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Chemoenzymatic Method of 1,2,4-Triazole Nucleoside Synthesis: Possibilities and Limitations

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Abstract—Possibilities and limitations of chemoenzymatic synthesis of novel structural analogues of an antiviral preparation of Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) were established. A synthesis of various amides of 1*H*-1,2,4-triazole-3-carboxylic acid and its 5-substituted analogues—potential substrates of purine nucleoside phosphorylase—has been described. Comparative efficiency of preparation methods of these amides, as well as the methods of introduction of functional groups to the C5 position of heterocyclic system, were investigated. Novel analogues of Ribavirin containing various substitutes in the carboxamide group were synthesized. A biotechnological method was developed for the preparation of 1-β-D-ribofuranozyl-1,2,4-triazole-3-carbonitryl, an intermediate in the synthesis of Viramidine, the modern analogue of Ribavirin.

Keywords: 1H-1,2,4-triazole-3-carboxamid, Ribavirin, Viramidine, transglycosylation reaction, nucleoside phosphorylases from E. coli.

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INTRODUCTION

The constantly growing interest in medications developed on the basis of modified nucleosides is due to their use in novel possibilities of therapy for a series of difficult-to-treat viral and autoimmune diseases and tumors of human blood-forming organs. The preparations introduced in the last decade in medical practice (Azidothymidine, Nelarabine, Cladribine, Fludarabine, Vidarabine and others) are officially recognized as the first-choice preparations for the treatment of a number of difficult-to-treat human diseases.

Ribavirin (**I**, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamid, Virazole) represents a modified nucleoside effective against a wide spectrum of RNA- and DNA- viruses. It demonstrates pronounced efficiency in treatment of hepatitis C and Lassa fever, as well as influenza of A- and B-types [1–4]. An interest in

Virazole increased significantly after its efficiency was established in the treatment of respiratory-scintillating viral infection, bronchiolitis and pneumonia in children in their first year of life, as well as for treatment of haemorrhagic fever with kidney syndrome [5].

The combination preparation of recombinant interferon alpha-2b with Ribavirin gained the highest popularity in the world's medical practice for treatment of chronic hepatitis C [6, 7]. This therapeutic standard is intended for patients that show recurrence after interferon use.

Interest in Ribavirin increased again in 2011 after approval by the FDA (Food and Drug Administration, United States) of a novel complex preparation for the therapy of hepatitis C that consisted of the proteinase inhibitor telaprevir (Vertex Pharmaceutical) in combination with interferon and Ribavirin. The use of the triple combination proved to be extremely promising for the treatment of both new patients and those who demonstrated resistance to the double therapy of hepatitis [8, 9].

Abbreviations: HCB, heterocyclic base; RSA, X-ray structure analysis; TCA, 1*H*-1,2,4-triazole-3-carboxamid; ATC, 1*H*-1,2,4-triazole-3-carboxylic acid; CDI, carbonyldiimidazole; NP, nucleoside phosphorylase; PNP, purine nucleoside phosphorylase.

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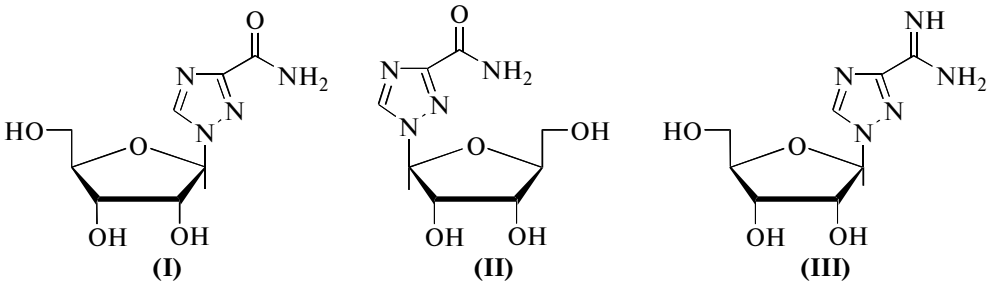


Formulas 1. Ribavirin and its new therapeutic analogues.

Ribavirin, however, has serious side effects in the blood-forming organs of patients: it causes haemolytic anemia, which motivates researchers to search for safer therapeutic preparations with less systemic toxicity. In this regard, ribavirin analogues—levovirin (**II**) and viramidine (**III**)—hold some promise [10, 11]. Levovirin is an L-enantiomer of ribavirin, which possesses immune-modulating activity similarly to ribavirin, but is not phosphorylated by kinases and, hence, does not cause hemolysis. Viramidine, a prodrug of ribavirin, is transformed into an active form in the liver and does not accumulate in erythrocytes.

Efficiency and safety of these two systems of antiviral therapy (pegintron + viramidine and pegintron + ribavirin) were compared in clinical studies of patients with hepatitis C. The lesser frequency of virologic response was observed in the first group in comparison with the traditional therapy (38% versus 52%), however, haemolytic anemia developed in significantly fewer cases in patients treated with viramidine than in ribavirin group (5% versus 24%) [12, 13].

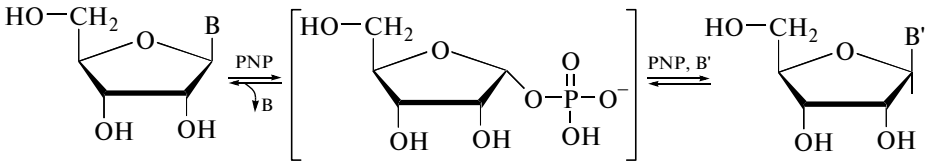
Significant progress in the preparation of biologically important analogues of natural nucleosides was achieved through the rational combination of chemical methods and biochemical transformations. Application of recombinant nucleoside phosphorylases (NP) as biocatalysts of the synthesis of natural nucleosides and their modified analogues is of significant interest for the development of modern technological processes [14–16].

The chemoenzymatic (biotechnological) approach to the synthesis of ribavirin and its analogues displaces the current multistage chemical processes and allows conducting the key transformations regio- and stereoselectivity with high efficiency [14, 15].

Moreover, testing novel derivatives of 1,2,4-triazole as substrates of pyridine nucleoside phosphorylase (PNP) can reveal structural features of heterocyclic bases defining the potential for conducting the reaction of the synthesis of modified nucleosides on their basis in the active site of the enzyme, which in turn widens our view of the mechanism of the function and synthetic abilities of the enzyme.

RESULTS AND DISCUSSION

A reaction of transglycosylation, an enzymatic reaction of the transfer of ribose from the natural nitrogenous base (guanine, hypoxanthine, etc) to 1*H*-1,2,4-triazole-3-carboxamid (TCA), is the basis of the biotechnological approach to ribavirin synthesis. Unlike human PNP, the bacterial enzyme is capable of accepting TCA and its structural analogues as a substrate. The mechanism of the transglycosylation reaction is presented in scheme 1 [17, 18].

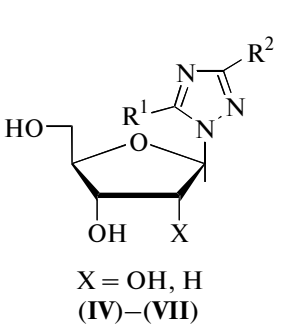


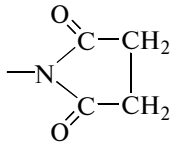
B = natural heterocyclic base;
B' = modified heterocyclic base.

Scheme 1. Mechanism of transglycosylation reaction.

Enzymatic synthesis of ribavirin is conducted with bacterial nucleoside phosphorylases from a variety of cell cultures using cell suspensions, lysates of the strains, or recombinant enzymes from *Escherichia coli (E. coli), Erwinia carotovora, Bacillus brevis, Brevibacterium fuscum, Sarcina lutea, Arthrobacter oxydans, Achromobacter dendriticum*, and others [17–19]. We have reported earlier the possibility of synthesis of six analogues of ribavirin (**IV**)–(**VII**) [20–22] using recombinant purine nucleoside phosphorylase from the

strain producer *E. coli* BL21(DE3)/pERPUPHHO1 (E.C. 2.4.2.1) [23, 24].

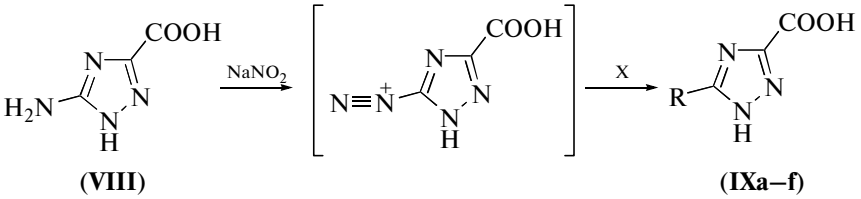


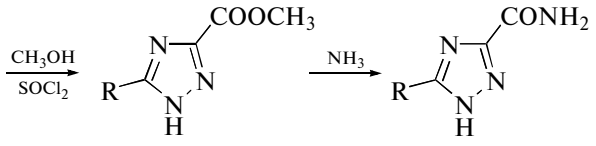
R^1	R^2	X	Compound
CH_3	-C(O)-NH_2	OH	**(IVa)**
		H	**(IVb)**
H	-NH_2	OH	**(V)**
		OH	**(VIa)**
		H	**(VIb)**
	-NH-C(O)-CH_3	OH	**(VII)**

Formulas 2. Ribavirin analogues.

Unfortunately, from all these analogues only 5-methylribavirin (**IV**) possesses antiviral activity comparable with ribavirin and demonstrating similar toxicity in experiments *in vitro* [21].

It was interesting to investigate possibilities and limitations of the enzymatic method of synthesis of novel ribavirin analogues, to determine the requirements for the structure of heterocyclic bases (derivatives of 1,2,4 triazole) defining their normal binding with the active center of PNP *E. coli* and the transglycosylation reaction. It was essential to obtain a large variety of differently substituted derivatives of 1,2,4-triazole in order to determine substrate specificity of PNP, hence, the development of the method for routine synthesis of two types of compounds, namely, the C5-derivatives of 1*H*-1,2,4-triazole-3-carboxamid and derivatives with their amide groups substituted, was the first goal of the study. 1*H*-1,2,4-triazole-3-carboxylic acid (ATC) (**IX**) and a series of substituted analogues (**IXb**)–(**IXe**) were synthesized with the Sandmeier reaction from 3-amino-1*H*-1,2,4-triazole-5-carboxylic acid (**VIII**) using a method described earlier (Scheme 2) [25–28]. See Table 1.





a: X = CH_3OH, R = H; **b**: X = HCl, R = Cl; **c**: X = HBr, R=Br; **d**: X = H_2SO_4, R = OH; **e**: X = HNO_3, R = NO_2; **f**: X = CuCN, R = CN.

Scheme 2. Synthesis of TCA (**XIa**) and its 5-substituted analogues.

