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PURINE NUCLEOSIDE PHOSPHORYLASE: INHIBITION BY PURINE *N*(7)- AND *N*(9)-ACYCLONUCLEOSIDES; AND SUBSTRATE PROPERTIES OF 7-β-D-RIBOFURANOSYLGUANINE AND 7-β-D-RIBOFURANOSYLHYPOXANTHINE

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Abstract—A series of 10 *N*(7)- and *N*(9)-acyclonucleosides of guanine and 8-substituted guanines (8-Br, 8-SH and 8-NH_2), and two *N*(7)-acyclonucleosides of hypoxanthine, were tested for their ability to inhibit purine nucleoside phosphorylase (PNP) (E.C. 2.4.2.1) from human erythrocytes and rabbit kidney. The acyclic chains contained a nitrogen in place of a carbon at the 3′, 4′ or 5′ position and, in one case, an ether oxygen at the 2′ position. Most striking was the finding that one of the *N*(7)-acyclonucleoside analogues, 7-[(1,3-dihydroxypropyl-2)amino]ethylguanine, proved to be a 3-fold more effective inhibitor than its corresponding *N*(9) counterpart, with K_i = 5 vs 14 μM for the human enzyme and 0.7 vs 2.3 μM for the rabbit enzyme. Both analogues, as well as the others examined, inhibited phosphorolysis competitively with respect to nucleoside substrates (inosine with the human enzyme and guanosine with the rabbit enzyme). The foregoing logically led to the finding that the 7-β-D-ribosides of guanine (N^7Guo) and hypoxanthine (N^7Ino) were weak substrates of PNP from human erythrocytes, calf spleen and *E. coli*. With the human enzyme the pseudo-first-order rate constants (V_{max}/K_m) for phosphorolysis of N^7Guo and N^7Ino were 0.08 and 0.02% that for Ino. The Michaelis constants (K_m) for N^7Guo were 27 (calf PNP), 108 (human PNP) and 450 μM (*E. coli* PNP). For N^7Ino the corresponding K_m values were 1.52, 1.26 and 0.64 mM. Four previously well-characterized *N*(9)-acyclonucleoside inhibitors of calf spleen PNP were found to inhibit phosphorolysis of N^7Ino by the same enzyme 2–10-fold more effectively than the parent Ino. The overall results, along with the known excellent substrate properties of N(7)-alkyl- Guo and Ino (Bzowska *et al. J Biol Chem* **263**, 9212–9217, 1988), were examined in relation to present concepts regarding binding of substrates and inhibitors at the active site(s) of these enzymes.

Key words: purine nucleoside phosphorylase; acyclonucleosides; inhibitors/substrates; 7-β-D-guanosine; 7-β-D inosine; binding sites; kinetics

PNP¶ (E.C. 2.4.2.1.) catalyses the reversible phosphorolysis of purine nucleosides, such as inosine and guanosine, in eucaryotes, as well as adenosine in some procaryotes, e.g. *E. coli* and *S. typhimurium*:

purine nucleoside + phosphate ⇌ purine base + ribose-1-phosphate

Because of the known relationship between PNP deficiency and certain immunological disorders [1, 2], and the propensity of this enzyme to enzymatically cleave chemotherapeutically active nucleoside analogues, which are thus rendered inactive [3], considerable effort is currently being devoted to development of potent inhibitors, particularly of the human enzyme [3–22]. These approaches are accompanied by intensive studies on the mechanism of action of the enzyme from various sources [23–30].

In a continuation of earlier studies in this field [8, 25, 26] the potential inhibitory properties of some synthetic *N*(7)-acyclonucleosides, namely acyclonucleoside analogues in which the acyclic chain is linked to the N(7) of a purine, have been examined. Surprisingly, and in contrast to some previous unpublished observations with other *N*(7)-acyclonucleosides, one of these turned out to be a more effective inhibitor than its N(9)-counterpart; this in turn, led to the finding that the N(7)-isomers of Ino and Guo (Fig. 1), i.e. N^7Ino and N^7Guo, are weak substrates of the enzyme from various sources.

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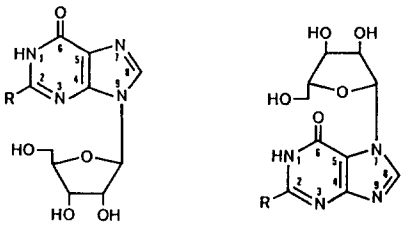
¶ Abbreviations: PNP, purine nucleoside phosphorylase; N^7Ino, 7-β-D-ribofuranosylhypoxanthine; N^7Guo, 7-β-D-ribofuranosylguanine; Ino, inosine; Guo, guanosine; br^8Gua, 8-bromoguanine; $nh_2{}^8$Gua, 8-aminoguanine; sh^8Gua, 8-thioguanine; m^7Hx, 7-methylhypoxanthine; m^9Hx, 9-methylhypoxanthine; m^7Gua, 7-methylguanine; m^9Gua, 9-methylguanine; CML, chronic myelogenous leukemia.



R = H Ino, N^7Ino

R = NH_2 Guo, N^7Guo

Fig. 1. Structures of N^7Ino and Ino (R = H), and N^7Guo and Guo (R = NH_2).

MATERIALS AND METHODS

Enzymes

Calf spleen PNP (25 U/mg), human erythrocyte PNP (20 U/mg) and xanthine oxidase from buttermilk (1 U/mg) were from Sigma (St Louis, MO, U.S.A.). Purified *E. coli* PNP (90 U/mg, 27°) was kindly supplied by Dr George Koszalka [31, 32]. Homogenous PNP from rabbit kidney (38 U/mg, 37°) was purified as described earlier [33].

Inosine and guanosine were from Sigma. Ethanolamine, 2-amino-propandiol-1,3 and trihydroxymethylaminomethane were from Fluka (Buchs, Switzerland) and Merck (Darmstadt, GFR). 7-β-D-ribofuranosylguanine [34] was a kind gift of Dr P. Garner. Compounds *2*, *3*, *9*, *10*, *11* and *12* (see Table 2), the synthesis of which will be described later, were kindly supplied by the Institute of Organic Chemistry, Latvian Academy of Sciences.

The synthesis of 9-(2′-chloroethyl)guanine and 7-(2′-chloroethyl)guanine was as described previously [35], and 9-(3′-chloro-2′-hydroxypropyl)guanine was obtained according to Piper *et al.* [36]. Syntheses of the remaining compounds are described below.

Ultraviolet absorption spectrophotometry

This was performed with Zeiss (Jena, GFR) Specord UV–VIS M40 and Kontron (Vienna, Austria) Uvikon 940 spectrophotometers, both with thermostatically controlled cell compartments, using 2, 5 and 10 mm path length cuvettes.

Kinetic constants

These were calculated from data obtained by continuous monitoring of phosphorolysis with a PDP11 minicomputer, using a program described previously [37, 38]. Inhibition constants, using the initial velocity method, were determined with a program for non-linear and weighted linear regression according to Cleland [39], with the aid of a Hewlett Packard 1000 minicomputer.

Chemical syntheses

Melting points (uncorrected) were determined with a Boetius microscope hot stage. Elemental analyses were performed with a Carlo Elementary Analizator EA 1106 (Erba, Italy) by the Chromatography Laboratory, Institute of Organic Chemistry, Latvian Academy of Sciences. NMR spectra were recorded in DMSO on a Brucker WH-90/BS spectrometer; chemical shifts are in ppm vs internal trimethylsilane. Mass spectra were obtained with an MS-50 (Kratos) spectrometer equipped with a Tech. Ltd FAB 11 NF source of argon as reagent gas.

