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ACYCLONUCLEOSIDE ANALOGUE INHIBITORS OF MAMMALIAN PURINE NUCLEOSIDE PHOSPHORYLASE

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Abstract—A series of about 60 purine acyclonucleosides, most with guanine as the aglycone and a 4-carbon chain as the acyclic moiety, was examined for ability to inhibit purine nucleoside phosphorylase from human erythrocytes and calf spleen. Compounds with shorter and longer acyclic chains were less effective inhibitors. Synthetic procedures are described. About 25 of the analogues were competitive inhibitors (relative to inosine or 7-methylguanosine as substrates) with K_i values in the range of 2 to 100 μM. The more potent ones (K_i 2–5 μM) included guanine as the aglycone, with various substituents at C(2′) of the acyclic chain and hydroxyls at C(3′) and C(4′). In one instance, 9-(2-fluoro-3,4-dihydroxybutyl)guanine, the (+)erythro enantiomer was 10-fold more effective than its (−) counterpart (2.5 μM vs 27 μM). Replacement of guanine by 8-bromo- or 8-aminoguanine enhanced affinity for the enzyme by an order of magnitude or more; 7-deazaacyclovir was also 10-fold more effective than acyclovir. With some of the inhibitors, K_i (human)/K_i (calf) varied over the range 0.4 to 4, reflecting differences between the two enzymes; nonetheless, the much more stable, and commercially available, calf spleen enzyme is recommended for preliminary screening of potential inhibitors of the human or other unstable enzymes. The overall results provide useful indications for the synthesis of potentially more potent inhibitors of the enzyme, by simultaneous modifications of the aglycone and the acyclic chains.

Purine nucleoside phosphorylase (PNP‖, purine nucleoside: orthophosphate ribosyltransferase, EC 2.4.2.1) catalyses the reversible phosphorolysis of the ribo- and deoxyribonucleosides of guanine and hypoxanthine. There is an urgent need for effective inhibitors of the phosphorolytic (degradative) pathway of this enzyme, since (a) potentially useful chemotherapeutic purine nucleoside analogues are readily cleaved intracellularly, with concomitant loss of activity [1], and (b) PNP deficiency is associated with defective cellular immunity [2, 3], suggesting that good inhibitors of this enzyme may serve as specific immunosuppressive agents [4, 5]. Several reasonably effective inhibitors have now been reported, e.g. acyclonucleosides of guanosine and their 8-amino congeners [6–8], with K_i values in the range 0.1 to 1 μM. However, more potent inhibitors are considered desirable to effectively inhibit the high levels of PNP in human erythrocytes [9]. One such inhibitor, acyclovir pyrophosphate, which probably acts as a multisubstrate analogue [10], is at the moment of rather limited value, since its high negative charge precludes transport through the cell membrane.

We now describe the pattern of *in vitro* inhibition of PNP from calf spleen, and from human erythrocytes, by about 40 structural analogues of acyclovir. The resultant structure–activity relationships are expected to be useful in continuing efforts to design more potent inhibitory agents and to study the nature of enzyme–inhibitor interactions.

MATERIALS AND METHODS

Materials. Calf spleen purine nucleoside phosphorylase (25 units/mg), xanthine oxidase (1 unit/mg), HEPES, m^{7}Guo and Ino were products of the Sigma Chemical Co. (St Louis, MO, U.S.A.). Sodium cacodylate was from BDH (Poole, U.K.).

Human erythrocyte PNP (98 units/mg with 500 μM Ino at pH 7.5 and 30°) was isolated by the method of Osborne [11].

We are indebted to The Wellcome Foundation for a sample of acyclovir, to Prof. F. Seela for 7-deazaacyclovir, and to Dr E. J. Reist for 9-(3-ethylphosphono-1-propyloxymethyl)guanine.

The following were synthesized according to published procedures: 9-(4-hydroxybutyl)guanine [12]; (*R*) and (*S*)-9-(3,4-dihydroxybutyl)guanine [13]; 6-deoxyacyclovir [14]; *N*(7)-methylguanine acyclonucleosides [15]; 8-bromo- [16] and 8-aminoguanine acyclonucleosides [17].

Acyclovir monophosphate and the 4′-mono-

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‖ Abbreviations: PNP, purine nucleoside phosphorylase (EC 2.4.2.1); ACV, acyclovir, 9-(2-hydroxyethoxymethyl)-guanine; carba-ACV, HBG, 9-(4-hydroxybutyl)-guanine; DHPG, 9-(1,3-dihydroxy-2-propoxy-methyl)guanine; carba-DHPG, 9-(4-hydroxy-3-hydroxymethylbutyl)guanine; m^{7}Guo, 7-methylguanosine; DHBG, 9-(3,4-dihydroxybutyl)guanine; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid).

phosphate of 9-(3,4-dihydroxybutyl)guanine were prepared by enzymatic phosphorylation of the respective acyclonucleosides with the wheat shoot nucleoside phosphotransferase system [18, 19].

Chemistry—miscellaneous. Melting points reported are uncorrected. Elemental analyses were performed by Mikro Kemi AB, Uppsala, Sweden. Thin-layer chromatography made use of Merck (Darmstadt, F.R.G.) precoated glass plates of silica gel 60 F254, and Merck Kieselgel 60 (0.040–0.063 mm) was used for column chromatography. NMR spectra were recorded on a Jeol JNM-FX200 instrument, and chemical shifts, δ, are in ppm vs tetramethylsilane as internal standard. Mass spectra were obtained with a Jeol DX-300/DA 5000 spectrometer. For the FAB spectra, 6 kV Xe atoms were used: for the CI spectra, isobutane was employed as the reagent gas.

Chemistry. Synthetic procedures for compounds **14**, **17**, **27**, **29**: The 9-(2-fluoro-3,4-dihydroxybutyl)- and 9-(2-azido-3,4 dihydroxybutyl)purine analogues were prepared as in Scheme 1.

cis-Butane-2,3-epoxide-1,4-diol was treated with hydrogen fluoride in pyridine, or with sodium azide and ammonium chloride, to give 2-deoxy-2-fluorothreitol and 2-deoxy-2-azidothreitol, respectively. The vicinal hydroxyl groups were protected and, after conversion of the primary hydroxyl to bromo with *N*-bromosuccinimide and triphenylphosphine, the 4-carbon units were condensed with 6-chloropurine or 2-amino-6-chloropurine to give mixtures of the isomeric N-9 and N-7 substituted purine derivatives. The N-9 isomers predominated and were isolated by chromatography on silica gel. Acid hydrolysis then afforded the desired N-9 substituted hypoxanthine and guanine (±)*threo* derivatives (Scheme 1 [compounds **14**, **17**, **27**, **29**]).

Analogously, (±)-*erythro*-9-(2-fluoro-3,4-dihydroxybutyl)guanine (**14**) was obtained by starting from *trans*-butane-2,3-epoxide-1,4-diol. Chromatography of (±)-*erythro*-2-amino-6-chloro-9-(2-fluoro-3,4-*O* - isopropylidene - 3,4 - dihydroxybutyl)purine on a triacetylcellulose column, eluted with ethanol, led to complete separation of the two *erythro* enantiomers. These were hydrolysed to (+)-*erythro*-(**14**) and (−)-*erythro*-9-(2-fluoro-3,4-dihydroxybutyl)guanine (**14**).

(±)-2-Deoxy-2-fluorothreitol and (±)-2-deoxy-2-fluoroerythritol have previously been prepared by separation from a mixture of (±)-2-deoxy-2-fluoroerythrols, obtained by reduction of (±)-2-fluoro-3-hydroxysuccinic acid diethylester [20, 21].

Treatment of (±)-*threo*-9-(2-fluoro-3,4-dihydroxybutyl)guanine with methyl iodide afforded the N-7 methylated analogue **28**. The other guanine acyclonucleosides in the present study were prepared by condensation of 2-amino-6-chloropurine with the appropriately substituted 4-bromobutyl units. The synthesis of these and other acyclic purine and pyrimidine derivatives, prepared by conventional condensation reactions, was carried out at Astra Alab AB by Dr K. Eklind, Dr C. E. Hagberg, Ms S. Kovacs, Dr B. Lindborg, Mr J. O. Norén and Mr G. Stening, and will be reported in detail elsewhere.

