Synthesis and Biological Evaluation of N⁴-Substituted Imidazo- and v-Triazolo[4,5-d]pyridazine Nucleosides†

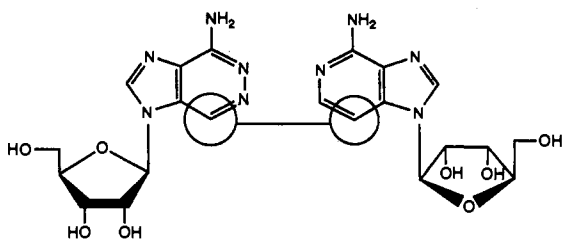
Jacqueline C. Bussolari,‡ Kakarla Ramesh,‡ Johanna D. Stoeckler,§ Shih-Fong Chen,⊥ and Raymond P. Panzica*,‡

Departments of Medicinal Chemistry and Chemistry, University of Rhode Island, Kingston, Rhode Island 02881, Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912, and The Cancer Group, DuPont Merck Pharmaceutical Company, Glenolden, Pennsylvania 19036

Received March 22, 1993•

The chemical synthesis of certain N⁴-substituted imidazo[4,5-*d*]pyridazine and *v*-triazolo[4,5-*d*]-pyridazine nucleosides is described. In both series, the 4-chloro analogues, *i.e.*, 4-chloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazine (**5a**) and 4-chloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-*v*-triazolo[4,5-*d*]pyridazine (**5b**), were used as synthons to the target nucleosides. Nucleoside **5b** was far more reactive toward nucleophilic displacements than **5a**. Attempted deprotection of **5b** was always accompanied with displacement of the 4-chloro substituent, whereas **5a** was conveniently deacetylated without loss of the chloro group. Biological evaluation of the title nucleosides included antitumor studies and substrate/inhibition studies with certain purine-metabolizing enzymes. The corresponding adenosine analogues, *i.e.*, 2-aza-3-deazaadenosine (**6a**) and 2,8-diaza-3-deazaadenosine (**6b**), were very slowly reacting substrates and weak inhibitors of bovine adenosine deaminase, whereas the inosine analogues were highly resistant to human purine nucleoside phosphorylase. The 4-benzylamino derivatives were weak inhibitors of adenosine transport into human erythrocytes. The inosine, adenosine, and selected N⁴-substituted analogues exhibited no *in vitro* toxicity toward murine L1210 leukemia and B16 melanoma cells.

Adenosine nucleosides,[1] their aza and deaza counterparts,[2] and their respective exocyclic N-substituted derivatives[3] continue to attract attention as potential therapeutic agents. Their application in the treatment of disease states covers a wide range and includes cancer, viral infections,[4] parasitic diseases,[5] and cardiovascular disorders.[6] This broad spectrum of activity undoubtedly reflects a large number of pharmacological sites of action, *e.g.*, adenosine receptors and enzymes involved in purine metabolism. An inherent drawback associated with the adenine nucleosides is that many are substrates for and inactivated by adenosine deaminase (ADA). A strategy for preventing deamination by ADA is to remove one of the purine-ring nitrogens, *e.g.*, 1-deaza-, 3-deaza-, and 7-deazaadenosine (tubercidin) all lack substrate activity.[7]



2-aza-3-deazaadenosine 3-deazaadenosine

Such chemical modification of the aglycone to avert deamination and possibly enhance activity prompted us to prepare "adenosine-type" analogues possessing the imidazo[4,5-*d*]pyridazine ring system. In this system, the N3 and C2 of purine are interchanged to produce nucleosides which resemble 3-deazaadenosine. On the basis of their X-ray crystallographic structure of murine ADA complexed with 6-hydroxy-1,6-dihydropurine riboside (HDPR), Quiocho and co-workers[8] postulated a stereospecific S_N2 catalytic mechanism. The ADA–HDPR complex showed that a hydrogen bond between N3 of the purine and Gly[184] of the enzyme is a key factor in holding the substrate in position at the active site and enhancing catalysis during the hydrolytic deamination. Failure to form a Gly[184]–N3 hydrogen bond is probably the reason why 3-deazaadenosine is resistant to ADA. The amino-type ribonucleosides of the imidazo[4,5-*d*]pyridazine ring system are patterned after 3-deazaadenosine but are isomeric with purine.

Due to problems encountered in their syntheses, the imidazo[4,5-*b*]pyridazine nucleosides have received limited attention.[9-11] Recently, we have developed an efficient, three-step synthesis of 1-(β-D-ribofuranosyl)imidazo-[4,5-*d*]pyridazin-4(5*H*)-one (**3a**),[12] and this intermediate, on further modification, now provides easy access to a variety of 4-substituted β-N1 nucleosides in this series. For the current study, 4-chloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazine[13] (**5a**) and its deblocked counterpart, **13**, were selected as synthons for the targeted N⁴-substituted imidazo[4,5-*d*]pyridazine nucleosides. Earlier, Otter and co-workers[11] elected to use 4-(methylthio)-1-(β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazine as their precursor but found that the 4-methylthio substituent was somewhat resistant to nucleophilic displacement. In the formation of the adenosine analogue **6a**, drastic conditions had to be employed, conditions which lead to glycosyl bond cleavage. Thus, in an effort to circumvent these problems, we focused our attention on **5a** and **13**.

