

Article

Multi-Enzymatic Cascades in the Synthesis of Modified Nucleosides: Comparison of the Thermophilic and Mesophilic Pathways

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Citation: Fateev, I.V.; Kostromina, M.A.; Abramchik, Y.A.; Eletskaia, B.Z.; Mikheeva, O.O.; Lukoshin, D.D.; Zayats, E.A.; Berzina, M.Y.; Dorofeeva, E.V.; Paramonov, A.S.; et al. Multi-Enzymatic Cascades in the Synthesis of Modified Nucleosides: Comparison of the Thermophilic and Mesophilic Pathways. *Biomolecules* **2021**, *11*, 586. <https://doi.org/10.3390/biom11040586>

Academic Editor: Jesús Fernández Lucas

Received: 13 January 2021

Accepted: 4 April 2021

Published: 16 April 2021

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Abstract: A comparative study of the possibilities of using ribokinase → phosphopentomutase → nucleoside phosphorylase cascades in the synthesis of modified nucleosides was carried out. Recombinant phosphopentomutase from *Thermus thermophilus* HB27 was obtained for the first time: a strain producing a soluble form of the enzyme was created, and a method for its isolation and chromatographic purification was developed. It was shown that cascade syntheses of modified nucleosides can be carried out both by the mesophilic and thermophilic routes from D-pentoses: ribose, 2-deoxyribose, arabinose, xylose, and 2-deoxy-2-fluoroarabinose. The efficiency of 2-chloroadenine nucleoside synthesis decreases in the following order: Rib (92), dRib (74), Ara (66), F-Ara (8), and Xyl (2%) in 30 min for mesophilic enzymes. For thermophilic enzymes: Rib (76), dRib (62), Ara (32), F-Ara (<1), and Xyl (2%) in 30 min. Upon incubation of the reaction mixtures for a day, the amounts of 2-chloroadenine riboside (thermophilic cascade), 2-deoxyribosides (both cascades), and arabinoside (mesophilic cascade) decreased roughly by half. The conversion of the base to 2-fluoroarabinosides and xylosides continued to increase in both cases and reached 20–40%. Four nucleosides were quantitatively produced by a cascade of enzymes from D-ribose and D-arabinose. The ribosides of 8-azaguanine (thermophilic cascade) and allopurinol (mesophilic cascade) were synthesized. For the first time, D-arabinosides of 2-chloro-6-methoxypurine and 2-fluoro-6-methoxypurine were synthesized using the mesophilic cascade. Despite the relatively small difference in temperatures when performing the cascade reactions (50 and 80 °C), the rate of product formation in the reactions with *Escherichia coli* enzymes was significantly higher. *E. coli* enzymes also provided a higher content of the target products in the reaction mixture. Therefore, they are more appropriate for use in the polyenzymatic synthesis of modified nucleosides.

Keywords: purine nucleoside phosphorylase; biocatalysis; enzyme cascade; expression of recombinant enzymes

1. Introduction

Nucleoside analogs can be synthesized by chemical or enzymatic methods or by a combination of these methods [1,2]. Chemical synthesis is a long multi-stage process involving the introduction and removal of various protective groups in the carbohydrate residue and heterocyclic base, which leads to a significant decrease in the efficiency of the process. Despite the use of selective glycosylation methods, racemic mixtures are formed in the synthesis of nucleosides, which complicates the isolation of target compounds. Enzymatic synthesis has a number of advantages over chemical synthesis: mild reaction

conditions, high stereo- and regioselectivity, the minimal use of polluting chemicals and organic solvents, high efficiency, and the absence of undesirable impurities [1,3,4].

Biocatalytic reactions can involve either one enzyme that catalyzes one specific reaction or several enzymes that act sequentially in a cascade of reactions [3]. In recent years, the terms “cascade reactions” or “tandem reactions” have been used to denote poly-enzymatic reactions [5].

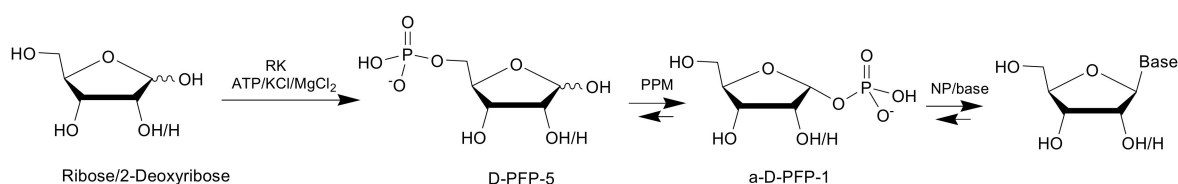
Bacterial glycosyltransferases that catalyze the transfer of the pentofuranosyl group to purine or pyrimidine bases are successfully used in the synthesis of various natural nucleoside analogs of biological and pharmaceutical significance. The donor of the pentofuranosyl residue can be a natural nucleoside or its derivative, while natural or modified heterocyclic bases serve as acceptors [3]. Nucleoside phosphorylases (NP) include purine nucleoside phosphorylases (PNP) and pyrimidine nucleoside phosphorylases (PyNP): uridine phosphorylases (UP) and thymidine phosphorylases (TP). One of the most studied polyenzymatic cascades in the synthesis of modified nucleosides is the sequential use of two NPs. The combination of PNP and PyNP (UP or TP) is often used in the enzymatic synthesis of nucleoside analogs. Such a combination allows obtaining purine nucleosides from pyrimidine ones and vice versa [6]. In addition, some enzymes are involved in the biosynthesis of uridyl peptide antibiotics, such as nikkomycin or tunicamycin [7]. One of the enzymes involved in the biosynthesis of the uridyl peptide antibiotic pacidamycin, dehydratase Pac13, can be used as a biocatalyst for the preparation of 3'-deoxynucleosides [8].

The use of nucleoside phosphorylase-producing microorganisms in the synthesis of modified nucleosides of biological and pharmaceutical significance has proven to be highly effective [9,10]. The transglycosylation reaction is usually carried out at 60 °C to inhibit other enzymes—for example, deaminases. At this temperature, some NPs retain most of their activity. However, reactions involving TP are usually carried out at 45 °C, since thymidine phosphorylase loses activity at temperatures above 50 °C.

Temperature restrictions can be overcome by using thermophilic microorganisms such as *Geobacillus stearothermophilus* [11] and *Thermus thermophilus* [4,12,13]. Several strains of *T. thermophilus* that can synthesize purine nucleosides from adenine or hypoxanthine have been selected [12]. An example of pyrimidine nucleoside synthesis is the enzymatic synthesis of 5-methyluridine from inosine and thymine with the participation of immobilized PNP and PyNP *Bacillus stearothermophilus* JTS 859 [14]. A similar approach has been used in the synthesis of several modified nucleosides: cladribine (2-chloro-2'-deoxyadenosine), fludarabine (2'-fluoradenine arabinoside), vidarabine (9-β-D-arabinosyladenine), and others. Partially purified preparations of PNP and PyNP *G. stearothermophilus* B-2194 immobilized on aminopropylated macroporous glass have shown high enzymatic activity and stability at 70 °C and reusability up to 20 times [15]. Immobilized thermophilic nucleoside phosphorylases have been used in the polyenzymatic synthesis of several halogenated nucleoside analogs: cladribine [4], 2-fluoro-2'-deoxyadenosine, and fludarabine [16]. The synthesis of halogenated nucleosides is of particular interest, since they exhibit a broad spectrum of biological activity [9,17].

Recently published data on the activity of the hyperthermophilic PyNP from *Thermus thermophilus* should be noted [13]. This enzyme works in a wide pH range (4–9) at 100 °C and relatively high concentrations of organic solvents (up to 80% v/v DMSO and ethylene glycol).

In the case of unnatural nucleoside synthesis, the cascade of ribokinase (RK) → phosphopentomutase (PPM) → *E. coli* nucleoside phosphorylase (NP) appears to be promising [18]. This cascade transforms D-pentose through 5-phosphate into 1-α-phosphate and then into the nucleoside (Scheme 1):



Scheme 1. One-pot enzymatic transformation of D-pentoses into nucleosides.

Phosphopentomutases catalyze the reversible transfer of the phosphate group between the C1 and C5 atoms of ribose or deoxyribose [19]. The resulting α -D-ribose-1-phosphate is a substrate for nucleoside phosphorylases in the synthesis of nucleosides. Tandem enzymatic cascades of nucleoside synthesis using two enzymes (*E. coli* PPM and NP combination) have been adapted for the synthesis of D-ribose, D-arabinose, and 2-deoxy-D-ribose nucleosides [18,20]. The possibility of the polyenzymatic synthesis of 2'-deoxy- β -D-ribofuranosides-8-aza-purine and 8-aza-7-deazapurine from 2-deoxyribose using ribokinase, PPM, and NP has been shown [21].

Cascades of thermophilic microorganism enzymes can also be used for the synthesis of modified nucleotides. Therefore, in 2016, a cascade of thermophilic enzyme *Thermus* species 2.9 was presented for the synthesis of nucleotides from D-pentoses [22].

For the thermophilic cascade synthesis of nucleosides, we previously obtained and described ribokinase from *Thermus* species 2.9 (*Tsp*RK, gene QT17_05185) [23], purine nucleoside phosphorylases from *Thermus thermophilus* HB27 (*Tth*PNPI, gene TT_RS05405, and *Tth*PNPII, gene TT_RS00985) [24].

The synthesis of natural nucleosides using various nucleoside phosphorylases yields similar results. However, with modified heterocyclic bases or different carbohydrates, the results may vary significantly. It would be promising to obtain recombinant *Tth*PPM and try the complete cascade of thermophilic $\text{RK} \rightarrow \text{PPM} \rightarrow \text{PNP}$ to synthesize modified nucleosides. It is essential to carry out a comparative study of the features and efficiency of the cascade syntheses of nucleosides using mesophilic and thermophilic enzymes. In addition, some heterocyclic bases, such as 2-chloroadenine and 2-fluoroadenine, have low water solubility, so the use of thermophilic enzymes might be preferable. Carrying out the reaction with thermophilic enzymes at the operating temperatures of mesophilic enzymes reduces the benefits of this approach; therefore, the reactions with *Thermus thermophilus* enzymes were carried out at a higher temperature.

2. Materials and Methods

2.1. General Procedures

Tris-HCl, acetic acid, sodium chloride, glycerol, acrylamide, N,N'-bisacrylamide, ATP disodium salt trihydrate, bromophenol blue, agarose, ethylenediaminetetraacetic acid (EDTA), isopropyl β -D-1-thiogalactopyranoside (IPTG), imidazole, and dimethylformamide (DMF) were purchased from Panreac (Barcelona, Spain). Ethanol was purchased from MedChemProm (Balashikha, Russia). Coomassie Brilliant Blue R-250 was purchased from Bio-Rad. Bacto yeast extract, bacto tryptone, and bacto agar were purchased from Becton Dickinson Biosciences (Franklin Lakes, NJ, USA). NaOH, HCl, pyridine, POCl₃, Et₂NPh, and hydrogen fluoride pyridine (HF/Py) (70%) were purchased from Merck (Darmstadt, Germany). Sodium persulfate, tetramethylethylenediamine (TEMED), ethidium bromide, and sodium azide were purchased from Helicon (Moscow, Russia). deoxynucleoside triphosphate mix (dNTP) was purchased from Fermentas (Waltham, MA, USA). Dithiothreitol (DTT), phenylmethylsulfonylchloride, magnesium chloride, nickel sulfate, potassium dihydroorthophosphate, 8-azaguanine, allopurinol, 2-chloroadenine, tert-butyl nitrite, and Ni-IDA Sepharose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecylsulfate was purchased from Serva (Heidelberg, Germany). Ampicillin was purchased from AppliChem GmbH (Darmstadt, Germany). Polyethersulfone membranes were purchased from Millipore (Burlington, MA, USA).