(±)-2-Deoxy-2-fluorothreitol (II) (cf. Ref. 20). The *cis*-butane-2,3-epoxide-1,4-diol (I) was obtained from *cis*-2,3-buten-1,4-diol by oxidation with 3-chloroperbenzoic acid in acetonitrile [21]. HF in pyridine (70%, 3 mL) was injected into a dried and closed plastic vial containing the epoxide-diol (2 g, 18.8 mmol). The solution was stirred at room temperature for 2.5 hr *in vacuo*. The gummy residue was neutralized with 40% aqueous K_2CO_3, treated with an ion exchanger (Amberlite IR-120, H^+) and the solution evaporated *in vacuo*. The resulting solid was extracted with boiling ethyl acetate–ethanol (3:2.4 × 50 mL), the extracts were filtered, dried ($MgSO_4$) and evaporated *in vacuo* to yield a pale brown, gummy residue of crude (±)-2-deoxy-2-fluorothreitol [22] (II, 84.4%). ^{13}C NMR (D_2O), δ (ppm): 61.81, 62.25 (CH_2-CF); 62.71, 62.83 (CH_2COH); 71.08, 71.44 (*C*HOH); 93.07, 96.50 (*C*HF). MS, Chem. Ioniz. (+): 125.0639 (70%). $C_4H_{10}FO_3$, mass 125.0627.

(±)-2-Deoxy-2-azidothreitol (III). *cis*-Butane-2,3-epoxide-1,4-diol (I, 2 g, 18.8 mmol), sodium azide (6.88 g, 96.9 mmol) and ammonium chloride (2.3 g, 42.6 mmol) were dissolved in methanol–water (6:1, 60 mL) and heated at reflux for 8 hr, after which the solvent was evaporated *in vacuo* and the residue was extracted with tetrahydrofurane–methanol–ethyl acetate (2:1:2, 3 × 50 mL). The solution was dried (Na_2SO_4) and evaporated *in vacuo* to yield an oily residue of crude (±)-2-deoxy-2-azidothreitol (III, 2.3 g, 81.3%). ^{13}C NMR (CD_3OD), δ(ppm): 64.43 (CH_2-CN_3); 66.21 (CH_2CHOH); 69.10, (*C*H-N_3); 72.80 (*C*H-OH). n_D^{23} 1.4581. MS, FAB(−) (glycerol–water): 146.0590 (5%) $C_4H_8N_3O_3$, mass 146.0566.

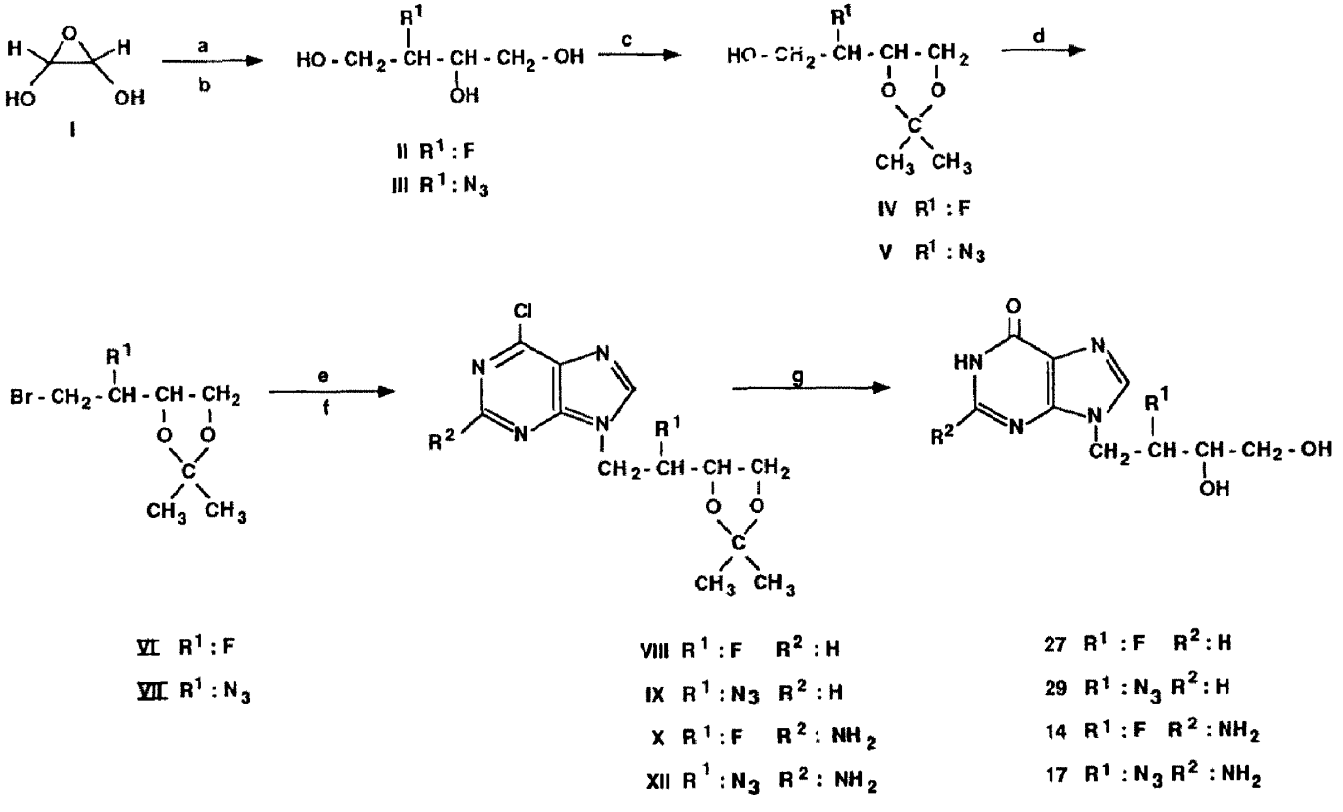
(±) - 2 - Deoxy - 2 - azido - 3,4 - O - isopropylidenethreitol (V). (±)-2-Deoxy-2-azidothreitol (III, 1.15 g) was stirred in acetone with a small amount of *p*-toluenesulfonic acid at ambient temperature for 16 hr. After neutralization with potassium carbonate, the mixture was filtered, the solution was evaporated and the residue purified by chromatography on silica (hexane–ethyl acetate, 1:1) to yield (±)-2-deoxy-2-azido-3,4-*O*-isopropylidenethreitol (V, 0.8 g, 55%).

^1H NMR ($CDCl_3$), δ:1.37 (s, 3H, CH_3); 1.47 (s, 3H, CH_3); 3.43 (t, 2H, CH_2OH); 3.85 (q, 1H, CHN_3); 4.00 (s, 1H, OH); 4.11 (q,2H, CH_2O); 4.23 (q, 1H, CHO). ^{13}C NMR ($CDCl_3$) δ:25.13, 26.22 (2 CH_3); 62.18 (CH_2OH); 64.19 (CHN_3); 66.17 (CH_2O); 75.87 (CHO); 109.83 ($C(CH_3)_2$). MS, FAB(−) (glycerol–PEG200–acetone): 186.0888 (6%). $C_7H_{12}N_3O_3$, mass 186.0839.

(±) - 2 - Deoxy - 2 - fluoro - 3,4 - O - isopropylidenethreitol (IV). The compound was prepared from (±)-2-deoxy-2-fluorothreitol (II) as described for the analogous 2-azido compound (V).

^1H NMR ($CDCl_3$), δ:1.37 (s, 3H, CH_3); 1.42 (s, 3H, CH_3); 3.19 (s, 1H, OH); 3.72–3.80 (m, 2H, CH_2OH); 3.90–4.14 (m, 2H, CH_2O); 4.21–4.37 (m, 1H, CHO); 4.33–4.66 (m, 1H, CHF). ^{13}C NMR ($CDCl_3$) δ:26.08, 27.37 ($2CH_3$); 61.93, 62.34 (CH_2OH); 64.83, 64.97 (CH_2O); 74.65, 75.04 (CHO); 94.33, 96.78 (CHF); 110.07 ($C(CH_3)_2$). MS, electron impact (+): 165.0926 (100%). $C_7H_{14}FO_3$, mass 165.0927.

(±) - 1,2 - Dideoxy - 1 - bromo - 2 - fluoro - 3,4 - O - isopropylidenethreitol (VI). (±)-2-Deoxy-2-fluoro-3,4-*O*-isopropylidenethreitol (IV, 1.09 g) and triphenylphosphine (2.98 g) in dichloromethane



a HF/pyridine (70%) ; 20°C;2.5 hrs.

b NaN_3 /NH_4 Cl/CH_3 OH; reflux; 8 hrs.

c Acetone, p-TSOH; 20°C; 16 hrs.

d NBS / Ph_3 P / CH_2 Cl_2 ;20°C; 3 hrs.

e 2 - Amino - 6 - chloropurine / K_2CO_3 / NaI / DMF; 20°C; 3 days.

f 6 - Chloropurine / K_2CO_3 / NaI / DMF; 20°C; 3 days.

g 2 N HCl aq; 100°C; 2 hrs.

Scheme 1.

