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Use of Nucleoside Phosphorylases for the Preparation of Purine and Pyrimidine 2′-Deoxynucleosides

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Abstract. Enzymatic transglycosylation - a transfer of the carbohydrate moiety from one heterocyclic base to another - is being actively developed and applied for the synthesis of practically important nucleosides. This reaction is catalyzed by nucleoside phosphorylases (NPs), which are responsible for reversible phosphorolysis of nucleosides to yield the corresponding heterocyclic bases and monosaccharide 1-phosphates.

We found that 7-methyl-2′-deoxyguanosine (7-Me-dGuo) is an efficient and novel donor of the 2-deoxyribose moiety in the enzymatic transglycosylation for the synthesis of purine and pyrimidine 2'-deoxyribonucleosides in excellent yields. Unlike 7-methylguanosine, its 2′-deoxy derivative is dramatically less stable. Fortunately, we have found that 7-methyl-2′-deoxyguanosine hydroiodide may be stored for 24 h in Tris-HCl buffer (pH 7.5) at room temperature without significant decomposition.

In order to optimize the reagent ratio, a series of analytical transglycosylation reactions were conducted at ambient temperature. According to HPLC analysis of transglycosylation reactions, the product 5-ethyl-2′-deoxyuridine (5-Et-dUrd) was obtained in high yield (84-93%) by using a small excess (1.5 and 2.0 equiv.) of 7-Me-dGuo over 5-ethyluracil (5-Et-Ura) and 0.5 equiv. of inorganic phosphate. Thymidine is a less effective precursor of α-D-2-deoxyribofuranose-1-phosphate (dRib-1p) compared to 7-Me-dGuo.

We synthesized 2'-deoxyuridine, 5-Et-dUrd, 2'-deoxyadenosine and 2'-deoxyinosine on a semi-preparative scale using the optimized reagents ratio (1.5:1:0.5) in high yield.

Unlike other transglycosylation reactions, the synthesis of 2-chloro-2'-deoxyadenosine was performed in heterogeneous medium because of the poor solubility of initial 2-chloro-6-aminopurine. Nevertheless, this nucleoside was prepared in good yield. The developed enzymatic procedure for the preparation of 2'-deoxynucleosides may compete with the known chemical approaches.

Keywords: enzymes, nucleoside phosphorylases, nucleosides, phosphorolysis, transglycosylation.

Introduction

Several drugs based on modified nucleosides and possessing antitumor (Cladribine, Fludarabine, Pentostatin, Nelarabin, Vidaza, Decitabin, etc.) or antiviral (hepatitis C, herpes, HIV, etc.) activity (Ribavirin, Zidovudine, Lamivudine, etc.) are currently used in clinical practice.[1, 2] Two main methods are employed to prepare nucleoside analogues. One of them is based on the modification of natural compounds. In another one, heterocycle or monosaccharide are modified followed by the formation of *N*-glycosidic bond.[3] The glycosylation method developed by Vorbrüggen et al. almost completely replaced the old procedures.[4] This method consists in the condensation of trimethylsilyl derivatives of heterocyclic bases with peracylated monosaccharides in the presence of Lewis acids. Stereoselectivity of the reaction is determined by the 2-*O*-acyl group, which is involved in the formation of the acyloxonium ion. Thus, natural β-nucleosides, in which the heterocyclic base is in the *trans* position to the 2-*O*-acyl group, are produced in the case of D-ribose, whereas the absence of the 2-*O*-acyl group, as in 2-deoxyribose, results in the formation of a mixture of α,β-isomers. This greatly complicates the purification of the target compounds.

Enzymatic methods for the formation of a glycosidic bond complement chemical procedures and, in some

cases, have obvious advantages.[5-7] The ability of NPs to cleave 2'-deoxynucleosides and β-D-arabinofuranosyl nucleosides and to catalyze reversible phosphorolysis[5] is widely used for the preparation of nucleosides and their analogues.[8-9] The equilibrium of these reactions is shifted towards nucleosides,[10] the shift being more pronounced for purine nucleoside. It may be considered as a driving force for the overall transglycosylation process.

NPs are involved in the salvage pathway of nucleoside biosynthesis. This class of enzymes includes thymidine phosphorylase (TP; EC 2.4.2.4), uridine phosphorylase (UP; EC 2.4.2.3), and purine nucleoside phosphorylase (PNP; EC 2.4.2.1). These enzymes are found in virtually all organisms and share similar primary structures. The most substrate-specific enzyme is TP, which is active only towards thymidine. UP catalyzes the phosphorolysis of both thymidine and uridine, PNP may cleave the *N*-glycosidic bond in purine ribo- and 2'-deoxyribonucleosides. [11, 12]

The enzymatic transglycosylation, a transfer of the carbohydrate moiety from one heterocyclic base to another, has been applied for the synthesis of nucleosidic drugs (Cladribine, Fludarabine, Nelarabin, and Vidarabin) (Fig. 1). [8, 9] The term "transglycosylation" was adopted from the carbohydrate field to describe the enzymatic transfer of sugar from an oligosaccharide to another carbohydrate acceptor. [13]

