Synthesis of 6-Aryloxy- and 6-Arylalkoxy-2-chloropurines and Their Interactions with Purine Nucleoside Phosphorylase from *Escherichia coli*

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Dedicated to Professor Frank Seela on the occasion of his 60th birthday

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The phase transfer method was applied to perform the nucleophilic substitution of 2,6-dichloropurines by modified arylalkyl alcohol or phenols. Since under these conditions only the 6-halogen is exchanged, this method gives 2-chloro-6-aryloxy- and 2-chloro-6-arylalkoxy-purines. 2-Chloro-6-benzylthiopurine was synthesized by alkylation of 2-chloro-6-thiopurine with benzyl bromide. The stereoisomers of 2-chloro-6-(1-phenyl-1-ethoxy)purine were obtained from *R*- and *S*-enantiomers of *sec.*-phenylethylalcohol and 2,6-dichloropurine.

All derivatives were tested for inhibition with purified hexameric *E. coli* purine nucleoside phosphorylase (PNP). For analogues showing $IC_{50} < 10$ μM, the type of inhibition and inhibition constants were determined. In all cases the experimental data were best described by the mixed-type inhibition model and the uncompetitive inhibition constant, K_{iu}, was found to be several-fold lower than the competitive inhibition constant, K_{ic}. This effect seems to be due to the 6-aryloxy- or 6-arylalkoxy substituent, because a natural PNP substrate adenine, as well as 2-chloroadenine, show mixed type inhibition with almost the same inhibition constants K_{iu} and K_{ic}.

The most potent inhibition was observed for 6-benzylthio-2-chloro-, 6-benzyloxy-2-chloro-, 2-chloro-6-(2-phenyl-1-ethoxy), 2-chloro-6-(3-phenyl-1-propoxy)- and 2-chloro-6-ethoxypurines (K_{iu} = 0.4, 0.6, 1.4, 1.4 and 2.2 μM, respectively). The *R*-stereoisomer of 2-chloro-6-(1-pheny-1-ethoxy)purine has K_{iu} = 2.0 μM, whereas inhibition of its *S* counterpart is rather weak ($IC_{50} > 12$ μM). More rigid (e.g. phenoxy-), non-planar (cyclohexyloxy-), or more bulky (2,4,6-trimethylphenoxy-) substituents at position 6 of the purine base gave less potent inhibitors (IC_{50} = 26, 56 and >100 μM, respectively). The derivatives are selective inhibitors of hexameric "high-molecular mass" PNPs because no inhibitory activity *vs.* trimeric *Cellulomonas sp.* PNP was detected.

By establishing the ligand-dependent stabilization pattern of the *E. coli* PNP it was shown that the new derivatives, similarly as the natural purine bases, are able to form a dead-end ternary complex with the enzyme and orthophosphate. It was also shown that the derivatives are substrates in the reverse synthetic direction catalyzed by *E. coli* PNP.

Abbreviations: PNP, purine nucleoside phosphorylase; Ino, inosine; m^7Guo, 7-methylguanosine; Hepes, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]); LB, Lineweaver-Burk; EH, Eadie-Hofstee; K_i, inhibition constant in uncompetitive or noncompetitive inhibiton models; K_{iu} and K_{ic}, inhibition constants in a mixed-type inhibition model; K_m, Michaelis constant; v_o, initial velocity; c_o, initial substrate concentration; I, inhibitor concentration; V_{max}, maximal velocity; IC_{50}, concentration of an inhibitor causing 50% inhibition under experimental conditions employed; nc, noncompetitive inhibition, uc, uncompetitive inhibition; U, enzyme activity unit; A. U. arbitrary units.

Introduction

The ubiquitous enzyme purine nucleoside phosphorylase (PNP, E. C. 2.4.2.1.) catalyzes the reversible phosphorolysis of purine nucleosides, as follows:

β-purine nucleoside + orthophosphate ↔ purine base + α-D-pentose-1-phosphate.

The "high-molecular mass" hexameric enzymes, found in some bacteria (e.g. *E. coli, S. typhimurium*), have a broad specificity towards purines

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and purine nucleosides (Jensen and Nygaard, 1975; Doskocil and Holy, 1977; Bzowska *et al.*, 1990), while the "low-molecular mass" trimeric, mainly mammalian, PNPs are specific in both binding and catalysis for 6-oxopurine nucleosides (Stoeckler, 1984; Bzowska *et al.*, 1990; Montgomery, 1993). Such differences in specificity between the hexameric and trimeric PNPs are in agreement with different architectures of the active centres and differences in specific interactions between the purine bases and the enzyme, observed in the crystal structures of *E. coli*, calf spleen and human erythrocyte phosphorylases (Mao *et al.*, 1997, 1998; Koellner *et al.*, 1997, 1998; Narayana *et al.*, 1997).

The PNP enzyme family is an important target for drug design (Stoeckler, 1984; Montgomery, 1993). Potent inhibitors of mammalian phosphorylases may be useful in many circumstances, e.g. to prevent host-*versus*-graft response in organ transplantation, to treat gout and to enhance therapeutic effect of drugs that are purine nucleosides and therefore are cleaved by PNP before reaching their target. Selective inhibitors of the PNP enzyme present in parasites could prevent spread of parasitic infection. Finally, the *E. coli* PNP has recently been shown to be useful in gene therapy of some types of tumours (Sorscher *et al.*, 1994; Hughes *et al.*, 1995). The method is based on the fact that specificities of *E. coli* and human enzymes are different and therefore, in contrast to the human PNP, the *E. coli* enzyme is capable to convert some nontoxic deoxyadenosine analogues to highly toxic adenine analogues. For example, the expression of *E. coli* PNP in fewer than 1% of cells in human colonic carcinoma cell lines was found to be efficient enough to kill virtually all bystander cells after treatment with 6-methylpurine-2'-deoxyribofuranoside. These findings have opened new directions in studies of the *E. coli* PNP, which beforehand had been studied much less extensively than the phosphorylases isolated from mammals, and stimulated broad experimental research (e.g. Bzowska and Kazimierczuk, 1995; Wielgus-Kutrowska *et al.*, 1997; Mao *et al.*, 1997; Kierdaszuk *et al.*, 1997; Bzowska *et al.*, 1998a; Koellner *et al.*, 1998).