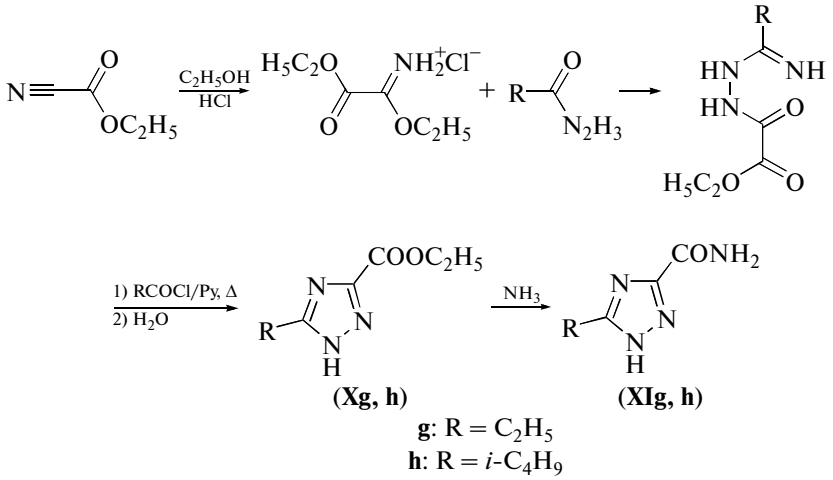
Methyl esters (**Xa**)–(**Xe**) were obtained with the traditional method (in absolute methanol in the presence of thionyl chloride), amides (**XIa**)–(**XIe**) were synthesized by subsequent ammonolysis of methyl esters (Tables 2 and 3). The same method was used for synthesis of 5-iodo-1*H*-1,2,4-triazole-3-carboxylic acid and its derivatives which were found to be unstable over storage for several days.

Synthesis of methyl ester (**Xf**) and amide (**XIf**) of of 5-cyano-1*H*-1,2,4-triazole-3-carboxyl acid in preparative amounts with the similar method failed (see scheme 2), probably due to the side reactions of the nitrile group in the given conditions. Ethyl esters of 5-alkyl-substituted analogues of ATC (**Xg**), (**Xh**) and the respective amides (**XIg**), (**XIh**) were obtained with the synthetic approach proposed earlier for the synthesis of 5-methyl-1*H*-1,2,4-triazole-3-carboxamide [20, 29] (Scheme 3).

Table 1. Preparation of ATC and 5-substituted analogues

Compound	Yield, %	T_m, °C	Physico-chemical characteristics	Method of synthesis
(IXa)	65	137 (decomp.), (137–138 ref. [25])	IR (suspension in vaseline oil), cm^{-1}: 520, 644, 724, 768, 968, 1112, 1204, 1380, 1656, 1740 (COOH); *m/z*, 114.0470 $[M + H]^+$ (calc. 114.0298); λ_{max}: <200 nm; Purity 98.8%, R_t = 2.1 min (variant 2). Found, %: C 31.24; H 2.84; N 36.94. Calculated, %: C 31.87; H 2.67; N 37.16.	[25]
(IXb)	42	70 (decomp.), (70 ref. [25])	^{13}C NMR (D_2O, δ, ppm): 168.8; 162.0; 148.4. Found, %: C 24.06; H 1.51; N 28.06. Calculated, %: C 24.43; H 1.37; N 28.48.	[25]
(IXc)	37	136 (decomp.)	^{13}C NMR (D_2O, δ, ppm): 169.1; 162.7; 154.7. Found, %: C 18.55; H 1.17; N 21.64. Calculated, %: C 18.77; H 1.05; N 21.89.	[25]*
(IXd)	56	201–203 (decomp.), (205 ref. [27])	^{13}C NMR (D_2O, δ, ppm): 159.5; 153.7; 143.8. Found, %: C 27.94; H 2.30; N 32.71. Calculated, %: C 27.92; H 2.34; N 32.56. *m/z*, 128.0080 $[M - H]^-$ (calc. 128.0102), 257.0247 $[2M - H]^-$ (calc. 257.0270). λ_{max}: 234.4 nm. Purity 96.2 %, R_t = 1.92 min (variant 5).	[27]
(IXe)	46	100 (decomp.), (100–102 ref. [25])	^{13}C NMR (D_2O, δ, ppm): 167.2; 161.4; 147.2. Found, %: C 18.55; H 3.05; N 29.01. Calculated, %: C 18.56; H 3.12; N 28.86 (dihydrate).	[25]
(IXf)	68	98–99 (decomp.), (98–100 ref. [28])	^{13}C NMR (D_2O, δ, ppm): 162.3; 137.5; 132.4; 112.2. Found, %: C 34.35; H 1.49; N 40.37. Calculated, %: C 34.79; H 1.46; N 40.57.	[28]

Note: similarly to **(IXb)** from 3-amino-ATC and 48% HBr in the presence of CuBr.

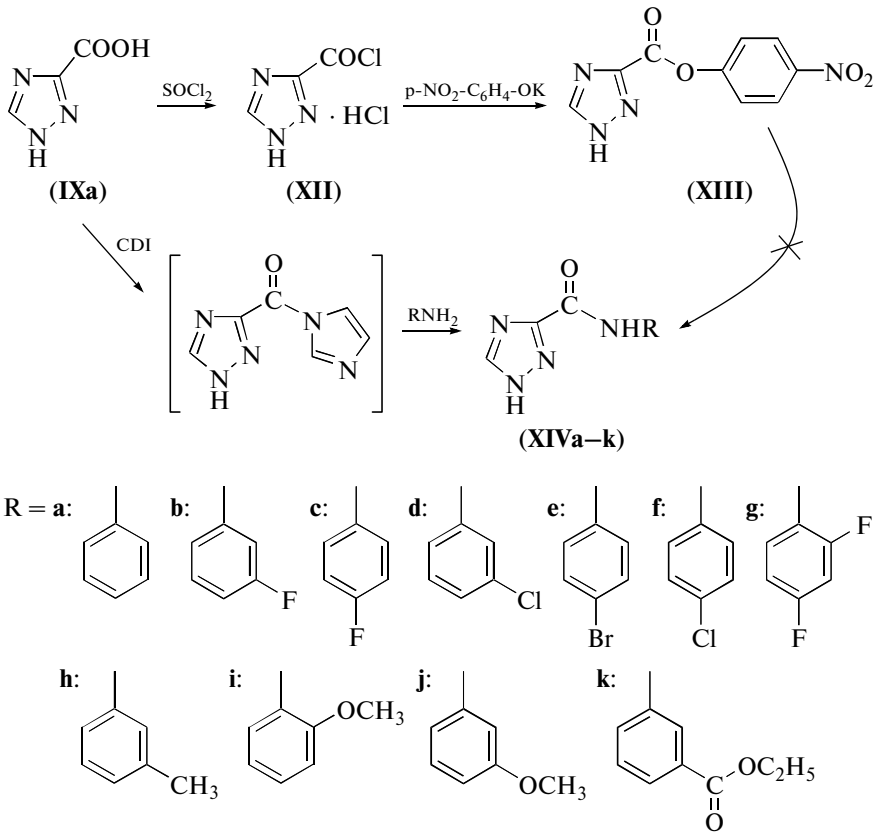


Scheme 3. Synthesis of 5-ethyl **(XIg)** and 5-isobutyl **(XIh)** substituted analogues of TCA.

Table 2. Preparation of methyl esters of ATC and its 5-substituted derivatives

Compound	Yield, %	T_m, °C	Physico-chemical characteristics
(**Xa**)	80	184 (decomp.), (184–185 ref. [25])	^{1}H NMR (acetone-d_6, δ, ppm): 8.51 (s, 1 H, ArH, 4.33 (s, 3 H, Me) Found %: C 37.71; H 4.06; N 33.10. Calculated, %: C 37.80; H 3.97; N 33.06.
(**Xb**)	83	121–122 (decomp.), 121 (ref. [25])	^{1}H NMR (acetone-d_6, δ, ppm): 4.12 (s, 3 H, Me) Found, %: C 30.01; H 2.51; N 25.73. Calculated, %: C 29.74; H 2.50; N 26.01. λ_{max}: 232.2 nm. Purity 92.93%, R_t = 10.61 min (variant 6).
(**Xc**)	77	above 340	^{1}H NMR (acetone-d_6, δ, ppm): 4.14 (s, 3 H, Me) Found, %: C 23.35; H 1.87; N 20.64. Calculated, %: C 23.32; H 1.96; N 20.40. λ_{max}: 233.2 nm. Purity 91.6%, R_t = 10.7 min (variant 6).
(**Xd**)	73	above 340	^{1}H NMR(DMSO-d_6, δ, ppm): 3.82 (s, 3 H, Me) ^{13}C NMR (DMSO-d_6, δ, ppm): 157.0; 155.3; 137.9; 52.6. Found, %: C 33.44; H 3.60; N 29.13. Calculated, %: C 33.57; H 3.52; N 29.36. *m/z*, 144.0416 $[M + H]^+$, (calc. 144.0404), 166.0228 $[M + Na]^+$, (calc. 166.0223); λ_{max} 245.0 nm Purity 98.5%, R_t = 5.31 min (variant 5).
(**Xe**)	90	133–134 (decomp.), (134 ref. [25])	^{1}H NMR (acetone-d_6, δ, ppm): 4.04 (s, 3 H, Me) Found, %: C 27.81; H 2.46; N 32.73. Calculated, %: C 27.92; H 2.34; N 32.55.

The synthesis of substituted amides of ATC is not a trivial task due to some properties of the initial compound. Acid (**IXa**) tends to decarboxylate even at moderate heating and is practically insoluble in the majority of organic solvents. In this connection it was impossible to use standard protocols for obtaining the amide bond. The major part of amide derivatives reported earlier was synthesized with ammonolysis of the methyl or ethyl ester of ATC. This approach, however, is justified only in the case of some highly active primary amines, methylamine and ammonia in particular. The reaction rate is too slow for the preparation of secondary amines and more so for aromatic amines. Traditional approaches to activation of the carboxyl group (chloroanhydride method, active esters method) have not been used previously for ATC. Chloroanhydride of 1*H*-1,2,4-triazole-3-carboxylic acid (**XII**) was obtained in a form of monohydrochloride on heating of the acid in thionyl chloride with 68% yield. It has not been possible to use it effectively for amide synthesis because of the side reactions: *N*-acylation in the N1 position of the triazole ring and accompanying polymerization (the yield of the target product was 14%). *n*-Nitrophenyl ester of 1*H*-1,2,4-triazole-3-carboxylic acid (**XIII**) was obtained with 96% yield from chloroanhydride through reaction with potassium *n*-nitrophenolate (Scheme 4).



Scheme 4. Synthesis of anilide derivatives of TCA.

