[[(Guaninylalkyl)phosphinico]methyl]phosphonic Acids. Multisubstrate Analogue Inhibitors of Human Erythrocyte Purine Nucleoside Phosphorylase

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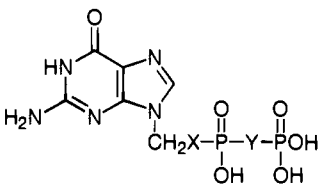
A series of [[(guaninylalkyl)phosphinico]methyl]phosphonic acids, **2**, was synthesized and tested as inhibitors of human erythrocyte purine nucleoside phosphorylase (PNPase). The target (phosphinicomethyl)phosphonic acids **2** were synthesized in six or seven steps from alkenylphosphonates **4**. The latter were converted to the intermediate alkylmesylates **9** in a series of steps that included (1) conversion of the diethyl phosphonates **4** to the (phosphinoylmethyl)-phosphonates **7** and (2) conversion of the terminal double bond of [(alkenylphosphinoyl)methyl]-phosphonates **7** to the alkylmesylates **9**. The pure 9-isomers **2** were obtained by alkylation of 2-amino-6-(2-methoxyethoxy)-9*H*-purine with alkylmesylates **9** followed by hydrolysis of the protecting groups with concentrated hydrochloric acid and ion exchange chromatography to give **2** as hydrated ammonium salts. The most potent inhibitor of human erythrocyte PNPase, [[[5-(2-amino-1,6-dihydro-6-oxo-9*H*-purin-9-yl)pentyl]phosphinico]methyl]phosphonic acid (**2b**), was a multisubstrate analogue inhibitor with a K_i' of 3.1 nM. Optimum PNPase inhibitory activity required the presence of zinc ions in the assay medium. These potent inhibitors of PNPase exhibited only weak activity against human leukemic T-cells *in vitro*.

Introduction

Purine nucleoside phosphorylase (purine nucleoside: orthophosphate ribosyltransferase, EC 2.4.2.1; PNPase) catalyzes the reversible phosphorolysis of purine nucleosides such as inosine, 2′-deoxyinosine, guanosine, and 2′-deoxyguanosine to the purine and α-ribose or 2-deoxy-α-ribose 1-phosphate.[1,2] Patients who are genetically deficient in PNPase exhibit clinical manifestations of immunodeficiency.[2] These individuals suffer from impairment of the T-cell component (cellular immunity) of their immune system but have normal B-cell function (humoral immunity).[2] Potential inhibitors of PNPase should be T-cell selective, immunosuppressive agents with potential clinical utility in the treatment of human T-cell leukemia and autoimmune disorders and in the prevention of transplant rejection.[3–6] Inhibitors of PNPase may also be beneficial in protecting therapeutically useful purine nucleoside analogues from rapid *in vivo* cleavage.[2] Consequently, extensive drug discovery research has been devoted to the design and synthesis of inhibitors of PNPase.[5,6]

Interest in PNPase as a target for chemotherapy has persisted for many years during which compounds of diverse structure have been synthesized and tested as candidate inhibitors. Examples include early work on a series of substituted purines and purine analogues by Hitchings' group,[7] studies on bulk tolerance with a variety of substituted purines,[8] and an investigation of stereoelectronic requirements for binding to PNPase.[9] More recently, a series of acyclic nucleosides[10–12] and acyclic nucleotides[10] were examined for their ability to inhibit PNPase; quinazoline-based irreversible inhibitors also have been reported.[13] Potent, nonionic inhibitors that have been reported include 8-aminoguanine,[14] 8-amino-9-benzylguanine,[15] 8-amino-9-(2-thienylmethyl)guanine,[16] and 9-substituted-9-deazaguanines.[17] The 9-deazaguanines constitute an example of enzyme structure-based inhibitor design utilizing X-ray data on the native enzyme and the enzyme-inhibitor complexes.[17,18]

Several phosphonic acids are multisubstrate analogue inhibitors of PNPase.[19,20] These include 9-(phosphonoalkyl) derivatives of hypoxanthine[21] and guanine,[22] 9-(difluorophosphonoalkyl)guanines,[23] and 9-[(phosphonoalkyl)benzyl]guanines.[24] The diphosphates of acyclovir (**1**)[25,26] and ganciclovir[10] are potent inhibitors of PNPase with K_i's of 8.7 and 9.0 nM, respectively.



1: X = OCH_2CH_2O, Y = O
2: X = $(CH_2)_n$, Y = CH_2

Because nucleoside diphosphates exhibit poor cellular permeability and have short plasma half-lives, there was little expectation that the diphosphate **1** would be active *in vivo*.[26] Compounds containing stable mimics of the diphosphate moiety inhibit squalene synthetase,[27] and stable mimics have been investigated in the pursuit of other types of biologically active molecules.[28] We envisaged the preparation of a stable mimic of diphosphate **1** in which the side chain oxygens are replaced with methylenes, as for (phosphinicomethyl)phosphonic acid **2**. These compounds should be stable to plasma phosphatases and might effectively mimic the potent PNPase inhibition properties of acyclovir diphosphate (**1**). The synthesis and PNPase inhibition properties of several [[(guaninylalkyl)phosphinico]methyl]phosphonic acids are described nerein.

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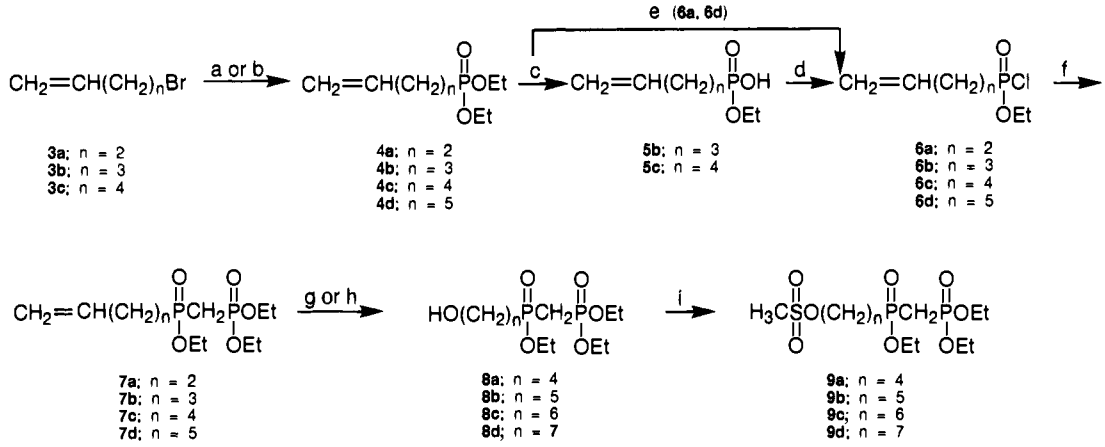
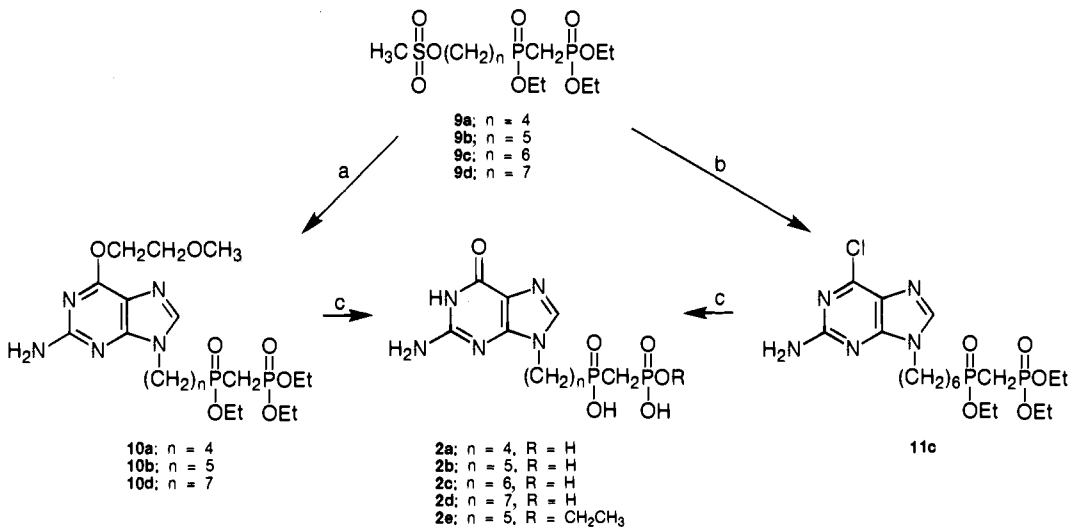
Scheme 1[a]



[a] (a) $P(OEt)_3$; (b) $CH_3P(O)(OEt)_2$, *n*-BuLi/hexane, THF; (c) 85% aq KOH, EtOH; (d) oxalyl chloride/DMF, CH_2Cl_2; (e) PCl_5, CH_2Cl_2; (f) $CH_3P(O)(OEt)_2$, *n*-BuLi/hexane, THF, aq NH_4Cl; (g) BH_3/THF, aq NaOH/30% H_2O_2; (h) BH_3/THF, $NaBO_3$; (i) mesyl chloride, Et_3N, CH_2Cl_2.

Scheme 2[a]



[a] (a) 2-Amino-6-(2-methoxyethoxy)-9*H*-purine, Cs_2CO_3, DMF; (b) 2-amino-6-chloropurine, K_2CO_3, DMF; (c) conc HCl.