Recently we have shown that substitution of the base with chlorine at position C-2 markedly enhances the affinity of purine and of 7-deazapurine nucleosides to *E. coli* PNP (Bzowska and Kazimierczuk, 1995; Bzowska *et al.*, 1998a). We have also observed that 6-benzyloxy-2-chloro-9-β-D-(2'-deoxyribofuranosyl)purine is a very good and selective substrate of *E. coli* PNP and one of the most potent competitive inhibitors of inosine phosphorolysis with an inhibition constant K_i = 0.5 μM (Bzowska and Kazimierczuk, 1995). Therefore we decided to synthesize some purine analogues of this inhibitor substituted at both crucial positions, namely a series of 6-aryloxy-2-chloropurines and 6-arylalkoxy-2-chloropurines, and to investigate their kinetic properties with hexameric *E. coli* PNP and with more sepcific trimeric PNP from *Cellulomonas sp.*

Materials and Methods

Materials

Inosine, 7-methylguanosine, adenine, guanine, hypoxanthine, ribose-1-phosphate, Hepes, and xanthine oxidase from buttermilk (1 U/mg) were from Sigma (St. Louis, Mo., USA). 2-Chloroadenine was synthesized according to Brown and Weliky (1958). *E. coli* PNP (60 U/mg) was a kind gift of Dr. G. Koszalka (Wellcome Research Laboratories, Research Triangle Park, NC, USA), and was purified by affinity chromatography to 104 U/mg as described elsewhere (Bzowska *et al.*, 1998a). *Cellulomonas sp.* PNP (8 U/mg) was from Toyobo Company (Japan) and was purified by ion-exchange chromatography as previously described (Wielgus-Kutrowska *et al.*, 1998; Bzowska *et al.*, 1998b). All solutions were prepared with high-quality MilliQ water.

One unit of PNP is the amount of enzyme that will cause phosphorolysis of one μmol of inosine to hypoxanthine and ribose-1-phosphate per min at 25 °C with 0.5 mM inosine and 50 mM Na-phosphate buffer pH 7.0 (see also section *Enzyme activity*).

Ultraviolet spectroscopy was performed with a Kontron (Vienna, Austria) Uvikon 940 UV-VIS spectrophotometer fitted with thermostatically controlled cell compartments and with the use of 2, 5 or 10 mm path length cuvettes. The concentrations of substrates and inhibitors were determined spectrophotometrically from their molar extinction coefficients, which are listed in Table I for analogues synthesized in this study, and were com-

plied from literature for other substrates: $\varepsilon(260\ nm) = 13\ 350\ M^{-1}cm^{-1}$ for adenine, $\varepsilon(264\ nm) = 12\ 000\ M^{-1}cm^{-1}$ for 2-chloroadenine, $\varepsilon(260\ nm) = 8\ 500\ M^{-1}cm^{-1}$ for m^7Guo at pH 7, and $\varepsilon(249\ nm) = 12\ 300\ M^{-1}cm^{-1}$ for inosine (Dawson *et al.,* 1969). Thin-layer chromatography was performed on silicagel plates with the solvent consisting of chloroform-methanol 9:1 (by vol.). Melting points (uncorr.) were measured on a Boetius microscope hot storage (Radebeul, Germany). NMR spectra were recorded on Bruker AC 250 (Karlsruhe, Germany). Measurements of pH were carried out with Beckman pH-meter equipped with a combination semimicro electrode and temperature sensor. Gel filtration FPLC chromatography was carried out on Pharmacia LKB Biotechnology (Uppsala, Sweden) equipment.

For linear regression analysis of the kinetic data, the Cricket Graph program for the Macintosh computer was used. For nonlinear regression analysis, the Leonora program (version 1.0) run on a PC computer (A. Cornish-Bowden, 1995) was employed.

The phase transfer method was applied to perform the nucleophilic substitution of 2,6-dichloropurines by modified arylalkyl alcohols or phenols. Since under these conditions only the 6-halogen was exchanged, the method produces 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines. 6-Benzylthio-2-chloropurine was synthesized by alkylation of 2-chloro-6-thiopurine with benzyl bromide. The stereoisomers of 2-chloro-6-(1-phenyl-1-ethoxy)purine were obtained from *R*- and *S*-enantiomers of *sec.*-phenylethylalcohol and 2,6-dichloropurine. Structures of the 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloro- analogues are presented in Figure 1. The details of the synthetic procedures are given below. All new purine derivatives were characterized by elemental analysis, melting points, TLC chromatography, UV and NMR spectra. The data are presented in Tables I and II.

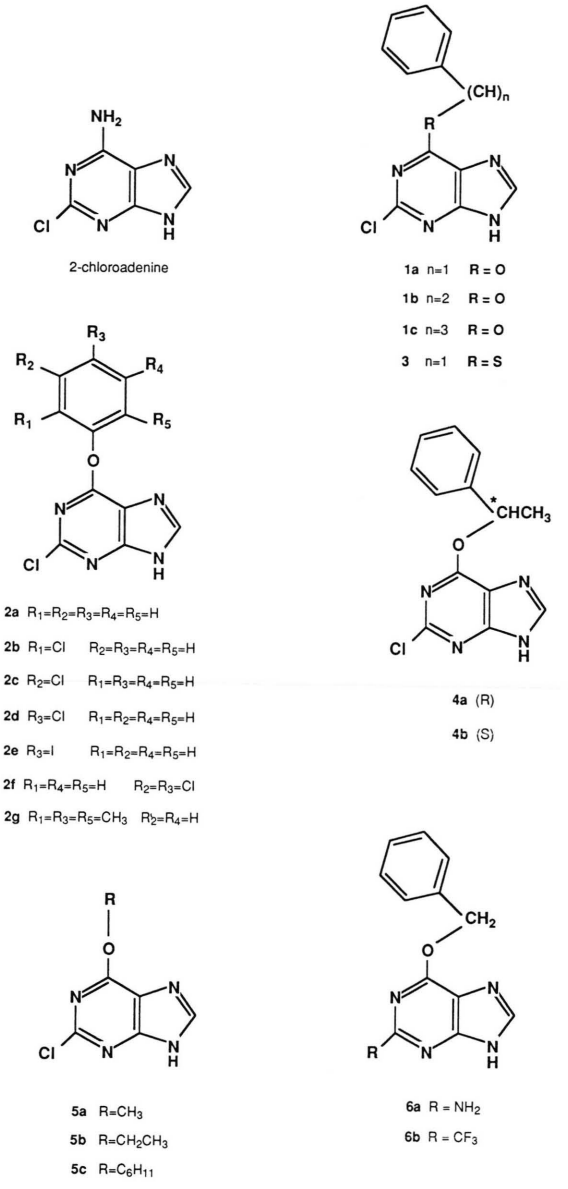

Fig. 1. Structures of 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines.

