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7-Deazapurine 2′-deoxyribofuranosides are noncleavable competitive inhibitors of *Escherichia coli* purine nucleoside phosphorylase (PNP)[°]

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A series of 7-deazapurine 2′-deoxyribofuranosides were synthesized according to already known procedures and their substrate and inhibitor properties with purified *E. coli* purine nucleoside phosphorylase were examined. In agreement with previous findings, substrate activity was not detected for any of the compounds tested. Most of the nucleosides showed weak inhibition in the preliminary screening, i.e. at a concentration of about 100 μM. However some combinations of 6-chloro, 6-amino or 6-methoxy substituents with bulky hydrophobic groups at position 7 of the base and/or chloro, amino, methoxy or methylthio group at position 2 markedly enhanced affinity of such modified nucleosides for the *E. coli* enzyme. The most potent inhibition was observed for two nucleosides: 6-chloro- and 2-amino-6-chloro-7-deazapurine 2′-deoxyribofuranosides that show inhibition constants K_i = 2.4 and 2.3 μM, respectively. Several other compounds were also found to be good inhibitors, with inhibition constants in the range 5–50 μM. In all instances the inhibition was competitive *vs.* the nucleoside substrate 7-methylguanosine. Inhibition constants for 7-deazapurine nucleosides are in general several-fold lower than those observed for their purine counterparts. Therefore 7-deaza modification together with substitutions at positions 2, 6 and 7 of

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Abbreviations: PNP, purine nucleoside phosphorylase; Ino, inosine; 3-isoAdo, 3-(β-D-ribofuranosyl)adenine; m^7Guo, 7-methylguanosine; 2CldAdo (cladribine, leustatin), 2-chloro-2′-deoxyadenosine; dPu, 9-(2′-deoxy-β-D-ribofuranosyl)purine; 7-deaza-2CldAdo, 4-amino-2-chloro-7-(2′-deoxy-β-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine; 7-deaza-6CldPu, 4-chloro-7-(2′-deoxy-β-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine, with similar connotations for other analogues.

the base is a very promising approach to obtain competitive noncleavable inhibitors of *E. coli* PNP that may bind to the enzyme with inhibition constants in the μM range.

The ubiquitous enzyme purine nucleoside phosphorylase (PNP, purine nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) catalyzes the reversible phosphorolysis of purine nucleosides, such as inosine and guanosine in eukaryotes, and additionally of adenosine in some (e.g. *E. coli* and *Salmonella typhimurium*), but not all, prokaryotes, as follows:

$$\text{Purine nucleoside} + P_i \leftrightarrow \text{Purine} + \text{(Deoxy)Ribose-1-phosphate}$$

Phosphorylase from enteric bacteria (*E. coli*) is responsible for degradation (phosphorolysis) of the drug 2-chloro-2′-deoxyadenosine (2CldAdo, cladribine, leustatin) that has been used for treatment of hairy cell leukemia and other hematological malignancies [1, 2], as well as autoimmunoaggressive diseases such as multiple sclerosis [3]. Because of the phosphorolysis and also acid hydrolysis [4–6] of cladribine in the human digestive system the oral administration of the drug is not as effective as the intravenous one.

In our previous paper we have shown that 2CldAdo is a relatively good substrate of *E. coli* purine nucleoside phosphorylase with a low Michaelis constant, K_m = 5.3 μM, and maximal velocity V_{max} = 15% of that for the natural substrate inosine [6]. Several base-modified derivatives of cladribine were also studied and from their enzymatic properties some conclusions were drawn. We have suggested that it would be very desirable to search for some modifications of cladribine that would make the drug resistant against glycosidic bond cleavage by both acid and *E. coli* PNP. Another approach could be based on the simultaneous administration of the drug and a potent *E. coli* PNP inhibitor to prevent degradation of cladribine by enteric bacteria in the patient's digestive system. Two groups of nucleosides could be considered as prospective *E. coli* PNP inhibitors, i.e. 9-deazapurine and 7-deazapurine derivatives.

It is known that some 9-deazapurine nucleosides bind better in the active site of mammalian phosphorylases than their purine counterparts. For example, 9-deazainosine shows K_i of 2 μM *vs.* human PNP [7], more than an order of magnitude lower than K_m for inosine, 30–46 μM [8]. For *E. coli* PNP there is no experimental data regarding inhibition by 9-deazapurine nucleosides, but it is known that 8-aza-9-deazapurine nucleosides, i.e. formycin A and B, are potent inhibitors with K_i = 5.3 and 4.6 μM, respectively [9]. It should be noted that 9-deazapurine and 8-aza-9-deazapurine nucleosides have a C–C (not N–C) glycosidic bond.

