Bioorganic & Medicinal Chemistry 20 (2012) 1222–1230

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis of purine N^9-[2-hydroxy-3-*O*-(phosphonomethoxy)propyl] derivatives and their side-chain modified analogs as potential antimalarial agents

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ARTICLE INFO

Article history:
Received 27 October 2011
Revised 15 December 2011
Accepted 17 December 2011
Available online 30 December 2011

Keywords:
Acyclic nucleoside phosphonates
Malaria
Plasmodium falciparum
Plasmodium vivax
Hypoxanthine-guanine-(xanthine)-phosphoribosyltransferase
5-Phospho-α-D-ribosyl-1-pyrophosphate

ABSTRACT

6-Oxopurine acyclic nucleoside phosphonates (ANPs) have been shown to be potent inhibitors of hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRT), a key enzyme of the purine salvage pathway in human malarial parasites. These compounds also exhibit antimalarial activity against parasites grown in culture. Here, a new series of ANPs, hypoxanthine and guanine 9-[2-hydroxy-3-(phosphonomethoxy)propyl] derivatives with different chemical substitutions in the 2′-position of the aliphatic chain were prepared and tested as inhibitors of *Plasmodium falciparum* (*Pf*) HGXPRT, *Plasmodium vivax* (*Pv*) HGPRT and human HGPRT. The attachment of an hydroxyl group to this position and the movement of the oxygen by one atom distal from N^9 in the purine ring compared with 2-(phosphonoethoxy)ethyl hypoxanthine (PEEHx) and 2-(phosphonoethoxy)ethyl guanine (PEEG) changes the affinity and selectivity for human HGPRT, *Pf*HGXPRT and *Pv*HGPRT. This is attributed to the differences in the three-dimensional structure of these inhibitors which affects their mode of binding. A novel observation is that these molecules are not always strictly competitive with 5-phospho-α-D-ribosyl-1-pyrophosphate. 9-[2-Hydroxy-3-(phosphonomethoxy)propyl]hypoxanthine (iso-HPMP-Hx) is a very weak inhibitor of human HGPRT but remains a good inhibitor of both the parasite enzymes with K_i values of 2 μM and 5 μM for *Pf*HGXPRT and *Pv*HGPRT, respectively. The addition of pyrophosphate to the assay decreased the K_i values for the parasite enzymes by sixfold. This suggests that the covalent attachment of a second group to the ANPs mimicking pyrophosphate and occupying its binding pocket could increase the affinity for these enzymes.

1. Introduction

The two most virulent and widespread species of *Plasmodium* that infect humans are *Plasmodium falciparum* (*Pf*) and *Plasmodium vivax* (*Pv*). Each year there are approximately 350–500 million cases of malaria, resulting in an estimated one to three million fatalities, the majority of whom are children in sub-Saharan Africa.[1,2] The antimalarial drugs currently in use have very different chemical structures and presumably target different metabolic pathways. For example, chloroquine and quinine inhibit polymerization of heme while pyrimethamine, proguanil, cycloguanil and sulfadoxine inhibit dihydrofolate reductase.[3,4] However, the precise mechanism of action of many of these drugs remains speculative even today. The fast spread of drug-resistant *Pf* and *Pv* requires an increased effort to develop new antimalarial drugs[5] including the search for new targets especially those that would be difficult for the parasite to mutate without compromising its own existence.

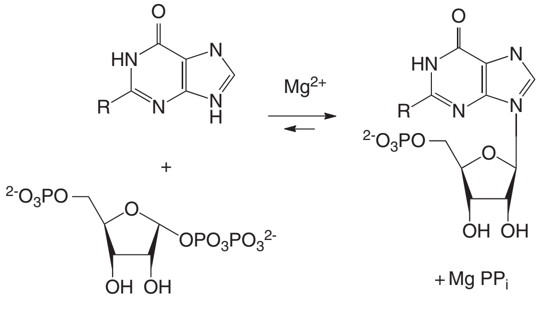

Figure 1. The reaction catalyzed by HG(X)PRT. R = -H (Hypoxanthine); $-NH_2$ (guanine); =0 (xanthine).

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doi:10.1016/j.bmc.2011.12.034

One such target for antimalarial drug discovery is hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRT). This enzyme is responsible for converting preformed 6-oxopurine bases transported from its host cell into the nucleoside monophosphates essential for DNA/RNA production. Therefore, its catalytic activity is essential for the survival and replication of the parasite. The reaction catalyzed by this enzyme is shown in Figure 1.

This enzyme is also present in human cells. Therefore, the design of selective inhibitors of *Pf*HGXPRT and *Pv*HGPRT, which do not exert toxic side-effects on the human host is a goal. This goal is assisted by the fact that, though the malarial parasites are auxotrophic for the purine ring, humans are not and possess a second de novo pathway to synthesize purine nucleoside monophosphates. A class of inhibitors which meet these criteria and target HG(X)PRT are the acyclic nucleoside phosphonates (ANPs). Such compounds have a broad spectrum of biological activities, derivatives of which are currently used in clinical practice as antivirals.[6] These antiviral agents differ from the ANPs in this study as they contain a 6-aminopurine or a pyrimidine base attached to the phosphonate containing moiety and target DNA polymerases or RNA transcriptases in the viruses. However, the common structural attributes of these compounds are a nucleobase attached to an aliphatic side-chain containing a phosphonomethyl linker that replaces the sugar moiety found in a nucleotide and the phosphonate group. Initial structure–activity-relationship (SAR) studies showed that ANPs are the first compounds which have selectivity towards *Pf*HGXPRT and *Pv*HGPRT compared to human HGPRT and that these compounds can arrest the growth of *Pf* in cell culture with IC_{50} values as low as 1 μM.[7,8] The crystal structures of three ANPs in complex with human HGPRT have suggested chemical modifications which could lead to increased potency and selectivity for the parasite enzymes. In this report, we describe the synthesis of a number of new ANPs some of which show >50-fold selectivity towards *Pf*HGXPRT and *Pv*HGPRT compared with human HGPRT (Fig. 2).

2. Chemistry

For the synthesis of designed compounds we selected 9-[2-hydroxy-3-(phosphonomethoxy)propyl] derivatives of 6-chloro- or 2-amino-6-chloropurine (**3a, 3b**) as starting material. Both compounds are easily accessible by nucleophilic opening of the epoxide ring in diethyl [(oxiranylmethoxy)methyl]phosphonate.[9] Reaction details are described in our previous work on 'iso-HPMP' derivatives.[10] Introduction of a two-carbon chain to the 2′-hydroxyl was performed using ethyl bromoacetate (Scheme 1). To avoid undesired elimination of this secondary hydroxy group in basic conditions, we had to pay special attention to the selection of reaction conditions, especially temperature. To avoid elimination, −40 °C was optimal to synthesize **4a** and **4b** in sufficient yields (30–40%) while the unreacted starting material could be recovered almost quantitatively. The obtained 6-chloropurine derivatives were converted to their hypoxanthine or guanine counterparts (**5a** and **5b**) by the treatment with acetic acid. This process leads partly to hydrolysis of carboxylic ester groups to give free carboxylic acids. Carboxylic ethyl ester group containing phosphonates can be also transformed to corresponding amides by heating with methanolic ammonia in an autoclave. The final products, free phosphonic acids **7a**,**b**–**9a**,**b** were obtained from the corresponding ethyl esters by transsilylation reaction with bromotrimethylsilane followed by hydrolysis.

Besides this reaction scheme, we tried several alternative procedures for modification of the 2′-position. One such process, starting from 6-chloropurine phosphonomethyl derivative **3a** is outlined in Scheme 2. The first reaction step, bromination in 2′-position was performed successfully using tetrabromomethane and triphenylphosphine. The 2′-bromo derivative **10** was subjected under different conditions to nucleophilic base catalyzed reaction with ethylene glycol to introduce a hydroxyethyl residue. These experiments lead exclusively to elimination products (**11–13**). Elimination was observed also in reactions starting from the 2′-bromohypoxanthine derivative **14**, the compound prepared from **3a** by the treatment with acetic acid. The attempts to substitute its 2′-hydroxyl with ethylene glycol or reaction with other nucleophilic agents (e.g., ammonia) always resulted in elimination products. Analogous reactions starting from the 6-chloropurine derivative **3a** and 2-chloroethanol in basic conditions preferentially resulted in cyclization between the phosphonate functional group and the free 2′-OH group, in parallel with a substitution reaction of the chloro atom in the C-6 position leading to a product of a dimeric structure. After acidic hydrolysis of 6-chloro derivatives to their hypoxanthine counterparts and deprotection of ester groups, compounds **16** and **17** were isolated as final products, together with a small amount of iso-HPMPHx (**1**) resulting from unreacted **3a** (Scheme 2). The most advantageous synthetic approach to iso-HPMPHx is deamination of its adenine counterpart, iso-HPMPA[10] using isoamyl nitrite.

