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Fluorophosphonate Derivatives of N^9-Benzylguanine as Potent, Slow-Binding Multisubstrate Analogue Inhibitors of Purine Nucleoside Phosphorylase

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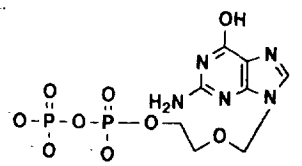
Abstract: The phosphonate derivatives of N^9-benzylguanine (**4a-4h**) have been prepared as purine nucleoside phophorylase inhibitors. Enzyme inhibition studies with PNP from calf spleen or human erythrocyte show that compounds **4b** and **4c** are among the best PNP inhibitors ever reported, demonstrating further the importance of fluorines in such type of inhibitors.

Purine nucleoside phosphorylase (PNP ; EC. 2.4.2.1) catalyzes the reversible phosphorolysis of nucleosides such as inosine, guanosine (and their 2'-deoxy forms) to their respective free base and ribose 1-phosphate (or deoxyribose-1-phosphate)[1]. PNP inhibitors might be useful as immunosuppressive agents as well as in the treatment of T-cell proliferative diseases (such as T-cell leukaemia), in the suppression of host-vs-graft response in organ transplant patients, in the treatment of gout and in the treatment of some parasitic diseases such as malaria[2-4]. In addition, PNP inhibitors may protect purine nucleosides used as chemotherapeutic agents such as 2',3' dideoxyinosine (dd I) against PNP metabolism[5].

Consequently, extensive drug discovery research has been devoted to the design and synthesis of PNP inhibitors during the last 10 years. In particular, numerous phosphonate derivatives of guanine or hypoxanthine have been prepared as multisubstrate analogue inhibitors of PNP[6-13]. The interest for these compounds was based on the finding that the diphosphate derivative of acyclovir **1** was a very potent inhibitor of the human enzyme, with the lowest K_i value described at that time (K_i = 8.7 nM at 1 mM orthophosphate concentration) for a PNP inhibitor[14]. The first metabolically stable analogues of **1** were 9-phosphono alkyl derivatives of hypoxanthine or guanine, the most potent compound **2a** in the series having a K_i value of 170 nM[7]. We recently described[8] some difluorophosphonate analogues which were found significantly more potent than the previous corresponding methylene phosphonates. For instance, compound **2b** was found to inhibit human erythocyte PNP with a K_i value of 15 nM. Interestingly, also phosphonate derivatives of N^9-benzyl guanine have recently been identified[13] as potent PNP inhibitors, for example compound **3** has a K_i = 1.1 nM.



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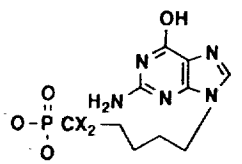
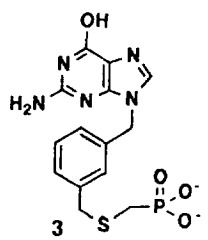


2a X = H

2b X = F



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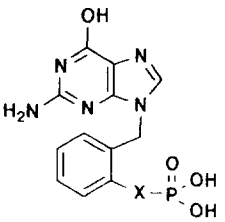
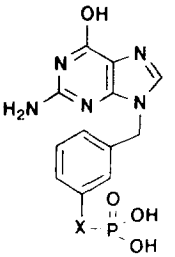
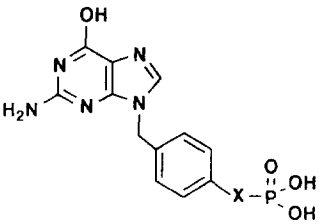
Here, we extend and develop preliminary data presented previously[11] and we further demonstrate the superiority of fluorophosphonates over phosphonates as stable substitutes for phosphates in "multisubstrate analogue" inhibitors. Thus, compounds **4a-4h** were synthesized in order to study the effect of fluorine atoms in N^9-benzyl phosphonate derivatives of guanine on the binding properties and some kinetic parameters of PNP inhibition.







4a X= CH_2CF_2 **4d** X= (E) CH=CH **4g** X= $CHFCF_2$ **4h** X= CH_2CF_2

4b X= $CHFCF_2$ **4e** X= $O(CH_2)_2CF_2$

4c X= (E) CH=CF **4f** X= $O(CH_2)_2CH_2$

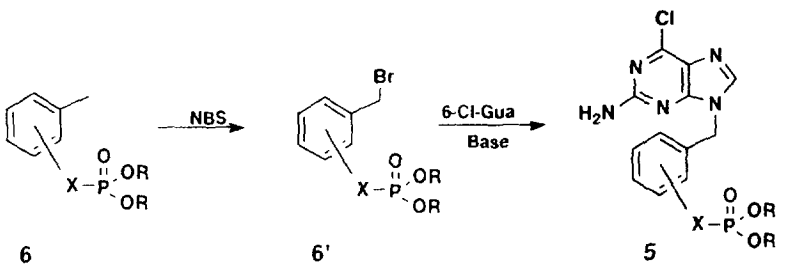
RESULTS AND DISCUSSION

Synthesis of fluorophosphonates 4. The phosphonic acid derivatives **4a-h** suitable for biochemical evaluation have all been obtained from the corresponding diethyl or di-isopropyl esters **5a-h** upon treatment with trimethylsilylbromide in acetonitrile or dichloromethane followed by acid aqueous hydrolysis (1N HCl, 18 h).

