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INHIBITION OF PURINE NUCLEOSIDE PHOSPHORYLASE BY 9-(PHOSPHONOALKYL)HYPOXANTHINES

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Purine nucleoside phosphorylase (EC 2.4.2.1; PNP) has been recognized as a target for antitumor and immunosuppressive agents [1, 2]. PNP catalyzes the reversible phosphorolysis reaction:

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

This reaction proceeds via a ternary complex of enzyme, nucleoside, and orthophosphate [1, 2]; therefore, a compound which incorporates the features of both an appropriate purine base and orthophosphate has the potential to strongly inhibit PNP [3]. We are interested in the possibility of developing "multisubstrate" analog inhibitors of PNP which contain a phosphonic acid moiety. Methyl- and ethylphosphonate are capable of replacing orthophosphate in the phosphorolysis reaction catalyzed by PNP [4]. In contrast, the "isosteric" methylenephosphonate analog of α-D-ribose 1-phosphate is not an inhibitor of PNP [5]. In view of this latter result, 9-phosphonoalkylhypoxanthines were chosen as probes to determine whether or not phosphonic acids could function as "multisubstrate" analog inhibitors of PNP. These phosphonates may be easily synthesized, and they bear a close structural resemblance to the phosphate esters of acyclovir which are known potent inhibitors of PNP [6]. We report here the synthesis of a series of 9-phosphonoalkylhypoxanthines and their favorable inhibitory activity towards PNP obtained from human erythrocytes.

MATERIALS AND METHODS

General methods. Ultraviolet spectra were taken on a Perkin-Elmer model 402 spectrophotometer. NMR spectra were obtained using a Varian XL-300 spectrophotometer. Chemical shifts are recorded in ppm downfield of an internal standard of tetramethylsilane. Elemental analyses were performed by the Baron Consulting Co., Orange, CT. Melting points are not corrected. Where TLC grade silica gel is specified for column chromatography, Kieselgel 60 G was used [7].

Diethyl 3-bromopropylphosphonate ($\mathbf{I}_{n=3}$) [8]. Triethylphosphite (8.3 g, 50 mmoles) was added with stirring over 30 min to a flask containing 1,3-dibromopropane (50.5 g, 250 mmoles) at 150°, and the mixture was stirred for a further 60 min. The flask was equipped with a short path distilling column to allow the bromoethane produced during the reaction to escape. The excess dibromide was removed by distillation under reduced pressure leaving an oily residue that was chromatographed on a column of silica gel (100–200 mesh) using pentane followed by ethyl acetate/ether giving 9.0 g (69%) of homogeneous (TLC, NMR) $\mathbf{I}_{n=3}$ as a pale yellow oil: NMR ($CDCl_3$) 1.34 (t, 6H, J=7 Hz, CH_3), 1.91 (m, 2H, 1-CH_2), 2.16 (m, 2H, 2-CH_2), 3.48 (t, 2H, J=6 Hz, 3-CH_2), and 4.11 (m, 4H, OCH_2).

6-Chloro-9-[3-(diethoxyphosphinyl)propyl]purine ($\mathbf{II}_{n=3}$) [9]. A suspension of 6-chloropurine (3.09 g, 20 mmoles), $\mathbf{I}_{n=3}$ (5.18 g, 20 mmoles) and anhydrous potassium bicarbonate (3.04 g, 22 mmoles) in freshly distilled dimethyl sulfoxide (200 ml) was stirred at room temperature for 20 hr. The solvent was removed by distillation under reduced pressure, and the residue was washed (3 x 50 ml) with ethyl acetate. The ethyl acetate washes were combined, the solvent was removed, and the remaining material was chromatographed on a column of TLC grade silica gel (130 g, 4 x 18 cm) using methylene chloride/ethanol (96:4) to give 3.5 g (53%) of homogeneous (TLC, NMR) $\mathbf{II}_{n=3}$ as a viscous

pale yellow oil: u.v. (EtOH) λ_{max} 265 nm (8500); NMR ($CDCl_3$) 1.33 (t, 6H, J=7 Hz, CH_3), 1.74 (dt, 2H, J=7, 18.5 Hz, 3-CH_2), 2.28 (m, 2H, 2-CH_2), 4.12 (m, 4H, OCH_2), 4.47 (t, 2H, J=7 Hz, 1-CH_2), 8.26 (s, 1H, purine), and 8.75 (s, 1H, purine).