For the second group considered, i.e. 7-deazapurine nucleosides, it has been shown that 7-deaza modification of the natural substrates inosine and adenosine gives analogues noncleavable by *E. coli* PNP and with approximately 2-fold higher affinity for the enzyme [10]. Additionally, in contrast to purine nucleosides, 7-deazapurine nucleosides are highly stable against acid hydrolysis [11]. The mechanisms of acid hydrolysis and probably also of the PNP-catalyzed enzymatic phosphorolysis [12–14] require protonation of the ring N(7), which is not possible in the case of 7-deaza nucleosides. These features, i.e. resistance to both acid hydrolysis and enzymatic phosphorolysis of the N-glycosidic bond, and potential good affinity to the enzyme, make 7-deazapurine nucleosides a prospective class of *E. coli* PNP inhibitors.

7-Deazapurine nucleosides are of a considerable interest from the biological point of view. The adenosine analogue tubercidin exhibits a profound cytotoxic effect on mammalian cell lines *in vitro* and shows a significant antitumor activity *in vivo* [15–21]. It also affects multiplication of DNA and RNA viruses [22]

and inhibits the growth of a variety of microorganisms [23].

In this paper we describe substrate and inhibitor properties of a series of 7-deazapurine 2'-deoxyribofuranosides *vs. E. coli* PNP. The results may help to find inhibitors that may serve as biochemical modifiers in chemotherapy with cladribine by minimizing its cleavage and inactivation in the human digestive system. These studies could also give some more details about the active center of the *E. coli* enzyme and make it possible to compare the kinetic data with structural information from X-ray crystallography [24, 25].

MATERIALS AND METHODS

Materials

Inosine, 7-metylguanosine and xanthine oxidase from butter milk (1 U/mg) were from Sigma (St. Louis, MO., U.S.A.). *E. coli* PNP (60% pure, 56 U/mg) was a kind gift of Dr. G. Koszalka (Wellcome Research Laboratories, Research Triangle Park, NC, U.S.A.) and was further purified as described below. 3-isoAdo was prepared using the method of Leonard & Laursen [26] and was kindly supplied by Dr. N.E. Poopeiko (Institute of Bioorganic Chemistry Byelorussian Academy of Sciences, Minsk, Byelorussia). Epoxy-activated Sepharose 6B was from Pharmacia (Uppsala, Sweden).

7-Deazapurine 2'-deoxyribofuranosides were synthesized as previously described [27–34]. Concentrations of substrates and inhibitors were determined spectrophotometrically. Extinction coefficients are listed in Table 1 and were compiled from literature [27–36] or determined in the present study.

Ultraviolet spectroscopy was performed with a Kontron (Vienna, Austria) Uvikon 940 UV-VIS spectrophotometer fitted with thermostatically controlled cell compartments and using 5- or 10-mm path length cuvettes. Thin-layer chromatography was performed on silica gel plates with the solvent consisting of 9:1 (v/v) chloroform/methanol.

Purfication of *E. coli* PNP

All operations were carried out at 4°C. The enzyme (60% pure, specific activity 56 U/mg) was purified on Sepharose 6B containing covalently bound formycin B. Formycin B was linked to the epoxy activated Sepharose 6B as described by Schrader *et al.* [37] for linkage of adenosine. The binding capacity of 50 ml of formycin B-Sepharose slurry (obtained from 15 g of dry epoxy-activated Sepharose 6B) was at least 80 mg *E. coli* PNP. After each use the affinity column was washed with 300–400 ml of 100 mM Tris/HCl buffer, pH 8.5, + 0.5 M NaCl + 1 mM β-mercaptoethanol, then with 300–400 ml of 100 mM Na-acetic buffer, pH 4.5 + 0.5 M NaCl + 1 mM β-mercaptoethanol and finally with 300–400 ml of 50 mM Tris/HCl buffer, pH 7.6, + 1 mM β-mercaptoethanol (buffer A).

The application of affinity chromatography, in which the affinity ligand was formycin B, to the purification of *E. coli* PNP has already been reported by Lehikoinen *et al.* [38] but no details of the procedure were given.

In the present study, the enzyme (40 mg in about 0.35 ml) was diluted to 5 ml with buffer A and washed with the same buffer 3–5 times on two 30 kDa cut-off Centricons ultrafiltration devices (Amicon) until the absorbance at 260 nm of the effluent was lower than 0.05. After the final wash the enzyme was diluted to 10 ml (buffer A) and pumped through a column of the affinity gel (2 cm × 16 cm) at 10 ml/h. The column was washed with 200 ml of buffer A (at 10 ml/h) and then with 150 ml of buffer B (100 mM Tris/HCl, pH 8.0 + 0.5 M NaCl + 1 mM β-mercaptoethanol). After this wash the bound enzyme was eluted (at 6–8 ml/h) from the column with buffer C (50 mM Na-phosphate buffer, pH 7.6, + 4 mM Ino + 1 mM β-mercaptoethanol). Fractions of 3–4 ml were collected every 30 min.