The 9-aryl phosphonate derivatives **5a-h** have been obtained by a common strategy from the aryl phosphonates **6a-i** (Scheme 1). The key step of the synthesis involves the condensation of the intermediate benzyl bromides **6'** with the 2-amino-6-chloropurine in the presence of potassium carbonate (method A) or sodium hydride (method B). This reaction gave 40 to 65 % yields of the adduct **5** and was found to proceed with a high control of N^9 regioselectivity (> 90 %) as determined by NMR analysis[15].

However, in the particular case of the trifluoroderivative **6i**, the yield of the expected product **5i** is very low (22 %) and the major identified reaction product was 9-ethyl-2-amino-6-chloropurine (60 % yield). The formation of this product is explained by a nucleophilic attack of the anion of 2-amino-6-chloropurine on the ethyl phosphonate ester. The reason why this side reaction prevails only with the trifluorophosphonate **6'i** can be the increased acidity of the phosphonic acid salt (which is formed during the reaction) due to the presence of three fluorine atoms. It is noteworthy that the formation of 9-ethyl-2-amino-6-chloropurine is only very marginal (< 5 %) in the other cases including the reaction giving the difluoro derivative **5a**. A similar side reaction was previously reported[16] during the condensation of 2-amino-6-benzyloxy-purine with diethyl 2-bromoethyl phosphonate. To avoid this undesired reaction, the bromination-alkylation sequence was studied starting from the di-isopropyl esters **6b** and **6g** under the same conditions; the expected condensation products **5b** and **5g** were then isolated in 65 and 56 % yields, respectively.

Scheme 1



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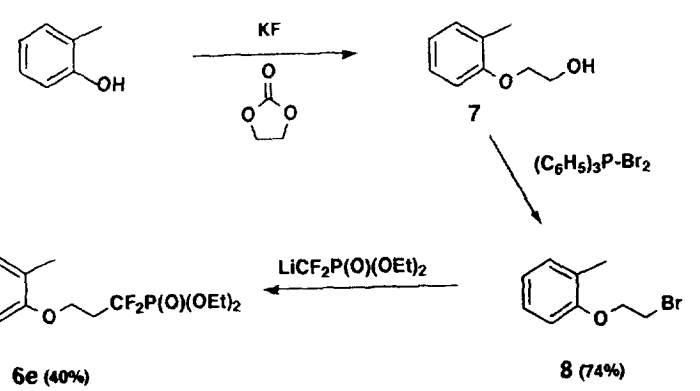
entry	X	R	Base	Yield (%)
a	*o*-CH_2CF_2	Et	NaH	42
b	*o*-$CHFCF_2$	*i*-Pr	K_2CO_3	65
c	*o*-CH=CF	Et	K_2CO_3	65
d	*o*-CH=CH	Et	K_2CO_3	56
e	*o*-$O(CH_2)_2CF_2$	Et	K_2CO_3	40
f	*o*-$O(CH_2)_2CH_2$	Et	K_2CO_3	45
g	*m*-$CHFCF_2$	*i*-Pr	K_2CO_3	56
h	*p*-CH_2CF_2	Et	K_2CO_3	54
i	*o*-$CHFCF_2$	Et	K_2CO_3	22

Aryl phosphonate derivatives **6a-d** and **6f-i** have been prepared according to known procedures[11,13]. The difluoropropyl phosphonate derivative of cresol **6e** was obtained in three steps as depicted in Scheme 2 : Intermediate 7 was easily prepared by condensing *o*-cresol with ethylene carbonate in the presence of potassium fluoride; then, after transformation of the alcohol 7 into the bromide **8**, condensation with diethyl phosphonyl difluoromethyllithium gave the expected difluorophosphonate **6e** in 40 % yield.

Scheme 2



6e (40%) **8** (74%)

Inhibition studies. Apparent inhibition constants (K_i) obtained from a Dixon plot for compounds **4** at pH 7.4, with inosine as the variable substrate and the orthophosphate concentration being fixed at 1mM, are

listed in Table 1. Compared to the other inhibitors reported here, compounds **4b**, **4c**, and **4d** have the highest affinity for PNP from human erythrocyte and calf spleen, their K_i values being in the nanomolar range.

Table 1. Inhibition constants for PNP inhibitors

COMPOUND	HUMAN ERYTHROCYTE PNP		CALF SPLEEN PNP	
	K_i (nM)[a] 1mM Pi	IC_{50} (nM)[b] 50mM Pi	K_i (nM)[a] 1mM Pi	IC_{50} (nM.)[b] 50 mM Pi
4a	13 ± 1	300	4 ± 1	200
4b	1.3 ± 0.1	85	0.6 ± 0.1	100
4c	1.8 ± 0.1	77	0.8 ± 0.3	60
4d	3.2 ± 0.3	155	0.8 ± 0.2	85
4e	210 ± 10	6000	250 ± 10	N.D.
4f	590 ± 50	24000	600 ± 100	23000
4g	160 ± 8	4600	225 ± 10	13000
4h	15000 ± 200	N.D.	3300 ± 900	N.D.

