SYNTHESIS AND ANTIVIRAL ACTIVITY OF HYDROPHOSPHORYLATED INOSINES

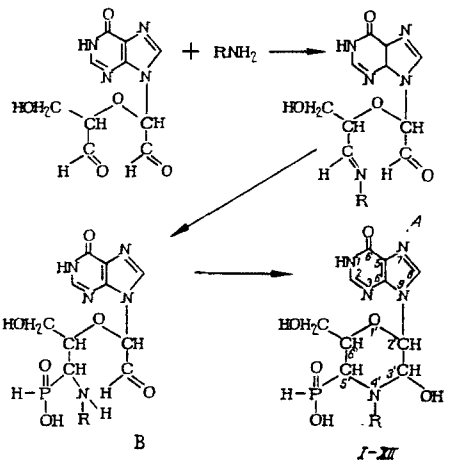
V. V. Belakhov, A. A. Levina, Yu. D. Shenin, B. I. Ionin, E. B. Shtil'bans, L. A. Rachkovskaya, É. V. Chekunova, S. S. Marennikova, and M. A. Shneider*

UDC 615.281:547.24
1].012.1.07

In recent years, the chemotherapy of viral infections has become an independent discipline, which has resulted in the development and introduction into medical practice of a number of highly active antiviral drugs (such as remantadine, virazol, and acyclovir). The most significant group of antiviral compounds to have found medical applications are nucleosides modified in the heterocyclic or carbohydrate moieties [2-4, 6, 10, 13, 14]. Increasing attention is now being paid to phosphorus-bearing nucleosides, which are of low toxicity and are highly active against DNA- and RNA-containing viruses [1, 6-8, 12].

In a search for organophosphorus derivatives of inosine with antiviral activity, we have examined the reactions of inosinedialdehyde (a dicarbonyl derivative of inosine) with primary amines and hypophosphorous acid. These reactions have given 9-[3′-hydroxy-4′-alkyl(and aryl)-5′-(P-hydroxy-P-phosphoryl)-6′-hydroxymethyl-1′,4′-morpholyl]hypoxanthines, i.e., the carbohydrate moiety has been converted into a morpholine ring bearing a phosphonite group at C(5′) and a primary amine residue at N(4′).

Clearly, the first stage involves addition of the primary amine to a C=O group of the inosinedialdehyde to give the azomethine (A), which then reacts with hypophosphorous acid at the C=N double bond. The resulting aminophosphonous acid derivative of inosinedialdehyde (B) then undergoes cyclization to give (I-XII).



R = CH_2COOH (I); $CH_3CHCOOH$ (II); *o*-$NO_2C_6H_4$ (III); P IC_6H_4 (IV); $(CH_3)_2CHCHCOOH$ (V); $C(NH_2)(O)(CH_2)_2CHCOOH$ (VI); $NH_2(CH_2)_4CHCOOH$ (VII); $C_6H_5CH_2CHCOOH$ (VIII); $C_2H_5CH(CH_3)CHCOOH$ (IX); P $OHC_6H_4CH_2CHCOOH$ (X); $CH_2(OH)CHCOOH$ (XI); $CH_3S(CH_2)_2CHCOOH$ (XII).

*Deceased.

All-Union Research Institute for the Technology of Antibiotics and Enzymes for Medicinal Use, Leningrad. Translated from Khimiko-farmatsevticheskii Zhurnal, Vol. 24, No. 9, pp. 44-48, September, 1990. Original article submitted October 3, 1989.

