, CH_2Gua),

4.79m (1H, CH-iPr), 4.31m (4H, CH_2OP, CH_2-Et), 3.83dd (2H, J 3.7, 4.7, CH_2OC), 1.30–1.36m (9H, CH_3-iPr, CH_3-Et). ^{31}P-NMR (CD_3OD): −4.87dt ($^3J_{P,CH_2} \sim {}^3J_{P,CH}$ 7.1).

Acyclovir Carboxyphosphonate, Ammonium Salt (V). A solution of 1 M sodium hydroxide (600 μL, 0.6 mmol) was dropped to a precooled (5°C) solution of phosphonate **II** (70 mg, 0.19 mmol) in water (5 mL). After stirring at 20°C for 18 h the reaction mixture was loaded on a Dowex-50 (NH_4^+-form) column (3.5 × 10 cm) and eluted with water. The eluate was evaporated, and the residue was purified on a LiChroprep RP-18 column (2 × 18 cm) eluting with water. The target fractions were freeze-dried to give 46 mg (74%) of compound **V**; retention time 11 min (gradient A). UV (H_2O, pH 7): λ_{max} 254 nm. ^{1}H-NMR (D_2O): 7.95s (1H, H-8), 5.53s (2H, CH_2Gua), 4.03m (2H, CH_2OP), 3.78m (2H, CH_2OC). ^{31}P-NMR (D_2O): 1.48br.s.

Experiments with Cell Cultures

Vero cells culture (African green monkey kidney cells) was from the Laboratory of Tissue Culture, Institute of Virology. The cells were maintained on Eagle's medium supplemented with 5% fetal calf serum (Fluka).

HSV-1/L_2 strain from the virus collection of the Institute of Virology was used. The mutant strain HSV-1/L_2/R resistant to acyclovir was obtained by serial passages in the presence of increasing concentrations of **ACV** at low multiplicity of infection (0.01 PFU/cell). The resulting viral strain was used to obtain the viral clone resistant to **ACV** (for the protocols, see Ref.[11]). Clinical isolate of acyclovir-resistant HSV-1 strain was obtained by infecting Vero cells with virus – containing material that was taken from patients according to Ref.[12]. Viral strains were passed in a 1:1 mixture of Eagle's and 199 media supplemented with 2% fetal calf serum. For cell cultivation and infection, 96-well plastic plates (Linbro, Flow Lab., UK) were used.

The antiviral activity was measured by the capacity of the compounds under study to inhibit the development of virus-induced cytopathogenic effect (CPE) by 50% (ID_{50}) and 95% (ID_{95}) as compared with CPE in the control infected cultures.[13] The multiplicity of infection was 0.1 PFU/cell. The infection titer was determined after 48-h incubation of the infected culture.

Cytotoxicity was measured by the capacity of dead cells to be stained with Trypan Blue.[14] The CTD_{50} value was determined as the concentration of the compound that provided 50% viability of the cells after 72-h contact with the compound under study.

Hydrolysis of Compounds in PBS and Human Serum

Hydrolysis of compounds was studied in the PBS (pH 7.2) (0.14 M NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, and 8.1 mM Na_2HPO_4). The compound under study (5 μL, 10 mM aqueous solution) was added to the PBS (95 μL) and incubated at 37°C. After a certain interval (Table 1) an aliquot (15 μL) was concentrated on an Automatic Speed-Vac Concentrator AS260 (USA). The residue was diluted with 5 mM sodium phosphate buffer (10 μL, pH 4.8) and analyzed by HPLC. For compounds **I**, **II**, **V** gradient A and for esters **III** **IV** gradient B were used.

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Human blood serum (95 μL) was added to 10 mL aqueous solution of the tested compound (5 μL), and the mixture was incubated at 37°C. An aliquot (15 μL) was taken, MeOH (45 μL) was added, and the mixture was kept for 20 min at −20°C. The mixture was centrifuged on an Eppendorf Centrifuge 5415 (Germany) at 15,800 g, the supernatant was concentrated on an Automatic Speed-Vac Concentrator AS260. The sample was diluted with 5 mM sodium phosphate buffer (10 μL, pH 4.8) and analyzed by HPLC.

ABBREVIATIONS

ACV	acyclovir
HSV-1/L_2	herpes simplex virus type 1
PivCl	pivaloyl chloride
TPSCl	2,4,6-triisopropylbenzenesulfonyl chloride
PBS	the phosphate buffer solution

ACKNOWLEDGMENT

The work was supported by the Russian Foundation for Basic Research, projects No. 01-04-48627, No. 02-04-49009, and No. 02-04-06525.

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Received August 14, 2002
Accepted January 27, 2003

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