(20 mL) were cooled to 0° and *N*-bromosuccinimide (1.82 g) was added. The solution was stirred at room temperature for 3 hr, after which hexane (40 mL) was added. The solution was decanted from the precipitate, which was triturated with hexane (2 × 40 mL). The combined extracts were washed with ice-cooled H_2O (2 × 40 mL), dried (Na_2SO_4) and concentrated to precipitate triphenylphosphine oxide, which was filtered off. The solvent was evaporated *in vacuo* to yield (±)-1,2-dideoxy-1-bromo-2-fluoro-3,4-*O*-isopropylidenethreitol (VI, 0.85 g, 56%).

^{1}H NMR ($CDCl_3$), δ:1.36 (s, 3H, CH_3); 1.42 (s, 3H, CH_3); 3.49–3.61 (m, 2H, CH_2Br); 4.08–4.35 (q, 1, CHO); 4.36–4.78 (m, 1H, CHF). ^{13}C NMR ($CDCl_3$) δ:25.47, 26.17 (2 CH_3); 29.05, 29.58 (CH_2Br); 65.00, 65.17 (CH_2O); 74.87, 75.26 (CHO); 89.25, 92.80 (CHF); 110.46 ($C(CH_3)_2$). MS, FAB(−) (glycerol–acetone): 228.0009 (2.6%), 226.0044 (2.4%). $C_7H_{12}BrFO_2$, mass 227.9982, 226.0004.

(±)-1,2-Dideoxy-1-bromo-2-fluoro-3,4-O-isopropylideneerythreitol (VI). The compound was prepared in 52% yield from (±)-2-deoxy-2-fluoro-3,4-*O*-isopropylideneerythreitol, obtained by a synthetic sequence analogous to the one described above for threo compounds II and IV, but starting with *trans*-butane-2,3-epoxide-1,4-diol [21].

^{13}C NMR ($CDCl_3$), δ:24.96, 26.76 (2 CH_3); 31.60, 32.04 (CH_2Br); 62.47, 62.54 (CH_2O); 70.83, 71.34 (CHO); 88.54, 92.09 (CHF).

(±)-1,2-Dideoxy-1-bromo-2-azido-3,4-O-isopropylidenethreitol (VII). The title compound was obtained from (±)-2-deoxy-2-azido-3,4-*O*-isopropylidenethreitol (V, 2 g) as described for the analogous 2-fluoro compound VI. Yield 1.85 g (65%).

^{1}H NMR ($CDCl_3$), δ:1.36 (s, 3H, CH_3); 1.48 (s, 3H, CH_3); 3.50 (m, 2H, CH_2Br); 3.88 (q, 1H, CHN_3); 4.08 (t, 2H, CH_2O); 4.36 (m, 1H, CHO). ^{13}C NMR ($CDCl_3$) δ:24.88, 26.13 (2 CH_3); 30.63 (CH_2Br); 63.25 (CHN_3); 66.02 (CH_2O); 26.21 (CHO); 110.29 ($C(CH_3)_2$). MS, electron impact (+): 235.9866 (100%), 233.9856 (94%). $C_6H_9BrN_3O_2$ (M-CH_3), mass: 235.9857, 233.9878.

*6-Chloro-9-[(2-RS, 3RS)-2-fluoro-3,4-O-isopropylidene-3,4-dihydroxybutyl]purine (*threo, *VIII).* 6-Chloropurine (185 mg), (±)-1,2-dideoxy-1-bromo-2-fluoro-3,4-*O*-isopropylidenethreitol (VI, 192 mg), anhydrous potassium carbonate (203 mg) and sodium iodide (82 mg) in dry dimethylformamide (DMF, 8 mL) were stirred at ambient temperature for 72 hr. After addition of water (15 mL), the mixture was extracted with *n*-hexane (3 × 15 mL) and dichloromethane (3 × 15 mL). The dichloromethane solution was dried ($NaSO_4$), the solvent evaporated *in vacuo* and the residue purified on a column of silica, using ethyl acetate-hexane (1:1) as eluant, to give 6-chloro-9-((±)-1,2-dideoxy-2-fluoro-3,4-*O*-isopropylidenethreitol)purine (VIII, 87 mg, 31%), m.p. 125°.

^{1}H NMR ($CDCl_3$), δ:1.38 (s, 3H, CH_3); 1.48 (s, 3H, CH_3); 4.10 (m, 2H, CH_2N); 4.16–4.37 (m, 2H, CH_2O); 4.71–4.87 (m, 1H, CHO); 4.72–4.75 (m, 1H, CHF); 8.74, 8.76 (2s, 2×1H, H2, H8). ^{13}C NMR ($CDCl_3$) δ:25.28, 26.01 (2 CH_3); 45.00, 45.46 (CH_2N); 64.85 (CH_2O); 74.22, 74.61 (CHO); 87.13, 89.81 (CHF); 110.63 ($C(CH_3)_2$); 129.20 (C5); 145.95, 150.06, 152.08, 152.58 (C2, C4, C6, C8).

*6-Chloro-9-[(2RS, 3RS)-2-azido-3,4-O-isopropylidene-3,4-dihydroxybutyl]purine (*threo, *IX).* This was prepared as for the 2-fluoro compound (VIII) from (±)-1,2-dideoxy-1-bromo-2-azido-3,4-*O*-isopropylidenethreitol (VII, 0.21 g). Purification on a column of silica, using chloroform–methanol (10:1) as eluant, gave a yield of 135 mg (49%), m.p. 124–125°.

^{1}H NMR (DMSO-d6), δ:1.31, 1.38 (2s, 2 × 3H, CH_3); 3.92–4.03 (m, 2H, CH_2N); 4.10–4.14 (m, 1H, CHN_3); 4.18–4.46 (m, 2H, CH_2O); 4.47–4.50 (m, 1H, CHO); 8.75, 8.82 (2s, 2 × 1H, H2, H8). ^{13}C NMR (DMSO-d6) δ:25.08, 26.37 (2 CH_3); 44.47 (CH_2N); 61.79 (CHN_3); 65.78 (CH_2O); 75.97 (CHO); 109.51 ($C(CH_3)_2$); 129.50 (C5); 147.85, 150.60, 151.86, 157.50 (C2, C4, C6, C8). MS, FAB(+) (DMSO–PEG400–PEG200): 326.0941 (15%), 324.0956 (55%). $C_{12}H_{15}ClN_7O_2$, mass: 326.0950 (34%), 324.0976 (100%).

*2-Amino-6-chloro-9-[(2RS, 3RS)-2-fluoro-3,4-O-isopropylidene-3,4-dihydroxybutyl]purine (*threo, *X) and 2-amino-6-chloro-7-[(2RS, 3RS)-2-fluoro-3,4-O-isopropylidene-3,4-dihydroxybutyl]purine (*threo, *XI).* The reaction was performed as described for compound VIII, starting from (±)-1,2-dideoxy-1-bromo-2-fluoro-3,4-*O*-isopropylidenethreitol (VI, 300 mg) and 2-amino-6-chloropurine (310 mg). After separation on a column of silica, using chloroform–methanol (15:1) as eluant, and recrystallization from diethyl ether–hexane (1:1), the two regioisomeric title compounds were obtained; the 9-regioisomer X, 186 mg (46%), m.p. 149–150° and the 7-regioisomer XI, 37 mg (9%), m.p. 179–180°.

Compound X. ^{1}H NMR ($CDCl_3$); δ:1.38, 1.48 (2s, 2 × 3H, CH_3); 4.04–4.17 (m, 2H, CH_2N); 4.34–4.50 (m, 2 × 2H, CH_2O, CHO); 4.70, 4.95 (2m, 1H, CHF); 5.67 (s, 2H, NH_2); 7.86 (s, 1H, H8). ^{13}C NMR ($CDCl_3$) δ: 25.30, 26.00 (2 CH_3); 44.27, 44.76 (CH_2N); 64.71, 64.80 (CH_2O); 74.19, 74.58 (CHO); 87.18, 90.83 (CHF); 110.51 ($C(CH_3)_2$); 125.01 (C5); 142.89 (C8); 151.40 (C6); 153.86 (C4); 159.38 (C2). Anal. calcd. (found) for $C_{12}H_{15}N_5ClFO_2 \times 1/3H_2O$, C44.80 (45.1); H4.90 (4.7); N21.80 (21.5).

Compound XI. ^{13}C NMR (DMSO-d6), δ:25.35, 26.15 (2 CH_3); 46.95, 47.37 (CH_2N); 64.44, 64.54 (CH_2O); 74.15, 74.51 (CHO); 89.13, 92.76 (CHF); 109.49 ($C(CH_3)_2$); 115.20 (C5); 138.97 (C6); 150.11 (C8); 160.16 (C2); 170.70 (C4). MS, FAB(+) (DMSO–PEG400): 318.0951 (32%); 316.0963 (100%); $C_{12}H_{16}N_5ClFO_2$: mass 318.0946 (34%); 316.0977 (100%).