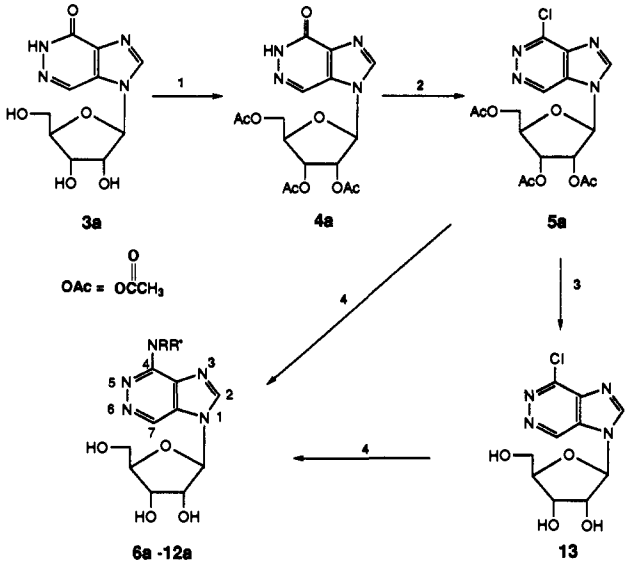
Results and Discussion

Chemistry. Nucleoside **5a** was prepared in sufficient quantity and in consistent yield (*ca.* 80%) from 1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazin-4(5*H*)-one (**4a**). The chlorination procedure involved

‡ University of Rhode Island.
§ Brown University.
⊥ DuPont Merck Pharmaceutical Co.
† This manuscript is dedicated to Prof. Leroy B. Townsend on the occasion of his 60th birthday.
• Abstract published in *Advance ACS Abstracts*, November 15, 1993.

0022-2623/93/1836-4113$04.00/0

Scheme I[a]

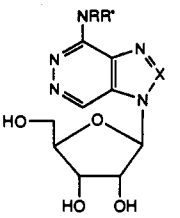


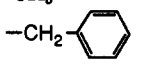
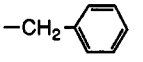
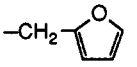
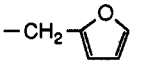
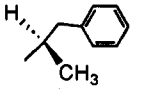
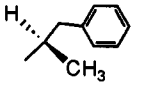
[a] Reagents: (1) Ac_2O, pyridine; (2) $POCl_3$, *N,N*-diethylaniline, *n*-Bu_4NCl, CH_3CN; (3) NH_3, MeOH; (4) HNRR′, absolute EtOH.

adding **4a** to a mixture of phosphorus oxychloride ($POCl_3$/**4a**, 6:1), *N,N*-diethylaniline, and tetrabutylammonium chloride (*n*-Bu_4NCl) in anhydrous acetonitrile.[14] The reaction mixture was heated to 90 °C for 1 h and worked up, and the resulting residue was purified by flash chromatography to provide **5a** as a foam (Scheme I). The syntheses of chlorinated imidazo[4,5-*d*]pyridazines have been problematic. Castle and Seese[15] were able to prepare 4,7-dichloroimidazo[4,5-*d*]pyridazine, but in very low yield (17%), and often, this heterocycle was contaminated with 4(7)-chloroimidazo[4,5-*d*]pyridazin-7(4)-one. Efforts in our laboratory to prepare the monochloro analogue, 4-chloroimidazo[4,5-*d*]pyridazine, by ring closure of 3-chloro-4,5-diaminopyridazine[16] met with failure. We used a variety of known, mild annelation procedures, but in the majority of these reactions, the only product isolated was imidazo[4,5-*d*]pyridazin-4-one, which led us to believe that the desired heterocycle was extremely reactive and the chloro group easily displaced. An attempt to prepare **5a**[11] using Vilsmeier reagent, a very mild chlorinating agent used in the preparation of other chloro nucleosides,[17] was not successful. Thus, the preparation of **5a** with the aforementioned $POCl_3$ procedure was unexpected.

Unlike 4-chloro-1-(2,3,5-tri-*O*-acetyl-D-ribofuranosyl)-pyrazolo[3,4-*d*]pyrimidine, which is a very reactive and unstable chloro nucleoside,[17] 4-chloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazine (**5a**) is quite stable and does not require any special conditions for storage. In fact, **5a** can be deblocked with methanolic ammonia at room temperature without causing displacement of the 4-chloro group. Like the aforementioned 4-methylthio analogue,[11] elevated temperatures were required to synthesize **6a–12a** (Table I). Both **5a** and **13** were used in their preparation. The latter nucleoside was employed with the more expensive amines such as D-amphetamine. The 4-benzylamino- (**9a**), 4-furfurylamino- (**10a**), 4-cyclohexylamino- (**11a**), and 4-((*R*)-1-phenyl-2-propyl)amino- (**12a**) derivatives were of particular interest to us, since their purine counterparts had displayed activity as either antitumor agents[18] or A_1 adenosine agonists.[6] They were all obtained in good yield by heating **5a** (or **13**) with an excess of the desired amine in ethanol.