6-Chloro-9-(3-phosphonopropyl)purine ($III_{n=3}$) [10]. A solution of $II_{n=3}$ (3.19 g, 9.6 mmoles) and bromotrimethylsilane (4.41 g, 28.8 mmoles) was stirred for 60 min at room temperature. The reaction was placed under a vacuum and, after volatile material had ceased to evolve, the remaining orange syrup was dissolved in acetonitrile (20 ml). Water (2 ml) was added to the acetonitrile solution which initiated the precipitation of 2.18 g (82%) $III_{n=3}$ with m.p. 180°, softens with decomposition: u.v. (0.1 N HCl) λ_{max} 265 nm (8400), (0.1 N NaOH) λ_{max} 266 nm (8500); NMR (d_6-DMSO) 1.51 (m, 2H, 3-CH_2), 2.07 (m, 2H, 2-CH_2), 4.37 (t, 2H, J=7 Hz, 1-CH_2), 8.72 (s, 1H, purine), and 8.78 (s, 1H, purine).

9-(3-Phosphonopropyl)hypoxanthine ($IV_{n=3}$). A suspension of $III_{n=3}$ (1.2 g, 4.34 mmoles) in 0.1 N HCl (20 ml) was heated to 120°. Within a few minutes the $III_{n=3}$ had dissolved and the solution was refluxed for 2.5 hr. The solution was cooled, titrated to pH 8.0 with 1 N NaOH, and applied to a 2 x 20 cm DEAE Sephadex A-25 column (HCO_3^- form) equilibrated in 25 mM triethylammonium bicarbonate buffer. The column was washed with the same buffer (60 ml) and eluted with a 25-500 mM linear gradient of the same buffer (400 ml). Fractions containing the major peak were pooled, the water and triethylammonium bicarbonate were removed under reduced pressure, and the residue was crystallized from 0.1 N HCl to give 0.65 g (58%) of $IV_{n=3}$. An analytical sample from dilute HCl had m.p. 110°, softens with decomposition: u.v. (0.1 N HCl) λ_{max} 250 nm (10,500), (0.1 N NaOH) λ_{max} 255 nm (11,900); NMR (d_6-DMSO) 1.47 (m, 2H, 3-CH_2), 1.98 (m, 2H, 2-CH_2), 4.20 (t, 2H, J=7 Hz, 1-CH_2), 8.05 (s, 1H, purine), and 8.10 (s, 1H, purine).

Anal. Calc. for $C_8H_{11}N_4O_4P$ (258.18): C, 37.21; H, 4.30; N, 21.70; P, 12.00. Found: C, 36.98; H, 4.37; N, 21.82; P, 11.82.

9-(5-Phosphonopentyl)hypoxanthine ($IV_{n=5}$). Compound $IV_{n=5}$ was prepared by the methods described above except that the hydrolysis of $III_{n=5}$ to $IV_{n=5}$ was conducted in acetic acid/0.1 N HCl (1:4 v/v). An analytical sample from dilute HCl had m.p. 115°, softens with decomposition: u.v. (0.1 N HCl) λ_{max} 250 nm (9,700), (0.1 N NaOH) λ_{max} 255 nm (10,800); NMR (d_6-DMSO) 1.32 (m, 2H, 3-CH_2), 1.42-1.57 (4H, alkyl), 1.79 (m, 2H, 2-CH_2), 4.13 (t, 2H, J=7 Hz, 1-CH_2), 8.04 (s, 1H, purine), and 8.11 (s, 1H, purine).

Anal. Calc. for $C_{10}H_{15}N_4O_4P$ (286.23): C, 41.96; H, 5.28; N, 19.58; P, 10.82. Found: C, 41.68; H, 4.96; N, 19.29; P, 11.27.

9-(6-Phosphonohexyl)hypoxanthine ($IV_{n=6}$). An analytical sample of $IV_{n=6}$ from dilute HCl had m.p. 195-198°: u.v. (0.1 N HCl) λ_{max} 250 nm (10,500), (0.1 N NaOH) λ_{max} 255 nm (11,900); NMR (d_6-DMSO) 1.15-1.55 (8H, alkyl), 1.77 (m, 2H, 2-CH_2), 4.12 (t, 2H, J=7 Hz, 1-CH_2), 8.03 (s, 1H, purine), and 8.11 (s, 1H, purine).