Its interaction with various amines in absolute ethyl acetate produced the desired amides with acceptable yields (40–70%). Rather complex chromatographic isolation did not allow the extension of the method to significant number of various substrates. Similar problems arose on application of dicyclohexylcarbodiimide as a condensation agent. The activated derivative of 1*H*-1,2,4-triazole-3-carboxylic acid was found to be unexpectedly stable and, hence, non-active.

The imidazole method for the production of anilides was found to be the most effective. An acid (**IXa**) is sufficiently soluble in absolute ethyl acetate for the formation of imidazolide on interaction with CDI *in situ* (Scheme 4). Target amides (**XIVa**)-(**XIVk**) were obtained with 58–92% yield in reaction of imidazolide with various anilines at 40–50°C, which we were able to isolate from the reaction mixture with high purity (no less than 90% according to ^{1}H-NMR and HPLC data, see Table 4) by precipitation with water.

Unfortunately, it was impossible to extend this method to aliphatic amides due to two reasons: the high solubility of the target products in water and the similar chromatographic mobility of such amides and imidazole, the main side product of the reaction. Isolation of each aliphatic amide obtained by the imidazole method required an individual approach.

The mixed anhydride method was used for batch production of aliphatic amides of ATC (**XIVa**)–(**XIVk**) (Scheme 5).

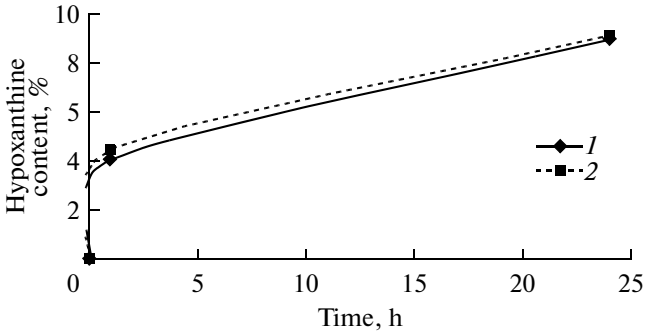
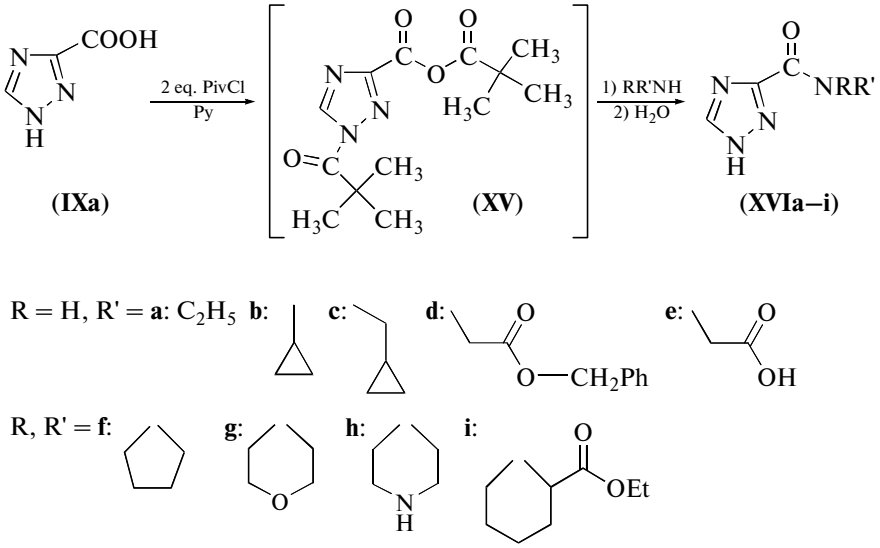


Fig. 1. Phosphorolysis of inosine in the absence (*1*, control) and presence of the amide of 5-ethyl-1*H*-1,2,4-triazole-3-carboxylic acid (**XIg**) (*2*).

Table 3. Preparation of 5-substituted analogues of ATC

Compound	Yield, %	T_m, °C	Physico-chemical characteristics
(XIb)	96	above 340	λ_{max}: 233.4 nm; *m/z*, 147.0081/149.0050 $[M + H]^+$ (Cl^{35}/Cl^{37}, 3/1), (calc. 147.0068). Found, %: C 24.61; H 2.00; N 38.47. Calculated, %: C 24.59; H 2.06; N 38.23. Purity 93.7% according to HPLC data, R_t = 5.9 min (variant 1).
(XIc)	quantitative	above340	λ_{max}: 233.2 nm; *m/z*, 190.9558/192.9561 $[M + H]^+$ (Br^{79}/Br^{81}, 1/1), (calc.190.9563); 212.9401/214.9380 $[M + Na]^+$; (calc. 212.9382). Found, %: C 19.05; H 1.67; N 29.28. Calculated, %: C 18.87; H 1.58; N 29.34. Purity 97.6% according to HPLC data, R_t = 6.11 min (variant 1).
(XId)	quantitative	above 340	^{1}H NMR spectrum (DMSO-d_6, δ, ppm): 11.21 (2 H, br. s, N<u>H</u> triazole); 7.62 (1 H, s, CON<u>H</u>); 7.40 (1 H, s, CON<u>H</u>). ^{15}N NMR spectrum (DMSO-d_6, δ, ppm): 100.7 (CO<u>N</u>H_2). λ_{max}: 204.4, 245.8 nm. *m/z*, 129.0423 $[M + H]^+$, (calc. 129.0407), 151.0240 $[M + Na]^+$, (calc. 151.0226). Found, %: C 28.01; H 3.16; N 44.10. Calculated, %: C 28.13; H 3.15; N 43.74. Purity 71.5% according to HPLC data, R_t = 2.1 min (variant 5).
(XIe)	84*	above 340	λ_{max}: 218.2, 282.4 nm. *m/z*, 156.0124 $[M - H]^-$ (calc. 156.0163); 113.0069 [*M*-CONH-H]$^-$ (calc. 113.0105) Found, %: C 22.81; H 2.00; N 44.16. Calculated, %: C 22.94; H 1.92; N 44.58. Purity 93.36% according to HPLC data, R_t = 4.4 min (variant 1).

Note: * after recrystallization from ethanol.



Scheme 5. Synthesis of aliphatic amides of ATC.

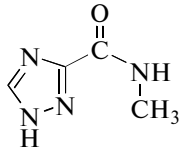
A mixed anhydride of 1-pivaloyl-1*H*-1,2,4-triazole-3-carboxylic acid with 2-trimethylacetic acid (**XV**) was obtained *in situ* by addition of 2 eq. of pivaloyl chloride to the ATC suspension in absolute pyridine, which was treated with 1 eq. of the respective amine. The pivaloyl group in the triazole ring was cleaved after the end of the reaction through the addition of a small quantity of water, the solvents were removed under vacuum, and target amides (**XVI**) were isolated with flash-chromatography on silica gel. Yields were from 69 to 88% (Table 5). An amide derivative of TCA with glycine (**XVIe**) was obtained from the compound (**XVId**) by cleavage of the benzyl group with hydrogen on a palladium catalyst.

Table 4. Preparation of anilides of 1,2,4-triazole-3-carboxylic acid

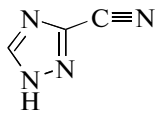
Compound	Yield, %	T_m, °C	Physico-chemical characteristics
(XIVa)	92	250	^{1}H NMR (DMSO-d_6, δ, ppm): 6.94 (1 H, m, PhH); 7.26 (2 H, m, PhH); 7.48 (2 H, m, PhH); 7.62 (1 H, s, NH); 9.13 (1 H, s, ArH). λ_{max}: 205, 255 nm. Purity 88.7%, R_t = 13.5 min (variant 4).
(XIVb)	58	185	^{1}H NMR (DMSO-d_6, δ, ppm): 6.79 (1 H, m, PhH); 7.12 (1 H, m, PhH); 7.30 (1 H, m, PhH); 7.47 (1 H, m, PhH); 7.86 (1 H, s, NH); 8.99 (1 H, s, ArH). λ_{max}: 255.0 nm. Purity 90.3% according to HPLC data, R_t = 16.58 min (variant 4).
(XIVc)	81	234	^{1}H NMR (DMSO-d_6, δ, ppm): 7.10 (2 H, m, PhH); 7.44 (2 H, m, PhH); 8.67 (1 H, s, ArH). λ_{max}: 262.2 nm. Purity 98.91% according to HPLC data, R_t = 14.87 min (variant 4).
(XIVd)	80	240	^{1}H NMR (DMSO-d_6, δ, ppm): 7.02 (1 H, m, PhH); 7.27 (2 H, m, PhH); 7.67 (1 H, m, PhH); 8.95 (1 H, s, ArH) λ_{max}: 259.1 nm. Purity 94.8% according to HPLC data, R_t = 18.90 min (variant 4).
(XIVe)	75	above 250	^{1}H NMR (DMSO-d_6, δ, ppm): 7.45 (4 H, m, PhH); 8.26 (1 H, s, NH); 9.72 (1 H, s, ArH). λ_{max}: 249.0 nm. Purity 99.7% according to HPLC data, R_t = 19.42 min (variant 4).
(XIVf)	86	above 250	^{1}H NMR (DMSO-d_6, δ, ppm): 7.32 (2 H, m, PhH); 7.46 (2 H, m, PhH); 8.83 (1 H, s, ArH). λ_{max}: 243.2 nm. Purity 98.07% according to HPLC data, R_t = 18.54 min (variant 4).
(XIVg)	67	215	^{1}H NMR (DMSO-d_6, δ, ppm): 6.98 (1 H, m, PhH); 7.24 (1 H, m, PhH); 8.03 (1 H, m, PhH); 8.85 (1 H, s, ArH). λ_{max}: 261, 278 nm. Purity 95.9% according to HPLC data, R_t = 16.37 min (variant 4).
(XIVh)	68	218	^{1}H NMR (DMSO-d_6, δ, ppm): 2.26 (3 H, s, CH_3); 6.77 (1 H, m, PhH); 7.13 (1 H, m, PhH); 7.19 (1 H, m, PhH); 7.29 (1 H, m, PhH); 8.53 (1 H, s, ArH). λ_{max}: 264.3 nm. Purity 98.4% according to HPLC data, R_t = 16.15 min (variant 3).
(XIVi)	70	184	^{1}H NMR (DMSO-d_6, δ, ppm): 3.85 (3 H, s, OCH_3); 6.93 (3 H, m, PhH); 8.07 (1 H, m, PhH); 8.86 (1 H, s, ArH) λ_{max}: 262.0, 292.0 nm. Purity 93.4% according to HPLC data, R_t = 15.8 min (variant 4).
(XIVj)	83	179	^{1}H NMR (DMSO-d_6, δ, ppm): 3.72 (3 H, s, OCH_3); 6.54 (1 H, m, PhH); 7.91 (1 H, m, PhH); 7.16 (2 H, m, PhH); 8.63 (1 H, s, ArH) λ_{max}: 262.0, 283.1 nm. Purity 91.5% according to HPLC data, R_t = 14.46 min (variant 4).
(XIVk)	81	175	^{1}H NMR (DMSO-d_6, δ, ppm): 1.32 (3 H, t, J 7, OCH_2CH_3); 4.31 (2 H, q, J 7, OCH_2CH_3); 7.42 (1 H, m, PhH); 7.57 (1 H, m, PhH); 7.66 (1 H, m, PhH); 8.15 (1 H, s, PhH); 8.96 (1 H, s, ArH) λ_{max}: 248.0 nm. Purity 98.17% according to HPLC data, R_t = 17.91 min (variant 4).

