PHOSPHORYLATED ETHYLENEAMIDE DERIVATIVES OF URACILS, THEIR PREPARATION AND BIOLOGICAL ACTIVITY

E. I. Besyadetskaya, T. N. Semenyuk, A. I. Potopal'skii,
S. V. Ivasivka, L. F. Savchuk, A. A. Tsegel'skii,
D. A. Shchesyuk, D. D. Lutsevich, L. S. Kontsevich,
B. O. Pinyazhko, L. I. Basiliya, and F. D. Onishchuk

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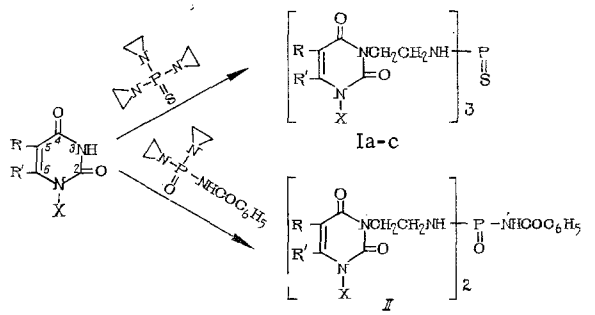
In the search for compounds with antitumoral activity, we studied the reaction of uracil and its derivatives, such as 6-methyl- and 5-nitrouracil with triethylene-thiophosphamide and N-benzoyl-N',N''-diethylenephosphoric acid triamide (benzotef).

Data are given in the literature [1, 2] on the alkylation of uracils and thiopyrimidine nucleosides with ethyleneimine. Direct amination of uracils and related compounds by phosphoric acid amides has also been described [3-5].

The alkylation of uracil and methyluracil with thiophosphamide or benzotef was carried out in an aqueous medium at pH 10.0, and of nitrouracil in a neutral medium. The ethyleneimine rings of the reagent were opened, and the N-phosphorylated 2-aminoethyl residue entered into the 3-position of the uracil ring to form the corresponding derivatives. Thus, N,N',N''-tris[β-(3-uracil)ethyl]thiophosphoric acid triamine (Ia), N,N',N''-tris[β-(6-methyl-3-uracil)-ethyl]thiophosphoric acid triamide (Ib) (both in the form of trisodium salts), N,N',N''-tris-[β-(5-nitro-3-uracil)ethyl]thiophosphoric acid triamide (Ic), and N-benzoyl-N,N''-bis[β-(6-methyl-3-uracil)ethyl]phosphoric acid triamide disodium salt (II) were obtained.



Ia-c

II

R = H or NO_2; R' = H or CH_3; X = H or Na.

The individuality of the compounds obtained was confirmed by TLC or paper chromatography, the composition by elemental analysis, and the structure by spectral data.

We found that acid hydrolysis of Ia or Ib leads to the evolution of hydrogen sulfide. Products were isolated which were identical with the initial uracils. To identify the substituent at the 3-position of the uracil ring, we studied the dependence of the UV absorption spectra of the compounds obtained on the pH of the medium. It is known that on transition from an acid to neutral and alkaline media, the N'-substituted pyrimidine bases do not cause a bathochromic shift of the absorption maximum [6, 7]. Compounds, Ia, b and II obtained have one absorption maximum in methanol (Table 1), which is practically identical with the absorption maximum of the initial uracils. The initial 5-nitrouracil has two absorption maxima in methanol: in the 235-236 and 297 nm regions. Its thiophosphamide derivative Ic is also characterized by two absorption maxima in the 229-235 and 331-338 nm regions (in methanol), i.e., a bathochromic shift of the absorption maximum of the second band is observed. Transition from acid to neutral or alkaline media causes a bathochromic shift of the absorption

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TABLE 1. Phosphorylated Ethyleneamide Derivatives of Uracils