Anal. Calc. for $C_{11}H_{17}N_4O_4P$ (300.26): C, 44.00; H, 5.71; N, 18.66; P, 10.32. Found: C, 43.84; H, 6.02; N, 18.41; P, 10.02.

9-(7-Phosphonoheptyl)hypoxanthine ($IV_{n=7}$). An analytical sample of $IV_{n=7}$ from dilute HCl had m.p. 205-207°: u.v. (0.1 N HCl) λ_{max} 250 nm (10,300), (0.1 N NaOH) λ_{max} 255 nm (11,400); NMR (d_6-DMSO) 1.15-1.55 (10H, alkyl), 1.77 (m, 2H, 2-CH_2), 4.12 (t, 2H, J=7 Hz, 1-CH_2), 8.04 (s, 1H, purine), and 8.12 (s, 1H, purine).

Anal. Calc. for $C_{12}H_{19}N_4O_4P$ (314.28): C, 45.86; H, 6.09; N, 17.83; P, 9.86. Found: C, 45.66; H, 6.40; N, 17.59; P, 10.15.

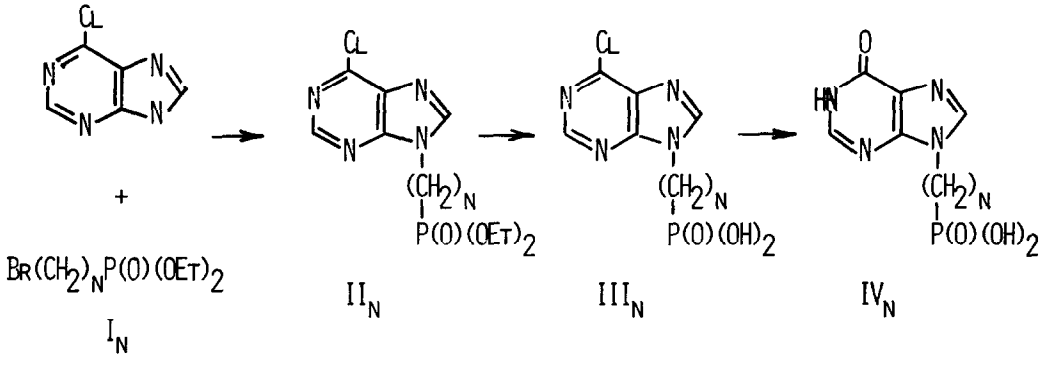
9-Propylhypoxanthine (V). An analytical sample of V from ethanol had m.p. 254-255° (lit. 259-260° [11]): u.v. (0.1 N HCl) λ_{max} 250 nm (9,800), (0.1 N NaOH) λ_{max} 255 nm (10,900); NMR (d_6-DMSO) 0.84 (t, 3H, J=7 Hz, 3-CH_3), 1.80 (q, 2H, J=7 Hz, 2-CH_2), 4.10 (t, 2H, J=7 Hz, 1-CH_2), 8.04 (s, 1H, purine), and 8.10 (s, 1H, purine).

Anal. Calc. for $C_8H_{10}N_4O$ (178.19): C, 53.92; H, 5.66; N, 31.44. Found: C, 53.63; H, 5.37; N, 31.70.

Enzyme assay. Partially purified human erythrocytic PNP (sp. act. = 1.0) [12] was obtained from the New England Enzyme Center, Boston, MA. Using inosine as substrate, PNP activity was determined spectrophotometrically by a xanthine oxidase coupled assay [13]. The increase in absorbance at 293 nm was monitored with a Gilford model 240 spectrophotometer. The assay contained 0.1 M HEPES/NaOH buffer, pH 7.4, 0.04 units xanthine oxidase (Sigma Chemical Co., St. Louis, MO), ca. 0.02 units PNP, 1 mM sodium phosphate, and appropriate concentrations of inosine and inhibitor in a total volume of 1.0 ml at 30°.

Inhibition of PNP was measured at eight concentrations of inosine ranging from 12.5 to 100 μ M. Slopes, obtained by linear regression analysis of double reciprocal plots of initial velocity vs inosine concentration, were replotted against two or three inhibitor concentrations to give the apparent inhibition constant (K_i'). The inhibitor concentrations used included a concentration at least two times the value of the K_i' that was obtained. The inhibitors did not appear to affect the xanthine oxidase in the assay.