N^7Ino synthesis (V). N^7Ino was synthesized as described in Fig. 2. Hypoxanthine (5.0 g, 36.7 mmol) was refluxed in hexamethyldisilazane (70 mL) and $(NH_4)_2SO_4$ (20 mg) until it dissolved completely. The reaction mixture was evaporated to dryness *in vacuo*. The residue was dissolved in a mixture of 1,2-dichloroethane (90 mL) and acetonitrile (30 mL), acetate *II* (9.74 g, 30.6 mmol) was added and the solution cooled to 0°. After addition of $SnCl_4$

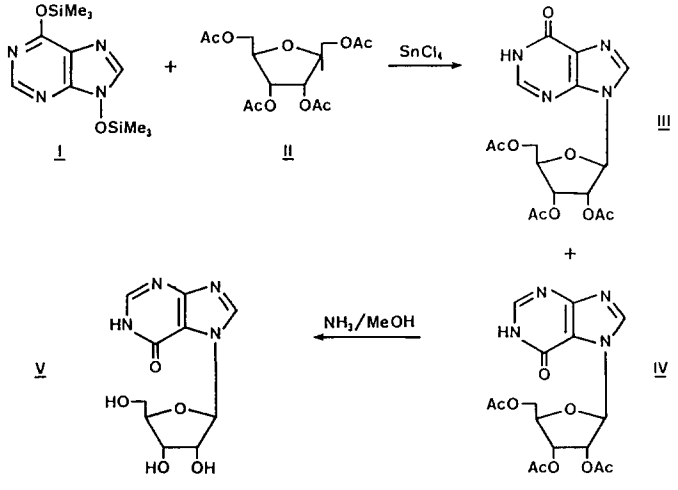


Fig. 2. Procedure for synthesis of N^7Ino.

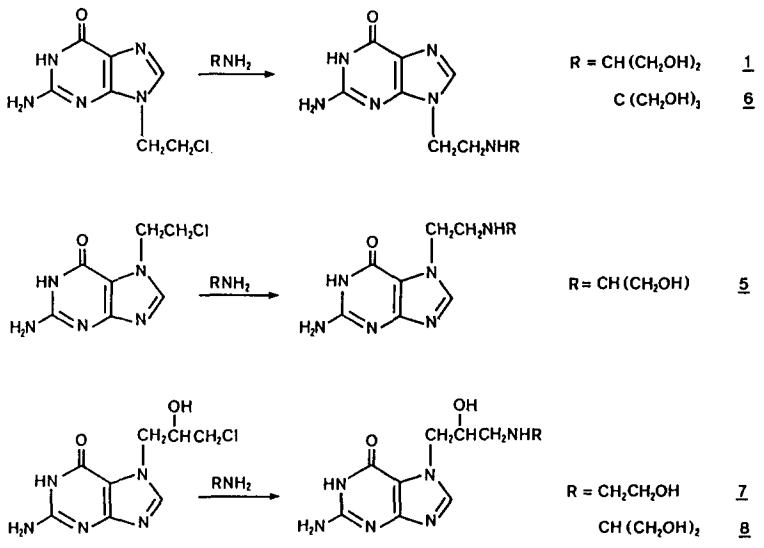


Fig. 3. Procedures for synthesis of *N*(7)- and *N*(9)-acyclonucleosides.

(3.58 mL, 30.6 mmol) the reaction mixture was left at room temperature for 72 hr. The clear solution was extracted with 100 mL aqueous $NaHCO_3$, washed with water (3 × 100 mL), dried (Na_2SO_4) and evaporated to dryness. The residue was purified on a silica gel (L 100/160, Chech) column by elution with chloroform (300 mL) and then with chloroform–ethanol–acetic acid (98:2:0.3). Fractions containing nucleoside *IV* were evaporated and treated with methanolic ammonia (100 mL) for 24 hr. Recrystallization from 85% ethanol gave *V* (3.28 g, yield 40%), m.p. 214–216°, lit. 216–218° [40]. ^{1}H-NMR (500 MHz, DMSO) δ H8 = 8.61(s), δ H2 = 8.02(s), δ H1′ = 6.19 (*d*, $J_{H1'H2'}$ = 5.5 Hz), δ OH2′ = 5.44 (*d*, $J_{OH2'H2'}$ = 5.9 Hz), δ OH3′ = 5.16 (*d*, $J_{OH3'H3'}$ = 4.9 Hz), δ OH5′ = 5.05(t), δ H2′ = 4.39 (*q*, $J_{H2'H3'}$ = 5.1 Hz), δ H3′ = 4.11 (*q*, $J_{H3'H4'}$ = 3.9 Hz), δ H4′ = 3.93 (*q*), δ H5′ = 3.68(m) ($J_{H5'H5''}$ = 12.0 Hz, $J_{OH5'H5'}$ = 4.8 Hz, $J_{H4'H5'}$ = 3.7 Hz), δ H5″ = 3.35(m) ($J_{OH5'H5''}$ = 5.8 Hz, $J_{H4'H5''}$ = 3.8 Hz).

Synthetic procedures for compounds 1 *and* 4–8. These are shown in Fig. 3. 9-[(1,3-dihydroxypropyl-2)amino]ethylguanine (1). 9-(2′-chloroethyl)guanine (0.43 g, 2 mmol) and 2-aminopropandiol-1,3 (0.54 g, 6 mmol) were dissolved in 2 mL dry butanol and heated at 120° for 5 hr. After addition of ethanol (15 mL) the mixture was left in a refrigerator for 12 hr. The precipitate was filtered and recrystallized from water, then ethanol. Yield 85%; m.p. 165° dec.; MS, FAB (M + 1) 269; anal. calcd. (found) for $C_{10}H_{16}N_6O_3H_2O$: C 41.9 (41.7), H 6.3 (5.9), N 29.4 (29.0)%; ^{1}H-NMR δ: 8-H 7.66, NH_2 6.40.

8 - Bromo - 9 - [(1,3 - dihydroxypropyl - 2)amino]-ethylguanine (4). To a solution of *1* (1.4 g, 5 mmol) in 10 mL H_2O bromine water was added dropwise until the orange colour persisted. The solvent was evaporated *in vacuo* and the residue recrystallized from ethanol. Yield 90%; m.p. 250° dec.; MS, FAB (M + 1) 429; anal. calcd. (found) for $C_{10}H_{15}N_6O_3Br$: C 28.1 (28.1), H 3.8 (3.8), N 19.6 (19.6)%; ^{1}H-NMR δ: NH_2 6.07.

7 - [(1,3 - dihydroxypropyl - 2)amino]ethylguanine (5). This was obtained as described for compound *1*, starting from 9-(2′-chloroethyl)guanine and 2-aminopropandiol-1,3. Yield 90%; MS, FAB (M + 1) 269; anal. calcd. (found) for $C_{10}H_{16}N_6O_3$: C 44.8 (44.7), H 6.0 (6.2), N 31.3 (31.3)%; ^{1}H-NMR δ: 8-H 7.84, NH_2 6.07.

9 - [(1,3 - dihydroxy - 2 - hydroxymethylpropyl - 2)-amino]ethylguanine (6). 9-(2′-Chloroethyl)-guanine (0.43 g, 2 mmol) and trihydroxymethyl-aminomethane (0.72 g, 6 mmol) were dissolved in 2 mL dry DMSO and heated at 120° for 5 hr. After addition of ethanol (15 mL) the mixture was left in a refrigerator for 12 hr. The precipitate was filtered and recrystallized from water and then ethanol. Yield 74%; m.p. 243–244°; MS, FAB (M + 1) 299; anal. calcd. (found) for $C_{11}H_{18}N_6\,O_40.5\,H_2O$: C 41.9 (41.7), H 6.1 (6.2), N 27.4 (27.4)%; ^{1}H-NMR δ: 8-H 7.64, NH_2 6.40.