Synthesis of 6-aryloxy and 6-arylalkoxy 2-substituted purines

A mixture of 6-chloro-2-substituted purine (2.5 mmol), finely powdered KOH (350 mg appropriate phenol or arylalkyl alcohol (5 mmol), tetrabutyl hydrosulfite (50 mg) and acetonitrile (2.5 ml) was vigorously stirred at 80 °C (oil bath) for 2 h. Then 3 ml of water was added and the mixture was neutralized by acetic acid. The precipitate formed after few h storage at low temperature was filtered and crystallized from ethanol/water mixtures. The yields were 55–85% with respect to starting purine derivative.

Table I. Ultraviolet spectral data at pH 7.0 in water containing 10% methanol (v/v), R_f values (silica gel plates, $CHCl_3$-CH_3OH, 9:1, v/v) and melting points (m.p., uncorrected) for 6-aryloxy and 6-arylalkoxy 2-substituted purines used in this study.

	Purine base	λ_{max} [nm]	ε_{max} [$M^{-1}cm^{-1}$]	m.p. [°C]	R_f
1a	6-benzyloxy-2-chloro	261	10900	202–203	0.21
1b	2-chloro-6-(2-phenyl-1-ethoxy)	259	10900	208	0.23
1c	2-chloro-6-(3-phenyl-1-propoxy)	259	10700	200–202	0.25
2a	2-chloro-6-phenoxy	264	12450	256–258	0.20
2b	2-chloro-6-(o-chlorophenoxy)	264	11850	215–217	0.20
2c	2-chloro-6-(m-chlorophenoxy)	266	10400	224–226	0.20
2d	2-chloro-6-(p-chlorophenoxy)	265	14200	260	0.20
2e	2-chloro-6-(p-iodophenoxy)	266	15500	270–281[a]	0.24
2f	2-chloro-6-(3,4-dichlorophenoxy)	266	13800	257–259	0.23
2 g	2-chloro-6-(2,4,6-trimethylophenoxy)	262	12800	257–259	0.26
3	6-benzylthio-2-chloro	299	18000	236–239	0.29
4a	2-chloro-6-((R)-1-phenyl-1-ethoxy)	261	14300	174–175	0.21
4b	2-chloro-6-((S)-1-phenyl-1-ethoxy)	261	14300	174–176	0.21
5a	2-chloro-6-methoxy	259[b]	10100[b]	254[b]	0.16
5b	2-chloro-6-ethoxy	259	9800	260–262	0.18
5c	2-chloro-6-cyclohexyloxy	260	11400	320[c]	0.22
6a	2-amino-6-benzyloxy	240, 281	6800, 5900	198–199	0.13
6b	6-benzyloxy-2-trifluoromethyl	255	11300	174–175	0.19

a Decomposition.
[b] Coburn *et al.* (1965).
[c] Decomposition > 280 °C.
[d] Elemental analyses CHN ± 0.25%.

Table II. Chemical shifts (in ppm *vs.* internal TMS) of 6-alkoxy-, 6-aryloxy and 6-arylalkoxy-2-substituted purines in D_6DMSO at 20 °C.

Compound	arom. H	8-H	CH_2	CH_3	CH	NH_2
1a	7.20–7.95 m	8.30 s	5.53 s	–	–	–
1b	7.20–7.40 m	8.39 s	3.13 t, 4.72 t	–	–	–
1c	7.20–7.40 m	8.42 s	2.76 t, 3.30 m, 4.50 t	–	–	–
2a	7.20–7.65 m	8.56 s	–	–	–	–
2b	7.30–7.80 m	8.60 s	–	–	–	–
2c	7.10–7.65 m	8.58 s	–	–	–	–
2d	7.35–7.65 m	8.57 s	–	–	–	–
2e	7.10–7.90 m	8.57 s	–	–	–	–
2f	7.30–7.90 m	8.57 s	–	–	–	–
2 g	6.97 s	8.56 s	–	2.01 s, 2.82 s	–	–
3	7.25–7.55 m	8.48 s	4.63 s	–	–	–
4a	7.30–7.60 m	8.38 s	–	1.72 d	6.46 q	–
4b	7.30–7.60 m	8.39 s	–	1.73 d	6.46 q	–
5a	–	8.15 s		4.04 s	–	–
5b	–	8.42 s	4.58 q	1.42 t	–	–
5c	–	8.39 s	1.25–2.05 5m	–	5.25 m	–
6a	7.30–7.60 m	7.80 s	5.64 s	–	–	6.27 bs
6b	7.25–7.65 m	8.62 s	5.64 s	–	–	–

Synthesis of 6-benzylthio-2-chloropurine

A solution of 2-chloro-6-thiopurine (370 mg, 2 mmol) and potassium carbonate (280 mg, 2 mmol) in 20 ml of a water/methanol mixture (7:3, v/v) was stirred at room temperature. Benzyl chloride (285 µl, 2.5 mmol) was added and the resulting solution was stirred overnight at room temperature and finally neutralized with acetic acid. The precipitate was separated and crystallized from 50% methanol. Light yellow needles (330 mg, 63%) with m.p. 236–239 °C were obtained.

Enzyme activity

The specific activity of purine nucleoside phosphorylase was measured spectrophotometrically with the use of Kalckar's method, where inosine was the variable substrate and the product (hypoxanthine) oxidized by xanthine oxidase to form uric acid (Kalckar, 1947; Stoeckler *et al.*, 1978). One unit of phosphorylase is defined as the amount of enzyme that will cause phosphorolysis of one μmol of inosine to hypoxanthine and ribose-1-phosphate per min at 25 °C with 0.5 mM inosine and 50 mM sodium phosphate buffer pH 7.0.

Enzymatic procedures, determination of IC_{50} and selectivity of inhibition

In preliminary experiments, all compounds were tested for inhibitory activity *vs.* the *E. coli* PNP with 7-methylguanosine as the variable substrate (Kulikowska *et al.*, 1986; Bzowska *et al.*, 1990), and in conditions of saturation with the second substrate of phosphorolysis, i.e. with orthophosphate. The inhibition was measured at 25 °C, in a pH 7.0 and 50 mM sodium phosphate buffer, at the initial substrate concentration c_o = 20 μM, and for at least three different inhibitor concentrations. The phosphorolysis was monitored spectrophotometrically at 260 nm (Kulikowska *et al.*, 1986). The initial velocity of the phosphorolysis was calculated by linear regression of absorbance *vs.* time. The IC_{50} values were calculated from plots of the inhibition ratio (v_i/v_o) *vs.* inhibitor concentration I, 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 type of inhibition and the inhibition constants K_{iu} and K_{ic} were then determined only for analogues showing good inhibitory properties in the initial screening (IC_{50} < 10 μM) with exception of adenine for which for the purpose of comparison full analysis was done regardless its known poor inhibitory properties (Jensen, 1976; Doskocil and Holy, 1977).