3. Inhibition of the PRTases

The chemical structures of 9-[2-hydroxy-3-(phosphonomethoxy)propyl]hypoxanthine (iso-HPMP-Hx) (compound **1**) and 9-[2-hydroxy-3-(phosphonomethoxy)propyl]guanine (iso-HPMPG) (compound **2**) are based on 2-(phosphonoethoxy)ethyl hypoxanthine (PEEHx) and 2-(phosphonoethoxy)ethyl guanine (PEEG) (Fig. 2) which are good inhibitors of *Pf*HGXPRT with K_i values of 0.1 ± 0.02 and 0.3 ± 0.04 μM (Ref. 7), respectively and have selectivity ratios, K_i(Hu)/K_i(*Pf*) of 10 and 12. PEEHx and PEEG were chosen as models because not only are they good inhibitors of *Pf*HGXPRT but they also exhibit selectivity in favor of the parasite enzyme. There are two chemical differences in the phosphonate moiety between compound **1**, compound **2** and the PEE compounds. These are the location of the oxygen atom and the addition of a hydroxyl group. Compound **1** inhibits both *Pf*HGXPRT and *Pv*HGPRT with K_i values of 2 ± 0.4 and 5 ± 0.6 μM, respectively but does not inhibit the human enzymes at concentrations ⩽30 μM. Compound **2** has a K_i value for human of 4 ± 0.4 μM and 1.4 ± 0.3 and 10 ± 1 μM for *Pf*HGXPRT and *Pv*HGPRT, respectively, (Table 1). Thus, for this phosphonate moiety, the human enzyme

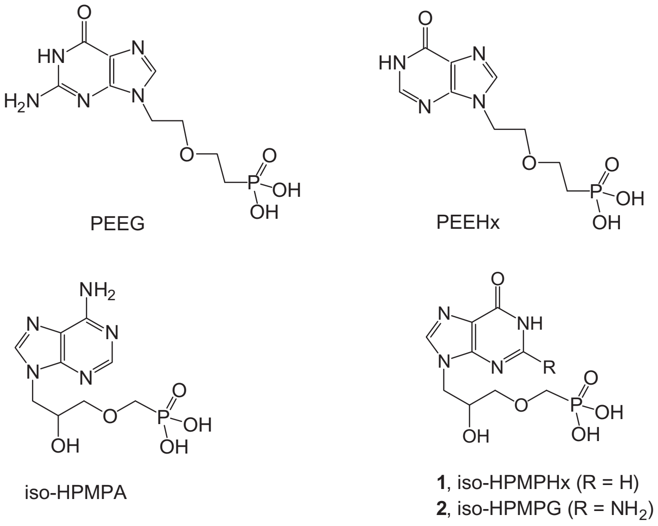
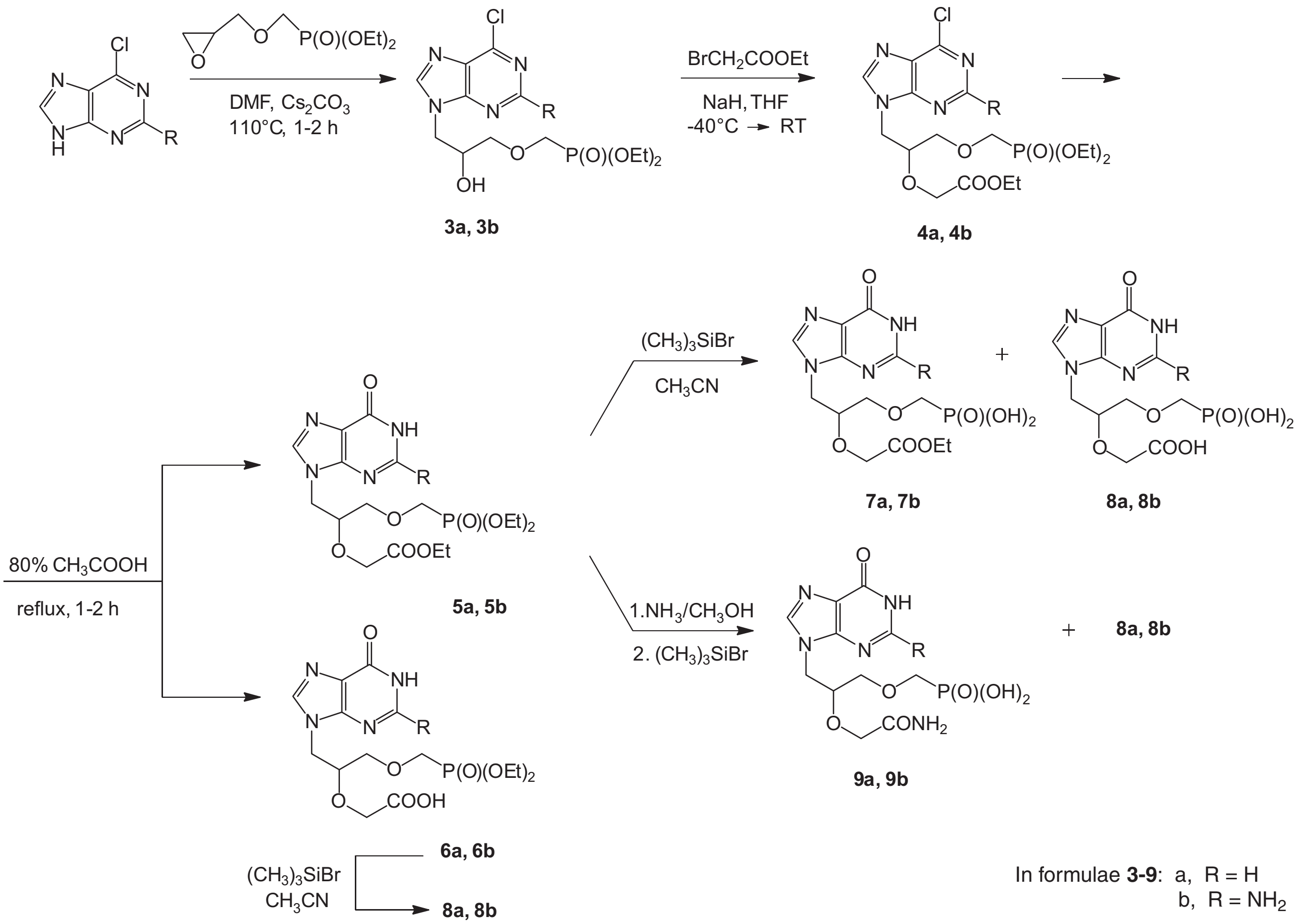


Figure 2. Structures of selected PEE and iso-HPMP derivatives.



Scheme 1. Introduction of carboxylic (and functionalized carboxylic) function containing chains to 2′-position of iso-HPMP derivatives.

prefers guanine as the base while the parasite enzymes are not as discriminatory. Though compounds **1** and **2** are not as strong inhibitors of the parasite enzyme as the PEE compounds, compound **1** does have increased selectivity of at least 50 (*Pf*) and 25 (*Pv*). These two compounds are not strictly competitive inhibitors of the parasite enzymes indicating that they are not orientated in the active site in the same way as the substrate, *P*Rib-*PP*.

Pyrophosphate (PP$_i$), at concentrations of 52 μM, does not inhibit either of the two parasite enzymes. However, when this concentration of PP$_i$ is added to the reaction mixture in the presence of 2.4 μM of compound **1**, the K_i for *Pf*HGXPRT and *Pv*HGPRT decreases to 0.3 ± 0.08 and 0.9 ± 0.09 μM, respectively, and strictly competitive inhibition kinetics is observed. Thus, the presence of PP$_i$ has a marked influence not only on the mode of binding of this compound but also in increasing its affinity.

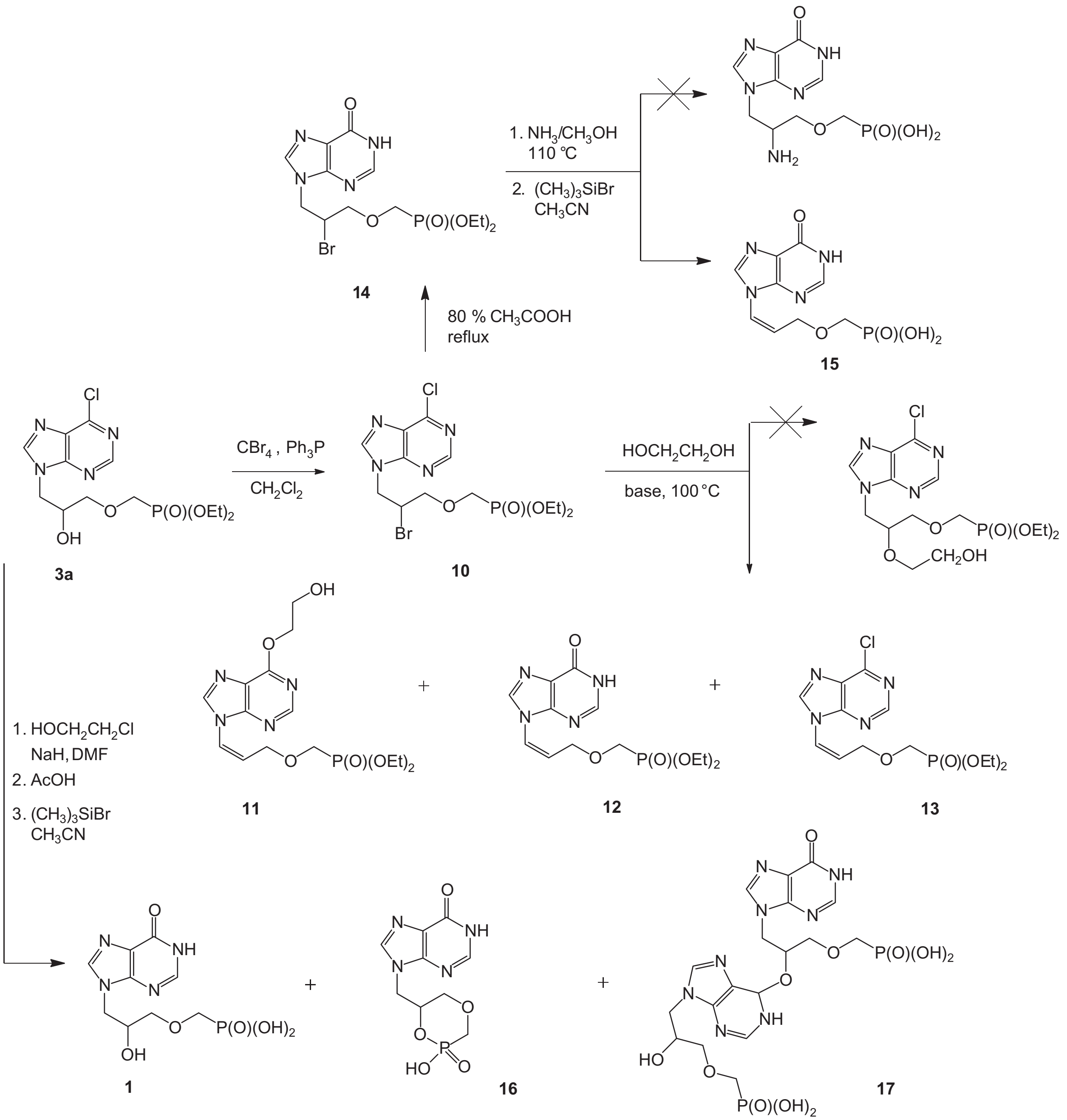
This data lead to the design and synthesis of a new series of compounds where a second group was covalently attached to either compounds **1** or **2**. It was postulated that if this second group could be made to occupy the PP$_i$ binding pocket, increased interactions between the ligand and the enzyme should result in a decrease in K_i. The chemical structure of these new compounds is given in Figure 4. Compound **8a**, where the attachment ends in a carboxyl group and the base is hypoxanthine, does not inhibit any of the three enzymes at the concentrations used (K_i > 100 μM). However, if the purine base is changed to guanine (compound **8b**), inhibition is observed with K_i values for the human, *Pf* and *Pv* enzymes being 5, 5 and 27 μM, respectively. These compounds are strictly competitive with *P*Rib-*PP*.