Unless otherwise noted, the materials were obtained from commercial suppliers and used without any purification.

9-(2,3,5-Tri-O-acetyl- β -D-ribofuranosyl)-2,6-dichloropurine was synthesized as described in [25,26]. 9-(2,3,5-Tri-O-acetyl- β -D-ribofuranosyl)-2-amino-6-chloropurine was synthesized as described in [27].

NMR spectra were recorded on Bruker Avance II 700 spectrometers (Bruker BioSpin, Rheinstetten, Germany) in DMSO- d_6 at 303 K. Chemical shifts in ppm (δ) were measured relative to the residual solvent signals as internal standards (2.508 ppm). Coupling constants (J) were measured in Hz; s—singlet, br.s.—broad signal, d—doublet, m—multiplet, t—triplet. NMR spectra data were provided in the Supplementary Materials.

Liquid chromatography-mass spectrometry was performed on the Agilent 6210 TOF LC/MS system (Agilent Technologies, Santa Clara, CA, USA).

UV spectra were recorded on a Hitachi U-2900 spectrophotometer (Hitachi, Tokyo, Japan).

2.2. Purification of Recombinant Enzymes

In this study, the following previously obtained recombinant enzymes were used: ribokinase from *Escherichia coli* (EcRK, gene AAA51476) [18,28], phosphopentomutase from *E. coli* (EcPPM, gene AAA97279) [18], purine nucleoside phosphorylase from *E. coli* (EcPNP, gene AAA24401) [29], ribokinase from *Thermus* species 2.9 (TspRK, gene QT17_05185) [23], purine nucleoside phosphorylases from *Thermus thermophilus* HB27 (*Tth*PNPI, gene TT_RS05405, and *Tth*PNPII, gene TT_RS00985) [24]. Using the protocols described in their respective publications, batches of purified enzymes were prepared in quantities sufficient for all the experiments.

2.3. Cloning, Expression, and Purification of Recombinant *Tth*PPM

The TT_RS08405 gene encoding the phosphopentomutase from *Thermus thermophilus* HB27 was amplified from genomic DNA by PCR with primer PPM-forward (5'-GGTGGTC ATATGAAGCGGTGGCCATCGTTTTG-3') and PPM-reverse (5'-GGTGGTGCGGCCCGC ACGAGGCTCGTTCCGGGG-3') and cloned into the pET-23a+ expression vector at the NdeI and NotI restriction sites. The resulting plasmid pER-PPM-Tth contained the gene encoding the *Tth*PPM with the C-terminal His-tag. The producing strain *E. coli* NiCo21 (DE3)/pER-PPM-Tth was obtained. Cultivation of the strain was carried out in a lysogeny broth (LB) medium containing 100 μ g/mL ampicillin. After reaching an absorbance of $A_{595} = 0.8$, the cultures were added with IPTG to a final concentration of 0.4 mM, and cultivation was continued for 4 h at 37 °C. After culturing, the cell biomass was separated by centrifugation (2.8 g of wet biomass per liter).

The cell biomass (5.6 g) was disrupted in 50 mM KH_2PO_4 , pH 6.8, 10 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF) in a ratio of 1:10 (*w/v*) using an ultrasonicator. The clarified cell lysate was heat-treated at 80 °C for 10 min to precipitate the contaminated proteins and DNA. To purify *Tth*PPM, a two-stage technique was developed. In the first step, the protein was purified by anion exchange chromatography using an XK 16/20 column packed with 10 mL of DEAE Sepharose Fast Flow resin and equilibrated with 10 mM KH_2PO_4 and 1 mM PMSF, pH 6.8. The target protein was eluted with a linear gradient from 0- to 0.4-M NaCl (100 mL, 2 mL/min). The chromatography eluate was loaded onto an XK 16/20 column packed with 20 mL of Chelating Sepharose Fast Flow and equilibrated with 50 mM Tris-HCl, pH 8.5. The second chromatography was performed on an XK 16/20 column packed with 20 mL of Chelating Sepharose Fast Flow and equilibrated with 50 mM Tris-HCl, pH 8.5, and 5 mM EDTA in a linear gradient of 50–200 mM imidazole (100 mL, 2 mL/min). The eluate was concentrated using a 30-kDa cut-off polyethersulfone membrane and then loaded on a HiLoad 16/60 Superdex 75-g size-exclusion column equilibrated with 20 mM Tris-HCl, pH 8.0, supplemented with 50 mM NaCl, 5% glycerol, and 0.04% (*w/v*) NaN_3 . Fractions containing a target enzyme with purity higher than 90% were combined and concentrated to the final concentration of 10.0 ± 0.5 mg/mL.

2.4. Analytical Methods

The protein concentration was determined by the Lowry method, using bovine serum albumin (BSA) as the standard [30]. Protein purity was determined using protein electrophoresis in polyacrylamide gel under denaturing conditions [31].

Oligomeric organization of enzymes was assessed by size-exclusion chromatography (SEC) using a Superdex 200 10/300 GL analytical column (GE Healthcare, USA). The analysis parameters were as follows: eluent 25 mM Na₂HPO₄, pH 8.0, 150 mM NaCl, and 0.04% (*w/v*) NaN₃; flow rate 0.6 mL/min; time 60 min; detection 220 nm; oven temperature 23 °C; and sample injection volume is 100 µL (50 µg). Gel Filtration Markers MWGF200 and MWGF1000 (Sigma-Aldrich, St. Louis, MO, USA) were used for plotting the calibration curve and calculating the molecular weight.

The analysis of the efficiency of nucleoside synthesis cascades was carried out using the following chromatographic systems:

HPLC system 1: Hitachi Chromaster, column YMC Triart-C18, 50×3.0 mm, 3 µm, eluent 0.1% aqueous trifluoroacetic acid (TFA), detection at 254 nm, and flow rate 0.4 mL/min.

HPLC system 2: Hitachi Chromaster, column Supelcosil LC-18-T, 150×4.6 mm, 5 µm, eluent 0.1M KH₂PO₄ pH 6.0, detection at 254 nm, and flow rate 0.4 mL/min.

HPLC system 3: Waters system (Waters 1525, Waters 2489, Breeze 2), column Nova Pak C₁₈, 4.6×150 mm, 5 µm, and flow rate 0.5 mL/min. Eluent A: 0.1% TFA/H₂O and eluent B: 70% MeCN in 0.1% TFA/H₂O, gradient 0–50% B, 20 min, detection at 254 nm.

HPLC system 4: Waters system (Waters 1525, Waters 2489, Breeze 2), column Nova Pak C₁₈, 4.6×150 mm, 5 µm, and flow rate 1.0 mL/min. Eluent A: 0.1% TFA/H₂O and eluent B: 70% MeCN in 0.1% TFA/H₂O, gradient 0–50% B, 20 min, detection at 254 nm.

HPLC system 5: Waters system (Waters 1525, Waters 2489, Breeze), column Nova Pak C₁₈, 4.6×150 mm, 5 µm, and flow rate 1.0 mL/min. Eluent A: 0.1% TFA/H₂O and eluent B: 70% MeCN in 0.1% TFA/H₂O, gradient 0–100% B, 20 min, detection at 254 nm.

2.5. Enzyme Assay

Phosphopentomutase Thermus thermophilus HB27 (*TthPPM*). Each reaction mixture (0.25 mL, 20 mM Tris·HCl, pH 8.0) contained 0.5 mM D-ribose 5-phosphate, 0.5 mM adenine, 0.5 mM MnCl₂, 0.2 µg *TthPPM*, and 1.4 µg *TthPNPII*. The reaction mixtures were incubated at 80 °C. Substrate and product quantities were determined using HPLC system 1.

Phosphopentomutase E. coli (*EcPPM*). Each reaction mixture (0.25 mL, 20 mM Tris·HCl, pH 7.5) contained 0.5 mM D-ribose 5-phosphate, 0.5 mM adenine, 0.1 mM MnCl₂, 0.26 µg *EcPPM*, and 1.1 µg *EcPNP*. The reaction mixtures were incubated at 37 °C. Substrate and product quantities were determined using HPLC system 1.

Purine nucleoside phosphorylase I Thermus thermophilus HB27 (*TthPNPI*). Each reaction mixture (0.25 mL, 25 mM KH₂PO₄, pH 8.0) contained 1 mM inosine and 0.025 µg *TthPNPI*. The reaction mixtures were incubated at 80 °C. Substrate and product quantities were determined using HPLC system 1.

Purine nucleoside phosphorylase II Thermus thermophilus HB27 (*TthPNPII*). Each reaction mixture (0.25 mL, 25 mM KH₂PO₄, pH 8.0) contained 1 mM adenosine and 0.03 µg *TthPNPII*. The reaction mixtures were incubated at 80 °C. Substrate and product quantities were determined using HPLC system 1.

Purine nucleoside phosphorylase E. coli (*EcPNP*). Each reaction mixture (0.25 mL, 25 mM KH₂PO₄, pH 7.0) contained 1 mM inosine and 0.11 µg *EcPNP*. The reaction mixtures were incubated at 37 °C. Substrate and product quantities were determined using HPLC system 1.

Ribokinase Thermus species 2.9 (*TsprRK*). Each reaction mixture (0.25 mL, 20 mM Tris·HCl, pH 8.0) contained 0.4 mM ATP, 1.2 mM D-ribose, 5 mM MgCl₂, 50 mM KCl, and 0.3 µg *TsprRK*. The reaction mixtures were incubated at 80 °C. Substrate and product quantities were determined using HPLC system 2.

Ribokinase E. coli (*EcRK*). Each reaction mixture (0.25 mL, 20 mM Tris·HCl, pH 8.0) contained 0.4 mM ATP, 1.2 mM D-ribose, 5 mM MgCl₂, 50 mM KCl, and 0.02 µg *EcRK*.

The reaction mixtures were incubated at 37 °C. Substrate and product quantities were determined using HPLC system 2.

2.6. Determination of Kinetic Parameters of Phosphopentomutase *Thermus Thermophilus* HB27 (*TthPPM*)

Each reaction mixture (100 µL, 20 mM Tris·HCl, pH 8.0) contained 1 mM adenine, 0.5 mM MnCl₂, and 0.25 µM α-D-glucose 1,6-bisphosphate, from 0.011 to 1.8 mM D-ribose 5-phosphate or 2-deoxy-D-ribose 5-phosphate, 0.02 or 0.05 µg *TthPPM*, and 0.5 µg *TthPNPII*. The reaction mixtures were incubated 2 min at 80 °C. HPLC system 1. Each experiment was repeated three times. Kinetic parameters were determined by nonlinear regression analysis using SciDAVis v2.3.0 software. Catalytic constants (k_{cat}) were calculated per 1 subunit (43.2 kDa, calculated based on amino acid sequences).

2.7. Cascade Reactions

Testing of D-pentoses. Each reaction mixture (0.5 mL, 20 mM Tris·HCl, pH 8.0) contained D-pentose (2 mM D-ribose or 2-deoxy-D-ribose or 60 mM D-arabinose or D-xylose or 5 mM 2-deoxy-2-fluoro-D-arabinose); 0.34 mM 2-chloroadenine; 2.5 mM ATP; 2.5 mM MnCl₂; 50 mM KCl; 2 mM KH₂PO₄; and thermophilic or mesophilic recombinant enzymes (ribokinase, phosphopentomutase, and purine nucleoside phosphorylase). The reaction mixtures were incubated at 80 or 50 °C. Substrate and product quantities were determined using HPLC system 1.

Testing of heterocyclic bases. Each reaction mixture (0.5 mL, 20 mM Tris·HCl, pH 8.0) contained 2 mM D-ribose, 0.5 mM heterocyclic base (allopurinol or 8-azaguanine), 2.5 mM ATP, 2.5 mM MnCl₂, 50 mM KCl, 2 mM KH₂PO₄, and thermophilic or mesophilic recombinant enzymes (ribokinase, phosphopentomutase, and purine nucleoside phosphorylase). The reaction mixtures were incubated at 80 or 50 °C. Substrate and product quantities were determined using HPLC system 1.