*2-Amino-6-chloro-9-[(2RS, 3SR)-2-fluoro-3,4-O-isopropylidene-3,4-dihydroxybutyl]purine (*erythro, *X) and enantiomers [(+)-*erythro, *X and (−)-*erythro, *X].* The racemic compound was prepared in 39% yield from *erythro*-VI as described for compound *threo*-X.

^{13}C NMR ($CDCl_3$), δ:25.03, 26.83 (2 CH_3); 44.47, 44.88 (CH_2N); 66.38, 66.43 (CH_2O); 73.75, 74.31 (CHO); 89.25, 92.80 (CHF); 110.73 ($C(CH_3)_2$); 142.93 (C8); 149.65 (C6); 154.10 (C4); 159.38 (C2).

Chromatography on a triacetylcellulose column with 95% ethanol as eluent afforded a clean

separation of (+)- and (−)-enantiomers, $[\alpha]_D^{25}$ +30.6° and −31.3°, respectively.

2-*Amino*-6-*chloro*-9-[(2RS,3RS)-2-*azido*-3,4-O-*isopropylidene*-3,4-*dihydroxybutyl*]*purine* (threo, *XII*). The reaction was performed as described for compound VIII starting from (±)-1,2-dideoxy-1-bromo-2-azido-3,4-*O*-isopropylidenethreitol (VII, 710 mg) and 2-amino-6-chloropurine (600 mg). Purification (silica gel, chloroform–methanol, 10:1) gave 350 mg (37%) of the title compound XII, m.p. 179–180.5°. ^{1}H NMR (DMSO-d6), δ:1.31, 1.42 (s, 2 × 3H, CH_3); 3.92–4.05 (m, 2H, CH_2N); 4.06–4.13 (m, 1H, CHN_3); 4.17–4.20 (m, 2H, CH_2O); 4.21–4.24 (m, 1H, CHO); 6.97 (s, 2H, NH_2); 8.17 (s, 1H, H8). ^{13}C NMR (DMSO-d6), δ:25.13, 26.35 ($2CH_3$); 43.76 (CH_2N); 61.62 (CHN_3); 65.80 (CH_2O); 75.90 (CHO); 109.49 ($C(CH_3)_2$); 127.90 (C5); 143.45 (C8); 150.71 (C6); 157.41 (C4); 160.04 (C2). MS, FAB(+) (DMSO-PEG200+400): 339.1085 (44%) $C_{12}H_{16}N_8ClO_2$: mass 339.1085.

(2RS, 3RS) - 9 - (2 - *Fluoro* - 3,4 - *dihydroxybutyl*) *hypoxanthine* (threo, **27**). 6-Chloro-9-[(*2RS, 3RS*)-2-fluoro-3,4-*O*-isopropylidene-3,4-dihydroxybutyl] purine (VIII, 87 mg) in 2 M hydrochloric acid (1 mL) was heated at 100° for 2 hr, after which the solvent was evaporated *in vacuo*. The residue was dissolved in water (10 mL), a weak anion exchange resin (OH) was added, the mixture heated, filtered while still warm, treated with activated carbon, filtered and the solvent evaporated *in vacuo*. The residue was recrystallized from water (1 mL) to yield (*2RS, 3RS*)-9-(2-fluoro-3,4-dihydroxybutyl)hypoxanthine (**27**), 44 mg (63%, m.p.), 150–151°. ^{13}C NMR (DMSO-d6), δ:44.46, 44.90 (CH_2N); 61.10, 61.20 (CH_2O); 69.95, 70.30 (CHO); 88.86, 92.41 (CHF); 123.86 (C5); 140.64 (C8); 145.60 (C2); 148.50 (C4); 156.58 (C6); $C_6H_{11}FN_4O_3 \times 1/3\ H_2O$. Anal. calcd. (found): C43.55 (43.3); H 4.74 (4.7); N 22.57 (22.5).

(2RS, 3RS) - 9 - (2 - *Azido* - 3,4 - *dihydroxybutyl*) *hypoxanthine* (threo, **29**). Hydrolysis of 6-chloro-9-[(*2RS, 3RS*) - 2 - azido - 3,4 - *O* - isopropylidene - 3,4-dihydroxybutyl]purine (IX, 60 mg) was performed as described for compound **27**. After a similar work-up procedure (*2RS, 3RS*)-9-(2-azido-3,4-dihydroxybutyl)hypoxanthine (**29**) was obtained, 25 mg (47%), m.p. 179–180.5°. ^{13}C NMR (DMSO-d6), δ:44.50 (CH_2N); 62.30 (CHN_3); 62.64 (CH_2OH); 71.67 (CHOH); 126.30 (C5); 140.65 (C8); 145.73 (C2); 148.75 (C4); 156.68 (C6). MS.FAB(+) (DMSO-PEG200): 266.1026 (100%), $C_9H_{12}N_7O_3$, mass 266.1002.

(2RS, 3RS) - 9 - (2 - *Amino* - 3,4 - *dihydroxybutyl*) *hypoxanthine* (threo, **29a**). This was obtained by electrochemical reduction of the azido analogue **29** as described elsewhere for the electrochemical reduction of 3′-azido-3′-deoxythymidine, AZT [23]. The reaction was quantitative, as determined by chromatography and the unchanged UV spectrum of the product.

(2RS, 3RS) - 9 - (2 - *Fluoro* - 3,4 - *dihydroxybutyl*) *guanine* (threo, **14**). Starting from 2-amino-6-chloro-9- [(*2RS, 3RS*)-2-fluoro- 3,4-*O*-isopropylidene-3,4-dihydroxybutyl]purine (X, 165 mg) and using the same reaction conditions as described for compound **27**, the title compound **14** was obtained, 84 mg (62%), m.p. 277–8°. ^{13}C NMR (DMSO-d6), δ: 43.76, 44.23 (CH_2N); 61.42, 61.52 (CH_2O); 70.18, 70.56 (CHOH); 88.94, 92.49 (CHF); 116.69 (C5); 137.93 (C8); 151.57 (C4); 153.84 (C2); 157.07 (C6). Anal. calcd. (found) for $C_9H_{12}FN_5O_3 \times 1/3\ H_2O$: C 41.06 (41.3); H 4.85 (4.6); N 26.61 (26.4).

(±)-(2RS, 3SR) - 9 - (2 - *Fluoro* - 3,4 - *dihydroxybutyl*)*guanine* [(±)-erythro, **14**] *and enantiomers* [(+)-erythro, **14** *and* (−)-erythro, **14**]. The racemic compound was prepared from (±)-*erythro* X as described for compound **14** (*threo*). Yield 65%, m.p. > 300°.

^{13}C NMR (DMSO-d6); δ:43.38, 43.81 (CH_2N); 61.98, 62.11 (CH_2O); 70.77, 71.20 (CHOH); 89.84, 93.31 (CHF); 116.57 (C5); 137.85 (C8); 151.50 (C4); 153.71 (C2); 156.88 (C6).

The enantiomers of compound **14** (*erythro*) were synthesized by the same method; (+)-*erythro*-X led to (+)-*erythro*-**14** and (−)-*erythro*-X to (−)-*erythro*-**14**.

(2RS, 3RS)-7-(2-*Fluoro*-3,4-*dihydroxybutyl*)*guanine* (threo, XIII). Starting from 2-amino-6-chloro-7-[(*2RS, 3RS*)-2-fluoro-3,4-*O*-isopropylidene-3,4-dihydroxybutyl]purine (XI) and using the same reaction conditions as described for compound **27**, the title compound XIII was obtained. MS, FAB(+) (DMSO-PEG200): 258.0995 (12%). $C_9H_{13}FN_5O_3$, mass: 258.1003.

(2RS, 3RS)-9-(2-*Azido*-3,4-*dihydroxybutyl*)*guanine* (threo, 17). Starting from 2-amino-6-chloro-9-[(*2RS, 3RS*)-2-azido-3,4-*O*-isopropylidene-3,4-dihydroxybutyl]purine (XII, 105 mg) and using the same reaction conditions as described for compound **27**, the title compound **17** was obtained, 55 mg (64%), m.p. 204.5–206°. ^{13}C NMR (DMSO-d6), δ: 43.72 (CH_2N); 61.50 (CHN_3); 62.57 (CH_2OH); 71.30 (CHOH); 116.69 (C5); 137.78 (C8); 153.96 (C4); 156.88 (C2); 159.72 (C6). Anal. calcd. (found) for $C_9H_{12}N_8O_3 \times 1/3\ H_2O$: C 37.77 (37.9); H 4.46 (4.3); N 39.16 (39.0). MS, FAB(+) (DMSO-PEG200+400): 281.1120 (74%). $C_9H_{13}N_8O_3$, mass: 281.1111.