7 - {2 - hydroxy - 3[(2 - hydroxyethyl)amino]propyl-1}guanine (7). This was obtained as described for compound *6*, starting from 9-(3′-chloro-2′-hydroxypropyl)guanine and ethanolamine. Yield 92%; m.p. 230° dec.; MS, FAB (M + 1) 269; anal. calcd. (found) for $C_{10}H_{16}N_6O_3H_2O$: C 41.9 (42.0), H 6.7 (6.3), N 29.4 (29.3)%; ^{1}H-NMR δ: 8-H 7.71, NH_2 6.18.

7-{2-hydroxy-3-[(1,3-dihydroxypropyl-2)amino]-propyl-1}guanine (8). 9-(3′-Chloro-2′-hydroxy-propyl)guanine (0.86 g, 4 mmol) and 2-amino-propandiol-1,3 (0.54 g, 6 mmol) were dissolved in 2 mL ethanol and heated at 120° for 5 hr. After addition of ethanol (15 mL) the mixture was left in a refrigerator for 12 hr. The precipitate was filtered and recrystallized from water and then ethanol. Yield 85%; m.p. 208–209°; MS, FAB (M + 1) 299; anal. calcd. (found) for $C_{11}H_{18}N_6O_4H_2O$: C 41.8 (41.6), H 6.4 (6.4), N 26.6 (26.5)%; ^{1}H-NMR δ: 8-H 7.76, NH_2 6.11.

Inhibitor properties

Compounds were tested for inhibition of phos-

Table 1. Spectral data for absorption, pH 7, of neutral forms of N^7Ino and N^7Guo, and spectral changes at λ_{obs} ($\Delta\varepsilon$) accompanying complete phosphorolysis of both nucleosides

Compound	λ_{max} (nm)	ε_{max} ($M^{-1}cm^{-1}$)	λ_{min} (nm)	ε_{min} ($M^{-1}cm^{-1}$)	pK_a	λ_{obs} (nm)	$\Delta\varepsilon$ ($M^{-1}cm^{-1}$)
N^7Ino	255*	8300*	229†	4070†	9.2‡	249§	4650§
						293‖	12,300‖
N^7Guo	286¶	9250¶	258	4830	10.0**	258§	3540§
	240(sh)¶	7950¶					

* Montgomery and Thomas [40] report a value of 8480 at 256 nm.
† From [40].
‡ Literature values: Ino pK_a 8.9; m^7Hx, 9.0; m^9Hx, 9.3 [50, 51].
§ Direct method.
‖ Phosphorolysis coupled with xanthine oxidase.
¶ From [34].
** Literature values: Guo, pK_a 9.2; m^7Gua, 9.95; m^9Gua, 9.8 [50–52].

phorolysis of Ino or Guo. Reactions were followed spectrophotometrically in 50 mM phosphate buffer, pH 7, either by the coupled xanthine oxidase procedure with Ino as substrate [41] or directly with Guo as substrate.

Kinetic parameters for Guo as substrate were determined by the initial velocity method only because of potent inhibition by the liberated guanine [42].

With Ino as substrate both methods, continuous monitoring and initial velocity, were employed. With continuous monitoring phosphorolysis was followed to completion and kinetic constants were evaluated by fitting the integrated form of the Michaelis–Menten equation [43, 44] to 10–20 experimental points by the least-squares method, as described previously [38]. Inhibition constants were then calculated using the kinetic parameters for Ino phosphorolysis ($K_m = 28\ \mu M$ for human PNP) as standard, as described previously [8].

Using the initial velocity method inhibition constants were determined by a program based on non-linear and weighted linear regression analysis, according to Cleland [39].

Substrate properties

Phosphorolysis of N^7Ino and N^7Guo was monitored spectrophotometrically. For N^7Guo the direct method was employed, while for N^7Ino both methods, i.e. direct spectrophotometric and coupling with xanthine oxidase [41], were used. The xanthine oxidase procedure profited from the fact that hypoxanthine is liberated during the course of the reaction of N^7Ino with PNP, as for phosphorolysis of Ino. Kinetic parameters for phosphorolysis were evaluated by the initial velocity method for both N^7Guo and N^7Ino [43], and the continuous monitoring method for N^7Ino [25, 44]. Spectral properties of both new substrates are listed in Table 1.

One unit of PNP is the amount of enzyme that converts 1 μmol of inosine to hypoxanthine/min at 25° in the presence of 50 mM phosphate buffer, pH 7.5, and an excess of xanthine oxidase.

RESULTS

All the synthetic acyclonucleoside analogues embraced in this study (see Table 2 and Fig. 4) were initially tested for inhibition of phosphorolysis of Ino (with the human erythrocyte enzyme, 50 μM inhibitor concentration, 25°) or Guo (with the rabbit kidney enzyme, 100 μM inhibitor concentration, 37°). For those which inhibited phosphorolysis by more than 50% under these conditions, inhibition constants, K_i, were determined kinetically with Ino as substrate by the continuous method (with human PNP) or by the initial velocity method (with rabbit PNP). It was first established that, at a concentration of 100 μM, none of the compounds affected the xanthine oxidase activity used in the coupled assay with Ino as substrate.

The overall results (Table 2) show that only compounds *1–6* (see Fig. 4) exhibited values of $K_i < 50\ \mu M$ for both the human and rabbit enzymes.

Position of acyclic chain

The most striking result is that analogue *5*, with the same acyclic chain as *1*, but located at N(7) of guanine, exhibits a K_i value 3-fold lower than its counterpart (*1*) ($K_i = 5$ cf 14 μM with human PNP, and $K_i = 0.7$ cf 2.3 μM with the rabbit enzyme). The *N*(7)-acyclonucleoside, like its N(9) counterpart, inhibited phosphorolysis competitively with respect to substrates.

Length and nature of acyclic chain

Compounds *7–10*, with longer acyclic chins at N(7) of guanine, are either moderate (compound *10*) or much less effective inhibitors. This was apparently in accord with previous observations that, in a series of guanine *N*(9)-acyclonucleoside inhibitors of PNP, extension of the acyclic chain led to marked decreases in affinity for the enzyme [8]. But compound *1* (see Fig. 4), which is an extended chain analogue of the known PNP inhibitor carba-2′-nordeoxyguanosine [7, 8], by insertion in the latter of an NH between C(2′) and C(3′), shows K_i values of 14 (25°) and 2.3 μM (37°) vs the human and rabbit enzymes, respectively. This is a significant improvement in

Table 2. Inhibition constants of some acyclic nucleosides with human erythrocyte PNP (Ino as variable substrate) and rabbit kidney PNP (Guo as variable substrate) in presence of 50 mM phosphate buffer, pH 7

Analogue	Base	Position of acyclic chain (or ribose)	Acyclic chain (or ribose)	K_i(μM) Human erythrocyte PNP*	Rabbit kidney PNP†
1	Gua	N(9)	$(CH_2)_2NHCH(CH_2OH)_2$	14‡ ± 4	2.3
2	nh_2^8Gua	N(9)	$(CH_2)_2NHCH(CH_2OH)_2$	~11	5
3	sh^8Gua	N(9)	$(CH_2)_2NHCH(CH_2OH)_2$	2.2 ± 0.9	0.6
4	br^8Gua	N(9)	$(CH_2)_2NHCH(CH_2OH)_2$	2.5 ± 1.0	0.6
5	Gua	N(7)	$(CH_2)_2NHCH(CH_2OH)_2$	5§ ± 2	0.7
6	Gua	N(9)	$(CH_2)_2NHC(CH_2OH)_3$	~50	15
7	Gua	N(7)	$CH_2CHOHCH_2NHCH(CH_2OH)_2$	‖	¶
8	Gua	N(7)	$CH_2CHOHCH_2NHCH_2CH_2OH$	‖	¶
9	Gua	N(7)	$[CH_2CH_2N(CH_3)_2CH_2CH_2OH]^+Cl^-$	‖	¶
10	Gua	N(7)	$CH_2O(CH_2)_2NHCH(CH_2OH)_2$	~60	82
11	Hx	N(9)	$(CH_2)_2NHCH(CH_2OH)_2$	**	¶
12	Hx	N(7)	$(CH_2)_2NHCH(CH_2OH)_2$	**	¶
–	Guo	N(7)	Ribose	~300††	

* K_i determined at 25° with the continuous spectrophotometric assay from increase of K_m and decrease of V_{max}/K_m for Ino phosphorolysis in the presence of inhibitor (see Materials and Methods).
† K_i at 37° from initial velocity method (see Materials and Methods).
‡ K_i^{app} in presence of 1 mM phosphate and 50 mM Tris buffer, pH 7, was (41 ± 7) μM.
§ K_i^{app} in presence of 1 mM phosphate and 50 mM Tris buffer, pH 7, was (18 ± 5) μM.
‖ No inhibition at 50 μM concentration of the analogue.
¶ Inhibition of guanosine phosphorolysis less than 50% at 100 μM concentration of the analogue.
** Not determined.
†† Inhibition constant determined by initial velocity method with calf spleen enzyme and Ino as a substrate.