Thus, though this second attachment has not increased the affinity (cf. compound **8b** vs **1**or **2**), it has affected the mode of binding. Substitution of the carboxyl group with an amine, has no effect on the binding constants and these values are unchanged. Therefore, when guanine is the purine base, inhibition is observed but selectivity is lost. When Hx is the purine base, no inhibition is observed at the maximum concentration of inhibitor able to be used in the spectrophotometric assay. The compound **8b** was manually docked (Fig. 3) into the active site of human HGPRT; the docking study shows that purine base and the phosphonate group should occupy the same site as in the crystal structure of PEEG in complex with this enzyme. However, it can be seen that the second group attached to the phosphonate does not extend into the PP$_i$ binding pocket and thus suggests why there is no decrease in K_i. The attachment of this second group does not appear to result in any new strong interactions which contribute to its binding. Thus, it can only be speculated that there are weaker interactions that contribute to the orientation of this ANP so that it occupies the same location as *P*Rib-*PP* as it is competitive with this substrate.

Compound **15** is also a chemical modification of compound **2**. In this compound, the hydroxyl group has been removed and a double bond introduced (Scheme 2). This compound does not inhibit either human HGPRT, *Pf*HGXPRT or *Pv*HGPRT. The reason for this may be that the double bond has made the phosphonate tail less flexible and it cannot adapt to fit into the active site.

4. Conclusions

An efficient synthetic methodology for preparation of a new class of acyclic nucleoside phosphonates derived from the 'iso-HPMP' scaffold has been developed. The new ANPs provide insight into the factors which contribute to binding to the enzyme. These



Scheme 2. Elimination reactions in an aliphatic chain during attempts at modification of 2′-position by amino or 2-hydroxyethyl groups.

studies should lead to the design of new ANPs which are both potent and selective inhibitors of the *Plasmodium* enzymes. The active compounds will be further transformed into prodrug forms to increase their uptake to cells and to facilitate their activity studies directly in the malarial parasites.

5. Experimental

5.1. General

Unless stated otherwise, solvents were evaporated at 40 °C/2 kPa and compounds were dried at 13 Pa. Melting points were determined on a Kofler block and are uncorrected. Analytical TLC was performed on Silica Gel 60 F_{254} plates (Merck KGaA, Darmstadt, Germany); chromatographic systems are described in text. Column chromatography was performed on Silica Gel 60 μm (Fluka). Preparative reverse phase HPLC separations were performed on a Waters Delta 600 instrument with a Waters 2487 Dual λ Absorbance Detector using a column Luna Phenomenex® C-18 (10 μm, 10 × 150 mm), flow 5 ml/min. ^1H and ^{13}C NMR spectra were measured on a Bruker Avance 500 spectrometer (^1H at 500 MHz, ^{13}C at 125.7 MHz) in D_2O, CD_3OD, DMSO-d_6 or $CDCl_3$. The numbering system for assignment of NMR signals is outlined in Figure 4. The spectra were referenced to TMS, residual solvent system (δ 3.31 and 49.0 for CD_3OD, δ 77.0 for $CDCl_3$, 2.5 and 39.7 for DMSO-d_6) or to dioxane in D_2O (δ 3.75 and 67.19). The assignment of chemical shifts was done with the help of 2D NMR experiments (COSY, HSQC and HMBC). Mass spectra were measured by ESI technique. Most of chemicals and ion-exchange resins (Dowex 1X2-400) were purchased from Sigma–Aldrich. Syntheses

Table 1
Comparison of the K_i values and selectivity for PfHGXPRT, PvHGPRT and human HGPRT in compounds **1**,**2**, **8a** and **8b**

Compound	K_i (μM)						Selectivity ratio[a]	
	(−)PP$_i$			(+)PP$_i$[b]				
	Hu[c]	*Pf*[d]	*Pv*[e]	Hu[c]	*Pf*[d]	*Pv*[e]	Hu/*Pf*	Hu/*Pv*
1	NI[f]	2 ± 0.4	5 ± 0.6	NI[f]	0.3 ± 0.08	0.9 ± 0.09	⩾50	⩾25
2	4 ± 0.4	1.4 ± 0.3	10 ± 1	NI[g]	0.5 ± 0.06	2.8 ± 0.7	2.8	0.4
8a[g]	NI[h]	NI[h]	NI[h]	NI[h]	NI[h]	NI[h]	NI[h]	NI[h]
8b	5 ± 0.8	5 ± 0.9	27 ± 6	NI[f]	NI[f]	NI[f]	1	0.2

[a] Selectivity ratio in the absence of PP$_i$.
[b] K$_i$ values in the presence of 52 μM PP$_i$.
[c] Human HGPRT.
[d] *Pf*HGXPRT.
[e] *Pv*HGPRT.
[f] No inhibition at concentrations of ⩾ 30 μM.
[g] Not determined.
[h] No inhibition at concentrations of ⩾100 μM.

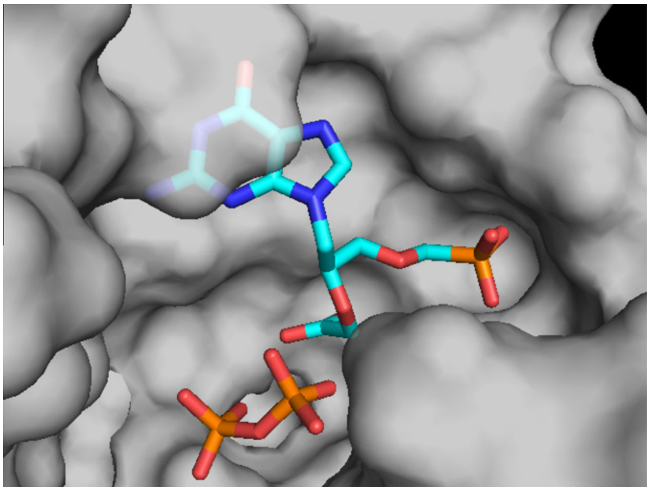


Figure 3. Docking of compound **8b** in the active site of human HGPRT. This figure suggests that the purine base and phosphonate should be orientated in nucleotide binding site in the same way as PEEG. Superimposed is the location of the pyrophosphate (stick model) as it appears in the human HGPRT structure (1BZY pdb code). There are no hydrogen bonding interactions between the 2′ extension and the enzyme.

of iso-HPMPA, iso-HPMPG (**2**) and compounds **3a**, **3b** are described in Ref. 10

5.2. 9-[2-Hydroxy-3-(phosphonomethoxy)propyl]hypoxanthine (1)

Isoamyl nitrite (0.16 mL) was added to a solution of iso-HPMPA[10] (55 mg; 0.18 mmol) in 80% acetic acid (1.5 mL). The mixture was set aside for 2 days at rt, followed by 2 days at 5 °C and evaporated. The residue was coevaporated with water (3 × 2 mL), applied onto a column of Dowex 50 (H$^+$ form, 20 mL) and the column eluted slowly with water. After initial elution of yellow colored impurities (with no UV absorption), elution of the product commenced. Pure UV absorbing fractions were evaporated, the residue coevaporated with absolute ethanol and dried in vacuo. Yield: 30 mg (55%) of a white solid. ^{1}H NMR (D$_2$O, ppm) δ: 3.59–3.72 (m, 4H, H-3′, PCH$_2$), 4.24 (m, 1H, H-2′), 4.32 (dd, 1H, $J_{1'a,2'}$ = 8.1, J_{gem} = 14.5, H-1′a), 4.43 (dd, 1H, $J_{1'b,2'}$ = 3.8, J_{gem} = 14.4, H-1′b), 8.13 (s, 1H, H-8), 8.17 (s, 1H, H-2). ^{13}C NMR (D$_2$O, ppm) δ: 47.23 (C-1′), 68.30 (d, $J_{P,C}$ = 156.5, P–C), 68.98 (C-2′), 74.25 (d, $J_{P,C}$ = 12.2, C-3′), 123.78 (C-5), 143.41 (C-8), 146.25 (C-2), 149.65 (C-4), 159.20 (C-6). ESI-MS, *m/z*: 303.2 [M−H]$^-$. ESI-HRMS calcd for $C_9H_{12}O_6N_4P$ 303.0500, found: 303.0499 [M−H]$^-$.

5.3. Reaction of compounds 3a, 3b with ethyl bromoacetate. General procedure

Ethyl diisopropyl amine (1.7 mL) followed by 60% sodium hydride suspension in oil (520 mg, 13 mmol) were added at −40 °C to a stirred solution of **3a** or **3b** (12.1 mmol) in THF (30 mL). After 10 min of stirring at −40 °C, ethyl bromoacetate (1.42 mL, 13 mmol) was added, the mixture let warm slowly to room temperature and the stirring continued for 24 h. The reaction was quenched by addition of 99% acetic acid (approx. 8–10 drops, to pH 7) and ethanol (6 mL) and the mixture evaporated. The residue was partitioned between water and chloroform (150 mL each), the chloroform extract dried with magnesium sulfate and evaporated. The residue was chromatographed on a column of silica gel (400 mL) in system ethyl acetate/acetone/ethanol/water (18:3:1:1) to give pure **4a** or **4b** followed by the rest of unreacted **3a** or **3b** (20–25%).