(2RS, 3RS) - 9 - (2 - *Amino* - 3,4 - *dihydroxybutyl*) *guanine* (threo, **17a**). Electrochemical reduction of **17**, as described above for **29**, led to quantitative conversion to **17a**, confirmed by chromatography and the unchanged UV spectrum of the product.

2-*Amino*-6-*chloro*-9-[(2RS, 3RS)-2-*fluoro*-3,4-*dihydroxybutyl*]*purine* (*XIV*). 2-Amino-6-chloro-9-[(*2RS, 3RS*)-2-fluoro-3,4- *O*-isopropylidene-3,4-dihydroxybutyl]purine (X, 26 mg) was dissolved in 0.5 M hydrochloric acid (1.5 mL) and stirred at ambient temperature for 1 hr. Water (8 mL) was added and the solution neutralized with a weak anion exchange resin (OH^-), briefly heated and filtered while hot. On cooling the title compound XIV precipitated (15 mg, 87%). Analysis for $C_9H_{15}ClFN_2O_2 \times 1/3\ H_2O$, calcd. (found): C 38.37 (38.8); H 4.17 (3.9); N 24.87 (24.4).

(2 - RS, 3RS) - 7 - *Methyl* - 9 - (2 - *fluoro* - 3,4 - *dihydroxybutyl*)*guanine* (**28**). (*2RS, 3RS*)-9-(2-Fluoro-3,4-dihydroxybutyl)guanine (**14**, 50 mg) and methyl iodide (55 mg) in dimethylformamide (0.5 mL) was heated at 35° for 30 hr, and then filtered through a small Celite pad. Methanol (2.5 mL) and petroleum ether (200 mL) were added, the solution was

evaporated *in vacuo*, and the solid residue recrystallized from water–methanol (2:1) to give (2*RS*, 3*RS*)-7-methyl-9-(2-fluoro-3,4-dihydroxybutyl)guanine (**28**, 20 mg, 44%). MS, FAB(+) (DMSO-PEG200): 272.1188 (10%). $C_{10}H_{15}FN_5O_3$, mass: 272.1159.

Enzymatic procedures. Ultraviolet absorption spectrophotometry was performed with a Zeiss (Jena, F.R.G.) Specord UV-VIS M40 recording instrument, or a Zeiss VSU-2P spectrophotometer, fitted with thermostatically controlled cell compartments, and using 1–10 mm path length cuvettes.

Measurements and control of pH were with a Mera-Elwro pH-meter (Wrocław, Poland) equipped with a combination semi-micro electrode.

Calculations of kinetic constants were performed with a PDP11 minicomputer, using a program previously described [24, 25], and available on request to us, or from the BBA Data Bank (citing Ref. BBA/DD279/31851/786 1984 170).

Compounds were tested for inhibition of phosphorolysis of Ino and, in some instances, m^7Guo. Reactions were followed in 50 mM phosphate buffer pH 7 at 25°, spectrophotometrically by the coupled xanthine oxidase procedure with Ino as substrate [26] and/or, directly spectrophotometrically or fluorimetrically, with m^7Guo as substrate [27].

Several compounds were also tested for inhibitory properties in the presence of 1 mM phosphate, the incubation medium in such instances being buffered at pH 7 with 100 mM cacodylate or HEPES buffers.

Stock solutions of the human erythrocyte enzyme, in 10 mM aqueous DTT, were used for not more than 3 days because of slow but significant loss of activity (in 5 days at 4° the specific activity decreased 6%). Aqueous solutions of the calf enzyme, by contrast, showed no loss of activity over a period of 2 weeks.

Concentrations of substrates and inhibitors were determined spectrophotometrically from their molar extinction coefficients.

With continuous monitoring of the reaction, the kinetic parameters were evaluated by fitting the integrated form of the Michaelis–Menten equation [28, 29] to 10–20 experimental points by the least squares method as previously described [25]. K_i values where then calculated, using the kinetic parameters for Ino and m^7Guo (K_m = 28 and 15 μM, respectively, for human PNP and 13 and 15 μM for the calf enzyme) determined independently for each series of experiments, from the equations [28]:

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

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

where K_m and V_{max} are the parameters for phosphorolysis of Ino or m^7Guo, $K_m{}^{app}$ and $(V_{max}/K_m)^{app}$ the parameters in the presence of inhibitor, and [I] the inhibitor concentration.

In almost all instances both equations led to the same values of K_i, and the inhibition may be described as competitive. For several of the more potent inhibitors of the human enzyme, which exhibits a rather high K_m (~ 30 μM) relative to initial substrate concentration ($c_0 \leq 120$ μM), the procedure based on Eqn 1 posed some difficulties because of the low solubility of uric acid [30] produced in the xanthine oxidase coupled assay, as well as substrate activation [31]. In these cases K_i values were determined only by the second method (see Results). Each K_i value is the mean of at least three independent experiments with inhibitor concentrations in the range from K_i to $5K_i$.

No substrate activation was observed for phosphorolysis of Ino by calf spleen PNP [32, 33].

With the initial velocity method, Ino concentrations were in the range 10 to 500 μM. The K_m and V_{max} parameters were determined by linear regression analysis from Eadie–Hofstee plots of v_0 vs v_0/c_0 [28]. However, in the case of the human erythrocyte enzyme, which exhibits substrate activation [31], only the linear portion of the plot was utilized.

RESULTS

The structures of the synthetic acyclonucleosides embraced in this study, with the acyclic chains depicted to show their possible conformational resemblance to the pentose ring of nucleosides, are exhibited in Fig. 1. Note that, with the exception of ACV (**32**) and its derivatives (**33**, **34** and **35**), they all possess a carbon chain which lacks the ether oxygen present in the ACV analogues previously reported as inhibitors of human erythrocyte PNP by Tuttle and Krenitsky [10].

All compounds (about 60) were initially screened for inhibitory activity at a concentration of 100 μM vs the calf spleen enzyme with Ino as substrate. Those exhibiting at least 50% inhibition under these conditions were then further tested against both the human erythrocyte and calf spleen enzymes, in all cases with Ino, and in some also with m^7Guo, as substrates. For purposes of convenience, the resulting K_i values for competitive inhibition (which prevailed in all instances) of phosphorolysis of Ino (unless otherwise indicated) by human PNP are included in Fig. 1. The overall data, with both substrates and for both enzymes, as well as the generic names of the various compounds, are listed in Table 1.

Acycloguanosines with 4-carbon chains

We consider first 9-butyl guanines with a hydroxyl at C(3′) and various substituents at C(4′) (**1** and **2**, see also below). With an additional hydroxyl at C(4′), the resulting (*S*)-**1**, with K_i 11.4 μM, is twice as effective as (*R*)-**1**, for which K_i is 21 μM. With the C(4′)-hydroxyl replaced by an amino group to give **2**, the K_i is 18 μM. However, **2** is a racemate, and it is therefore likely that one of the enantiomers may be an even better inhibitor than (*S*)-**1**. Introduction at C(4′) of other substituents (not shown), such as CO_2H or $CO_2C_2H_5$, gave products with very low or non-detectable inhibitory activity, even at 100 μM.

The chains with a hydroxyl at C(4′) were then further modified at C(3′) to give compounds **4**–**9** (cf. with **1** and **3**). This group includes carba-ACV (**3**, K_i 52 μM) and carba-DHPG (**6**, K_i 36 μM), as compared to values of 42 and 36 μM reported by Stein *et al.* [7]. Note that **6**, in which the C(3′)-H of **1** is replaced by a CH_2OH, is appreciably less active than the former. The most interesting appears to be