[a]Determination of K_i values was performed as described in Experimental Section. [b]Values of IC_{50} (concentration of inhibitor giving 50 % of enzyme inhibition) was measured in the presence of 50 μM inosine and 50 mM Pi.

The phosphonate derivatives of 9-benzylguanine, **4a-4h**, present inhibitory properties closely related to position of their phenyl ring substitution, thus confirming similar results from Kelley et al.[13]. Compound **4g**, the *meta* analog of **4b**, is 100-fold less potent than its isomer as an inhibitor of PNP from human erythrocyte and calf spleen ; compound **4h**, the para analog of **4a**, is 1000-fold less potent than **4a** when tested on the same enzymes. Recently, Montgomery et al.[17] prepared 9-benzyl-9-deazaguanine derivatives and showed in crystallographic studies that the position of the phenyl ring substitution was also critical on the interaction of the deazaguanine moiety with the purine binding site. As our compounds are multisubstrate analogs, a misplacement of the substitution on the phenyl ring may result in a general displacement of the components of the inhibitor from their optimal binding positions. Another important conclusion which confirms and extends previous findings[8,11] can be drawn from Table 1 regarding the superiority of fluorophosphonates over their phosphonate counterparts: compound **4b** is about 10 times more potent than **4a** whereas **4c** and **4e** are twice more potent than their non fluorinated homologs **4d** and **4f**, respectively.

As shown for other phosphonates[8], inhibition decreased with increasing concentrations of orthophosphate. At 50 mM orthophosphate, IC_{50} values are substantially higher than K_i values measured at 1 mM phosphate (Table 1). Such data demonstrate further that compounds **4** are multisubstrate analogs.

Interestingly, we found **4b**, **4c** and **4d** to behave as slow-binding inhibitors of PNP from both human erythrocyte and calf spleen, the steady state being reached in their presence after several minutes. The rate constants k_{on} and k_{off} were measured (see Experimental section) and $K_i = k_{off} / k_{on}$ was calculated for the three slow-binding inhibitors (Table 2). There is a good agreement between these K_i values and the K_i values of Table 1 calculated from a Dixon plot based on the steady state velocities.

Table 2. Inhibition constants for slow-binding PNP inhibitors[a]

COMPOUND	Source of PNP	k_{on} ($M^{-1} s^{-1}$)	k_{off} (s^{-1})	$K_i = k_{off} / k_{on}$ (nM)
4 b	human erythrocyte	5.0×10^5	1.3×10^{-3}	2.6
	calf spleen	1.0×10^6	5.5×10^{-4}	0.55
4 c	human erythrocyte	1.4×10^6	2.6×10^{-3}	1.85
	calf spleen	2.1×10^6	4.3×10^{-4}	0.2
4 d	human erythrocyte	2.2×10^6	5.7×10^{-3}	2.6
	calf spleen	1.2×10^6	1.0×10^{-3}	0.8

[a]Determination of K_i values was performed as described in Experimental Section.

In conclusion, compounds **4b** and **4c** are among the most potent inhibitors of PNP from both human erythrocyte and calf spleen reported so far, demonstrating further the importance of fluorines in our inhibitors[8,11].

EXPERIMENTAL SECTION

Synthesis. Reagents. All chemical reagents are from Janssen Chimica (Beerse, Belgium). They were used without further purification.

General Methods. Melting points were obtained on a Büchi SMP-20 melting point apparatus and are uncorrected. Proton and fluorine NMR spectra were recorded on either a Varian EM 390 spectrometer (90 MHz) or a Bruker instrument (360 MHz). Chemical shifts are reported in δ units, parts per million (ppm) downfield from TMS (external standard) for ^1H NMR and from sodium trifluoroacetate (δ_{CFCl3} = δ CF_3CO_2H + 77 ppm) or hexafluorobenzene ($\delta_{CFCl3} = \delta_{C6F6}$ + 163 ppm) for ^{19}F NMR. Mass spectra were recorded on a Finnigan TSQ46 apparatus. Thin-layer chromatography was performed on silica gel 60F-254 plates (Merck; 0.2 mm layer). Flash column chromatography utilized silica gel 60 as the solid phase (230-400 mesh) from E. Merck laboratories.