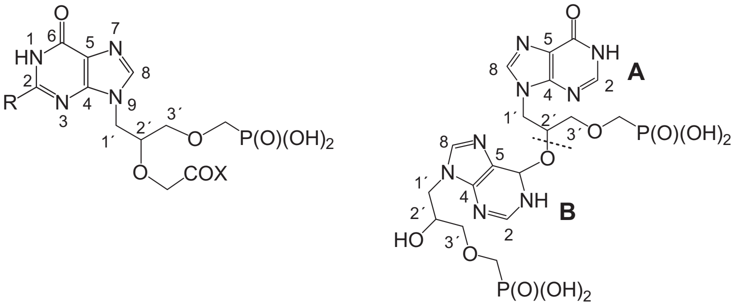


Figure 4. The numbering system for assignment of NMR signals.

5.3.1. Ethyl 2-({1-(6-chloro-9*H*-purin-9-yl)-3-[(diethoxy phosphoryl)methoxy]propan-2-yl}oxy)acetate (4a)

Yield: 2.4 g (43%) as a colorless syrup. ^{1}H NMR ($\mathrm{CDCl_3}$, ppm) δ: 1.20 (t, 3H, $J_{\mathrm{CH3,CH2}} = 7.1$, $\mathrm{COOCH_2}\mathit{CH_3}$), 1.36 (t, 6H, $J_{\mathrm{CH3,CH2}} = 7.1$, $\mathrm{POCH_2}\mathit{CH_3}$), 3.69 (dd, 1H, $J_{3'\mathrm{a},2'} = 5.3$, $J_{\mathrm{gem}} = 10.3$, H-3′a), 3.73 (dd, 1H, $J_{3'\mathrm{b},2'} = 4.9$, $J_{\mathrm{gem}} = 10.3$, H-3′b), 3.78–3.87 (m, 2H, $\mathrm{PCH_2}$), 4.00 (m, 1H, H-2′), 4.11 (q, 2H, $J_{\mathrm{CH2,CH3}} = 7.1$, $\mathrm{COOCH_2}$), 4.15–4.23 (m, 6H, $\mathrm{POCH_2}$, $\mathrm{OCH_2COO}$), 4.42 (dd, 1H, $J_{1'\mathrm{a},2'} = 6.8$, $J_{\mathrm{gem}} = 14.5$, H-1′a), 4.64 (dd, 1H, $J_{1'\mathrm{b},2'} = 3.7$, $J_{\mathrm{gem}} = 14.5$, H-1′b), 8.37 (s, 1H, H-8), 8.73 (s, 1H, H-2). ^{13}C NMR ($\mathrm{CDCl_3}$, ppm) δ: 14.06 ($\mathrm{COOCH_2}\mathit{CH_3}$), 16.47 and 16.51 ($\mathrm{POCH_2}\mathit{CH_3}$), 44.91 (C-1′), 61.07 ($\mathrm{COO}\mathit{C}\mathrm{H_2CH_3}$), 62.49 ($\mathrm{POCH_2}$), 65.65 (d, $J_{\mathrm{P,C}} = 166.4$, P–C), 67.54 ($\mathrm{O}\mathit{C}\mathrm{H_2COO}$), 72.26 (d, $J_{\mathrm{P,C}} = 9.7$, C-3′), 76.94 (C-2′), 131.36 (C-5), 146.90 (C-8), 150.84 (C-6), 151.78 (C-2), 152.12 (C-4), 169.77 (COO). ESIMS, *m/z*: 487.2 $(\mathrm{M+Na})^{+}$ (100), 465.2 $(\mathrm{MH})^{+}$ (23). HRMS (ESI): For $\mathrm{C_{17}H_{27}ClN_4O_7P}$ $(\mathrm{MH})^{+}$ calculated: 465.1300; found: 465.1299. Anal. Calcd for $\mathrm{C_{17}H_{26}ClN_4O_7P}$: C, 43.93; H, 5.64; Cl, 7.63; N, 12.05; P, 6.66. Found: C, 43.55; H, 5.57; Cl, 7.33; N, 11.70; P, 6.58.

5.3.2. Ethyl 2-({1-(2-amino-6-chloro-9*H*-purin-9-yl)-3-[(diethoxyphosphoryl)methoxy]propan-2-yl}oxy)acetate (4b)

Yield: 2.59 g (33%) as a colorless syrup. ^{1}H NMR ($\mathrm{CDCl_3}$, ppm) δ: 1.24 (t, 3H, $J_{\mathrm{CH3,CH2}} = 7.2$, $\mathrm{COOCH_2}\mathit{CH_3}$), 1.36 (t, 6H, $J_{\mathrm{CH3,CH2}} = 7.1$, $\mathrm{POCH_2}\mathit{CH_3}$), 3.64 (dd, 1H, $J_{3'\mathrm{a},2'} = 5.3$, $J_{\mathrm{gem}} = 10.2$, H-3′a), 3.68 (dd, 1H, $J_{3'\mathrm{b},2'} = 5.1$, $J_{\mathrm{gem}} = 10.2$, H-3′b), 3.80–3.86 (m, 2H, $\mathrm{PCH_2}$), 3.93 (m, 1H, H-2′), 4.12–4.23 (m, 8H, $\mathit{CH_2}\mathrm{COO}\mathit{CH_2}$, $\mathrm{PO}\mathit{CH_2}$), 4.26 (dd, 1H, $J_{1'\mathrm{a},2'} = 6.2$, $J_{\mathrm{gem}} = 14.5$, H-1′a), 4.40 (dd, 1H, $J_{1'\mathrm{b},2'} = 3.8$, $J_{\mathrm{gem}} = 14.5$, H-1′b), 5.40 (bs, 2H, $\mathrm{NH_2}$,), 7.96 (s, 1H, H-8). ^{13}C NMR ($\mathrm{CDCl_3}$, ppm) δ: 14.05 ($\mathrm{COOCH_2}\mathit{CH_3}$), 16.45 (d, $J_{\mathrm{P,C}} = 5.5$, $\mathrm{POCH_2}\mathit{CH_3}$), 44.21 (C-1′), 61.03 ($\mathrm{COO}\mathit{C}\mathrm{H_2}$), 62.46 ($\mathrm{POCH_2}$), 65.66 (d, $J_{\mathrm{P,C}} = 167.2$, P–C), 67.74 ($\mathrm{O}\mathit{C}\mathrm{H_2COO}$), 72.16 (d, $J_{\mathrm{P,C}} = 11.0$ C-3′), 77.08 (C-2′), 124.79 (C-5), 143.81 (C-8), 151.02 (C-6), 154.04 (C-4), 159.14 (C-2), 169.88 (COO). ESIMS, *m/z*: 502.1 $(\mathrm{M+Na})^{+}$ (100), 480.1 $(\mathrm{MH})^{+}$ (88). HRMS (ESI): For $\mathrm{C_{17}H_{28}ClN_5O_7P}$ $(\mathrm{MH})^{+}$ calculated: 480.14094; found: 480.14084. Anal. Calcd for $\mathrm{C_{17}H_{27}ClN_5O_7P}$: C, 42.55; H, 5.67; Cl, 7.39; N, 14.59; P, 6.45. Found: C, 42.87; H, 5.83; Cl, 7.30; N, 14.26; P, 6.58.

5.4. Ethyl 2-({1-[(diethoxyphosphoryl)methoxy]-3-(hypoxanthin-9-yl)propan-2-yl}oxy)acetate (5a)

A solution of **4a** (1.29 g, 2.8 mmol) in 80% acetic acid (140 mL) was refluxed for 2 h, then cooled to ambient temperature and evaporated. The residue was coevaporated with water (2 × 50 mL), followed by ethanol (50 mL) and chromatographed on silica gel (200 mL) in system ethyl acetate/acetone/ethanol/water (15:3:4:3) to give **5a** as a colorless foam. Yield: 310 mg (25%). ^{1}H NMR ($\mathrm{CDCl_3}$, ppm) δ: 1.24 (t, 3H, $J_{\mathrm{CH3,CH2}} = 7.1$, $\mathrm{COOCH_2}\mathit{CH_3}$), 1.35–1.37 (m, 6H, $\mathrm{POCH_2}\mathit{CH_3}$), 3.69 (dd, 1H, $J_{3'\mathrm{a},2'} = 5.3$, $J_{\mathrm{gem}} = 10.4$, H-3′a), 3.74 (dd, 1H, $J_{3'\mathrm{b},2'} = 4.7$, $J_{\mathrm{gem}} = 10.4$, H-3′b), 3.83 (dd, 1H, $J_{\mathrm{P,CHa}} = 8.1$, $J_{\mathrm{gem}} = 13.9$, $\mathrm{PCH_a}$), 3.88 (dd, 1H, $J_{\mathrm{P,CHb}} = 8.1$, $J_{\mathrm{gem}} = 13.9$, $\mathrm{PCH_b}$), 3.98 (m, 1H, H-2′), 4.15 (q, 2H, $J_{\mathrm{CH2,CH3}} = 7.1$, $\mathrm{COOCH_2}$), 4.17–4.23 (m, 6H, $\mathrm{POCH_2}$, $\mathrm{OCH_2COO}$), 4.34 (dd, 1H, $J_{1'\mathrm{a},2'} = 6.7$, $J_{\mathrm{gem}} = 14.5$, H-1′a), 4.52 (dd, 1H, $J_{1'\mathrm{b},2'} = 4.0$, $J_{\mathrm{gem}} = 14.5$, H-1′b), 8.01 (s, 1H, H-8), 8.23 (s, 1H, H-2), 13.08 (br s, 1H, NH). ^{13}C NMR ($\mathrm{CDCl_3}$, ppm) δ: 14.06 ($\mathrm{COOCH_2}\mathit{CH_3}$), 16.45 (d, $J_{\mathrm{P,C}} = 5.6$, $\mathrm{POCH_2}\mathit{CH_3}$), 44.66 (C-1′), 61.00 ($\mathrm{COO}\mathit{C}\mathrm{H_2CH_3}$), 62.47 and 62.54 ($\mathrm{POCH_2}$), 65.55 (d, $J_{\mathrm{P,C}} = 166.3$, P–C), 67.77 ($\mathrm{O}\mathit{C}\mathrm{H_2COO}$), 72.50 (d, $J_{\mathrm{P,C}} = 10.2$, C-3′), 77.39 (C-2′), 124.11 (C-5), 141.58 (C-8), 145.09 (C-2), 149.28 (C-4), 159.02 (C-6), 169.92 (COO). ESIMS, *m/z*: 469.1 $(\mathrm{M+Na})^{+}$ (100), 447.2 $(\mathrm{MH})^{+}$ (45). HRMS (ESI): For $\mathrm{C_{17}H_{28}N_4O_8P}$ $(\mathrm{MH})^{+}$ calculated: 447.1639; found: 447.1639.