N-Methylamide of 1*H*-1,2,4-triazole-3-carboxylic acid (**XVII**) was synthesized with the reaction of ATC methyl ester (**Xa**) with an excess of methylamine in methanol.

A compound (**XVIII**) was obtained according to a previously described method [30] through dehydrogenation of TCA with trifluoroacetic anhydride in dioxane-pyridine system with 60% yield. 1*H*-1,2,4-Triazole-3-carbonitrile (**XVIII**) synthesized from TCA (**XIa**) can be easily transformed into another derivative of 1,2,4-triazole or used in the chemical synthesis of ribavirine and viramidine directly.



(**XVII**)



(**XVIII**)

Formulas 3. Hetercyclic bases.

Table 5. Prepared aliphatic amides of 1,2,4-triazole-3-carboxylic acid

Compound	Yield, %	T_m, °C	Physico-chemical characteristics
(XVIa)	71	181–185	^{1}H NMR*: 8.65 (1 H, br. s, ArH); 3.25 (2 H, q, *J* 7, NCH$_2$CH$_3$); 1.08 (3 H, t, *J* 7, NCH$_2$CH$_3$); *m/z*, 141.0774 $[M + H]^+$ (calc. 141.0771); 163.0594 $[M + Na]^+$ (calc. 163.0590); λ_{max}: 226.2 nm; Purity 95.26% according to HPLC data, R_t = 4.8 min (variant 2).
(XVIb)	82	200–206	^{1}H NMR: 8.28 (1 H, br. s, ArH); 2.82 (1 H, m, cyclo-Pr); 0.63 (4 H, m, cyclo-Pr); *m/z*, 153.0780 $[M + H]^+$ (calc. 153.0771); 175.0590 $[M + Na]^+$ (calc. 175.0590); λ_{max}: 226.6 nm; Purity 94.9% according to HPLC data, R_t = 5.2 min (variant 2).
(XVIc)	88	200–204	^{1}H NMR: 8.66 (1 H, br. s, ArH); 3.09 (2 H, m, NCH$_2$); 1.03 (1 H, m, cyclo-Pr); 0.38 and 0.22 (4 H, 2 m, cyclo-Pr); *m/z*, 167.0932 $[M + H]^+$ (calc. 167.0927); 189.0750 $[M + Na]^+$ (calc. 189.0747); λ_{max} (nm): 230.2; Purity 98.2% according to HPLC data, R_t = 8.51 min (variant 2).
(XVId)	52	204–206	^{1}H NMR: 8.40 (1 H, br. s, ArH); 7.37 (5 H, m, Ph); 5.20 (2 H, s, PhCH$_2$); 4.25 (2 H, d, *J* 6.1, N-CH$_2$); *m/z*, 261.0998 $[M + H]^+$ (calc. 261.0982); λ_{max} (nm): 232.2; Purity 90.6% according to HPLC data, R_t = 10.18 min (variant 4).
(XVIe)	84	214–216 degr.	^{1}H NMR: 8.51 (1 H, s, ArH); 4.15 (2 H, d, *J* 6.1, N-CH$_2$); *m/z*: 171.0528 $[M + H]^+$ (calc. 171.0518); λ_{max}: 235.4 nm; Purity 99.47% according to HPLC data, R_t = 3.98 min (variant 2).
(XVIf)	80	170–174	^{1}H NMR (D_2O): 8.44 (1 H, s, ArH); 3.70 and 3.53 (4 H, 2 m, β-CH$_2$ pyrrolidine); 1.88 (4 H, m, α-CH$_2$ pyrrolidine); *m/z*, 167.0929 $[M + H]^+$ (calc. 167.0927); 189.0748 $[M + Na]^+$ (calc. 189.0747); λ_{max}: 235.6 nm; Purity 97.2% according to HPLC data, R_t = 7.9 min (variant 2).
(XVIg)	74	189–193	^{1}H NMR (D_2O): 8.53 (1 H, s, ArH); 3.85–3.68 (8 H, m, morpholine); *m/z*, 183.0873 $[M + H]^+$ (calc. 183.0877); 205.0692 $[M + Na]^+$ (calc. 205.0696); λ_{max}: 231.2 nm; Purity 94.2% according to HPLC data, R_t = 4.96 min (variant 2).
(XVIh)	43	255 (subl.)	^{1}H NMR: 14.66 (1 H, br. s, NHCH$_2$); 8.52 (1 H, s, C5-H); 3.87, 3.19 (8 H, 2 m, NCH$_2$CH$_2$NH); *m/z*, 182.1031 $[M + H]^+$ (calc. 182.1036); λ_{max}: 235.2 nm; Purity 97.75% according to HPLC data, R_t = 2.0 min (variant 1).
(XVIi)	69	132–136	**^{1}H NMR (D_2O): 8.56 and 8.51(1 H, 2 s, ArH); 5.32 and 5.16 (1 H, m, α-CHCO-OEt); 4.44; 3.98; 3.25; 2.95; 2.36–2.23; 1.80–1.35 (8 H, 6 m, CH$_2$); 4.27 and 4.22 (2 H, 2 q, *J* 7 Hz, OCH$_2$CH$_3$); 1.28 and 1.23 (3 H, 2 t, *J* 7, OCH$_2$CH$_3$); *m/z*, 253.1297 $[M + H]^+$ (calc. 253.1295); 275.1109 $[M + Na]^+$ (calc. 275.1115); λ_{max}: 229.4 nm; Purity 93.9% according to HPLC data, R_t = 13.02 min (variant 3).
(XVII)	73	238–240	^{1}H NMR: 8.50 (1 H, s, ArH); 4.24 (3 H, d, *J* 6, N-Me); *m/z:* 127.0625 $[M + H]^+$ (calc. 127.0620), 149.0442 $[M + Na]^+$ (calc. 149.0434); λ_{max}: 233.6 nm; Purity 100% according to HPLC data, R_t = 4.2 min (variant 2).

Notes: * NMR was recorded in DMSO-d_6 if not mentioned otherwise.

** Doubling of all signals was observed in the spectrum of **(XVII)** compound due to the hindered rotation about C(O)–N< bond.

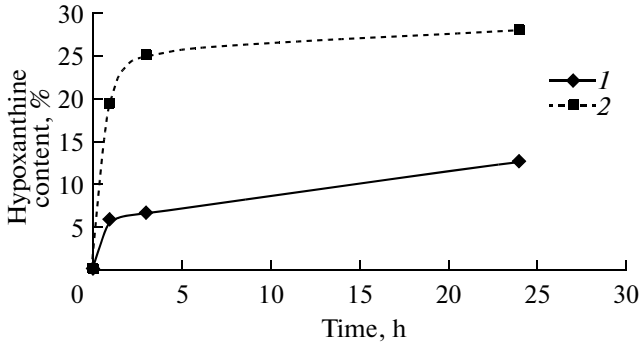


Fig. 2. Phosphorolysis of inosine in the absence (*1*, control) and presence of the benzyl ester od *N*-(1*H*-1,2,4-triazole-3-yl-carbonyl)glycine (**XVId**) (*2*).

In the second step of the experimental study, substrate-specific properties of the synthesized compounds in relation to *E. coli* PNP were investigated.

Three possible results are expected usually in the studies of transglycosylation reaction with new bases: the test compound is an inhibitor of PNP, a substrate of PNP, or neither an inhibitor, nor a substrate of the enzyme.

The standard reaction mixtures contained for the *control* natural nucleoside inosine and for the test, a modified heterocyclic base and inosine in a molar ratio of 1 : 2. Two units of PNP per mole of the substrate (base) was added to each reaction mixture. Enzymatic reactions were conducted in 2 mM KH_2PO_4 buffer, pH 7.0 at 55°C. The duration of the process was up to five days. Aliquots were taken after 1 h and further once a day. The extent of the conversion of initial compounds was monitored with HPLC.

The conclusions on the substrate specificity of 1,2,4-triazole analogues were based on the comparison of the amount of hypoxanthine (product of phosphorolysis of inosine) in the control and test mixture, as well as on the appearance of new nucleosides in the test reaction mixture (according to HPLC and mass-spectrometry data).

An equal amount of hypoxanthine in the control and test (containing modified base) mixtures indicates that the tested compound is neither the substrate not the inhibitor of nucleoside phosphorylases. This process is presented graphically in Fig. 1. The data of the reaction containing amide of 5-ethyl-1*H*-1,2,4-triazole-3-carboxylic acid (**XIg**) are given as an example.

If the phosphorolysis reaction rate of natural nucleoside is lower in the test system than in the control, or phosphorolysis is not observed in the presence of the modified base, the test compound is an inhibitor of PNP. Unfortunately no such PNP inhibitors were found among all the synthesized heterocyclic bases (HCB).

New compounds appear in the mixture in the case where the modified base is a PNP substrate, furthermore, the rate of hypoxanthine production in the test system is higher than in the control, which indicates the realization of a coupled process, the transfer of the formed α-D-ribosyl phosphate to the modified base-acceptor. This process is presented graphically in Fig. 2. The data of the reaction containing the heterocyclic base (**XVId**) are given as an example.

The results of a series of experiments showed that the presence of a nitrogen atom (amide or nitrile group) in the C3-position of the heterocyclic base of the substitute is required for interaction of 1,2,4-triazole bases with the active center of the enzyme: ATC (**IXa**) and its methyl ester (**Xa**) are not PNP substrates.

We have conducted a series of calculations of the electron structures (*ab initio, 6-31G**, HyperChem v.8.0.6*) of the investigated heterocycles and hypoxanthine (as a standard of comparison). Analysis of the data presented in Table 6 allows us to suggest that the substrate properties of hypoxanthine are defined by a combination of the electron properties of N1- and O7-atoms. This factor is present in the TCA molecule, but not in ATC and its methyl ester, which can explain the lack of substrate properties in the latter.

None of the synthesized 5-substituted triazole derivatives (**IXb–h**)–(**XIb–h**) are PNP substrates.