Table I. Substitution Modification on the N4 Amido Group of Imidazo- and *v*-Triazolo[4,5-*d*]pyridazine Nucleosides

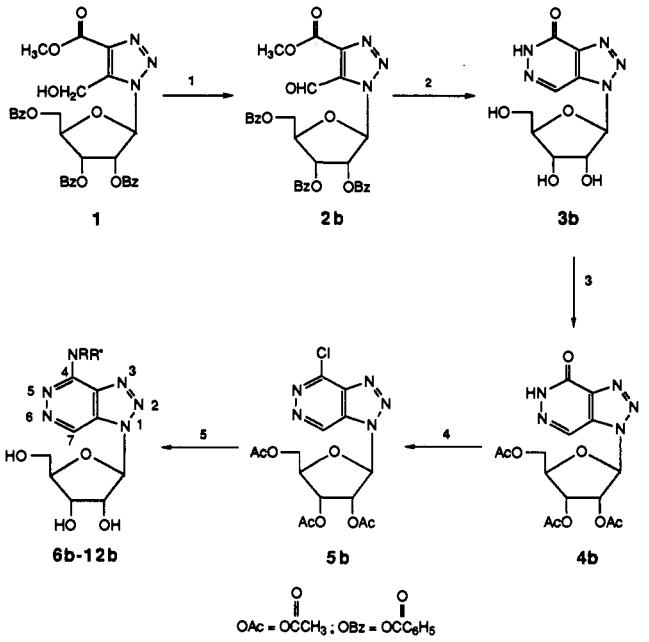


compd	X	R	R′
6a	CH	H	H
6b	N	H	H
7a	CH	CH_3	H
7b	N	CH_3	H
8a	CH	CH_3	CH_3
8b	N	CH_3	CH_3
9a	CH	H	
9b	N	H	
10a	CH	H	
10b	N	H	
11a	CH	H	
11b	N	H	
12a	CH	H	
12b	N	H	

Having accomplished the synthesis of the targeted N^4-substituted nucleosides possessing the imidazo[4,5-*d*]-pyridazine aglycone, we turned our attention to the preparation of their corresponding 3-aza analogues, *i.e.*, the *v*-triazolo[4,5-*d*]pyridazine nucleosides. Replacement of the methine (CH) in the 8 position of purine is a popular modification and has led to a number of biologically active nucleosides. For example, 8-azaadenosine, which is deaminated faster than adenosine by ADA, shows excellent activity against P388 leukemia cells, *in vitro*, when coadministered with 2′-deoxycoformycin, a potent inhibitor of this enzyme.[19] 8-Azainosine, the expected deamination product of 8-azaadenosine, was shown to be active against lymphoid leukemia L1210 and adenocarcinoma 755, *in vivo*.[20]

This work represents the first disclosure of nucleosides of the *v*-triazolo[4,5-*d*]pyridazine ring system. Our synthetic strategy for the preparation of the 3-aza nucleosides followed that developed for **3a**, *i.e.*, beginning with ring closure of a suitably substituted azole nucleoside.[12] The starting material for this facet of our work was methyl 5-(hydroxymethyl)-1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-*v*-triazole-4-carboxylate (**1**). This triazole nucleoside had been previously synthesized by Earl and Townsend.[21] We modified the ensuing oxidation step (to **2b**) by replacing MnO_2 with pyridinium chlorochromate which afforded the 5-formyl derivative **2b** in quantitative yield (Scheme II). Subsequent ring closure of **2b** with anhydrous hydrazine in ethanol provided 1-(β-D-ribofuranosyl)-*v*-tri-

Scheme II[a]



[a] Reagents: (1) PCC, CH_2Cl_2; (2) NH_2NH_2, absolute EtOH; (3) Ac_2O, pyridine; (4) $POCl_3$, *N,N*-diethylaniline, *n*-Bu_4NCl, CH_3CN; (5) HNRR′, Absolute EtOH.

azolo[4,5-*d*]pyridazin-4(5*H*)-one (2,8-diaza-3-deazainosine, **3b**) in 90% yield. Acetylation of **3b** followed by chlorination furnished the synthon 4-chloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-v-triazolo[4,5-*d*]pyridazine (**5b**) in an overall 67% yield from 1. Unlike **5a**, **5b** required cold storage (freezer) under anhydrous conditions. The 4-chloro substituent of **5b** was far more reactive than that of **5a** and could be displaced with ease at room temperature. With the exception of temperature, the reaction conditions established for **6a–12a** were used to prepare nucleosides **6b–12b** (Table I). All were obtained in good yield. A higher temperature was employed with D-amphetamine to ensure complete deacetylation of **5b**. Heating **5b** with this amine in ethanol gave **12b** in 75% yield.

Biology. Previously, only two imidazo[4,5-*d*]pyridazine nucleosides, *i.e.*, **6a** and its 6-ribosyl isomer, were evaluated for biological activity. 2-Aza-3-deazaadenosine (**6a**) was shown to be inactive toward H. Ep. No. 2 cells; whereas, its 6-β-ribosyl isomer was cytotoxic.[22] The adenosine analogue **6a** was found to be a good substrate, but a poor inhibitor, of *S*-adenosylhomocysteine hydrolase from beef liver.[23] This analogue was also reported to resist deamination by ADA, but no experimental details were provided.[23] We now have examined a number of the title nucleosides for their potential use as biological probes and chemotherapeutic agents.