2.8. Nucleosides Synthesis

2.8.1. 2-Chloro-6-methoxy-9-(β-D-ribofuranosyl)purine (2)

To a solution of MeONa in MeOH (376 mg, 16.2 mmol Na in 8 mL of MeOH), 1.0 g (2.2 mmol) of dichloride (1) was added at room temperature under stirring. In 2 h, the reaction mixture was cooled up to 0 °C, and conc. HCl was added up to pH 7.0. NaCl was filtered off, and the precipitate was washed by hot MeOH (3 × 3 mL). The filtrate was evaporated to a quarter of the volume, 1 mL of H₂O was added, and the solution was heated to boiling. The clear solution was left at room temperature. The crystals were filtered off and washed with *i*PrOH and Et₂O. Yield 560 mg, purity 74%. The desired product was purified by chromatography on silica gel (3 × 30 cm, elution by gradient of MeOH in CHCl₃ (0–20%), 1 L, with a flow rate of 6 mL/min. Yield 310 mg (0.97 mmol, 44%), purity 92.9% (HPLC system 5).

HRMS (ESI+): m/z [M + H]⁺ calc. for C₁₁H₁₄O₅N₄Cl 317.0654/319.0624 Cl³⁵/Cl³⁷, found 317.0636/319.0609, and [Base + H]⁺ calc. for C₆H₆N₄OCl 185.0230/187.0200 Cl³⁵/Cl³⁷, found 185.0221/187.0189.

¹H NMR (700 MHz): δ = 8.67 (s, 1H, H-8), 5.93 (d, $J_{H1,H2}$ = 5.5, 1 H, H-1'), 5.64 and 5.35 (br. s, 2 H, OH-2', and OH-3'), 5.06 (m, 1 H, OH-5'), 4.52 (t, J = 5.1, 1 H, H-2'), 4.16 (t, J = 4.2, 1 H, H-3'), 4.12 (s, 3 H, OMe), 3.98 (m, 1 H, H-4'), 3.68 (dd, J = 4.0, 12.2, 1 H, H-5'a), 3.58 (dd, J = 3.7, 12.0, 1 H, H-5'b). ¹³C NMR (176 MHz): δ = 160.78 (C-6), 152.84 (C-4), 151.43 (C-2), 142.72 (C-8), 120.19 (C-5), 87.59 (C-1'), 85.63 (C-4'), 73.85 (C-2'), 70.05 (C-3'), 61.00 (C-5'), 54.89 (OCH₃). ¹⁵N NMR (71 MHz): δ = 241.3 (N-7), 172.47 (N-9).

2.8.2. 2-Fluoro-6-chloro-9-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)purine (5)

A 70% solution of HF in pyridine (7.5 mL) was cooled to −18 °C, and 3.20 g (7.4 mmol) of monochloride (4) was added. Some tert-butyl (2.15 mL) (28.5 mmol) was added dropwise within 8 min, and the reaction mixture was stirred at −18 °C for 30 min and at room

temperature for 20 min. The mixture was poured into a glass with ice (150 g) and CaCO₃ (15 g), filtered off, and the precipitate was washed by CHCl₃ (150 mL). The organic layer was washed by water (100 mL), 5% NaHCO₃ (100 mL), water (150 mL), dried by MgSO₄, and concentrated. The desired product was isolated by column chromatography on silica gel (2 × 9 cm; 7 cm of silica gel covered by 2 cm of activated carbon, elution by 200 mL of CHCl₃, then by 5% MeOH in CHCl₃, with a flow rate 0.9 mL/min). Yield 1.49 g (3.4 mmol, 46%), purity 88.7% (HPLC system 5).

HRMS (ESI+): m/z [M + H]⁺ calc. for C₁₆H₁₇O₇N₄FCI 431.0764/433.0735 Cl³⁵/Cl³⁷, found 431.0766/433.0736.

¹H NMR (700 MHz): δ = 8.90 (s, 1 H, H-8), 6.31 (d, J = 5.2, 1 H, H-1'), 5.92 (t, J = 5.5, 1 H, H-2'), 5.61 (t, J = 5.6, 1 H, H-3'), 4.44 (m, 1 H, H-4'), 4.40 (dd, J = 3.4, 12.2, 1 H, H-5'a), 4.29 (dd, J = 5.4, 12.2, 1 H, H-5'b), 2.13 (s, 3 H, Me-Ac3'), 2.06 (s, 3 H, Me-Ac2'), 2.03 (s, 3 H, Me-Ac5'). ¹³C NMR (176 MHz): δ = 169.88 (C=O_{Ac5'}), 169.24 (C=O_{Ac3'}), 169.09 (C=O_{Ac2'}), 156.01 (d, C-2, $J_{C2,F2}$ = 215.1), 153.12 (d, C-4, $J_{C4,F2}$ = 16.6), 150.95 (d, C-6, $J_{C6,F2}$ = 17.3), 146.79 (C-8), 130.65 (C-5), 86.09 (C-1'), 79.70 (C-4'), 72.01 (C-2'), 69.63 (C-3'), 62.51 (C-5'), 20.29 (CMe_{Ac5'}), 20.22 (CMe_{Ac3'}), 20.07 (CMe_{Ac2'}). ¹⁵N NMR (71 MHz): δ = 243.03 (N-7), 169.79 (N-9).

2.8.3. 2-Fluoro-6-methoxy-9-(β -D-ribofuranosyl)purine (6)

To a solution of MeONa in MeOH (376 mg, 16.2 mmol Na in 8 mL of MeOH), 1.0 g (2.3 mmol) of nucleoside (5) was added at room temperature under stirring. In 2 h, the reaction mixture was cooled up to 0 °C, and conc. HCl was added up to pH 7.0. NaCl was filtered off, and the precipitate was washed by hot MeOH (3 × 3 mL) and concentrated. The desired product was isolated by column chromatography on silica gel (4.6 × 40 cm, elution by a gradient of MeOH in CHCl₃ (0–30%, 1 L, flow rate 6 mL/min). Yield 358 mg (1.2 mmol, 50%), purity 97.0% (HPLC system 5).

HRMS (ESI+): m/z [M + H]⁺ calc. for C₁₁H₁₄O₅N₄F 301.0949, found 301.0906, [Base + H]⁺ calc. for C₆H₆N₄OF 169.0525, found 169.0504.

¹H NMR (700 MHz): δ = 8.63 (s, 1 H, H-8), 5.89 (d, $J_{H1,H2}$ = 5.5, 1 H, H-1'), 4.52 (t, J = 5.3, 1 H, H-2'), 4.16 (t, J = 4.3, 1 H, H-3'), 4.13 (s, 3 H, OMe), 3.97 (m, 1 H, H-4'), 3.68 (dd, J = 4.1, 12.0, 1 H, H-5'a), 3.57 (dd, J = 4.1, 12.0, 1 H, H-5'b). ¹³C NMR (176 MHz): δ = 162.16 (d, $J_{C6,F}$ = 18.0, C-6), 156.97 (d, $J_{C2,F}$ = 210.5, C-2), 152.89 (d, $J_{C4,F}$ = 18.8, C-4), 142.53 (d, $J_{C8,F}$ = 2.0, C-8), 119.35 (d, $J_{C5,F}$ = 5.0, C-5), 87.57 (C-1'), 85.54 (C-4'), 73.73 (C-2'), 70.01 (C-3'), 60.98 (C-5'), 54.96 (OCH₃). ¹⁵N NMR (71 MHz): δ = 241.76 (N-7), 172.78 (N-9).

2.8.4. 5-Amino-3-(β -D-ribofuranosyl)triazolo [4,5-d]pyrimidin-7-one (8)

8-Azaguanine (28 mg, 0.184 mmol), D-ribose (42.6 mg, 0.284 mmol), MnCl₂ (44.7 mg, 0.355 mmol), KCl (529 mg, 7.1 mmol), and KH₂PO₄ (38.6 mg, 0.284 mmol) were dissolved in Tris·HCl (20 mM, 142 mL, pH 8.0) under stirring and heating at 80 °C. ATP disodium salt trihydrate (215 mg, 0.355 mmol), TspRK (0.6 mg, 11.4 units, concentration in the reaction mixture 4.2 μ g/mL), TthPPM (0.2 mg, 2.8 units, concentration in the reaction mixture 1.4 μ g/mL), and TthPNPI (0.063 mg, 11.4 units, concentration in the reaction mixture 0.44 μ g/mL) were added. The reaction mixture was incubated at 80 °C for 24 h (monitored by HPLC system 1). The solution was neutralized with 2 N HCl and concentrated *in vacuo* to ca. 35 mL. The precipitate was filtered off, and the filtrate was applied to the column (octadecyl-Si 100 polyol (0.03 mm); 4 × 14 cm). The desired product was eluted with 15% ethanol in water. Yield 36.1 mg (0.127 mmol; 69%), purity 98.5% (HPLC system 4).

HRMS (ESI+): m/z [M + H]⁺ calc. for C₉H₁₃N₆O₅ 285.0947; found 285.0930; [Base + H]⁺ calc. for C₄H₅N₆O 153.0525; found 153.0515.

¹H NMR (700 MHz): δ = 6.98 (br. s, 2 H, NH₂), 5.87 (d, J = 4.8, 1 H, H-1'), 5.50 (s, 1 H, OH-2'), 5.15 (br. s, 1 H, OH-3'), 4.78 (m, 1 H, OH-5'), 4.70 (t, J = 4.9, 1 H, H-2'), 4.24 (t, J = 4.8, 1 H, H-3'), 3.94 (m, 1 H, H-4'), 3.59 (dd, J = 4.6, 11.9, 1 H, H-5'a), 3.46 (dd, J = 5.9, 11.7, 1 H, H-5'b). ¹³C NMR (176 MHz): δ = 151.73 (C-4), 87.75 (C-1'), 85.28 (C-4'), 72.47 (C-2'), 70.30 (C-3'), 61.62 (C-5'). ¹⁵N NMR (71 MHz): δ = 234.90, 156.95.

2.8.5. 1-(β -D-Ribofuranosyl)pyrazolo[3,4-d]pyrimidine-4-one (9)

Allopurinol (25 mg, 0.184 mmol), D-ribose (42.6 mg, 0.284 mmol), MnCl_2 (44.7 mg, 0.355 mmol), KCl (529 mg, 7.1 mmol), and KH_2PO_4 (38.6 mg, 0.284 mmol) were dissolved in Tris-HCl buffer (20 mM, 142 mL, pH 8.0) under stirring and heating at 50 °C. ATP disodium salt trihydrate (215 mg, 0.355 mmol), *Ec*RK (0.018 mg, 5.7 units, concentration in the reaction mixture 0.13 $\mu\text{g}/\text{mL}$), *Ec*PPM (0.57 mg, 2.8 units, concentration in the reaction mixture 4 $\mu\text{g}/\text{mL}$), and *Ec*PNP (0.052 mg, 11.4 units, concentration in the reaction mixture 0.37 $\mu\text{g}/\text{mL}$) were added. The reaction mixture was incubated at 50 °C for 24h (monitored by HPLC system 4). The solution was neutralized with 2-N HCl and concentrated *in vacuo* to ca. 35 mL. The precipitate was filtered off, and the filtrate was applied to the column (octadecyl-Si 100 polyol (0.03 mm); 4 \times 14 cm). The desired product was eluted with 10% ethanol in water. Yield 41.6 mg (0.155 mmol; 84%), 98.5% purity (HPLC system 4).

UV (H_2O , pH 7.0) λ_{max} , nm (ϵ , $\text{M}^{-1}\text{cm}^{-1}$): 250 (7600), 206 (27600); λ_{min} , nm (ϵ , $\text{M}^{-1}\text{cm}^{-1}$): 232 (5100).