The fractions showing enzyme activity were pooled, washed several times on two 30 kDa cut-off Amicon ultrafiltration devices with buffer D (10 mM Na-citric buffer, pH 6.9, + 1 mM β-mercaptoethanol) until the absorbance of the effluent was lower than 0.01 at 249 nm (absorbance of the substrate – inosine and the product – hypoxanthine). In the final wash the enzyme was concentrated to give the protein at about 100 mg/ml.

The purity of the phosphorylase was tested by denaturating polyacrylamide gel electrophoresis. After this step of purification the enzyme was > 98% pure and could be crystallized according to an already known procedure [25, 39]. The specific activity of the purified enzyme was 104 U/mg. One unit of phosphorylase is the amount of enzyme that will cause phosphorolysis of one μmole of inosine to hypoxanthine and ribose-1-phosphate per minute at 25°C in the presence of 0.5 mM inosine and 50 mM Na-phosphate buffer, pH 7.0. The specific activity of PNP was measured spectrophotometrically by coupling with the xanthine oxidase reaction [40, 41].

Protein concentration

The PNP concentration was measured spectrophotometrically in buffer A. The extinction coefficient at 278 nm (maximum of absorbance) was determined using the Lowry method with human serum albumin as standard [42], enhanced alkaline copper method with bovine serum albumin as a standard [43], and by absorbance at 205 nm [43]. The results are $\varepsilon_{278}^{1\%} = 2.4$ (Lowry method), $\varepsilon_{278}^{1\%} = 2.6$ (enhanced copper), and $\varepsilon_{278}^{1\%} = 3.2$ (absorbance at 205). The mean value ($\varepsilon_{278}^{1\%} = 2.7$) was used for protein determination.

The value obtained experimentally is close to the theoretical one ($\varepsilon_{278}^{1\%} = 3.4$) calculated for 6 tyrosine chromophores per subunit of PNP [44], subunit molecular mass of 23.7 kDa [45] and the extinction coefficient for unionized tyrosine residue $\varepsilon = 1340\ M^{-1}cm^{-1}$ [46].

Inhibitor properties

In preliminary experiments several compounds were tested for inhibitory activity at a concentration of at least 100 μM *vs. E. coli* enzyme with inosine as substrate. The inhibition was measured at 25°C, in 50 mM phosphate buffer, pH 7.0, and inosine concentration was 90 μM. The phosphorolysis was monitored spectrophotometrically by coupling with the xanthine oxidase reaction [40, 41]. Unfortunately, it turned out that many of the compounds tested are inhibitors of xanthine oxidase, and in those cases inhibition constants could not be determined spectrophotometrically with Ino as substrate by the coupled assay. Because of that the screening for all 7-deazapurine nucleosides was then performed with 7-methylguanosine as substrate using a direct spectrophotometric assay (λ_{obs} = 260 nm) [35, 36].

The initial velocity (v_o and v_i) of the phosphorolysis was calculated by linear regression analysis of plots of absorbance (λ_{obs} = 260 nm) *vs.* time, where v_o is the initial velocity of phosphorolysis with no inhibitor added and v_i is the initial velocity in the presence of an analogue. The inhibition constants were then determined only for analogues showing in the preliminary experiments, at a concentration of about 100 μM, at least 50% reduction of the initial velocity, i.e. $v_i/v_o < 0.5$.

The inhibition constants were determined with m^7Guo as a substrate for at least three different concentrations of the inhibitor, with the aid of the initial velocity method, using the kinetic parameters for m^7Guo as standards. The K_m^{app} and V_{max}^{app} for phosphorolysis in the presence of an inhibitor were determined by linear regression analysis from Eadie-Hofstee plots of v_o *vs.* v_o/c_o, where c_o is initial substrate concentration. The inhibition constants were calculated from the equations 1 and 2 [47]:

$$K_i = [I](K_m^{app}/K_m - 1)^{-1} \quad (1)$$

$$K_i = [I][(V_{max}/K_m)/(V_{max}^{app}/K_m^{app}) - 1]^{-1} \quad (2)$$

where K_m (32 μM) and V_{max} (100%) are the Michaelis constant and maximal velocity for m^7Guo phosphorolysis catalyzed by the *E. coli* PNP, and [I] is the inhibitor concentration. In all instances both equations led to similar values of inhibition constants, indicating that the inhibition may be described as competitive.

Additionally, the type of inhibition was analyzed by the Dixon (i.e. $1/v_i$ *vs.* [I]), Webb (i.e. $v_o/(v_o - v_i)$ *vs.* 1/[I]) [48], and c_o/v_o *vs.* [I] plots [49].