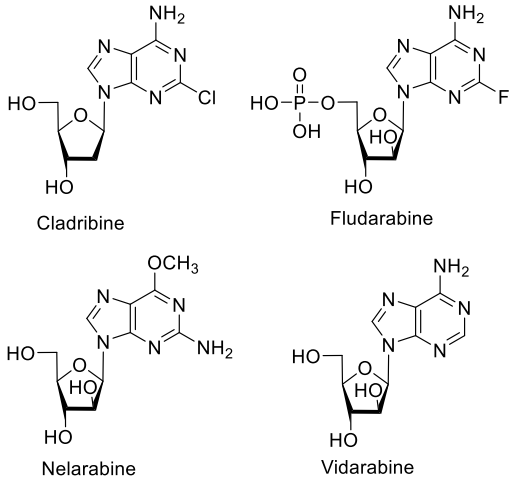
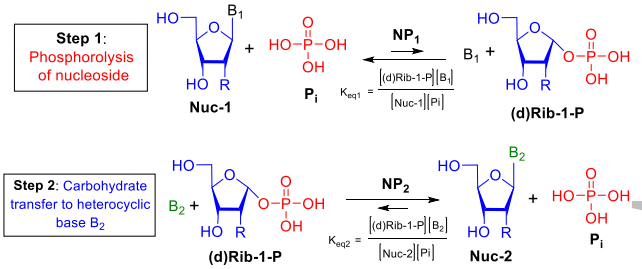


Figure 1. Structures of Cladribine, Fludarabine, Nelarabine, and Vidarabine.

Here, we developed a new effective method for the preparation of 2'-deoxynucleosides by NP-catalyzed transglycosylation starting from 7-methyl-2′-deoxyguanosine.

Results and Discussion

The overall transglycosylation process may be represented as two consecutive equilibrium reactions (Scheme 1), where K_{eq1} and K_{eq2} are the equilibrium constants of the corresponding phosphorolysis reactions. One or two different NPs may be used in this process; their choice depends on the starting nucleoside (Nuc-1) and heterocyclic base (B2) and the resulting nucleosides (Nuc-2) (Scheme 1).



Scheme 1. Enzymatic phosphorolysis of nucleosides and transglycosylation. NP_1, NP_2 are nucleoside phosphorylases, B_1 and B_2 are heterocyclic bases, R - H or OH.

The analysis of these reactions shows that the highest yield of Nuc-2 may be expected when the equilibrium of step 1 is shifted towards the formation of α-D-2-deoxyribose-1-phosphate (dRib-1-p), and the equilibrium of step 2 is shifted towards the target nucleoside (Nuc-2). Therefore, dRib-1-p is the optimal substrate for the preparation of the target nucleoside in the highest yield. There are three sources of the key dRib-1-p:

1. Commercially available dRib-1-p; [14]
2. dRib-1-p synthesized according to known procedures; [15, 16]
3. dRib-1-p synthesized by the enzymatic reaction through the intermediate formation of D-2-deoxyribose-5-phosphate; [17, 18]

Methods 2 and 3 are rather laborious; besides, commercially available dRib-1-p is rather expensive. We supposed that dRib-1-p may be prepared by the enzymatic phosphorolysis of 7-Me-dGuo in the presence of PNP (step 1, Scheme 1). The resulting dRib-1-p was used without the isolation, in the condensation with the heterocyclic base B2 in the presence of NP (step 2, Scheme 1).

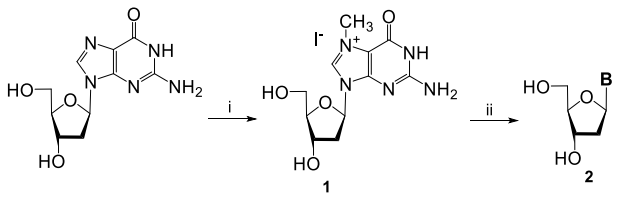
We suggest this procedure as the most simple and cost-effective one. The further advantage of this method is that it allows the combination of steps 1 and 2 (Scheme 1) without the isolation of labile dRib-1-p.

7-Methylguanosine is often used for the preparation of purine ribonucleosides. [18-22] It was shown that phosphorolysis of 7-methylguanosine in the presence of PNP produces α-D-ribofuranose-1-phosphate and 7-methylguanine (7-Me-Gua) in quantitative yields. [23] Thus, 7-methylguanosine is a low-cost replacement for α-D-ribose-1-phosphate. We

supposed that its phosphorolysis will proceed in a similar way affording phosphate in a high yield.

To the best of our knowledge, the use of 7-methyl-2′-deoxyguanosine for the enzymatic synthesis of nucleosides is not described in the literature probably because of its instability in aqueous solutions. [24]

The methylation of guanosine using an original method [25] gave, in our hands, 7-methyl-2′-deoxyguanosine hydroiodide (**1**) with some visible impurities. Therefore, we modified this procedure by performing the methylation in *N*,*N*-dimethylformamide in the presence of barium carbonate. The purity of **1** was estimated by ^{1}H NMR spectroscopy at > 98%. The developed protocol allowed the preparation of this compound on a multigram scale (Scheme 2, step i).



Scheme 2. Synthesis of 2′-deoxyribonucleosides by transglycosylation. *Reagents and conditions.* (i) MeI/$BaCO_3$, DMF, 5.5 h, r.t., 65%. (ii) heterocyclic base **B**, potassium dihydrophosphate, Tris-HCl buffer, pH 7.5, *E. coli* PNP (*E. coli* TP for pyrimidine bases Ura, 5-F-Ura, 5-Et-Ura), r.t., 24 h, isolated yields: dAdo, 93%; dIno, 95%; 2-Cl-dAdo, 70%; dUrd, 80%; 5-F-dUrd, 81%; 5-Et-dUrd, 81%.