Typical procedures for the preparation of the phosphonate diester derivatives 5:

Synthesis of {2-[2-[(2-amino1,6-dihydro-6-chloro-9H-purin-9-yl)-methyl]phenyl]-1,1-difluoroethyl} phosphonic acid diethyl ester (5a). A suspension of *N*-bromo-succinimide (3 mmol, 0.53 g), benzoyl peroxide (5 mg), and **6a** (3 mmol, 0.88 g) in 20 mL of carbon tetrachloride is heated (with a UV lamp) at refluxing temperature for 90 min. The reaction mixture is filtered to remove succinimide and the filtrate is evaporated under reduced pressure to give 1.1 g of an oil which is then added to a stirred solution of the sodium salt of 2-amino-6-chloropurine [prepared by adding 3.5 mmol (592 mg) of 2-amino-6-chloropurine to 3.5 mmol of NaH (140 mg of a 60 % suspension in oil freshly washed with hexane) in 8 mL of DMF. The reaction mixture is stirred at 20 °C for 20 hours, evaporated to dryness and purified by flash chromatography on silica gel (using ethylacetate and increasing amounts of methanol as eluents) to give 1.15 g of product **5a** (42 % yield) as a colorless oil. ^1H NMR ($CDCl_3$) : 7.95 (s, H_8), 7.15-7.45 (m, 4H arom.), 5.40 (s, CH_2N), 4.0-4.40 (m, 4H, OCH_2), 3.32 (dt, CH_2CF_2, J_{H-F} = 21 Hz, J_{H-P} = 4 Hz), 1.35 (t, 6H, OCH_2CH_3). MS (CI, NH_3): 460 (MH^+), 477 (MNH_4^+), 291. Anal. Calcd for $C_{18}H_{21}N_5PO_3F_2Cl$: C, 47.02; H, 4.60; N, 15.23. Found : C, 46.88; H, 4.51; N, 15.60.

Synthesis of {2-[2-[(2-amino-1,6-dihydro-6-chloro-9H-purin-9-yl)methyl]phenyl]-1,1,2-trifuoroethyl}-phosphonic acid diisopropylester (5b). A suspension of *N*-bromosuccinimide (12.35 mmol, 2,2 g), benzoyl peroxide (10 mg), and **6b** (10.3 mmol, 3,48 g) in 40 mL of carbon tetrachloride is heated (with a UV lamp) at refluxing temperature for 90 min. The reaction mixture is filtered to remove succinimide and the filtrate is evaporated under reduced pressure to give 4.5 g of a light-brown oil which is added to a stirred suspension of potassium carbonate (1,68 g, 12.2 mmol) and 2-animo-6-chloropurine (2 g, 12.2 mmol) in 30 mL of

anhydrous DMF at 20 °C under argon. The reaction mixture is stirred at 20 °C for 20 hours, evaporated under pressure and purified by flash chromatography on silica gel using ethyl acetate and increasing amounts of methanol as eluents to give 3.4 g of expected product **5b** (oil) (65 %) yield. ^{1}H NMR ($CDCl_3$): 7.82 (s, H_8), 7.60 (m, 1H arom.), 7.4-7.5 (m, 2H arom.) 7.35 (m, 1H arom.), 7.00 (ddd, CHF_A, J_{H-FA} = 41 Hz, J_{H-FB} = 21 Hz, J_{H-FC} = 5 Hz), 5.30 (AB system, CH_2N), 5.50 (m, NH_2), 4.80 (m, 2H, OCH), 1.45 (m, 12H, CH *(Me)*$_2$). ^{19}F NMR ($CDCl_3$, C_6F_6) : 43 (F_C, ddd, J_{FC-FB} = 324 Hz, J_{FC-FA} = 18 Hz, J_{FC-P} = 101 Hz), 35.5 (F_B, dddd, J_{FB-FC} = 324 Hz, J_{FB-FA} = 18 Hz, J_{FB-H} = 21 Hz, J_{FB-P} = 102 Hz), - 30 (F_A, dt, J_{FA-H} = 41 Hz, J_{FA-FBC} = 18 Hz). MS (CI, NH_3), 506 (MH^+), 202, 170.

(E)-{2-[2-[(2-amino-1,6-dihydro-6-chloro-9H-purin-9-yl)methyl]-phenyl]-1-fluoroethenyl} phosphonic acid diethyl ester (5c). ^{1}H NMR ($CDCl_3$) : 8.20 (dd, H_{vinyl}, J_{H-F} = 42 Hz, J_{H-P} = 11 Hz), 7.85 (s, H_8), 7.90-7.80 (m, 1H arom.), 7.30-7.50 (m, 3H arom.), 5.90-6.00 (m, NH_2), 5.30 (s, CH_2N), 4.10-4.35 (m, 4H, OCH_2), 1.40 (t, 6H, OCH_2CH_3). ^{19}F NMR ($CDCl_3$, C_6F_6) : 36.2 (dd, J_{F-P} = 99 Hz, J_{F-H} = 42 Hz). MS (CI, NH_3): 440 (MH^+), 457 (MNH_4^+), 170. Anal. Calcd for $C_{18}H_{20}N_5O_3PClF$: C, 49.15; H, 4.58; N, 15.92. Found : C, 49.30; H, 4.46; N, 15.68.