TABLE 1. Hydrophosphoryl Derivatives of Inosine

Compound	Yield, %	mp, °C (decomp.)	Empirical formula	IR spectrum, ν, cm^{-1}	UV spectrum, λ, nm
I	49	201—206	$C_{10}H_{16}N_5O_8P$	1215 (P=O), 1705 (C=N), 2385 (P—H)	225, 250
II	62	123—128	$C_{13}H_{20}N_5O_9P$	1226 (P=O), 1698 (C=N), 2382 (P—H)	221, 248
III	37	245—251	$C_{16}H_{19}N_6O_9P$	1232 (P=O), 1595 (o-$NO_2C_6H_4$) 1703 (C=N), 2374 (P—H)	221, 249, 265
IV	46	217—222	$C_{16}H_{19}JN_5O_7P$	1219 (P=O), 1603 (p-JC_6H_4), 1690 (C=N), 2379 (P—H)	228, 250, 259
V	57	208—213	$C_{15}H_{24}N_5O_9P$	1227 (P=O) 1695 (C=N), 2392 (P—H)	228, 249
VI	63	233—238	$C_{15}H_{23}N_6O_{10}P$	1239 (P=O) 1715 (C=N), 2405 (P—H)	228, 250
VII	47	167—171	$C_{16}H_{27}N_6O_9P$	1244 (P=O), 1703 (C=N), 2386 (P—H)	224, 251
VIII	42	198—203	$C_{19}H_{24}N_5O_9P$	1229 (P=O), 1591 (C_6H_5), 1697 (C=N), 2400 (P—H)	225, 241, 250
IX	56	209—212	$C_{16}H_{26}N_5O_9P$	1241 (P=O), 1711 (C=N), 2387 (P—H)	
X	38	223—229	$C_{19}H_{24}N_5O_{10}P$	1249 (P=O), 1598 (p-OHC_6H_4) 1707 (C=N), 2389 (P—H)	228, 250, 262
XI	47	185—189	$C_{13}H_{20}N_5O_{10}P$	1223 (P=O), 1713 (C=N), 2407 (P—H)	223, 249
XII	31	172—177	$C_{15}H_{24}N_5O_9P$	1237 (P=O), 1717 (C=N), 2396 (P—H)	227, 249

Note. The R_f values of (I-XII) were determined by thin layer chromatography in the systems butanol—ethanol—water (4:1:5), 2-propanol—ammonia (conc.)—water (7:1:2), and butanol—acetic acid—water (5:2:3). They were: 0.52; 0.80; 0.69 (I); 0.61; 0.72; 0.52 (II); 0.63; 0.69; 0.57 (III), 0.58; 0.76; 0.63 (IV); 0.51; 0.64; 0.57 (V); 0.59; 0.65; 0.46 (VI); 0.55; 0.66; 0.50 (VII); 0.53; 0.72; 0.65 (VIII); 0.56; 0.69; 0.74 (IX); 0.58; 0.67; 0.43 (X); 0.54; 0.63; 0,48 (XI); 0.67; 0.78; 0.51 (XII).

The hydrophosphorylating agent used was hypophosphorous acid, which is highly reactive and selective, is of low toxicity, and has an acidic group attached to phosphorus, thus enabling water-soluble derivatives to be obtained which are convenient for biological studies [1, 7]. The following primary amines were employed: 1) aromatic amines (o-nitroaniline and p-iodoaniline), and 2) aminoacids (glycine, DL-α-alanine, L-glutamine, DL-lysine, DL-valine, DL-β-phenyl-α-alanine, L-isoleucine, DL-tyrosine, DL-serine, and DL-methionine.

The hydrophosphorylated inosines obtained (I-XII) were solids which crystallized readily from water or methanol—water (1:1). The compounds (I-XII) did not have sharp melting points, and decomposed on heating. They were readily soluble in dimethyl sulfoxide and dimethylformamide, soluble in water, sparingly soluble in alcohols (methanol, ethanol, propanol, etc.), and insoluble in acetone, chloroform, ether, benzene, hexane, and carbon tetrachloride.

The physicochemical constants and spectral data for the compounds obtained are given in Table 1.

The structures of the products were established by ^1H and ^{31}P NMR, IR, and UV spectroscopy.

The PMR spectra of the hydrophosphorylated inosines (I-XII) showed signals for the protons of the purine base and the morpholine ring, the methylene group (as a doublet, OCH_2), a doublet for the proton at phosphorus with a coupling constant J(PH) of 537 Hz, and signals for the protons of the amine moiety at the N(4′) nitrogen. The signals for the hydroxylic protons and the proton of the acid function were seen at low field (δ 10-12 ppm). In the PMR spectrum of (III), (IV), and (X) the benzene ring, substituted at the para- or the ortho-position, gave rise to two pairs of chemically equivalent, but magnetically

TABLE 2. Toxicity and Antiviral Activity of Hydrophosphorylated Inosines (I-XII)