2'-Deoxynucleoside **1** is stable in the solid state for several weeks at ambient temperature and for at least 6 months at -20°C. Compound **1** is highly soluble in water and buffer solutions at ambient temperature that makes it possible to use **1** as a substrate for enzymatic reactions, where aqueous media and mild conditions are preferable. According to the literature data, the 7-methylated guanosine derivative is stable in aqueous solutions at high temperature up to 90°C in the pH range from 2 to 6. The *N*-glycosidic bond is cleaved in strong acids (pH<2). The cleavage of the imidazole ring of guanine proceeds under basic conditions (pH >8) [24] just above its pK_a that is close to 7.1. [26] 7-Methyl-2'-deoxyguanosine (**1**) is dramatically less stable compared to 7-methylguanosine that limits its use for the synthesis of nucleosides. Moreover, dRib-1-p is also unstable under acidic conditions and at elevated temperatures (30-60ºC). [27]

We have investigated the stability of **1** by HPLC and UV spectroscopy (Figures 10-17, Supporting Info). Compound **1** appeared to be unstable in aqueous solution at pH 4.1 and 20°C. According to HPLC analysis, compound **1** proved to be quite stable at 20°C in 50 mM Tris-HCl buffer, pH 7.5; the degree of decomposition was about 30% after two days at 20°C (Figure 16, Supporting Info).

As follows from the analysis of UV spectra, the decomposition of compound **1** in Tris-HCl buffer at pH 7.5 is similar in character to its phosphorolysis in the presence of PNP (Figures 13-14, Supporting Info) and significantly differs from the cleavage of the imidazole ring of **1** under basic conditions (pH >7.5, Figure 15, Supporting Info). Thus, it can be concluded that the imidazole ring of **1** is stable in Tris-HCl buffer at pH 7.5, while the *N*-glycosidic bond is slowly hydrolyzed under such conditions.

The stock solution of **1** remains stable during storage at 7°C in 50 mM Tris-HCl buffer, pH 7.5 (its hydrolysis is *ca*. 5% after one week, Figure 17, Supporting Info). The transglycosylation conditions were chosen based on the properties of **1**. Thus, the conditions of choice are room temperature and Tris-HCl buffer (pH 7.5). The degree of decomposition of **1** increases at elevated temperature (37ºC).

The use of **1** as the initial substrate makes it possible to simplify the general scheme of enzymatic transglycosylation (Scheme 1) because step 1 becomes irreversible. Compound **1** was nearly quantitatively converted into dRib-1-p in the presence of PNP (Scheme 1).

To optimize the reagent ratio, a series of analytical transglycosylation reactions were conducted at room temperature (Table 1). The formation of purine nucleosides proceeded in the presence of PNP, and the formation of pyrimidine nucleosides requires the presence of two enzymes, namely PNP and TP. When using 5-Et-Ura at a concentration of about 0.2 mM, the reaction is usually completed within 1 h. In the presence of a large excess of inorganic phosphate over heterocyclic base (≥5 equiv.), the yield of the target nucleoside decreased. This reflects the reversible feature of 5-Et-Urd production. On the other hand, the phosphate concentration should be sufficient for the transglycosylation rate to overcome the hydrolysis rate of **1** or dRib-1-p.

According to the HPLC data, 5-Et-dUrd can be obtained in high yield (84-93%) by using a small excess (1.5 and 2.0 equiv.) of **1** over 5-Et-Ura in the presence of 0.5 equiv. of inorganic phosphate (Table 1).

The use of a large excess of **1** results in a quantitative yield of the product (Table 1, entry 1). However, a large excess of **1** complicates the purification of the product and is not cost-effective. Therefore, a small excess of **1** (1.5 equiv.) was chosen for the further development of the translycosylation protocol. Thymidine (Thd) is a less effective precursor of dRib-1-p compared to **1** (Table 1, entries 2,4). A low yield of transglycosylation when using Thd as a glycosyl donor is apparently associated with

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reversibility of its phosphorolysis reaction (Step 1 in Scheme 1).
The conduction of enzymatic transglycosylation using a small excess of **1** (Table 1, entries 6 and 7) makes the chromatographic purification of the target products easier and reduces their cost.
These conditions were extended to the preparative synthesis of several purine and pyrimidine nucleosides on a larger scale (Table 2).

Table 1. Analytical reactions resulting in the 5-Et-dUrd formation from 5-Et-Ura, **1** (or Thd) and potassium dihydrophosphate at 20°C at different reagent ratios for 1 h [a)]

Entry	Glycosyl donor	Yield, %	Reagent ratio 1 (Thd):5-Et-Ura:Pi
1	**1**	100	5:1:0.5
2	**Thd**	80	5:1:0.5
3	**1**	95	3:1:0.5
4	**Thd**	56	1.5:1:0.5
5	**1**	78	2:1:1
6	**1**	93	2:1:0.5
7	**1**	84	1.5:1:0.5
8	**1**	65	1:1:0.5

a) The reactions were performed in 1 mL volume at 0.2 μM concentration of 5-Et-Ura and 20°C for 1 h until the equilibrium of the transglycosylation reaction was established; HPLC analysis was performed on the reversed-phase sorbent Luna® C_{18}.