Chemistry

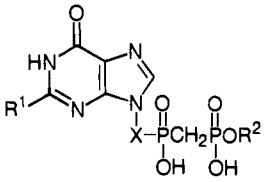
The (phosphinicomethyl)phosphonic acids **2a–d** were prepared in six or seven synthetic steps from alkenylphosphonates **4a–d** (Scheme 1). The latter were prepared via the Arbuzov reaction with triethyl phosphite **4a,b** or via alkylation of the anion of diethyl methylphosphonate **4c,d**. The phosphonates **4a–d** were converted to (phosphinoylmethyl)phosphonates **7a–d** via a method reported by Biller et al.[27] for preparation of isoprenoid phosphonates. Esters **4b,c** were hydrolyzed to the monoacids **5b,c** with aqueous potassium hydroxide; this was followed by chlorination of the monoacids with oxalyl chloride to give **6b,c**. The phosphonochloridates **6a,d** were prepared more conveniently in one step by treatment of the diethyl phosphonates **4a,d** with phosphorus pentachloride in dichloromethane. Reaction of the lithium salt of diethyl methylphosphonate with **6a–d** provided the triesters **7a–d**.

The primary alcohols **8a–d** were obtained from the olefins **7a–d** by hydroboration. Initial attempts to convert the olefin **7b** to **8b** with 9-borabicyclo[3.3.1]-nonane (9-BBN) were not successful because it was difficult to separate residual 1,5-cyclooctanol from product **8b**. However, hydroboration with diborane in tetrahydrofuran followed by oxidation with sodium hydroxide–hydrogen peroxide or sodium perborate gave high yields of **8a–d**.

The primary alcohols **8a–d** were converted to the mesylates **9a–d** for use in alkylation of the purines. Alkylation of 2-amino-6-chloropurine with **9c** gave a mixture of the 9-isomer **11c** with the 7-isomer (Scheme 2). These isomers were not separable by column chromatography, a technique that is usually successful with 9-benzyl and acyclic nucleoside derivatives of 2-amino-6-chloropurine.[29,30] The mixture was hydrolyzed with concentrated hydrochloric acid to give acid **2c** as a mixture with 12% of the 7-isomer even after purification by ion exchange chromatography.

An improved entry to the pure 9-isomers **2** was developed by use of 2-amino-6-(2-methoxyethoxy)-9*H*-purine[31] in reactions with mesylates **9a,b,d**. Although a mixture of 7- and 9-isomers was formed, separation by chromatography on silica gel was successful, which provided **10a,b,d** as pure 9-isomers in 34–37% yields. Hydrolysis of **10a,b,d** with concentrated hydrochloric acid followed by ion exchange chromatography on DEAE Sephadex A-25 gave the (phosphinicomethyl)phosphonic acids **2a,b,d** as the hydrated ammonium salts.

Table 1. Inhibition of Human Erythrocyte Purine Nucleoside Phosphorylase by [[(Guaninylalkyl)phosphinico]methyl]phosphonic Acids[a]



no.	R^1	X	R^2	K_i', μM ± SE (n)[b,c] $ZnCl_2$	K_i', μM ± SE (n)[b,c] Na_2EDTA
2a	H_2N	$(CH_2)_4$	H	0.10 ± 0.01 (4)	1.4 ± 0.1 (6)
2b	H_2N	$(CH_2)_5$	H	0.0031 ± 0.0003 (6)	0.9 ± 0.1 (6)
2c	H_2N	$(CH_2)_6$	H	0.0037 ± 0.0003 (4)	0.39 ± 0.01 (5)
2d	H_2N	$(CH_2)_7$	H	0.010 ± 0.001 (4)	1.1 ± 0.2 (4)
2e	H_2N	$(CH_2)_5$	CH_2CH_3	1.1 ± 0.1 (4)	14 ± 2 (4)
12	HO	$(CH_2)_5$	H	1.4 ± 0.1 (4)	270 ± 10 (3)
1	acyclovir	diphosphate		0.0042 ± 0.0001 (9)	0.011 ± 0.001 (17)

[a] The enzyme was purified from human erythrocytes and assayed spectrophotometrically via a xanthine oxidase-coupled assay as described in the Experimental Section. [b] Apparent inhibition constants (K_i') of these compounds were determined from the fractional inhibition of inosine phosphorolysis at 1 mM phosphat as described in the Experimental Section. The number of determinations (n), the mean K_i' value, and the standard error of the mean (SE) are tabulated. [c] Compounds were assayed in the presence of 2 μM zinc chloride or 0.1 mM Na_2EDTA to assure a zinc-free medium.

The half-ester **2e** was prepared by careful hydrolysis of the 6-(2-methoxyethoxy)purine **10b** with 1 N hydrochloric acid followed by hydrolysis with aqueous potassium hydroxide and purification by ion exchange chromatography. The 9-alkylated xanthine[32] derivative **12** (Table 1), which was produced by hydrolysis of the 2-amino group during preparation of **2b**, was isolated from the ion exchange chromatography purification.

The dissociation constants of the (phosphinicomethyl)-phosphonic acid moiety were determined by pH titration using ^{31}P NMR chemical shift values to monitor the change in the ionization state of the acid. Neither spectrophotometric nor potentiometric methods were applicable to pK_a determination because a suitable chromophore was absent, and we anticipated similarity with the guanine ionizations. Since the ^{31}P chemical shifts of phosphorus acids are correlated with their degree of ionization,[33] the ^{31}P resonances of **2b** were measured at various pH's in water to determine the three (phosphinicomethyl)phosphonic acid dissociation constants. The two pK_a's of the phosphonic acid moiety are 1.61 and 8.45, and the pK_a of the phosphinic acid is 3.2. The third phosphoric acid pK_a of the nucleoside diphosphate GDP is 7.19.[34] Thus, these (phosphinicomethyl)phosphonic acids are not as acidic as nucleoside diphosphates but exist primarily as dianionic species at physiological pH.

Biological Results and Discussion

Inhibition of Purine Nucleoside Phosphorylase. The compounds in Table 1 were tested for inhibition of human erythrocyte purine nucleoside phosphorylase (PNPase), as described in the Experimental Section. In addition to enzyme, the assay mixtures contained inhibitor, inosine, Tris-hydrochloride buffer, potassium phosphate, and zinc chloride or ethylenediaminetetraacetic acid disodium salt (Na_2EDTA). Because inhibition of PNPase by phosphates and phosphonic acids is inversely proportional to the concentration of inorganic phosphate,[10,21,25] the apparent inhibition constants (K_i') were determined at 1 mM phosphate, which is the approximate intracellular concentration.

Compounds that contained a (phosphinicomethyl)-phosphonic acid moiety attached to the 9-position of guanine inhibited PNPase (Table 1). [[(Guaninylpentyl)phosphinico]methyl]phosphonic acid **2b** was a potent inhibitor of PNPase with a K_i' of 0.0031 μM (plus 2 μM zinc chloride). The butyl analogue **2a** with an alkyl chain one methylene shorter than **2b** was 30-fold less inhibitory. The six-methylene analogue **2c** was essentially as potent an inhibitor as **2b**, with a K_i' = 0.0037 μM, but inhibitory potency began to diminish with the seven-methylene homologue **2d**, which was 3-fold less active. Substantial inhibition was lost with the monoethyl ester **2e** of phosphonic acid **2b**, which was 350-fold less potent with a K_i' = 1.1 μM. Changing the 2-amino group of **2b** to a 2-oxo group (**12**) decreased inhibitory potency 450-fold. Thus, the best inhibitory activity resides in [[(guaninylalkyl)phosphinico]methyl]-phosphonic acids with alkyl chains of five or six methylenes.

The [(alkylphosphinico)methyl]phosphonic acids effectively function as stable mimics of acyclovir diphosphate (**1**) when evaluated as inhibitors of PNPase. Tuttle and Krenitsky reported K_i's of 0.0087, 0.015, and 0.071 μM for **1** (five-atom chain) and its propoxy (six-atom chain) and butoxy (seven-atom chain) homologues, which is the same rank order of potency of acids **2b–d**.[26] Under our assay conditions, **1** has a K_i of 0.0042 μM, which is comparable to the K_i of **2b**. The inhibition constants and relative potencies of [(alkylphosphinico)-methyl]phosphonic acids **2b–d** are similar to those of acyclovir diphosphate (1) and its one- and two-methylene homologues.[25,26] Thus, acid **2b**, which is a carbon isostere of acyclovir diphosphate (**1**), is an effective mimic with respect to inhibition of PNPase.

Since our studies were done before X-ray crystallographic structural information on the PNPase enzyme–inhibitor complex was published,[17,18] the chain lengths for bridging the (phosphinicomethyl)phosphonic acid moiety to N-9 of guanine were based on the structure of acyclovir diphosphate (**1**), a potent inhibitor of PNPase.[25,26] Our most potent inhibitor, **2b**, contains the five-atom chain length which allows it to closely overlay the X-ray enzyme–inhibitor complex of **1**, which also contains a five-atom spacer between N-9 of guanine and the diphosphate moiety. Compound **2c**, which has a six-atom spacer, is also a potent inhibitor of the enzyme.

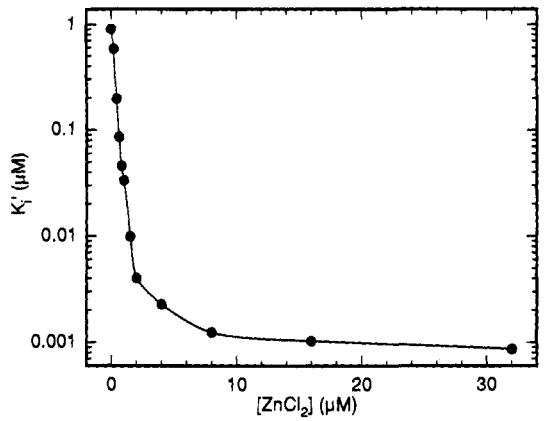


Figure 1. Effect of zinc chloride concentration on the apparent inhibition constant (K_i') for **2b**. K_i' values at the indicated zinc chloride concentrations were determined from the fractional inhibition of inosine phosphorolysis at 1 mM phosphate as described in the Experimental Section. The concentration of **2b** used in the assays varied from 0.0030 μM (at 32 μM zinc chloride) to 4.0 μM (no zinc chloride).

The extra length of the six-atom spacer may be accommodated by the hydrophobic region in the ribose binding site. Thus, our results are entirely compatible with the X-ray crystallographic data on the enzyme–inhibitor complex with acyclovir diphosphate (**1**).