With 3-isoAdo and Ino as substrates the inhibition constants were determined by the initial velocity method using equation (2).

Substrate properties

The substrate properties of 7-deazapurine 2′-deoxyribofuranosides with *E. coli* PNP were checked by TLC and spectrophotometrically. The experiments were carried out at 25°C. For TLC experiments, the reaction mixture contained 50 mM phosphate buffer, pH 7.0, 1 mM nucleoside and 1.5 μg of *E. coli* PNP in a total volume of 40 μl. Samples (1 ml) were taken at time 0, 4 h and 24 h and applied onto silica gel plates. As a control 2′-deoxyadenosine was used.

For spectrophotometric experiments, the reaction mixture contained 50 mM phosphate buffer, pH 7.0, 0.1 mM nucleoside and 6 μg of *E. coli* PNP in a total volume of 1 ml. UV spectra were recorded at 5 min intervals.

RESULTS AND DISCUSSION

The structure of cladribine (2CldAdo), its 7-deaza-counterpart (7-deaza-2CldAdo) and the natural PNP substrate adenosine (Ado), are presented in Fig. 1 together with their systematic ring numbering. For convenience, in this paper we will be using purine numbering for both purine and 7-deazapurine 2′-deoxy-ribosides. The structures of nucleosides investigated in this study are shown in Fig. 2.

The experimentally determined inhibition properties of 7-deazapurine nucleosides with *E. coli* PNP are presented in Table 1. The inhibition constants were determined with m^7Guo as a substrate (see Materials and Methods) [35, 36]. The method has already been tested for other PNP inhibitors [35, 36,

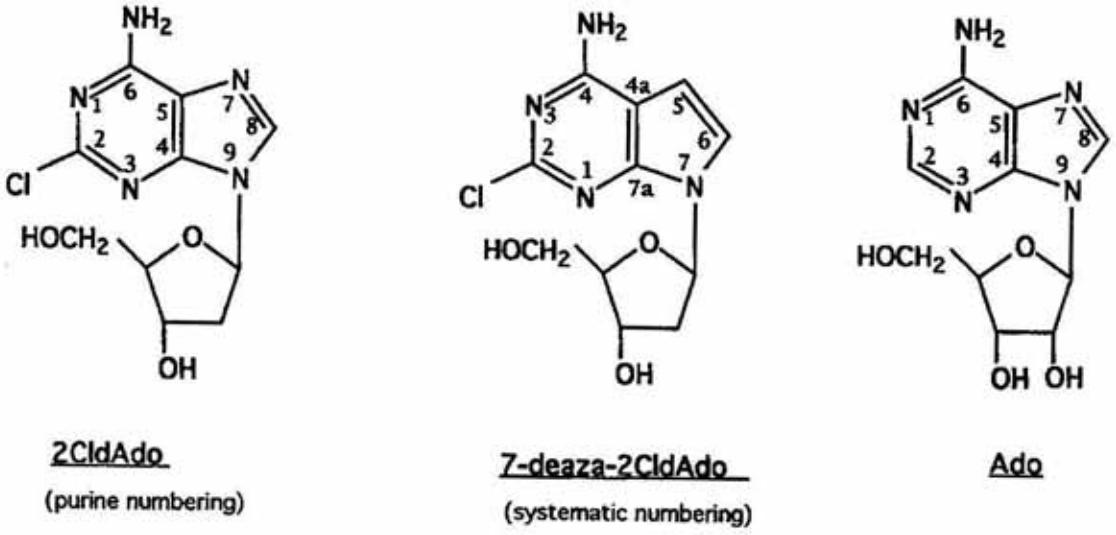
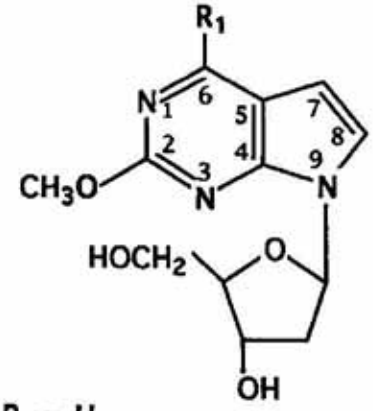
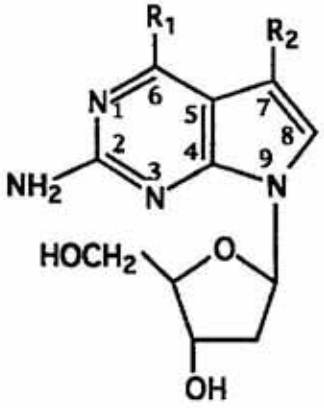


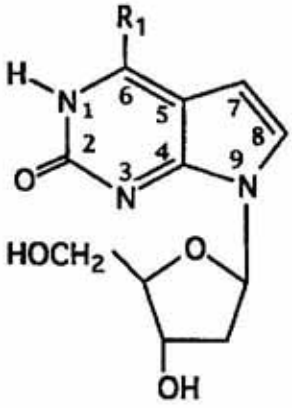
Figure 1. Structure of 2CldAdo – a good unusual substrate of *E. coli* purine nucleoside phosphorylase, 7-deaza-2CldAdo (Fig. 2, 6c) – a good inhibitor, and Ado – a typical substrate.