The enzymatic reactions were controlled using the reversed-phase HPLC sorbent Dr. Maisch 5μm Reprosil-Pur C_{18}-AQ 120 Å (Fig.2). In the preparative reactions, the equilibrium was established within 20 h at room temperature.
The only one peak of 5-ethyluracil was observed in a chromatogram before the addition of the enzyme (Fig. 2A, t_0). Compound **1** gave a very broad peak on a reversed-phase sorbent Luna® C_{18} column with RT ~ 4.00-5.50 that is difficult to integrate (Figure 18, Supporting Info). We could not detect **1** on the reversed-phase sorbent Dr. Maisch 5μm, Reprosil-Pur C_{18}-AQ 120Å (Figure 11, Supporting Info). Therefore, we performed reaction analysis using a pair of 5-ethyluracil and 5-ethyl-2′-deoxyuridine.
We have successfully employed the developed protocol for the preparation of 2'-deoxyuridine, 5-ethyl-2'-deoxyuridine, 2'-deoxyadenosine, and 2'-deoxyinosine on a semi-preparative scale using the optimized reagent ratio (1.5:1:0.5) in high yield. (Table 2). 7-Me-Gua is practically insoluble in water and precipitates from the reaction mixture. The formation of 7-methylguanine and 5-ethyl-2′-deoxyuridine was accompanied by a decrease in the intensity of the 5-ethyluracil signal as the equilibrium was established (Fig. 2B). We have not observed any formation of 5-isopropyluridine starting from 5-isopropyluracil (entry 3, Table 2) probably due to the steric interactions of the bulky substituent with the catalytic site of TP.
This protocol was tested on the synthesis of cladribine (Table 2, entry 5). Because of the low solubility of initial 2-chloro-6-aminopurine in Tris-HCl buffer, the reaction was performed in heterogeneous medium (see the Experimental Section) as opposed to the other transglycosylation reactions, which were performed in homogeneous solutions. According to this procedure, cladribine was isolated in 70% yield.

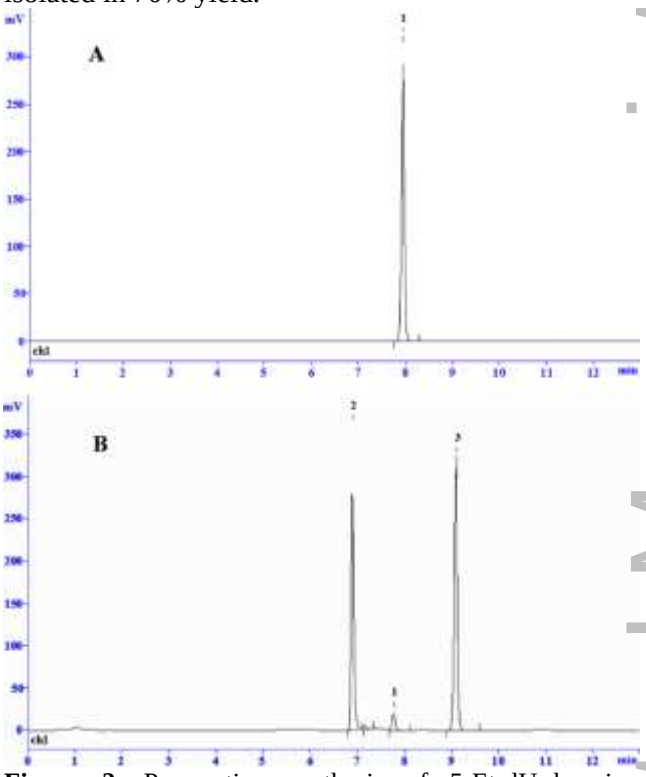


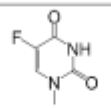
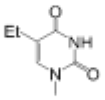
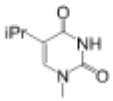
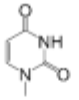
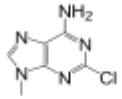
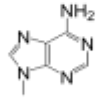
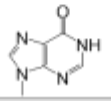
Figure 2. Preparative synthesis of 5-Et-dUrd using transglycosylation reaction. HPLC analysis of the reaction mixture was performed on the reversed-phase sorbent Dr. Maisch 5μm, Reprosil-Pur C_{18}-AQ 120Å (**A**) before the addition of PNP and TP (t_0); (**B**) after the establishment of the equilibrium (t_{eq} 24 h). **1**, 5-Et-Ura; **2**, 7-Me-Gua; **3**, 5-Et-dUrd in a linear gradient of acetonitrile in deionized water from 2 to 12% for 10 min at a flow rate of 1 ml/min with UV detection at a wavelength of 260 nm.

A variety of enzymatic methods for cladribine synthesis using 2-chloro-6-aminopurine and 2′-deoxynucleosides as a starting compounds has been described in the literature.[28-34] According to HPLC the analytical yields were determined in the range from 80 to 95%. At the same time the isolated yields of cladribine were substantially lower (35-88%). The best results were obtained when using 2-chloro-6-aminopurine and 2′-deoxyguanosine in the presence of intact cells of GA *E. coli* BMT 4D/1A strain (81%)

[31] and using dRib-1-p in the presence of *E. coli* PNP (88%). [35]

We prepared cladribine using a small excess of glycosyl-donor **1** in 70% yield within the range reported in the literature. From our point of view, the main problem of cladribine synthesis by transglycosylation is a low solubility of 2-chloro-6-aminopurine in aqueous media. [32] Analysis of patent literature has revealed that one of a possible improvements might be the dropwise addition of alkali solution of 2-chloro-6-aminopurine to the buffered reaction mixture. [36] Even in this case the yield does not exceed 80% and is comparable with the method described above (Table 2).