Compound	Yield, %	mp, °C	Found, %				Empirical formula	Calculated, %				R_f (system)	UV spectrum					
			N	S	P	Na		N	S	P	Na		0.1 N HCl		methanol		0.1 N NaOH	
													λ_{max}, nm	lg ε	λ_{max}, nm	lg ε	λ_{max}, nm	lg ε
Ia	64,7	~230, (dec)	21,04	5,73	5,44	10,92	$C_{19}H_{21}N_6O_6PSNa_2$	21,30	5,4	5,24	11,66	0,51 (A)	258—260	4,35	259—261	4,36	271—277	4,23
Ib	42,9	264—6, (dec)	19,96	4,42	5,47	10,28	$C_{21}H_{17}N_9O_6PSNa_3$	19,79	5,01	4,89	10,86	0,59—0,61 (A)	259—262	4,48	259—262	4,49	277	4,32
Ic	74,2	116—120, (dec)	25,15	4,85	4,70	—	$C_{18}H_{21}N_{12}O_{12}PS$	25,43	4,85	4,69	—	—	234—238 296—300	4,20 4,29	229—235 331—338	4,30 4,22	267—272 281—288	4,23 4,26
II	81,2	~200.0, (dec)	17,59	—	5,63	8,64	$C_{21}H_{21}N_8O_6PNa_2$	17,92	—	5,66	8,10	0,41 (B)	260—261	4,35	258—260	4,52	350—357	4,19

TABLE 2. Cytotoxic Activity of Phosphorylated Ethyleneamide Derivatives of Uracils

Compound	Dilution				
	1:200	1:500	1:1000	1:2000	1:3000
	% of dead Ehrlich tumor cells after incubation for 1 h				
Ia			90,66	84,62	77,60
Ib	94,50	89,50	85,10	Coagulation	Coagulation
Ic	99,80	98,30	97,80	100,00	76,58
II			74,51	64,22	58,77

maxima of the initial uracils and the compounds obtained (Table 1), the band intensity of the latter compounds increasing more than the intensity of the initial uracils.

Comparison of the UV absorption spectra of the compounds obtained with the spectra of the known 3-alkyluracils [1] also confirms the presence of a substituent at the 3-position of the molecule of the phosphorylated ethyleneamide derivatives of uracils Ia-c, II.

EXPERIMENTAL CHEMICAL SECTION

TLC was carried out on Silufol UV-254 plates in n-butanol—methanol—water—ammonia 60:20:20:1 (A) and n-butanol—acetic acid—water 4:1:5 (B) systems of solvents. Paper chromatography was carried out on Filtrak FN1 paper in the isopropanol—hydrochloric acid—water 680:164:156 (C) system. The compounds were dissolved in 50% aqueous ethanol or methanol. Development was carried out in UV light, and the initial thiophosphamide was developed by iodine vapors. The UV absorption spectra were obtained on SF-4 spectrophotometer in methanol or ethanol at pH 1.1 (0.1 N HCl) and 12.2 (0.1 N NaOH). The content of sodium was determined titrimetrically.

General Method for Preparation of Sodium Salts of 3-Substituted Phosphorylated Ethyleneamide Uracils Ia, b and II. A 0.015 mole portion of uracil or 6-methyluracil is dissolved in 50 ml of 0.03 M sodium hydroxide, and then 0.005 mole of thiophosphamide or 0.075 mole of benzotef is added and the reaction mixture is held in a thermostat at 37°C for 7 days. The reaction mixture is shaken with activated charcoal, filtered, extracted many times by ether from the unreacted impurities, and subjected to lyophilic drying. The Ia powder obtained is washed again from the initial compounds with a minimal amount of 50% ethanol and ether, and Ib and II are washed by ether.

Preparation of N,N',N''-Tris[β-(5-nitro-3-uracil)ethyl]thiophosphoric Acid Triamide (Ic). A 2.36 g portion (0.015 mole) of 5-nitrouracil is dissolved in 250 ml of distilled water heated to 50°C, and after cooling, 0.95 g (0.005 mole) of thiophosphamide are added. The reaction mixture is held in a thermostat at 37°C for 12 days. The color of the solution becomes thus yellow. The purification and isolation of the product is carried out as described for Ic and II.

The compounds obtained are colorless or yellow crystalline powders, which are soluble in water, DMSO, 0.1 N HCl, 50% ethanol, and 50% acetone, and are isoluble in ether. The yields, physical characteristics, and analytical data are given in Table 1.