SCHEME 1



N = 3, 5, 6, AND 7.

RESULTS AND DISCUSSION

9-Phosphonoalkylhypoxanthines (IV_n) were synthesized according to the standard procedures illustrated in Scheme 1. Each of these phosphonates was found to inhibit PNP. Under the assay conditions described in Materials and Methods, double reciprocal plots of initial velocity vs inosine concentration were linear as were secondary plots of slope vs inhibitor concentration. Inhibition was competitive with inosine as variable substrate. Apparent inhibition constants (K_i') obtained with inosine as variable substrate and orthophosphate concentration fixed at 1 mM are listed in Table 1. No inhibition was observed for any of these phosphonates at 50 mM phosphate under conditions essentially identical to those used to obtain the data shown in Table 1. These results suggest that all of these phosphonate inhibitors interact with both the purine and phosphate binding sites on the enzyme.

Table 1. Apparent inhibition constants for purine nucleoside phosphorylase

Inhibitor		K_i' (M)
9-Propylhypoxanthine	(V)	0.6×10^{-3}
9-(3-Phosphonopropyl)hypoxanthine	($IV_{n=3}$)	2.7×10^{-3}
9-(5-Phosphonopentyl)hypoxanthine	($IV_{n=5}$)	1.1×10^{-6}
9-(6-Phosphonohexyl)hypoxanthine	($IV_{n=6}$)	2.2×10^{-6}
9-(7-Phosphonoheptyl)hypoxanthine	($IV_{n=7}$)	0.9×10^{-6}

The K_i' values determined for phosphonates $IV_{n=5,6,and\ 7}$ were approximately 400-fold lower than the value obtained for 9-propylhypoxanthine (V). Interaction of PNP with the phosphonate moiety of these inhibitors resulted in increased affinity. In contrast, the K_i' determined for phosphonate $IV_{n=3}$ was 4-fold higher than that of compound V; therefore, no binding advantage was obtained. The shorter length and/or the fewer degrees of rotational freedom of the methylene linkage in $IV_{n=3}$ (as compared to $IV_{n=5,6,or\ 7}$) do not allow optimal occupancy of the hypoxanthine and phosphonate moieties to their respective binding sites on the enzyme. The K_i' values of PNP for $IV_{n=5,6,\ and\ 7}$ were essentially identical. These results suggest that the distance and the orientation of the phosphate binding site of PNP, relative to the purine binding site, are restricted to a locus described by the configurations of compound $IV_{n=5}$.

It is noteworthy that the K_i' value of compound $IV_{n=5}$ (1.1 μM) is of the same order of magnitude as the K_i' value of acyclovir monophosphate (6.6 μM [6]) obtained under

similar conditions. The key feature of these inhibitors is that an acceptable purine moiety is connected to a phosphate-like moiety. The type of connection is less important since both oxygens of the acyclo portion of acyclovir monophosphate may be substituted by methylene groups without adversely affecting inhibitory activity. Thus, the binding of acyclovir monophosphate to PNP does not appear to involve specific interactions of the enzyme with either of the oxygens in the acyclo side chain. The connection may be lengthened without reducing the efficacy of these phosphonate inhibitors; however, the K_i' values do not approach that of acyclovir diphosphate (8.7 nM [6]) in spite of similar lengths between purine and phosphate-like moieties. The 100-fold lower K_i' of acyclovir diphosphate, relative to that of $\mathbf{IV_{n=7}}$, may result from specific interactions of the enzyme with the polar substituents of the second phosphate. It is likely that attaching a phosphate to compound $\mathbf{IV_{n=5}}$ via a phosphate ester linkage would produce an inhibitor of PNP as potent as acyclovir diphosphate.

Few reactions catalyzed by enzymes require that orthophosphate approach C_1' of a nucleoside; therefore, a compound that contains a phosphate-like moiety connected to an appropriate purine base has the potential of being a potent and highly specific inhibitor of PNP. There are some interesting potential "multisubstrate" analog inhibitors of PNP in which a phosphate moiety (but not a phosphonate moiety) would be unstable. It is encouraging to find in this initial study that a methylenephosphonate group may be used as a phosphate-like moiety as this is not always the case [4, 14]. Appropriate modification of the inhibitors reported here should lead to inhibitors of the other purine and pyrimidine phosphorylases.

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