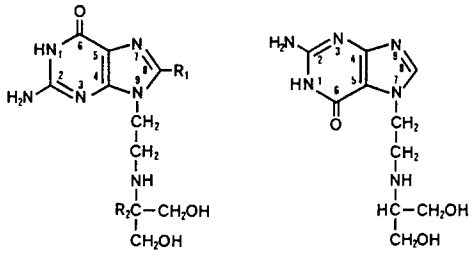


Fig. 4. Structures of some synthetic acyclonucleoside inhibitors of PNP. Figures in brackets are K_i values (μM) for inhibition of human erythrocyte PNP.

affinity for the human enzyme over carba-2′-nordeoxyguanosine (K_i = 36–38 μM at 25° [7, 8]).

It should also be noted that compounds *1–5* contain a chiral centre at C(4′), so that each is a racemic mixture of two enantiomers. If one of these is more active as an inhibitor, as appears most likely [8, 15], these compounds may be even more potent than indicated in Table 2 and Fig. 4.

Role of aglycon

For two of the compounds with good inhibitory properties (*1* and *5*) it is of interest that replacement of the guanine aglycon by hypoxanthine, to give *11* and *12* (Table 2), led to a dramatic drop in inhibitory activity. This is in accord with reports that the C(2) amino group of guanine is a hydrogen-bond donor in N(9) substrates and inhibitors [10, 24, 28] of the mammalian enzymes. It is also consistent with the authors' proposal (see below) concerning the mode of binding of unusual N(7) substrates and inhibitors at the active site of PNP.

Role of an aglycon 8-substituent

It is well known that some purine 8-substituents, especially NH_2, may enhance affinity of an inhibitor for the enzyme [3, 4, 6, 14, 16, 21]. Therefore, analogues of *1* with NH_2 (*2*), SH (*3*) and Br (*4*) groups at C(8) of guanine were prepared. It was seen (Table 2 and Fig. 4) that two of these, SH and Br, led to more potent inhibitors, with K_i values 4- to 6-fold lower than that for *1* (see also Table 4), while an NH_2 substituent did not modify affinity for either the human or rabbit enzymes.

Effect of phosphate concentration

It was first noted by Tuttle and Krenitsky [18] and Krenitsky *et al.* [19] that the moderate inhibition of human erythrocyte PNP by acyclovir pyrophosphate is dramatically enhanced when the phosphate

concentration in the incubation medium is decreased from the usually employed saturating 50 mM to 1 mM (intracellular phosphate concentration). This was ascribed to the comportment of acyclovir pyrophosphate as a bisubstrate analogue inhibitor. More recently Montgomery *et al.* [21] and Secrist *et al.* [22] prepared a series of 9-arylmethyl and 9-cykloalkylmethyl derivatives of 9-deazaguanine with IC_{50} values for inhibition of calf spleen PNP in the micromolar range at a phosphate concentration of 50 mM. Reduction of the phosphate concentration to 1 mM led to decreases in K_i values of up to 20-fold. For two of our inhibitors (*5* and *1*), which differ only in that the acyclic chain is at N(7) or N(9) of guanine, respectively, it was found that the K_i values increased 3-fold at the lower phosphate concentration, from 5 to 18 μM for *5*, and from 14 to 41 μM for *1*.

Inhibition of phosphorolysis of Ino by N⁷Guo

The products of phosphorolysis of Ino and Guo, Hx and Gua, are potent inhibitors of the reaction as well as substrates in the reverse reaction [23, 45, 46]. By contrast, alkylated purines are relatively poor inhibitors. Elsewhere it has been shown that, with the calf spleen enzyme, the K_i values for inhibition of phosphorolysis of Ino and 7-alkylGuo by a series of 7-alkyalguanines are quite high, in the range of 0.45–0.65 mM [25]. This further testifies to the specificity of the *N*(7)-acyclonucleosides as inhibitors of the phosphorolytic reaction and raises the question as to whether N⁷Guo is an inhibitor. When the latter was tested for potential inhibition of Ino phosphorolysis with the calf spleen enzyme, it yielded a K_i of ~0.3 mM, significantly lower than the K_i values of the 7-aklylguanines.

Substrate properties of N⁷Ino and N⁷*Guo*

The low, but significant, inhibition of Ino phosphorolysis by N⁷Guo is in line with the most striking finding of the present study, namely the behaviour of compounds *1* [with the acyclic chain at N(9) of guanine] and *5* [with the acyclic chain at N(7) of guanine]. The observation that an *N*(7)-acyclonucleoside of guanine may bind to mammalian PNP more effectively than its N(9) counterpart (see Table 2) pointed to the desirability of examining possible substrate activity of N⁷Ino and N⁷Guo.

Using standard spectrophotometric techniques for following phosphorolysis of purine nucleosides, it was initially noted that exposure of both N⁷Ino and N⁷Guo to mammalian (calf spleen and human erythrocyte), as well as bacterial (*E. coli*), enzymes led to their conversion to products with absorption spectra corresponding to hypoxanthine and guanine, respectively.

The reaction of N⁷Ino with *E. coli* PNP, pH 7 (data not shown), led to a shift in the absorbance maximum from 255 to 250 nm and an increase in the height of the absorbance maximum with an isosbestic point at 258 nm, all consistent with formation of hypoxanthine ($\lambda_{max} = 249.5$ nm, $\varepsilon_{max} = 10{,}700\ M^{-1}\ cm^{-1}$ for Hx as compared to $\lambda_{max} = 255$ nm and $\varepsilon_{max} = 8300\ M^{-1}\ cm^{-1}$ for N⁷Ino, see Table 1). Due to potent inhibition by the liberated hypoxanthine

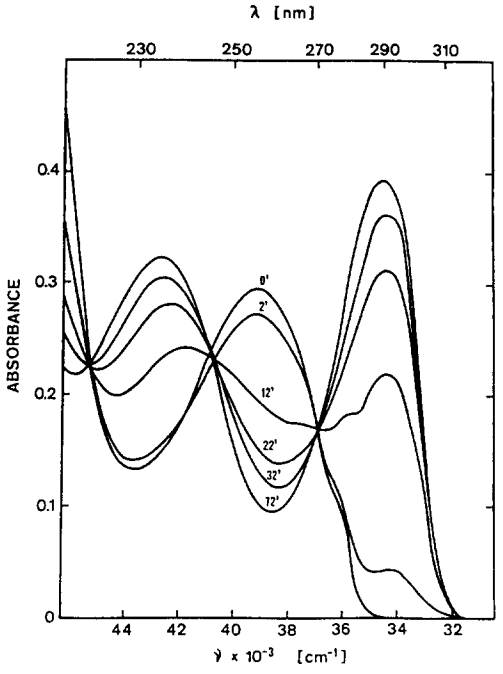


Fig. 5. Enzymatic phosphorolysis of N⁷Ino catalysed by calf spleen PNP (0.015 U/mL) in 50 mM phosphate buffer, pH 7, at 25°. Phosphorolysis was coupled with xanthine oxidase (0.15 U/mL), so that the reaction went to completion. Quantitative formation of uric acid from hypoxanthine liberated in the course of phosphorolysis of N⁷Ino is shown by isosbestic points at 221, 246 and 270 nm. The curve marked "0" is that for N⁷Ino; figures beside the other curves represent the time of phosphorolysis (min).