Effect of Zinc. These (phosphinicomethyl)phosphonic acids must be assayed in the presence of micromolar concentrations of zinc ions to exhibit optimum PNPase inhibitory activity. When the three most potent phosphonic acids, **2b–d**, were assayed in the absence of 2 μM zinc chloride, with Na_2EDTA added to assure a metal-free medium, the K_i' values for inhibition of PNPase were 100–300-fold higher (Table 1). In contrast, the K_i' for acyclovir diphosphate (**1**) was increased only 2.4-fold. The effect of varying the concentration of zinc chloride on the K_i' of **2b** is illustrated in Figure 1. As the concentration of zinc chloride was increased from 0 to 8 μM, the K_i' decreased about 1000-fold. The increase in potency due to zinc leveled at concentrations above 8 μM. The effect of zinc on the K_i' values of the other compounds listed in Table 1 was qualitatively similar to that shown for **2b** (Figure 1). The decrease in the K_i' values for these compounds appeared to level off at zinc chloride concentrations above 8 μM. At 8 μM zinc chloride, the K_i' values for **2a** (0.024 μM), **2b** (0.0012 μM), **2c** (0.0012 μM), **2d** (0.0032 μM), **2e** (0.54 μM), **12** (0.37 μM), and **1** (0.0018 μM) were one-fourth to one-half their value at 2 μM zinc chloride (Table 1). This 100-fold potentiation effect of zinc on the K_i' of PNPase inhibitors **2b–d** was not observed with several other classes of inhibitors. No potentiation by zinc was found with guanine and guanosine analogues, monophosphates of acyclic guanosine analogues, (phosphonoalkyl)-guanines, nor [(phosphonoalkyl)benzyl]guanines.[35]

An apparent potentiation of an inhibitor's potency by zinc would be expected if the addition of zinc chloride to the assay mixtures were to reduce the concentration of substrates (inosine or phosphate) with which the inhibitor competes or to reduce the affinity of these substrates for the enzyme. However, such an apparent potentiation by zinc should be observed with all inhibitors. Since other classes of inhibitors were not potentiated by zinc, as noted above, this mechanism cannot explain the potentiation by zinc observed with the inhibitors in Table 1. Moreover, the affinity of the substrates for the enzyme was unaffected by zinc since the K_m' values for inosine (0.057 mM) and phosphate (0.28 mm) measured in the absence (plus 0.1 mM Na_2-EDTA) and presence of 2 μM zinc chloride were identical. The effects of other metals on inhibitor K_i' were examined, but out of 14 di- and trivalent cations (see Experimental Section), none enhanced the inhibitory potency as markedly as zinc.

Although the mechanism of this effect is not clear, we speculate that zinc may form a chelate with the (phosphinicomethyl)phosphonic acid moiety to create a molecular species that exhibits enhanced affinity for the phosphate-binding domain. Five-membered chelates were proposed to explain the increased complex stability of several metals to 9-[2-(phosphonylmethoxy)ethyl]-adenine, an antiviral agent.[36] If a similar phenomenon prevails with these (phosphinicomethyl)phoshponic acids, it is not a strongly associated chelate since zinc concentrations some 500-fold greater than inhibitor concentration are necessary for optimum activity. Nonetheless, micromolar concentrations of zinc have a profound effect on (phosphinicomethyl)phosphonic acid K_i', whereas there is only a minor effect on the K_i' of acyclovir diphosphate (**1**).

Competition with Inosine and Phosphate. The effect of inosine and phosphate on the most potent inhibitor, **2b**, was studied in more detail. When inosine was used as the variable substrate, as described in the Experimental Section, **2b** displayed competitive kinetics with respect to inosine both in the presence and absence of zinc. Likewise, when phosphate was used as the variable substrate, **2b** displayed competitive kinetics with respect to phosphate both in the presence and absence of zinc. K_i' values were obtained for **2b** by a weighted, least-squares fit[37] of the rate at each substrate concentration to the rate equation for competitive inhibition. The K_i' values obtained for **2b** using phosphate as the variable substrate were the same as those obtained using inosine as the variable substrate and similar to those determined from fractional inhibition (Table 1).

The competition with both inosine and phosphate displayed by **2b** suggests that this compound possesses binding determinants for both the purine- and phosphate-binding domains of the enzyme. The X-ray crystallography work of Montgomery et al. shows that the guanine and the terminal phosphate of acyclovir diphosphate bind to the purine and phosphate sites of PNPase.[17] Since the [[(guaninylalkyl)phosphinico]methyl]-phosphonic acids are serving as stable mimics of acyclovir diphosphate (**1**), they probably bind in a similar configuration with **1**. Compound **2b** contains guanine and a phosphate mimic linked via a chain of methylenes of optimum length to give a molecule possessing the binding stabilization of the individual substrates in one molecule. These characteristics are consistent with **2b** being a multisubstrate analogue inhibitor of PNPase.[19,20]

Effects on Growth of Lymphocytes. Each of the [[(guaninylalkyl)phosphinico]methyl]phosphonic acids **2a–d** was tested for its ability to selectively potentiate the cytotoxicity of 2′-deoxyguanosine, a PNPase substrate and a precursor of the cytotoxic metabolite dGTP. The compounds were evaluated in a growth inhibition

Table 2. Effect of Purine Nucleoside Phosphorylase Inhibitors on Growth of Human Leukemic T- and B-Cell Lines *in Vitro*[a,b]

no.	CEM, %	Molt-4, %	IM9, %
2a	120	150	130
2b	95	70	110
2c	72	93	85
2d	63	39 (68 μM)	110
8-aminoguanosine[c]	39 (5 μM)	21 (1.6 μM)	150

[a] Human leukemic T-cell (CEM, Molt-4) or B-cell (IM9) lines were used as in a previously described assay system[38] but with the addition of 20 μM 2′-deoxyguanosine. [b] Values are growth in the presence of 100 μM compound as a percentage of growth in the absence of compound. Parenthetical values are concentrations giving 50% inhibition of growth. [c] 8-Aminoguanosine inhibited PNPase in the presence of 2 μM zinc chloride with a K_i' of 0.5 μM.

assay with three human leukemic cell lines[38] (Table 2). CEM cells and Molt-4 cells exhibit T-cell markers, while IM9 cells exhibit B-cell markers. In control experiments with 20 μM 2′-deoxyguanosine, no inhibition of T-cell growth occurred. However, inhibition of T-cell growth occurred with the addition of 8-aminoguanosine, a reversible inhibitor of PNPase that is nonionic at physiological pH.[14] T-cell cytotoxicity is dependent on 2′-deoxyguanosine, since 8-aminoguanosine was not toxic in the absence of 2′-deoxyguanosine. At 100 μM of these potent PNPase inhibitors and 20 μM 2′-deoxyguanosine, only **2d** exhibited significant inhibition of T-cell growth. Phosphonic acid **2d** had an IC_{50} of 68 μM against Molt-4 cells under conditions where 8-aminoguanosine had an IC_{50} of 1.6 μM. Thus, although these phosphonic acids are potent inhibitors of PNPase, they only weakly potentiate the cytotoxicity of 2′-deoxyguanosine toward T-lymphocytes.

At least two factors may contribute to the absence of significant *in vitro* cytotoxicity. Optimum inhibition of PNPase is dependent on the availability of 2 μM zinc chloride. Although the cell growth medium contained approximately 4 μM zinc arising from the serum supplement, the availability of this zinc for complex formation with the test compounds is unknown. Furthermore, these [(alkylphosphinico)methyl]phosphonic acids may poorly penetrate into cells since these compounds are dianionic at physiological pH. Thus, the cell penetration of **2a–d** is expected to be limited by analogy with the poor cellular penetration of nucleotide diphosphates.

Conclusions

We synthesized a series of [[(guaninylalkyl)phosphinico]methyl]phosphonic acids, **2**, that are potent inhibitors of human erythrocyte purine nucleoside phosphorylase. The most potent compounds, **2b,c**, which are stable mimics of the diphosphate moiety of **1**, have K_i's of 3–4 nM when assayed in the presence of zinc chloride. Compound **2b** exhibits inhibition kinetics with inosine and phosphate that are consistent with its characterization as a multisubstrate analogue inhibitor of PNPase. However, these potent inhibitors exhibited only weak activity against human leukemia T-cells *in vitro*.

Experimental Section

NMR spectra were recorded on a Varian XVR-200 or Varian XVR-300 (^{1}H NMR, 200 MHz or 300 MHz; ^{13}C NMR, 75.43 MHz; ^{31}P NMR, 121.42 MHz) spectrometer. Chemical shift values are reported in parts per million. UV spectra were recorded on a Hewlett Packard 8452A diode array or Perkin-Elmer 751 spectrophotometer. UV data were analyzed by an IBM PC-AT. Mass spectra (~50 MA/s) were obtained from Oneida Research Services, Whitesboro, NY, using a Finnegan 4500 TFQ mass spectrometer. FAB^+ mass spectra were obtained on a VG70 SQ mass spectrometer (VG Ltd., Manchester, England) using a cesium ion source and a glycerol/hydrogen chloride matrix. Elemental microanalyses were determined by Atlantic Microlabs, Atlanta, GA, and gave combustion values for C, H, N, Cl, and S within 0.4% of theoretical values. Compounds analyzed for fractional amounts of solvent showed the appropriate solvent impurity signals in the ^{1}H NMR spectra. Preparative flash chromatography[39] was performed using silica gel 60 (40–63 μm, E.M. Science 9385-9). Analytical thin-layer chromatography was done using silica gel 60A (250 μm) MKGF (Whatman) plates. Preparative ion exchange chromatography was performed using DEAE Sephadex (A-25; Pharmacia-LKB) in a Michel-Miller glass chromatography column (21 mm × 300 mm; Ace Glass). An approximately linear gradient of aqueous ammonium bicarbonate (0–1 M, 2 L total volume) was generated with a two-chamber gradient apparatus and pumped with an FMI pump Model RP-lCSC and a PD-60-LF pulse dampener. Ultraviolet detection of the effluent and fraction collection were with an ISCO UA-5 monitor (2 mm path cells, 254 nm) and an ISCO 1850 fraction collector. Melting points were determined with a Thomas Hoover or Mel-Temp capillary melting point apparatus and are uncorrected. Diethyl 4-pentenylphosphonate (**46**) was available from Aldrich Chemical Co.