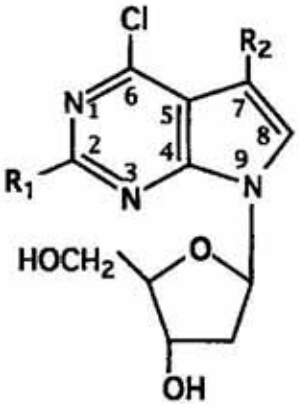
1a R_1 = H
1b R_1 = OCH_3
1c R_1 = NH_2



2a R_1 = OCH_3 R_2 = H
2b R_1 = OCH_3 R_2 = CH_3
2c R_1 = OCH_3 R_2 = I
2d R_1 = H R_2 = C≡CH



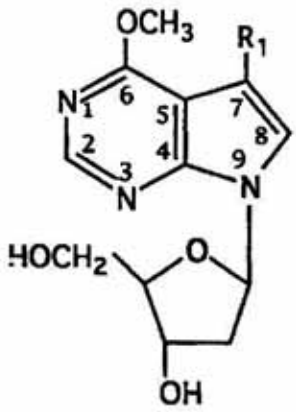
3a R_1 = H
3b R_1 = NH_2



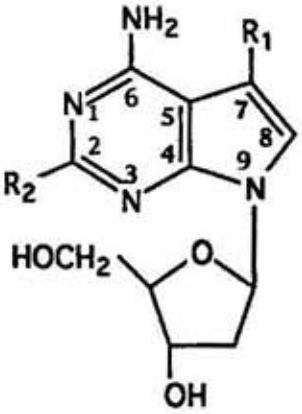
4a R_1 = H R_2 = H
4b R_1 = SCH_3 R_2 = H
4c R_1 = H R_2 = CH_3
4d R_1 = NH_2 R_2 = H

Figure 2. Legend to the figure on the next page

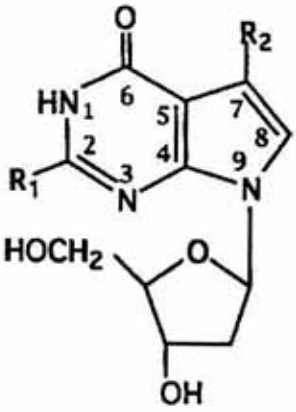
50]. To test the applicability of the method for 7-deazapurine nucleosides, the inhibition constants were checked for one of them, 2,6-dimethoxy-7-deazapurine nucleoside (1b), which does not affect xanthine oxidase activity even at a very high concentration (> 500 μM). The inhibition constants were determined with both Ino and m^7Guo as substrates and shown to be similar (see Table 2). For 2,6-dimethoxy- and 6-amino-2-chloro-7-deazapurine 2′-deoxyribosides (1b and 6c) the inhibition constants were also determined with 3-isoAdo as a substrate [51] and shown to be similar to those obtained for phosphorolysis



5a R_1 = H
5b R_1 = Cl



6a R_1 = H R_2 = H
6b R_1 = I R_2 = H
6c R_1 = H R_2 = Cl
6d R_1 = H R_2 = SCH_3
6e R_1 = $C{\equiv}CC_6H_5$ R_2 = H
6f R_1 = $C{\equiv}CC_6H_{11}$ R_2 = H
6g R_1 = $C{\equiv}CC_6H_4CH_3$ R_2 = H



7a R_1 = H R_2 = H
7b R_1 = OCH_3 R_2 = H
7c R_1 = NH_2 R_2 = H
7d R_1 = NH_2 R_2 = I

Figure 2. Structures of 7-deazapurine nucleosides used in this study.

The numbering of 7-deazapurine, i.e. pyrrolo[2,3-d]pyrimidine, ring atoms is given as for the purine ring to facilitate comparison of 7-deazapurine inhibitors with typical *E. coli* PNP purine nucleoside substrates and inhibitors. See Fig. 1 for a comparison of purine ring numbering and systematic numbering of pyrrolo[2,3-d]pyrimidine ring atoms.

of m^7Guo (see Table 2). Thus it may be concluded that the inhibition constants determined with the m^7Guo are similar to those obtained with other substrates of *E. coli* PNP.

Table 1. Inhibition of *E. coli* PNP by 7-deazapurine nucleosides.