5.5. Ethyl 2-({1-(guanin-9-yl)-3-[(diethoxyphosphoryl)methoxy]propan-2-yl}oxy)acetate (5b)

The compound was prepared from **4b** (2.59 g, 5.4 mmol) as described for **5a**.Yield: 947 mg (38%) of amorphous solid. ^{1}H NMR ($\mathrm{CDCl_3}$, ppm) δ: 1.22 (t, 3H, $J_{\mathrm{CH3,CH2}} = 7.2$, $\mathrm{COOCH_2}\mathit{CH_3}$), 1.35 (t 6H, $J_{\mathrm{CH3,CH2}} = 7.1$, $\mathrm{POCH_2}\mathit{CH_3}$), 3.71–3.76 (m, 2H, H-3′), 3.82–3.90 (m, 2H, $\mathrm{PCH_2}$), 3.93 (m, 1H, H-2′), 4.09–4.22 (m, 9H, H-1′a, $\mathit{CH_2}\mathrm{COO}\mathit{CH_2}$, $\mathrm{POCH_2}$), 4.28 (dd, 1H, $J_{1'\mathrm{b},2'} = 3.9$, $J_{\mathrm{gem}} = 14.6$, H-1′b), 7.11 (bs, 2H, $\mathrm{NH_2}$), 7.70 (s, 1H, H-8), 12.01 (bs, 1H, NH). ^{13}C NMR ($\mathrm{CDCl_3}$, ppm) δ: 14.09 ($\mathrm{COOCH_2}\mathit{CH_3}$), 16.46 (d, $J_{\mathrm{P,C}} = 5.6$, $\mathrm{POCH_2}\mathit{CH_3}$), 44.36 (C-1′), 60.96 ($\mathrm{COO}\mathit{C}\mathrm{H_2CH_3}$), 62.67 (m, $\mathrm{PO}\mathit{C}\mathrm{H_2}$), 65.56 (d, $J_{\mathrm{P,C}} = 167.0$, P–C), 67.90 ($\mathrm{O}\mathit{C}\mathrm{H_2COO}$), 72.97 (d, $J_{\mathrm{P,C}} = 11.8$, C-3′), 77.53 (C-2′), 116.15 (C-5), 138.76 (C-8), 151.74 (C-4), 153.99 (C-2), 159.14 (C-6), 170.16 (COO). ESIMS, *m/z*: 484.1 $(\mathrm{M+Na})^{+}$ (100), 462.2 $(\mathrm{MH})^{+}$ (18). HRMS (ESI): For $\mathrm{C_{17}H_{29}N_5O_8P}$ $(\mathrm{MH})^{+}$ calculated: 462.17483; found: 462.17465.

5.6. 2-({1-[(Diethoxyphosphoryl)methoxy]-3-(hypoxanthin-9-yl)propan-2-yl}oxy)acetic acid (6a)

The compound was obtained after chromatographic isolation of **5a** by further elution of the column with methanol. Yield: 918 mg (75%) as a white solid. ^{1}H NMR ($\mathrm{CD_3OD}$, ppm) δ: 1.33–1.36 (m, 6H, $\mathrm{CH_3}$), 3.67–3.73 (m, 2H, H-3′), 3.89–3.96 (m, 2H, $\mathrm{PCH_2}$), 4.03 (m, 1H, H-2′), 4.11–4.22 (m, 6H, $\mathit{CH_2}\mathrm{COO}$, $\mathit{CH_2}\mathrm{CH_3}$), 4.39 (m, 1H, H-1′a), 4.53 (m, 1H, H-1′b), 8.04 (s, 1H, H-2), 8.17 (bs, 1H, H-8). ^{13}C NMR ($\mathrm{CD_3OD}$, ppm) δ: 16.78 (d, $J_{\mathrm{P,C}} = 5.1$, $\mathrm{CH_2}\mathit{CH_3}$), 45.84 (C-1′), 64.20 ($\mathit{CH_2}\mathrm{CH_3}$), 65.95 (d, $J_{\mathrm{P,C}} = 166.0$, P–C), 69.0 ($\mathit{CH_2}\mathrm{COOH}$), 73.71 (d, $J_{\mathrm{P,C}} = 11.3$, C-3′), 78.28 (C-2′), 143.5 (C-8), 146.57 (C-2), 150.77 (C-4), 158.1 (C-6), 172.4 (COO). ESIMS, *m/z*: 441.3 $(\mathrm{M+Na})^{+}$ (100), 419.3 $(\mathrm{MH})^{+}$ (32). HRMS (ESI): For $\mathrm{C_{15}H_{24}N_4O_8P}$ $(\mathrm{MH})^{+}$ calculated: 419.1326; found: 419.1326.

5.7. 2-({1-[(Diethoxyphosphoryl)methoxy]-3-(guanin-9-yl)propan-2-yl}oxy)acetic acid (6b)

The compound was obtained after chromatographic isolation of **5b** by further elution of the column with methanol. Yield: 491 mg (21%) as a white solid. ^{1}H NMR ($\mathrm{CD_3OD}$, ppm) δ: 1.34 (m, 6H, $\mathrm{CH_3}$), 3.61–3.75 (m, 2H, H-3′), 3.84–4.00 (m, 5H, H-2′, $\mathit{CH_2}\mathrm{COOH}$, $\mathrm{PCH_2}$), 4.25 (m, 5H, $\mathit{CH_2}\mathrm{CH_3}$, H-1′b), 4.31 (m, 1H, H-1′a), 7.82 (s, 1H, H-8). ^{13}C NMR ($\mathrm{CD_3OD}$, ppm) δ: 16.79 (d, $J_{\mathrm{P,C}} = 5.6$, $\mathrm{CH_3}$), 45.07 (C-1′), 64.15–64.22 (m, $\mathit{C}\mathrm{H_2CH_3}$), 65.95 (d, $J_{\mathrm{P,C}} = 166.0$, P–C), 69.50 ($\mathit{C}\mathrm{H_2COOH}$), 73.88 ($J_{\mathrm{P,C}} = 11.2$, C-3′), 78.34 (C-2′), 117.18 (C-5), 140.78 (C-8), 152.5 (C-4), 155.30 (C-2), 159.48 (C-6), 176.8 (COO). ESIMS, *m/z*: 456.1 $(\mathrm{M+Na})^{+}$ (82), 434.1 $(\mathrm{MH})^{+}$ (20). HRMS (ESI): For $\mathrm{C_{15}H_{25}N_5O_8P}$ $(\mathrm{MH})^{+}$ calculated: 434.14353; found: 434.14341.

5.8. {[2-(2-Ethoxy-2-oxoethoxy)-3-(hypoxanthin-9-yl)propoxy]methyl}phosphonic acid (7a)

Bromotrimethylsilane (0.65 mL, 5 mmol) was added to a solution of **5a** (428 mg, 0.96 mmol) in acetonitrile (5 mL), the mixture stirred for 24 h at 20 °C in the dark and evaporated. The residue was coevaporated with acetonitrile, dissolved in water (5 mL) and applied onto a column of Dowex 50 (40 mL, $\mathrm{H^{+}}$ form). The resin was washed with water (800 mL), elution of the mixture **7a** and **8a** was then commenced. 2.5% Aqueous ammonia was then applied resulting in the elution of more of the product. Products containing solutions were evaporated and applied onto a column of Dowex 1 (30 mL, acetic form). Elution was performed with water (200 mL) followed by 20% formic acid. UV absorbing fraction was evaporated and purified by preparative HPLC with isocratic elution with water.

Compound **7a** was eluted first (retention time 2–4 min), followed by **8a** (5–9 min). Both products were lyophilized. Yield of **7a**: 35 mg (22%), white amorphous solid. ^{1}H NMR (D_2O, ppm) δ: 1.14 (t, 3H, $J_{CH3,CH2}$ = 7.1, CH_3), 3.69–3.78 (m, 3H, PCH_2, H-3′a), 3.83 (dd, 1H, $J_{3'b,2'}$ = 4.3, J_{gem} = 11.0, H-3′b), 4.06 (q, 2H, $J_{CH2,CH3}$ = 7.2, CH_2), 4.12 (m, 1H, H-2′), 4.23 and 4.29 (2 × d, 2 × 1H, J_{gem} = 16.9, C*H*$_2$COO), 4.56 (dd, 1H, $J_{1'a,2'}$ = 8.1, J_{gem} = 14.9, H-1′a), 4.67 (dd, 1H, $J_{1'b,2'}$ = 3.5, J_{gem} = 14.9, H-1′b), 8.30 (s, 1H, H-2), 8.80 (bs, 1H, H-8). ^{13}C NMR (D_2O, ppm) δ: 13.80 (CH_3), 46.35 (C-1′), 62.72 (*C*H$_2$CH$_3$), 67.38 (O*C*H$_2$COO), 67.73 (d, $J_{C,P}$ = 157.9, P–C), 71.68 (d, $J_{3',P}$ = 11.5, C-3′), 77.33 (C-2′), 119.75 (C-5), 142.7 (C-8), 148.11 (C-2), 149.2 (C-4), 157.05 (C-6), 172.67 (COO). ESIMS, *m/z*: 389.2 (M−H)$^-$ (100). HRMS (ESI): For $C_{13}H_{18}N_4O_8P$ (M−H)$^-$calculated: 389.0868; found: 389.0871.