[23, 45, 46], the latter stage of phosphorolysis proceeds very slowly, but addition of an excess of xanthine oxidase to the reaction mixture (0.029 U/mL) instantly converts the product of the reaction to uric acid, $\lambda_{max} = 293$, $\varepsilon_{max} = 12{,}300\ M^{-1}\ cm^{-1}$, proving that hypoxanthine is formed in the course of the reaction of N⁷Ino with PNP.

Figure 5 presents the spectral changes accompanying complete phosphorolysis of N⁷Ino catalysed by calf spleen PNP. The reaction is coupled with xanthine oxidase, so that phosphorolysis proceeds to completion. Quantitative formation of uric acid from N⁷Ino is shown by isosbestic points at 221, 246 and 270 nm, proving once again that the glycosidic bond of N⁷Ino is phosphorolysed by the calf enzyme with liberation of hypoxanthine and ribose-1-phosphate. Similar results were obtained for human erythrocyte and *E. coli* PNP (data not shown).

Continuous monitoring at 293 nm of phosphorolysis of N⁷Ino catalysed by human erythrocyte PNP (0.8–3.2 U/mL) and *E. coli* PNP (0.4–2.8 U/mL) shows: (1) first-order kinetics with $V_{max}/K_m = 0.07$ and 0.23% that for inosine, respectively (Table 3), indicating that the K_m values for the calf enzyme are high relative to initial substrate concentrations employed ($c_0 = 40$ and 30 μM,

Table 3. Substrate properties of N^7Ino and N^7Guo with calf spleen, human erythrocyte and *E. coli* purine nucleoside phosphorylases

	K_m(μM)			V_{max}(%)			V_{max}/K_m(%)		
Substrate	Calf	Human	*E. coli*	Calf	Human	*E. coli*	Calf	Human	*E. coli*
Ino	13*	~28*	32†	100*	100*	100†	100*	100*	100†
N^7Ino†	1520	1260	640	26	2.3	3.4	0.23	0.08	0.17
							0.26‡	0.07§	0.23‖
Guo	11	46¶	~20	220	90¶		260	80¶	110
N^7Guo**	27	108	~450	0.6	0.09	33	0.3	0.02	2

* Continuous monitoring method, phosphorolysis coupled with xanthine oxidase.
† Initial velocity method, phosphorolysis coupled with xanthine oxidase.
‡ With initial substrate concentration, $c_0 = 35$ μM pseudo-first order kinetics observed, indicating $K_m \gg c_0$.
§ With $c_0 = 40$ μM pseudo-first order kinetics observed, indicating $K_m \gg c_0$.
‖ With $c_0 = 30$ μM pseudo-first order kinetics observed, indicating $K_m \gg c_0$.
¶ At pH 7.5, from [18].
** Initial velocity method, phosphorolysis monitored directly.

respectively); (2) linear dependence of the rate constant on enzyme concentration, in accord with enzyme-catalysed phosphorolysis of N^7Ino.

With the initial velocity method K_m and V_{max} values were determined for N^7Ino as a substrate of all three enzymes. Linear regression analysis from Eadie-Hofstee plots of initial velocities v_0 *vs* v_0/c_0 gave: $K_m = 1260$ μM and $V_{max} = 2.3\%$ for Ino with the human enzyme; $K_m = 1520$ μM and $V_{max} = 26\%$ with the calf spleen enzyme; and $K_m = 640$ μM and $V_{max} = 3.4\%$ with *E. coli* PNP (Table 3).

Figure 6A presents spectral modifications accompanying phosphorolysis of N^7Guo catalysed by calf spleen PNP. These are consistent with guanine formation ($\lambda_{max} = 276$, $\varepsilon_{max} = 8200\ M^{-1}\ cm^{-1}$ and $\lambda_{max} = 246$, $\varepsilon_{max} = 10{,}700\ M^{-1}\ cm^{-1}$ as compared to $\lambda_{max} = 286$ nm and $\varepsilon_{max} = 9250\ M^{-1}\ cm^{-1}$ for N^7Guo, Table 1), although phosphorolysis proceeds very slowly in the last stage of the reaction due to very potent inhibition by the liberated guanine [23, 45]. Quantitative formation of guanine from N^7Guo is shown by isosbestic points at 231 and 280 nm. Similar changes were observed with the human and *E. coli* enzymes (data not shown).

With the initial velocity method, K_m and V_{max} were determined for N^7Guo and three of the enzymes used in this study. Linear regression analysis of Eadie–Hofstee plots for initial velocities v_0 *vs* v_0/c_0 gives: $K_m = 108$ μM and $V_{max} = 0.09\%$ for Ino with the human enzyme (Fig. 6B) and $K_m = 27$ μM and $V_{max} = 0.6\%$ for Ino for the calf spleen enzyme (data not shown). For *E. coli* PNP the initial velocity method gave V_{max}/K_m 2% that for Ino, and a rather high K_m (~450 μM) in comparison with mammalian phosphorylases.

Quantitative data for phosphorolysis of Ino, N^7Ino, Guo and N^7Guo with calf spleen, human erythrocyte and *E. coli* phosphorylases are listed in Table 3.

Inhibition of N^7Ino phosphorolysis by N(9)-acyclonucleoside inhibitors of Ino phosphorolysis

In view of the foregoing findings, it appeared desirable to examine the effect on phosphorolysis of N^7Ino of some known *N*(9)-acyclonucleoside inhibitors of phosphorolysis of Ino. Using the calf spleen enzyme, four previously reported inhibitors of Ino phosphorolysis [8] were tested for potential inhibition of N^7Ino phosphorolysis (results listed in Table 4). It will be seen that all four were more effective inhibitors of phosphorolysis of N^7Ino than of Ino, from 2-fold for compounds *a* and *d* to as much as 10-fold for compound *c*. An additional, rather striking, feature is the behaviour of *d*, the 8-bromo analogue of *a*, which is 3.5-fold more potent than *a* in inhibiting phosphorolysis of both Ino and N^7Ino. This is qualitatively in accord with the observation (Table 2 and Fig. 4) that compound *4*, the 8-bromoanalogue of *1*, is a 5-fold more potent inhibitor of Ino phosphorolysis.

DISCUSSION

It has previously been shown [8] that the 3′-position of the acyclic chains plays an important role in the binding of guanine *N*(9)-acyclonucleosides to mammalian phosphorylases, e.g. a hydroxyl substituent at C(3′) substantially lowers the inhibition constants. It has been demonstrated in this work that enhanced affinity for the enzyme also results from replacement of C(3′) by an NH group (analogue *1*), and that this affinity is further increased by introduction of substituents such as Br or SH at the C(8) ring of the aglycon.

Most striking, however, is the observation that displacement of the same chain from the normal N(9) position (analogue *1*) to the N(7) position, to give analogue *5*, resulted in a 3-fold increase in affinity. This points to the possible development of additional *N*(7)-acyclonucleosides as a new class of PNP inhibitors.