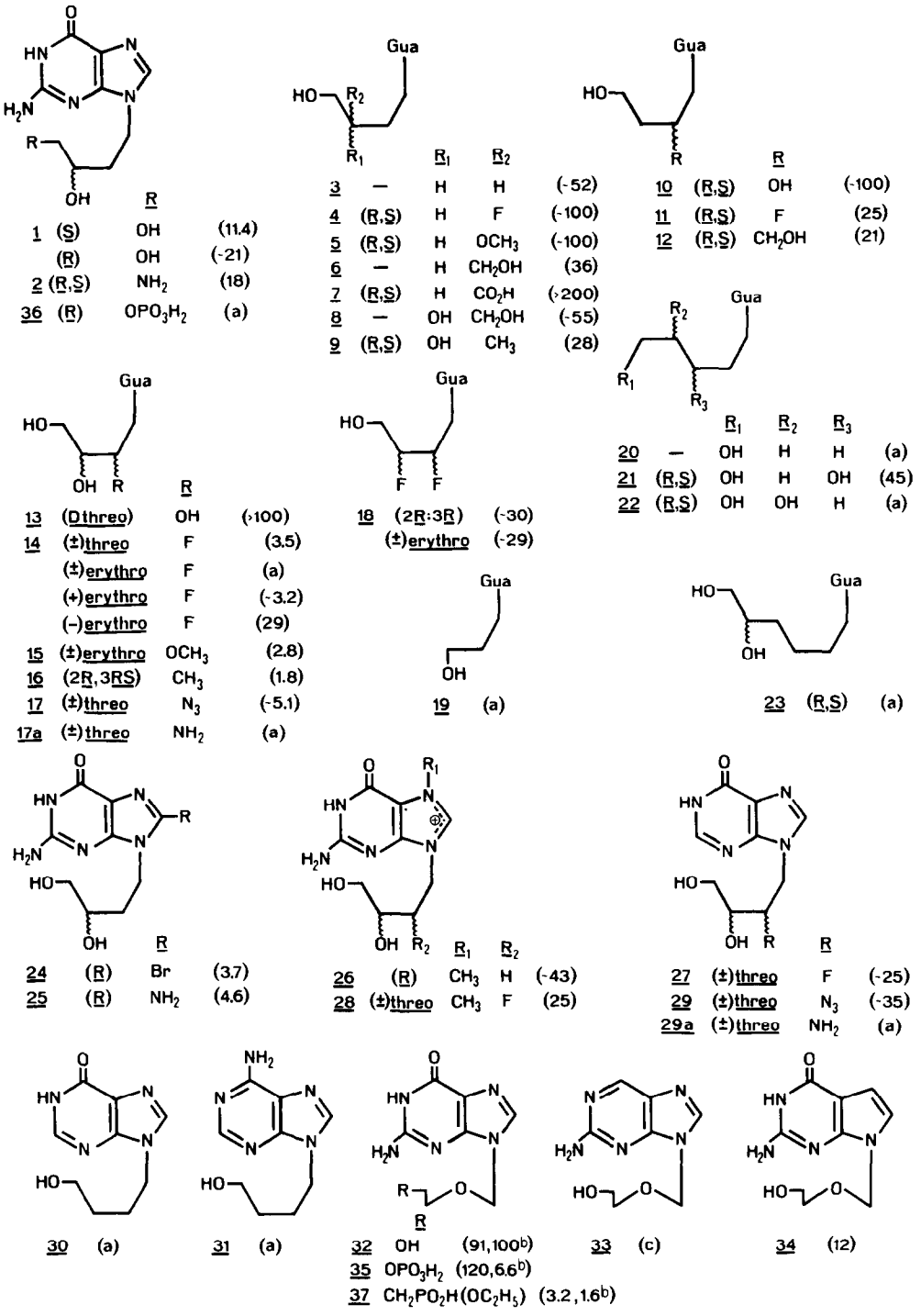


Fig. 1. Structures of the various synthetic acyclo-nucleosides and -nucleotides employed as potential inhibitors of PNP. Figures in brackets are K_i values (in μM) for inhibition of human erythrocyte PNP. (a) K_i only for calf enzyme (Table 1); (b) with 1 mM phosphate; (c) no inhibition at 300 μM.

9, where the C(3′)-substituents are $-OH$ and $-CH_3$, with K_i 28 μM; but this is also a racemate, and, by comparison with the two enantiomers of **1**, it is most probable that the more active enantiomer is (*S*)-**9**. In general, a C(3′)-hydroxyl appears to confer good binding properties to the enzyme. Other C(3′) substituents, such as fluoro (**4**), methoxy (**5**), carboxy (**7**) markedly reduce inhibitory activity.

Attention was then directed to several analogues with substituents at C(2′) instead of C(3′) (**10–12**), and to a number substituted at both C(2′) and C(3′) (**13–17**). With two exceptions (**10** and **13**), these are comparable or much better inhibitors than ACV (**32**), carba-ACV (**3**), and the two enantiomers of **1**. Note, in particular, that all analogues with a hydroxyl at C(3′) and various substituents at C(2′), such as

Table 1. Inhibition constants of some acyclic nucleosides and nucleotides with calf spleen PNP and human erythrocyte PNP at pH 7 and 25° in the presence of 50 mM phosphate (unless otherwise indicated)

	Compound		K_i(μM) with calf spleen PNP and		K_i(μM) with human PNP and
			m^7Guo as substrate	Ino as substrate	Ino as substrate
1	9-(3,4-Dihydroxybutyl)guanine (DHBG)	(*S*)	17	16	11.4
		(*R*)		24, 7.3*	~21
2	9-(3-Hydroxy-4-aminobutyl)guanine	(*R,S*)		~30	18
3	9-(4-Hydroxybutyl)guanine (HBG)	—	81	77	~52, 42†
4	9-(3-Fluoro-4-hydroxybutyl)guanine	(*R,S*)		150	~100
5	9-(3-Methoxy-4-hydroxybutyl)guanine	(*R,S*)		~120	~100
6	9-(3-Hydroxymethyl-4-hydroxybutyl)guanine	—	28	21	36, 38†
7	9-(3-Carboxy-4-hydroxybutyl)guanine	(*R,S*)		>200	>200
8	9-(3-Hydroxymethyl-3,4-dihydroxybutyl)guanine	(*R,S*)		~100	~55
9	9-(3-Methyl-3,4-dihydroxybutyl)guanine	(*R,S*)		~43	28
10	9-(2,4-Dihydroxybutyl)guanine	(*R,S*)		~90	~100
11	9-(2-Fluoro-4-hydroxybutyl)guanine	(*R,S*)	7.5	29	25
12	9-(2-Hydroxymethyl-4-hydroxybutyl)guanine	(*R,S*)	80	5.3	21
13	9-(2,3,4-Trihydroxybutyl)guanine	D-*threo*		>200	>100
14	9-(2-Fluoro-3,4-dihydroxybutyl)guanine	(±)*threo*		2.7	3.5
		(±)*erythro*	~4.2	4.3	
		(+)*erythro*		2.2	~3.2
		(−)*erythro*		26	29‡
15	9-(2-Methyl-3,4-dihydroxybutyl)guanine	(±)*erythro*		2.5	2.8‡
16	9-(2-Methyl-3,4-dihydroxybutyl)guanine	(*2R,3RS*)		4.2	1.8‡
17	9-(2-Azido-3,4-dihydroxybutyl)guanine	(±)*threo*		~3.6	~5.1
17a	9-(2-Amino-3,4-dihydroxybutyl)guanine	(±)*threo*		~13.8	
18	9-(2,3-Difluoro-4-hydroxybutyl)guanine	(*2R,3R*)		12.2	~30
		(±)*erythro*		9.7	~29
19	9-(3-Hydroxypropyl)guanine	—		>150	
20	9-(5-Hydroxypentyl)guanine	—		>150	
21	9-(3,5-Dihydroxypentyl)guanine	(*R,S*)		55	45
22	9-(4,5-Dihydroxypentyl)guanine	(*R,S*)		>100	
23	9-(5,6-Dihydroxyhexyl)guanine	(*R,S*)		>150	
24	8-Bromo-9-(3,4-dihydroxybutyl)guanine	(*R*)	6.2	7.3	3.7
25	8-Amino-9-(3,4-dihydroxybutyl)guanine	(*R*)		11.8	4.6, 4.5§
26	7-Methyl-9-(3,4-dihydroxybutyl)guanine	(*R*)		>250	~43, 43§
27	9-(2-Fluoro-3,4-dihydroxybutyl)hypoxanthine	(±)*threo*		11.5	~25
28	7-Methyl-9-(2-fluoro-3,4-dihydroxybutyl)guanine	(±)*threo*		38	25, 19§
29	9-(2-Azido-3,4-dihydroxybutyl)hypoxanthine	(±)*threo*		~11	~35
29a	9-(2-Amino-3,4-dihydroxybutyl)hypoxanthine	(±)*threo*		>100	
30	9-(4-Hydroxybutyl)hypoxanthine	—		>200	
31	9-(4-Hydroxybutyl)adenine	—		>200	
32	Acyclovir (ACV)	—	64, 61*		91‖, 100*‖
33	6-Deoxyacyclovir	—		¶	
34	7-Deazaacyclovir(7-deaza-ACV)	—		16	12
35	Acyclovir monophosphate	—	69	1.7*	120‖, 6.6*‖
36	9-(3,4-Dihydroxybutyl)guanine 4-monophosphate	(*R*)		11, 2.2*	
37	9-(3-Ethylphosphono-1-propyloxymethyl)guanine	—		9.8	3.2, 1.6*

Values represent the average of at least three experiments.
Unless otherwise indicated (~), error <30%.
* Phosphate concentration 1 mM.
† From Stein *et al.* [7].
‡ K_i evaluated from decrease in V_{max}/K_m only (see Materials and Methods).
§ K_i from initial velocity method.
‖ From Tuttle and Krenitsky [10].
¶ No inhibition at a concentration of 300 μM.

fluoro (**14**), methoxy (**15**), methyl (**16**) or azido (**17**), exhibit lower K_i values in the range 2 to 5 μM, hence are as effective as guanine, hypoxanthine or 8-aminoguanosine [5, 31], and bind more strongly to the enzyme than the parent guanosine [31]. It is of interest that the (+)*erythro* enantiomer of **14** is 12-fold more effective than its (−)*erythro* counterpart, but equal in potency to the (±)*threo* enantiomers: it is consequently to be anticipated that one of the *threo* enantiomers may be an even more potent inhibitor than its (+)*erythro* counterpart.