Adenosine Deaminase. 2-Aza-3-deazaadenosine (**6a**) proved to be a very poor substrate for calf intestinal mucosa ADA. Incubation of a 1 mM solution with 10 units/mL ADA for 18 h at 37 °C caused only 14% conversion to the inosine analogue, **3a**. The earlier finding of resistance to deamination[23] may have resulted from testing with a lower enzyme concentration. Fivefold higher, but still low, substrate activity was observed with **6b**, which showed 70% conversion to the deamination product **3b** after 18 h. Both **6a** and **6b** were weak inhibitors of ADA at 100 μM concentration, causing only 15% and 7% inhibition, respectively, when adenosine was used near its K_m concentration (25 μM). Thus, the interchange of C7 and N6, *i.e.*, C2 and N3 in purine numbering, in **6a** and **6b** leads to a reduction in activity comparable to that seen with 3-deazaadenosine.[7] This severe impairment of substrate activity confirms that N3 of purine is an essential hydrogen-bond acceptor for binding and catalysis with ADA, as suggested by the X-ray crystal structure.[8] The moderately higher substrate activity and lower affinity of **6b** *vs* **6a** parallel the (2–4.6)-fold higher relative V_{max} and (3–5)-fold higher K_m values reported for 8-azaadenosine *vs* adenosine with both calf intestinal and human erythrocytic ADA.[24] Similarly, 2-azacoformycin was found to be a 2-fold or more less potent ADA inhibitor than coformycin.[25] However, due to the increased ability of 8-azanebularine to hydrate across the 1,6-bond, it proved to be 400-fold more potent than nebularine.[25] It is worth noting that **6b** showed moderate activity (apparent K_I = 1.41 ± 0.17 mM) as an inhibitor of human plasma ADA, designated as ADA_2.[26a,b] In comparison, the apparent K_I for adenosine was 2.18 ± 0.51 mM.[26b]

Purine Nucleoside Phosphorylase (PNP). The inosine analogues, **3a** and **3b**, were found to have low or undetectable substrate and inhibitory activities with human erythrocytic PNP. Neither analogue was cleaved when incubated at 200 μM concentration with 0.02 unit/mL PNP for 1 h at room temperature. However, an 18-h incubation at 37 °C of 625 μM **3a** with 0.5 unit/mL PNP and an 80-fold excess of phosphate caused 65% conversion to imidazo[4,5-*d*]pyridazin-4(5*H*)-one. An earlier study[27] had shown that the aglycone of **3a** is also a slowly reacting substrate of PNP. Inhibition studies revealed that **3a** and **3b** have very low affinities for PNP, since 1 mM **3a** or 300 μM **3b** did not inhibit phosphorolysis when the substrate was present at approximately the K_m concentration. Higher concentrations of **3b** interfered with the spectrophotometric assay. The aglycone of **3a** has higher affinity for PNP, since an equimolar concentration (50 μM) caused 12% inhibition of guanosine phosphorolysis.[27]

The poor substrate activities of these analogues cannot be attributed to the loss of hydrogen bonding at the C7 (purine N3) position. X-ray crystallographic analysis of human erythrocytic PNP–ligand complexes revealed hydrogen-bonding interactions with N1 and N7 of purine but no available hydrogen-bond donor for N3.[28] On the basis of recent studies of site-directed mutants,[29] it has been postulated that a crucial hydrogen bond from N1 orients the base in the active site and another hydrogen bond to N7 assists phosphorolysis. Therefore, the low or absent substrate activities of **3a** and **3b** may reflect the influence of the adjacent nitrogens on the character of the hydrogen bonds at N5 and N3, *i.e.*, N1 and N7 in purine numbering.

Nucleoside Transport. Adenosine enters human erythrocytes by facilitated diffusion *via* a single, nonspecific carrier system that is very sensitive to inhibition by certain 6-substituted nucleosides.[30] Under the present assay conditions, ((nitrobenzyl)thio)inosine and ((nitrobenzyl)thio)formycin cause 99% and 90% inhibition, respectively, at 0.1 μM concentration.[31] In comparison, analogues **9a** and **9b** caused only 45% and 34% inhibition, respectively, at the highest concentration tested, 10 μM. The low activity of these analogues is attributable primarily to the 1000-fold lower affinity of the benzylamino substituent *vs* the 4-(nitrobenzyl)thio group.[30] On the basis of results with the 4-(nitrobenzyl)thio counterpart of **9a**,

the 2-aza-3-deazapurine ring system also reduces affinity, but to a lesser degree.[32]

Adenosine Receptors. Analogues **6a** and **6b** were evaluated as agonists and antagonists for adenosine A_2 receptors, which stimulate cyclic AMP production in human promyelocytic leukemia HL-60 cells. When cells were exposed for 15 min to either 2 or 250 μM concentrations in the presence of a phosphodiesterase inhibitor, cyclic AMP concentrations were not raised above control levels. When the analogues were preincubated with the cells for 5 min, a 2 μM concentration (final) did not inhibit subsequent stimulation by 2 μM adenosine (the EC_{50} concentration). An early study that used the cyclic AMP-dependent inhibition of blood platelet aggregation to monitor A_2 receptor stimulation[33] found 2-azaadenosine to be 40–100% as effective as adenosine. Subsequently, 2-azaadenosine was shown to be as efficacious as adenosine in stimulating A_2 receptors in VA13 human fibroblasts[34] and to have a 2-fold lower EC_{50}. With these cells, 3-deazaadenosine was completely inactive, while 8-azaadenosine was a very weak agonist. Thus, the lack of activity of **6a** and **6b** with A_2 receptors in HL-60 cells confirms that N3 is required for agonist and antagonist binding.