5.9. {[(3-(Guanin-9-yl)-2-(2-ethoxy-2-oxoethoxy)propoxy]methyl}phosphonic acid (7b)

Prepared from **5b** (750 mg, 1.63 mmol) by the same method as described for **7a**. The final HPLC purification was performed immediately after elution of the product from Dowex 50. Yield: 273 mg (41%) of a white amorphous solid. ^{1}H NMR (D_2O, ppm) δ: 1.16 (t, 3H, $J_{CH3,CH2}$ = 7.1, CH_3), 3.66–3.73 (m, 2H, PCH_2), 3.74 (dd, 1H, $J_{3'a,2'}$ = 4.2, J_{gem} = 11.0, H-3′a), 3.80 (dd, 1H, $J_{3'b,2'}$ = 4.6, J_{gem} = 11.0, H-3′b), 4.08 (q, 2H, $J_{CH2,CH3}$ = 7.1, CH_2), 4.09 (m, 1H, H-2′), 4.23–4.32 (m, 2H, OC*H*$_2$COO), 4.42 (dd, 1H, $J_{1'a,2'}$ = 8.0, J_{gem} = 14.8, H-1′a), 4.54 (dd, 1H, $J_{1'b,2'}$ = 3.4, J_{gem} = 14.8, H-1′b), 8.86 (bs, 1H, H-8). ^{13}C NMR (D_2O, ppm) δ: 13.80 (CH_3), 46.03 (C-1′), 62.75 (COO*C*H$_2$), 67.25 (O*C*H$_2$COO), 68.03 (d, $J_{C,P}$ = 157.0, P–C), 71.41 (d, $J_{3',P}$ = 11.7, C-3′), 76.79 (C-2′), 155.78 and 156.41 (C-4, C-6), 172.71 (COO). ESIMS, *m/z*: 404.0 (M−H)$^-$ (100). HRMS (ESI): For $C_{13}H_{19}N_5O_8P$ (M−H)$^-$ calculated: 404.09767; found: 404.09754.

5.10. 2-{[1-(Hypoxanthin-9-yl)-3-(phosphonomethoxy)propan-2-yl]oxy}acetic acid (8a)

The compound was obtained as by-product during preparation of **7a**. Yield: 63 mg (17%), white amorphous solid. ^{1}H NMR (D_2O, ppm) δ: 3.71–3.80 (m, 3H, PCH_2, H-3′a), 3.85 (dd, 1H, $J_{3'b,2'}$ = 4.3, J_{gem} = 11.0, H-3′b), 4.13 (m, 1H, H-2′), 4.24 and 4.30 (2xd, 2x1H, J_{gem} = 17.1, C*H*$_2$COO), 4.60 (dd, 1H, $J_{1'a,2'}$ = 8.0, J_{gem} = 14.9, H-1′a), 4.71 (dd, 1H, $J_{1'b,2'}$ = 3.5, J_{gem} = 14.8, H-1′b), 8.33 (s, 1H, H-2), 9.00 (bs, 1H, H-8). ^{13}C NMR (D_2O, ppm) δ: 46.63 (C-1′), 66.97 (O*C*H$_2$COO), 67.55 (d, $J_{C,P}$ = 158.1, P–C), 71.74 (d, $J_{3',P}$ = 11.7, C-3′), 77.02 (C-2′), 118.25 (C-5), 142.1 (C-8), 148.7 (C-4), 148.74 (C-2), 156.27 (C-6), 174.50 (COOH). ESIMS, *m/z*: 361.0 (M−H)$^-$ (100). HRMS (ESI): For $C_{11}H_{14}N_4O_8P$ (M−H)$^-$ calculated: 361.0555; found: 361.0552.

5.11. 2-{[1-(Guanin-9-yl)-3-(phosphonomethoxy)propan-2-yl]oxy}acetic acid (8b)

A suspension of **6b** (500 mg, 1.15 mmol) in acetonitrile (5 mL) was incubated for three days with bromotrimethylsilane (0.75 mL, 5.6 mmol) in the dark at 24 °C. The following purification was performed as described for **7a**. Elution of the product from Dowex 1 was obtained by 1 M formic acid. The fractions containing the desired product were evaporated and the residue coevaporated with water (3 × 30 mL) and absolute ethanol to give **8b** as a white amorphous solid. Yield: 90 mg (21%). ^{1}H NMR (D_2O, ppm) δ: 3.63–3.69 (m, 3H, PCH_2, H-3′a), 3.72 (dd, 1H, $J_{3'b,2'}$ = 4.8, J_{gem} = 10.9, H-3′b), 4.02 (m, 1H, H-2′), 4.02 and 4.09 (2xd, 2x1H, J_{gem} = 16.1, C*H*$_2$COO), 4.27 (dd, 1H, $J_{1'a,2'}$ = 7.1, J_{gem} = 14.8, H-1′a), 4.37 (dd, 1H, $J_{1'b,2'}$ = 4.2, J_{gem} = 14.8, H-1′b), 8.06 (br s, 1H, H-8). ^{13}C NMR (D_2O, ppm) δ: 44.76 (C-1′), 68.05 (d, $J_{C,P}$ = 156.5, P–C), 68.73 (*C*H$_2$COO), 71.78 (d, $J_{3',P}$ = 11.9, C-3′), 77.00 (C-2′), 152.1 (C-4), 154.57 (C-2), 158.96 (C-6), 176.83 (COOH). ESIMS, *m/z*: 376.0 (M−H)$^-$ (100), 398 (M_{Na}−H)$^-$ (58). HRMS (ESI): For $C_{11}H_{15}N_5O_8P$ (M−H)$^-$ calculated: 376.06637; found: 376.06645.

5.12. {[2-(2-Amino-2-oxoethoxy)-3-(hypoxanthin-9-yl)propoxy]methyl}phosphonic acid (9a)

A solution of **5a** (580 mg, 1.3 mmol) in methanolic ammonia (30 mL) was heated at 110 °C for 8 h in an autoclave. After cooling, the mixture was evaporated, the residue dissolved in acetonitrile (10 mL) and bromotrimethylsilane (1 mL, 7.7 mmol) was then added before incubation for 24 h at ambient temperature. The solution was evaporated. The residue was coevaporated with acetonitrile (5 mL) and in aqueous solution applied onto a column of Dowex 50 (H^+ form, 30 mL). Elution was performed with water (400 mL) followed by 2.5% aqueous ammonia. UV absorbing fractions were evaporated and the residue purified by preparative HPLC using isocratic elution with water. After elution of **8a** (retention time 3 min, 90 mg, 19%), the desired product **9a** was eluted (retention time 5 min). The product was lyophilized from water. Yield: 80 mg (17%) of a white solid. ^{1}H NMR (D_2O, ppm) δ: 3.63 (dd, 1H, $J_{3'a,2'}$ = 5.7, J_{gem} = 10.9, H-3′a), 3.65 (dd, 1H, $J_{P,CHa}$ = 8.9, J_{gem} = 13.2, PCH_a), 3.69 (dd, 1H, $J_{P,CHb}$ = 8.8, J_{gem} = 13.2, PCH_b), 3.79 (dd, 1H, $J_{3'b,2'}$ = 3.9, J_{gem} = 10.9, H-3′b), 4.00 and 4.10 (2 × d, 2 × 1H, J_{gem} = 15.9, C*H*$_2$CON), 4.13 (m, 1H, H-2′), 4.47 (dd, 1H, $J_{1'a,2'}$ = 7.0, J_{gem} = 15.0, H-1′a), 4.56 (dd, 1H, $J_{1'b,2'}$ = 4.0, J_{gem} = 15.0, H-1′b), 8.20 (s, 2H, H-2, H-8). ^{13}C NMR (D_2O, ppm) δ: 45.12 (C-1′), 68.06 (d, $J_{C,P}$ = 156.7, P–C), 68.94 (*C*H$_2$CON), 72.03 (d, $J_{3',P}$ = 12.0, C-3′), 78.36 (C-2′), 123.80 (C-5), 143.7 (C-8), 146.45 (C-2), 149.9 (C-4), 159.24 (C-6), 175.95 (*C*ONH$_2$). ESIMS, *m/z*: 360.21 (M−H)$^-$ (100). HRMS (ESI): For $C_{11}H_{15}N_5O_7P$ (M−H)$^-$ calculated: 360.0715; found: 360.0717.

5.13. {[2-(2-Amino-2-oxoethoxy)-3-(guanin-9-yl)propoxy]methyl}phosphonic acid (9b)

This compound was synthesized from **5b** (1.2 g, 2.6 mmol) by the same procedure as described for **9a**. No formation of carboxylic acid **8b** occurred. The crude product was purified on Dowex 50 (H^+ form, 50 mL) using elution with water. Elution of pure **9b** occurred after 300 mL of water. The fractions containing the compound were concentrated to ca. 5 mL of volume and lyophilized. Yield: 419 mg (43%) of a white solid. ^{1}H NMR (D_2O, ppm) δ: 3.75–3.90 (m, 4H, H-3′, PCH_2), 4.19–4.28 (m, 3H, H-2′, C*H*$_2$CONH$_2$), 4.55 (dd, 1H, $J_{1'a,2'}$ = 6.4, J_{gem} = 14.9, H-1′a), 4.65 (dd, 1H, $J_{1'b,2'}$ = 4.0, J_{gem} = 14.9, H-1′b), 8.97 (s, 1H, H-8). ^{13}C NMR (D_2O, ppm) δ: 45.92 (C-1′), 67.83 (d, $J_{C,P}$ = 157.6, P–C), 68.77 (*C*H$_2$CON), 71.70 (d, $J_{3',P}$ = 12.0, C-3′), 77.14 (C-2′), 108.78 (C-5), 139.08 (C-8), 151.18 (C-4), 155.98 and 156.11 (C-2, C-6), 175.81 (*C*ONH$_2$). ESIMS, *m/z*: 375.1 (M−H)$^-$ (100). HRMS (ESI): For $C_{11}H_{16}N_6O_7P$ (M−H)$^-$ calculated: 375.08236; found: 375.08243.