Compound	Toxicity, mg/embryo		PI %					
			pox vaccine virus		RSV		influenza virus	
	LD_{50}	MTD	prophylactic mode	therapeutic mode	prophylactic mode	therapeutic mode	type A	type C
I	4,5	3,0	62,7	41,5	62,9	71,4	51,6	33,0
II	4,5	3,0	51,7	35,7	53,2	51,7	39,7	20,0
III	4,5	3,5	72,4	68,5	60,0	61,0	57,5	47,3
IV	4,0	3,0	78,5	41,8	60,7	74,9	0	0
V	4,5	3,5	86,5	11,5	56,7	19,6	60,6	47,1
VI	4,0	2,5	82,7	55,6	82,8	56,8	58,0	43,3
VII	4,0	3,0	65,1	57,8	61,7	47,5	0	0
VIII	4,0	3,0	85,2	58,9	70,6	47,5	20,0	0
IX	4,5	4,0	80,9	51,8	65,0	45,8	33,0	0
X	4,5	3,0	60,2	39,4	49,0	33,0	0	0
XI	4,0	3,0	78,0	45,8	57,4	55,9	0	0
XII	4,0	3,0	80,2	51,3	56,7	61,9	0	0
Ribamide	3,0	2,0	98,0	85,9	78,0	86,0	—	—
Remantadine	4,0	—	—	—	—	—	78,0	—

nonequivalent nuclei (ABA'B'), or a multiplet when no substituent was present (VIII). The IR spectra of (I-XII) showed absorption characteristic of the P–H, P=O, C=N, C_6H_4R, and other groups of the purine base, the morpholine ring, and the amine residues.

In the UV spectra, all the compounds showed a maximum at 248-251 nm, characteristic of nitrogenous bases. The compounds incorporating a phenyl substituent showed an absorption maximum in the 241-265 nm region.

Chemical reactions have not been used to characterize these compounds, since such work has already been carried out for inosinedialdehyde derivatives [8].

EXPERIMENTAL (CIIEMICAL)

^1H and ^{31}P NMR spectra were obtained on a Bruker AC-200 (West Germany) instrument, operating frequency 200 MHz. The compounds were examined as the 25% solutions in $(CD_3)_2SO$, internal standard hexamethyldisiloxane, or as the 40-45% solutions in D_2O with an external standard. The δ_{31_P} chemical shifts were measured from 85% H_3PO_4 as external standard. IR spectra were recorded on a Specord IR-75 spectrophotometer (East Germany) in KBr disks. UV spectra were obtained on a Specord UV-VIS (East Germany). The purity of the products was checked by thin layer chromatography on Silufol UV-254 plates (Czech SSR) in the systems butanol–ethanol–water (4:1:5, upper layer), 2-propanol–25% aqueous ammonia–water (7:1:2), and butanol–acetic acid–water (5:2:3). The compounds were visualized on the chromatograms by UV.

The elemental analyses were in satisfactory agreement with the calculated values.

Inosinedialdehyde was obtained by oxidizing inosine with periodic acid, as in [11]. Hypophosphorous acid, available as the 50% aqueous solution, was dried by lyophilization before use.

9-[3'-Hydroxy-4'-alkyl(or aryl)-5'-(P-hydroxy-P-hydrophosphoryl)-6'-hydroxymethyl-1',4'-morpholyl]hypoxanthine (I-XII). To 0.01 mole of inosinedialdehyde in 10 ml of water was added with stirring 0.012 mole of the amine (an aminoacid or aromatic amine) in methanol. The reaction was carried out at 30°C for 1-2 h. To the reaction mixture was then added 0.012 mole of hypophosphorous acid in methanol, and the reaction continued at 55-60°C for 4-6 h. When reaction was complete, the mixture was cooled, filtered, and the filtrate treated with an excess of acetone. The solid which separated was filtered off, washed with acetone and ether, and dried at 50°C for 4-5 h. The compounds (X-XII) were obtained as finely crystalline solids of varying color.