Compunds with IC_{50} < 10 μM were tested also for inhibiton *vs.* the *Cellulomonas* PNP. The experimental conditions were as descibed above for the *E. coli* PNP. Concentrations of the analogues employed in this case were at least ten fold higher that IC_{50} values determined for the *E. coli* PNP.

Enzymatic procedures – inhibition type and inhibition constants

For the most potent inhibitors, i.e. those with IC_{50} < 10 μM, the type of inhibition and the inhibition constants were determined by the initial velocity method with 7-methylguanosine as the variable substrate (see above). The initial velocities v_o, and v_i measured in the absence and in the presence of an inhibitor, correspondingly, were obtained from linear regression of absorbance (λ_{obs} = 260 nm for m^7Guo) *vs.* time. At least five initial substrate concentrations (in the range 5–100 μM), and typically four or five different inhibitor concentrations (including no inhibitor added) were analyzed, so the typical data set consisted of initial velocity values measured for 20 or 25 combinations of c_o and I. The measurements were conducted twice for each pair (c_o, I).

A. Linear regression

To determine the inhibition type, the data were plotted in the form of Lineweaver-Burk (LB), $1/v_o$ *vs.* $1/c_o$; Eadie-Hofstee (EH), v_o *vs.* v_o/c_o and Hanes, c_o/v_o *vs.* c_o, plots (Keleti, 1986). A linear regression analysis was performed. Replots of the slopes and y-intercepts of the LB plot *vs.* I were analyzed to classify the inhibition type according to Cleland (1963). In case of doubts on the inhibition type, additional plots were analyzed and compared with the literature (Keleti, 1986), i.e. the Dixon, $1/v_o$ *vs.* I and Hunter-Down, $v_oI/(v_o-v_i)$ *vs.* c_o, plots.

For adenine and 2-chloroadenine, the inhibition constants were determined with the use of the model of noncompetitive inhibition, as a mean of the values obtained as: the slope and y-intercept of the replots of the LB and Hanes plots, and the x- and y-intercepts of the replots of the EH plot *vs.* inhibitor concentration I (Keleti, 1986). For all 2-chloro-6-aryloxy- and 2-chloro-6-arylalkoxypurines the uncompetitive inhibition model was used and the inhibition constants were determined as a mean of the values obtained from the replots of the LB, EH and Hanes plots *vs.* either the inhibitor concentration I (the x- and y-intercepts of the LB plot and the slope of the Hanes plot), or the reciprocal of inhibitor concentration 1/I (the x-intercept of the Hanes plot, the slope and y-intercept of EH plot) (Keleti, 1986).

B. Nonlinear regression

All experimental data for inhibition of 7-methylguanosine phosphorolysis by 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines, and by adenine and 2-chloroadenine, were additionally analyzed with the use of the weighted least-squares non-linear regression (Leonora numerical program, Cornish-Bowden, 1995). Models of uncompetitive ($K_{ic} = \infty$, in Eqn. (1)) and mixed-type inhibition (Eqn. (1)) were compared by analysis of variance. The data were finally fitted to the mixed-type inhibition model described by the following equation with two inhibition constants, K_{iu} and K_{ic}:

$$v_o(c_o) = \frac{V_{max}Co}{c_o(1+I/K_{iu})+K_m(1+I/K_{ic})} \quad (1)$$

The inhibition constants presented in Table III were determined by fitting equation (1) to the whole data set obtained for one analogue, i.e. for all triplets (v_o, c_o, I).

Substrate properties

The direct spectrophotometric method was employed to study the substrate activity of the 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines in the reverse synthetic direction of the PNP-catalyzed reaction. The reaction mixture (1 ml volume, kept at 25 °C) contained 1 mM ribose-1-phosphate, 50 mM Hepes buffer pH 7.0, and a substrate in a concentration of approximately 100 μM (or lower in the case of analogues 6-benylthio-2-chloro, **3**, 6-(*p*-iodo-phenoxy), **2e**, and 6-((*R*)-1-phenyl-1-ethoxy, **4a**, due to their poor solubility in water). The reaction was initiated by addition of the enzyme (0.1 μg of *E. coli* PNP), and UV absorption spectra in the range 220–360 nm were recorded every 2 min until the reaction reached equilibrium. If the reaction seemed very slow in these conditions, 2 μg of PNP was added to 1 ml of the reaction mixture after recording of several spectra. The natural substrates of the *E. coli* enzyme, adenine and guanine, were used in control experiments conducted in a similar way as described above.

The rates of synthesis were determined for three analogues: 6-methoxy-, 6-ethoxy- and 6-benzyloxy-2-chloropurines (**5a**, **5b** and **1a**), for which 2'-deoxyribonucleosides had already been synthesized, and spectral data for them were available (Kazimierczuk *et al.*, 1992; Bzowska and Kazimierczuk, 1995). The absorbance at λ_{obs} was plotted *vs.* time and the initial velocity of the synthesis was calculated by linear regression of the initial linear portion of the plots A(t). The experimental conditions were the following: c_o = 200 μM (i.e. equal to K_m, Jensen and Nygaard, 1975), λ_{obs} = 260 nm and $\Delta\varepsilon$ = +1700 $M^{-1}cm^{-1}$ for adenine (Dawson *et al.*, 1969), c_o = 100 μM, λ_{obs} = 249 nm, $\Delta\varepsilon$ = +5050 $M^{-1}cm^{-1}$ for 2-chloro-6-methoxy (**5a**), c_o = 100 μM, λ_{obs} = 245 nm, $\Delta\varepsilon$ = +3400 $M^{-1}cm^{-1}$ for 2-chloro-6-ethoxypurine (**5b**), (Bzowska and Kazimierczuk, 1995), and c_o = 60 μM, λ_{obs} = 250 nm, $\Delta\varepsilon$ = +3000 $M^{-1}cm^{-1}$ for 6-benzyloxy-2-chloropurine (**1a**).

Enzyme stabilization pattern research

To determine the ligand-induced stabilization of the enzyme, the *E.coli* PNP was additionally purified with the use of gel filtration FPLC chromatography method (Superose 12, HR 10/30 column) in order to remove traces of phosphate. The residual phosphate could be present in the enzyme sample purified by the affinity chromatography because the phosphate buffer is used in this method to remove the enzyme from the affinity column (Bzowska *et al.*, 1998a). The column for the gel filtration was equilibrated and eluted (flow rate 0.4 ml min^{-1}) with 50 mM Hepes buffer pH 7 and 0.2 ml fractions each were collected.