Antitumor Activity. Selected nucleosides in both series (**3a,b** and **6a,b–10a,b**) were evaluated *in vitro* for growth inhibitory activity against murine L1210 leukemia and B16 melanoma cells. With the exception of **9b**, all of the nucleosides evaluated had an $ID_{50} > 50$ μg/mL in both cell lines. Nucleoside **9b** showed no apparent activity against L1210 but exhibited weak growth inhibitory activity against the B16 melanoma cell line with an ID_{50} value of 35.8 μg/mL. Anticancer agents such as methotrexate, arabinosylcytosine (ara-C), and 5-fluorouracil (5-FU) are routinely tested under the same conditions, and they exhibit ID_{50} values <0.1 μg/mL against both lines.

Summary

2-Aza-3-deazainosine (**3a**) and 2,8-diaza-3-deazainosine (**3b**) are highly resistant to catabolism by PNP, while their adenosine counterparts, **6a** and **6b**, are highly resistant to ADA. Neither **6a** nor **6b** interact with A_2-type adenosine receptors on HL-60 cells. Such characteristics would be advantageous if future studies identify chemotherapeutic targets for this class of analogues.

Experimental Section

Melting points were determined on a Buchi 535 melting-point apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on either a Varian EM 390 or Bruker AM-300 spectrometer, as indicated, using Me_4Si (TMS) as an internal standard. Optical rotations were measured on a Perkin-Elmer Model 141 automatic digital readout polarimeter. UV absorption spectra were recorded with a Beckman DU-64 spectrophotometer. All moisture-sensitive reactions were performed using flame-dried glassware. Methylene chloride (CH_2Cl_2) and acetonitrile were dried over CaH_2 and distilled. Anhydrous THF was obtained by distillation over sodium benzophenone ketyl. Evaporations were performed under diminished pressure using a Buchi rotary evaporator unless stated otherwise. Davison silica gel (grade H, 60–200 mesh), purchased from Fisher Scientific, was used for flash column chromatography. Thin-layer chromatography was performed on precoated silica gel plates (60-F254, 0.2 mm) manufactured by E. M. Science, Inc., and short-wave ultraviolet light (254 nm) was used to detect the UV-absorbing compounds. All solvent proportions are by volume unless stated otherwise. Elemental analyses were performed by MHW Laboratories, Phoenix, AZ.

Methyl 5-Formyl-1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-*v*-triazole-4-carboxylate (2b). Alcohol **1** (11.45 g, 19.0 mmol) and pyridinium chlorochromate (PCC, 20.53 g, 95 mmol) were heated at reflux in dry CH_2Cl_2 (350 mL) for 2 h. The mixture was cooled to room temperature and poured onto a silica gel pad (15 × 8 cm), and the pad was washed thoroughly with CH_2Cl_2/ethyl acetate (3:1). Evaporation of the filtrate afforded **2b** (11.43 g, 99% yield) as a white foam whose physical data were identical to those previously reported in the literature:[21] ^1H NMR (300 MHz, $CDCl_3$) δ 4.04 (s, 3H, CH_3), 4.57,4.76 (ABX, 2H, $J_{5'a,5'b}$ = 12.3, $J_{5'a,4'}$ = 4.92, $J_{5'b,4'}$ = 3.69 Hz, H-5'a, H-5'b), 4.94 (dddd, 1H, H-4'), 6.25 (dd, 1H, $J_{3',4'}$ = 7.11, $J_{3',2'}$ = 5.5 Hz, H-3'), 6.46 (dd, 1H, $J_{2',1'}$ = 2.1, $J_{2',3'}$ = 5.4 Hz, H-2'), 7.09 (d, 1H, J = 2.07 Hz, H-1'), 7.35–8.02 (m, 15H, C_6H_5), 10.54 (s, 1H, CHO).