Diethyl 5-Hexenylphosphonate (4c). This compound was prepared in a manner analogous to **4d**, with **3b** used in place of **3c**. Fractional distillation gave 21.25 g (78%) of **4c** as a clear oil: bp 89–90 °C at 0.1 Torr; ^{1}H NMR ($CDCl_3$) δ 5.87–5.62 (m, 1H, CH_2=C*H*), 5.05–4.88 (m, 2H, CH_2=CH), 4.15–3.95 (m, 4H, POCH_2), 2.04 (dt, 2H, J = 7.0 and 6.8 Hz, CH_2=CHCH_2), 1.80–1.68 (m, 2H, PCH_2), 1.68–1.65 (m, 2H, $PCH_2CH_2CH_2$), 1.54–1.34 (m, 2H, PCH_2CH_2), 1.29 (t, 6H, J = 7.0 Hz, $POCH_2CH_3$); MS (CI, CH_4) *m/e* 221 (MH^+). Anal. ($C_{10}H_{21}O_3P$) C, H.

Diethyl 6-Heptenylphosphonate (4d). To a solution of diethyl methylphosphonate (13.99 g, 92.0 mmol) in anhydrous tetrahydrofuran (20 mL) at −60 °C under a nitrogen atmosphere was added a solution of *n*-butyllithium in hexane (1.6 M) (57.5 mL, 92.0 mmol). The solution was stirred at −55 °C for 20 min, and then a solution of **3c** (15 g, 92.0 mmol) in anhydrous tetrahydrofuran (30 mL) was added in a dropwise manner. The solution was allowed to slowly warm to ambient temperature during 18 h of stirring. The solution was concentrated to 60 mL *in vacuo*, diluted with water (100 mL), and extracted with dichloromethane (4 × 300 mL). The organic layer was spin evaporated *in vacuo*, and the residual oil was purified by fractional distillation to give 12.77 g (59%) of **4d**: bp 155–168 °C at 17 Torr; ^{1}H NMR ($CDCl_3$) δ 5.80–5.70 (m, 1H, CH_2=C*H*), 5.00–4.87 (m, 2H, CH_2=CH), 4.10–3.99 (m, 4H, $POCH_2$), 2.07–1.95 (m, 2H, CH_2=CHCH_2), 1.72–1.55 (m, 4H, PCH_2 and CH_2=CHCH_2CH_2), 1.40–1.33 (m, 4H, $PCH_2CH_2CH_2$), 1.28 (t, 6H, J = 7.0 Hz, $POCH_2CH_3$); ^{31}P NMR (DMSO-d_6) δ 33.43 (s, phosphonyl); MS (CI, CH_4) *m/e* 235 (MH^+). Anal. ($C_{11}H_{23}O_3P$) C, H.

Ethyl Hydrogen 4-Pentenylphosphonate (5b). Diethyl 4-pentenylphosphonate (**4b**) (26.0 g, 0.126 mol) and 85% aqueous potassium hydroxide (33.3 g, 0.504 mol) were combined in ethanol (250 mL) and refluxed under a nitrogen atmosphere for 1.5 h. The reaction solution was spin evaporated *in vacuo* to one-half its volume, diluted to 800 mL with distilled water, and cooled in an ice bath. The solution was made acidic with concentrated hydrochloric acid (50 mL) and extracted with dichloromethane (3 × 250 mL). The dichloromethane extracts were concentrated by spin evaporation *in vacuo* to give 23.75 g (quantitative yield) of **5b** as a clear oil, which was used as such in the next reaction: ^{1}H NMR ($CDCl_3$) δ 9.66 (s, 1H, OH), 5.8–5.6 (m, 2H, CH_2=C), 5.05–4.92 (m, 1H, CH), 4.15–3.95 (m, 2H, OCH_2), 2.15–2.05 (m, 2H, CH_2), 1.80–1.62 (m, 4H, PCH_2CH_2), 1.31 (t, 3H, J = 6.9 Hz, CH_3).

Ethyl Hydrogen 5-Hexenylphosphonate (5c). This compound was prepared in a manner analogous to **5b**, with **4c** used in place of **4b**. The dichloromethane extracts were concentrated *in vacuo* to give 12.91 g (86%) of **5c** as a clear

oil: ^{1}H NMR ($CDCl_3$) δ 11.95 (s, 1H, OH), 5.84–5.70 (m, 1H, CH), 5.04–4.90 (m, 2H, CH_2=CH), 4.07 (dq, 2H, J_{HH} = 7.12 Hz, J_{PH} = 7.88 Hz, $POCH_2CH_3$), 2.05 (dt, 2H, J = 6.8 and 7.2 Hz, CH_2=$CHCH_2$), 1.81–1.72 (m, 2H, PCH_2), 1.71–1.55 (m, 2H, $PCH_2CH_2CH_2$), 1.55–1.43 (m, 2H, PCH_2CH_2), 1.31 (t, 3H, J = 7.0 Hz, $POCH_2CH_3$); MS (FAB$^+$) *m/e* 193 (MH^+). Anal. ($C_8H_{17}O_3P$) C, H.

Ethyl (3-Butenylphosphono)chloridate (6a). Phosphorus pentachloride (6.9 g, 30.2 mmol) was added in one portion to a solution of **4a** (4.8 g, 25.2 mmol) in dichloromethane (40 mL). The reaction mixture was sealed and stirred at ambient temperature for 4 h. Fractional distillation gave 2.43 g (53%) of **6a**, which was used as such in the next reaction: bp 123–125 °C at 15 Torr; ^{1}H NMR ($CDCl_3$) δ 5.85–5.70 (m, 1H, CH_2=C*H*), 5.15–4.95 (m, 2H, CH_2=CH), 4.35–4.10 (m, 2H, $POCH_2$), 2.48–2.35 (m, 2H, CH_2=$CHCH_2$), 2.25–2.10 (m, 2H, PCH_2), 1.35 (t, 3H, J = 7.1 Hz, $POCH_2CH_3$).

Ethyl (4-Pentenylphosphono)chloridate (6b). To a stirred solution of **5b** (5.0 g, 0.028 mol) in dichloromethane (50 mL) at 0 °C were added dimethylformamide (0.5 mL) and oxalyl chloride (6.4 g, 0.0505 mol). The reaction mixture was allowed to come to room temperature without external heat and was stirred for 72 h with protection from moisture. The volatiles were removed by spin evaporation *in vacuo* at <40 °C with the addition of dichloromethane to aid in codistillation to give 5.55 g (quantitative yield) of **6b**, which was used without further purification in the next reaction: ^{1}H NMR ($CDCl_3$) δ 5.85–5.66 (m, 2H, CH_2=C), 5.10–4.95 (m, 1H, CH), 4.44–4.05 (m, 2H, OCH_2), 2.25–2.03 (m, 4H, PCH_2 and CH_2C=C), 1.90–1.62 (m, 2H, CH_2), 1.40–1.25 (t, 3H, J = 7.3 Hz, CH_3).

Ethyl (5-Hexenylphosphono)chloridate (6c). This compound was prepared in a manner analogous to **6b** from **5c**. Evaporation of the dichloromethane under reduced pressure left 12.7 g (quantitative yield) of **6c** as a clear oil contaminated with *N,N*-dimethylformamide (0.21 mol equiv), which was used as such in the next reaction: ^{1}H NMR ($CDCl_3$) δ 8.09 (br s, 0.21H, C*H*O), 5.9–5.7 (m, 1H, CH_2=C*H*), 5.1–4.9 (m, 2H, CH_2=CH), 4.4–4.1 (m, 2H, $POCH_2CH_3$), 3.03 (s, 0.63H, NCH_3), 2.94 (s, 0.63H, NCH_3), 2.2–2.0 (m, 4H, PCH_2 and CH_2=$CHCH_2$), 1.8–1.6 (m, 2H, $PCH_2CH_2CH_2$), 1.60–1.45 (m, 2H, PCH_2CH_2), 1.38 (t, 3H, J = 7.1 Hz, $POCH_2CH_3$).

Ethyl (6-Heptenylphosphono)chloridate (6d). This compound was prepared in a manner analogous to **6a** from **4d**. Evaporation of the dichloromethane under reduced pressure left 10.5 g (92%) of **6d** as a clear oil, which was used as such in the next reaction: ^{1}H NMR ($CDCl_3$) δ 5.80–4.89 (m, 1H, CH_2=C*H*), 5.00–4.89 (m, 2H, CH_2=CH), 4.35–4.15 (m, 2H, $POCH_2$), 2.16–1.90 (m, 4H, PCH_2 and CH_2=$CHCH_2$), 1.77–1.64 (m, 2H, CH_2=$CHCH_2CH_2$), 1.47–1.37 (m, 4H, PCH_2 and CH_2CH_2), 1.36 (t, 3H, J = 7.1 Hz, $POCH_2CH_3$).

Diethyl [[(3-Butenyl)ethoxyphosphinoyl]methyl]phosphonate (7a). This compound was prepared in a manner analogous to **7b** from **6a**. Fractional distillation gave 4.42 g (21%) of **7a** hemihydrate: bp 166–167 °C at 0.15 Torr; ^{1}H NMR ($CDCl_3$) δ 5.90–5.70 (m, 1H, CH), 5.10–4.93 (m, 2H, CH_2=C), 4.20–3.95 (m, 6H, OCH_2), 2.35 (dd, 2H, J_{PH} = 16.5 and 20.8 Hz, PCH_2P), 2.45–2.20 (m, 2H, CH_2), 2.10–1.90 (m, 2H, PCH_2), 1.29 (t, 3H, J = 7.0 Hz, CH_3); ^{31}P NMR ($CDCl_3$) δ 47.14 (d, 1P, J_{PH} = 5.4 Hz, phosphinyl), 22.25 (d, 1P, J_{PH} = 5.4 Hz, phosphonyl); MS (CI, CH_4) *m/e* 299 (MH^+). Anal. ($C_{11}H_{24}O_5P_2 \cdot 0.5H_2O$) C, H.