In stabilization experiments the *E.coli* enzyme (0.056 mg/ml, 2.3 μM in a total volume of 100 μl) was incubated for 10 min at 56 °C in the absence (control experiment) and presence of the ligands. Before and after the heat treatment the specific activity of the enzyme was determined as described above, i.e. with Ino as the variable substrate (0.5 mM) at 50 mM phosphate pH 7.0 and 25 °C (see section *Enzyme activity*). The ligands were present at concentrations indicated in Table IV. The heat treatment was conducted at pH 7.0 (50 mM Hepes buffer), i.e. close to the pH value optimal for the stability of the enzyme (Krenitsky *et al.*, 1981), and the same as used for all kinetic experiments. In some cases (see Table IV) 5% MeOH was present in the incubation mixture. The addition of methanol was necessary

in the case of experiments with 6-benzylthio-2-chloropurine because its solubility in water is very low. For this reason for other ligands the stabilization patterns in the absence and presence of methanol were determined (see Table IV). The stabilization patterns are in principle similar in the absence and in the presence of 5% MeOH, although the enzyme is slightly less stable in the latter case.

Results

Inhibition properties with Escherichia coli and Cellulomonas phosphorylases

The direct spectrophotometric method with m^7Guo as substrate was employed to study the inhibitory properties of the 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines. The most typical spectrophotometric assay for the PNP enzyme, i.e. the method of Kalckar based on coupling of the PNP catalyzed reaction with the xanthine oxidase reaction (Kalckar 1947; Stoeckler *et al.*, 1978), is very sensitive to the presence of purine bases in the reaction mixture (because many of purine base analogues are inhibitors or substrates of xanthine oxidase), and therefore could not be employed in this study.

In preliminary experiments the IC_{50} value was determined for all the compounds *vs.* the *E. coli* PNP (see Table III). All derivatives demonstrated some inhibitory activity with the enzyme, although difference between the best inhibitor, 6-benzylthio-2-chloropurine, **3**, IC_{50} = 0.5 μM, and the one showing the weakest activity, 2-chloro-6-(2,4,6-trimethylphenoxy)purine, **2 g**, IC_{50} >100 μM, was more than 2 orders of magnitude. However, all new analogues show more potent inhibitory activity than the two natural PNP substrates hypoxan-

Table III. Inhibition of 7-methylguanosine phosphorolysis catalyzed by *E. coli* PNP by 6-aryloxy and 6-arylalkoxy 2-substituted purines, at 25 °C and in 50 mM sodium phosphate buffer pH 7.0.
The IC_{50} were determined for all analogues as described in Materials and Methods. The type of inhibition and the inhibition constants, K_{iu} and K_{ic} were determined only for analogues showing good inhibitory properties in the initial screening (IC_{50} < 10 μM). Unless otherwise indicated mixed inhibition model was fitted (Eqn. (1), Methods) and the error of the K_i determination i.e. (±SD) was 15% or less.

	Purine base	IC_{50} [μM]	K_{iu} [μM]	K_{ic} [μM]
	6-keto (hypoxanthine)	>150[a]	nd[b]	nd
	6-amino (adenine)	>150	200	140
	6-amino-2-chloro (2-chloroadenine)	2.3	3.1	2.3
1a	6-benzyloxy-2-chloro	0.8	0.59	4 ± 3
1b	2-chloro-6-(2-phenyl-1-ethoxy)	1.0	1.4	12 ± 6
1c	2-chloro-6-(3-phenyl-1-propoxy)	1.1	1.4	5 ± 2
2a	2-chloro-6-phenoxy	26	nd	nd
2b	2-chloro-6-(o-chlorophenoxy)	10	nd	nd
2c	2-chloro-6-(m-chlorophenoxy)	19	nd	nd
2d	2-chloro-6-(p-chlorophenoxy)	22	nd	nd
2e	2-chloro-6-(p-iodophenoxy)	7	10	40 ± 13
2f	2-chloro-6-(3,4-dichlorophenoxy)	14	nd	nd
2 g	2-chloro-6-(2,4,6-trimethylophenoxy)	>100[c]	nd	nd
3	6-benzylthio-2-chloro[d]	0.5	0.37	6 ± 4
4a	2-chloro-6-((R)-1-phenyl-1-ethoxy)[d]	3.2	2.0	9 ± 2
4b	2-chloro-6-((S)-1-phenyl-1-ethoxy)	>12[d,e]	nd	nd
5a	2-chloro-6-methoxy	12	6.3	34 ± 7
5b	2-chloro-6-ethoxy	3	2.2	9 ± 4
5c	2-chloro-6-cyclohexyloxy	56[f]	nd	nd
6a	2-amino-6-benzyloxy	~50	nd	nd
6b	6-benzyloxy-2-trifluoromethyl	>60	nd	nd

[a] 40% inhibition in a concentration of 130 μM of the analogue.
[b] Not determined.
[c] 30% inhibition in a concentration of 70 μM of the analogue.
[d] In the presence of 3% MeOH in the reaction mixture.
[e] No determination of IC_{50} possible due to poof solubility of the derivative in water solution.
[f] In the presence of 15% MeOH in the reaction mixture.

thine and adenine (see Table III). Two analogues that lack the 2-chloro substituent, i.e. 2-amino-6-benzyloxy- (**6a**) and 6-benzyloxy-2-trifluoromethylpurine (**6b**), have much higher IC_{50} than their corresponding 2-chloro counterpart (**1a**) (see Table III).

The compounds showing IC_{50} < 10 μM were tested also for inhibition *vs.* the *Cellulomonas* PNP. None of them caused detectable decrease of the enzyme activity in a concentration of about ten fold higher than IC_{50} determined with the *E. coli* enzyme. *Cellulomonas* PNP is a homotrimeric enzyme with molecular mass typical for the "low-molecular mass" PNPs. Therefore it may be concluded that derivatives synthesized in this study are probably selective inhibitors of the "high-molecular mass" hexameric phosphorylases.

The compounds showing IC_{50} < 10 μM in the initial screening *vs.* the *E. coli* PNP were then analyzed more extensively to determine the inhibition type and inhibition constants (8 analogues, see Table III). In addition, for the purpose of comparison the inhibition by adenine and 2-chloroadenine were analyzed with the same methods.

The kinetic data were transformed to the Lineweaver-Burk, Hanes of Eadie-Hofstee coordinates, and linear regression was performed. The uncompetitive type of inhibition was found to be in agreement with the experimental data for all 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines, while the simple noncompetitive model described the inhibition caused by adenine and 2-chloroadenine. The data for 2-chloro-6-(3-phenyl-1-propoxy)purine, **1c** and 2-chloroadenine, displayed as a Lineweaver-Burk plot of $1/v_o$ vs. $1/c_o$, are shown in Figure 2A and 2B, respectively. In the case of inhibitor **1c,** a set of almost parallel lines were obtained, so the inhibition may be described as uncompetitive. The inhibition constant obtained from the replot of slopes *vs.* I is K_i = 1.1 μM. Also other plots (Hanes, Eadie-Hofstee) of the same data set were in agreement with the uncompetitive inhibition type (data not shown), and the inhibition constant obtained as the mean form all replots is K_i = 1.4 ± 0.9 μM. While similar plots (data not shown) were also obtained for other 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines, in some cases the independence of the slopes of the LB plot on I was not so clear. So for the new analogues synthesized in this study it may be concluded that linear regression analysis of the data suggests the uncompetitive model of inhibition, although errors of K_i determination are high (see below) and the mixed-inhibition type with a much higher value of the second inhibition constant cannot be ruled out.