1-(β-D-Ribofuranosyl)-*v*-triazolo[4,5-*d*]pyridazin-4-one (3b). To a solution of **2b** (11.43 g, 19.03 mmol) in absolute ethanol (200 mL) was added anhydrous hydrazine (6.10 g, 190 mmol). The reaction mixture was stirred at reflux overnight, cooled to room temperature, and concentrated. The resulting residue was dissolved in H_2O (50 mL) and washed with CH_2Cl_2 (5 × 250 mL). The aqueous solution was then cooled to 0 °C in an ice bath and acidified to pH 4 using Amberlite IR-120 acid resin. Filtration and subsequent lyophilization of the solution afforded **3b** (4.58 g, 90% yield) as a white solid: mp 93–94 °C; $[\alpha]^{24}_D$ –60.8° (*c* = 1.71, EtOH); ^1H NMR (DMSO-d_6) δ 3.43–3.65 (dq, 2H, H-5', H-5''), 4.01–4.08 (m, 1H, H-4'), 4.15–4.23 (dd, 1H, H-3'), 4.52–4.60 (dd, 1H, H-2'), 5.00 (b s, 1H, D_2O exchangeable, OH), 5.33 (b s, 1H, D_2O exchangeable, OH), 5.68 (b s, 1H, D_2O exchangeable, OH), 6.26 (d, 1H, $J_{1',2'}$ = 6.0 Hz, H-1'), 8.83 (s, 1H, H-7), 12.84 (s, 1H, D_2O exchangeable, NH); ^{13}C NMR (DMSO-d_6) δ 61.2 (C5'), 70.4 (C3'), 74.8 (C2'), 86.8 (C4'), 92.6 (C1'), 126.7 (C7), 133.3 (C7a), 139.9 (C3a), 156.6 (C4). Anal. ($C_6H_{11}N_5O_5$) C, H, N.

1-(2,3,5-Tri-*O*-acetyl-β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazin-4-one (4a). A solution of the methanolate, **3a**[12] (5.00 g, 17.59 mmol), acetic anhydride (10 mL), and anhydrous pyridine (10 mL) was stirred at room temperature for 2 h. The solution was poured over cracked ice/water and extracted with CH_2Cl_2 (2 × 100 mL). The organic layers were combined, washed with water (2 × 100 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated. The resulting residue was purified by flash column chromatography using CH_2Cl_2/ethyl acetate (2:1) as the eluant to give **4a** (6.94 g, quantitatively) as a white foam: mp 100 °C dec; ^1H NMR (300 MHz, $CDCl_3$) δ 2.12 (s, 3H, CH_3), 2.15 (s, 3H, CH_3), 2.16 (s, 3H, CH_3), 4.39–4.52 (dq, 2H, $J_{5',5''}$ = 11.0, $J_{4',5'}$ = 2.8 Hz, H-5', H-5''), 4.55–4.60 (m, 1H, H-4'), 5.42 (dd, 1H, $J_{2',3'}$ = 5.4, $J_{3',4'}$ = 5.4 Hz, H-3'), 5.48 (dd, 1H, $J_{2',1'}$ = 5.4, $J_{2',3'}$ = 5.4 Hz, H-2'), 6.16 (d, 1H, $J_{1',2'}$ = 5.4 Hz, H-1'), 8.27 (s, 1H, H-7), 8.46 (s, 1H, H-2), 12.11 (s, 1H, D_2O exchangeable, NH); ^{13}C NMR ($CDCl_3$) δ 20.4 (CH_3), 20.5 (CH_3), 20.8 (CH_3), 62.7 (C5'), 69.9 (C3'), 74.3 (C2'), 81.1 (C4'), 88.0 (C1'), 127.0 (C7), 131.9 (C7a), 137.8 (C3a), 141.3 (C2), 159.1 (C4), 169.5 (C=O), 169.7 (C=O), 170.3 (C=O). Anal. ($C_{16}H_{16}N_4O_8$) C, H, N.

1-(2,3,5-Tri-*O*-acetyl-β-D-ribofuranosyl)-*v*-triazolo[4,5-*d*]pyridazin-4-one (4b). A solution containing compound **3b** (3.87 g, 14.4 mmol), dry pyridine (10 mL), and acetic anhydride (5 mL) was stirred under N_2 at room temperature overnight. Workup of the reaction mixture as described for the preparation of **4a** followed by flash column chromatography using CH_2Cl_2/ethyl acetate (4:1) as the eluant provided **4b** (5.52 g, 96% yield) as a white foam: mp 92–94 °C; ^1H NMR ($CDCl_3$) δ 1.99 (s, 3H, CH_3), 2.12 (s, 3H, CH_3), 2.15 (s, 3H, CH_3), 4.23–4.40 (dq, 2H, H-5', H-5''), 4.55–4.60 (m, 1H, H-4'), 5.63 (dd, 1H, H-3'), 6.03 (dd, 1H, H-2'), 6.37 (d, 1H, $J_{1',2'}$ = 3.8 Hz, H-1'), 8.40 (s, 1H, H-7), 11.38 (s, 1H, D_2O exchangeable, NH). Anal. ($C_{15}H_{17}N_5O_8 \cdot 0.5\ H_2O$) C, H, N.