Diethyl [[Ethoxy(4-pentenyl)phosphinoyl]methyl]phosphonate (7b). A solution of *n*-butyllithium (1.6 M in hexane) (87.5 mL, 0.140 mol) was added dropwise with stirring to diethyl methylphosphonate (21.3 g, 0.140 mol) in 200 mL of dry tetrahydrofuran at −60 °C under a nitrogen atmosphere. After 10 min, **6b** (11.0 g, 0.056 mol) in dry tetrahydrofuran (40 mL) was added slowly and stirring was continued. After 2.5 h at −60 °C, the excess base was neutralized with aqueous ammonium chloride. The reaction solution was diluted with water (600 mL) and extracted with dichloromethane (3 × 200 mL). The dichloromethane layers were combined and spin evaporated *in vacuo*. The residue was distilled to give 9.30 g (53%) of **7b**, which was used as such in the next reaction: bp 155–165 °C at 0.1 Torr; ^{1}H NMR ($CDCl_3$) δ 5.80–5.65 (m, 2H, CH_2=C), 5.10–4.93 (m, 1H, CH), 4.20–4.00 (m, 6H, CH_2), 2.36 (dd, 2H, J_{PH} = 16.5 and 20.8 Hz, PCH_2P), 2.14 (br q, 2H, J = 7.0 Hz, C=CCH_2), 2.0–1.84 (m, 2H, PCH_2), 1.80–1.65 (m, 2H, CH_2), 1.32 (t, 6H, J = 7.1 Hz, CH_3), 1.309 (t, 3H, J = 7.1 Hz, CH_3).

Diethyl [[Ethoxy(5-hexenyl)phosphinoyl]methyl]phosphonate (7c). This compound was prepared in a manner analogous to **7b** from **6c**. Fractional distillation gave 5.7 g (30%) of **7c**: bp 160–180 °C at 0.07 Torr; ^{1}H NMR ($CDCl_3$) δ 5.80–5.62 (m, 1H, CH_2=C*H*), 5.00–4.88 (m, 2H, CH_2=CH), 4.20–4.00 (m, 6H, $POCH_2$), 3.51 (dd, 2H, J_{PH} = 16.5 and 20.7 Hz, PCH_2P), 2.08–1.99 (m, 2H, CH_2=$CHCH_2$), 1.98–1.85 (m, 2H, PCH_2), 1.68–1.53 (m, 2H, CH_2=$CHCH_2CH_2$), 1.51–1.40 (m, 2H, PCH_2CH_2), 1.31 (t, 4H, J = 7.0 Hz, $POCH_2CH_3$), 1.29 (t, 3H, J = 7.0 Hz, $POCH_2CH_3$); MS (CI, CH_4) *m/e* 327 (MH^+). Anal. ($C_{13}H_{28}O_5P_2$) C, H.

Diethyl [[Ethoxy(6-heptenyl)phosphinoyl]methyl]phosphonate (7d). This compound was prepared in a manner analogous to **7b**, with **6d** used in place of **6b**. Fractional distillation gave 6.16 g (41%) of **7d**: bp 160–170 °C at 0.2 Torr; ^{1}H NMR ($CDCl_3$) δ 5.82–5.72 (m, 1H, CH_2=C*H*), 5.0–4.89 (m, 2H, CH_2=CH), 4.20–4.05 (m, 6H, $POCH_2CH_3$), 2.37 (dd, 2H, J_{PH} = 16.4 and 20.7 Hz), 2.10–2.00 (m, 2H, CH_2=$CHCH_2$), 2.00–1.86 (m, 2H, PCH_2), 1.70–1.55 (m, 2H, CH_2=$CHCH_2CH_2$), 1.45–1.3 (m, 2H, $PCH_2CH_2CH_2$), 1.33 (t, 6H, J = 7.0 Hz, $POCH_2CH_3$), 1.32 (t, 3H, J = 7.0 Hz, $POCH_2CH_3$); ^{31}P NMR (DMSO-d_6) δ 48.01 (d, 1P, J_{PP} = 5.12 Hz, phosphinyl), 22.66 (d, 1P, J = 5.12 Hz, phosphonyl); MS (CI, CH_4) *m/e* 341 (MH^+). Anal. ($C_{14}H_{30}O_5P_2 \cdot 0.6H_2O$) C, H.

Diethyl [[Ethoxy(4-hydroxybutyl)phosphinoyl]methyl]phosphonate (8a). This compound was prepared in a manner analogous to **8b**, with **7a** used in place of **7b**. The residue obtained from concentration of the ethyl acetate extracts was dissolved in dichloromethane (100 mL), filtered, and spin evaporated *in vacuo*, with the addition of dichloromethane (2 × 100 mL), to give 11.64 g (81%) of **8a**·1.2 hydrate as a clear oil: ^{1}H NMR (DMSO-d_6) δ 3.90–4.10 (m, 6H, $POCH_2CH_3$), 3.39 (t, 2H, J = 5.9 Hz, CH_2OH), 2.62 (dd, 1H, J_{PH} = 16.8 and 20.2 Hz, PC*H*HP), 2.61 (dd, 1H, J_{PH} = 16.4 and 20.4 Hz, PCH*H*P), 1.90–1.76 (m, 2H, PCH_2), 1.58–1.42 (m, 4H, $PCH_2CH_2CH_2$), 1.24 (t, 6H, J = 7.0 Hz, $POCH_2CH_3$), 1.22 (t, 3H, J = 7.0 Hz, $POCH_2CH_3$); ^{31}P NMR (DMSO-d_6) δ 48.08 (d, 1P, J_{PH} = 5.3 Hz, phosphinyl), 22.66 (d, 1P, J = 5.3 Hz, phosphonyl); MS (CI, CH_4) *m/e* 317 (MH^+). Anal. ($C_{11}H_{26}O_6P_2 \cdot 1.2H_2O$) C, H.

Diethyl [[Ethoxy(5-hydroxypentyl)phosphinoyl]methyl]phosphonate (8b). Phosphonate **7b** (5.0 g, 0.016 mol) in anhydrous tetrahydrofuran (15 mL) was slowly added to 1.0 M borane in tetrahydrofuran (16 mL, 16 mmol) in anhydrous tetrahydrofuran (10 mL) at −10 °C under a nitrogen atmosphere. The solution was allowed to warm to ambient temperature (22 °C) over 2 h and then cooled to 10 °C. Very slowly, in a dropwise manner to control frothing, 3 N aqueous sodium hydroxide (5.25 mL) was added followed by 30% hydrogen peroxide (5.25 mL), while maintaining the temperature in an ice bath. The reaction mixture was then heated to 50 °C for 1.5 h and cooled to 25 °C. The excess peroxide was reduced with 5% aqueous sodium bisulfite. The volatiles were then removed by spin evaporation *in vacuo*. Dichloromethane was added during the evaporation to assist in removal of the residual water. The damp solid residue was extracted with ethyl acetate (3 × 150 mL), which was then dried with magnesium sulfate, filtered, and concentrated *in vacuo* to give 4.68 g (88%) of **8b** as a clear oil, which was used as such in the next reaction: ^{1}H NMR ($CDCl_3$) δ 4.25–4.00 (m, 6H, OCH_2), 3.64 (t, 2H, J = 6.0 Hz, $HOCH_2$), 2.38 (dd, 2H, J_{PH} = 16.6 and 20.7 Hz, PCH_2P), 2.05–1.85 (m, 2H, PCH_2), 1.75–1.40 (m, 6H, $CH_2CH_2CH_2$), 1.337 (t, 6H, J = 7.0 Hz, CH_3), 1.326 (t, 3H, J = 7.0 Hz, CH_3).

Diethyl [[Ethoxy(6-hydroxyhexyl)phosphinoyl]methyl]phosphonate (8c). This compound was prepared in a manner analogous to **8b**, with **7c** used in place of **7b**. The ethyl acetate extraction solutions were concentrated *in vacuo*. The residue was dissolved in dichloromethane and chromatographed through a pad of silica gel 60 (3 cm × 3 cm) using a mixture of methanol and dichloromethane (1:20). The eluent

solution was concentrated *in vacuo* with the addition of dichloromethane to give 4.96 g (85%) of **8c**·monohydrate as a clear oil: ^{1}H NMR ($CDCl_3$) δ 4.22–4.01 (m, 6H, $POCH_2$), 3.63 (t, 2H, J = 6.4 Hz, CH_2OH), 2.38 (dd, 2H, J = 16.4 and 20.7 Hz, PCH_2P), 2.00–1.88 (m, 4H, CH_2CH_2OH and PCH_3), 1.7–1.6 (m, 2H, PCH_2CH_2), 1.6–1.5 (m, 2H, $HOCH_2CH_2CH_2$), 1.45–1.37 (m, 2H, $PCH_2CH_2CH_2$), 1.34 (t, 6H, J = 7.0 Hz, $POCH_2CH_3$), 1.33 (t, 3H, J = 7.0 Hz, $POCH_2CH_3$); MS (CI, CH_4) *m/e* 345 (MH^+). Anal. ($C_{13}H_{30}O_6P_2$·H_2O) C, H.

