Enzymatic Synthesis of 2′-Deoxy-β-D-ribonucleosides of 8-Azapurines and 8-Aza-7-deazapurines

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Abstract: The enzymatic synthesis of 8-azapurine and 8-aza-7-deazapurine 2′-deoxyribonucleosides has been studied. Two methods have been used: (i) transglycosylation employing 2′-deoxyguanosine, 2′-deoxycytidine, 2′-deoxyuridine, and 2′-deoxythymidine as 2-deoxy-D-ribofuranose donors and recombinant *E. coli* purine nucleoside phosphorylase (PNP) as biocatalyst, and (ii) one-pot synthesis from 2-deoxy-D-ribose and nucleobases employing recombinant *E. coli* ribokinase (RK), phosphopentomutase (PPM) and PNP as biocatalysts. Good substrate activity was observed for all bases studied except 2-amino-8-aza-6-chloro-7-deazapurine, which afforded the desired N^9-nucleoside in moderate yield due to very low solubility of the base and partial replacement of C6-chloro atom of the base and formed nucleoside with a hydroxy group. The participation of Ser90 O^{γ} of *E. coli* PNP in the binding of 8-aza-7-deazapurines in the catalytic center of PNP followed by the formation of a productive complex and glycosidic bond is suggested.

Key words: azapurine, nucleosides, enzyme catalysis, glycosylation, phosphorylases

Nucleosides with 8-azapurines and 8-aza-7-deazapurines (purine numbering throughout) are applicable as drugs[1] and tools in chemistry, chemical biology, and molecular diagnostics.[2–5] Chemical syntheses of these nucleoside shape mimics suffer from the formation of mixtures of isomers, which makes it necessary to conduct time-consuming separation procedures. As a result, the desired nucleosides are obtained in moderate or low yields (for example see refs.[4,6] and those in the Supporting Information).

Nucleoside phosphorylases (NP), in particular *E. coli* NP, are very efficient biocatalysts in glycosylation reactions.[7] Doskocil and Holy[8] have shown that 8-azaguanine is a good ribosyl acceptor in the enzymatic reaction catalyzed by *E. coli* purine nucleoside phosphorylase (PNP; product of *deoD* gene; EC 2.4.2.1;[7,9]). Later, Votruba et al. studied alginate gel entrapped cells of an auxotrophic thymine-dependent strain of *E. coli* as a biocatalyst for the transfer of the 2′-deoxy-D-ribofuranosyl moiety of 2′-deoxyuridine to purine and pyrimidine bases including aza and deaza analogues.[10] The reaction proceeds regio- and stereospecifically, affording 8-aza-2′-deoxyadenosine and 8-aza-2′-deoxyguanosine. Furthermore, it was found that the presence of nitrogen-7 of purines and their isosteric analogues is a prerequisite for the reaction.[10] However, there are several exceptions; 5-aza-7-deazaguanine[11] (**1**), 5-aza-7-deazaisoguanine[12] (**2**) and *N*-(1,3,4-thiadiazol-2-yl)-cyanamide[13] (**3**) are substrates for bacterial purine nucleoside phosphorylases (Figure 1).

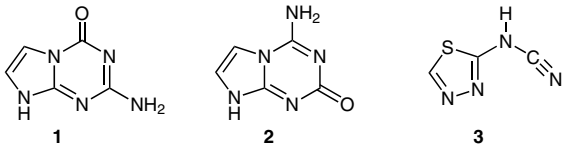

Figure 1 Some unusual substrates of *E. coli* purine nucleoside phosphorylase in synthetic reactions

We applied the enzyme-catalyzed glycosylation to diverse range of heterocyclic bases belonging to the classes of 8-azapurines and 8-aza-7-deazapurines. The work presented here reports on the use of various donors of the 2′-deoxy-D-ribofuranosyl residue and recombinant *E. coli* PNP[14] as a biocatalyst (transglycosylation reaction; Scheme 1, path A) and enzymatic cascade transformation of 2-deoxy-D-ribose into the nucleosides (Scheme 1, path B). This cascade reaction uses recombinant *E. coli* ribokinase (RK),[15] phosphopentomutase (PPM),[16] and PNP[14] (one-pot synthesis[16]). We studied the enzymatic synthesis of N^9-2′-deoxy-β-D-ribonucleosides of a number of 8-azapurines (**4** and **5**) and 8-aza-7-deazapurines (**6**–**9**) (Figure 2).