HRMS (ESI+): m/z $[\text{M} + \text{H}]^+$ calc. for $\text{C}_{10}\text{H}_{13}\text{N}_4\text{O}_5$ 269.0886; found 269.0849; $[\text{Base} + \text{H}]^+$ calc. for $\text{C}_5\text{H}_5\text{N}_4\text{O}$ 137.0463; found 137.0444.

^1H NMR (700 MHz): δ = 12.25 (s, 1 H, NH), 8.14 (s, 1 H, H-3), 8.12 (s, 1 H, H-6), 6.06 (d, J = 4.53, 1 H, H-1'), 4.55 (dt, J = 4.57; < 0.5, 1 H, H-2'), 4.21 (t, J = 4.8, 1 H, H-3'), 3.91 (m, 1 H, H-4'), 3.58 (dd, $J_{5'a,4}$ = 4.57; $J_{5'a,5'b}$ = 11.65, 1 H, H-5'a), 3.43 (dd, $J_{5'b,4}$ = 5.96; $J_{5'b,5'a}$ = 11.78, 1 H, H-5'b). ^{13}C NMR (176 MHz): δ = 157.45 (C-4), 153.98 (C-7a), 148.86 (C-6), 135.37 (C-3), 106.23 (C-4a), 88.38 (C-1'), 85.25 (C-4'), 73.31 (C-2'), 70.81 (C-3'), 62.27 (C-5'). ^{15}N NMR (71 MHz): δ = 302.20 (N-2), 210.47 (N-7), 204.80 (N-1), 173.67 (N-5).

2.8.6. 9-(β -D-Arabinofuranosyl)-2-chloro-6-methoxypurine (10)

Eighty milligrams (0.25 mmol) of riboside (2) were dissolved in 5 mL of 10 mM KH_2PO_4 (pH 7.0). Fifty microliters of 20 mM Na_2HAsO_4 and *Ec*PNP (0.045 mg, 10 units, concentration in the reaction mixture 9 $\mu\text{g}/\text{mL}$) were added. The solution was heated up to 50 °C and kept for 3 h until the complete conversion of riboside (2) to base (3) (monitored by HPLC System 3, R_t (2) = 7.6 min, R_t (3) = 6.0 min). The reaction mixture was cooled down to 4 °C and kept for 12 h. The precipitate was filtered off, washed with cold (4 °C) 10 mM KH_2PO_4 (pH 7.0) (2 \times 5 mL), and suspended in 167 mL of 20 mM Tris-HCl, pH 8.0.

D-Arabinose (1.5 g, 10 mmol), MnCl_2 (52.5 mg, 0.417 mmol), KCl (622 mg, 8.35 mmol), and KH_2PO_4 (44.7 mg, 0.334 mmol) were added under stirring and heating at 50 °C. ATP (253 mg, 0.417 mmol), *Ec*RK (5.4 mg, 1670 units, concentration in the reaction mixture 32 $\mu\text{g}/\text{mL}$), *Ec*PPM (68.2 mg, 334 units, concentration in the reaction mixture 408 $\mu\text{g}/\text{mL}$), and *Ec*PNP (13.6 mg, 3000 units, concentration in the reaction mixture 82 $\mu\text{g}/\text{mL}$) were added, and the reaction mixture was incubated at 50 °C for 48 h (monitored by HPLC System 3). The solution was neutralized with 2-N hydrochloric acid and concentrated *in vacuo* to ca. 25 mL. The precipitate was filtered off. The filtrate was applied to the column (octadecyl-Si 100 polyol (0.03 mm); 4 \times 16 cm), and the product was eluted with a gradient of acetonitrile in water (0–50%, 1 L, flow rate 6 mL/min). Yield 43 mg (0.136 mmol, 54%), white amorphous powder, purity 97.0% (HPLC System 3, R_t = 9.4 min).

UV (H_2O , pH 7.0) λ_{max} , nm (ϵ , $\text{M}^{-1}\text{cm}^{-1}$): 270 (10400).

HRMS (ESI+): m/z $[\text{M} + \text{H}]^+$ calc. for $\text{C}_{11}\text{H}_{14}\text{N}_4\text{O}_5\text{Cl}$ 317.0654/319.0624 $\text{Cl}^{35}/\text{Cl}^{37}$, found 317.0636/319.0609; $[\text{Base} + \text{H}]^+$ calc. for $\text{C}_6\text{H}_6\text{N}_4\text{OCl}$ 185.0230/187.0200 $\text{Cl}^{35}/\text{Cl}^{37}$, found 185.0221/187.0189.

^1H NMR (700 MHz): δ = 8.48, (s, 1 H, H-8), 6.27 (d, J = 4.8 Hz, 1 H, H-1'), 5.64 (br.s., 0.6 H, 2'-OH), 5.57 (br.s, 1 H, 3'-OH), 5.11 (br.s, 1 H, 5'-OH), 4.24 (m, 1 H, H-2'), 4.14 (m, 1 H, H-3'), 4.12 (s, 3 H, O- CH_3), 3.81(m, 1 H, H-4'), 3.69 (m, 1 H, H-5'a), 3.67 (m, 1 H, H-5'b). ^{13}C NMR (176 MHz): δ = 160.63 (C-6), 152.90 (C-4), 151.23 (C-2), 143.57 (C-8), 119.49 (C-5), 84.12 (C-4'), 84.10 (C-1'), 75.56 (C-2'), 74.23 (C-3'), 60.44 (C-5'), 54.87 (O CH_3). ^{15}N NMR (71 MHz): δ = 239.64 (N-7), 170.65 (N-9).

2.8.7. 9-(β-D-Arabinofuranosyl)-2-fluoro-6-methoxypurine (11)

Thirty-six milligrams (0.12 mmol) of riboside (6) were dissolved in 10 mL of 10 mM KH_2PO_4 (pH 7.0). Fifty microliters of 20 mM Na_2HAsO_4 and *Ec*PNP (0.068 mg, 15 units, concentration in the reaction mixture 6.8 $\mu\text{g}/\text{mL}$) were added. The solution was heated up to 50 °C and kept for 3 h until the complete conversion of riboside (6) to base (7) (monitored by HPLC System 3, RT(6) = 7.9 min, RT(7) = 6.2 min). The reaction mixture was concentrated twice, cooled down to 4 °C, and kept for 16 h. The supernatant was removed, and the precipitate was washed with cold (4 °C) 10 mM KH_2PO_4 (pH 7.0) (2 × 5 mL) and suspended in 80 mL of 20 mM Tris·HCl, pH 8.0. D-Arabinose (720 mg, 4.8 mmol), MnCl_2 (25.2 mg, 0.2 mmol), KCl (298 mg, 4 mmol), and KH_2PO_4 (21.4 mg, 0.16 mmol) were added under stirring and heating at 50 °C. ATP (121 mg, 0.2 mmol), *Ec*RK (2.58 mg, 800 units, concentration in the reaction mixture 32 $\mu\text{g}/\text{mL}$), *Ec*PPM (32.7 mg, 160 units, concentration in the reaction mixture 408 $\mu\text{g}/\text{mL}$), and *Ec*PNP (6.55 mg, 1440 units, concentration in the reaction mixture 82 $\mu\text{g}/\text{mL}$) were added, and the reaction mixture was incubated at 50 °C for 48 h (monitored by HPLC System 3). The solution was neutralized with 2-N hydrochloric acid and concentrated *in vacuo* to ca. 35 mL. The precipitate was filtered off. The filtrate was applied to the column (octadecyl-Si 100 polyol (0.03 mm); 4 × 16 cm), and the product was eluted with a gradient of acetonitrile in water (0–50%, 1 L, flow rate 6 mL/min). Yield 15 mg (0.05 mmol, 42%), white amorphous powder, purity 99.0% (HPLC System 3, R_t = 7.7 min).

UV (H_2O , pH 7.0) λ_{max} , nm (ϵ , $\text{M}^{-1}\text{cm}^{-1}$): 254 (7200), 244 (6050).

HRMS (ESI+): m/z $[\text{M} + \text{H}]^+$ calc. for $\text{C}_{11}\text{H}_{14}\text{N}_4\text{O}_5\text{F}$ 301.0943; found 301.0927; $[\text{Base} + \text{H}]^+$ calc. for $\text{C}_6\text{H}_6\text{N}_4\text{OF}$ 169.0520; found 169.0503.

^1H NMR (700 MHz): δ = 8.46, (s, 1 H, H-8), 6.22 (d, J = 5.4, 1 H, H-1'), 5.72 (br. s., 1 H, OH-2'), 5.63 (br. s, 1 H, OH-3'), 5.11 (br. s, 1 H, OH-5'), 4.23 (t, J = 5.2, 1 H, H-2'), 4.14 (t, J = 5.4, 1 H, H-3'), 4.12 (s, 3 H, O-CH₃), 3.80(m, 1 H, H-4'), 3.70 (dd, J = 3.7, 12.0, 1 H, H-5'a), 3.66 (dd, J = 4.8, 12.0, 1 H, H-5'b). ^{13}C NMR (176 MHz): δ = 161.98 (d, $J_{\text{C}_6,\text{F}}$ = 17.0 Hz, C-6), 156.98 (d, $J_{\text{C}_2,\text{F}}$ = 210.8 Hz, C-2), 153.00 (d, $J_{\text{C}_4,\text{F}}$ = 18.8 Hz, C-4), 143.38 (C-8), 118.64 (d, $J_{\text{C}_5,\text{F}}$ = 4.5 Hz, C-5), 84.09 (C-4'), 84.05 (C-1'), 75.59 (C-2'), 74.18 (C-3'), 60.44 (C-5'), 54.97 (OCH₃). ^{15}N NMR (71 MHz): δ = 239.9 (N-7), 200.11 (N-3), 170.71 (N-9).

3. Results

To create a polyezymatic cascade for the synthesis of modified nucleosides, it was necessary to obtain a sufficient amount of recombinant ribokinase, phosphopentomutase, and nucleoside phosphorylases. We believe that the enzymes from *Escherichia coli* and thermophilic bacteria *Thermus* species 2.9 and *Thermus thermophilus* HB27 have the best prospects for research and subsequent use. The purifications of all the enzymes (except *Tth*PPM) were obtained earlier, the methods of their isolation were optimized, and the substrate characteristics were studied [21–24,28,29].

To obtain *Tth*PPM, an expression vector was constructed. The TT_RS08405 gene obtained by the amplification of genomic DNA from *Thermus thermophilus* HB27 was cloned into the pET-23a+ vector. A strain *E. coli* NiCo21(DE3)/pER-PPM-Tth producing a soluble-form enzyme was created, and a purification protocol was developed. The technique includes the stages of heat precipitation of contaminating proteins and DNA, anion exchange, metal chelate affinity, and size-exclusion chromatography.

Preparations of purified enzymes were characterized by the purity, the content of oligomeric forms, and enzymatic activity (Table 1).

Table 1. The characteristics of the purified recombinant enzymes.

Name	Enzyme	Gene	Concentration *, mg/mL	Oligomer form **	Purity, % ***
TspRK	Ribokinase <i>Thermus</i> species 2.9	QT17_05185	13.4	63 kD adimer	94.3
TthPPM	Phosphopentamutase <i>Thermus thermophilus</i> HB27	TT_RS08405	10.1	43 kD amonomer	70.5
TthPNPI	Purine nucleoside phosphorylase I <i>Thermus thermophilus</i> HB27	TT_RS05405	9.8	153 kD ahexamer	96.4
TthPNPII	Purine nucleoside phosphorylase II <i>Thermus thermophilus</i> HB27	TT_RS00985	10.3	179 kD ahexamer	95.0
EcRK	Ribokinase <i>E. coli</i> K12	AAA51476.1	6.9	66 kD adimer	83.2
EcPPM	Phosphopentamutase <i>E. coli</i> K12	AAA97279.1	11.0	44 kD amonomer	85.4
EcPNP	Purine nucleoside phosphorylase <i>E. coli</i> K12	AAA24401.1	30.1	155 kD ahexamer	95.8

* Analysis by Lowry method. ** Analysis by size exclusion chromatography. *** Analysis by protein electrophoresis in SDS-PAGE.