4-Chloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazine (5a). A solution containing **4a** (1.19 g, 3.03 mmol), *N,N*-diethylaniline (0.50 mL, 3.03 mmol), tetrabutylammonium chloride (*n*-Bu_4NCl, 1.68 g, 6.05 mmol), freshly distilled phosphorus oxychloride ($POCl_3$, 1.7 mL, 18.15 mmol), and anhydrous acetonitrile (15 mL) was stirred at 90 °C for 1 h. The reaction mixture was cooled and poured over cracked ice/water (*ca.* 50 mL), and the aqueous solution was extracted with CH_2Cl_2 (3 × 50 mL). The organic layers were combined, washed successively with water (2 × 50 mL) and saturated aqueous

$NaHCO_3$ (50 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated. Purification of the residue by flash chromatography using CH_2Cl_2/ethyl acetate (4:3) as the eluant afforded **5a** (1.08 g, 86% yield) as a white foam: mp 45–46 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 2.14 (s, 3H, CH_3), 2.18 (s, 3H, CH_3), 2.20 (s, 3H, CH_3), 4.40–4.60 (m, 3H, H-4′, H-5′), 5.39 (dd, 1H, $J_{3',2'}$ = 6 Hz, H-3′), 5.47 (dd, 1H, $J_{2',1'}$ = 3, $J_{2',3'}$ = 6 Hz, H-2′), 6.20 (d, 1H, $J_{1',2'}$ = 3.0 Hz, H-1′), 8.46 (s, 1H, H-2), 9.64 (s, 1H, H-7); ^{13}C NMR ($CDCl_3$) δ 20.3 (CH_3), 20.5 (CH_3), 20.8 (CH_3), 62.4 (C5′), 69.7 (C3′), 74.3 (C2′), 81.3 (C4′), 88.3 (C1′), 131.8, 137.7, 140.4, 144.2, 149.5, 169.4 (C=O), 169.5 (C=O), 170.0 (C=O). Anal. ($C_{16}H_{17}N_4O_7Cl$) C, N, Cl; H: calcd, 4.15; found, 4.60.

4-Chloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-v-triazolo-[4,5-*d*]pyridazine (5b). To a solution of **4b** (0.88 g, 2.22 mmol) in anhydrous acetonitrile (10 mL) were added *N,N*-diethylaniline (0.35 mL, 2.22 mmol), *n*-Bu_4NCl (1.23 g, 4.44 mmol), and freshly distilled $POCl_3$ (1.23 mL, 13.32 mmol). This mixture was stirred at 70 °C for 12 h under N_2. Workup of the reaction mixture as described for the preparation of **5a** followed by flash column chromatography using CH_2Cl_2/ethyl acetate (9:1) as the eluant gave **5b** (0.72 g, 78% yield) as a yellow solid: mp 45–46 °C (Note: compound **5b** decomposes upon standing at room temperature.); ^1H NMR (300 MHz, $CDCl_3$) δ 2.00 (s, 3H, CH_3), 2.14 (s, 3H, CH_3), 2.17 (s, 3H, CH_3), 4.28–4.41 (dq, 2H, $J_{5',5''}$ = 12, $J_{5',4'}$ = 3 Hz, H-5′, H-5″), 4.62–4.66 (m, 1H, H-4′), 5.63 (dd, 1 H, $J_{3',2'}$ = 6, $J_{3',4'}$ = 6 Hz, H-3′), 6.10 (dd, 1H, $J_{2',1'}$ = 6, $J_{2',3'}$ = 6 Hz, H-2′), 6.57 (d, 1H, $J_{1',2'}$ = 6 Hz, H-1′), 9.75 (s, 1H, H-7). Anal. ($C_{15}H_{16}N_5O_7Cl$) C, H, N.

4-Amino-1-(β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazine (6a). Method A: A solution of **5a** (0.50 g, 1.21 mmol) in absolute ethanol (5 mL) and liquid ammonia (20 mL) was heated at 150 °C for 6 h in a glass-lined steel reaction vessel. The reaction mixture was cooled to room temperature and the ammonia gas vented. The crude product was purified by flash column chromatography using CH_2Cl_2/MeOH (9:1) as the eluant. Recrystallization from 95% ethanol provided **6a** (0.10 g, 31% yield) as a white solid: mp 225 °C (lit.[11] mp 225–226 °C); $[\alpha]^{25}_D$ −49.1° (*c* = 0.61, MeOH); ^1H NMR (DMSO-d_6) δ 3.33–3.70 (m, 2H, H-5′, H-5″), 4.00–4.35 (m, 3H, H-4′, H-3′, H-2′), 4.80 (b s, 3H, D_2O exchangeable, 3 O*H*), 5.90 (d, 1H, *J* = 6.0 Hz, H-1′), 6.50 (b s, 2H, D_2O exchangeable, NH_2), 8.55 (s, 1H, H-2), 9.10 (s, 1H, H-7) [lit.[11] ^1H NMR (DMSO-d_6) δ 3.70 (m, 2H, H-5′, H-5″), 4.08 (m, 2H, H-3′, H-4′), 4.31 (m, 1H, H-2′), 5.90 (d, 1H, *J* = 6.4 Hz, H-1′), 6.59 (b s, 2H, NH_2), 8.55 (s, 1H, H-2), 9.11 (s, 1H, H-7)]. Anal. ($C_{10}H_{13}N_5O_4$) C, H, N.