EXPERIMENTAL (BIOLOGICAL)

The toxicities of the hydrophosphorylated inosines (I-XII) were determined with 10-11 day old developing chick embryos, by introducing the compounds in a range of concentrations into the allantoic cavity, or on the choronoallantoic membrane (CAM). Five embryos were used for each dilution, and they were incubated in a thermostat at 37°C until they hatched, ovoscopy being carried out every other day.

TABLE 3. Viral Inhibitory Activity of Hydrophosphorylated Inosines Against Pox Vaccine Virus at Various Doses

Compound	Percentage succession of Pocks at a dose of (μg/embryo)				ED_{50}	CTI	K
	1000	500	250	125			
III	74,1	62,3	44,5	39,7	327,5	10,7	13,7
V	86,5	50,0	18,4	6,7	500,0	7,0	9,0
VI	80,4	61,8	60,7	8,0	221,0	11,3	18,1
VIII	90,2	72,7	45,0	—	295,0	10,2	13,5
IX	80,0	77,1	20,7	—	379,8	10,5	11,8
Ribamide	98,0	76,0	49,8	43,2	251,9	7,9	11,9

Note. CTI is the chemotherapeutic index, and K is an indicator of the breadth of pharmacological activity of the test compounds, equal to the ratio of the LD_{50} to the ED_{50}.

TABLE 4. Antiviral Activity of Hydrophosphorylated Inosines in a Model Pox Vaccine Virus Infection in Cotton Rats

Compound	Route of administration	PI, %
III	Oral	30,8
	Subcutaneous	33,3
	Intraperitoneal	7,7
V	Oral	23,1
	Subcutaneous	25,0
	Intraperitoneal	14,3
IX	Oral	27,8
	Subcutaneous	33,3
	Intraperitoneal	21,0
Ribamide	Oral	33,3

The antiviral activity of (I-XII) was assessed *in ovo* against the DNA-bearing pox vaccine virus (strain L-IVP 01.72). The working titers of the pox virus vaccine were 10^{-6}-10^{-8} PFU/ml. The virus-containing material was prepared on MacIlvaine's solution. The test compounds were administered to the CAM of 10-11-day old chick embryos in prophylactic mode (one hour before introduction of the virus) and in therapeutic mode (one hour after infection with the virus). The embryos were incubated for 48-72 h at 37°C. The pock count was carried out on the embryonic CAM.

Compounds (III), (V), and (IX), which showed the greatest antiviral activity against the pox virus *in ovo*, were further examined in an experimental orthopox infection of cotton rats weighing 50-70 g. The animals were infected intranasally with pox virus (strain L-IVP, infectious activity $1.6 \cdot 10^9$ PFU/ml, constituting 10 LD_{50} doses). Compounds (III), (V), and (IX) were administered orally, subcutaneously, and intraperitoneally in a dose of 100 mg/kg four hours before infection and on the second day following infection of the animals. The tests were assessed only when all the control animals had died.

The antiviral activity of (I-XII) was also examined against RNA viruses, namely oncogenic Rous sarcoma virus (RSV) (strain RSV-RAV-1) and infectious influenza viruses types A [strain A_2/Odessa 2882/82 (H_3N_2)] and type C (strain C/USSR/69) in developing chick embryos. Following measurement of their toxicities, the compounds were administered (with the RSV) to the CAM of 10-11-day old developing chick embryos in the prophylatic and therapeutic modes. The number of foci of neoplastic transformation on the embryonic CAM were assessed on the eighth day of incubation at 37°C.

Antiinfluenzal activity was assessed by introducing the compounds (I-XII) into the chorionoallantoic cavity of 10-11-day old developing chick embryos in prophylactic mode, i.e., 1-1.5 h before inoculation with 10-100 EID_{50} of virus. The results were assessed when the embryos had been incubated for 48 h at 37°C in the case of type A influenza virus, and after 72 h at 34°C in the case of the type C virus. The presence of virus in the embryos was determined by the hemagglutination reaction with chick erythrocytes in the allantoic fluid of the test and control animals. The control embryos were treated with physiological sodium chloride solution in place of the test compounds.

The examination of the antiviral activity of the test compounds (I-XII) against the pox vaccine virus and RSV *in ovo* was carried out in comparison with the well known antiviral drug ribamide, while in the case of type A influenza virus the reference drug was remantadine. In the experimental orthopox virus infection of cotton rats, the reference drug used was ribamide, given orally in a dose of 100 mg/kg in the standard therapeutic/prophylactic mode.