The foregoing also suggested conceivable binding of N(7)-nucleosides by PNP. And, notwithstanding that N^7Guo is a poor inhibitor of Ino phosphorolysis, both N^7Guo and N^7Ino proved to be substrates, albeit weak ones, of the enzyme from several different sources (Table 3). Although N(7) is probably not a binding site, but is necessary only for

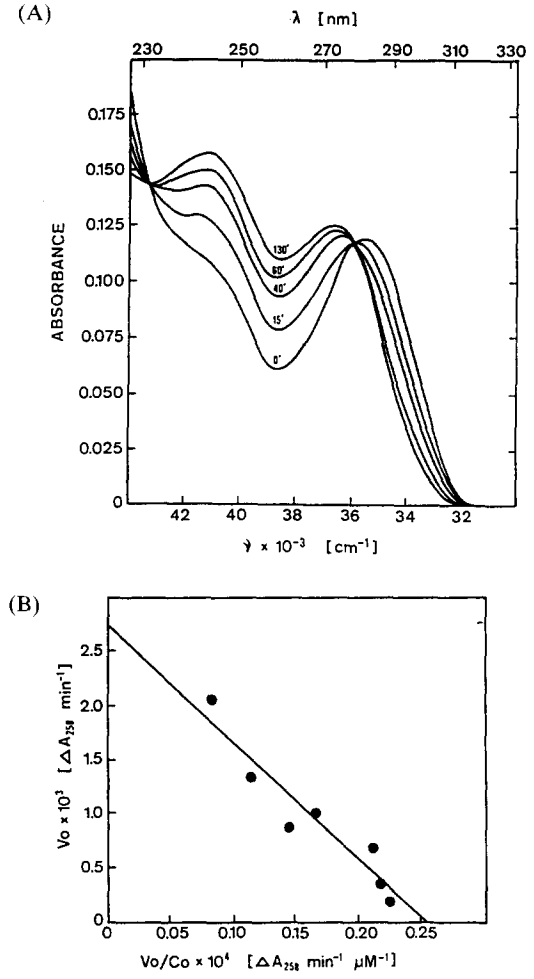


Fig. 6(A). Enzymatic phosphorolysis of N^7Guo catalysed by calf spleen PNP (0.061 U/mL) in 50 mM phosphate buffer, pH 7, at 25°. Quantitative formation of guanine is shown by isosbestic points at 231 and 280 nm. The curve marked "0" is that for N^7Guo; figures beside the other curves represent the time of phosphorolysis (min) (see text for further details). (B) Eadie–Hofstee plot for phosphorolysis of N^7Guo catalysed by human erythrocyte PNP in 50 mM phosphate buffer, pH 7 at 25°. The concentration of the enzyme was 0.84 U/mL and the absorption was monitored at 258 nm. The estimated values of kinetic parameters from this plot are: K_m = 108 μM and V_{max} = 0.09% of that for inosine.

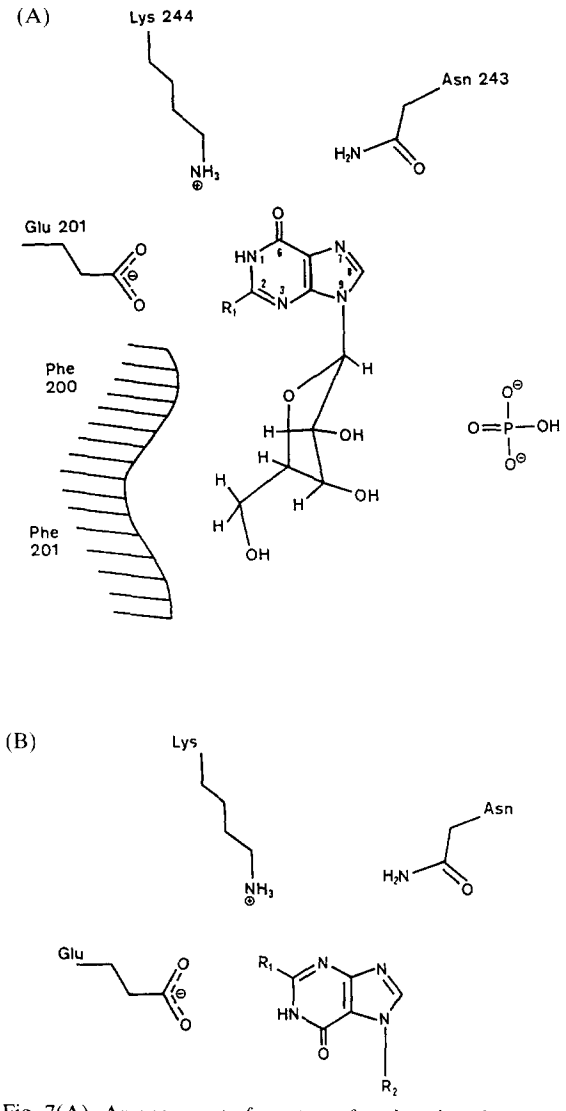


Fig. 7(A). Arrangement of reactants for phosphorolysis in the active site of human erythrocyte PNP (adapted from [10]). R_1 = H for Ino and R_1 = NH_2 for Guo. (B) Possible arrangement of N(7)-analogues in the active site of PNP. R_1 = H for N^7Ino and R_1 = NH_2 for N^7Guo; R_2 = ribose for nucleosides and R_2 = acyclic chain for acyclonucleosides.

Table 4. K_i values for inhibition of phosphorolysis (calf spleen enzyme in 50 mM phosphate buffer, pH 7) of Ino and N^7Ino by some N(9) acyclonucleosides

Analogue	Base	Acyclic chain	Enantiomer	K_i(μM) vs Ino* as substrate	K_i(μM) vs N^7Ino† as substrate
a	Gua	$(CH_2)_2CHCH_2OH$	(*R*)	24	11.4 ± 0.5
b	Gua	$CH_2CHN_3CHOHCH_2OH$	(±)threo	3.6	0.8 ± 0.3
c	Gua	$CH_2CHFCH_2CH_2OH$	(*R,S*)	29	2.8 ± 0.8
d	br^{8}Gua	$(CH_2)_2CHCH_2OH$	(*R*)	7.3	3.2 ± 1.0

* From [8]; K_i determined at 25° by the continuous spectrophotometric assay from the increase in K_m and decrease of V_{max}/K_m for Ino phosphorolysis in the presence of inhibitor (see Materials and Methods).

† K_i determined at 25° by the continuous spectrophotometric assay from the decrease of V_{max}/K_m for N^7Ino phosphorolysis in the presence of inhibitor (see Materials and Methods).

substrate activity [25, 26], this appears, at first sight, inconsistent with X-ray diffraction data [10, 27, 28] for binding, by human erythrocyte PNP, of the guanine and hypoxanthine aglycon of substrates and inhibitors, with the exocyclic O^6 and the ring N(7) hydrogen-bonded to the amino hydrogens of Asn^{243}, as shown in Fig. 7A [28]. Ribose at N(7) would sterically impede such interaction (see below).

However, bearing in mind that phosphate is also a substrate, which must bind in the vicinity of C(1′) to catalyse phosphorolysis of the glycosidic bond (Fig. 7A), it necessarily follows that the N(7)-nucleoside should be in an "upside-down" form which formally resembles the normal N(9)-nucleoside substrates, as in Fig. 7B.

Human erythrocyte PNP is considered to bind the pyrimidine moiety of the purine aglycone *via* three (inosine) or four (guanosine) hydrogen bonds [24, 27, 28], with O^6 as acceptor of two hydrogen bonds and the hydrogens of N(1)-H and C(2)-NH_2 as donors (Fig. 7A). With N(7)-nucleoside substrates in an "upside-down" form, it is equally possible that there are two hydrogen bonds with N(1)-H and the ring N(3) of N^7Ino and a third with the C(2)-NH_2 of N^7Guo (Fig. 7B). This is consistent with the lower K_m values of N^7Guo relative to N^7Ino with all mammalian enzymes examined (see Table 3). Furthermore, the NH_2 of Asn^{243} may form a hydrogen bond to N(9) of the N(7)-nucleosides (Fig. 7B), but the same Asn^{243} may compete with the NH_3^{-+} of Lys^{244} for hydrogen bonding to the ring N(3).