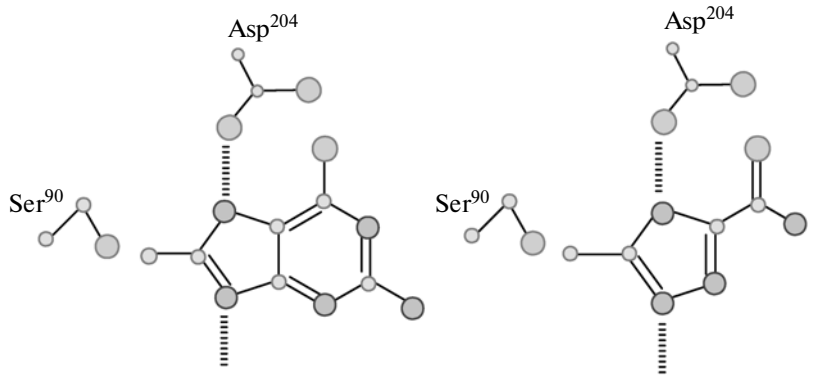
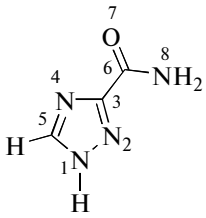
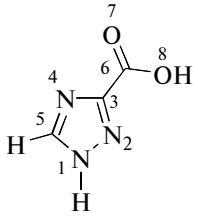


Fig. 3. Model of 8-methylguanine (left) and 5-methyl TCA (right) binding in the active center of PNP from *E. coli*.

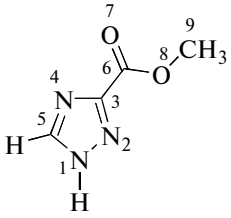
Table 6. Calculation data for electron structures of the investigated heterocycles and hypoxanthine (*ab initio, 6-31G**, Hyper Chem v.8.0.6*)*



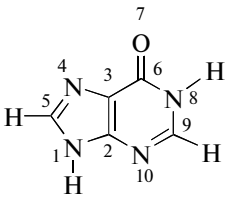
(XIa)



(IXa)



(Xa)



Hypoxanthine

Compound	Charges			
	N1	N4	H-N1	O7
TCA (**XIa**)	–0.422	–0.538	0.342	–0.579
ATC (**IXa**)	–0.421	–0.537	0.344	–0.535
(**Xa**)	–0.422	–0.538	0.343	–0.550
Hypoxanthine	–0.724	–0.520	0.334	–0.586

* Full calculation data are available from the authors upon request.

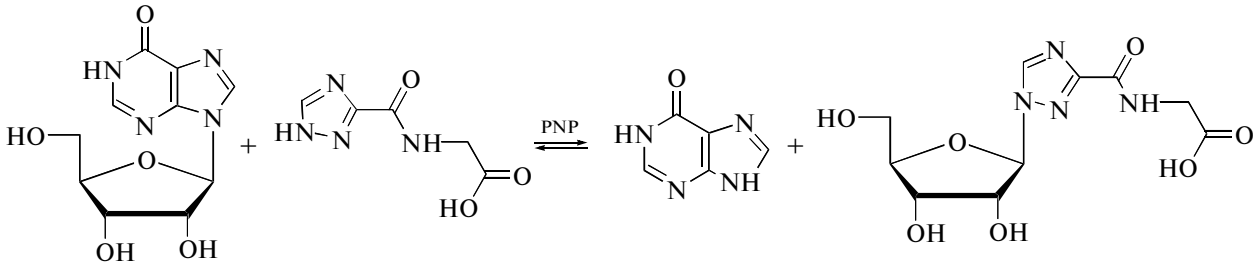
Introduction of voluminous substitutes in the 5-position of TCA (larger than methyl in volume) results in steric difficulties in linking the base with the enzyme active center (compounds **XIg–h**). The fact that the 5-chloro- (**XIb**) and 5-bromo-derivative (**XIc**) of TCA are not PNP substrates, though 5-methyl TCA is, is in agreement with the literature data reported for purine bases. The data from X-ray structure analysis (RSA) suggest that the 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide is positioned in the active center of enzyme in such a way that the fifth position of triazole ring is located where the eighth position of purine usually is (Fig. 3) [31].

Investigation of substrate specificity of C8-substituted guanosines [32] showed that while the presence of a methyl or amino group in the 8-position of purine decreases the efficiency of their interaction with the enzyme in comparison with guanosine itself, they nevertheless remain sufficiently good substrates. The availability of chlorine or bromine atoms in the eighth position makes the interaction of the base with the active center of the enzyme practically impossible. *Ab initio* geometry optimization method was used to estimate the most energy efficient 1,2,4-triazole tautomers of the compounds in the (**XI**) series. The calculation showed that a N^1,N^4-dihydro-5-keto-1,2,4-triazole-3-carbamide tautomer of 5-hydroxy triazole carboxamide (**XId**) (Fig. 4) is the most thermodynamically stable, which can explain the lack of substrate properties for this compound due to the impossibility of hydrogen bond formation between the N^4-atom of the base and Asp^{204} of the PNP from *E. coli*, because the hetercyclic base bound to the active center should have an unprotonated N4 atom (or N7 in the case of purine base). The aromatic phenyl ring probably interferes with hydrophobic non-specific π–π interaction of Phe^{159} and Tyr^{160} with HCB. However, if the voluminous aromatic substituent is 'pushed' from triazole by the introduction of a glycine spacer (see compound **XVId**), such a heterobase becomes the perfect PNP substrate.

Unfortunately, there are no RSA data available for the complex of PNP from *E. coli* with any triazole analogue. Currently, only RSA data were obtained for the active site of *E. coli* PNP with the inhibitor formycin [17]. Orientation of the heterocyclic base in the enzyme active center is achieved through hydrogen bonds between its N^7 atom, C6 amino- or hydroxyl groups, and Asp^{204} residue. According to our data, other hydrogen bonds between the nitrogen atom of the substituent at the C3 atom of 1,2,4-triazole and amino acids of the active site of the enzyme should exist: if the interaction is restricted only to Asp^{204}, then the 1,2,4-triazole-3-carboxylic acid (**IXa**) should be a good substrate (Fig. 5), but this is not observed in our experiments (Fig. 5). It is also unlikely that the single Asp^{204} amino acid 'reaches' to both the N^4 atom of triazole and the nitrogen atom of the carbonitrile group of the (**XVIII**) heterocycle or piperidinyl substituent (**XVIh**).

Aliphatic and cyclic *N*-alkyl triazole carboxamides (**XVIa**)–(**XVIi**) were found to be good substrates among the substituted amide derivatives of TCA with the extent of conversion to the respective nucleosides up to 99% (Table 6). The affinity of the substrate to the enzyme active center (the rate of synthesis of new nucleosides) directly correlates with the rate of hypoxanthine formation in the test reaction mixture: experimental data for some derivatives are presented graphically in Fig. 6. The pyrrolidone derivative of TCA (**XVIf**) was found to be the best substrate among amides (**XVIa–i**).

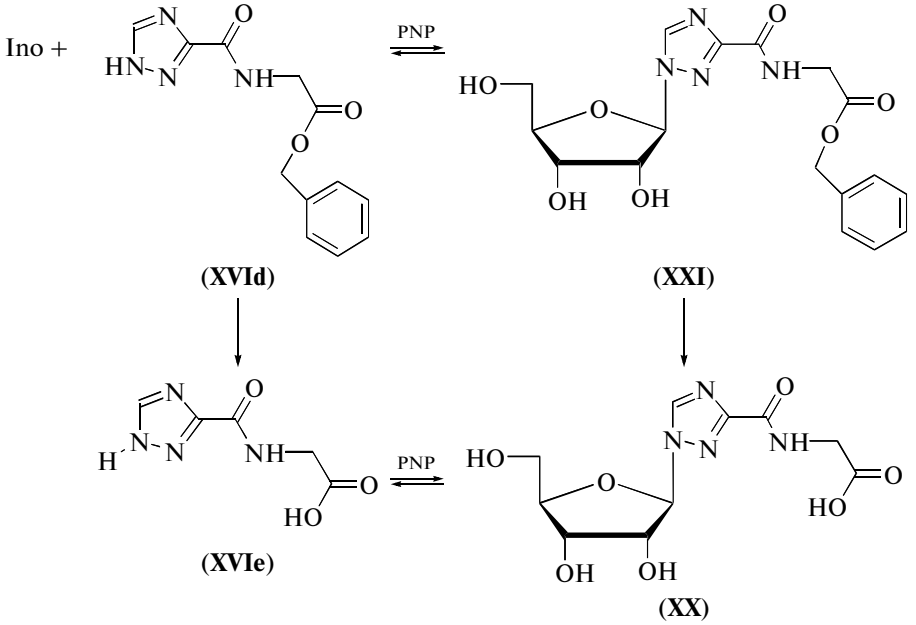
The surprising thing was that the glycine-containing base (**XVIe**) became an acceptor of ribose in the active site of the enzyme even with the conversion to nucleoside being as low as 17% (Scheme 6, Table 7).



Scheme 6. Synthesis of riboside (**XX**).

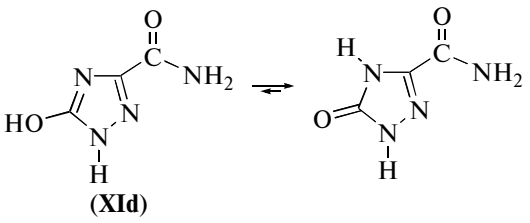
Most probably the voluminous negatively charged carboxyl group located in the C3 position of triazole ring of ATC (**IXa**) prevents its binding to the active site of the enzyme. In the case of the base (**XVIe**), the carboxyl group is 'pushed' from the triazole ring by the spacer group, which results in the emergence of substrate properties in HCB.

Replacement of the carboxyl group of base (**XVIe**) by the benzene carbonyl one in HCB (**XVId**) improved the situation even more: the conversion of the base (**XVId**) to riboside (**XXI**) was 91% after one day. However, two additional compounds were found in the reaction mixture, which were later identified as compounds (**XVIe**) and (**XX**) (Scheme 7) according to the retention times in HPLC (Fig. 7) and mass-spectrometry data (Table 8). It was found that at the chosen conditions (pH 7.0 and temperature 50°C) hydrolysis of the benzene protecting group of the carboxyl residue (**XVId**) occurs. The content of riboside (**XX**) in the reaction mixture was 24% (Fig. 7); hence, the efficiency of the enzymatic process of the (**XX**) synthesis was twice as high as in the case of Scheme 6. In this manner, nucleoside (**XX**) can be synthesized quantitatively by an alternative method of varying the pH of the reaction mixture.