Diethyl [[Ethoxy(7-hydroxyheptyl)phosphinoyl]methyl]phosphonate (8d). A solution of **7d** (2.0, 5.69 mmol) in anhydrous tetrahydrofuran (5 mL) was added dropwise to a solution of borane (1.0 M in tetrahydrofuran) (5.7 mL, 5.7 mmol) in tetrahydrofuran (10 mL) at −10 °C under a nitrogen atmosphere. The solution was stirred for 2 h while it warmed to ambient temperature. To this solution was added, dropwise, distilled water (6 mL) followed by sodium perborate·$4H_2O$ (2.62 g, 17 mmol) in portions. The mixture was stirred for 2 h and then spin evaporated *in vacuo* to dryness. Dichloromethane was added (3 × 150 mL) during the evaporation to remove the last traces of water. The white residue was leached with ethyl acetate (3 × 150 mL). The ethyl acetate solution was filtered and concentrated *in vacuo* to give 2.18 g (100%) of **8d**·0.75 hydrate as a thick oil: ^{1}H NMR ($CDCl_3$) δ 4.22–4.05 (m, 6H, $POCH_2$), 3.32 (t, 2H, J = 6.4 Hz, CH_2OH), 2.37 (dd, 2H, J_{PH} = 16.5 and 20.8 Hz, PCH_2P), 2.05–1.80 (m, 3H, PCH_2 and OH), 1.80–1.50 (m, 4H, PCH_2CH_2 and $HOCH_2CH_2$), 1.48–1.30 (m, 6H, $PCH_2CH_2CH_2CH_2CH_2$), 1.33 (t, 6H, J = 7.0 Hz, $POCH_2CH_3$), 1.32 (t, 2H, J = 7.0 Hz, $POCH_2CH_3$); ^{31}P NMR (DMSO-d_6) δ 47.79 (d, 1P, J_{PP} = 5.2 Hz, phosphinyl), 22.39 (d, 1P, J_{PP} = 5.2 Hz, phosphonyl); MS (CI, CH_4) *m/e* 359 (MH^+). Anal. ($C_{14}H_{32}O_6P_2$·0.75H_2O) C, H.

Diethyl [[Ethoxy[4-[(methylsulfonyl)oxy]butyl]phosphinoyl]methyl]phosphonate (9a). This compound was prepared in a manner analogous to **9b**, with **8a** used in place of **8b**. The chromatography solutions were spin evaporated *in vacuo* to give 3.43 g (58%) of **9a** as a clear oil, which was used as such in the next reaction: ^{1}H NMR ($CDCl_3$) δ 4.23 (t, 2H, J = 6.1 Hz, $SOCH_2$), 4.20–4.06 (m, 6H, OCH_2), 3.00 (s, 3H, CH_3S), 2.37 (dd, 2H, J_{PH} = 16.7 and 20.6 Hz, PCH_2P), 2.05–1.70 (m, 6H, $CH_2CH_2CH_2$), 1.33 (t, 6H, J = 6.3 Hz, CH_3), 1.32 (t, 3H, CH_3); ^{31}P NMR (DMSO-d_6) 47.77 (d, 1P, J = 5.4 Hz, phosphinyl), 22.56 (d, 1P, J = 5.4 Hz, phosphonyl).

Diethyl [[Ethoxy[5-[(methylsulfonyl)oxy]pentyl]phosphinoyl]methyl]phosphonate (9b). Methanesulfonyl chloride (3.04 mL, 39.4 mmol) was slowly added to a stirring solution of **8b** (13.0 g, 39.4 mmol) and triethylamine (5.5 mL, 39.4 mmol) in dichloromethane (50 mL) at −60 °C. After 3 h of stirring, the reaction mixture was applied to a chromatography column (5 cm × 20 cm) of silica gel 60 wet with dichloromethane and was purified by chromatographic elution (eluent: methanol gradient in dichloromethane, 0–10%, 3 L). Evaporation of the appropriate fractions at <40 °C gave 8.75 g (54%) of **9b** as a clear oil: ^{1}H NMR ($CDCl_3$) δ 4.25–4.00 (m, 8H, OCH_2), 2.99 (s, 3H, SCH_3), 2.36 (dd, 2H, J_{PH} = 16.6 and 20.7 Hz, PCH_2P), 2.05–1.4 (m, 8H, $PCH_2CH_2CH_2CH_2$), 1.33 (t, 3H, J = 7.03 Hz, CH_3), 1.31 (t, 3H, J = 7.03 Hz, CH_3); MS (CI, CH_4) *m/e* 409 (MH^+). Anal. ($C_{13}H_{30}O_8P_2S$·0.5H_2O) C, H.

Diethyl [[Ethoxy[6-[(methylsulfonyl)oxy]hexyl]phosphinoyl]methyl]phosphonate (9c). This compound was prepared in a manner analogous to **9b**, with **8c** used in place of **8b**. Evaporation of the chromatography solutions *in vacuo* gave 2.48 g (100%) of **9c** as a clear oil, which was used as such in the next reaction: ^{1}H NMR ($CDCl_3$) δ 4.22 (t, 2H, J = 6.5 Hz, $SOCH_2$), 4.25–4.15 (m, 6 H, $POCH_2$), 3.00 (s, 3H, CH_3S), 2.38 (dd, 2H, J_{PH} = 16.4 and 20.7 Hz, PCH_2P), 2.02–1.89 (m, 2H, PCH_2), 1.80–1.70 (m, 2H, $SO_3CH_2CH_2$), 1.70–1.60 (br m, 2H, PCH_2CH_2), 1.5–1.4 (m, 4H, $PCH_2CH_2CH_2CH_2$), 1.35 (t, 6H, J = 7.0 Hz, $POCH_2CH_3$), 1.34 (t, 3H, J = 7.0 Hz, $POCH_3CH_3$).

Diethyl [[Ethoxy[7-[(methylsulfonyl)oxy]heptyl]phosphinoyl]methyl]phosphonate (9d). This compound was prepared in a manner analogous to **9b**, with **8d** used in place of **8b**. Evaporation of the chromatography solution gave 3.0 g (80%) of **9d** monohydrate as a thick oil: ^{1}H NMR ($CDCl_3$) δ 4.25–4.00 (m, 8H, SO_2OCH_2 and $POCH_2$), 3.00 (s, 3H, CH_3SO_3), 2.37 (dd, 2H, J_{PP} = 16.6 and 20.7 Hz, PCH_2P), 2.05–1.80 (m, 2H, PCH_2), 1.80–1.50 (m, 6H, $CH_2CH_2CH_2CH_2CH_2CH_2P$), 1.5–1.3 (m, 4H, $CH_2CH_2CH_2CH_2P$), 1.33 (t, 6H, J = 7.0 Hz, $POCH_2CH_3$), 1.32 (t, 3H, J = 7.0 Hz, $POCH_2CH_3$); ^{31}P NMR (DMSO-d_6) δ 47.78 (d, 1P, J_{PP} = 5.2 Hz, phosphinyl), 22.39 (d, 1P, J_{PP} = 5.2 Hz, phosphonyl); MS (CI, CH_4) *m/e* 437 (MH^+). Anal. ($C_{15}H_{34}O_8P_2S$·H_2O) C, H.

Diethyl [[[4-[2-Amino-6-(2-methoxyethoxy)-9*H*-purin-9-yl]butyl]ethoxyphosphinoyl]methyl]phosphonate (10a). This compound was prepared in a manner analogous to **10b**, with **9a** used in place of **9b**. The chromatography solution was concentrated *in vacuo* to give 1.52 g (37%) of **10a** as a clear oil: ^{1}H NMR ($CDCl_3$) δ 7.59 (s, 1H, purine H-8), 4.91 (br s, 2H, NH_2), 4.64 (t, 2H, J = 5.0 Hz, OCH_2), 4.20–4.05 (m, 6H, OCH_2), 4.07 (t, 2H, J = 7.2 Hz, NCH_2), 3.80 (t, 2H, J = 5.0 Hz, OCH_2), 3.43 (s, 3H, CH_3), 2.38 (dd, 2H, J_{PH} = 16.7 and 20.7 Hz, PCH_2P), 2.12–1.90 (m, 4H, CH_2CCH_2), 1.75–1.55 (m, 2H, CH_2), 1.34 (t, 6H, J = 7.0 Hz, CH_3), 1.30 (t, 3H, J = 7.0 Hz, CH_3); ^{31}P NMR (DMSO-d_6) δ 47.75 (d, 1P, J_{PH} = 5.15 Hz, phosphinyl), 22.57 (d, 1P, J_{PH} = 5.15 Hz, phosphonyl); MS (CI, CH_4) *m/e* 508 (MH^+). Anal. ($C_{19}H_{35}N_5O_7P_2$·0.5CH_2Cl_2) C, H, N.

Diethyl [[[5-[2-Amino-6-(2-methoxyethoxy)-9*H*-purine-9-yl]pentyl]ethoxyphosphinoyl]methyl]phosphonate (10b). Methylsulfonyl phosphonate **9b** (13.66 g, 33.45 mmol), 2-amino-6-(2-methoxyethoxy)-9*H*-purine[31] (7.0 g, 33.45 mmol), and anhydrous cesium carbonate (21.7 g, 66.9 mmol) in 200 mL of anhydrous dimethylformamide (freshly distilled from calcium hydride) were stirred at 80 °C for 3 h under a nitrogen atmosphere. The dimethylformamide was evaporated *in vacuo*. The residue was dissolved in dichloromethane, filtered, and purified by flash chromatography on a column (5 cm × 25 cm) of silica gel 60 wet with dichloromethane (eluent: methanol gradient in dichloromethane, 0–10%, 4 L). Evaporation of the appropriate fractions at <40 °C gave 6.04 g (34%) of **10b** as a pale oil, which was used in the next reaction: ^{1}H NMR ($CDCl_3$) δ 7.57 (s, 1H, H-8), 4.94 (br s, 2H, NH_2), 4.63 (t, 2H, J = 5.1 Hz, OCH_2), 4.25–4.00 (m, 8H, NCH_2 and $POCH_2$), 3.80 (t, 2H, J = 5.1 Hz, OCH_2), 3.42 (s, 3H, OCH_3), 2.37 (dd, 2H, J_{PH} = 16.6 and 20.7 Hz, PCH_2P), 2.00–1.60 (m, 6H, PCH_2 and CH_2CH_2), 1.48–1.324 (m, 2H, CH_2), 1.32 (t, 3H, J = 7.03 Hz, CH_3), 1.31 (t, 3H, J = 7.03 Hz, CH_3).