(E)-{2-[2-[(2-amino-1,6-dihydro-6-chloro-9H-purin-9-yl)methyl]-phenyl]-ethenyl} phosphonic acid diethyl ester (5d). ^{1}H NMR ($CDCl_3$) : 8.80 (dd, H_{Bvinyl}, J_{H-P} = 23.7 Hz, J_{H-H} = 17 Hz), 7.81 (s, H_8), 7.60 (m, 1H arom.), 7.30-7.45 (m, 3H arom.), 6.25 (dd, H_{Avinyl}, J_{H-P} = 19 Hz, J_{H-H} = 17 Hz), 5.50 (s, 2H, CH_2N), 4.45 (m, 4H, OCH_2) , 1.35 (t, 6H, OCH_2CH_3). MS (CI,NH_3) : 422 (MH^+), 170.

{3[2-[(2-amino-1,6-dihydro-6-chloro-9H-purin-9-yl)methyl]phenoxy]-1,1-difluoropropyl} phosphonic acid diethyl ester (5e). ^{1}H NMR ($CDCl_3$) : 7.85 (s, H_8), 7.30 (m, 2H arom.), 6.95 (m, 2H arom.), 5.30 (s, CH_2N), 5.20 (m, NH_2), 4.30 (m, 6H, OCH_2), 2.60 (m, 2H, CH_2CF_2), 1.35 (t, 6H, OCH_2CH_3). ^{19}F NMR ($CDCl_3$, C_6F_6) : 50.6 (dt, J_{H-F} = 19 Hz, J_{F-P} = 105 Hz). MS (CI, NH_3) : 490 (MH^+), 170.

{3-[2-[(2-amino-1,6-dihydro-6-chloro-9H-purin-9-yl)methyl]phenoxy]-propyl} phosphonic acid diethyl ester (5f). ^{1}H NMR ($CDCl_3$) : 7.85 (s, H_8), 7.15-7.40 (m, 2H arom.), 6.75-7.15 (m, 2H arom.), 5.90 (m, NH_2), 5.25 (s, CH_2N), 3.90-4.40 (m, 6H, CH_2O), 1.70-2.30 (m, 4H, CH_2CH_2P), 1.25 (t, 6H, OCH_2CH_3). MS (CI, NH_3) : 454 (MH^+), 471 (MNH_4^+).

{2-[3-[(2-amino-1,6-dihydro-6-chloro-9H-purin-9-yl)methyl]phenyl]1,1,2-trifluoroethyl} phosphonic acid diisopropyl ester (5g). ^{1}H NMR ($CDCl_3$) : 7.80 (s, H_8), 7.25-7.5 (m, 4H arom.), 5.75 (ddd, CHF_A, J_{H-FA} = 43 Hz, J_{H-FB} = 18 Hz, J_{H-FC} = 4 Hz), 4.85 (m, 2H, OCH), 1.40 (m, 6H, $OCH(CH_3)_2$). MS (CI, NH_3) : 506 (MH^+), 170. Anal. Calcd for $C_{20}H_{24}N_5ClF_3PO_3$: C, 47.49; H, 4.78; N, 13.84. Found : C, 47.67 ; H,4.96; N, 13.68.

{2-[4-[(2-amino-1,6-dihydro-6-chloro-9H-purin-9-yl)methylphenyl]1,1-difluoroethyl]phosphonic acid} diethyl ester (5h). ^{1}H NMR ($CDCl_3$) : 7.90 (s, H_8), 7.25 (s, 4H arom.), 5.30 (s, CH_2N), 4.00-4.35 (m, 4H, OCH_2), 3.35 (dt, CH_2CF_2, J_{H-F} = 20 Hz, J_{H-P} = 5 Hz), 1.35 (t, 6H, OCH_2CH_3). MS (CI, NH_3) : 460 (MH^+).

Typical procedure for the preparation of the phosphonic acid derivatives 4a - h

Synthesis of 4d. Freshly distilled trimethylsilyl bromide (2.1 mL, 16.4 mmol) is added dropwise to a stirred suspension of **5d** (1.73 g, 4.11 mmol) in 10 mL of anhydrous acetonitrile at 20 °C under argon. After stirring for 20 hours, the reaction mixture is evaporated under reduced pressure. The residue is dissolved in acetonitrile (10 mL) and treated with 0.5 mL of methanol. The white yellow solid isolated after filtration is dissolved in 1 N aqueous HCl (10 mL) containing 3 mL of THF and heated at 80 °C for 18 hours. A white solid is formed and isolated after filtration at 20 °C. Recristallization from hot water gives 842 mg of product 5d (60 % yield for both steps) after lyophilisation.

{2-[2-[(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methyl]phenyl]-1,1-difluoroethyl} phosphonic acid (4a). ^{1}H NMR (D_2O, NaOD) : 7.70 (s, H_8), 7.48 (d, 1H arom.), 7.38 (t, 1H arom.), 7.28 (t, 1H arom.), 6.75 (d, 1H arom.), 5.45 (s, CH_2N), 3.6 (t, CH_2CF_2, J_{H-F} = 21 Hz). ^{19}F NMR (D_2O, NaOD) (ref. is CF_3CO_2Na). - 35.9 (dt, J_{F-H} = 21 Hz, J_{F-P} = 84 Hz). IR (KBr) (cm^{-1}) : 3438, 1696, 1641, 1178. UV (H_2O) [λ (cm) (ε)] 270 (10497), 253 (12888), 206 (29334). MS (FAB, argon): 386 (MH^+), 185. Anal. Calcd for $C_{14}H_{14}F_2N_5O_4P$, 0.5 HCl, H_2O : C, 39.89; H, 3.95; N, 16.62. Found : C, 40.21; H, 4.12; N, 16.46.