The two enantiomers of **18**, with fluorines at C(2′) and C(3′), both exhibit activity comparable to **11**, with a fluorine only at C(2′). It is clear that a fluorine at C(3′) results in poorer affinity for the enzyme as compared to hydroxyl or hydroxymethyl substituents (cf. also with **4** and **1**, **4** and **6**).

Analogues with shorter and longer acyclic chains

Only one analogue with a 3-carbon chain was available, 9-(3-hydroxypropyl)guanine (**19**), and this was a very poor inhibitor. Three analogues with 5-carbon chains (**20–22**), and one with a 6-carbon chain (**23**), were tested only against the calf spleen enzyme because of their poor activity, except for **21** with only moderate affinity for the enzyme (K_i 45 μM), comparable to those of carba-ACV and carba-DHPG.

Analogues with modified aglycones

A series of analogues was examined in which the guanine base was either modified or replaced by another base (**24–31**). Only three of these displayed higher affinity for the enzyme than their parent counterparts, namely 9-(3,4-dihydroxybutyl)-8-bromoguanine (**24**), with K_i 3.7 μM, as compared to 11.4 and 21 μM for its non-brominated parent compounds (**1**); 7-deaza-ACV (**34**), with K_i 12 μM, 10-fold lower than for ACV; and 8-amino-9-(3,4-dihydroxybutyl)guanine (**25**) with $K_i \sim 4.5$ μM.

Methylation of the guanine ring N(7) of (*R*)-**1** to give **26** led to a 50% decrease in affinity for the enzyme; similar methylation of (±)-*threo*-**14** to give **28** resulted in a 6-fold decrease in affinity.

Compounds with the guanine base replaced by hypoxanthine exhibited inhibitory activity an order of magnitude lower than the corresponding acyclonucleoside with a guanine aglycone (cf. **27** with **14** and **29** with **17**).

Replacement of guanine by bases such as adenine, 2,6-diaminopurine, 2-aminopurine, 6-chloropurine, 2-amino-6-chloropurine, and various pyrimidines all resulted in complete loss of inhibitory activity at concentrations as high as 100 μM.

Potential bisubstrate analogue inhibitors

Two acyclonucleoside monophosphates (**35, 36**) and one phosphonate (**37**) were examined as possible bisubstrate analogue inhibitors [10], by measurement of the K_i values in the presence of 50 mM phosphate and 1 mM phosphate.

The K_i of ACV phosphate (**35**) is strongly dependent on phosphate concentration with human PNP, 120 and 6.6 μM in the presence of 50 and 1 mM phosphate, respectively [10]. We have found a similar even more striking relationship with the calf-spleen enzyme, 69 and 1.7 μM.

By contrast, the K_i for 9-(3,4-dihydroxybutyl)guanine 4-phosphate (**36**) in the presence of 1 mM phosphate is only one-fifth that at high phosphate concentrations; the significance of even this small effect is diminished by the fact that a comparable effect is observed with the parent nucleoside (**1**). No such effect is observed with ACV.

In the case of 9-(3-ethylphosphono-1-propyloxymethyl)guanine (**37**), the inhibitory activity is almost independent of the phosphate concentration.

Inhibition pattern for calf spleen enzyme

PNP from calf spleen shows a similar pattern of inhibition by acyclic nucleosides described above as inhibitors of the human erythrocyte enzyme (see Table 1). The ratio of K_i (human) to K_i (calf) is in the range of 0.4 to 4.0.

Inhibitory activity with different substrates

Several analogues were tested as inhibitors of phosphorolysis of m^7Guo by the calf spleen enzyme, and the results compared with those for inhibition of Ino phosphorolysis. With six compounds (**1, 3, 6**, (±)-*erythro*-**14, 24**), the K_i values were similar with the two substrates. It was consequently surprising to find that two compounds, **11** and **12**, exhibited different K_i values with the two substrates. For **11**, the K_i values were 7.5 μM with m^7Guo as substrate and 29 μM with Ino as substrate; for **12** the values were 80 and 5.3 μM.

DISCUSSION

While the results of this investigation have not led to more potent inhibitors of PNP than several already known [7, 8, 34, 35], they do furnish some useful clues for further improvements, which will be referred to below. The present data should also prove useful in better delineating interactions of nucleosides with PNP, and distinguishing subtle differences in properties of the enzyme from different sources.

As previously noted by others [7, 36, 37], the presence of the ether oxygen in the acyclo chain of ACV and its analogues is dispensable. In fact, carba-ACV (**3**, HBG) exhibits a K_i 2-fold lower than that for ACV (**32**). Compound **10**, in which a hydroxyl-substituted carbon replaces the ether oxygen is as active as ACV, while **11** and **12**, in which substituted carbons replace the ether oxygen, are 4-fold more active than ACV with the human erythrocyte enzyme.

In contrast to the two enantiomers of 9-(3,4-dihydroxybutyl)guanine (**1**), (*R,S*)-9-(2,4-dihydroxybutyl)guanine (**10**) is a relatively poor inhibitor, although it is conceivable that one of the enantiomers may be more potent. More striking is the fact that the D-*threo* stereomer of 9-(2,3,4-trihydroxybutyl) guanine (**13**), the acyclic chain of which may conformationally resemble the "lower" portion of the ribose ring (see Fig. 1), is amongst the poorest inhibitors.

It was consequently a surprise to find that replacement of the C(2′)-hydroxyl of **13** by a variety of other substituents (fluorine, methyl, methoxy, azido) (**14–17**) enhanced inhibitory activity by two orders of magnitude, so that the K_i values are in the range 2 to 5 μM. This is a somewhat puzzling result, inasmuch as these four substituents differ significantly in such properties as van der Waals radii, electronegativity, polarizability and hydrophobicity.

A somewhat analogous situation prevails with the (*R,S*)-9-(2,4-dihydroxybutyl)guanine (**10**), also a poor inhibitor. Here again, replacement of the C(2′)-hydroxyl by fluorine or hydroxymethyl results in considerable enhancement of inhibitory activity of **11** and **12**, respectively. The two enantiomers of **1**, with no substituent at C(2′), exhibit K_i values more than 5-fold lower than the corresponding analogue with a C(2′) hydroxyl (**10** or **13**). This is somewhat unusual if we recall that 2′-deoxynucleosides are not superior substrates of PNP, relative to their ribo

counterparts, notwithstanding the higher lability of the glycosidic bonds of the former.

The differences in binding, by PNP, of nucleosides and corresponding acyclonucleosides are also reflected by the finding [38] that 2′-fluoro-2′-deoxy-Ino is poorly bound by human PNP (K_m 490 μM), and is barely detectably phosphorolysed (V_{max} 0.2% that for Ino). This has been ascribed [38] to the "unacceptable" conformation of the sugar ring, which is in the rarely encountered C(3′)*endo*-C(4′)*exo* form in the solid state [39]. However, this is at best a tenuous argument since the sugar ring will exhibit a conformational equilibrium in solution, which may be shifted on binding to the enzyme.

The difference in affinity between acyclonucleosides and nucleosides is more pronounced with 2′-azido and 2′-methoxy substituents, e.g. 2′-azido-2′-deoxy-Ino [38] and 2′-*O*-methyl-Guo [1] are neither substrates nor inhibitors of human PNP. By contrast, the acyclonucleosides with 2′-azido (**17**) and 2′-*O*-methyl (**15**) substituents are relatively good inhibitors with K_i values of 5.1 and 2.8 μM.

The foregoing situation is reversed for substituents at C(3′), in that a hydroxyl at this position confers higher inhibitory activity. Introduction of a hydroxyl at C(3′) of carba-ACV (**3**) leads to (*S*)-**1**, which is 4-fold more active, and (*R*)-**1** with 2-fold higher activity. Introduction of a hydroxymethyl at C(3′) of **3** to give **6** leads to only a small increase in activity against the human enzyme, but a 4-fold higher activity vs the calf spleen enzyme (see Table 1). By contrast, replacement of the C(3′) hydroxyl by fluorine (**4**), methoxy (**5**) or carboxy (**7**) leads to drastic increases in K_i.