3.1. Study of the Influence of Various Factors on TthPPM Activity

The effect of pH, reaction mixture temperature, divalent cations, and cofactor (glucose-1,6- biphosphate) on TthPPM activity was studied. The enzymatic activity of TthPPM was calculated, allowing for the content of impurity proteins.

The studied phosphopentomutase showed the maximal activity at pH 8.0 (Figure 1a). The enzyme was active in a wide temperature range; the highest activity was observed in the temperature range from 80 to 90 °C (Figure 1b).

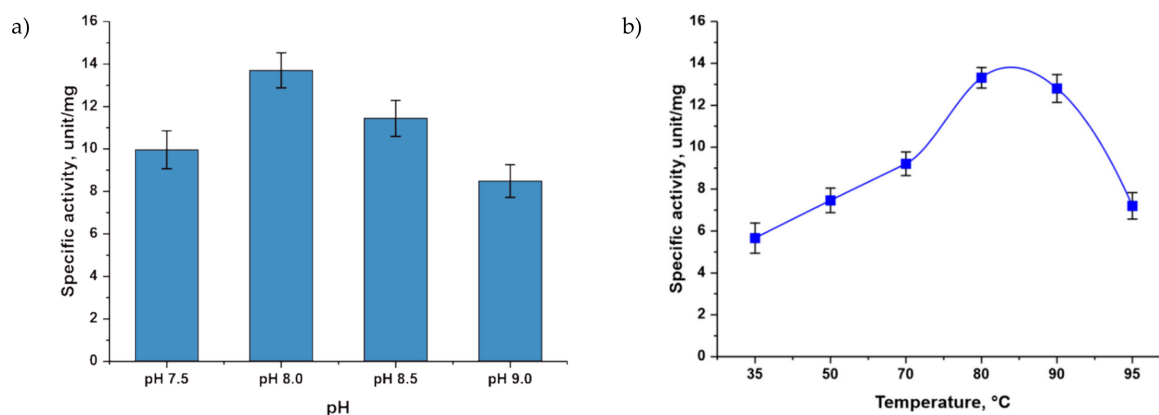


Figure 1. Dependence of TthPPM activity on various factors: (a) pH and (b) temperature. Reaction conditions: (a) 0.5 mM adenine, 0.5 mM D-ribose 5-phosphate, 0.5 mM MnCl₂, 20 mM Tris-HCl (pH from 7.5 to 9.0), 0.5 µg TthPPM, and 2.7 µg TthPNPII in a 0.25 mL reaction mixture, 80 °C and (b) 0.5 mM adenine, 0.5 mM D-ribose 5-phosphate, 0.5 mM MnCl₂, 20 mM Tris-HCl, pH 8.0, 0.1 µg TthPPM, and 2.7 µg TthPNPII in a 0.25 mL reaction mixture, temperature 35, 50, 70, 80, 90, and 95 °C.

The measurement of activity in the presence of chlorides of various metals showed that phosphopentomutase can bind various divalent cations, while the maximal activity was observed in the presence of a manganese cation. In the absence of divalent metal cations, the activity of the enzyme significantly decreased (Figure 2a). The studied phosphopentomutase exhibited maximal activity at a concentration of manganese ions of 0.5 mM (Figure 2b).

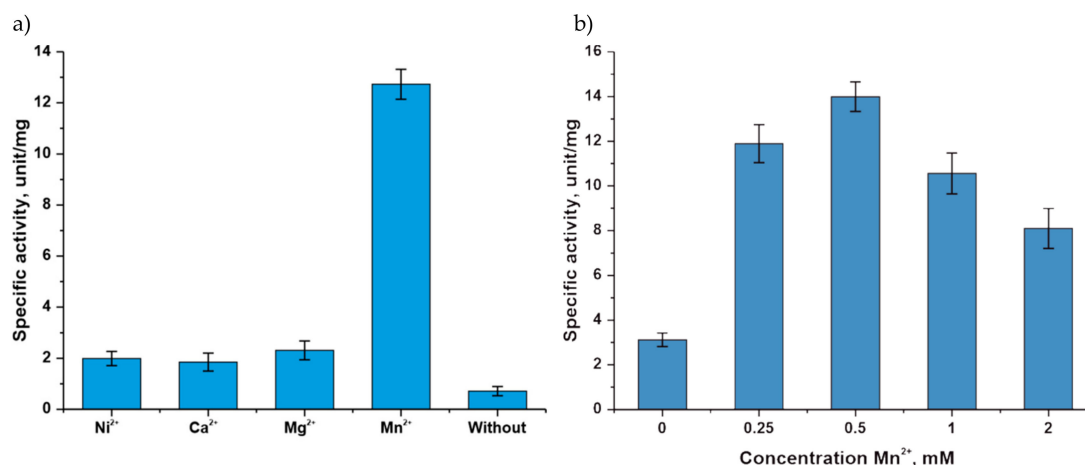


Figure 2. Dependence of *Tth*PPM activity on various factors: (a) divalent cations and (b) Mn²⁺ concentration. Reaction conditions: (a) 0.5 mM adenine; 0.5 mM D-ribose 5-phosphate; 0.5 mM MnCl₂, MgCl₂, NiCl₂, or CaCl₂ and without cation; 20 mM Tris-HCl, pH 8.0; 0.1 μg *Tth*PPM and 2.7 μg *Tth*PNPII in a 0.25 mL reaction mixture, temperature 80 °C and (b) 0.5 mM adenine, 0.5 mM D-ribose 5-phosphate, from 0- to 5 mM MnCl₂, 20 mM Tris-HCl, pH 8.0, 0.1 μg *Tth*PPM, and 2.7 μg *Tth*PNPII in a 0.25 mL reaction mixture, temperature 80 °C.

Glucose-1,6-bisphosphate increased the activity of *Tth*PPM (Figure 3), although its presence in the reaction was not necessary.

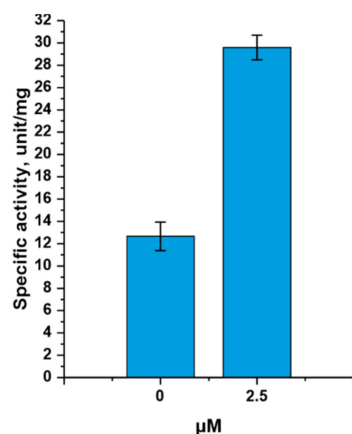


Figure 3. Dependence of *Tth*PPM activity on the cofactor concentration. Reaction conditions: 0.5 mM adenine, 0.5 mM D-ribose 5-phosphate, 0.5 mM MnCl₂, 20 mM Tris-HCl, pH 8.0, 0- or 2.5 μM glucose-1,6-bisphosphate, 0.2 μg *Tth*PPM, and 1.4 μg *Tth*PNPII in a 0.25 mL reaction mixture, temperature 80 °C.

3.2. Determination of the Kinetic Parameters for *Tth*PPM

The kinetic parameters were determined with natural substrates: D-ribose-5-phosphate (R5P) and 2-deoxy-D-ribose-5-phosphate (dR5P) (Table 2 and Figure 4).

The data obtained indicate that the affinity of D-ribose-5-phosphate and 2-deoxy-D-ribose-5-phosphate for the active site of *Tth*PPM differs insignificantly. Thus, the presence of an OH group in the second position does not significantly affect the binding to the active site. At the same time, the reaction rate in the active site for D-ribose-5-phosphate is 7.1 times higher.

The activity of all the enzymes used in this study was determined (Table 3). For thermophilic enzymes, the activity was determined at 80 °C and, for mesophilic enzymes, at 37 °C. As the activity of *Tsprk* was 16 times lower compared to *EcRk*, more proteins were added to the reaction mixture. In this case, the activity of phosphopentomutase and purine nucleoside phosphorylase II (with respect to adenosine) of *Thermus thermophilus* was

higher than that of *E. coli* enzymes, while the activity of *Tth*PNPI (with respect to inosine) and *Ec*PNP was the same.

Table 2. Kinetics of α -D-ribose 1-phosphate and 2-deoxy- α -D-ribose 1-phosphate synthesis by phosphopentomutase *Thermus thermophilus* HB27. Each reaction mixture (100 μ L, 20 mM Tris-HCl, pH 8.0) contained 1-mM adenine, 0.5 mM MnCl₂, and 0.25 μ M α -D-glucose 1,6-bisphosphate, from 0.011 to 1.8 mM D-ribose 5-phosphate or 2-deoxy-D-ribose 5-phosphate, 0.02 or 0.05 μ g *Tth*PPM, and 0.5 μ g *Tth*PNPII. The reaction mixtures were incubated 2 min at 80 °C.

Substrate	K _M , mM	V _{max} , μ mol/min·mg	k _{cat} , s ⁻¹	k _{cat} /K _M , s ⁻¹ ·M ⁻¹
D-ribose 5-phosphate	0.19 \pm 0.02	3.50 \pm 0.40	2.50 \pm 0.30	1.3 \times 10 ⁴
2-deoxy-D-ribose 5-phosphate	0.25 \pm 0.04	0.48 \pm 0.08	0.35 \pm 0.06	1.4 \times 10 ³

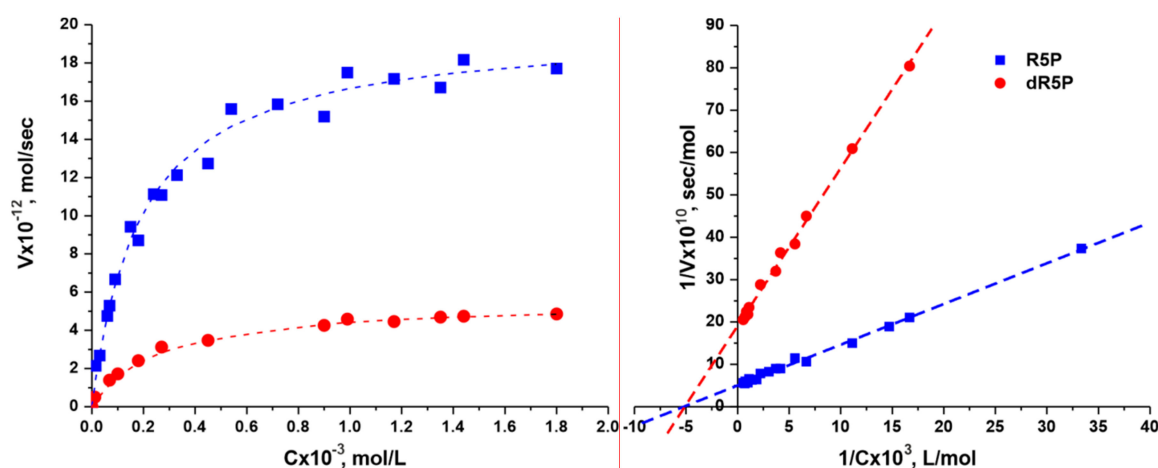


Figure 4. Michaelis–Menten and Lineweaver–Burk plots for α -D-ribose 1-phosphate and 2-deoxy- α -D-ribose 1-phosphate synthesis by *Tth*PPM.

Table 3. Specific enzymatic activity of the recombinant enzymes.