X-ray diffraction studies [10, 21, 22, 28] on human PNP and inhibitory activity of 9-substituted guanines pointed to the exocyclic O^6 and the ring N(7) as hydrogen-bond acceptors of the NH_2 protons of Asn^{243}. However, this scheme is not applicable to all substrates, since it has been shown elsewhere [25, 26] that N(7)-alkyl and N(7)-aryl guanosines are good, or even better, substrates then the parent Guo. These results are consistent with the theory which postulated that phosphorolysis of Ino and Guo proceeds via intermediate protonation of the ring N(7) [26]. With the N(7) blocked, and concomitant appearance of a positive charge distributed between the two imidazole ring nitrogens, hydrogen bonding to this position is excluded.

Relevant to the foregoing description is a report [47] that crude extracts of *E. coli* contain an activity that readily converts N^7Guo to guanine, and the 3-deaza analogue of N^7Guo, an active antibacterial agent, to 3-deazaguanine. With the aid of various criteria, this activity was identified as a purine nucleoside phosphorylase. Extracts from various mammalian cells were found to be inactive against 3-deaza-N^7Guo and, in contrast to the present findings, against N^7Guo. The latter result may have been due to the low specific activity of the crude extracts employed by these authors. Surprisingly, the *E. coli* extracts readily cleaved Guo, as expected, but not 3-deaza-Guo, whereas both were phosphorolysed by the mammalian extracts. This is most likely related to the reported differences in specificities between the bacterial and mammalian enzymes [24]. It is also relevant, in this context, to recall that 3-deazaIno is a substrate for both the *E. coli* and calf spleen enzymes [24].

Finally, attention should be drawn to the natural occurence, albeit uncommon, of purine N(7)-nucleosides, particularly underlined by the identification and isolation of N^7Ino in the urine of CML patients, but not from healthy individuals or those with other types of tumours [48]. The source of this metabolite has not been definitively established, but may be associated with the known abnormally high levels of bound vitamin B_{12} in the plasma of patients with myeloproliferative diseases such as CML. Some vitamin B_{12} related analogues have been reported to contain purine N(7)-ribosides [49]; and it has been demonstrated that pseudovitamin B_{12}, following deamination, is converted to a so-called factor G, containing the α-anomer of N^7Ino [49]. The N^7Ino isolated from the urine of CML patients may thus be derived from a vitamin B_{12} analogue present in their plasma [48]. It is, of course, also conceivable that the source of N^7Ino in CML patients may be some reaction catalysed by specific transglycosylases.

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REFERENCES

1. Giblett ER, Ammann AJ, Wara DW, Sandman R and Diamond LK, Nucleoside phosphorylase deficiency in a child with severely defective T-cell immunity and normal B-cell immunity. *Lancet* **1**: 1010–1013, 1975.
2. Ammann AJ, Immunological aberrations in purine nucleoside deficiency. *Found Symp* **68**: 55–75, 1978.
3. Stoeckler, JD, Cambor C, Kuhns V, Chu S-H and Parks Jr RE, Inhibitors of purine nucleoside phosphorylase: C(8) and C(5′) substitutions. *Biochem Pharmacol* **31**: 163–171, 1982.
4. Stoeckler JD, Ealick SE, Bugg CE and Parks Jr RE, Design of purine nucleoside phosphorylase inhibitors. *Fed Proc* **45**: 2773–2778, 1986.
5. Stoeckler JD, Ryden JB, Parks Jr RE, Chu M-Y, Lim M-I, Ren W-Y and Klein RS, Inhibition of purine nucleoside phosphorylase: Effects of 9-deazapurine ribonucleosides and synthesis of 5′-deoxy-5′-iodo-9-deazainosine. *Cancer Res* **46**: 1774–1778, 1986.
6. Shewach DS, Chern J-W, Pillote KE, Townsend LB and Daddona PE, Potentiation of 2′-deoxyguanosine cytotoxicity by a novel inhibitor of purine nucleoside phosphorylase, 8-amino-9-benzyl-guanine. *Cancer Res* **46**: 519–523, 1986.
7. Stein JM, Stoeckler JD, Li S-Y, Tolman RL, MacCoss M, Chen A, Karkas JD, Ashton WT and Parks Jr RE, Inhibition of human purine nucleoside phosphorylase by acyclic nucleosides and nucleotides. *Biochem Pharmacol* **36**: 1237–1244, 1987.
8. Bzowska A, Kulikowska E, Shugar D, Bing-yi C, Lindborg B and Johansson NG, Acyclonucleoside analogue inhibitors of mammalian purine nucleoside phosphorylase. *Biochem Pharmacol* **41**: 1791–1803, 1991.
9. Dempcy RO and Skibo EB, Rational design of quinazoline-based irreversible inhibitors of human