Preliminary X-ray diffraction data for human erythrocyte PNP [35, 40] led to the inference that the C(3′)-hydroxyl of the substrate is located in the vicinity of the enzyme-bound phosphate group, and that this is optimal for proper orientation of the two reactants. The present results with acyclonucleoside inhibitors, pointing to the importance of the C(3′)-hydroxyl for binding to the enzyme, are at least consistent with this proposal. With the availability of suitable crystals of PNP, it may be feasible to co-crystallize with an acyclonucleoside inhibitor to determine with precision the location of the binding sites on the enzyme.

Previous studies [1] with sugar-modified analogues of the PNP substrate, Ino, demonstrated that alterations of the steric configuration at C(2′) and/or C(3′) resulted in a very marked diminution, or disappearance, of both substrate and inhibitory activities. Similar, but less pronounced, effects may be noted with two of the acyclonucleoside analogues of the present series. The (*S*)-enantiomer of **1** is about 2-fold more active than its (*R*) form; and the (+)*erythro* enantiomer **14** is a 10-fold better inhibitor than its (−)*erythro* counterpart. These smaller effects resulting from changes in configuration, as against the more drastic ones observed with nucleosides, are clearly due to the greater flexibility of the acyclic chains. Nonetheless the 10-fold difference between the two *erythro* enantiomers of **14** may also occur with other acyclonucleosides, and should be exploited by the synthesis of additional acyclonucleoside enantiomers.

Influence of base modifications

The data in Table 1 and Fig. 1 underline the importance of the nature of the base moiety on inhibitory properties.

As previously noted with nucleosides [41–44] and bases, analogues with no oxygen at C(6) (6-deoxyacyclovir, **33**) or with the oxygen replaced by an amino group (**31**) are not bound by the enzyme. This is consistent with the proposal [44, 45] that O^6 is an acceptor in hydrogen bonding of substrate or inhibitor.

A variety of pyrimidine acyclonucleosides (results not shown), which are effective inhibitors of uridine phosphorylase [46], were virtually inactive as inhibitors of PNP. Conversely, none of our better inhibitors of PNP exhibited inhibitory activity vs uridine phosphorylase (A. Drabikowska, unpublished).

Replacement of the guanine moieties of (±)*threo*-**14** and **17** by hypoxanthine, to give **27** and **29**, resulted in a 5-fold decrease in inhibitory activity towards the calf spleen enzyme, and an 8-fold decrease towards the human enzyme. An analogous, even more pronounced effect, has been reported with the human enzyme, for which the K_i of 8-amino-9-benzylguanine is 0.2 μM as compared to 150 μM for 8-amino-9-benzylhypoxanthine [6]. This is in accord with the proposal [44] that the amino group at C(2) of a substrate is a donor in hydrogen bonding at the active site of the enzyme.

Note that the 8-amino analogue of (*R*)-**1**, i.e. **25**, is a 5-fold better inhibitor of human PNP than the parent (*R*)-**1**. This is in accord with a previous report that 8-amino-Guo is a good inhibitor [5, 47], and ascribed to involvement of the 8-amino group as a hydrogen bond donor in binding to the enzyme. However, the corresponding 8-bromo analogue of (*R*)-**1**, compound **24** is as effective, or slightly better, than **25**. This is in striking contrast to the fact that 8-bromo-Guo is neither a substrate nor inhibitor of PNP [48], presumed due to constrainment of the nucleoside to the *syn* conformation by the 8-bromo substituent [49], a situation which does not prevail for **24** because of the much greater flexibility of its acyclic chain.

The substantial decrease in inhibitory properties accompanying replacement of guanine by 7-methylguanine (cf. (*R*)-**1** and **26**, and (±)*threo*-**14** and **28**) appears at first sight surprising in view of the very good substrate properties of m^7Guo relative to Guo [27, 44, 48]. But it is consistent with the premise that the good substrate properties of m^7Guo are due more to labilization of the glycosidic bond [50] (due to the positive charge on the imidazole ring) than to enhanced affinity for the enzyme [48]. It should also be noted that the difference resulting from N(7)-methylation is less pronounced for the human, as compared to the calf spleen enzyme: this is in line with the substrate properties of m^7Guo, with the two enzymes, namely a lower K_m than for Guo with the human enzyme, and similar K_m values for both substrates with the calf spleen enzyme [27, 44, 48].

One of the more novel findings of the present investigation is the effect of replacement of guanine by 7-deazaguanine, in that 7-deaza-ACV (**34**) is

almost 10-fold more effective than the parent ACV (**32**). This is all the more interesting in that 7-deaza-Ino, which is not a substrate for either enzyme [44, 51], exhibits a lower affinity than Ino for both the calf spleen enzyme ($K_i \sim 60\,\mu M$) [44] and the human enzyme (K_i 330 μM) [51]. On the other hand, the high affinity of 7-deaza-ACV is in accord with a previous suggestion [48] that the purine ring N(7) is required, not so much for binding, as for facilitating phosphorolysis, which is postulated [52, 53] to proceed via intermediary protonation at N(7), as in the case of acid hydrolysis of the glycosidic bond.

The foregoing clearly suggests the desirability of synthesizing the corresponding 7-deaza analogues of the more potent acyclonucleoside, and perhaps acyclonucleotide, inhibitors listed in Table 1. Enhancement of inhibitory activity by an 8-amino substituent also suggests a combination with a 7-deaza ring, i.e. 8-amino-7-deaza-Gua as the aglycon of various acyclonucleosides.

The bisubstrate analogue inhibitors. The pyrophosphates of ACV and DHPG are presently the most potent *in vitro* PNP inhibitors, with $K_i \sim 10^{-8}$ M [7, 10], in the presence of limiting (1 mM) concentrations of P_i. The monophosphates **35** and **36**, and the monophosphonate **37**, are much less effective, undoubtedly because the distance between the phosphate and the aglycone is too short to span the region between the phosphate and aglycon binding sites on the enzyme. However, these analogues are, as such, clearly unsuitable for use *in vivo*, since their high negative charge precludes their traversal of the cell membrane. On the other hand, in cells infected with a virus which codes for a thymidine kinase, the acyclonucleosides may be phosphorylated to the monophosphates and, then, by cellular kinases, to the pyrophosphates [54], which may then exert a potent inhibitory effect vs PNP. Furthermore, both ACV and DHPG are phosphorylated to a low, but significant, extent in some non-infected cells [54], and, because of the low K_i values of these phosphorylated forms, may appreciably inhibit PNP in such cells as well. It would be desirable to prepare the pyrophosphates of acyclonucleosides with lower K_i values than ACV and DHPG, e.g. (+)*erythro*-**14**, the pyrophosphate of which could be an even better *in vitro* inhibitor than the pyrophosphates of ACV and DHPG. Finally, it should be noted that the monophosphonate of ACV (**32**), i.e. **37**, is a 60-fold better inhibitor than ACV and, if this were to more readily traverse the cell membrane at physiological pH (where it is in the monoanionized form), it would be more effective *in vivo* than ACV. It is worth noting, in this connection, that phosphonate and alkylphosphonates are capable of replacing P_i in the phosphorolytic reaction [55].

Differences between enzymes. Reverting once again to Table 1, attention should be drawn to the fact that, while the various inhibitors exhibit in general comparable patterns of inhibition with respect to the two mammalian enzymes, there are nonetheless exceptions, in some instances quite marked. In fact, the ratio K_i(human)/K_i(calf) varies over the range 0.4 to 4, which clearly reflects differences in the properties of the two enzymes. It is to be anticipated that such differences may be encountered with the enzyme from other sources. On the other hand, the much higher stability of the calf spleen enzyme recommends it as the enzyme of choice for at least initial screening of potential inhibitors of the human, and other inherently labile, enzymes.

Recapitulation of the foregoing results, in conjunction with findings reported by others [7, 10, 36], points to additional approaches to the design of more potent inhibitors, as follows: (a) use of 7-deaza-Guo, as aglycons; (b) use of 8-substituted guanines and hypoxanthines, particularly 8-bromo and 8-amino; (c) combination of the foregoing with 4-carbon C(3′), C(4′)-dihydroxy chains with various C(2′) substituents (fluoro, methyl, methoxy, azido), along with synthesis of the individual diastereomers of chiral acyclic chains; (d) phosphate and pyrophosphate analogues of the foregoing, not only as potential bisubstrate analogue *in vitro* inhibitors, but also for investigations on the mechanism(s) of interaction of various PNPs with substrates and inhibitors; (e) phosphonyl analogues, which may more readily traverse cell membranes, are relatively resistant to phosphatases, and can mimic the corresponding phosphorylated acyclonucleosides as bisubstrate analogue inhibitors.

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