{2-[2-[(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methyl]phenyl]-1,2,2-trifluoroethyl}phosphonic acid (4b). ^1H NMR (D_2O, NaOD) : 7.75 (s, H_8), 7.70 (d, 1H arom.), 7.30-7.40 (t, 1H arom), 6.80 (d, 1H arom.), 6.35 (dd, CHF, $J_{H\text{-}FA}$ = 44 Hz, $J_{H\text{-}FB}$ = 22 Hz), 5.45 (q, CH_2N). ^{19}F NMR (D_2O, NaOD) (ref. is CF_3CO_2Na). - 39.5 (FC, ddd, $J_{FC\text{-}FB}$ = 303 Hz, $J_{FC\text{-}P}$ = 77 Hz, $J_{FC\text{-}FA}$ = 14 Hz), - 51 (F_B, ddd, $J_{FB\text{-}FC}$ = 303 Hz, $J_{FB\text{-}P}$ = 82 Hz, $J_{FB\text{-}FA}$ = 15 Hz, $J_{FB\text{-}H}$ = 22Hz). - 113.7 (F_A, dt, $J_{FA\text{-}H}$ = 44 Hz, $J_{FA\text{-}FBC}$ = 14 Hz). IR (KBr) (cm^{-1}) : 3378, 3225, 1700, 1639, 1231, 1077. UV (H_2O) [λ (nm) (ε)] : 270 (10175), 253 (13143), 297 (28978). MS (FAB, Xenon) : 404 (MH^+). Anal. Calcd for $C_{14}H_{13}F_3N_5O_4P$, H_2O : C, 39.92; H, 3.59; N, 16.62. Found: C, 40.20; H, 3.53; N, 16.52.

(E)-{2-[2-[(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methyl]phenyl]-1-fluoroethenyl}phosphonic acid (4c). ^1H NMR (D_2O, NaOD) : 7.72 (s, H_8), 7.70 (d, 1H arom.), 7.35 (t, 1H arom), 7.25 (t, 1H arom.), 6.75 (d, 1H arom.), 6.55 (dd, CH = C, $J_{H\text{-}F}$ = 43 HZ, $J_{H\text{-}P}$ = 7 Hz), 5.35 (s, CH_2N). ^{19}F NMR (D_2O, NaOD) (ref. is CF_3CO_2Na). - 42.5 (dd, $J_{F\text{-}H}$ = 43 Hz, $J_{F\text{-}P}$ = 91 Hz). IR (KBr) (cm^{-1}) : 3408, 1704, 1659, 1599, 1387, 1224, 1174, 1109. UV (H_2O) [λ (nm) (ε)] : 281 (6700), 248 (21530), 203 (38030). MS (FAB, Xenon) : 366 (MH+), 185. Anal. Calcd for $C_{14}H_{13}FN_5O_4P$, 0.5 HCl, 0.5 H_2O : C, 42.84; H, 3.72; N, 17.84. Found : C, 42.93; H, 3.71; N, 17.84.

(E)-{2-[2-[(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methyl]phenyl]ethenyl}phosphonic acid (4d). ^1H NMR (D_2O, NaOD) : 7.75 (s, H_8), 7.70 (d, H arom.), 7.38 (dd, H_B vinyl, $J_{H\text{-}P}$ = 22 Hz, $J_{H\text{-}H}$ = 15 Hz), 7.35 (t, 1H arom.), 7.25 (t, 1H arom.), 6.70 (d, 1H arom.), 6.50 (dd, H_A vinyl, $J_{H\text{-}H}$ = 15 Hz, $J_{H\text{-}P}$ = 17 Hz), 5.45 (s, CH_2N). UV (H_2O) [λ (nm) (ε)] : 253 (30554), 203 (51600), 194 (53325). MS (FAB , Xenon) : 347 (MH^+). Anal. Calcd for $C_{14}H_{14}N_5O_4P$, 0.5 H_2O, 0.70 HBr : C, 40.72. H, 3.83; N, 16.96. Found : C, 40.66; H, 3.75; N, 17.31.