Table 2. Preparative synthesis of nucleosides from 7-methyl-2′-deoxyguanosine by transglycosylation[a)]

Entry	B	Nucleoside	Yield (HPLC)[b)]	Isolated yield
1		5-Fluoro-2'-deoxyuridine	95%	81%
2		5-Ethyl-2'-deoxyuridine	90%	81%
3		5-Isopropyl-2'-deoxyuridine	No reaction	-
4		2'- Deoxyuridine	90%	80%
5		2-Chloro-2'-deoxyadenosine	81%	70%
6		2'-Deoxyadenosine	100%	93%
7		2'-Deoxyinosine	100%	95%

[a)] Reactions were performed at 20°C for 20 h using 1.5:1:0.5 ratio of the initial substrates **1** : heterocyclic base : Pi until the equilibrium of transglycosylation was established. [b)] HPLC analysis was performed on the reversed-phase sorbent Dr. Maisch 5µm, Reprosil-Pur C_{18}-AQ 120Å.

The use of a small excess of **1** in preparative enzymatic transglycosylation reactions makes it possible to simplify the isolation and chromatographic purification of the target products and provides nearly quantitative yields in most cases. The structures of all products were confirmed by NMR and UV spectroscopy.

Conclusion

In conclusion, 7-methyl-2′-deoxyguanosine hydroiodide (**1**) was shown for the first time to be a source of 2′-deoxyribose in enzymatic synthesis of nucleosides. It was determined on the basis of analytical data, chromatographic purification and prime cost that the optimal ratio of the initial compounds 7-Me-dGuo : base : Pi is 1.5:1:0.5. This developed methodology allows the preparation of both purine and pyrimidine 2′-deoxyribonucleosides in high yield.

Experimental Section

The solvents and materials were of reagent grade and were used as received.

HPLC analysis was performed using an Akvilon (Russia) HPLC gradient system (2×Stayer pumps (2nd series), Stayer MS16 dynamic mixer, and Stayer 104M UV-Vis detector).

The stability of **1** was analyzed by HPLC on a 4.6×250 mm chromatographic column (sorbent Luna® NH_2, average particle diameter 5 µm, pore diameter 100 Å, catalogue number 00G-4378-E0, Phenomenex (USA)) equipped with a protective column of EC-standart (4×3.0 mm, average particle diameter 5 µm, sorbent Luna® NH_2, catalogue number AJ0-4302, Phenomenex (USA)), in a linear gradient of acetonitrile in deionized water from 2 to 12% for 10 min at a flow rate of 1 ml/min with UV detection at a wavelength of 280 nm.

HPLC analysis of transglycosylation reactions was performed on a 4×150 mm Dr. Maisch HPLC chromatographic column (5µm, Reprosil-Pur C_{18}-AQ 120 Å, Part No r15.aq.s1504, Dr. Maisch HPLC GmbH (Germany)) in a linear gradient of acetonitrile in deionized water from 2 to 12% (from 2 to 25% for cladribine) for 10 min (flushing with 12-80% acetonitrile/deionized water for 10-10.1 min, then 80-2% for 10.1-10.8 min for 5-ethyl-2′-deoxyuridine, 2′-deoxyadenosine) at a flow rate of 1 ml/min with UV detection at a wavelength of 260 nm.

The pH values were determined with a microprocessor-based pH (mV- C) bench meter 211 (Hanna Instruments, Germany) equipped with an HI1131B double junction combination pH electrode and a HI7662 stainless steel temperature sensor for pH compensation.

Preparative chromatography was performed using Kieselgel (0.040-0.063 mm, Merck).

TLC was carried out on Alugram SIL G/UV254 (Macherey-Nagel) with UV visualization (λ = 254 nm). UV spectra were recorded on a Cary 300 UV/VIS instrument (Varian, Australia).

^{1}H NMR spectra were recorded on a Bruker AMX 300 NMR instrument (Germany) at 300 MHz and 303 K. ^{13}C NMR spectra (with complete proton decoupling) were recorded on a Bruker AMX 400 NMR instrument (Germany) at 100 MHz and 303 K. Chemical shifts were measured in ppm relative to the residual solvent signals as internal standards (DMSO-d_6, ^{1}H: 2.50 ppm, ^{13}C: 39.5

ppm; D_2O, ^1H: 4.79 ppm). Spin-spin coupling constants (J) are given in Hz.

2-Chloro-6-aminopurine (2-chloroadenine) was prepared by amination of 2,6-dichloropurine. [37] 5-Ethyluracil and 5-isopropyluracil were prepared according to the literature procedure. [38]

Enzymes

E. coli purine nucleoside phosphorylase (PNP, 295 U/ml, 32 mg/ml solution) and *E. coli* thymidine phosphorylase (1197 U/ml, 17.2 mg/ml solution) were purchased from Sigma–Aldrich (United States).

7-Methyl-2′-deoxyguanosine hydroiodide (1)

To a solution of 2′-deoxyguanosine monohydrate (5 g, 17.5 mmol) in dry DMF (100 mL) barium carbonate (6.9 g, 35 mmol) was added. Then iodomethane (10.9 mL, 175 mmol) was added dropwise to the resulting suspension with vigorous stirring. The reaction mixture was stirred for 5.5 h at r.t. and then allowed to stand for 30 min in an open flask. The reaction mixture was filtered off from barium carbonate through celite (25 mL). The celite was washed with DMF (50 mL). The transparent liquid filtrates were collected, diluted with trichloromethane (0.5 L), and allowed to stand at 0°C for 16 h. The precipitate was filtered, washed with ethanol (100 mL) and trichloromethane (100 mL), and dried on a vacuum pump at r.t. for 1 h. Yield 4.6 g (65%) as a white powder that is stable on storage at -20°C for 6 months. R_f 0.1 (dichloromethane – ethanol, 1:1, v/v).