First, the substrate specificity of bases **4**–**8** was tested in the transglycosylation reaction performed under standard conditions using 2′-deoxyguanosine (**10**) as a glycosyl donor (Scheme 1, path A).[17] All the bases are satisfactory and the respective nucleosides were obtained in good yields. The structure of these nucleosides (**15**–**19**) was confirmed by ^{1}H, ^{13}C NMR and UV spectroscopic analysis and compared to previously published data (see the Supporting Information). We did not observe the formation of regioisomeric nucleosides.

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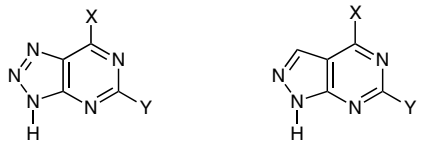


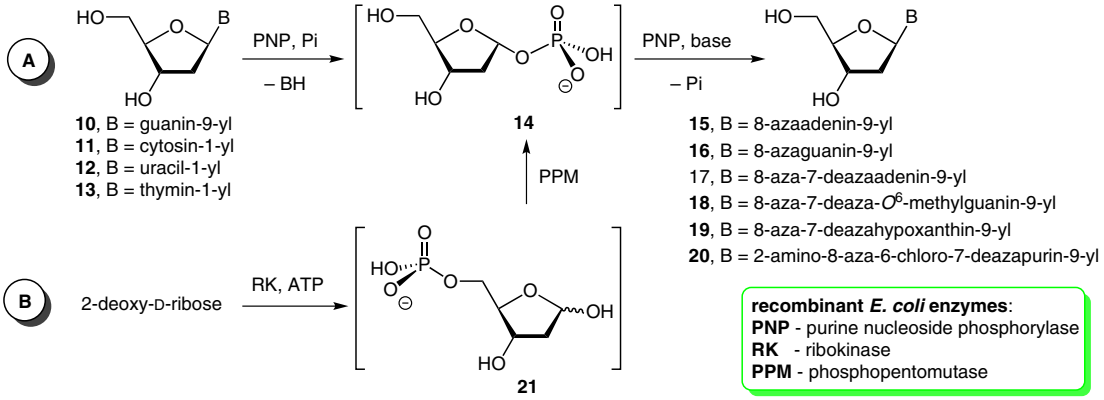
4, X = NH_2, Y = H
5, X = OH, Y = NH_2

6, X = NH_2, Y = H
7, X = OMe, Y = NH_2
8, X = OH, Y = H
9, X = Cl, Y = NH_2

Figure 2 The structures of 8-aza- and 8-aza-7-deazapurine bases studied in the synthesis of 2′-deoxy-β-D-ribonucleosides catalyzed by recombinant *E. coli* PNP

The regiospecific 2′-deoxy-D-ribosylation of 8-azaadenine (**4**) and 8-azaguanine (**5**) catalyzed by recombinant *E. coli* PNP is in line with published data.[8,9b,10] In contrast, glycosylation of anions of 8-azapurines with 2-deoxy-3,5-di-*O*-(4-toluoyl)-α-D-erythropentofuranosyl chloride gave a very complex mixture of regioisomers and their α,β-anomers.[4,18,19] There are a great number of publications devoted to the tautomerizm of 8-azapurines and the chemical reactivity of nitrogen atoms of nucleobase anions that are involved in the formation of the glycosyl bond (reviewed in refs.[20,21]). The nucleobase anions are one of the activated species in the chemical glycosylation of 8-azapurinrs existing in a mixture of different tautomers; the other method of activation of purine bases involves the preparation of trimethylsilyl derivatives, -C–$N^{9(7)}$(H)- → -C–$N^{9(7)}$(TMS)-, leading to the enhancement of nucleophilicity of nitrogen atoms of the sp^2 hybridized imino -C=$N^{7(9)}$- tautomers.[21] An interplay of the equilibrium of tautomeric forms of anions or imino tautomers, on the one hand, and nucleophilicity of the activated nitrogen atoms, on the other, are responsible for the formation of regioisomeric nucleosides. In the case of the *E. coli* PNP catalyzed coupling of base and pentofuranose, activation of natural purines involves fixing a specific sp^2 hybridized imino -C=N^9- tautomer of base in the catalytic site.[7b,22] From a chemical viewpoint, the formation of the glycosyl bond results from nucleophilic attack of a nitrogen atom of the base on the electrophilic C1-carbon atom of 2-deoxy-D-ribofuranose 1-phosphate (**14**). The regioselectivity of the enzymatic glycosylation is governed by the binding mode of base in the catalytic center of the enzyme enabling nucleophilic attack of a nitrogen atom of the sp2 hybridized imino -C=N^9- tautomer on the electrophilic center of the co-substrate. An analogous mode of binding can be assumed with high probability for natural purine bases as well as for 8-azapurines **4** and **5**, leading to the exclusive formation of N^9-glycosides. However, the formation of nucleosides **17**–**19** was rather unexpected in the light of the earlier data pointing to the crucial importance of nitrogen-7 of purines and their isosteric analogues in the synthetic reaction catalyzed by *E. coli* PNP.[7–10]