Diethyl [[[7-[2-Amino-6-(2-methoxyethoxy)-9*H*-purin-9-yl]heptyl]ethoxyphosphinoyl]methyl]phosphonate (10d). This compound was prepared in a manner analogous to **10b**, with **9d** used in place of **9b**. The chromatography solution was concentrated *in vacuo* to give 1.43 g (37%) of **10d** as a thick oil, which was used as such in the next reaction: ^{1}H NMR (DMSO-d_6) δ 7.85 (s, 1H, purine 8-H), 6.36 (br s, 2H, NH_2), 4.50 (t, 2H, J = 4.7 Hz, OCH_2), 4.10–3.82 (m, 8H, $POCH_2$ and NCH_2), 3.67 (t, 2H, J = 4.7 Hz, CH_2OCH_3), 3.30 (s, 3H, OCH_3), 2.55 (dd, 2H, J_{PH} = 16.5 and 19.2 Hz, PCH_2P), 1.90–1.60 (br m, 2H, NCH_2CH_2), 1.5–1.3 (br m, 2H, PCH_2), 1.30–1.00 (br m, 17H, $PCH_2CH_2CH_2CH_2CH_2$ and $POCH_2CH_3$); ^{31}P NMR (DMSO-d_6) δ 46.28 (d, 1P, J_{PP} = 5.1 Hz, phosphinyl), 21.6 (d, 1P, J_{PP} = 5.1 Hz, phosphonyl).

[[[4-(2-Amino-1,6-dihydro-6-oxo-9*H*-purine-9-yl)butyl]phosphinico]methyl]phosphonic Acid (2a). This compound was prepared in a manner analogous to **2b**, with **10a** used in place of **10b**. Lyophilization of the chromatography solution gave 0.348 g (29%) of **2a**·1.7 ammonium salt·1.7 hydrate: mp 173–176 °C; UV (0.1 N hydrochloric acid) λ_{max} 254 nm (ϵ 11 600), 279 (ϵ 7800), λ_{min} 270 (ϵ 7400), (pH 7 buffer) λ_{max} 254 (ϵ 12 300), λ_{sh} 271 (ϵ 9300), (0.1 N sodium hydroxide) λ_{max} 270 (ϵ 10 500), λ_{sh} 258 (ϵ 9200); ^{1}H NMR (D_2O) (HOD resonance was reduced by presaturation with the decoupler channel) δ 7.91 (br s, 1H, purine 8-H), 4.08 (t, 2H, J = 7.0 Hz, NCH_2), 2.00 (dd, 2H, J_{PH} = 17 and 18 Hz, PCH_2P), 1.86 (quintet, 2H, J = 7.1 Hz, NCH_2CH_2), 1.72 (br s, 2H, PCH_2), 1.51 (br s, 2H, PCH_2CH_2); ^{31}P NMR (D_2O) δ 39.16 (br s, 1P, phosphinyl); MS (FAB$^+$) *m/e* 366 (MH^+). Anal. ($C_{10}H_{17}N_5O_6P_2$·1.7NH_3·1.7H_2O) C, H, N.

[[[5-(2-Amino-1,6-dihydro-6-oxo-9*H*-purin-9-yl)pentyl]phosphinico]methyl]phosphonic Acid (2b). Phosphonate **10b** (4.0 g, 0.00567 mol) was hydrolyzed by heating in hydrochloric acid (12 M) (40 mL) at 100 °C for 18 h. The

solution was concentrated by spin evaporation *in vacuo*. The residue was dissolved in water and purified by anion exchange chromatography on DEAE Sephadex A-25 (HCO_3^- form) with an aqueous ammonium bicarbonate buffer gradient (0–1 M). The appropriate fractions were combined, spin evaporated *in vacuo* to a small volume, and lyophilized to give 2.09 g (71%) of **2b** as a white powder: mp 152–155 °C; UV (0.1 N hydrochloric acid) λ_{max} 253 nm (ϵ 10 900), 278 (ϵ 7300), λ_{min} 227 (ϵ 2700), 270 (ϵ 7000), (pH 7 buffer) λ_{max} 271 (ϵ 8400), 252 (ϵ 10 900), λ_{min} 228 (ϵ 3800), 267 (ϵ 8300), (0.1 N sodium hydroxide) 270 (ϵ 9500), λ_{min} 233 (ϵ 4600), λ_{sh} 257 (ϵ 8700); ^{1}H NMR (DMSO-d_6) δ 7.69 (s, 1H, H-8), 6.6 (br s, 2H, NH_2), 4.5 (vbr s, NH_4^+ and H_2O) 3.91 (t, 2H, J = 6.8 Hz, NCH_2), 1.80–1.60 (m, 4H, PCH_2P and CH_2), 1.58–1.40 (m, 4H, PCH_2 and CH_2, 1.38–1.20 (m, 2H, CH_2); ^{31}P NMR (DMSO-d_6) δ 35.205 (s, 1P, phosphinyl), 14.484 (s, 1P, phosphonyl); MS (FAB$^+$) *m/e* 380 (MH^+). Anal. ($C_{11}H_{19}N_5O_6P_2{\cdot}2NH_3{\cdot}2.25H_2O$) C, H, N.

[[[6-(2-Amino-1,6-dihydro-6-oxo-9*H*-purin-9-yl)hexyl]phosphinico]methyl]phosphonic Acid (2c). To a mixture of 2-amino-6-chloropurine (3.11 g, 18.4 mmol) and potassium carbonate (5.0 g, 36 mmol) in *N*,*N*-dimethylformamide (15 mL), which had been dried by distillation from calcium hydride under nitrogen, was added a solution of **9c** (2.54 g, 6.1 mmol) in *N*,*N*-dimethylformamide (5 mL). The reaction mixture was heated to 80 °C for 18 h while being protected from moisture. The solvent was removed by spin evaporation *in vacuo* to give crude **11c**. The residue was dissolved in concentrated hydrochloric acid (25 mL) and refluxed for 24 h. The solution was concentrated by spin evaporation *in vacuo*; distilled water (50 mL) was added and removed by spin evaporation to remove residual hydrochloric acid. The residue was dissolved in water (25 mL), and the pH was adjusted to 7 with potassium carbonate. After cooling in an ice bath for 5 h, the precipitate was removed by filtration, and the filtrates were diluted to 0.5 L. Ion exchange column chromatography on QAE Sephadex A-25 ion exchange media (HCO_3^- form, 2.5 cm × 55 cm column, 0.02–1.0 M sodium carbonate buffer (pH 9.9), 2 L) was used for preliminary purification. Final purification was performed on DEAE Sephadex A-25 ion exchange media (HCO_3^- form, 2.5 cm × 55 cm column, 0–1 M ammonium bicarbonate buffer, 2 L). The chromatography solution was lyophilized to give 0.615 g (23%) of **2c** (87.5% plus 12.5% of the 7-purine isomer): mp 173–175 °C; UV (0.1 N hydrochloric acid) λ_{max} 278 nm (ϵ 8200), 253 (ϵ 12 200), λ_{min} 227 (ϵ 3300), 270 (ϵ 7900), (pH 7 buffer) λ_{max} 271 (ϵ 12 000), 278 (ϵ 8200), λ_{min} 228 (ϵ 4700), 268 (ϵ 9300), (0.1 N sodium hydroxide) λ_{max} 270 (ϵ 10 600), λ_{min} 233 (ϵ 5400), λ_{sh} 257 (ϵ 9600); ^{1}H NMR (D_2O) (HOD resonance was reduced by presaturation using the decoupler channel) δ 8.36 (br s, 0.125H, NH of 7-isomer), 8.07 (br s, 0.875H, NH of 9-isomer), 4.33 (t, 0.25H, J = 7.0 Hz, NCH_2 of 7-isomer), 4.08 (t, 175H, J = 7.0 Hz, NCH_2 of 9-isomer), 2.10 (dd, 2H, J_{PH} = 16.6 and 9.2 Hz, PCH_2P), 1.95–1.60 (m, 4H, PCH_2 and NCH_2CH_2), 1.60–1.20 (m, 6H, $PCH_2CH_2CH_2CH_2$); ^{31}P NMR (H_2O) δ 39.39 (s, 1P, phosphinyl), 15.98 (s, 1P, phosphonyl); MS (FAB) *m/e* 392 ($(M - H)^-$). Anal. ($C_{12}H_{21}N_5O_6P_2{\cdot}1.5NH_3{\cdot}0.5H_2O$) C, H, N.

[[[7-(2-Amino-1,6-dihydro-6-oxo-9*H*-purin-9-yl)heptyl]phosphinico]methyl]phosphonic Acid (2d). This compound was prepared in a manner analogous to **2b**, with **10d** used in place of **10b**. Lyophilization of the chromatography solution gave 0.497 g (61%) of **2d**·1.3 ammonium salt·0.85 hydrate as white crystals: mp 140–145 °C; UV (0.1 N hydrochloric acid) λ_{max} 252 nm (ϵ 11 700), 278 (ϵ 7800), λ_{min} 267 (ϵ 7600), (pH 7 buffer) λ_{max} 252 (12 200), λ_{sh} 271 (ϵ 9300), (0.1 N sodium hydroxide) λ_{max} 268 (10 600), λ_{sh} 256 (9800); ^{1}H NMR (DMSO-d_6) δ 7.68 (s, 1H, purine H-8), 6.52 (br s, 2H, NH_2), 5.00 (vbr s, NH_4^+ and H_2O), 3.91 (t, 2H, J = 7.1 Hz, NCH_2), 1.80–1.60 (br m, 4H, PCH_2P and NCH_2CH_2), 1.50–1.35 (br m, 4 H, PCH_2 and NCH_2CH_2), 1.30–1.15 (br s, 6H, $PCH_2CH_2CH_2CH_2$); ^{31}P NMR (D_2O) δ 43.13 (br s, 1P, phosphinyl), 15.31 (br s, 1P, phosphonyl); MS (FAB$^+$) *m/e* 408 (MH^+). Anal. ($C_{13}H_{23}N_5O_6P_2{\cdot}1.3NH_3{\cdot}0.85H_2O$) C, H, N.