Inhibition constants (K_i) for good inhibitors were determined as described in Methods, at pH 7.0 and 25°C, in 50 mM phosphate buffer, with m^7Guo as substrate, for three or more inhibitor concentrations. For compounds showing weak ($v_i/v_o < 0.5$) or no inhibition in the preliminary screening at about 100 μM, inhibition constants were not determined. Extinction coefficients are for water solutions (MeOH solutions for compounds 1a, 4c and 6d) and were compiled from literature (see Methods) or determined in the present study.

Substituents of 7-deazapurine 2'-deoxyribofuranoside		λ_{max} (nm)	ε_{max} ($M^{-1}cm^{-1}$)	K_i ±S.D. (μM)
1a	2-methoxy	224 290	30700 5900	no inhibition
1b	2,6-dimethoxy	257 272	7300 7400	38 ± 10
1c	6-amino-2-methoxy	261 271	9600 9600	weak inhibition
2a	2-amino-6-methoxy	285	7400	9 ± 3
2b	2-amino-6-methoxy-7-methyl	264	9800	46 ± 5
2c	2-amino-7-iodo-6-methoxy	266 288	6600 6050	weak inhibition
2d	2-amino-7-ethinyl	279	10100	weak inhibition
3a	2-oxo	260 330	3100 2900	weak inhibition
3b	6-amino-2-oxo	255 305	7600 7200	weak inhibition
4a	6-chloro	273	4700	2.4 ± 0.8
4b	6-chloro-2-methylthio	276 310	6300 6500	7 ± 2
4c	6-chloro-7-methyl	230 272sh* 297sh	10300 1300 2850	weak inhibition
4d	2-amino-6-chloro	258 316	4300 5800	2.3 ± 0.4
5a	6-methoxy	263	7700	47 ± 12
5b	7-chloro-6-methoxy	266sh	9200	45 ± 10
6a	6-amino (2'-deoxytubercidin)	270	11900	weak inhibition
6b	6-amino-7-iodo	283	5800	weak inhibition
6c	6-amino-2-chloro (7-deaza-2CldAdo)	274	12500	8 ± 2
6d	6-amino-2-methylthio	281	15000	weak inhibition
6e	6-amino-7-phenylethinyl	257 296	13800 24300	5 ± 1
6f	6-amino-7-cyclohexylethinyl	280	11200	11 ± 2
6g	6-amino-7-p-methylphenylethinyl	295	19600	19 ± 5
7a	6-oxo	260	9300	no inhibition
7b	2-methoxy-6-oxo	253	9800	weak inhibition
7c	2-amino-6-oxo	259	9800	weak inhibition
7d	2-amino-7-iodo-6-oxo	267	11200	weak inhibition

*sh, shoulder.

All 7-deazapurine 2'-deoxyribofuranosides tested in this study may be in general divided into three groups according to their affinity to the enzyme. The first group of compounds includes nucleosides showing either weak or no inhibition in the preliminary screening, i.e. at a concentration of about 100 μM. For such compounds the inhibition constants were not determined (see Table 1). Fourteen analogues from 26 tested belong to this group indicating that regardless of the broad specificity of *E. coli* PNP [10, 36], some special substitutions at the heterocyclic nucleus are necessary to make the nucleoside even moderately inhibitory for the enzyme.

The second group includes compounds showing moderate inhibition properties (K_i/K_m in the range 0.3–1.5, where K_m = 32 μM is that for m^7Guo), i.e. with inhibition constants in the range 10–50 μM. Six 7-deazapurine 2'-deoxyribofuranosides modified on the heterocyclic nucleus belong to this group: 6-amino-7-cyclohexylethinyl (6f), 6-amino-7-*p*-methylphenylethinyl (6g), 2,6-dimethoxy (1b), 2-amino-6-methoxy-7-methyl (2b), 6-methoxy (5a) and 7-chloro-6-methoxy (5b). Inhibition constants are 11 μM and 19 μM for compounds 6f and 6g, respectively. This shows that bulky hydrophobic substituent, such as cyclohexylethinyl or methylphenylethinyl placed at position 7 of the base, may notably enhance the affinity for the enzyme of modified 7-deazapurine nucleosides. The 6-amino derivative (6a) that lacks such substituent at position 7 belongs to the first group, i.e. it has only a small effect on the enzyme activity at 100 μM concentration (see Table 1). Four other compounds from the second group are slightly less potent inhibitors; the inhibition constants are 38 μM and 47 μM for 2,6-dimethoxy- (1b) and 6-methoxy-7-deazapurine nucleosides (5a), and 45 μM and 46 μM for 7-chloro-6-methoxy- (5b) and 2-amino-6-methoxy-7-methyl-7-deazapurine nucleosides (2b). It should be concluded that all compounds belonging to the second group have either an amino or a methoxy substituent at position 6 of the base, but additional substituents at ring positions 2 or 7 are in general necessary to enhance the affinity of the analogue to the *E. coli* PNP.