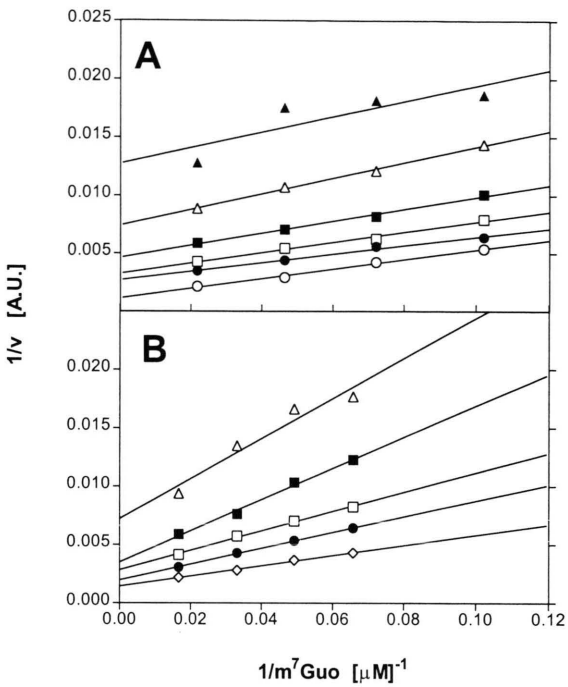

Fig. 2. Lineweaver-Burk plot for inhibition of 7-methylguanosine phosphorolysis catalyzed by the *E. coli* PNP at pH 7.0 and 25 °C, in 50 mM phosphate buffer by 2-chloro-6-(3-phenyl-1-propoxy)purine, **1c** (panel A) and 2-chloroadenine (panel B). Inhibitor concentrations are 0 μM (○), 0.49 μM (●), 0.99 μM (□), 1.97 μM (■), 3.89 μM (△) and 7.77 μM (▲) in panel A, and 0 μM (○), 1.13 μM (●), 2.27 μM (□), 4.54 μM (■) and 9.08 μM (△) in panel B. Inhibition constant determined for inhibitor **1a** from the replot of y-intercepts of the LB plot *vs.* inhibitor concentrations I is K_i = 1.1 μM, and the slope of the LB plot is almost independent from I, so in the inhibition may be described as uncompetitive. For 2-chloroadenine inhibition constants K_i^{slope} = 2.2 μM and $K_i^{intercept}$ = 3.6 μM were determined from the replots of slopes and y-intercepts of the LB plot *vs.* I, respectively.

The inhibition constants obtained from linear regression analysis are in agreement with the preliminary screening results (IC_{50}, see Table III), and show the following order of influence of the aryloxy and arylalkoxy substituents on the inhibitory activity *vs. E. coli* PNP of the following 6-substituetd-2-chloropurines: 6-benzylthio (**3**) K_i = 0.45 ± 0.09 μM, 6-benzyloxy (**1a**) K_i = 1.0 ± 0.5 μM, 6-

(2-phenyl-1-ethoxy) (**1b**) K_i = 1.3 ± 0.6 μM, 6-(3-phenyl-1-propoxy) (**1c**) K_i = 1.4 ± 1.0 μM, 6-ethoxy (**5b**) K_i = 1.9 ± 0.4 μM, 6-((*R*)-1-phenyl-1-ethoxy) (**4a**) K_i = 2.3 ± 0.8 μM, 6-methoxy (**5a**) K_i = 7.7 ± 1.3 μM and 6-(p-iodophenoxy) (**2e**) K_i = 10.2 ± 2.8 μM. By constrast, *S*-stereoisomer of compound **4a**, showed only very weak inhibiton in comparison with its *R*-counteropart (see Table III). It should be noted that K_i values obtained by linear regression assuming uncompetitive inhibition type are similar to K_{iu} values (see Table III) determined finaly using the equation for the mixed type inhibition (Eqn. (1)) which was found to be a better model (see next section).

In contrast, for inhibition caused by 2-chloroadenine, both the slopes and the y-intercepts of the LB plot vary with I (see Fig. 2B). The inhibition constants obtained from replots of the slopes and y-intercepts are K_i^{slope} = 2.2 μM and $K_i^{intercept}$ = 3.6 μM, respectively. Also Hanes and Eadie-Hofstee plots obtained for the same data set agree in first approximation with the pure noncompetitive inhibition; the inhibition constant obtained with the assumption of the noncompetitive type of inhibition was K_i = 2.8 ± 0.5 μM. The same type of dependence was obtained for inhibition caused by adenine (data not shown), although the value of inhibition constant was much higher, K_i = 197 ± 16 μM.