erythrocyte purine nucleoside phosphorylase. *Biochemistry* **30**: 8480–8487, 1991.
10. Ealick SE, Babu YS, Narayana SVL, Cook WJ and Bugg CE, Design of purine nucleoside phosphorylase inhbitors using X-ray crystallography. In: *Crystallographic and Modelling Methods in Molecular Design* (Eds. Bugg CE and Ealick SE), pp. 43–55. Springer-Verlag, New York, 1990.
11. Nakamura CE, Chu S-H, Stoeckler JD and Parks Jr RE, Inhibition of purine nucleoside phosphorylase by phosphonoalkylpurines. *Nucleosides Nucleotides* **8**: 1039–1040, 1989.
12. Nakamura CE, Chu S-H, Stoeckler JD and Parks Jr RE, Inhibition of purine nucleoside phosphorylase by 9-(phosphonoalkyl)hypoxanthines. *Biochem Pahrmacol* **35**: 133–136, 1986.
13. Gilbertsen RB, Josula U, Sircar JC, Dong MK, Wu W-S, Wilbrun DJ and Conroy MC, Comparative *in vitro* and *in vivo* activities of two 9-deazaguanine analog inhibitors of purine nucleoside phosphorylase, CI-972 and PD 141955. *Bichem Pharmacol* **44**: 996–999, 1992.
14. Woo PWK, Kostlan CR, Sircar JC, Dong MK and Gilbertsen RB, Inhibitors of human purine nucleoside phosphorylase. Synthesis and biological activities of 8-amino-3-benzylhypoxanthine and related analogues. *J Med Chem* **35**: 1451–1457, 1992.
15. Ealick SE, Babu YS, Bugg CE, Erion MD, Guida WC, Montgomery JA and Secrist III JA, Application of crystallographic and modeling methods in the design of purine nucleoside phosphorylase inhibitors. *Proc Natl Acad Sci* **88**: 11540–11544, 1991.
16. Sircar JC, Kostlan CR, Pinter GW, Suto JM, Bobovski TP, Capiris T, Schwender CF, Dong MK, Scott ME, Bennett MK, Kossarek LM and Gilbertsen RB, 8-Amino-9-substituted guanines: Potent purine nucleoside phosphorylase (PNP) inhibitors. *Agents Actions* **21**: 253–256, 1987.
17. Sircar JC, Kostlan CR, Gilbertsen RB, Bennett MK, Dong MK and Cetenko WJ, Inhibitors of purine nucleoside phosphorylase. Synthesis of pyrrolo[3, 2-d] pyrimidines, a new class of purine nucleoside phosphorylase inhibitors as potentially T-cell selective immunosuppressive agents. Description of 2, 6-diamino-3,5-dihydro-7-(3-thienylmethyl)-4H-pyrrolo-[3, 2-d]pyrimidin-4-one. *J Med Chem* **35**: 1605–1609, 1992.
18. Tuttle JV and Krenitsky TA, Effects of acyclovir and its metabolites on purine nucleoside phosphorylase. *J Biol Chem* **259**: 4065–4069, 1984.
19. Krenitsky TA, Tuttle JV, Miller WH, Moorman AR, Orr GF and Beauchamp L, Nucleotide analogue inhibitors of purine nucleoside phosphorylase. *J Biol Chem* **265**: 3066–3069, 1990.
20. Sheen MR, Kim BK and Parks Jr RE, Purine nucleoside phosphorylase from human erythrocytes. III. Inhibition by the inosine analogue formycin B of the isolated enzyme and of nucleoside metabolism in intact erythrocytes and Sarcoma 180 cells. *Mol Pharmacol* **4**: 293–299, 1968.
21. Montgomery JA, Niwas S, Rose JD, Secrist III JA, Babu YS, Bugg CE, Erion MD, Guida WC and Ealick SE, Structure-based design of inhibitors of purine nucleoside phosphorylase. 9-(Arylmethyl) derivatives of 9-deazaguanine. *J Med Chem* **36**: 55–69, 1993.
22. Secrist III JA, Niwas S, Rose JD, Babu S, Bugg CE, Erion MD, Guida WC, Ealick SE and Montgomery JA, Structure-based design of inhibitors of purine nucleoside phosphorylase. 2. 9-Alicyclic and 9-heteroalicyclic derivatives of 9-deazaguanine. *J Med Chem* **36**: 1847–1854, 1993.
23. Stoeckler JD, Purine nucleoside phosphorylase: a target for chemotherapy. In: *Developments in Cancer Chemotherapy* (Ed. Glazer RI), pp. 35–60 CRC Press Inc., Boca Raton, FL, 1984.
24. Bzowska A, Kulikowska E and Shugar D, Properties of purine nucleoside phosphorylase (PNP) of mammalian and bacterial origin. *Z Naturforsch* **45c**: 59–70, 1990.
25. Bzowska A, Kulikowska E, Darzynkiewicz E and Shugar D, Purine nucleoside phosphorylase: Structure-activity relationships for substrate and inhibitor properties of N-1, N-7 and C-8-substituted analogues; Differentiation of mammalian and bacterial enzymes with N-1 methylinosine and guanosine. *J Biol Chem* **263**: 9212–9217, 1988.
26. Bzowska A, Kulikowska E and Shugar D, Linear free energy relationships for N(7)-substituted guanosines as substrates of calf spleen purine nucleoside phosphorylase. Possible role of N(7)-protonation as an intermediary in phosphorolysis. *Z Naturforsch* **48c**: 803–811, 1993.
27. Cook WJ, Ealick SE, Bugg CE, Stoeckler JD and Parks Jr., RE, Crystallization and preliminary X-ray investigation of human erythrocytic purine nucleoside phosphorylase. *J Biol Chem* **256**: 4079–4080, 1981.
28. Ealick SE, Rule SA, Carter DC, Greenhough TJ, Babu YS, Cook WJ, Habash, J., Halliwell JR, Stoeckler JD, Parks, Jr., RE, Chen SF and Bugg CE, Three-dimensional structure of human erythrocytic purine nucleoside phosphorylase at 3.2 A resolution. *J Biol Chem* **265**: 1812–1820, 1990.
29. Porter DJT, Purine nucleoside phosphorylase. Kinetic mechanism of the enzyme form calf spleen. *J Biol Chem* **267**: 7342–7351, 1992.
30. Kline PC and Schramm VL, Purine nucleoside phosphorylase. Inosine hydrolysis, tight binding of the hypoxanthine intermediate, and third-the-sites reactivity. *Biochemistry* **31**: 5964–5973, 1992.
31. Krenitsky TA, Koszalka GW and Tuttle JV, Purine nucleoside synthesis, an efficient method employing nucleoside phosphorylase. *Biochemistry* **20**: 3615–3621, 1981.
32. Lehikoinen PK, Sinnott ML and Krenitsky TA, Investigation of α-deuterium isotope effects on the purine nucleoside phosphorylase reaction by the equilibrium-perturbation technique. *Biochem J* **257**: 355–359, 1989.
33. Ananiev AV, Bezirjian Kh O and Akopian Zh I, A comparative study of purine nucleoside phosphorylase from rabbit kidney, spleen, liver and embryos. *Biokhimya* **52**: 2022–2028, 1987.
34. Garner P and Ramakanth S, A regiocontrolled synthesis of N^7- and N^9-guanine nucleosides. *J Org Chem* **53**: 1294–1298, 1988.
35. Ramzaeva N, Alksins E, Goldberg Yu and Lidakas M, Alkylation of 6-methylthio and 6-benzyloxyguanine under phase-transfer conditions. *Synth Commun* **19**(18): 3121–3128, 1989.
36. Piper JR, Laseter AG and Montgomery JA, Synthesis and potential inhibition of hypoxanthine-guanine phosphoribosyl-transferase for testing for antiprotozoal agents. I. 7-substituted 6-oxopurines. *J Med Chem* **23**: 357–364, 1980.
37. Wierzchowski J and Shugar D, Sensitive fluorometric assay for adenosine deaminase with formycin as substrate; and substrate and inhibitor properties of some pyrazolopyrimidines and related analogues. *Z Naturforsch* **38c**: 67–73, 1983.
38. Wierzchowski J, Lassota P and Shugar D, Continuous fluorometric assay of 5′-nucleotidase with formycin 5′-phosphate as substrate, and its application to properties of substrates and inhibitors. *Biochim Biophys Acta* **786**: 170–178, 1984.
39. Cleland WW, The statistical analysis of enzyme kinetic data. *Adv Enzymol* **29**: 1–32, 1967.

40. Montgomery JA and Thomas HJ, Ribosyl derivatives of hypoxanthine. *J Org Chem* **34**: 2646–2650, 1969.
41. Stoeckler JD, Agarwal RP, Agarwal KC, Parks Jr RE, Purine nucleoside phosphorylase from human erythrocytes. *Methods Enzymol* **51**: 530–538, 1978.
42. Kim BK, Cha S and Parks Jr RE, Purine nucleoside phosphorylase. 1. Purification and properties. *J Biol Chem* **243**: 1763–1770, 1968.
43. Segal IH, *Enzyme Kinetics*. John Wiley and Sons, New York, 1975.
44. Wierzchowski J, Sierakowska H and Shugar D, Continuous fluorometric assay of nucleotide pyrophosphatase. Kinetics, inhibition and extention to dinucleotide oligophosphates. *Biochim Biophys Acta* **828**: 109–115, 1985.
45. Krenitsky TA, Elion GB, Henderson AM and Hitchings GH, Inhibition of human purine nucleoside phosphorylase. Studies with intact erythrocytes and purified enzyme. *J Biol Chem* **243**: 2876–2881, 1968.
46. Kulikowska E, Bzowska A, Wierzchowski J and Shugar D, Properties of two unusual, and fluorescent substrates of purine nucleoside phosphorylase: 7-methylguanosine and 7-methyl-inosine. *Biochim Biophys Acta* **874**: 355–363, 1986.
47. Streeter DG, Miller M, Matthews TR, Robins RK and Miller JP, 7-Ribosyl-3-deazaguanine—mechanism of antibacterial action. *Biochem Pharmacol* **29**: 1791–1797, 1980.
48. Chheda GB, Dutta SP, Mittelman A, Montgomery JA, Sethi SK, McCloskey JA and Patrzyc HB, Isolation and characterization of a novel nucleoside, 7-β-D-ribofuranosyl-hypoxanthine from the urine of a chronic myelogenous leukemia patient. *Cancer Res* **45**: 5958–5963, 1985.
49. Bonnett R, The chemistry of the vitamin B12 group. *Chem Rev* **63**: 537–605, 1963.
50. *Data for Biochemical Research* (Eds. Davson RMC, Elliott DC, Elliott WH and Jones KM). Oxford University Press, London, 1969.
51. Lister JH, Fused Pyrimidines Part. II. Purines (Ed. Brown DJ). Wiley Interscience, New York, 1971.
52. Pfleiderer W, Purine IV1, Uber die methylierung des 9-methyl-guaninas und die structur des herbopolins. *Ann Chem* **647**: 167–173, 1961.