The third group of compounds includes the best inhibitors of the *E. coli* enzyme that show inhibition constants in the range of several μM, i.e. lower than Michaelis constants observed for three natural substrates of *E. coli* PNP: Ado, Guo and Ino (K_m = 12 μM, 20 μM and 47 μM, respectively) [36, 51]. The most potent inhibition is observed for 6-chloro- (7-deaza-6CldPu, 4a) and 2-amino-6-chloro (4d) analogues, K_i = 2.4 ± 0.8 μM and 2.3 ± 0.4 μM, respectively. These values are even lower than those for formycins A and B, the selective non-cleavable inhibitors of *E. coli* PNP, that have inhibition constants K_i = 5.3 μM and 4.6 μM, respectively [9].

Table 2. Comparison of inhibition constants, K_i obtained with Ino, m^7Guo and 3-isoAdo as substrates.

Inhibition constants were determined as described in Methods, at pH 7.0 and 25°C, in 50 mM phosphate buffer.

		K_i ±S.D. (μM)	
	Analogue	1b	6c
Substrate	Substituents	2,6-dimethoxy	6-amino-2-chloro
m^7Guo		38 ± 10	8 ± 2
3-isoAdo		42 ± 13	12 ± 4
Ino		36 ± 4	a

a, Not determined due to inhibition of xanthine oxidase (see Methods).

Four other compounds also belong to the third group; 6-amino-7-phenylethinyl- (6e), 6-chloro-2-metylthio- (4b), 6-amino-2-chloro- (7-deaza-2CldAdo, 6c) and 2-amino-6-methoxy-7-deazapurine nucleosides (2a), and show inhibition constants 5, 7, 8 and 9 μM, respectively (see Table 1). It should be noted that in addition to the 6-amino and 6-methoxy substituents that were present in compounds belonging to the second group, the 6-chloro substitution also markedly enhances the affinity of 7-deazapurine 2'-deoxyribosides for the *E. coli* enzyme.

Nucleosides from the last two groups are competitive inhibitors of *E. coli* PNP *vs.* m^7Guo. Data for compounds 6f (7-deaza-7-cyclohexylethinyl-dAdo) and 4a (7-deaza-6CldPu) are displayed in Figs. 3 A and B, respectively, in the form of plots of c_o/v_o *vs.* inhibitor concentration [I]. The parallel lines observed for various substrate concentrations are indicative of competitive inhibition [49]. Similar plots were recorded for other analogues for which inhibition constants were determined (not shown).

None of the 7-deazapurine nucleosides tested was a substrate for *E. coli* PNP. The lack of phosphorolysis was demonstrated by both TLC and spectrophotometric methods (see Materials and Methods). It was already noted by Doskočil & Holý [10] that the replacement of the N^7atom of natural PNP substrates, Ado and Ino, by a methine group in tubercidin (7-deazaadenosine) and 7-deazainosine resulted in the inhibitory nonsubstrates. The inhibition constants for such modified analogues were 3-fold and 1.5-fold lower than those for natural substrates (120 μM and 350 μM for the pair tubercidin and Ado, and 250 μM and 350 μM for 7-deazaIno and Ino) [10]. The same tendency was in general observed in the present study. Data for the comparison is available for compounds 3b, 4a and 5a, although it should be noted that, in the case of N^7 nucleosides, not 2'-deoxyribo but a ribo form was studied [10]. The 6-chloro substituted nucleoside 7-deaza-6CldPu (4a) shows K_i of 2.4 μM and 17 μM [10] in 7-deaza and N^7 form, the 6-methoxy substituted one (5a), 47 μM and 150 μM [10] in 7-deaza and N^7 form, respectively, while for the very weak inhibitor, 6-amino-2-oxo-7-deazapurine nucleoside (3b), K_i > 2300 μM was reported for the N^7 form [10] and a weak inhibition (v_i/v_o > 0.5, see Methods) at 130 μM was observed in the present study. For 2CldAdo (cladribine)