Ethyl Hydrogen [[[5-(2-Amino-1,6-dihydro-6-oxo-9*H*-purine-9-yl)pentyl]phosphinico]methyl]phosphonate (2e). A solution of **10b** (0.400 g, 0.78 mmol) in ethanol (5 mL) and hydrochloric acid (1 N) (10 mL) was heated to reflux for 1 h. The cooled reaction solution was spin evaporated *in vacuo*. The residue was dissolved in ethanol (10 mL) containing potassium hydroxide 85% (1 g). The solution was heated to reflux for 2 h. The cooled solution was diluted with hydrochloric acid (1 N) (20 mL) and spin evaporated *in vacuo*. The residue was dissolved in water (400 mL) and purified by anion exchange chromatography on DEAE Sephadex A-25 (HCO_3^- form, 21 mm × 250 mm column) with an aqueous ammonium bicarbonate buffer (0–1 M gradient, 2 L). The appropriate fractions were combined and spin evaporated *in vacuo*. The residue was dissolved in water and lyophilized to give 0.174 g (50%) of **2e** as the monoammonium salt: mp 130–140 °C; UV (0.1 N hydrochloric acid) λ_{max} 277 nm (ϵ 8300), 252 (ϵ 12 400), λ_{min} 269 (ϵ 8200), 227 (ϵ 3100), (pH 7 buffer) λ_{max} 251 (ϵ 13 300), λ_{min} 225 (ϵ 3400), λ_{sh} 270 (ϵ 10 000), (0.1 N sodium hydroxide) λ_{max} 268 (ϵ 11 400), λ_{min} 231 (ϵ 4900), λ_{sh} 256 (ϵ 10 700); ^{1}H NMR (DMSO) δ 7.69 (s, 1H, H-8), 6.50 (br s, 2H, NH_2), 4.8 (vbr s, NH_4^+ and H_2O), 3.91 (t, 2H, J = 7.1 Hz, NCH_2), 3.72 (dq, 2H, J_{PH} = 7.1 Hz, J_{HH} = 7.1 Hz, OCH_2), 1.76 (dd, 2H, J_{PH} = 15.4 and 17.4 Hz, PCH_2P), 1.75–1.6 (m, 2H, CH_2), 1.60–1.40 (m, 4H, CH_2 and CH_2), 1.35–1.2 (m, 2H, CH_2), 1.10 (t, 3H, J = 7.0 Hz, CH_3); MS (FAB$^+$) *m/e* 408 (MH^+). Anal. ($C_{13}H_{23}N_5O_6P_2{\cdot}NH_3{\cdot}1.15H_2O$) C, H, N.

[[[5-(1,2,3,6-Tetrahydro-2,6-dioxo-9*H*-purin-9-yl)pentyl]phosphinico]methyl]phosphonic Acid (12). Compound **12** was isolated from the mixture obtained from acid hydrolysis of **10b**. Fractions containing a product that eluted just prior to compound **2b** from the preparative DEAE Sephadex ion exchange chromatography column were combined, spin evaporated *in vacuo* to a small volume, and lyophilized to give 0.649 g (19%) of **12** as a white solid: mp 162–166 °C; UV (0.1 N hydrochloric acid) λ_{max} 260.5 nm (ϵ 10 200), 236 (ϵ 6700), λ_{min} 243 (ϵ 6200), (pH 7 buffer) λ_{max} 274.5 (ϵ 9100), 246 (ϵ 9300), λ_{min} 260 ϵ 6700), (0.1 N sodium hydroxide) λ_{max} 277.5 (ϵ 10 100), 246 (ϵ 10 100), λ_{min} 261 (ϵ 6700); ^{1}H NMR (DMSO-d_6) δ 7.59 (s, 1H, H-8), 4.6 (vbr s, NH_4^+ and H_2O), 3.97 (m, 2H, NCH_2), 1.80–1.60 (m, 4H, PCH_2P and CH_2), 1.60–1.40 (m, 4H, PCH_2 and CH_2), 1.40–1.20 (m, 2H, CH_2); ^{31}P NMR (DMSO-d_6) δ 34.360 (s, 1P, phosphinyl), 13.863 (s, 1P, phosphonyl); MS (FAB$^+$) *m/e* 381 (MH^+). Anal. ($C_{11}H_{18}N_4O_7P_2{\cdot}2.5NH_3{\cdot}1.5H_2O$) C, H, N.

Determination of pK_a of 2b. Since the ^{31}P chemical shifts of phosphorus acids exhibit a dependence on ionization state, we estimated the pK_a's of phosphorus acids of compound **2b** by observing the shifts in the ^{31}P NMR chemical shift in water as the pH was changed. The ^{31}P NMR spectra were acquired with a Varian XVR-300 NMR spectrometer using a 10 mm probe designed for use with ionic sample solutions. The ^{31}P observed frequency was 121.42 MHz. Each spectrum was acquired at 23.2 °C with broad-band proton decoupling and referenced to external 85% H_3PO_4 at 0 ppm. The data were obtained using four acquisition transients with an interpulse delay of 4 s and an acquisition time of 1 s. The spectrometer was operated in a nonlock stabilized magnet mode to eliminate any field changes due to changes of a lock signal resulting from changes in pH or ionic strength of the observed solution. Natural isotope abundance water was used so no corrections for solvent deuteration were required.

Compound **2b** (12 mg) was dissolved in 15 mL of distilled water. The pH of the solution was measured using a pH meter (Orion Research Model 301 with a combination pH electrode). The ^{31}P NMR spectra were obtained using a portion of this solution. The contents of the NMR tube were combined with the remaining solution, and the pH was lowered approximately 0.25 pH unit with the addition of 1 N hydrochloric acid. The ^{31}P NMR spectra were taken again, and the process was repeated until the pH of the solution was below 0.5. An additional sample was prepared with distilled water, the pH of this solution was raised to above 12 with 1 N sodium hydroxide, and spectra were taken as before. A total of 37 spectra were recorded over a pH range of 0.42–12.2. The chemical shifts of the two phosphorus atoms were plotted against measured pH, and the resulting curves were fitted to the required mathematical function using nonlinear regression analysis of the two sets of data. Seven variables were used in the analysis of the phosphinate data, giving three pK_a values

of 1.69, 3.19, and 8.45. Five variables were used for the phosphonate data, giving two pK_a values of 3.44 and 8.46. When the phosphonate data were analyzed using seven variables, three pK_a values of 1.61, 3.43, and 8.47 were found, although no improvement in fit was observed over the analysis using five variables, so this analysis was not used. Thus the first and second ionizations of the phosphonic acid were found to have pK_a's of approximately 1.6 and 8.45, and the ionization of the phosphinic acid had a pK_a of approximately 3.2.

Enzyme Assay. PNPase was purified from human erythrocytes and assayed using a xanthine oxidase-coupled spectrophotometric assay as described previously.[25,26] In addition to enzyme, the assay mixtures contained inhibitor, 0.1 mM inosine, 100 mM Tris-hydrochloride buffer, 1.0 mM potassium phosphate, and 2 μM zinc chloride or 0.1 mM ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) at pH 7.4. The apparent inhibition constant (K_i') of a compound was determined from its ability to inhibit the phosphorolysis of inosine at 1 mM phosphate. Rates of inosine phosphorolysis in the presence (v_i) and absence (v_o) of a single concentration of inhibitor were measured using the spectrophotometric assay. The concentration of inhibitor (I) used was sufficient to inhibit the reaction rate about 50% at a substrate concentration (S) of 0.1 mM inosine (K_m' = 40 μM[25]). The K_i' value was calculated from the fractional inhibition ($i = 1 - (v_i/v_o)$) using the equation $K_i' = I[(1/i) - 1]/[1 + (S/K_m')]$, which was derived from the rate equation for competitive inhibition.[40]

Competition with Inosine and Phosphate. Rates of inosine phosphorolysis in the presence and absence of a single concentration of **2b** were measured using inosine or phosphate as the variable substrate. Rates were measured in the absence (plus 0.1 mM Na_2EDTA) and presence of 2 μM $ZnCl_2$. When inosine was varied, a minimum of seven concentrations, ranging from 5 to 100 μM, were used and the concentration of phosphate was maintained at 1 mM. When phosphate was varied, a minimum of five concentrations, ranging from 0.1 to 4 mM, were used and the concentration of inosine was maintained at 0.1 mM. Plots of reciprocal rate verses reciprocal substrate concentration were constructed, and the type of inhibition was determined from the pattern of intersecting lines. K_i' values were determined from a weighted, least-squares fit[37] of the rate at each substrate concentration to the rate equation for competitive inhibition, and the fitted data were analyzed[41] for conformity to the competitive model.

Effects of Metal Cations. K_i' values for **2c** in the presence and absence of 50 μM metal chloride were determined from the fractional inhibition of inosine phosphorolysis at 1 mM phosphate as described above. The ratio of the K_i' in the absence of metal (plus 0.1 mM Na_2EDTA) to the K_i' in the presence of metal was calculated for various di- and trivalent metals. This ratio, which is equal to the increase in potency of **2b** due to 50 μM metal cation, is listed for each metal tested as follows: Zn^{2+} (>370), Cu^{2+} (30), Co^{2+} (16), Cd^{2+} (6.2), Mn^{2+} (2.5), Ni^{2+} (1.2), Ca^{2+} (1.1), Ba^{2+} (1.0), Fe^{2+} (0.98), Mg^{2+} (0.95), Al^{3+} (0.98), Fe^{3+} (0.80), Cr^{3+} (0.79), and La^{3+} (0.64).

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