Acid Hydrolysis of Ia or Ib. A 0.0015-0.003 mole portion of Ia or Ib is dissolved in 10-15 ml of aqueous alcohol (1:2). To the solution 10 ml of concentrated hydrochloric acid are added, and the solution is boiled for 13-20 h. Hydrogen sulfide is evolved. When cool, the precipitate is filtered, washed with alcohol and ether, and recrystallized from an appropriate solvent.

In hydrolysis of Ia, 0.42 g (42.0%) of uracil is obtained, mp about 335°C (dec., from water), R_f 0.71 (system C). UV spectrum, λ_{max}, nm (log ε): at pH 1.1: 258-259 (3.94); ethanol, 257-259 (4.13); at pH 12.2: 283-285 (4.00). Found, %: N 25.96. $C_4H_4N_2O_2$. Calculated, %: N 25.87.

In hydrolysis of Ib, 0.3 g (53.5%) of 6-methyluracil is obtained, mp 308-310°C (dec., from aqueous methanol), R_f 0.78 (system C). UV spectrum, λ_{max} nm (log ε): at pH 1.1: 260

(3.96); methanol, 260 (3.94); at pH 12.2: 274-275 (4.13). Found, %: N 2216. $C_5H_6N_2O_2$. Calculated, %: N 22.22.

The R_f values of the products obtained in acid hydrolysis of Ia or Ib agree with those of the initial uracils (system C) and the UV spectra agree with the spectra of the initial compounds.

EXPERIMENTAL BIOLOGICAL SECTION

The biological activity of the compounds obtained was studied. The toxicity, potential antitumor and cytogenetic action, influence on the peripheral blood indexes, and state of reactivity of the organism were investigated.

The acute toxicity was studied with a single intraperitoneal administration. The results were summarized after one month of observation on animals. The LD_{50} was calculated graphically by an assay analysis according to Leachfield and Wilkinson [8].

The cytotoxic activity was studied in contact experiments with Ehrlich's ascites tumor cells by the Schreck method [9].

The chemotherapeutic experiments were carried out after a palpatory diagnosis of tumors in animals and thorough randomization. The tumors were transplanted under sterile conditions by conventional methods [10, 11]. The course of treatment consisted of 5-10 injections. The antitumoral effect was evaluated 5-7 days after discontinuation of injections to reveal possible aftereffects of the preparations, and was expressed in percent of tumor growth inhibition [12]. For a model of malignant growth, we used transplanted strains of Ehrlich's tumor, sarcoma 37, sarcoma 180 in mice, and Gueren, Ohya, and Pliss tumors in rats. In peripheral blood, we selectively studied the content of hemoglobin and the number of erythrocytes and leucocytes. For tests of reactivity of organism toward tumor carriers, we used the phagocytic activity of peripheral blood leucocytes [13] and the cancerolytic activity of the blood serum [14, 15].

The cytogenetic action of Ib and II was studied on bone marrow cells of male mice of the $C_{57}BL_6$ line. The compounds were dissolved in distilled water and administered intraperitoneally. The length of the experiments was 24 and 48 h after the administration of the compounds in a dose close to $^1/_2$ LD_{50}. In experiments with Ib, we determined the concentrational dependence with four more equally diminishing concentrations. The possible cumulation of cytogenetic action of the compound studied was evaluated in experiments with 5 daily administrations in a dose of $^1/_5$ LD_{50} [16]. In each variant of the experiment, no less than 5 mice were used, and 100 metaphases from each animal were studied. The preparations were prepared by the Ford method.

In the experiments, we used 250 male mice of the F_1(CBA × $C_{57}BL_6$) line, weighing 19-21.5 g each, and 140 male rats of the Wistar, Way, and August lines weighing 180-200 g each. The animals were kept under standard vivarium conditions.