Enzyme	Substrate	Activity, Units/mg
<i>Tsp</i> RK	D-ribose	19 \pm 3
<i>Tth</i> PPM	D-ribose 5-phosphate	14 \pm 3
	2-deoxy-D-ribose 5-phosphate	2.8 \pm 0.7
<i>Tth</i> PNPI	Inosine	180 \pm 30
<i>Tth</i> PNPII	Adenosine	200 \pm 30
<i>Ec</i> RK	D-ribose	310 \pm 40
<i>Ec</i> PPM	D-ribose 5-phosphate	4.9 \pm 0.9
<i>Ec</i> PNP	Inosine	220 \pm 30
	Adenosine	120 \pm 10

3.3. Synthesis of Modified Heterocyclic Bases for Cascade Synthesis of Nucleosides

The use of a thermophilic cascade could be advantageous in the synthesis of 2,6-disubstituted purine arabinosides, since the solubility of 2-chloro- and 2-fluoradenine (for example) is known to be under 0.4 mM at 50 °C. Model reactions to determine the effectiveness of the thermophilic and mesophilic cascades were performed using 2-chloroadenine as a pentose acceptor.

After identifying the polyenzymatic cascade conditions, it was useful to synthesize both already known nucleosides (8-azaguanosine and allopurinol riboside) and new modified nucleosides using the cascade approach.

We decided to synthesize two bases: 2-chloro- and 2-fluoro-6-methoxypurine, and to obtain the corresponding arabinosides by a cascade of enzymes. From our experience, the optimal approach to the synthesis of 2-chloro- and 2-fluoro-6-methoxypurines is chemical-enzymatic (Figures 5 and 6). This variant of the synthesis has not been previously described in the literature.

All chemical transformations of bases were carried out on ribonucleosides with acetyl protection of the ribose residue. In this case, the protected ribose acts as a protection for the highly reactive N9 in purine.

In addition, the solubility of the nucleosides (both protected and unprotected) in most organic solvents is significantly higher than the solubility of the corresponding bases.

3.3.1. Synthesis of 2-Chloro-6-methoxypurine (3)

The scheme of 2-chloro-6-methoxypurine (3) synthesis is shown in Figure 5.

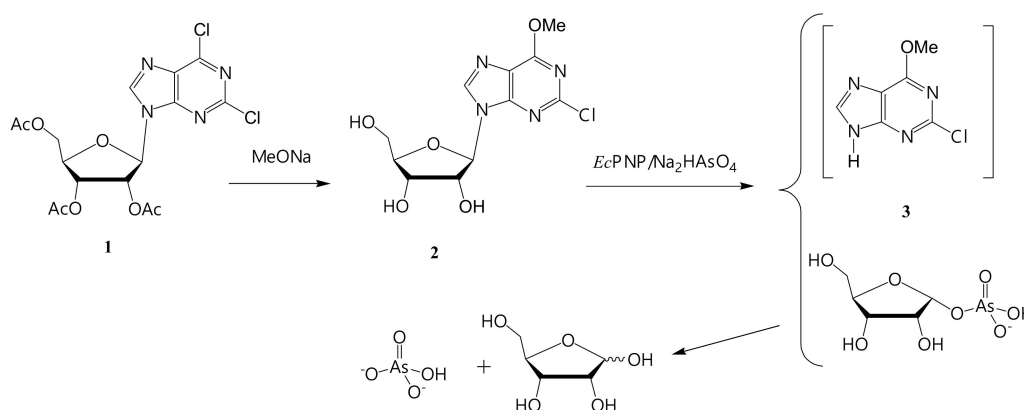


Figure 5. Synthesis of 2-chloro-6-methoxypurine (3).

Riboside (2) was prepared from 9-(2,3,5-tri-O-acetyl-D-ribofuranosyl)-2,6-dichloropurine (1) by a treatment with sodium methylate in methanol, followed by crystallization from the reaction mixture (after neutralization with HCl). Additional purification was performed by column chromatography. Riboside (2) was obtained with 44% yield and 92.9% purity (HPLC).

Base (3) was obtained from nucleoside (2) using an enzymatic arsenolysis of the glycosidic bond [32].

E. coli PNP can perform arsenolysis of a nucleoside bond. 1- α -Ribose arsenate is formed in the active site instead of 1- α -phosphate. Ribose arsenate is rapidly hydrolyzed to ribose and inorganic arsenate, and the equilibrium of the enzymatic reaction shifts towards the formation of base (3). *E. coli* PNP and Na_2HAsO_4 were added to the riboside (2) solution. The reaction mixture was incubated at 60 °C for several hours. Then, the mixture was concentrated to a minimal volume and kept at 4 °C for a while. The precipitate (2-chloro-6-methoxypurine (3)) was used in the synthesis of arabinoside without additional purification.

3.3.2. Synthesis of 2-Fluoro-6-methoxypurine (7)

The starting compound for the preparation of 2-fluoro-6-methoxypurine (7) was 2-amino-6-chloropurine riboside triacetate (4), obtained by the method [27]. The substitution of the 2-amino group with fluorine was performed by the action of 70% HF in pyridine (Figure 6).

Product (5) was isolated by column chromatography on silica gel. 2-Fluoro-6-methoxyriboside (6) was prepared by a treatment with sodium methylate in methanol according

to the procedure described above. The target riboside of 2-fluoro-6-methoxypurine was isolated by column chromatography on a silica gel. The yield of nucleoside (6) was 44%, and the byproduct, according to NMR data, was 2,6-dimethoxypurine riboside. The target product (7) was synthesized by the same method as base (3) and used in the subsequent transformation without purification.

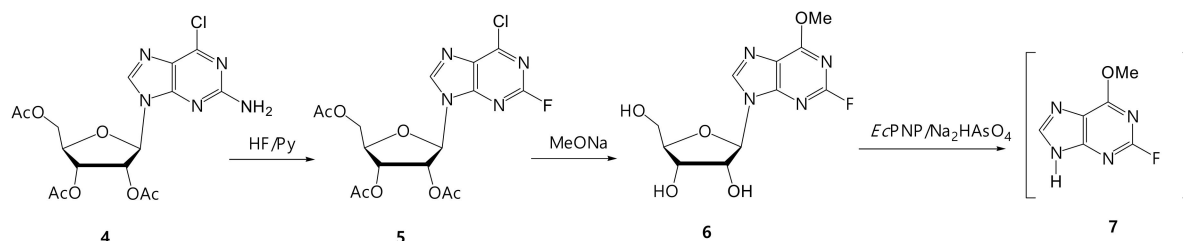


Figure 6. Synthesis of 2-fluoro-6-methoxypurine (7).

3.4. Conditions for Poly-Enzymatic Cascades Using Mesophilic and Thermophilic Enzymes

A series of poly-enzymatic cascades using recombinant thermophilic enzymes and *E. coli* enzymes was performed. The cascade of enzymatic reactions includes the sequential conversion of D-pentoses to 5-monophosphates, catalyzed by ribokinase (RK), the conversion of 5-phosphates to α -D-pentose-1-phosphates, catalyzed by phosphopentomutase (PPM), and the condensation of them with the corresponding base, catalyzed by purine nucleoside phosphorylase (PNP). This leads to the formation of the desired β -D-nucleoside (Figure 7). The reactions were monitored using the liquid chromatography–mass spectrometry (LC-MS) method.

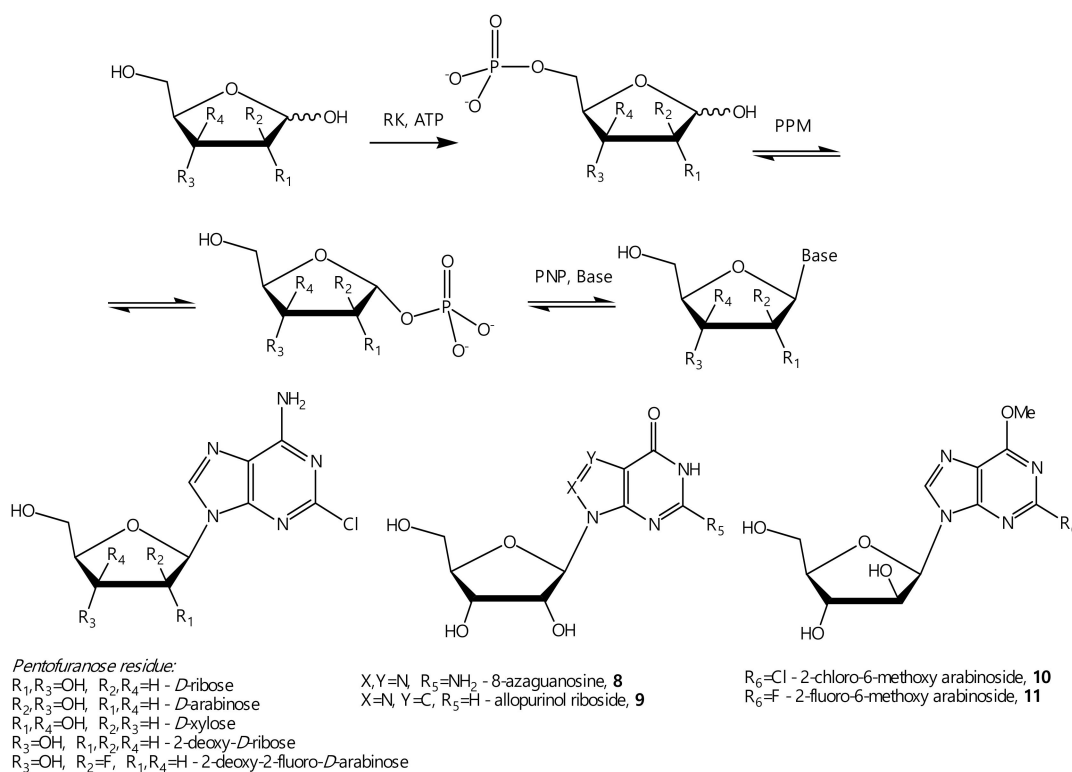


Figure 7. Synthesis of β -D-nucleosides from D-pentoses by nucleic and carbohydrate metabolism enzymes.

The conditions for the polyenzymatic cascade with mesophilic *E. coli* enzymes were determined earlier [18,33]: temperature 50 °C, pH 8.0, and the presence of potassium

chloride and manganese ions. In the case of thermophilic enzymes, the conditions differed only in temperature (80 °C).

The conditions for performing the poly-enzymatic cascade with recombinant thermophilic enzymes with various D-pentoses are presented in Table 4.

Table 4. Conditions for performing the polyenzymatic cascades of nucleoside synthesis with recombinant thermophilic and mesophilic enzymes with various D-pentoses.

Nucleoside	2ClAde Conc., mM	ATP Conc., mM	D-Pentose Conc.	Enzyme Ratio, Units			Max. Nucleoside Content, %
				RK	PPM	PNP	
<i>Thermus thermophilus</i>							
Ribo-2ClAde	0.34	2.5	2 mM D-ribose	0.3 *	0.1	0.5 **	78 (30 min)
dRibo-2ClAde			2 mM 2-deoxy-D-ribose	0.3 *	0.1	0.5 **	61 (30 min)
Ara-2ClAde			60 mM D-arabinose	11 *	1	10 **	52 (4 h)
Xylo-2ClAde			60 mM D-xylose	11 *	1	10 **	4 (24 h)
Clofarabine			10 mM 2-deoxy-2-fluoro-D-arabinose	6 *	1	10 **	19 (24 h)
<i>Escherichia coli</i>							
Ribo-2ClAde	0.34	2.5	2 mM D-ribose	0.2	0.1	0.4	91 (1 h)
dRibo-2ClAde			2 mM 2-deoxy-D-ribose	0.2	0.1	0.4	73 (30 min)
Ara-2ClAde			60 mM D-arabinose	5	1	9	67 (1 h)
Xylo-2ClAde			60 mM D-xylose	5	1	9	26 (24 h)
Clofarabine			10 mM 2-deoxy-2-fluoro-D-arabinose	6	1	4	46 (24 h)

* Ribokinase from *Thermus* species 2.9 (*Tsp*RK, gene QT17_05185) [23] was used. ** Purine nucleoside phosphorylase II (*Tth*PNPII) was used.