^1H NMR (300 MHz, DMSO-d_6): 11.63 br s (1H, NH 7-Me-Gua), 9.27 s (1H, H8 7-Me-Gua), 7.15 br s (2H, NH_2 7-Me-Gua), 6.19 t (1H, $J_{1',2'a}$=$J_{1',2'b}$=6.0, H1′), 5.50-5.25 m (1H, OH), 5.25-4.75 m (1H, OH), 4.37 ddd (1H, $J_{3',2'a}$= 5.0, $J_{3',2'b}$= 4.8, $J_{3',4'}$= 3.9, H3′), 4.00 s (3H, CH_3), 3.92 td (1H, $J_{4',3'}$= 3.9, $J_{4',5'a}$= 4.2, $J_{4',5'b}$ = 4.2, H4′), 3.62 dd (1H, $J_{5'a,4'}$ = 4.2, $J_{5'a,5'b}$ = -12.0, H5′a), 3.57 dd (1H, $J_{5'b,5'a}$ = 4.2, $J_{5'b,5'a}$ = -12.0, H5′b), 2.55-2.45 m (1H, H2′a), 2.40 ddd (1H, $J_{2'b,2'a}$ = -13.4, $J_{2'b,1'}$ = 6.0, $J_{2'b,3'}$ = 4.8, H2′b). UV (H_2O): λ_{max} (ε) = 256 nm (11000); λ_{max} (ε) = 281 nm (7600); UV (50 mM Tris-HCl buffer, pH 7.5): λ_{max} (ε) = 256 nm (7100); λ_{max} (ε) = 281 nm (7600).

Study of stability of 1 in aqueous solution. pH 4.1

To study the stability of **1** at 20°C, 10.2 mg of **1** were dissolved in 25 mL of deionized water (Milli-Q®). The pH value of the resulting 1 mM solution of **1** was estimated to be 4.1. After 1 h, 1, 2, 3, 4, and 7 days, aliquots were taken from the solution, diluted 50 times for UV spectroscopy and 5 times for HPLC analysis with deionized water, and analyzed by UV spectroscopy and HPLC at 20°C. Compound **1** was hydrolyzed by 73% after two days and by 96% in a week at 20°C according to HPLC. The retention time (RT) of 7-methylguanine is 6.50 min; compound **1** - 3.10 min on chromatographic column 4.6×250 mm (Luna® NH_2, 5 μM, 100 Å) in a linear gradient of acetonitrile in deionized water from 2 to 12% for 10 min at a flow rate 1 ml/min with UV detection at wavelength 280 nm, injection volume was 20 μL.

Study of stability of 1 in Tris-HCl buffer. pH 7.5

To study the stability of **1** at 20°C and 7°C, a 1mM stock solution of **1** (40.9 mg) in 100 mL of 50 mM Tris-HCl buffer at pH 7.5 was used. After 1 h, 1, 2, 5, and 7 days aliquots were taken from the solution, diluted 50-fold with Tris-HCl buffer (pH 7.5), and analyzed by UV spectroscopy and HPLC at 20°C. The retention time (RT) of 7-methylguanine is 6.50 min; compound **1** - 3.10 min on chromatographic column 4.6×250 mm (Luna® NH_2, 5 μM, 100 Å) in a linear gradient of acetonitrile in deionized water from 2 to 12% for 10 min at a flow rate 1 ml/min with UV detection at wavelength 280 nm, injection volume 20 μL.

5-Fluoro-2′-deoxyuridine

5-Fluorouracil (227 mg, 1.75 mmol) was dissolved in 100 mL of 50 mM Tris-HCl buffer (pH 7.5) on heating and cooled to r.t. Then potassium dihydrophosphate (119 mg, 0.875 mmol) and **1** (1.074 g, 2.62 mmol) were added to the solution, and the resulting mixture was diluted with 50 mM Tris-HCl buffer (pH 7.5) to a volume of 350 mL. Then solutions containing enzymes *E. coli* PNP (10 μl of 32 mg/ml solution of *E. coli* PNP (2.95 U)) and *E. coli* TP (10 μl of 17.2 mg/ml solution of *E. coli* TP diluted 10-fold (1.2 U)) were added to the mixture using a dispenser and the resulting mixture was carefully stirred for 5 min and allowed to stand overnight at r.t. without stirring. The reaction mixture was filtered through a Phenomenex membrane (0.2 μ, 47 mm) and concentrated *in vacuo* to the volume of *ca*. 50 mL. The suspension was repeatedly filtered off from the precipitate of 7-methylguanine through a Phenomenex membrane (0.2 μ, 47 mm) and concentrated *in vacuo* to near dryness. The residue was dissolved in ethanol (50 mL), and then silica gel (20 mL) was added to the solution. The resulting mixture was evaporated to dryness, co-evaporated with ethanol (2×50 mL), and the dry residue was applied on a chromatographic column (4×10 cm) packed with silica gel (150 mL) for purification. The column was washed with a mixture of dichloromethane and ethanol (95:5 v/v, 200 mL), the product was eluted with dichloromethane:ethanol