The mechanism of the synthetic reaction catalyzed by *E. coli* PNP nucleoside phosphorylases has not attracted the attention of researchers thus far, and many important details are not clear (see, for example the discussion in the literature[7b,c]). Thus, the mode of binding of the substrate or inhibitor in the catalytic center of the enzyme might shed light on the mechanism of the enzyme's functioning, and provide clues that could help to explain some unusual observations such as the good substrate activity of 8-aza-7-deazapurines. Detailed analysis of the mechanism of the phosphorolysis of purine nucleosides by *E. coli* PNP showed that the base binding site is formed mainly by Asp204 and, to some extent, by Phe159.[23–26] It is noteworthy that replacement of D-aspartic acid residue 204 by alanine resulted in complete loss of the catalytic activity of *E. coli* PNP (see note on page 1266 of the paper by Ealick et al.[26a] and the recent paper by Luic et al.[26b]). Taking into account that it is an equilibrium reaction, one can expect that the same amino acid residues make the main contribution to binding of the substrate in the synthetic reaction. Asp204 interacts with nitrogen-7 and the substituent at C6 of the purine base, giving rise, in all likelihood, to the optimal base orientation and to enhancement of the nucleophilic properties of nitrogen-9, i.e., activation of the substrate (Figure 3). It is, therefore, surprising that replacement of nitrogen-7 with a CH group did not abolish the substrate activity of bases **6**–**8**, which suggests a rather efficient contribution of the Asp204–C6 substituent interaction to correct binding and activation. To establish the role of such interaction, we investigated the substrate properties of base **9**, which has no groups that could imitate an interaction of natural purine substrates with Asp204. It was surprising to find that base **9** still retains



10, B = guanin-9-yl
11, B = cytosin-1-yl
12, B = uracil-1-yl
13, B = thymin-1-yl

15, B = 8-azaadenin-9-yl
16, B = 8-azaguanin-9-yl
17, B = 8-aza-7-deazaadenin-9-yl
18, B = 8-aza-7-deaza-O^6-methylguanin-9-yl
19, B = 8-aza-7-deazahypoxanthin-9-yl
20, B = 2-amino-8-aza-6-chloro-7-deazapurin-9-yl

recombinant *E. coli* enzymes:
PNP - purine nucleoside phosphorylase
RK - ribokinase
PPM - phosphopentomutase

Scheme 1 Synthesis of nucleosides **15**–**20** by the transglycosylation reaction (path A) and cascade one-pot transformation of 2-deoxy-D-ribose into nucleosides (path B)

moderate substrate activity, despite the absence of any interactions with Asp204 and the extremely low solubility.

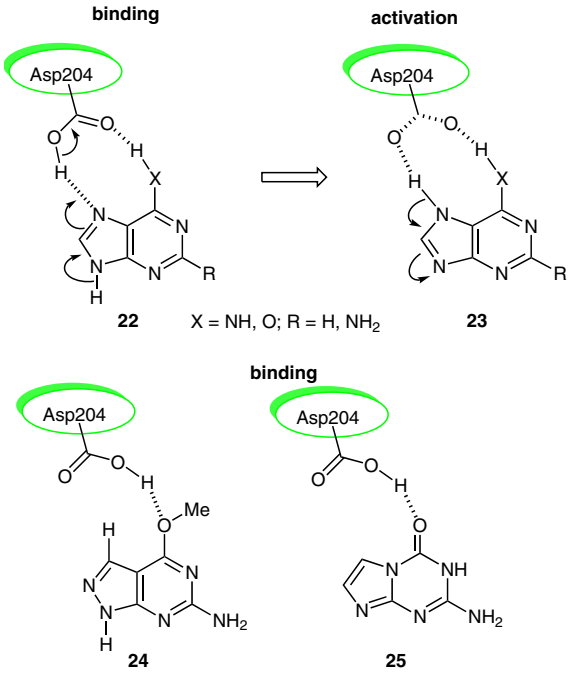


Figure 3 Schematic presentation of binding adenine, hypoxanthine and guanine (**22**), followed by activation (**23**), and a possible binding of 2-amino-8-aza-6-methoxy-7-deazapurine (**7**) and 5-aza-7-deazaguanine (**1**); the most populated tautomers are show for bases in structures **24** and **25**