5.14. Diethyl [2-bromo-3-(6-chloro-9*H*-purin-9-yl)propoxy]methylphosphonate (10)

A solution of **3a** (5.7 g, 15 mmol) and carbon tetrabromide (10.3 g, 31 mmol) in dichloromethane (10 mL) was cooled to 0 °C and triphenylphosphine (10 g, 38 mmol) was added slowly for 30 min. The mixture (dark paste) was then stirred at room temperature for 24 h, the solvent evaporated and the residue chromatographed on silica gel (400 mL), first in ethyl acetate (till complete elution of triphenylphosphine oxide), followed by system ethyl acetate/acetone/ethanol/water (18:3:1:1). Yield: 3.6 g (54%) of **10** as a yellowish syrup. ^{1}H NMR ($CDCl_3$, ppm) δ: 1.38 (t, 6H, $J_{CH3,CH2}$ = 7.1, CH_3), 3.85–3.95 (m, 4H, H-3′, PCH_2), 4.17–4.27 (m, 4H, $POCH_2$), 4.55–4.65 (m, 2H, H-1′a, H-2′), 4.89 (dd, 1H, $J_{1'b,2'}$ = 4.5,

J_{gem} = 14.2, H-1′b), 8.31 (s, 1H, H-8), 8.78 (s, 1H, H-2). ^{13}C NMR (CDCl$_3$, ppm) δ: 16.51 (d, 2C, $J_{P,C}$ = 5.6, CH$_3$), 46.66 (C-2′), 47.68 (C-1′), 62.59–62.69 (m, PO*C*H$_2$), 65.57 (d, $J_{P,C}$ = 166.9, P–C), 73.85 (d, $J_{P,C}$ = 9.7, C-3′), 131.51 (C-5), 145.85 (C-8), 151.23 (C-6), 151.80 (C-4), 152.06 (C-2). ESIMS, *m/z*: 441. 1 (MH)$^+$ (54, 463.0 (M+Na)$^+$ (80). HRMS (ESI): For $C_{13}H_{20}O_4N_4BrClP$ (MH)$^+$ calculated: 441.00886; found: 441.00884.

5.15. Base-catalyzed reaction of compound 10 with ethylene glycol

Cesium carbonate (30 mg) was added to a solution of **10** (317 mg, 0.71 mmol) and ethylene glycol (0.05 mL, 1 mmol) in DMF (2 mL). The mixture was stirred for 4 h at 100 °C. Ethylene glycol (0.1 mL) and ethyldiisopropyl amine (0.3 mL) were added and the heating continued for additional 1 h. After that time, DBU (1,8-diazabicycloundec-7-ene, 0.2 mL) was added and the mixture heated for 110 °C for an additional 3 h (till complete conversion of the staring material). The mixture was evaporated and the residue chromatographed using a preparative TLC on silica gel in system ethyl acetate/acetone/ethanol/water (18:3:2:2). Three products were obtained in the following order:

5.15.1. (*Z*)-Diethyl {(3-[(6-(2-hydroxyethoxy)-9*H*-purin-9-yl] allyl}oxymethylphosphonate (11)

R$_F$ 0.26. Yield: 77 mg (28%) of a white foam. ^{1}H NMR (DMSO-*d*$_6$, ppm) δ: 1.24 (t, 6H, $J_{CH3,CH2}$ = 7.1, CH$_3$), 3.80 (m, 2H, H-2″), 3.87 (d, 2H, $J_{P,CH}$ = 8.5, PCH$_2$), 4.02–4.09 (m, 4H, P-OCH$_2$), 4.27 (bdd, 2H, $J_{3',2'}$ = 6.4, $J_{3',1'}$ = 1.4, H-3′), 4.56 (m, 2H, H-1″), 5.00 (m, 1H, OH), 6.75 (dt, 1H, $J_{2',1'}$ = 14.5, $J_{2',3'}$ = 6.3, H-2′), 7.38 (dt, 1H $J_{1',2'}$ = 14.5, $J_{1',3'}$ = 1.4, H-1′), 8.57 (s, 1H, H-2), 8.73 (s, 1H, H-8). ^{13}C NMR (DMSO-*d*$_6$, ppm) δ: 16.57 (d, $J_{P,C}$ = 5.4, CH$_3$), 59.34 (C-2″), 62.00 (d, $J_{P,C}$ = 6.1, PO*CH$_2$*), 63.31 (d, $J_{P,C}$ = 163.8, P–C), 68.73 (C-1″), 70.42 (d, $J_{3',P}$ = 13.0, C-3′), 116.46 (C-2′), 121.28 (C-5), 123.78 (C-1′), 141.93(C-8), 151.21 (C-4), 152.42 (C-2), 160.49 (C-6). ESIMS, *m/z*: 387.1 (MH)$^+$ (23), 409.2 (M+Na)$^+$ (80). HRMS (ESI): For $C_{15}H_{24}N_4O_6P$ (MH)$^+$ calculated: 387.1428; found: 387.1429.

5.15.2. (*Z*)-Diethyl {3-[6-oxo-1*H*-purin-9(6*H*)-yl]allyl} oxymethylphosphonate (12)

R$_F$ 0.30. Yield: 61 mg (25%) of a white foam. ^{1}H NMR (CDCl$_3$, ppm) δ: 1.29 (t, 6H, $J_{CH3,CH2}$ = 7.1, CH$_3$), 3.78 (d, 2H, $J_{P,CH}$ = 8.7, PCH$_2$), 4.08–4.16 (m, 4H, P–OCH$_2$), 4.25 (bdd, 2H, $J_{3',2'}$ = 6.4, $J_{3',1'}$ = 1.4, H-3′), 6.38 (dt, 1H, $J_{2',1'}$ = 14.5, $J_{2',3'}$ = 6.4, H-2′), 7.20 (dt, 1H, $J_{1',2'}$ = 14.5, $J_{1',3'}$ = 1.4, H-1′), 7.86 (s, 1H, H-8), 8.29 (s, 1H, H-2). ^{13}C NMR (CDCl$_3$, ppm) δ: 16.49 (d, $J_{P,C}$ = 5.4, CH$_3$), 62.51 (d, $J_{P,C}$ = 6.4, PO*C*H$_2$), 64.03 (d, $J_{P,C}$ = 167.5, P–C), 71.17 (d, $J_{3',P}$ = 12.7, C-3′), 115.07 (C-2′), 123.91 (C-1′), 120.27 (C-5), 135.38 (C-8), 149.76 (C-4), 152.89 (C-2), 154.83 (C-6). ESIMS, *m/z*: (MH)$^+$ not observed.

5.15.3. (*Z*)-Diethyl {3-[(6-chloro-9*H*-purin-9-yl)allyl] oxymethyl) phosphonate (13)

R$_F$ 0.40. Yield: 52 mg (20%) of a colorless syrup. ^{1}H NMR (CDCl$_3$, ppm) δ: 1.38 (t, 6H, $J_{CH3,CH2}$ = 7.1, CH$_3$), 3.89 (d, 2H, $J_{P,CH}$ = 8.6, PCH$_2$), 4.18–4.25 (m, 4H, P-OCH$_2$), 4.39 (bdd, 2H, $J_{3',2'}$ = 5.8, $J_{3',1'}$ = 1.6, H-3′), 6.73 (dt, 1H, $J_{2',1'}$ = 14.5, $J_{2',3'}$ = 5.8, H-2′), 7.31 (dt, 1H, $J_{1',2'}$ = 14.5, $J_{1',3'}$ = 1.6, H-1′), 8.31 (s, 1H, H-8), 8.80 (s, 1H, H-2). ^{13}C NMR (CDCl$_3$, ppm) δ: 16.50 (d, $J_{P,C}$ = 5.6, CH$_3$), 62.55 (d, $J_{P,C}$ = 6.5, PO*C*H$_2$), 64.46 (d, $J_{P,C}$ = 167.7, P–C), 70.58 (d, $J_{3',P}$ = 11.9, C-3′), 118.30 (C-2′), 122.51 (C-1′), 132.11 (C-5), 142.87 (C-8), 150.74 (C-4), 151.49 (C-6), 152.52 (C-2). ESIMS, *m/z*: 361.0 (MH)$^+$ (36), 383.1 (M+Na)$^+$ (34), 742.5 (2 M+Na)$^+$ (100). HRMS (ESI): For $C_{13}H_{19}N_4O_4ClP$ (MH)$^+$ calculated: 361.0827; found: 361.0827.

5.16. Diethyl {2-bromo-3-[6-oxo-1*H*-purin-9(6*H*)-yl]propoxy} methylphosphonate (14)

A mixture of compound **10** (450 mg, 1.02 mmol) and 80% acetic acid (15 mL) was refluxed for 3 h. After cooling to room temperature, the mixture was evaporated, the residue coevaporated with ethanol (30 mL) and chromatographed on silica gel (40 mL) in ethyl acetate/acetone/ethanol/water (18:3:2:2) to give 240 mg (56%) of **14** as a colorless syrup. ^{1}H NMR (DMSO-*d*$_6$, ppm) δ: 1.24 (t, 6H, $J_{CH3,CH2}$ = 7.1, POCH$_2$*CH$_3$*), 3.84–3.91 (m, 2H, H-3′), 3.94 (d, 2H, $J_{P,CH}$ = 8.0, PCH$_2$), 4.04–4.10 (m, 4H, P-OCH$_2$), 4.51 (dd, 1H, $J_{1'a,2'}$ = 8.8, J_{gem} = 14.7, H-1′a), 4.62 (dd, 1H, $J_{1'b,2'}$ = 4.9, J_{gem} = 14.7, H-1′b), 4.74 (m, 1H, H-2′), 8.06 (s, 1H, H-2), 8.12 (s, 1H, H-8), 12.37 (bs, 1H, NH). ^{13}C NMR (DMSO-*d*$_6$, ppm) δ: 16.58 (d, $J_{P,C}$ = 5.5, CH$_3$), 46.86 (C-1′), 50.38 (C-2′), 62.14 (d, $J_{P,C}$ = 5.7, PO*CH$_2$*), 64.40 (d, $J_{P,C}$ = 161.9, P–C), 73.85 (d, $J_{P,C}$ = 11.9, C-3′), 76.94 (C-2′), 124.05 (C-5), 140.78 (C-8), 146.01 (C-2), 148.72 (C-4), 156.86 (C-6). ESIMS, *m/z*: 445.1 (M+Na)$^+$ (100), 423.1 (MH)$^+$ (40). HRMS (ESI): For $C_{13}H_{21}BrN_4O_5P$ (MH)$^+$ calculated: 423.0427; found: 423.0425. Anal. Calcd for $C_{13}H_{20}BrN_4O_5P$: C, 36.89; H, 4.76; Br, 18.88; N, 13.24; P, 7.32. Found: C, 37.09; H, 4.89; Br, 18.53; N, 12.95; P, 7.08.