(90:10 v/v) and dichloromethane : ethanol (85:15 v/v). The fractions containing the product were collected and evaporated *in vacuo* to dryness. The resulting residue was repeatedly purified on a chromatographic column (2×10 cm) packed with silica gel (50 mL). The column was washed with a dichloromethane : ethanol mixture (95:5 v/v, 200 mL), the product was eluted with dichloromethane : ethanol (90:10 v/v).
The fractions containing the product were collected, evaporated *in vacuo*, co-evaporated 5 times with dichloromethane and dried on a vacuum pump at r.t. for 1 hour. Yield 349 mg (81%) as white crystals. Yield (according to HPLC analysis) 95%. R_f 0.23 (dichloromethane : ethanol, 9:1, v/v). ^{1}H NMR (300 MHz, D_2O): 8.09 d (1H, $^3J_{H,F}$=6.5, H6 5-F-Ura), 6.32 td (1H, $J_{1',2'a}$=$J_{1',2'b}$=6.6, $^5J_{H,F}$=6.6, H1′), 4.51 ddd (1H, $J_{3',2'a}$= 6.5, $J_{3',2'b}$= 4.2, $J_{3',4'}$= 4.8, H3′), 4.10 ddd (1H, $J_{4',3'}$= 4.8, $J_{4',5'a}$= 3.5, $J_{4',5'b}$ = 4.9, H4′), 3.92 dd (1H, $J_{5'a,4'}$ = 3.5, $J_{5'a,5'b}$ = -12.5, H5′a), 3.82 dd (1H, $J_{5'b,5'a}$ = 4.9, $J_{5'b,5'a}$ = -12.5, H5′b), 2.48 ddd (1H, $J_{2'b,2'a}$ = -14.2, $J_{2'b,1'}$ = 6.6, $J_{2'b,3'}$ = 4.2, H2′b), 2.38 ddd ($J_{2'a,2'b}$ = -14.2, $J_{2'a,1'}$ = 6.6, $J_{2'a,3'}$ = 6.5, H2′a). ^{13}C NMR (100 MHz, D_2O): 162.41 d ($^2J_{C,F}$=26, 4-C=O), 153.02 (5-C=O), 143.49 d ($^1J_{C,F}$=233, 5-C), 128.44 d ($^2J_{C,F}$=34, C6), 89.57 (C1′), 88.40 (C4′), 73.17 (C3′), 63.92 (C5′), 41.58 (C2′). UV (H_2O): pH 2-7: λ_{max} (ε) = 269 nm (8200); pH 13: λ_{max} (ε) = 269 nm (6300).

5-Ethyl-2′-deoxyuridine

Following the procedure for the preparation of 5-fluoro-2′-deoxyuridine, the reaction of 5-ethyluracil (245 mg, 1.75 mmol) with **1** (1.074 g, 2.62 mmol) in the presence of potassium dihydrophosphate (119 mg, 0.875 mmol), *E. coli* PNP and *E. coli* TP at r.t. gave 363 mg (81%) of 5-ethyl-2′-deoxyuridine as a white powder. Yield (according to HPLC) 90%. R_f 0.31 (dichloromethane : ethanol, 9:1, v/v).

^{1}H NMR (300 MHz, D_2O): 7.68 t (1H, ^4J = 1.1, H6 5-Et-Ura), 6.36 t (1H, $J_{1',2'a}$=$J_{1'2'b}$=6.7, H1′), 4.53 td (1H, $J_{3',2'a}$=$J_{3',2'b}$=5.5, $J_{3',4'}$=4.4 H3′), 4.09 ddd (1H, $J_{4',5'a}$= 3.5, $J_{4',5'b}$=4.7, $J_{4',3'}$=4.4, H4′), 3.91 dd (1H, $J_{5'a,5'b}$= -12.5, $J_{5'a,4'}$= 3.5, H5′a), 3.83 dd (1H, $J_{5'b,5'a}$= - 12.5, $J_{5'b,4'}$= 4.7, H5′b), 2.44 dd (2H, $J_{2'a,1'}$=$J_{2'b,1'}$=6.7, $J_{2'a,3'}$=$J_{2'b,3'}$=5.5, H2′), 2.37 qd (2H, ^3J = 7.5, ^4J = 1.1, CH_2), 1.14 t (3H, ^3J = 7.5, CH_3). ^{13}C NMR (100 MHz, D_2O): 168.90 (C=O), 154.37 (C=O), 139.53 (C6), 119.84 (C5), 89.30 (C1′), 87.97 (C4′), 73.15 (C3′), 63.80 (C5′), 41.42 (C2′), 22.28 (CH_2), 14.68 (CH_3). UV (H_2O): pH 2-7: λ_{max} (ε) = 266 nm (9800); pH 13: λ_{max} (ε) = 266 nm (7400).

2′-Deoxyuridine

Following the procedure for the preparation of 5-fluoro-2′-deoxyuridine, the reaction of uracil (196 mg, 1.75 mmol) with **1** (1.074 g, 2.62 mmol) in the presence of potassium dihydrophosphate (119 mg, 0.875 mmol), *E. coli* PNP and *E. coli* TP at r.t. gave 319 mg (80%) of 2′-deoxyuridine as a white powder. Yield (according to HPLC) 90%. NMR and UV spectra are identical to those of the commercially available 2′-deoxyuridine.