It appears clear that binding of 2-amino-8-aza-6-methoxy-7-deazapurine (**7**) in the catalytic center of PNP cannot lead to activation of the substrate. Indeed, analysis of the tautomeric structures of base **7** by ab initio calculations using a Polack–Ribiere conjugate gradient (HyperChem 8.01) showed a strong preference for the sp^3-hybridized amino -C-N^9(H)- tautomer.[27,28] Remarkably, similar analyses using a semi-empirical method PM3 (in water box) for three possible tautomeric structures of 5-aza-7-deazaguanine (**1**) revealed a strong preference for the sp^2 hybridized imino -C=N^9- tautomer (Figure 3).[7b] This electronic structure is in harmony with satisfactory substrate activity of isosteric guanine analogue **1**, implying the important role of Asp204 and Phe159 residues in correct binding at the catalytic center of *E. coli* PNP.

Analysis of the crystal structure of the ternary complex of hexameric *E. coli* PNP with formycins A and B showed that Ser90 is involved in binding of the bases.[23,24] Moreover, crystallographic data for adenine binding to the active site of *E. coli* PNP clearly showed the close proximity of Ser90 O^γ to carbon-8 of the base.[25,26a] These data, together with the aforementioned considerations, suggest a possible explanation for the good substrate properties of bases **6**–**8** and moderate activity of base **9**; it is apparent that Ser90 O^γ is hydrogen bonded to nitrogen-8 (purine numbering) of the base in **26**, giving rise to the correct base orientation in the catalytic site of *E. coli* PNP, followed by activation of the nitrogen-9 in productive complex **27** (Figure 4). The contribution of Phe159 of *E. coli* PNP to both processes — binding and activation — appears to be analogous to that of the natural bases. Thus, in the case of 8-aza-7-deaza purine analogues, the Ser90 residue of the catalytic site of *E. coli* PNP takes the place of Asp204 in the case of natural purine substrates.

The spatial tautomeric structures of base **9** in complex with Ser90-O^γ have been analyzed by the restricted Hartree–Fock (RHF) ab initio method using basis set of 6-31** FIREFLY QC package,[29] which is partially based on the GAMESS (US)[30] source code. The files of MOPAC format containing Z-matrix of internal coordinates obtained by the PM3 geometry optimization[31] was used as starting approximation for the ab initio calculations. The following main dimensions were obtained for the respective structures **26**: Ser90-O^γH···N^8 (0.97 and 1.86 Å); Ser90-O^γ···H–N^9 (2.39 and 0.99 Å); =N^8–N^9(H)- (1.39 Å); **27**: Ser90-O^γ···H–N^8 (2.37 and 0.99 Å); Ser90-O^γH···N^9 (0.96 and 1.83 Å); -N^8(H)–N^9= (1.37 Å); these data are in fair agreement with strong hydrogen bonding of the base, enabling correct binding followed by activation.

In the next series of experiments, we studied the one-pot synthesis of nucleosides **15**–**19** with 2-deoxy-D-ribose and heterocyclic bases **4**–**8** in the presence of recombinant RK, PPM and PNP (Scheme 1, Path **B**).[7b,16] Under the reaction conditions employed, the formation of 8-aza-2′-deoxyadenosine (**15**) and 8-aza-2′-deoxyguanosine (**16**) proceeded slowly, affording the nucleosides in moderate yields. In contrast, 8-aza-7-deazapurines **6**–**8** showed satisfactory substrate activity and the respective nucleosides **17**–**19** were formed in yields of more than 50% after 20 hours (Scheme 1; see also the Supporting Information).