When selecting conditions for cascade reactions, previous experience with these enzymes was taken into account [18,22,33]. High concentrations of arabinose and xylose were due to their poor affinity for the active site of ribokinase. In addition, at ATP concentrations above 3 mM, ribokinase inhibition is observed.

As we expected, at first glance, the conditions for conducting polyenzymatic cascades of nucleoside synthesis are very similar, except for the temperature of reaction mixtures. However, there is a significant difference in synthesis efficiency and the stability of products under the conditions of obtaining 2-chloroadenine nucleosides.

3.5. Synthesis of Nucleosides (8–11)

We attempted to synthesize ribosides of two bases: 8-azaguanine (8-Aza-Gua) and allopurinol (Allop), using both mesophilic and thermophilic pathways. The conditions for the process are presented in Table 5.

For 8-azaguanine, the efficiency of the thermophilic cascade differed slightly from the mesophilic one. Therefore, we decided to synthesize 8-azaguanosine (8) using thermophilic enzymes, since the increased temperature provided acceptable solubility of the starting heterocycle. *E. coli* enzymes were used to obtain the allopurinol riboside (9), as they provide higher conversion of the base to riboside (96% per day).

The products were isolated from the reaction mixtures by column chromatography. The yields of nucleosides (**8**) and (**9**) were 69% and 84%, respectively.

Table 5. Conditions for performing the poly-enzymatic cascade with recombinant thermophilic and mesophilic enzymes with various heterocyclic bases.

Nucleoside	D-Rib Conc., mM	ATP Conc., mM	Base, 0.5 mM	Enzyme Ratio, Units			Max. Nucleoside Content, % (LC MS)
				RK	PPM	PNP	
<i>Thermus thermophilus</i>							
Ribo-8azaGua (8)	2	2.5	8-Aza-Gua	0.4 *	0.1	0.4 **	81 (24 h)
Ribo-Allop (9)			Allop	0.4 *	0.1	0.4 **	73 (8 h)
<i>Escherichia coli</i>							
Ribo-8azaGua (8)	2	2.5	8-Aza-Gua	0.2	0.1	0.4	86 (24 h)
Ribo-Allop (9)			Allop	0.2	0.1	0.4	96 (24 h)

* Ribokinase from *Thermus* species 2.9 (TspRK, gene QT17_05185) [23] was used. ** Purine nucleoside phosphorylase I (*Th*PNPI) was used.

The main conditions for the cascade synthesis of nucleosides (**8–10**) are shown in Table 6.

Table 6. Conditions for the cascade synthesis of nucleosides (**8–10**).

Comp.	D-Pentose, mg (mmol)	Base, mg (mmol)	Vol., mL	Enzymes, U			Time, h /Temp, °C	Isolated Yield, mg (%)
				RK	PPM	PNP		
<i>Thermus thermophilus</i>								
8	Ribose 42.6 (0.284)	8-Aza-Gua 28 (0.184)	142	11.4 *	2.8	11.4 **	24/80	36.1 (69)
<i>Escherichia coli</i>								
9	Ribose 42.6 (0.284)	Allop 25 (0.184)	142	5.7	2.8	11.4	24/50	41.6 (84)
10	Arabinose 1500 (10)	(2) 80 (0.25)	167	1670	334	3000	48/50	43.0 (54 ***)
11	Arabinose 720 (4.8)	(6) 36 (0.12)	80	800	160	1440	48/50	14.7 (41 ***)

* Ribokinase from *Thermus* species 2.9 (TspRK, gene QT17_05185) [23] was used. ** Purine nucleoside phosphorylase I (*Th*PNPI) was used.

*** Recalculated for the starting riboside.

The synthesis of 9-(β -D-Arabinofuranosyl)-2-chloro-6-methoxypurine (**10**) and 9-(β -D-Arabinofuranosyl)-2-fluoro-6-methoxy-purine (**11**) was performed without preliminary optimization of the conditions. Only the quantity of *E. coli* enzymes was doubled. This allowed achieving the conversion of the base into arabinoside for the 2-chloro derivative (**10**) of 65% and for the fluorinated analog (**11**) of 90% in two days.

The products were isolated by column chromatography. The yields of nucleosides (**10**) and (**11**) were 54% and 42%, respectively.

4. Discussion

To understand the peculiarities of the mesophilic and thermophilic cascades in the synthesis of 2-chloroadenine riboside, 2-deoxyriboside, arabinoside, 2-deoxy-2-fluoro-D-arabinoside, and xyloside, it is necessary to scrutinize the dynamics of nucleoside formation.

4.1. Possibilities of Using Various D-Pentoses in the Synthesis of 2-Chloroadenine Nucleosides

D-ribose, 2-deoxy-D-ribose, and their phosphates are natural substrates of nucleic acid and carbohydrate metabolism enzymes. Therefore, their conversion in a cascade with thermophilic enzymes proceeded quite intensively. The maximal content of 2-chloroadenosine (Ribo-2ClAde) was 78% after 30 min, and 2'-deoxy-2-chloroadenosine (dRibo-2ClAde) was 60% after 30 min (Figure 8). Interestingly, the equilibrium of the cascade synthesis in the synthesis of the 2-chloroadenine riboside by *E. coli* enzymes was shifted towards nucleoside formation. However, under the conditions of a thermophilic cascade, the maximal product concentration was observed after 30 min, and then, the equilibrium began to shift in the opposite direction, which led to a twofold decrease of product content in the reaction mixture. These results may be due to the increasing hydrolysis rate of α -D-ribose-1-phosphate with the temperature increasing [34].

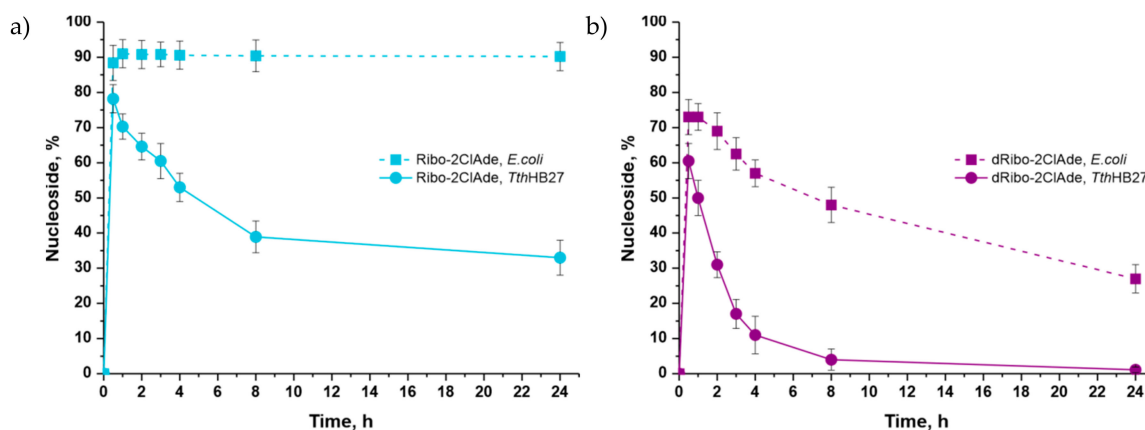


Figure 8. Accumulation of 2-chloroadenosine (a) and 2'-deoxy-2-chloroadenosine (Cladribine) (b) during cascades of enzymatic reactions with thermophilic and mesophilic enzymes (HPLC data).

The content of 2'-deoxy-2-chloroadenosine also sharply decreased over time due to the equilibrium shift towards the opposite reaction because of the hydrolysis of 2-deoxy- α -D-ribose-1-phosphate (Figure 8b).

In the cascade reaction with *E. coli* enzymes, the maximal content of 2-chloroadenosine (91%) was observed after one hour and 2'-deoxy-2-chloroadenosine (73%) after 30 min. The content of 2-chloroadenosine in the reaction mixture remained constant. The content of 2'-deoxy-2-chloroadenosine gradually decreased over time due to the shift of equilibrium towards the opposite reaction. However, the rate of the opposite reaction was noticeably lower than in the cascade with thermophilic enzymes.

The conversion of D-xylose in the thermophilic cascade was very slow, and the maximal conversion of 2-chloroadenine to 9-(β -D-xylofuranosyl)-2-chloroadenine was only 3.9% after 24 h (Figure 9a). The maximal content of 9-(β -D-xylofuranosyl)-2-chloroadenine was 26% after 24 h in the mesophilic cascade, which is much higher than in the cascade with thermophilic enzymes.

The formation of 9-(β -D-arabinofuranosyl)-2-chloroadenine from D-arabinose proceeded much more slowly (Figure 9a) compared to the formation of 2-chloroadenosine and 2'-deoxy-2-chloroadenosine (arabinoside content 48.1% after 24 h). The maximal content of 9-(β -D-arabinofuranosyl)-2-chloroadenine was higher in the mesophilic cascade than in the cascade with thermophilic enzymes (67% after an hour), but then, its content gradually decreased.

The conversion of 2-deoxy-2-fluoro-D-arabinose to 9-(2'-deoxy-2'-fluoro- β -D-Arabinofuranosyl)-2-chloroadenine (clofarabine) (Figure 9b) proceeded slower than the synthesis of arabinosides (the maximal content was 19% after 24 h, from a thermophilic cascade). The conversion of 2-deoxy-2-fluoro-D-arabinose to clofarabine proceeded much better in the mesophilic cascade than in the cascade with thermophilic enzymes (the maximal content of clofarabine was 46% versus 19% after 24 h).

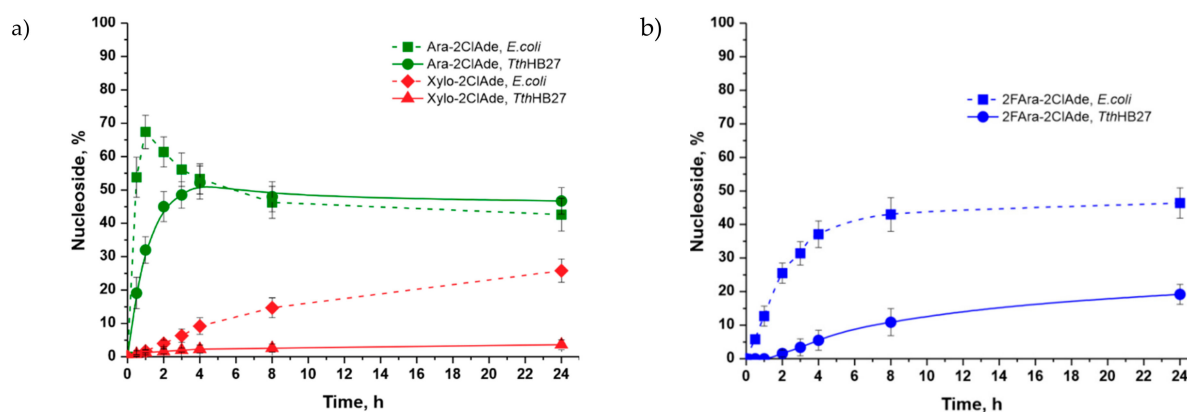


Figure 9. Accumulation of 2-chloroadenine arabinoside and xyloside (a) and 2-chloroadenine 2'-deoxy-2'-fluoro-D-arabinoside (Clofarabine) (b) during the cascades of enzymatic reactions with thermophilic and mesophilic enzymes.

Kamel with coworkers [35] investigated the stability of some pentose-1-phosphates at various temperatures. The stability of α -D-arabinose-1-phosphate was higher than that of α -D-ribose-1-phosphate and 2-deoxy- α -D-ribose-1-phosphate, but all these compounds were hydrolyzed. The stability of 2-deoxy-2-fluoro- α -D-arabinose-1-phosphate was much higher than that of all other pentose-1-phosphates. A slow decreasing of the product content after 24h in the cascade with D-arabinose and the absence of decreasing in the cascade with 2-deoxy-2-fluoro-D-arabinose may be due to a low rate of 1-phosphate intermediate hydrolysis.