The antiviral activity of the test compounds *in ovo* was assessed by the protective index (PI), calculated using the formula:

$$PI = \frac{\%\text{ virally infected embryos in controls} - \%\text{ virally infected test embryos}}{\%\text{virally infected embryos in controls}} \cdot 100.$$

The antiviral effects of (III), (V), and (IX) in the tests on cotton rats were assessed as the level of protection (LP), calculated using the formula:

$$LP = \frac{\text{Number of animals surviving}}{\text{Number of animals used in tests}} \cdot 100\%.$$

The test compounds were tested against all the model viruses in at least three series of experiments. The results obtained were treated statistically by the Fisher method, at a probability level of 95%.

The biological tests showed that *in ovo* all the compounds were of low toxicity, being less toxic than ribamide, and less toxic than or comparable with remantadine (Table 2). All the compounds were more active against pox vaccine virus when given in prophylactic mode than when given therapeutically. This behavior is most clearly apparent with (V), (VI), (VIII), and (IX). For example, when given prophylactically, the PI values of these compounds were 86.5, 82.7, 85.2, and 80.9% respectively, and when given therapeutically, 11.5, 55.6, 58.9, and 51.8% respectively ($p < 0.05$). The antiviral activity of (III), (V), (VIII), and (IX), which show the greatest inhibitory activity, was examined in a range of doses in the prophylactic mode (Table 3). The results showed at the therapeutic index (CTI) and the coefficient K (a measure of the breadth of the pharmacological activity of the test compounds, equal to the ratio of the LD_{50} to the ED_{50}) for compounds (III), (V), (VI), (VIII), and (IX) were quite high, ranging from 7.0 to 11.3 and from 9.0 to 13.7 respectively. It is worthy of note that the CTI values of the inosine derivatives were comparable with, or higher than, those for ribamide (Table 3).

Examination of the antiviral activity of (III), (V), and (IX) in a model lethal pox virus infection of cotton rats by various routes showed that these inosine derivatives have significant viral inhibitory activity when given by either the oral or subcutaneous routes (the LP values ranging from 27.8 to 33.3%; Table 4).

There has been a report of the possible protective effects of organophosphorus analogs of nucleosides in AIDS infections [9]. We have examined the viral inhibitory activity of the hydrophosphorylated inosines (I-XII) against the Rous sarcoma rotrovirus, this model having been recommended as adequate for the retroviral screening and examination of anti-AIDS drugs [5]. These tests have shown that most of the compounds tested were active against RSV, the most effective compound in suppressing the foci of neoplastic transformation induced by RSV being (VI), the PI value of which when given prophylactically was 82.8% (Table 2). In the therapeutic mode, (I) and (IV) were the most active, the PI values being 71.4 and 74.9% respectively.

These biological studies have shown that virtually all of the test compounds were of low activity against influenza, the greatest protective effect being found with (V), the PI of which was 60.6% against type A influenza virus, which is however 18% lower than that of remantadine (Table 2).

These hydrophosphorylated inosines are therefore of low toxicity, and show antiviral activity against RNA and DNA viruses. The results of these investigations have shown the need for further work on the chemical modification of nucleosides by phosphorylation, with the aim of identifying novel antiviral drugs.

LITERATURE CITED

1. D. Corbridge. Phosphorus: Fundamentals of its Chemistry, Biochemistry, and Technology [Russian translation from the English], Moscow (1982), pp. 424-429.