{3-[2-[(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methyl]phenoxyl]-1,1 difluoropropyl}phosphonic acid (4e). ^1H NMR (D_2O, NaOD) : 7.75 (s, H_8), 7.30-7.40 (m, 1H arom.), 7.15 (d, 1H arom.), 6.90-7.10 (m, 2H arom.), 5.25 (s,CH_2N), 4.35 (t, OCH_2), 2.55 (m, CH_2CF_2). ^{19}F NMR (D_2O, NaOD) (ref. is CF_3CO_2Na). - 35.40 (dt, $J_{H\text{-}F}$ = 20 Hz, $J_{F\text{-}P}$ = 104 Hz). UV (H_2O) [λ (nm) (ε)] : 271 (6279), 259 (5519), 205 (16151). MS (FAB-Xenon) : 416 (MH^+). Anal. Calcd for $C_{15}H_{18}N_5O_6P$, 0.6 HCl, 1.5 H_2O : C, 42.07; H, 5.08; N, 16.36. Found : C, 42.24; H, 4.94; N, 16.02.

{3-[2-[(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methyl]phenoxy]-propyl}phosphonic acid (4f). ^1H NMR (D_2O, NaOD) : 7.70 (s, H_8), 7.35 (m, 1H arom.), 7.15 (d, 1H arom.), 7.05 (m, 1H arom)., 6.90 (t, 1H arom.), 5.25 (s, CH_2N), 4.05 (t, OCH_2), 1.95 (m, CH_2P), 1.5 (m, CH_2). IR (KBr) (cm^{-1}) : 3349, 3127, 2945,1698,1636, 988, 939. UV (H_2O) [λ (nm) (ε)] : 271 (12900), 255 (10780), 215 (27850). MS (FAB-Xenon) : 380 (MH^+), 225, 152, 123 Anal. Calcd for $C_{15}H_{16}F_2N_5O_6P$, 0.5 H_2O : C, 42.46; H, 4.04; N, 16.51. Found : C, 42.49; H, 4.44, N, 16.22.

{2-[3-[(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methyl]phenyl]-1,1,2-trifluoroethyl}phosphonic acid (4g). ^1H NMR (D_2O, NaOD) : 7.75 (s, H_8), 7.4-7.6 (m, 3H arom.), 7.25 (d, 1H arom.), 5.85 (dd, CHF, $J_{H\text{-}FA}$ = 43 Hz, $J_{H\text{-}FB}$ = 22 Hz), 5.35 (s, CH_2N). ^{19}F NMR (D_2O, NaOD) (ref. is CF_3CO_2Na). - 40.5 (F_C, ddd, $J_{FC\text{-}FB}$ = 304 Hz, $J_{FC\text{-}P}$ = 78 Hz, $J_{FC\text{-}FA}$ = 12Hz) - 51.9 (F_B, dddd, $J_{FB\text{-}FC}$ = 304 Hz, $J_{FB\text{-}P}$ = 80 Hz, $J_{FB\text{-}FA}$ = 16 Hz, $J_{FB\text{-}H}$ = 22 Hz), - 114.2 (F_A, dt, $J_{FA\text{-}H}$ = 43 Hz, $J_{FA\text{-}FBC}$ = 16 Hz). IR (KBr) (cm^{-1}) : 3428, 3222, 1702, 1639, 1166, 1058. UV (H_2O) [λ (nm) (ε)] : 268 (11945), 253 (15420), 204 (34275) MS (FAB, Xenon) : 404 (MH^+). Anal. Calcd for $C_{14}H_{13}F_3N_5O_4P$, HCl : C, 38.24; H, 3.21; N, 15.93. Found : C, 37.75; H, 3.13, N, 15.98.

{2-[4-[(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methyl]phenyl]-1,1-difluoroethyl}phosphonic acid (4h). ^1H NMR (D_2O, NaOD) : 7.65 (s, H_8), 7.35 (d, 2H arom.), 7.15 (d, 2H arom.), 5.2 (s, CH_2N), 3.30 (t, CH_2CF_2, $J_{H\text{-}F}$ = 22 Hz). ^{19}F NMR (D_2O, NaOD) (ref. is CF_3CO_2Na). - 37.15 (dt, $J_{H\text{-}F}$ = 22 Hz, $J_{F\text{-}P}$ = 85 Hz) IR (KBr) (cm^{-1}) : 3315, 3155, 3009, 1693, 1682, 1572, 1150, 1089, 1039. UV (H_2O) [λ (nm) (ε)] : 268 (11630), 258 (11160), 212 (37431). MS (FAB, Xenon) : 386 (mH^+). Anal. Calcd for $C_{14}H_{14}F_2N_5O_4P$, 0.5 H_2O : C, 42.65; H, 3.83; N, 17.76. Found : C, 42.67; H, 3.60; H, 17.43.

Assay of PNP. PNP from human erythrocyte and calf spleen were purchased from Sigma Chemical Co., St. Louis, MO. PNP activity was determined spectrophotometrically by a xanthine oxidase coupled assay according to Kalckar[18] with inosine as substrate. The increase in absorbance at 293 nm was monitored with a Beckman DU-7 spectrophotometer. The typical assay contained 0.1 M HEPES/NaOH buffer (pH 7.4), 0.04 units of xanthine oxidase (Boehringer Mannheim Gmbh, West Germany), 1mM (or 50 mM) sodium

phosphate, appropriate concentrations of inosine (Sigma Chemical Co.) and PNP in a total volume of 1 ml at 37 °C. Under these conditions, in the presence of 50 mM sodium phosphate, the specific activities of the two PNP, expressed as micromoles per minute per milligram of protein were found to be 44 (human erythrocyte) and 116 (calf spleen).