2-Chloro-2′-deoxyadenosine (cladribine)

2-Chloro-6-aminopurine (21 mg, 0.124 mmol) was suspended in 150 mL of 50 mM Tris-HCl buffer (pH 7.5) in an ultrasonic bath at r.t. Then potassium dihydrophosphate (8.5 mg, 0.062 mmol) and **1** (76 mg, 0.186 mmol) were added to the suspension. Then a solution containing *E. coli* PNP (10 µl of 32 mg/ml solution of *E. coli* PNP (2.95 U)) was added to the mixture and the resulting suspension was carefully stirred for 6 days at r.t. The reaction mixture was filtered through a Phenomenex membrane (0.2 µ, 47 mm) and concentrated *in vacuo* to the volume of *ca.* 10 mL. The suspension was repeatedly filtered from the precipitate of 7-methylguanine through a Phenomenex membrane (0.2 µ, 47 mm) and concentrated *in vacuo* to near dryness. The residue was dissolved in ethanol (10 mL), and then silica gel (1.5 mL) was added to the solution. The resulting mixture was evaporated to dryness, co-evaporated with ethanol (2×10 mL), and the dry residue was applied on a chromatographic column (1×10 cm) packed with silica gel (5 mL) for purification. The column was washed with a mixture of dichloromethane and ethanol (95:5 v/v, 100 mL), the product was eluted with dichloromethane : ethanol (90:10 v/v). The fractions containing the product were collected, evaporated *in vacuo*, co-evaporated 5 times with dichloromethane.
The fractions containing the product were collected, evaporated *in vacuo*, co-evaporated 5 times with dichloromethane and dried on a vacuum pump at r.t. for 1 hour to obtain 25 mg (70%) of cladribine as white crystals.
Yield (according to HPLC) 81%. ^{1}H NMR (300 MHz, D_2O): 8.27 s (1H, H8 – 2-Cl-Ade), 6.38 dd (1H, $J_{1',2'a}$=7.4, $J_{1',2'b}$= 6.6, H1′), 4.68 ddd (1H, $J_{3',2'a}$=6.2, $J_{3',2'b}$=3.4, $J_{3',4'}$=3.0, H3′), 4.22 ddd (1H, $J_{4',3'}$=3.0, $J_{4',5'a}$=3.3, $J_{4',5'b}$=4.3 H4′), 3.90 dd (1H, $J_{5'a,5'b}$= -12.6, $J_{5'a,4'}$=3.3, H5′a), 3.82 dd (1H, $J_{5'b,5'a}$= -12.6, $J_{5'b,4'}$=4.3, H5′b), 2.81 ddd (1H, $J_{2'a,2'b}$= -13.9, $J_{2'a,1'}$=7.4, $J_{2'a,3'}$=6.2, H2′a), 2.60 ddd (1H, $J_{2'b,2'a}$= -13.9, $J_{2'b,1'}$=6.6, $J_{2'b,3'}$=3.4, H2′b). UV (H_2O): pH 2: λ_{max} (ε) = 265 nm (13600); pH 7-13: λ_{max} (ε) = 265 nm (14100).

2′-Deoxyadenosine

Following the procedure for the preparation of 5-fluoro-2′-deoxyuridine, the reaction of adenine (236

mg, 1.75 mmol) with **1** (1.074 g, 2.62 mmol) in the presence of potassium dihydrophosphate (119 mg, 0.875 mmol) and PNP at r.t. gave 407 mg (93%) of 2′-deoxyadenosine as a white powder. Yield (according to HPLC) 100%. NMR and UV spectra are identical to those of the natural compound.

2′-Deoxyinosine

Following the procedure for the preparation of 5-fluoro-2′-deoxyuridine, the reaction of hypoxanthine (238 mg, 1.75 mmol) with **1** (1.074 g, 2.62 mmol) in the presence of potassium dihydrophosphate (119 mg, 0.875 mmol) and PNP at r.t. gave 419 mg (95%) of 2′-deoxyinosine as a white powder. Yield (according to HPLC) 100%. NMR and UV spectra are identical to those of the natural compound.

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FULL PAPER

Use of nucleoside phosphorylases for the preparation of purine and pyrimidine 2′-deoxynucleosides

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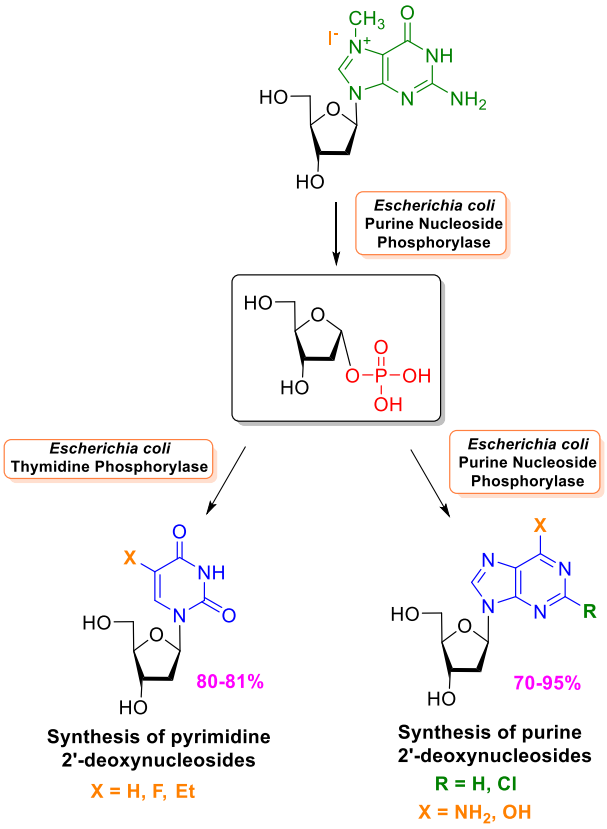

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