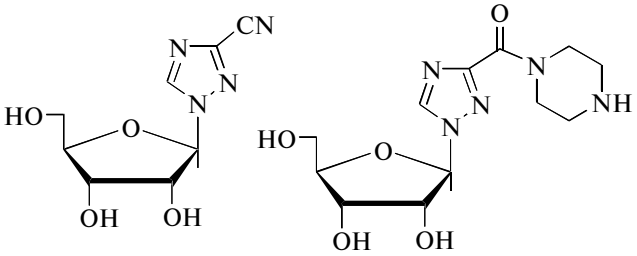
Scheme 7. Synthesis of riboside (**XXI**).

Two ribavirin analogues (**XXII**) and (**XXIII**) were prepared after optimization of the conditions of enzymatic reactions (the most effective ribose donor was identified, the nucleoside/base ratio and the optimal amount of enzyme were determined).



(**XId**)

Fig. 4. Tautomeric forms of 5-hydroxy-1*H*-1,2,4-triazole-3-carboxamide (**XId**). An analogous situation is observed for anilide derivatives (**XIVa-k**). Introduction of aromatic substituents in the carboxamide group of TCA prevents the interaction of HCB with the active center of PNP from *E. coli*.



(**XXII**) (**XXIII**)

Formulas 4. Ribavirin analogues obtained in preparative quantities.

The fact that 1*H*-1,2,4-triazole-3-carbonitrile (**XVIII**) was found to be a PNP substrate was considered very promising: the development of a biotechnological method of novel viramidine analogue (**III**) production became possible. The ribose derivative of 1,2,4-triazole-3-carbonitrile (**XXII**) was obtained through a transglycosylation reaction with a yield of 60%. Guanosine was chosen as a ribose donor.

A ribonucleoside of 3-carboxypiperasinyl-1,2,4-triazole (**XXIII**) was synthesized with a yield of 65% using a similar method.

Hence, the basic requirements for the structure of 1*H*-1,2,4-triazole-3-carboxamide allowing the performance of enzymatic synthesis of novel ribose and deoxyribose analogues of ribavirin were determined as a result of a series of experiments: voluminous substituents (larger than methyl) should not be present in the C5 position of HCB, the amide or nitrile group should be available in the C3 position of 1,2,4-triazole with aliphatic, *N*-cyclic, or carboxyl-containing (amino acids as an example), but not aromatic, substituents allowed in the amide group. The introduction of aromatic substituents in the amide group through the spacer is acceptable.

Investigation of antiviral activity of the two synthesized compounds in the models of hepatitis C, herpes simplex virus type 2, type A and B flu viruses, and human immunodeficiency virus is currently being conducted in the Ivanovskii Institute of Virology. The test results of antiviral activity of the synthesized compounds will be published later.

EXPERIMENTAL

Acetyl chloride, hydrazine hydrate (100%), ethyl esters and chloro-anhydrides of propionic, butyric, isobutyric, isovalerianic, cyclopropane carboxylic, and trimethylacetic acids (Sigma-Aldrich), solvents from a domestic manufacturer purified with standard procedures were used in the work.

Purine nucleoside phosphorylase (protein concentration determined with Bradford assay, 15 mg/mL; activity, 52 units/mg of protein) was obtained in the group of recombinant proteins (Institute of Bioorganic Chemistry, Russian Academy of Sciences, supervisor Dr. R.S. Esipov) [23, 24].

^{1}H NMR spectra (δ, ppm; *J*, Hz) were recorded in DMSO (if not stated otherwise) using a Bruker DPX-300 with operating frequency 300 MHz and a Bruker DRX-700 (Germany) with operating frequency 700 MHz

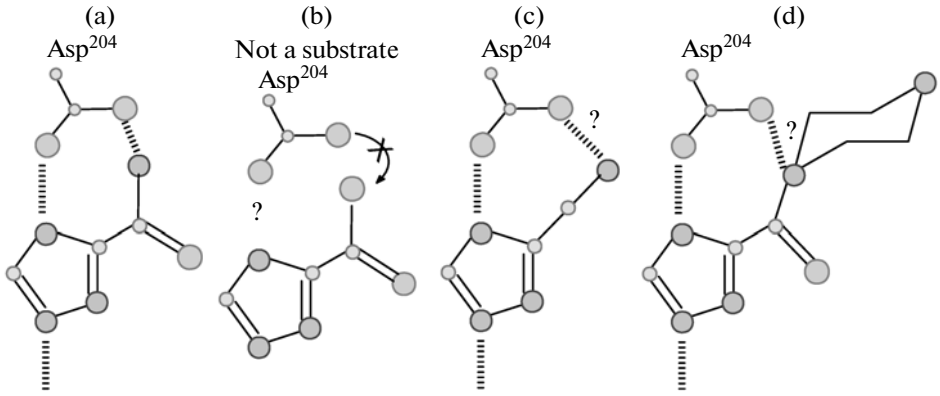


Fig. 5. Proposed model of binding of TCA (a), ATC (**IXa**) (b), 1*H*-1,2,4-triazole-3-carbonitrile (**XVIII**) (c), and 3-carboxypiperanyzyl-1*H*-1,2,4-triazole (**XVIh**) (d) in the active center of PNP from *E. coli*.

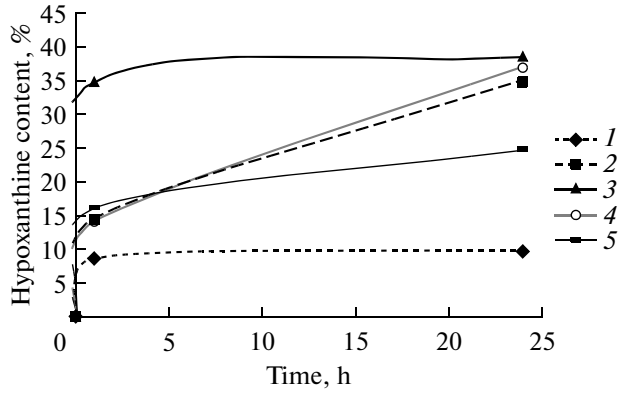


Fig. 6. Phosphorolysis of inosine in the absence (*1*, control) and presence of N-substituted bases of 1*H*-1,2,4-triazole-3-carboxylic acid (**XVIa**) (*2*), (**XVIf**) (*3*), (**XVIc**) (*4*), and (**XVIb**) (*5*).

(IBC RAS). Designations used in ^{1}H NMR spectra: s, singlet, d, duplet, t, triplet, q, quartet, m, multiplet, br., broad.

Mass spectra were recorded with an API 150EX (Perkin-Elmer Instruments) (ionization with electrospray) and with an Agilent 6224, ESI-TOF, LC/MS. IR spectra were recorded with a Specord 3000 instrument (Germany). UV spectra were recorded with a Shimadzu UV-160 spectrophotometer (Japan).

Silica gel Kiselgel F_{254} (Merck, Germany) was used for column chromatography; domestic plates Sorbfil and Merck F_{254} plates (Germany) were used for thin layer chromatography.

Enzymatic reactions were monitored using HPLC (chromatograph Waters 2487, Breeze, United States), column Nova-Pak C18, 4 μm, 4.6 × 150 mm. Eluent A: 0.1% TFA/water, eluent B: 70% acetonitrile in A. The flow rate was 1 mL/min, detection was conducted at 215, 225, 230, and 254 nm. Variants of elution gradient B in A: 1) gradient 0% B, 5 min, 0–15% B, 10 min, 215 and 254 nm; 2) gradient 0–30% B for 20 min, 225 and 254 nm; 3) gradient 0–50% B for 20 min, 230 and 254 nm; 4) gradient 0–100% B for 20 min, 230 and 254 nm; 5) 100% A, 215 and 254 nm; 6) gradient 0% B, 5 min, 0–50% B, 15 min, 230 and 254 nm.

Carboxylic acids hydrazides were obtained from the respective ethyl esters and hydrazine hydrate according to a standard method, hydrochloride of diethyl ester of carbonyl formimide acid was prepared according to [28], and triazole carboxamide, according to [27].

1*H*-1,2,4-triazole-3-carboxylic acid and its substituted analogues (IXa)–(IXf) were synthesized from 3-amino-1,2,4-triazole-5-carboxylic acid in accordance with the method described in the literature [25, 27, 28]. Physico-chemical characteristics of the compounds obtained were in agreement with the structure and literature data (Table 1).

Methyl esters of 1*H*-1,2,4-triazole-3-carboxylic acids (Xa)–(Xe). Thionyl chloride (1.8 g, 15 mmol) was added dropwise under stirring to a solution of 10 mmol of compound (**IXa**)–(**IXe**) in 20 mL of absolute methanol cooled to 10°C in a way that the temperature did not increase above 15°C. Next, the temperature was increased to room temperature; the mixture was stirred for 2 h followed by the solvent removal under vacuum. The residue was dissolved in chloroform and filtered through the silica gel pad. The solvent was removed under vacuum; the residue was dried under a vacuum of 1 mm Hg for 48 h. The physicochemical characteristics and yields of the compounds (**Xa**)–(**Xe**) are given in Table 2.

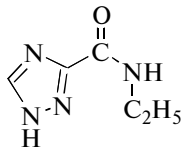
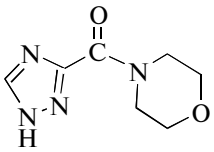
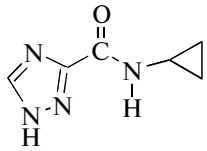
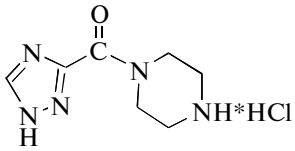
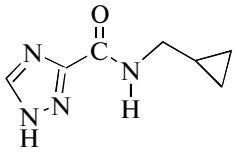
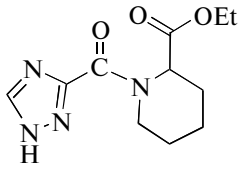
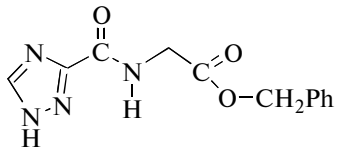
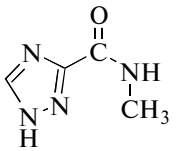
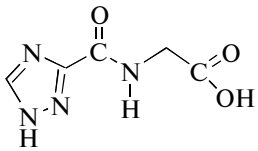
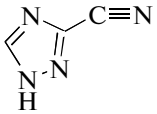
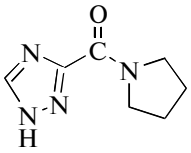
Amides of 1*H*-1,2,4-triazole-3 carboxylic acids (XIa)–(XIe). A solution of 1 mmol of (**Xa**)–(**Xe**) methyl ester in 5 mL of a saturated ammonia solution in absolute methanol was stirred for 48 h at room temperature. The solvent was removed under vacuum, the residue was dried at 1 mm Hg for 48 h. Physico-chemical characteristics and yields of the compounds (**XIb**)–(**XIe**) are presented in Table 2.