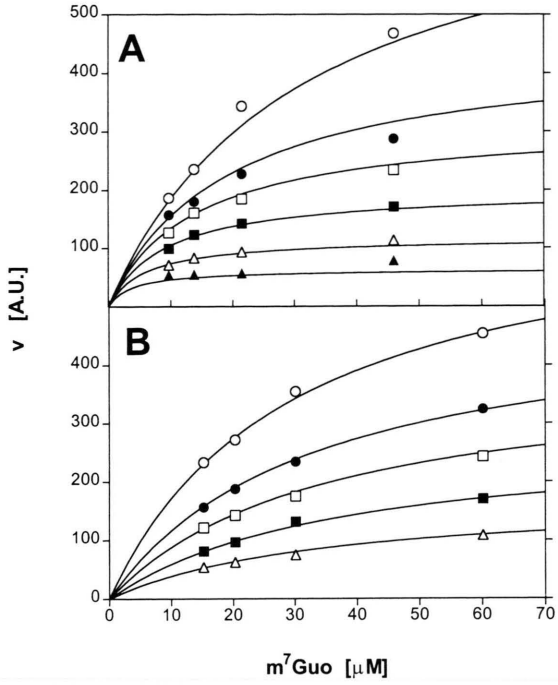

Fig. 3. Inhibition of 7-methylguanosine phosphorolysis catalyzed by the *E. coli* PNP at pH 7 and 25 °C, in 50 mM phosphate buffer by 2-chloro-6-(3-phenyl-1-propoxy)-purine, **1c** (panel A) and 2-chloroadenine (panel B). The sama data sets as those displayed in Fig. 2 in $1/v_o$ *vs.* $1/c_o$ coordinates, are included here in a form of direct plot v_o *vs.* c_o. Inhibitor concentrations were 0 μM (○), 0.49 μM (●), 0.99 μM (□), 1.97 μM (■), 3.89 μM (△) and 7.77 μM (▲) in panel A, and 0 μM (○), 1.13 μM (●), 2.27 μM (□), 4.54 μM (■) and 9.08 μM (△) in panel B. The equation (Eqn. (1), see Methods) for the mixed type inhibition was fitted to the whole data set for each inhibitor. Fitted values are: V_{max} = 760.4 A. U., K_m = 29.6 μM, K_{iu} = 0.70 μM, K_{ic} = 17.8 μM for inhibitor **1c**, and: V_{max} = 679.9 A. U., K_m = 31.4 μM, K_{iu} = 3.07 μM, K_{ic} = 2.23 μM for 2-chloroadenine.

Inhibition constants and type of inhibition

The data sets that were initially analyzed as described above were then analyzed once again with the use of the weighted non-linear regression least squares method (the Leonora numerical program, Cornish-Bowden, 1995). The main objective of this analysis was model discrimination between the uncompetitive and mixed types of inhibition (see Methods). On the basis of the F-values associated with the introduction of the fourth parameter (K_{ic}) to the three parameter model describing the uncompetitive inhibition (K_{iu}, K_m and V_{max}), it was concluded in the case of all 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines studied that the introduction of K_{ic} makes significant improvements to the fits, so the mixed-type inhibition is the proper model to describe the experimental data. For example, in Figs. 3A and 3B the data sets for 2-chloro-6-(3-phenyl-1-propoxy)purine, **1c** and 2-chloroadeninie are shown in a form of direct plot v_o *vs.* c_o, and the lines fitted with Eqn. (1) to the whole data set are included.

The inhibition constants determined by fitting Eqn. (1) (see Methods) are listed in Table III. However, it should be noted that for all 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines studied K_{iu} is much smaller than K_{ic} (sometimes even by an order of magnitude), so in the first approximation the inhibition may be described by the uncompetitive model, as it was done in the previous section. This discrepancy between the two inhibition constants was not observed for inhibition

caused by adenine and 2-chloroadenine, for which values for K_{iu} and K_{ic} were found to be almost identical (see Table III), and the inhibition at first sight appeared to be of a simple noncompetitive type.

Substrate properties

All derivatives synthesized in this study are purines-base analogues and therefore in principle may be substrates in the reverse synthetic direction of the reaction catalyzed by PNP (see Introduction, reaction scheme). Therefore their substrate activity was studied. In the proposed mechanism of the reaction catalyzed by *E. coli* PNP, ribose-1-phosphate is the first substrate to add to the free enzyme in a synthetic direction and purine bases add to the binary complex between the enzyme and ribose-1-phosphate (Jensen, 1976), so the conditions with the ribose-1-phosphate present in a saturation concentration (1 mM) were employed.

Three analogues 6-methoxy-, 6-ethoxy- and 6-benzyloxy-2-chloropurines (**5a**, **5b** and **1a**) for which 2'-deoxyribonucleosides have already been synthesized and described in the literature (Kazimierczuk *et al.*, 1992; Bzowska and Kazimierczuk, 1995) were chosen for more detailed studies. For all three purine-base analogues, changes in UV spectra after addition of the *E. coli* PNP were consistent with formation of a corresponding nucleoside, i.e. increase of absorbance in the region of 260-nm peak, a decrease of absorbance of the long-wavelength side of the peak, and small changes in λ_{max} were observed. The initial rates of the synthesis reaction were: 9% for 6-ethoxy-, **5b**, 4% for 6-methoxy-, **5a**, and 2% for 6-bezyloxy-2-chloropurine, **3**, when compared with the experimental rate for adenine taken as 50% (c_o for adenine was equal to K_m, so the measured rate may be assumed to be 50% of V_{max}). These values should be treated only as a rough approximation of the true rates since: (i) the extinction coefficients of 2'-deoxyribonucleosides, not of ribonucleosides, were used for calculations, (ii) a full kinetic analysis for the analogues **3**, **5a** and **5b** was not carried out, so the saturation conditions were not necessarily achieved, and the measured rates are not neccesarily equal to V_{max}.

The overall changes in the UV spectra of other purine-base analogues, for which spectral properties for the corresponding nucleosides are not known, were similar as those observed for analogues **5a**, **5b** and **1a**. Thus it may be concluded that all 6-aryloxy- and 6-arylalkoxy-2-chloropurines studied are substrates of the *E. coli* PNP in the reverse synthetic direction.

Ligand stabilization pattern

The effects of 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines on the thermal stability of *E. coli* PNP were established to determine the type of complexes with the PNP enzyme that the new analogues are able to form (binary or ternary complexes). Natural substrates (purine nucleosides, purine bases, phosphate and ribose-1-phosphate) were used in control experiments. By kinetic analysis, binding studies and stabilization pattern it has already been shown that purine nucleosides (inosine and adenosine), phosphate, and ribose-1-phosphate are able to form binary complexes with the free enzyme, while the purine bases are probably not capable to bind to the free enzyme and they form ternary complexes only: a catalytical one with enzyme/ribose-1-phosphate (see Introduction, the reaction scheme), and a dead-end complex with enzyme/orthophosphate (Jensen and Nygaard, 1975; Jensen, 1976; Krenitsky and Tuttle, 1982).

Table IV presents data obtained for 6-benzyloxy-2-chloropurine, **1a**, and 6-benzylthio-2-chloropurine, **3**. Similar data (not shown) were obtained for other 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines. The concentrations of ligands employed were chosen on the basis of the inhibition constants of the analogues obtained in the previous section (see Table III). The stabilization pattern determined here is similar to the pattern described by Krenitsky and Tuttle (1982): the purine bases are the only substrate of four that in principle are incapable of effectively stabilize the enzyme in the absence of other substrates. However, the combination of purine base and phosphate resulted in a stabilization (see Table IV). Similar data were obtained for 2-chloroadenine that had been chosen as an additional control, because its inhibition constant is similar to those found for many 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines (see Table III). It should be noted finally, that adenine present in

Table IV. Effects of ligands on the thermal stability of the *E.coli* PNP.
E.coli PNP purified on affinity column and FPLC (0.056 mg/ml, 2.3 μM) was assayed with Ino as variable substrate (0.5 mM) at 50 mM sodium phosphate buffer pH 7.0 as described in Methods. Ligands were present at concentrations indicated. Heat treatment was for 10 min at pH 7.0 in 50 mM Hepes buffer, and in some cases (see below) in 5% MeOH.