Determination of K_i values using a Dixon plot. Kinetics of steady-state inhibition were followed using four concentrations of inosine and six concentrations of inhibitor. Initial velocities were measured after a time sufficient to observe steady state kinetics and K_i values were determined by using a Dixon plot and a computer program developed in-house for linear regression analysis. It was verified that the inhibitors did not affect xanthine oxidase activity in the assay.

Determination of kinetic constants for slow-binding inhibition of PNP. Rate constants for slow-binding inhibitors (k_{on} and k_{off}) were determined from the progress curves of product formation versus time as described by Williams and Morrisson[19] using Enzfitter, a non-linear regression data analysis program[20] from Elsevier-Biosoft (Cambridge). The assays were initiated by the addition of enzyme.

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REFERENCES

1. Parks, R.E., Jr; Agarwal, R.P. In *The Enzymes*, 3rd Ed., Boyer, P.D., Ed.; Academic Press : New York, **1972**; *Vol. 7*, pp 483-514.
2. (a) Stoeckler J.D. In *Developments in Cancer Chemotherapy* ; Glazen, R.I. Ed.; CRC Press, Boca Raton, **1984**, pp 35-60.
   (b) Gilbertsen, R.B.; Sircar, J.C. In *Comprehensive Medicinal Chemistry*; Sammes, P.G.; Ed.; Pergamond Press: Oxford, **1990**; *Vol. 2*, pp 443-480.
3. Stoeckler, J.D.; Ealick, S.E.; Bugg, C.E.; Parks, R.E. *Jr. Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1986**, *45*, 2773-2778.
4. Montgomery, J.A. *Exp. Opin. Invest. Drugs* **1994**, *3*, 1303-1313.
5. Weibel, M.; Balzarini, J.; Bernhardt, A.; Mamont, P. *Biochem. Pharmacol.* **1994**, *48*, 245-252.
6. Nakamura, C.E.; Chu, S-H.; Stoeckler, J.D.; Parks, R.E. Jr., *Biochem. Pharmacol.* **1986**, *34*, 133-136.
7. Nakamura, C.E.; Chu, S- H.; Stoeckler, J.D.; Parks, R.E. Jr. *Nucleosides, Nucleotides* **1989**, *8*, 1039-1040.
8. Halazy, S.; Ehrhard, A.; Danzin, C. *J. Am. Chem. Soc.* **1991**, *113*, 315-317.
9. Elliott, R.D.; Niwes, S.; Riordan, J.M.; Montgomery, J.A.; Secrist J.A. *Nucleosides, Nucleotides* **1992**, *11*, 97-119.
10. Kelley, J.L.; Mc Lean, E.W.; Crouch, R.C.; Averett, D.R.; Tuttle, J.V. *J. Med. Chem.* **1995**, *38*, 1005-1014.
11. A preliminary account of the present work has been published as a communication : Halazy S.; Eggenspiller, A.; Ehrhard, A.; Danzin, C. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 407-410.
12. Erion, M.D.; Niwas, S.; Rose, J.D.; Ananthan, S.; Allen, M.; Secrist, J.A.; Babu, Y.S.; Bugg C.E.; Guida, W.C.; Ealick, S.E.; Montgomery, J.A. *J. Med. Chem.* **1993**, *36*, 3771-3783.
13. Kelley, J.L.; Linn, J.A.; Mc Lean, E.W.; Tuttle, J.V. *J. Med. Chem.* **1993**, *36*, 3455-3463.
14. Tuttle, J.V.; Krenitsky, T.A. *J. Biol. Chem.* **1984**, *259*, 4065-4069.
15. a) Kjellberg, K.; Johansson, N.G. *Tetrahedron* **1986**, *42*, 6541-6544.
    b) Geen, G.R.; Grinter, T.J.; Kincey, P.M.; Jarvest, R.L. *Tetrahedron* **1990**, *46*, 6903-6914.
16. Stein, J.M.; Stoeckler, J.D.; Li, S-Y.; Tolman, R.L.; Maccoss, M.; Chen, A.; Karkas, J.D.; Ashton, W.T.; Parks, R.E. *Biochem. Pharmacol.* **1987**, *36*, 1237-1244.
17. Montgomery, J.A.; Niwas, S.; Rose, J.D.; Secrist, J.A.; III, Babu, Y. S.; Bugg, C.E.;Erion, M.D.; Guida, W.C.; Ealick, S.E. *J. Med. Chem.* **1993**, *36*, 55-69.
18. Kalckar, H.M. *J. Biol. Chem.* **1947**, *167*, 429-443.
19. Williams, J.W., Morrison, J.F. *Methods enzymol.* **1979**, *63*, 437-467.
20. Leatherbarrows, R.J., ENZFITTER, a non-linear regression data analysis program for the IBM-PC, Elsevier-Biosoft, Cambridge, England (**1987**)

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