Recently, Mitsui Chemicals, Inc. reported an enzymatic method for the production of nucleosides.[32] Surprisingly they found that recombinant *E. coli* PNP is able to catalyze the reaction of cytosine and 2-deoxy-D-ribofuranose 1-phosphate in Tris·HCl buffer (100 mM, pH 8.0; 50 °C, 20 h) giving rise to the formation of 2′-deoxycytidine (**11**; dC) in 57.5% yield (HPLC). We have confirmed this finding and found that dC is phosphorolyzed under very mild conditions (nucleoside 0.05 mmol; 80 mM K,Na-phosphate buffer, pH 7.0; 27 units[14] PNP; 14–15°C), resulting in more than 60% product yield (Figure 5). Furthermore, 2′-deoxyuridine (**12**; dU) and, to a lesser extent, 2′-deoxythymidine (**13**; dT) are also cleaved. However, a plateau is reached in the phosphorolysis of dT implying two competing reactions: phosphorolysis and glycosylation. It is noteworthy that Pugmire and Ealick performed detailed structural analysis of NP and defined two distinct families of the enzymes: NP-I and NP-II.[33] The first family includes homotrimeric and hexameric enzymes from both prokaryotes and eukaryotes that accept purine (inosine, guanosine and adenosine) as well as pyrimidine (uridine) nucleosides as substrates. The second family encompasses bacterial pyrimidine phosphorylases and eukaryotic

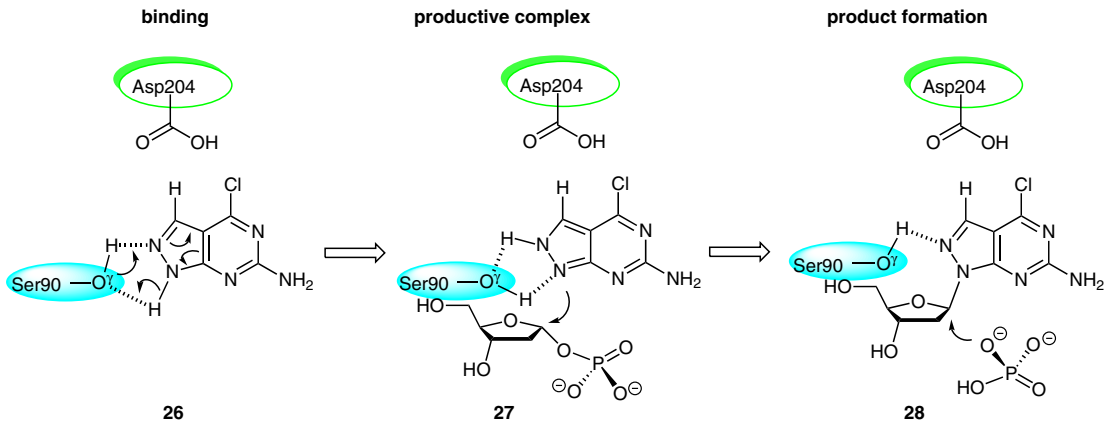


Figure 4 Schematic presentation of binding 2-amino-8-aza-6-chloro-7-deazapurine (**9**), and the formation of productive complex **27** and nucleoside **28**

thymidine phosphorylases. The activity of *E. coli* PNP against dU, dC and dT deserves detailed structural analysis. It should be stressed that the corresponding ribonucleosides showed no substrate activity with *E. coli* PNP under the aforementioned reaction conditions.

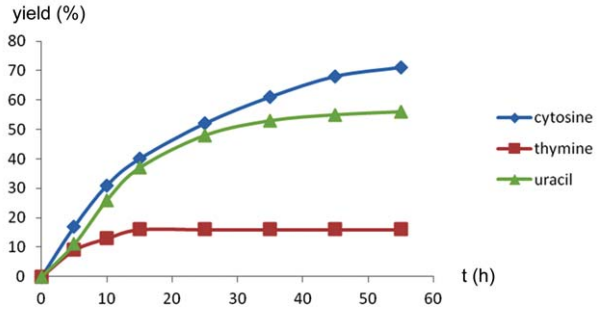


Figure 5 Phosphorolytic cleavage of 2′-deoxycytidine, 2′-deoxyuridine, and 2′-deoxythymidine with recombinant *E. coli* PNP (HPLC analysis of the formation of the corresponding bases).

Finally, the transglycosylation reaction of 8-azaadenine (**4**) and 8-aza-7-deazaadenine (**6**) with 2′-deoxycytidine (dC), 2′-deoxyuridine (dU) and 2′-deoxythymidine (dT) as sugar donors, and recombinant *E. coli* PNP as biocatalyst, was studied.[34] In the case of dC/base combination of substrates, the reaction proceeded smoothly and the nucleosides **15** and **17** were formed in 10 and 60% yields, respectively, after 72 hours. Under these reaction conditions, the formation of nucleosides was not observed in the case of dU/base and dT/base substrate combinations. The use of dU and dT as glycosyl donors in enzymatic synthesis of nucleosides is under study.