2. S. Ya. Mel'nik and M. N. Preobrazhenskaya, Itogi Nauki Tekhniki, Ser. Bio. Khim. (Science and Technology Summaries, Bioorganic Chemistry Series), Moscow (1984).
3. A. Mikel'son, The Chemistry of Nucleosides and Nucleotides [Russian translation from the English], Moscow (1966).
4. N. P. Chizhov, F. I. Ershov, and M. K. Indulen, Fundamentals of the Experimental Chemotherapy of Viral Infections [in Russian], Riga (1988).
5. M. A. Shneider, N. K. Rudenko, V. M. Kavsan, and R. Sh. Bibilashvili, Mol. Biol., **21**, No. 3, 837-846 (1987).
6. M. A. Shneider and N. P. Chizhov, Antibiotiki, **33**, No. 5, 386-394 (1988).
7. V. I. Yudelevich, E. V. Komarov, and B. I. Ionin, Khim. Farm. Zh., **19**, No. 6, 668-685 (1985).
8. V. I. Yudelevich, M. A. Shneider, V. V. Belakhov, et al., ibid., No. 11, 1340-1347.
9. K. Issleib, Nachr. Chem. Techn. Lab., **35**, No. 10, 1038-1042 (1987).
10. C. Martin, C. Dvorak, D. Smel, et al., J. Med. Chem., **26**, 759-761 (1983).
11. M. R. Murthy and P. A. Ranjekar, J. Chromatogr., **76**, 337-343 (1973).
12. I. R. Prous, Int. Publ. Ann. Drug Data Rep., **3**, 1-287 (1981); **4**, 1-265 (1982).
13. W. Shannon and F. M. Schabel, Jr., Pharmacol. Ther., **11**, 263-290 (1980).
14. C. Stuart-Harris, J. Antimicrob. Chemother., **15**, 387-397 (1985).

SYNTHESIS OF N-SUBSTITUTED 1-O-[1'-ALKENYL]2-2-ACETYL-sn-GLYCERO-3-PHOSPHOETHANOLAMINES AND THEIR EFFECTS ON THROMBOCYTE AGGREGATION

A. A. Dergousov, T. I. Kuznetsova, and V. I. Kulikov

UDC 615.273.015.4:612.111.7].07

The synthesis and study of analogs and antagonists of the thrombocyte aggregation factor (TAF) is a new area of investigation for the development of new drugs for the regulation of the functional activity of thrombocytes and other blood cells [3, 5].

It has previously been shown that certain plasmogenic analogs of TAF [1-O-(1'-alkenyl)-2-acetyl-sn-glycero-3-phosphocholine and 1-O-(1'-alkenyl)-2-acetyl-sn-glycero-3-phosphoethanolamine] inhibit the aggregation of thrombocytes induced by TAF [2, 6].

This report describes the synthesis of novel analogs of TAF (I-III) which are N-substituted derivatives of 1-O-(1'-alkenyl)-2-acetyl-sn-glycero-3-phosphoethanolamine, and an examination of their effects on the aggregation of human erythrocytes.

The compounds (I-III) were synthesized using a fraction of ethanolaminophosphoglycerides containing 50% of phosphatidylethanolamine, isolated from ox brain [4].

The synthesis of (I-III) was carried out in three steps, namely introduction of an aromatic substituent at the free amino group of the ethanolaminophosphoglycerides, cleavage of the fatty acids in the sn-2 position with alcoholic alkali, and acetylation of the appropriate lyso-derivatives of the phospholipids.

The structures of the compounds obtained were confirmed by UV, IR, and PMR spectroscopy. The UV spectra of (I-III) showed absorption for the aromatic residues with λ_{max} 225 (I), 347 (II), and 334 nm (III), characteristic of phospholipids containing benzoyl, 2,4-dinitrophenyl, and 2,4-dinitrophenylene residues respectively.

The IR spectra of (I-III) showed absorption for the ester bond (1725-1730 cm^{-1}), the P—O—C bond (1060-1070 cm^{-1}), and the aldehydrogenic bond —OCH=OCH— (1220-1250 cm^{-1}). The IR spectra of (II) and (III) also showed absorption for the NO_2 group (two bands) at 1310-1530 cm^{-1}, and that of (I) two bands for amide absorption at 1640 and 1540 cm^{-1}. In the PMR spectra of (I-III), the protons of the acetyl group were seen as a narrow singlet at 2.03-2.08 ppm, the protons involved in the formation of the aldehydrogenic bond at 4.10-5.35 ppm, and signals for the aromatic ring protons at 6.3-9.1 ppm.

Medical Biotechnology Center, Ministry of Public Health of the USSR, Moscow. Translated from Khimiko-farmatsevticheskii Zhurnal, Vol. 24, No. 9, pp. 48-50, September, 1990. Original article submitted September 19, 1989.

 0091-150X/90/2409-0656$12.50