RESULTS AND DISCUSSION

Study of Acute Toxicity of (Ia-c, II). Table 3 shows that the compounds obtained are slightly toxic, with the toxic effect beginning on the 3rd-5th to 10th day after administration. When completely lethal doses of Ib and II were used, the acute toxic pattern was the same, and was accompanied by general oppression, depression, and acrocyanosis. Directly after injections of Ib and II, during the next 1.5-2 h, the respiration frequency increased. Therapeutic doses of the compounds do not cause changes in the general appearance of the animals.

Cytotoxic Activity of (Ia-c, II). The data in Table 2 show that the contact action of the compounds obtained on cancer cells of Ehrlich's tumor is noticeable because of the presence of a fairly high percentage of dead cells in all dilutions studied.

From the results of the study on the cytotoxic effect and acute toxicity of the compounds obtained, we see that the chemotherapeutic experiments could be applied to different models of the tumor growth.

Chemotherapeutic Activity of Ia-c, II. The compounds were studied in doses of 50 mg/kg (II) and 100 mg/kg (Ia-c). A pronounced antitumor activity of the compounds was observed on several models of tumors (Table 3). A certain selectivity was observed in the spectrum of tested tumors, expressed in different degrees of suppression of the tumor growth.

TABLE 3. Biological Activity of Phosphorylated Ethyleneamide Derivatives of Uracils

Compound	Type, line of animals	LD_{50}, mg/kg	Time up to death of animals, hours, days	Strain of tumor	Therapeutic dose, mg/kg	Percent of tumor inhibition	Number of leucocytes, 10^9 liters	Indexes	
								cancerolytic	phagocytic
								$M \pm m$	
Ia	Mice of F_1 (CBA×C57BL/6) line	605 (550—700)	24—96—5 days	Sarcoma 37	100	62,0	27,65±4,93	—	—
	Rats of Wistar line			Gueren tumor	100	84,2	9,37±1,98	1,16±0,04*	4,43±0,22**
	Rats of Wistar line			Schwetz erythromyelosis	100	79,57	16,41±5,96		
	Rats of way line			Pliss lymphosarcoma	100	73,1		1,12±0,057**	5,47±0,83*
IIa	Mice of F_1 (CBA×C57BL/6) line	610 (484—769)	24—96—5 days						
	Rats of Wistar line			Gueren tumor	100	81,7	6,43±0,63***	1,13±0,06**	4,68±0,196**
	Rats of Wistar line			Schwetz erythromyelosis	100	99,98	9,46±4,62		
	Rats of August line			Ohya tumor	100	44,0			
IIb	Mice of F_1 (CBA×C57BL/6) line	1000	No death observed	Sarcoma 37	50	52,5	10,1±1,47**		
	Rats of Wistar line			Gueren tumor	50	74,6	4,48—1,60***	1,20±0,041****	5,20—0,245*
	Rats of Wistar line			Schwetz erythromyelosis	50	98,91	14,55±3,54		
	Rats of August line			Ohya tumor	50	51,0			
IIIa	Mice of F_1 (CBA×C57BL/6) line	1650 (1179—2310)	24—96—10 days						
	Rats of Wistar line			Gueren tumor	100	75,8		1,11±0,05*	4,70±0,255****
Control	Mice of F_1 (CBA×C57BL/6) line								
	Rats of Wistar line			Sarcoma 37			23,85±4,51		
	Rats of Way line			Gueren tumor			12,83±2,46	0,43±0,041	2,98±0,12
	Rats of Wistar line			Pliss lymphosarcoma			12,81±1,73	0,40±0,114	3,23±0,02
				Schwetz erythromyelosis			15,05±6,69		

Note. Fluctuation limits are shown in parentheses; one asterisk — $P < 0.001$, two asterisks — $P < 0.02$, three asterisks — $P < 0.05$, four asterisks — $P < 0.01$.

TABLE 4. Cytogenic Activity of Ib and II in Bone Marrow Cells of Mice

Compound	Length of investigation, h	Dose, mg/kg	Number of analyzed metaphases	Metaphases with aberrations	
				number	% ± M
Triethylene-thiophos-phoramide	24	5	500	182	36,40±2,15
Ib	24	300	600	60	10,00±2,37
Ib	24	150	500	23	4,60±0,87
Ib	24	75	500	9	1,80±0,50
Ib	24	37,5	500	5	1,00±0,45*
Ib	24	18,75	500	2	0,40±0,24*
Ib	24	300	500	21	4,20±0,66
Ib	48	120 (5 administrations)	500	6	1,20±0,49*
Benzotef	24	15	500	261	52,20±3,35
II	24	100	600	30	5,00±1,05
II	48	100	500	5	1,00±0,45*

*Data statistically unreliable.