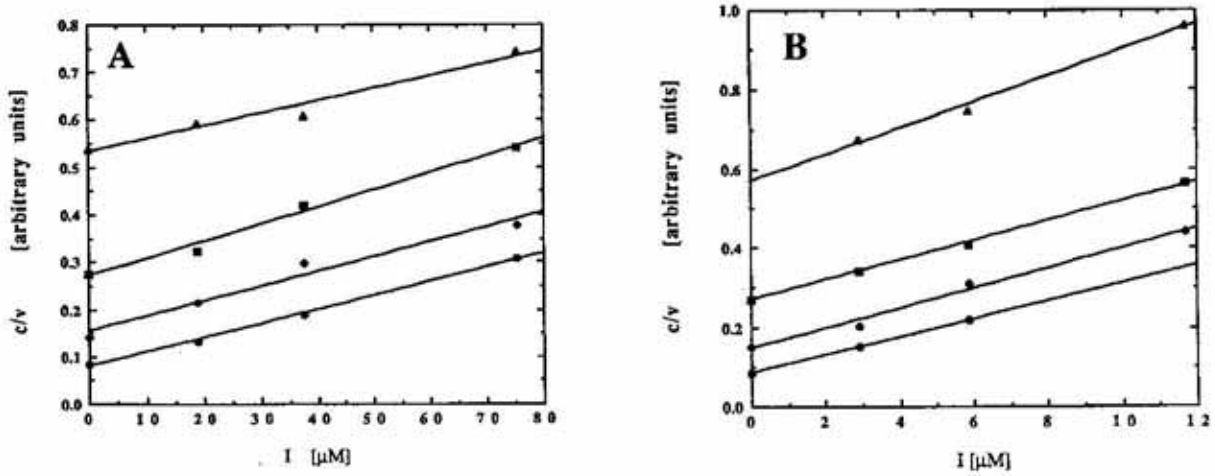


Figure 3. Plot of c_o/v_o *vs.* [I] for inhibition of m^7Guo phosphorolysis catalyzed by the *E. coli* PNP at pH 7 and 25°C, in 50 mM phosphate buffer by 7-deaza-7-cyclohexylethinyl-dAdo, 6f, (panel A) and 7-deaza-6CldPu, 4a, (panel B).

Substrate (m^7Guo) concentrations are (●) 18 μM, (◆) 48 μM, (■) 100 μM and (▲) 200 μM.

comparable values of inhibition constants for both 7-deaza and N^7 forms are observed, i.e. 8 μM for 7-deaza-2CldAdo (6c) (see Table 1) and 4.5 μM for 2ClAdo (cladribine) [6].

The results obtained in this study are in line with the broad specificity of *E. coli* PNP towards nucleoside substrates [10, 36], and agree with the interactions in the nucleoside binding site identified by X-ray crystallography in the three-dimensional structure of the enzyme [24, 25]. In general, many purine and even non purine nucleosides (e.g. riboside of benzimidazole [36]) are accepted as substrates by *E. coli* PNP, but their affinity to the enzyme is relatively low, typically K_i is in the range 100–1000 μM [10, 36]. This is in agreement with the fact that in *E. coli* PNP the base binding site is largely exposed to solvent and, in the case of most purine nucleosides, only a weak C(8)–H hydrogen bond to Ser90 and unspecific aromatic–aromatic interactions of the purine base with Phe159/Tyr160 are observed [25]. Therefore it is not surprising that most of the 7-deazapurine nucleosides tested in this study show only a weak inhibition at concentrations of about 100 μM. The severalfold higher affinity of 7-deazapurine derivatives in comparison with their N^7 counterparts observed typically may be attributed to the fact that in the case of 7-deazapurine nucleosides (not substituted at position C(7)), an additional weak C–H hydrogen bond could probably be formed *via* C(7)–H of the base to Asp204 of the protein [25].

It has already been noted that some modifications of the purine base may notably enhance the affinity of some nucleosides to *E.coli* PNP (e.g. [6, 9, 10]). The most potent noncleavable inhibitors of the *E. coli* enzyme reported up to now are formycins A and B, both being 8-aza-9-deaza nucleosides, with inhibition constants of 5.3 μM and 4.6 μM, respectively, and the neutral form of N(6)-methylformycin A with K_i = 0.3 μM [9]. Some of the inhibitors tested in the present study show comparable affinities for the *E. coli* enzyme: a 2-chloro and 6-chloro substitution as well as bulky hydrophobic groups located at position 7 of the base give compounds with inhibition constants of several μM (see Table 1). The base binding site in *E. coli* PNP is very hydrophobic [24] and one may speculate that in the case of analogues with bulky hydrophobic substituents hydrophobic interactions may be responsible for the potent binding. The influence of the 2-chloro- and 6-chloro substitutions could probably manifest itself by changing the electronic properties of the purine base and possibly enhancing the interactions in the base binding site, i.e. aromatic–aromatic interactions with Phe159/Tyr160 and C–H...O hydrogen bonds to Ser90 and Asp204.

Thus it may be summarized that replacing of the N^7 atom of purine nucleosides with a methine group that results in 7-deazapurine nucleosides is a promising approach to obtain competitive noncleavable inhibitors of *E. coli* PNP that may bind with the enzyme severalfold better than the parent purine nucleosides. Additionally some combination of substituents at positions 2, 6 and 7 of the base may result in analogues with inhibition constants of several μM or even lower.

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