Ligand	Concentration [mM]	% activity remaining after 10 min heat treatment at	
		57 °C	57 °C (5% MeOH)
None	-----	1	1
Adenosine	4	95	49
Adenine	4	22	3
Ribose-1-phosphate	4	100	91
6-Benzylthio-2-chloropurine (**3**)	0.034	nd	2
6-Benzyloxy-2-chloropurine (**1a**)	0.060	2	0
2-Chloroadenine	0.080	1	0
Inosine	4	62	13
Hypoxanthine	1.8	4	0
Phosphate	4.0	27	6
Adenine + phosphate	4 + 4	81	85
Hypoxanthine + phosphate	1.8 + 4.0	47	73
6-Benzylthio-2-chloropurine (**3**) + phosphate	0.034 + 4.0	nd	42
6-Benzyloxy-2-chloropurine (**1a**) + phosphate	0.060 + 4.0	76	47
2-Chloroadenine + phosphate	0.080 + 4.0	85	nd

4 mM concentration, by contrast to other purine bases, provides some protection for the *E. coli* enzyme (22% of activity remains after the heat treatment, see Table IV). It was not possible to check if presence of higher concentration of 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines is able to stabilize the enzyme due to poor solubility of these analogues in water.

Hence, it may be concluded that many 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines, similarly as the natural purine bases, are able to form the dead-end ternary complex with the *E. coli* enzyme and orthophosphate. However, binding in the case of these analogues, as well as in the case of 2-chloroadenine, is much stronger than observed for the natural purine bases, because lower concentration of ligands is enough to partially stabilize the enzyme.

Discussion

All new analogues synthesized in this study were shown to be selective inhibitors of the hexameric "high-molecular mass" PNP isolated from *E. coli*. They were inactive *vs.* trimeric enzyme from *Cellulomonas sp.*, although the later enzyme is less specific than typical trimeric "low-molecular mass" PNPs (Wielgus-Kutrowska *et al.*, 1998).

The inhibitory properties of many 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines with *E. coli* PNP markedly depend on the kind of substituents placed at positions 2 and 6 of the purine base. In agreement with the previous finding (Bzowska and Kazimierczuk, 1995; Bzowska *et al.*, 1998a), a substitution of the purine base with chlorine at position C-2 results in analogues with the inhibitory activity at least 2 orders of magnitude higher than the activity of their counterparts with no substituent (e.g. 2-chloroadenine as compared to adenine) or with other substituents at this position (e.g. 6-benzyloxy-2-chloropurine, **1a**, as compared with 2-amino-6-benzyloxy, **6a**, and 6-benzyloxy-2-trifluoromethylpurine, **6b**). In *E. coli* PNP the base binding site is largely exposed to solvent and for most purines only a weak C(8)-H hydrogen bond to Ser90 and unspecific aromatic-aromatic interactions of the purine base with Phe159/Tyr160 are observed (Mao *et al.*, 1997; Koellner *et al.*, 1998). The influence of the 2-chloro substitution could probably be explained as an enhancement of the aromatic-aromatic interactions between the purine ring

and Phe159/Tyr160 caused by changes of the electronic properties of the purine base introduced by the substituent.

The influence of the additional substituent placed at position 6 is in agreement with the results reported earlier (Bzowska and Kazimierczuk, 1995; Bzowska *et al.*, 1998a). From the series of derivatives synthesized in the present study, benzyloxy and benzylthio analoguess (**1a**, **3**) are the most potent inhibitors of the *E. coli* enzyme. The uncompetitive inhibition constants, K_{iu} are 0.59 and 0.37 μM, respectively. Therefore these two analogues, together with the neutral form of 6-methylformycin A and 6-benzyloxy-2-chloro-9-β-D-(2'-deoxyribofuranosyl)purine, with the inhibition constants 0.3 and 0.5 μM, respectively, are the best inhibitors of the *E. coli* PNP reported up to now. It should be stressed, however, that the derivatives synthesized in the present study are the only purine base analogues with affinity to the *E. coli* enzyme better than 1 μM.

Somehow surprising results were obtained during determination of the inhibition constants for the analogues synthesized in the present study. In contrast to the noncompetitive inhibition of adenine reported by Jensen (1976), for 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines at first sight inhibition seemed to be uncompetitive. However, a detailed analysis of the data for these analogues, and for inhibition by adenine and 2-chloroadenine measured in a control experiments, revealed that the reason of this discrepancy is the fact that the inhibition type is neither noncompetitive nor uncompetitive but mixed. Therefore two inhibition constants have to be introduced in all cases to describe the inhibition properly (Eqn. (1), see Methods). But in the case of adenine and 2-chloroadenine the two constants, K_{iu} and K_{ic}, have almost the same value (although the difference between the inhibitory activities of the analogues is two orders of magnitude, see Table III), so the inhibition appears noncompetitive. By contrast, for all 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines K_{iu} is several-fold lower than K_{ic}, so the inhibition seems to be uncompetitive.

It has been shown here that according to the reaction mechanism proposed by Jensen and Nygaard (1975), 6-aryloxy-2-chloro- and 6-arylalkoxy-2-chloropurines are able to form similar ternary complexes with the *E. coli* PNP as the natural substrates, i.e. catalytical complex with the enzyme/ribose-1-phosphate, and dead-end complex with the enzyme/orthophosphate. But it cannot be unequivocally concluded if these analogues are able to bind to the free enzyme. It should be noted that the same problem exists also in the case of the natural substrate adenine, which in a high concentration (4 mM) is able to partially but significantly protect the enzyme from thermal inactivation even in the absence of phosphate (see Table IV). This result does not agree with the proposed reaction mechanism for the *E. coli* PNP in which the purine bases are not able to bind to the free enzyme (Jensen and Nygaard, 1975). One explanation may be traces of phosphate in the incubation mixture. Another may be to assume that the proposed mechanism is an oversimplification of the true mechanism and that the purine bases have affinity, though very weak, to the free enzyme, and therefore are able to form binary complexes. The first explanation seems to be unlikely since traces of phosphate should result also in the stabilization by 6-benzylthio- and 6-benzyloxy-2-chloropurines, which was not observed (see Table IV). This problem could probably be resolved by a detailed kinetic analysis of the substrate and inhibitor properties of the purine bases in the synthetic and phosphorolytic directions of the PNP-catalyzed reaction or by methods that allow to determine the binding constants for possible complexes (binary and ternary ones) directly. Such studies, performed mainly with the use of fluorescence spectroscopy methods, are now underway.

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