In conclusion, efficient enzymatic methods for the preparation of N^9-2′-deoxy-β-D-ribonucleosides of 8-azapurines and 8-aza-7-deazapurines are described. One of the approaches involves pentofuranose transfer from 2′-deoxyguanosine or 2′-deoxycytidine to heterocyclic bases catalyzed by recombinant *E. coli* PNP.[7] The uses the one-pot transformation of 2-deoxy-D-ribose into the aforementioned nucleosides through intermediate formation of 2-deoxy-D-ribofuranose 5-phosphate (**21**), 2-deoxy-α-D-ribofuranose 1-phosphate (**14**), and finally nucleosides catalyzed by recombinant *E. coli* RK, PPM and PNP, respectively.[7c,16] The methods employed in the present work for the synthesis of 2′-deoxy-β-D-ribonucleosides are complementary to each other. We tested both methods to assess, first of all, the feasibility of the latter for the synthesis of base-modified nucleosides. It is clear that the choice of method depends on the availability of the enzymes and the starting pentose or its donor.

The participation of Ser90 O^γ of *E. coli* PNP in the binding of 8-aza-7-deazapurines in the catalytic center of PNP followed by the formation of productive complex and the glycosidic bond is suggested.

The reaction conditions in both types of enzymatic reactions have not been optimized. Despite this, the heterocyclic bases **4**–**8** showed satisfactory substrate properties and the desired N^9-2′-deoxy-β-D-ribofuranosyl nucleosides have been prepared in good yields.

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Supporting Information for this article is available online at http://www.thieme-connect.com/ejournals/toc/synlett.

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(17) **Standard reaction conditions A**: K,Na-phosphate buffer (10 mM, pH 7.0); donor/acceptor ratio was 1.5:1.0 (mol); 1000 units of *E. coli* PNP[14] was used per 1 mmol of base (ca. 0.10–0.15 mmol of base in 10–15 mL buffer was taken in the reaction); 50 °C, 48–50 h. The reaction mixture was filtered, silica gel (ca. 2 mL) was added to the filtrate and the mixture was evaporated to dryness and co-evaporated with EtOH. The silica gel with adsorbed product was put on the top of a silica gel column (ca. 50 mL) and product was eluted with $CHCl_3$–MeOH. HPLC data [Kromasil C18, 4.6 × 250 mm, 5 μm; elution with H_2O–MeOH–TFA, 90:10:0.1; flow rate 1 mL/min]: R_t (base/2′-deoxyriboside) = 4.7/6.8 (**4**/**15**), 7.8/13.6 (**5**/**16**), 4.2/8.4 (**6**/**17**), 10.7/17.0 (**7**/**18**), 5.6/12.2 (**8**/**19**), 6.1/14.3 (**9**/**20**) min. Yields for individual isolated products **15**–**19**: 66, 60, 77, 76 and 68%, respectively.
Synthesis of nucleoside 20: To a solution of 2′-deoxyguanosine (95 mg, 0.356 mmol) in K,Na-phosphate buffer (18 mM; pH 7.5) was added PNP (10 mg, 270 units) and the reaction mixture was stirred at 50 °C for 1 h. A solution of **9** (40 mg, 0.237 mmol) in DMSO (2 mL) was added dropwise over 1 h and the mixture was heated at 50 °C for 72 h (progress of the reaction monitored by HPLC). The mixture was cooled to r.t., the guanine formed and the remaining base **9** was filtered off, the filtrate was evaporated, and the desired product **20** was isolated by silica gel column chromatography (MeOH–$CHCl_3$, 1:10) to give **20** (17 mg, 25%) as an amorphous product. UV (H_2O): λ_{max} = 305 nm, λ_{min} = 228 nm.
Conditions B: Total volume: 2 mL; 2 mM ATP, 50 mM KCl, 3 mM $MnCl_2$, 20 mM Tris×HCl (pH 7.5); 1.3 mM 2-deoxy-D-ribose, 1 mM heterocyclic base; units of enzymes: RK[15] (9), PPM[16] (4), and PNP[14] (14); 40 °C. Yields by HPLC of nucleoside and reaction time: **15** (8 %; 15 h), **16** (23%; 25 h), **17** (52%; 25 h), **18** (50%; 20 h) and **19** (60%; 10 h).
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