Ethyl ester of 5-ethyl-1*H*-1,2,4-triazole-3-carboxylic acid (Xg). Two grams (11 mol) of hydrochloride of diethyl ester of carbonyl formimide acid was added upon stirring to the ice-cold solution of 0.97 g (11 mmol) of propionic acid hydrazide in 5 mL of non-aqueous methanol. Sodium hydrocarbonate (1 g, 12 mmol) dissolved in 10 mL of water was added to the reaction mixture upon the reaction progress (control with TLC, chloroform-methanol (9 : 1) system). Methanol from the reaction mixture was removed with a rotary evaporator. The white crystals formed were separated by filtration, carefully washed with water and diethyl ester, and air dried. The yield of ethyl-β-propionyl oxalic amiderazone was 0.43 g (21%). ^{1}H NMR: 1.24 (3 H, m, $OCH_2C\underline{H}_3$); 1.89 and 2.07 (1.3 H and 1.6 H, 2 s, $C(O)C\underline{H}_3$); 4.20 (2 H, m, $OC\underline{H}_2CH_3$); 6.39 (2 H, br.d., *J* 15.2 NHNH); 9.76 (1 H, br.d., *J* 9.4, =NH). Mass-spectrum, *m/z*: 188.1197 $[M + H]^+$; 210.0849 $[M + Na]^+$.

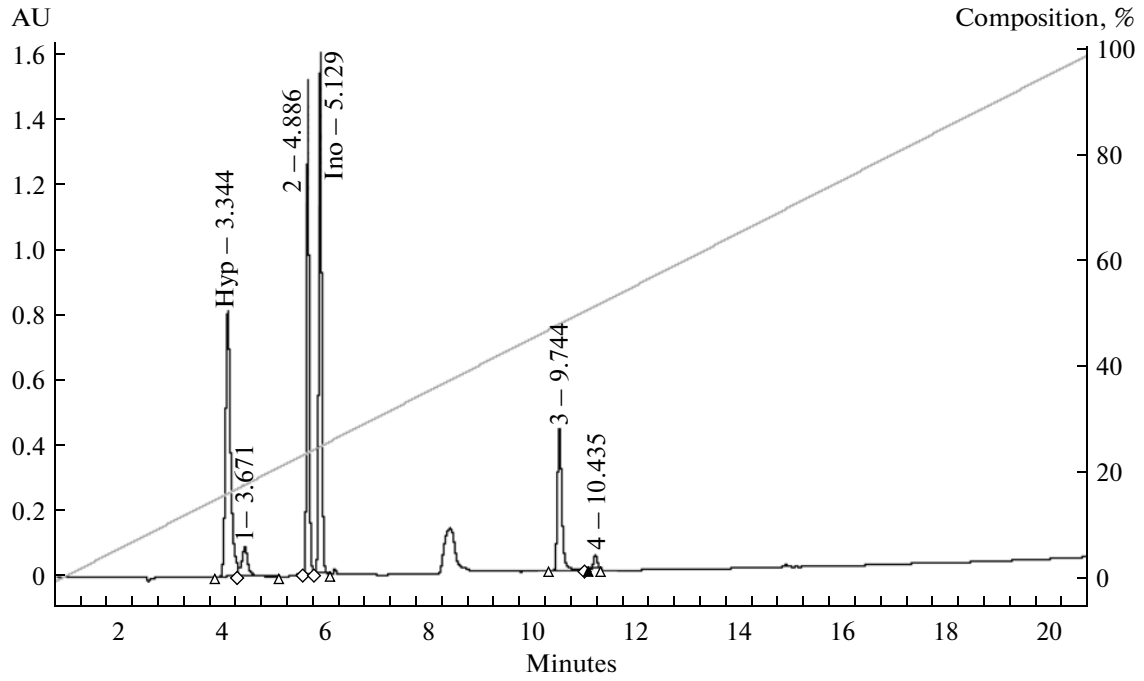
Chloro-anhydride of propionic acid (220 mg, 2.4 mmol) was added to the stirred suspension of 430 mg (2.3 mmol) of ethyl-β-propionyl oxalic amiderazone in 5 mL of absolute pyridine and heated to boiling. The mixture was boiled for 24 h followed by solvent removal under vacuum; the residue was chromatographed on the open silica gel column (eluent chloroform). Fractions containing the target product were combined, the solvent was removed under vacuum, the residue was dried under a vacuum of 10 mm Hg for 4 h. The yield was 331 mg (85%). ^{1}H NMR ($CDCl_3$): 1.33 (3 H, t, *J* 7.64, $-CH_2C\underline{H}_3$); 1.35 (3 H, t, *J* 7.14, $-OCH_2C\underline{H}_3$); 2.92 (2 H, q, *J* 7.64, $C\underline{H}_2CH_3$); 4.42 (2 H, q, *J* 7.14, $-OC\underline{H}_2CH_3$); *m/z*: 170.0934 $[M + H]^+$ (calc. 170.0903).

Amide of 5-ethyl-1*H*-1,2,4-triazole-3-carboxylic acid (XIg). A saturated aqueous ammonia solution

Table 7. Substrates of PNP*

Heterocyclic base	Ratio nucleoside/base	Heterocyclic base	Ratio nucleoside/base
 **(XVIà)**	95 : 5	 **(XVIg)**	99 : 1
 **(XVIb)**	97 : 3	 **(XVIh)**	70 : 30
 **(XVIc)**	86 : 14	 **(XVIi)**	91 : 9
 **(XVId)**	91 : 9	 **(XVII)**	86 : 14
 **(XVIe)**	17 : 83	 **(XVIII)**	45 : 55
 **(XVIf)**	99 : 1		

* Target nucleoside/HCB ratio is presented according to HPLC analysis of the final mixture of the enzymatic reaction.



Name of the peak	R_t, min	Area, %
Hyp	3.344	27.72
1*	3.671	3.97
2	4.886	23.89
Ino	5.129	30.10
3	9.744	13.08
4	10.435	1.24

Fig. 7. HPLC data of the reaction mixture of nucleoside (**XXI**) synthesis. * Components of the reaction mixture (on the 10th day) were identified with mass spectrometry: 1, (**XVIe**), 2, (**XX**), 3, (**XXI**), 4, (**XVId**).

(3 mL) was added to the solution of 300 mg (1.77 mmol) of compound (**Xg**) in 5 mL of methanol and boiled with an inverse refrigerator for 72 h adding equal portions of saturated aqueous ammonia solution (0.25 mL) every 12 h. The mixture was cooled on completion of the reaction, the precipitate was filtered, washed with water and diethyl ester, and air dried. The yield was 226 mg (91%). ^{1}H NMR: 1.33 (3 H, t, *J* 7.64, -$CH_2C\underline{H}_3$); 2.92 (2 H, q, *J* 7.64, $C\underline{H}_2CH_3$); 7.52–7.83 (0.5 H, br.m, $CONH_2$); 9.7 (0.6 H, s, 1-NH); *m/z*: 142.0642 $[M + H]^+$ (calc. 142.0617).

Amide of 5-isobutyl-1*H*-1,2,4-triazole-3-carboxylic acid (XIe). A compound (**XIh**) was prepared similarly to the compound (**XIg**) without isolation and characterization of an intermediate ester (**Xh**). After the removal of solvents, the reaction mixture containing (**Xh**) ester was diluted with ethanol, saturated aqueous ammonia solution was added, and the reaction conducted as described above. Yield of the ethyl-β-iso-valeriaryl oxalic amidrazone was 51% (from the hydrazide of isovalerianic acid), ^{1}H NMR: 0.88 (6 H, dd, J_1 6.63; J_2 3.47, -$CH(C\underline{H}_3)_2$); 1.23 and 1.24 (3 H, 2 t, J_1 7.06; J_2 7.10, $OCH_2C\underline{H}_3$); 1.96–2.10 (2 H, m, -$C\underline{H}_2CH(CH_3)_2$); 2.39 (1 H, d, *J* 7.03 -$C\underline{H}_2CH(CH_3)_2$); 4.15–4.25 (2 H, m, $OC\underline{H}_2CH_3$); 6.35–6.42 (2 H, br.m, -NH-NH-); 9.62–9.81 (1 H br. M, =NH). Yield of (**XIh**) amide was 90% (per chloro anhydride of isovalerianic acid). ^{1}H NMR: 0.88 (6 H, br. d, *J* 6.88, $CH(\underline{CH}_3)_2$), 1.88–2.07 (3 H, m, $\underline{CH}_2\underline{CH}(CH_3)_2$); 2.56 (2 H, br. d, *J* 6.88, -$\underline{CH}_2CH(CH_3)_2$); 7.38–7.77 (2 H, br. m, $CONH_2$); 9.61 (1 H, s, 1-NH); *m/z*: 169.1092 $[M + H]^+$ (calc. 169.1089); λ_{max}: 224.2 nm.

Chloro anhydride of 1*H*-1,2,4-triazole-3-carboxylic acid hydrochloride (XII). 1,2,4-triazole-3-carboxylic acid (**IXa**) (22.4 g, 0.2 mol) was boiled in 40 mL (0.55 mol) of thionyl chloride. The precipitate was filtered after 4 h and washed with diethyl ester (3 × 50 mL). Next, it was dried under a vacuum of 10 mm Hg for 4 h. The yield was 17.66 g (68%). IR spectrum (suspension in vaseline oil), cm^{-1}: 1012 (av., -COCl); 1136 (av., -COCl); 1152 (weak, -COCl). Found, %: C 21.69; H 1.88; N 25.29. Calculated, %: C 21.45; H 1.80; N 25.01.

***n*-Nitrophenyl ester of 1*H*-1,2,4-triazole-3-carboxylic acid (XIII).** Potassium hydroxide (78 mg, 1.44 mmol) was added to the solution of 97 mg (0.7 mmol) of *n*-nitrophenol in 5 mL of absolute methanol. The solvent was evaporated to dryness in 20 min and the residue was dried under a vacuum of 10 mm Hg at 70°C for 4 h. Absolute acetone (15 mL) and 118 mg (0.7 mmol) hydrochloride of *1H*-1,2,4-triazole-3-carboxylic acid chloro anhydride (**XII**) were added. The reaction mixture was stirred at room temperature for 12 h. The precipitate was filtered, the filtrate was evaporated under vacuum. The residue was dried under vacuum (10 mm Hg) for 4 h. the yield was 157 mg (96%). ^{1}H NMR spectrum: 7.62 (2 H, d, *J* 9.57, 2 and 6 PhH<u>H</u>); 7.62 (2 H. d. *J* 9.57, 3 and 5 PhH<u>H</u>); 8.81 (1 H, s, Ar<u>H</u>).