5.17. (*Z*)-{3-[6-Oxo-1*H*-purin-9(6*H*)-yl]allyl}oxymethylphosphonic acid (15)

A solution of **14** (980 mg, 2.3 mmol) in 30% methanolic ammonia (50 mL) was heated in an autoclave at 110 °C for 10 h. After cooling, the solution was evaporated to dryness, the residue dissolved in acetonitrile (15 mL) and stirred with bromotrimethylsilane (2.8 mL, 22 mmol) at room temperature in dark for 24 h. The solution was then evaporated, the residue coevaporated with acetonitrile (50 mL) and dissolved in a minimum amount of water. The solution was applied onto a column of Dowex 50 (H$^+$ form, 40 mL) using elution with water (800 mL). The first batch of **14** was eluted after washing with approx. 300 mL of water. Further amounts of **14** were eluted with 2.5% aqueous ammonia. Both product containing solutions were combined and evaporated and additionally purified on Dowex 1 (acetic form, 30 ml). Impurities were eluted first with a gradient of acetic acid (0–1 M, 500 mL). The product was then eluted with 20% formic acid. The eluate was evaporated and the residue crystallized from water to give 135 mg (20%) of **14** as white crystals. ^{1}H NMR (D$_2$O, ppm) δ: 3.57 (d, 2H, $J_{P,CH}$ = 8.7, PCH$_2$), 4.31 (dd, 2H, $J_{3',2'}$ = 6.3, $J_{3',1'}$ = 1.3, H-3′), 6.46 (dt, 1H, $J_{2',1'}$ = 14.5, $J_{2',3'}$ = 6.3, H-2′), 7.17 (bd, 1H, $J_{1',2'}$ = 14.5, H-1′), 8.18 (s, 1H, H-2), 8.25 (s, 1H, H-8). ^{13}C NMR (D$_2$O, ppm) δ: 68.83 (d, $J_{P,C}$ = 150.7, P–C), 70.57 (d, $J_{3',P}$ = 11.4, C-3′), 120.41 (C-2′), 123.10 (C-1′), 124.07 (C-5), 139.92 (C-8), 148.58 (C-2), 148.60 (C-4), 161.17 (C-6). ESIMS, *m/z*: 285.1 (M−H)$^-$ (100). HRMS (ESI): For $C_9H_{10}N_4O_5P$ (M−H)$^-$ calculated: 285.0394; found: 285.0389.

5.18. Reaction of 3a with 2-chloroethanol

A suspension of 60% sodium hydride (120 mg, 3 mmol) was added to a solution of **3a** (1.04 g, 2.8 mmol) in dimethylformamide (4 mL), followed by 2-chloroethanol (0.34 mL, 5 mmol) after 15 min stirring. This solution was stirred for 5 h at 24 °C, then neutralized with AcOH to pH 7 and evaporated. The residue was refluxed with 80% acetic acid for 4 h, the mixture evaporated and the resulting material coevaporated with water followed by absolute ethanol, and dried in vacuo for 2 h at 70 °C. Acetonitrile (10 mL) and bromotrimethylsilane (1.95 mL, 15 mmol) were added, the mixture was stirred at room temperature for 48 h in dark for and evaporated. The residue was dissolved in water (5 mL), neutralized with ammonia to pH 7 and applied to a column of DEAE Sephadex A25 (20 mL, activated with triethylammonium hydrogen carbonate–TEAB). The column was washed with water

(500 mL), followed by a linear gradient of TEAB (0–0.4 M, 2 L). The complex mixture of products **1** and **16** was eluted at 0.2 M TEAB, followed by **17** eluted at 0.3–0.4 M TEAB. Both fractions were desalted on activated charcoal and subsequently purified by a reverse phase HPLC using isocratic elution with water. The following products were isolated:

5.18.1. 9-[2-Hydroxy-3-*O*-(phosphonomethoxy)propyl] hypoxanthine (1)

Yield: 90 mg (11%) of a white solid. The spectral data of **1** are identical with those obtained in synthesis from 9-[2-hydroxy-3-*O*-(phosphonomethoxy)propyl]adenine[10] (See Section 5.2).

5.18.2. 9-[(2-Hydroxy-2-oxido-1,4,2-dioxaphosphinan-6-yl) methyl]hypoxanthine (16)

Yield: 303 mg (39%) of a white solid. ^{1}H NMR (D_2O, ppm) δ: 3.34 (dd, 1H, $J_{3'a,2'} = 10.0$, $J_{gem} = 12.5$, H-3′a), 3.57 (dd, 1H, $J_{P,CHa} = 2.0$, $J_{gem} = 13.9$, PCH_a), 3.88 (dd, 1H, $J_{P,CHb} = 8.9$, $J_{gem} = 13.9$, PCH_b), 4.01 (dm, 1H, $J_{gem} = 12.4$, H-3′b), 4.40–4.51 (m, 2H, H-1′), 4.77 (m, 1H, H-2′); 8.20 (s, 1H, H-2); 8.24 (s, 1H, H-8). ^{13}C NMR (D_2O, ppm) δ: 45.42 (d, $J_{P,C} = 6.0$, C-1′), 65.99 (d, $J_{P,C} = 143.3$, P–C), 68.88 (d, $J_{P,C} = 3.6$, C-3′), 76.88 (d, $J_{P,C} = 6.4$, C-2′), 123.42 (C-5), 143.40 (C-8), 146.56 (C-2), 149.74 (C-4), 159.12 (C-6). ESIMS, *m/z*: 285.1 $(M-H)^-$ (100). HRMS (ESI): For $C_9H_{10}N_4O_5P$ $(M-H)^-$ calculated: 285.0394; found: 285.0395.

5.18.3.6-{[1-(Hypoxanthin-9-yl)methyl-2-(phosphonometh oxy)]ethoxy}-9-[2-hydroxy-3-*O*-(phosphonomethoxy) propyl]hypoxanthine (17)

Isolated as a diastereoisomeric mixture. Yield: 350 mg (22%) of a white solid. ^{1}H NMR (D_2O, ppm) δ: 3.57–3.86 (m, 6H, PCH_2, H-3′$_B$), 4.06–4.13 (m, 2H, H-3′$_A$), 4.22 (m, 1H, H-2′$_B$), 4.37 and 4.49 (m, 2H, H-1′$_B$), 4.76–4.84 (m, 2H, H-1′$_A$), 6.19 (m, 1H, H-2′$_A$), 8.03–8.54 (m, 4H, H-2, H-8). ^{13}C NMR (D_2O, ppm) δ: 45.57 (C-1′$_A$), 47.40 and 47.46 (C-1′$_B$), 67.43–68.69 (m, P–C), 68.48 and 68.59 (C-2′$_B$), 71.73 and 71.82 (C-3′$_A$), 74.20 and 74.30 (C-3′$_B$), 74.36 and 74.38 (C-2′$_A$), 120.64 (C-5$_B$), 123.12 and 123.19 (C-5$_A$), 141.44 (C-8$_A$), 145.87 (C-8$_B$), 150.53 and 150.57 (C-4$_A$), 151.34 and 151.40 (C-2$_A$), 152.21 and 152.28 (C-4$_B$), 153.84 (C-2$_B$), 159.98 (C-6$_B$), 167.75 and 167.81 (C-6$_A$). ESIMS, *m/z*: 591.1 $(MH)^+$ (100),1180.9 $(2\,M+H)^+$ (42). ESIMS, *m/z*: 589.1 $(M-H)^-$ (100). HRMS (ESI): For $C_{18}H_{23}N_8O_{11}P_2$ $(M-H)^-$ calculated: 589.0967; found: 589.0961.

5.19. Purification of human HGPRT, *Pf*HGXPRT and *Pv*HGPRT

These recombinant enzymes were purified as previously described.[11,12]

5.20. Determination of K_i values

The K_i values for all three enzymes were determined in 0.1 M Tris–HCl, 0.01 M $MgCl_2$, pH 7.4, 25 °C as previously described.[7] The purine base was guanine and the concentration was constant at 60 μM. The concentration of the variable substrate, *P*Rib-*PP* ranged between 5 and 750 μM depending on the value for $K_{m(app)}$.

5.21. Manual docking

Manual docking of compound **8b** into the active site of human HGPRT was performed using the program COOT 0.6.[13] A model of compound **8b** was generated using the Prodrug DUNDEE server.[14] The purine ring and the phosphonate groups of this model were superimposed onto the equivalent atoms of PEEG in the crystal structure of PEEG in complex with human HGPRT (pdb code 3GEP). The rotatable dihedral angles for the second group were modified to avoid steric clashes.

Acknowledgments

This work is a part of the research project of the Institute OZ40550506. It was supported by the Centre of New Antivirals and Antineoplastics 1M0508 by the Ministry of Education, Youth and Sports of the Czech Republic, the Grant Agency of the Czech Republic (Grant No. P207/11/0108) and funds from the National Health and Medical Research Council, Australia (Grant Nos 569703 & 1030353). The authors′ thanks are due to the staff of the mass spectrometry (Dr. J. Cvačka, Head) and analytical departments of the Institute (Dr S. Matějková, Head). The excellent technical assistance of Mrs. Julie Křelinová is also acknowledged.

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