Compound Ia did not exhibit any activity towards the following models of tumors: sarcoma 180, solid form of Ehrlich's and Ohya tumors (48.0, 25.0, and 28.5% of inhibition respectively). Compound Ib was inactive towards sarcoma 37, the Pliss lymphosarcoma (24.0, 35.7% of inhibition), and compound Ic was inactive towards sarcoma 37 and the Pliss and Ohya lymphosarcomas (41.3, 45.5-12.3% of inhibition).

Influence of Ia-c; IIa, b; IIIa on Hematological Indexes. The compounds did not have any effect on the hemoglobin content and the number of erythrocytes in the peripheral blood of the animals treated. The number of leucocytes did not decrease when Ia was administered to mice with sarcoma 37, Ehrlich's carcinoma, and to rats with Gueren's tumor, Ib to rats with Pliss lymphosarcoma, and Ic to mice with sarcoma 37. In the remaining cases mild leucopenia was observed.

Reactivity of Organism and Tumor Growth after Administration of Ia-c, II. During the examination of the cancerolytic and phagocytic indexes, a correlation was observed between increase in these indexes and inhibition of tumor growth when all the preparations studied were introduced. Thus, administration of compound Ia led to a stimulation of the protective forces of the organism and inhibition up to 73.18% of tumor growth. The cancerolytic index was statistically reliably higher than in control, and was 1.12 ± 0.057 (P 0.02), and the phagocytic index was at the level of 5.47 ± 0.87 (P 0.001) (see Table 3).

Cytogenetic Action of IIa, b. The alkylation of 6-methyluracil was carried out by alkylating agents with a strongly expressed cytogenetic activity [17]. Compound Ib in a dose close to $^1/_2$ LD_{50} induces chromosome aberrations in 10.00 ± 2.37% of metaphases, i.e., in an amount smaller by a factor of 3.5 than in the case of triethylenethiophosphoramide used at a concentration with corresponding toxicity (Table 4).

The main form of the structural disturbances is chromatid-type aberrations, but cells with multiple defects of chromosomes, characteristic of the action of thiophosphoramide, are not observed. Experiments with introduction of different concentrations of the material showed a relationship between the frequency and spectrum of the defects and the dose of the compound.

When Ib was used in doses of 37.5 and 18.75 mg/kg, the number of structural aberrations of chromosomes was statistically unreliably different from the index in the control. The aberrations are thus represented by single fragments only. Experiments set up to clarify possible cumulation of the cytogenetic action of Ib (five daily administrations in a dose of 120 mg/kg) did not give any positive results.

Compound II in a dose of 100 mg/kg also exhibited a weaker cytogenetic action than benzotef.

Experimental data and clinical observations indicate that 6-methyluracil does not stimulate malignant growth and metastasis, and is little toxic; stimulation of phagocytosis and favorable results in the treatment of leukemia were noticed [18]. Its use in oncology can be broadened by using new modifications of the preparation.

According to known data [19], LD_{50} in mice for triethylenethiophosphoramide is 16.5 (12.1-22.4) mg/kg, and for benzotef, 35 (20.5-59.5) mg/kg [19]. By alkylation of uracils by thiophosphoramide or benzotef, slightly toxic compounds with pronounced antitumor activity could be obtained. These compounds retain the ability to activate phagocytosis and cancerolysis, correlated with an antitumor effect, and have either a moderate or no influence on leucopoiesis. It was also found that the phosphorylated 6-methyluracil derivatives cause less damage to hereditary structures of somatic cells than the alkylating compound included in their composition.

Thus, Ia-c and II can be characterized as slightly toxic compounds, which increase the protective forces of the organism with active inhibition of tumor growth in experiments.

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