Anilides of 1*H*-1,2,4-triazole-3-carboxylic acids (XIVa)–(XIVk). A carbonyldiimidazole (194 mg, 1.20 mmol) was added to a dispersion of 113 mg (1 mmol) of 1,2,4-triazole-3-carboxylic acid (**IXa**) in 4 mL of absolute ethyl acetate on intensive stirring. The respective anilide (0.8 mmol) was added to the mixture after 1 h. The reaction mixture was stirred for 3 h, evaporated to 0.5 mL volume, the precipitate was filtered, dried on the filter, and washed with distilled water (3 × 5 mL). The product was dried under vacuum (10 mm Hg) for 24 h. Physico-chemical characteristics and yields of the compounds (**XIVa**)–(**XIVk**) are presented in Table 4.

Substituted amides of 1*H*-1,2,4-triazole-3-carboxylic acid (XVIa)–(XVId) and (XVIf)–(XVIi). Pivaloyl chloride (0.42 mL, 3.54 mmol) was added to the dispersion of 200 mg (1.77 mmol) of 1,2,4-triazole-3-carboxylic acid (**IXa**) in absolute pyridine (5 mL) at 0–5°C. The respective amine (1.77 mmol) was added to the reaction mixture after 1.5 h. The mixture was stirred for 15 min followed by addition of several drops of distilled water. The reaction mass was evaporated under vacuum, the residue was re-evaporated with toluidine (5 mL). The product was purified with column flash-chromatography on silica gel (eluent: ethyl acetate). Fractions containing the target product were combined, the solvent was removed under vacuum, and the residue was dried under vacuum (1 mm Hg) for 4 h. The physico-chemical characteristics and yields of the compounds (**XVIa**)–(**XVId**) and (**XVIf**)–(**XVIi**) are presented in Table 5.

***N*-(1*H*-1,2,4-triazole-3-carbonyl)glycine (XVIe).** 10% Pd/C (30 mg) was added to a solution of 100 mg (0.38 mmol) of the compound (**XVId**) in 3 mL of methanol, and the mixture was saturated with hydrogen on mixing. After 1 h, when the calculated amount of hydrogen was adsorbed, the solution was filtered through a celite pad, and the solvent was removed under vacuum. The yield was 55 mg (84%). ^{1}H NMR spectrum: 8.51 (1 H, s, Ar<u>H</u>); 4.15 (2 H, d, *J* 6.1, N-C<u>H</u>$_2$); *m/z*: 171.0528 $[M + H]^+$ (calc. 171.0518), 193.0333 $[M + Na]^+$.

Table 8. Mass spectrometry data of the reaction mixture in the synthesis of the nucleoside (**XXI**)

Compound	R_t, min	*m/z*, $[M + H]^+$	Others
(**XVIe**)	3.67	171.0526	193.0333 $[M + Na]^+$
(**XX**)	4.89	303.0957	605.1803 $[2M - H]^+$; 171.0526 $[base]^+$
(**XXI**)	9.74	393.1412	785.2767 $[2M - H]^+$; 261.1009 $[base]^+$
(**XVId**)	10.43	261.1009	–

***N*-Methylamide of 1*H*-1,2,4-triazole-3-carboxylic acid (XVII).** Three mL of 4M solution of methylamine in methanol was added to a solution of 1.27 g (10 mmol) of the compound (**Xa**) in 20 mL of absolute methanol under stirring, the mixture was heated to boiling, and boiled for 1 h. The reaction mass was cooled, the solvent was removed under vacuum, and the residue was crystallized from ethyl alcohol. The yield was 0.92 g (73%). T_m = 238–240°C. ^{1}H NMR spectrum8.50 (1 H, s, Ar<u>H</u>); 4.24 (3 H, d, *J* 6, N-Me); *m/z*: 127.0625 $[M + H]^+$ (calc. 127.0620), 149.0442 $[M + Na]^+$ (calc. 149.0434). Purity 100% according to HPLC data, R_t = 4.2 min (variant 2); UV, λ_{max}: 233.6 nm.

1*H*-1,2,4-Triazole-3-carbonitrile (XVIII). 1,2,4-Triazole-3-carboxamide (**XIa**) (2.5 g, 22 mmol), 22 mL of absolute dioxane, and 8 mL (0.1 mol) of absolute pyridine were placed in a two-neck flask in a nitrogen atmosphere. The reaction mixture was cooled to 0°C followed by the dropwise addition of 7.1 mL (51 mmol) of trifluoroacetic anhydride within 10 min. The cooling was stopped, and the mixture was stirred at room temperature for the following 30 min. The target product was purified on the completion of the reaction with flash-chromatography on silica gel using ethyl acetate as an eluent. The yield was 1.25 g (60%). T_m = 185–187°C (lit. 187°C [30]); *m/z*: 117.0363 $[M + Na]^+$ (calc. 117.0358), λ_{max} 218.2 nm, purity 100%, R_t = 3.92 min (HPLC, variant 2). ^{1}H NMR spectrum: 8.87 (1 H, s, H5). ^{13}C NMR-spectrum: 113.05, 137.97, 146.15.

Substrate specificity of nucleoside phosphorylases. An aliquot of modified base (2 mmol) was dissolved in 1 mL of 2 mM KH_2PO_4 buffer solution, pH 7.0 (solution was heated till complete dissolution or up to 20% v/v of DMSO was added if required). A 4 mM solution of inosine in 2 mM KH_2PO_4 buffer solution, pH 7.0, was similarly prepared. The following mixtures were prepared: 1) 0.3 mL of 4 mM inosine solution and 0.3 mL of 2 mM KH_2PO_4 buffer solution (control reaction), and 2) 0.3 mL of 4 mM inosine solution and 0.3 mL of 2 mM solution of modified base (test reaction). PNP (5 μL) was added to the reaction mixtures and they were thermostated at 55°C. Samples were taken after 1 h and after each 24 h from the start of the reaction.

The reaction progress was monitored with HPLC: a detection wavelength for the control reaction was 254 nm, and for the test reactions was determined separately for each modified base with a UV spectrophotometer (hence, the detection was conducted at two wavelengths). A comparison of the hypoxanthine amount (% according to HPLC data at 254 nm) was conducted in the test and control mixtures.

1-β-D-Ribofuranozyl-1,2,4-triazole-3-carbonitrile (XXII). A (**XVIII**) base 20 mg (2 mmol), guanosine 451.97 mg (15 mmol), and KH_2PO_4 28.93 mg (2 mmol) were dissolved in 106.3 mL of distilled water. The pH of the solution was adjusted to 7.0 with 2 mM KOH. PNP (1.1 mL) was added to the reaction mixture. The solution was thermostated at 55°C for 4 d. The reaction mixture was placed in a refrigerator for 2 h for guanine precipitation. The precipitate was removed with centrifugation (2 min, 12000 rpm), the supernatant was separated, and the precipitate was dispersed in a minimal quantity of water followed by the centrifugation. Supernatants were combined and concentrated under vacuum (5 mmHg). A product was isolated with preparative chromatography on the Luna C18 column (Phenomenx) 100 μm, 250 × 21.20 mm. Fractions containing the product were combined, and eluent was removed under vacuum (5 mm Hg). The residue was dried in a vacuum desiccator. The yield was 27 mg (60%), main compound content 98.35%, R_t = 5.4 min (HPLC, variant 5), *m/z*: 227.0763 $[M + H]^+$ (calc. 227.0775). ^{1}H NMR spectrum: 9.15 (1 H, s, H5), 5.89 (1 H, d, $J_{1',2'}$ 3.7, H1'), 5.66 (1 H, br.s, $OH_{2'}$), 5.24 (1 H, br.s, $OH_{3'}$, 4.93 (1 H, br.t, *J* 4.5, $OH_{5'}$), 4.34 (1 H, dd, *J* 3.9, H'), 4.13 (1H, dd, $J_{3',2'}$ and $J_{3',4'}$ 4.4, H3'), 3.98 (1 H, dd, $J_{4',3'}$ 4.4, $J_{4',5'}$ 9.1, H4'), 3.62 (1 H, m, H5a'), 3.51 (1 H, m, H5b'). ^{15}N NMR: 307.7 (N2 triazole), 260.4 (N4 triazole), 237.2 (N1 triazole).

1-β-D-Ribofuranosyl-3-carboxpiperazinyl-1,2,4-triazole (XXIII). KH_2PO_4 (0.034 g) was dissolved in 61.54 mL of distilled water. The pH of the buffer solution was adjusted to 7.0 with 2 M KOH. Guanosine (0.262 g, 15 mM and base (**XVIh**) (0.050 g, 3.75 mM) were dissolved in the buffer. The reaction mixture was heated to 60°C to dissolve guanosine. Next, 0.06 mL of PNP was added to the reaction mixture. The solution was themostated for 7 d. Guanine (according to HPLC data) was filtered, and the filtrate was evaporated to a minimum volume. The residue was applied to a column with reverse-phase sorbent Silica gel 100 C_{18}-reversed phase (Fluka) (size of the column 25 × 170 mm) and eluted with water. Fractions containing more than 95% of the target product were combined, and the solvent was removed under vacuum (5 mm Hg). The product was dried in a desiccator over P_2O_5 for 24 h. The yield was 47 mg (65%). The content of the main compound was 98.28%, R_t = 2.22 min (HPLC, variant 1); *m/z*: 314.1452 $[M + H]^+$ (calc. 314.1459), 336.1269 $[M + Na]^+$. ^{1}H NMR: 8.86 (1 H, s, H%), 5.81 (1 H, d, $J_{1',2'}$ 3.7, H1'), 5.74 and 5.38 (2 H, m, OH3' and OH2'), 4.89 (1 H, m, OH5'), 4.32 (1 H, m, H2'), 4.13 (1 H, m, H3'), 3.94 (1 H, m, H4'), 3.61 (1 H, dd, $J_{4',5'}$ 3.8, H5a'), 3.54 (2 H, m, $NC\underline{H}'_2CH'_2NH$), 3.48 (1 H, m, $J_{4',5'}$ 4.89, H5'), 3.43 (2 H, m, $NC\underline{H}_2CH_2NH$), 2.72 (2 H, m, $NCH'_2C\underline{H}'_2NH$), 2.65 (2 H, m, $NCH_2C\underline{H}_2NH$). ^{13}C NMR: 160.55 (C(O)), 157.69 (C3), 144.83 (C5), 92.7 (C1'), 85.84 (C4'), 74.98 (C2'), 70.39 (C3'), 61.71 (C5'), 48.34 ($N\underline{C}H_2CH_2NH$), 46.60 ($NCH_2\underline{C}H_2NH$), 45.85 ($NCH'_2\underline{C}H'_2NH$), 43.35 ($N\underline{C}H'_2CH'_2NH$). ^{15}N NMR: 282.33 (N of piperazinyl), 254.38 (N4), 229.4 (N1), 122.44 (NH of piperazinyl).

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