The lower rate of D-arabinosides synthesis in comparison with D-ribosides and 2-deoxy-D-ribosides can be explained by the different conformations of carbohydrates in the active site of purine nucleoside phosphorylase. For this reason, the rate of D-arabinosides phosphorolysis is also much lower [36].

4.2. Non-Natural Heterocyclic Bases in the Cascade Synthesis of Nucleosides

To determine the possibility of practical use of thermophilic or mesophilic cascades, we tried to synthesize several non-natural nucleosides. We selected 8-azaguanine, allopurinol, 2-chloro-6-methoxypurine (3), and 2-fluoro-6-methoxypurine (7) as the heterocyclic bases, taking into account the prospect of subsequent biological testing of the corresponding modified nucleosides.

Synthesis of 8-Azaguanine and Allopurinol Ribosides

We investigated the formation of 9-(β -D-ribofuranosyl)-8-azaguanine and 9-(β -D-ribofuranosyl) allopurinol from 8-azaguanine and allopurinol, respectively. In both cases, the transformation proceeded at a high rate. The formation of 9-(β -D-ribofuranosyl) allopurinol (Figure 10a) in the reaction with *E. coli* enzymes proceeded at a high rate; its maximal content was 95% after eight hours and did not change over time. The maximal content of 9-(β -D-ribofuranosyl) allopurinol in the thermophilic cascade was 73% after eight hours; then, the content decreased. The rate of conversion of 8-azaguanine to 8-azaguanosine (8-azaGuo) in the mesophilic cascade was higher than in the cascade with thermophilic enzymes (Figure 10b). 8-Azaguanosine gradually accumulated in the reaction mixture with *E. coli* enzymes, and its maximum content was 89% after 24 h.

Using the parameters of the test cascade syntheses, four nucleosides were prepared and isolated. 8-Azaguanosine was synthesized by a thermophilic cascade with a 69% yield. Allopurinol riboside was obtained by a mesophilic cascade with an 84% yield. For the first time, 2-chloro-6-methoxypurine (3) and 2-fluoro-6-methoxypurine (7) D-arabinosides were synthesized using a mesophilic cascade (yields 54% and 41%, respectively).

All synthesized nucleosides were characterized by LC-MS data. The structure was confirmed by NMR spectra: ^1H , ^1H - ^1H -COSY, ^1H - ^{13}C -HSQC, ^1H - ^{13}C -HMBC, ^1H - ^{15}N -HSQC, and ^1H - ^{15}N -HMBC.

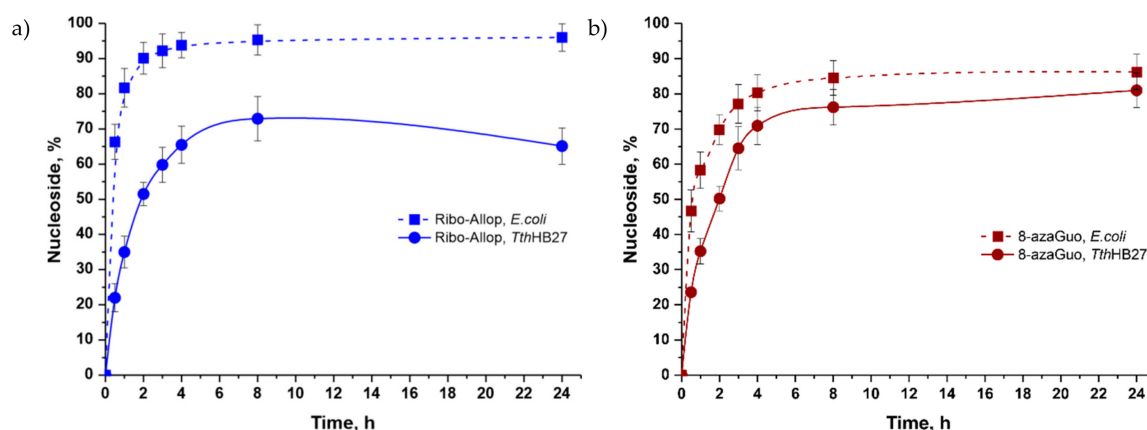


Figure 10. Comparative efficiency of allopurinol (a) and 8-azaguanine (b) ribosides synthesis in mesophilic and thermophilic cascades

The data obtained indicated that, despite the relatively small difference in temperatures when performing the cascade reactions (50 and 80 °C), the rate of product formation in the reactions with *E. coli* enzymes was much higher. The *E. coli* enzymes also provided a higher content of target products in the reaction mixture. Therefore, they were more appropriate for use in the polyenzymatic synthesis of modified nucleosides.

5. Conclusions

To carry out a comparative study of the possibilities of using the RK → PPM → NP cascade in the synthesis of modified nucleosides, sufficient quantities of genetically engineered enzymes of both *E. coli* and thermophilic *Thermus* species 2.9 and *Thermus thermophilus* HB27 were obtained. Recombinant phosphopentomutase PPM from *Thermus thermophilus* HB27 was obtained for the first time: a strain-producer of the soluble form of the enzyme was created, and a procedure for its isolation and chromatographic purification was developed. The influence of the pH, temperature, presence of divalent cations, and cofactor (glucose-1,6-bisphosphate) on the activity of *Tth*PPM was studied. The maximum activity was observed at pH 8.0. The enzyme is active over a wide temperature range, with the maximum activity in the interval between 80 and 90 °C. Glucose-1,6-bisphosphate significantly increased the activity of *Tth*PPM, although its presence in the reaction was not necessary.

The preparations of all purified enzymes were characterized by purity, the content of the oligomeric forms, and enzymatic activity.

It was shown that the cascade synthesis of the modified nucleosides from D-pentoses could be performed by both the mesophilic and thermophilic enzymes. The following D-pentoses were tested: ribose, 2-deoxyribose, arabinose, xylose, and 2-deoxy-2-fluoroarabinose. The efficiency of 2-chloroadenine nucleoside synthesis with mesophilic enzymes decreased in the following order: Rib (92), dRib (74), Ara (66), F-Ara (8), and Xyl (2%) in 30 min. For thermophilic enzymes, the order was Rib (76), dRib (62), Ara (32), F-Ara (<1), and Xyl (2%) in 30 min. After one day of incubation, the amounts of 2-chloroadenine riboside (thermophilic cascade), 2-deoxyribosides (both cascades), and arabinoside (mesophilic cascade) decreased roughly by half. Meanwhile, the conversion of the base to 2-fluoroarabinosides and xylosides continued to grow in both cases and reached 20–40%. That was quite acceptable for the preparative synthesis of nucleosides.

Four D-ribose and D-arabinose nucleosides were synthesized on a large scale by a cascade of enzymes. 8-Azaguanine riboside was synthesized by the thermophilic cascade with a 69% yield. Allopurinol riboside was obtained by the mesophilic cascade with an 84% yield. For the first time, 2-chloro-6-methoxypurine and 2-fluoro-6-methoxypurine D-arabinosides were synthesized using the mesophilic cascade (yields 54% and 42%, respectively). The data obtained indicated that, despite the relatively small difference

in temperatures when performing the cascade reactions (50 and 80 °C), the rate of the product formation in the reactions with *E. coli* enzymes was much higher. The *E. coli* enzymes also provided a higher content of products in the reaction mixtures. Therefore, they are more appropriate for use in the polyenzymatic synthesis of modified nucleosides. The use of thermophilic enzymes and a high reaction temperature might be preferable with low-soluble heterocyclic bases (2-chloroadenine and 2-fluoroadenine), as well as in the case of using carbohydrates that form 1-phosphates with high temperature stability (D-arabinose and 2-deoxy-2-fluoro-D-arabinose).

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/biom11040586/s1>: Figure S1: Synthesis of 2-chloro-6-methoxypurine (3), Figure S2: The ¹H NMR spectrum of compound 1, Figure S3: The ¹H NMR spectrum of compound 2, Figure S4: The ¹³C NMR spectrum of compound 2, Figure S5: The fragment of ¹H-¹⁵N-HMBC NMR spectrum of nucleoside 2, Figure S6: Synthesis of 2-fluoro-6-methoxypurine (7), Figure S7: The ¹H NMR spectrum of compound 4, Figure S8: The ¹H NMR spectrum of compound 5, Figure S9: The ¹³C NMR spectrum of compound 5, Figure S10: The fragment of ¹H-¹⁵N-HMBC NMR spectrum of nucleoside 5, Figure S11: The ¹H NMR spectrum of compound 6, Figure S12: The ¹³C NMR spectrum of compound 6, Figure S13: The fragment of ¹H-¹⁵N-HMBC NMR spectrum of nucleoside 6, Figure S14: The ¹H NMR spectrum of compound 8, Figure S15: The fragment of ¹H-¹³C-HSQC NMR spectrum of nucleoside 8, Figure S16: The fragment of ¹H-¹⁵N-HMBC NMR spectrum of nucleoside 8, Figure S17: The ¹H NMR spectrum of compound 9, Figure S18: The ¹³C NMR spectrum of compound 9, Figure S19: The fragment of ¹H-¹⁵N-HMBC NMR spectrum of nucleoside 9, Figure S20: The ¹H NMR spectrum of compound 10, Figure S21: The ¹³C NMR spectrum of compound 10, Figure S22: The fragment of ¹H-¹⁵N-HMBC NMR spectrum of nucleoside 10, Figure S23: The ¹H NMR spectrum of compound 11, Figure S24: The ¹³C NMR spectrum of compound 11, Figure S25: The fragment of ¹H-¹⁵N-HMBC NMR spectrum of nucleoside 11.

Author Contributions: Conceptualization, I.V.F., I.D.K., and R.S.E.; methodology, I.V.F., M.A.K., B.Z.E., M.Y.B. and A.L.K.; software, I.V.F. and M.A.K.; validation, B.Z.E., I.V.F., and M.A.K.; investigation, I.V.F., M.A.K., Y.A.A., B.Z.E., M.Y.B., O.O.M., D.D.L., E.A.Z., E.V.D., and A.S.P.; resources, I.V.F. and M.A.K.; data curation, I.D.K. and R.S.E.; writing—original draft preparation, I.V.F., M.A.K., B.Z.E., and I.D.K.; writing—review and editing, I.V.F., I.D.K., A.L.K.; visualization, I.V.F. and M.A.K.; supervision, I.D.K. and R.S.E.; and project administration, I.D.K. and R.S.E. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

a-D-PFP-1	1- α -phosphate D-pentofuranose
Allop	allopurinol
Ara	D-arabinose
Ara-2ClAde	2-chloradenine arabinoside
8-Aza-Gua	8-azaguanine
BSA	bovine serum albumin
dNTP	deoxynucleoside triphosphate mix
D-PFP-5	5-phosphate D-pentofuranose
dRibo, dRib	2-deoxy-D-ribose
DTT	dithiothreitol
Ec	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid

F-Ara	2-deoxy-2-fluoro-D-arabinose
HMBC	heteronuclear multiple bond correlation spectroscopy
HSQC	heteronuclear single quantum correlation spectroscopy
kDa	kilodalton
IPTG	isopropyl β -D-1-thiogalactopyranoside
NP	nucleoside phosphorylase
PMSF	phenylmethylsulphonyl fluoride
PNP	purine nucleoside phosphorylase
PPM	phosphopentomutase
PyNP	pyrimidine nucleoside phosphorylase
Ribo, Rib	D-ribose
RK	ribokinase
TEMED	tetramethylethylenediamine
TP	thymidine phosphorylase
Tsp	<i>Thermus species 2.9</i>
Tth	<i>Thermus thermophilus</i>
UP	uridine phosphorylase
Xyl	D-xylose
Xylo-2ClAde	2-chloroadenine xyloside

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