Method B: A solution of **13** (0.35 g, 1.22 mmol) in absolute ethanol (8 mL) and liquid ammonia (10 mL) was stirred at 150 °C for 6 h as described in method A. This procedure afforded **6a** (0.14 g, 44% yield) which was identical in all respects to **6a** synthesized from method A.[35]

4-Amino-1-(β-D-ribofuranosyl)-v-triazolo[4,5-*d*]pyridazine (6b). A solution of **5b** (0.50 g, 1.20 mmol) in absolute ethanol (8 mL) and liquid ammonia (10 mL) was stirred at room temperature for 5 h as described for the preparation of **6a**. The usual workup followed by flash column chromatography using ethyl acetate/MeOH (4:1) as the eluant and recrystallization of the product from 95% ethanol afforded **6b** (0.25 g, 80% yield) as a yellow solid: mp 182–183 °C; $[\alpha]^{25}_D$ −39.5° (*c* = 1.71, MeOH); ^1H NMR (DMSO-d_6) δ 3.39–3.76 (m, 2H, H-5′, H-5″), 3.89–4.26 (m, 2H, H-4′, H-3′), 4.62 (dd, 1H, H-2′), 6.26 (d, 1H, $J_{1',2'}$ = 6.0 Hz, H-1′), 7.66 (b s, 2H, D_2O exchangeable, NH_2), 9.27 (s, 1H, H-7). Anal. ($C_6H_{12}N_6O_4$) C, H, N.

4-(Methylamino)-1-(β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazine (7a). A solution containing **5a** (0.50 g, 1.21 mmol) in absolute ethanol (5 mL) and 40% aqueous methylamine (10 mL) was heated to 110 °C overnight in a glass-lined steel reaction vessel. The reaction mixture was cooled and concentrated under vacuum. The resulting residue was purified by flash column chromatography using CH_2Cl_2/MeOH (9:1) as the eluant to provide **7a** (0.20 g, 76% yield) as a white solid: mp 215–216 °C; $[\alpha]^{25}_D$ −53.5° (*c* = 0.96, MeOH); ^1H NMR (DMSO-d_6) δ 3.06 (s, 3H, CH_3), 3.53–3.73 (m, 2H, H-5′, H-5″), 3.93–4.37 (m, 3H, H-4′, H-3′, H-2′), 5.47 (b s, 3H, D_2O exchangeable, 3 O*H*), 5.96 (d, 1H, $J_{1',2'}$ = 6.0 Hz, H-1′), 7.97 (b s, 1H, D_2O exchangeable, N*H*), 8.65 (s, 1H, H-2), 9.12 (s, 1H, H-7). Anal. ($C_{11}H_{15}N_5O_4$) C, H, N.

4-(Methylamino)-1-(β-D-ribofuranosyl)-v-triazolo[4,5-*d*]pyridazine (7b). A solution containing **5b** (0.42 g, 1.01 mmol), absolute ethanol (5 mL), and 40% aqueous methylamine (5 mL) was stirred at room temperature overnight. The reaction mixture was concentrated *in vacuo*, and the residue was purified by flash column chromatography using ethyl acetate/MeOH (9:1) as the eluant. The product was crystallized from absolute ethanol to afford **7b** (0.21 g, 71% yield) as a white solid: mp 163–164 °C; $[\alpha]^{25}_D$ −82.7° (*c* = 0.63, EtOH); ^1H NMR (300 MHz, DMSO-d_6) δ 3.07 (d, 3H, *J* = 4.5 Hz, CH_3), 3.48–3.66 (m, 2H, H-5′, H-5″), 4.06 (m, 1H, H-4′), 4.22 (dd, 1H, $J_{3',4'}$ = 9.09, $J_{3',2'}$ = 4.74 Hz, H-3′), 4.62 (dd, 1H, $J_{2',3'}$ = 10.8, $J_{2',1'}$ = 5.4 Hz, H-2′), 5.08 (t, 1H, *J* = 5.1 Hz, D_2O exchangeable, O*H*), 5.37 (d, 1H, *J* = 5.17 Hz, D_2O exchangeable, O*H*), 5.67 (d, 1H, *J* = 6.03 Hz, D_2O exchangeable, O*H*), 6.30 (d, 1H, $J_{1',2'}$ = 5.35 Hz, H-1′), 8.01 (q, 1H, *J* = 4.5 Hz, D_2O exchangeable, N*H*), 9.34 (s, 1H, H-7). Anal. ($C_{10}H_{14}N_6O_4$) C, H, N.

4-(Dimethylamino)-1-(β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazine (8a). A solution containing **5a** (0.50 g, 1.21 mmol) in absolute ethanol (5 mL) and 40% aqueous dimethylamine (10 mL) was heated to 110 °C overnight as described for the preparation of **7a**. The usual workup followed by flash column chromatography using CH_2Cl_2/MeOH (9:1) as the eluant gave **8a** (0.19 g, 71% yield) as a white solid: mp 180–183 °C; $[\alpha]^{25}_D$ −44.2° (*c* = 0.31, MeOH); ^1H NMR (DMSO-d_6) δ 3.47 (s, 6H, CH_3), 3.57–3.73 (m, 2H, H-5′, H-5″), 3.93–4.37 (m, 3H, H-4′, H-3′, H-2′), 5.17 (b s, 3H, D_2O exchangeable, 3 O*H*), 5.90 (d, 1H, $J_{1',2'}$ = 6.0 Hz, H-1′), 8.58 (s, 1H, H-2), 9.12 (s, 1H, H-7). Anal. ($C_{12}H_{17}N_5O_4$) C, H, N.

4-(Dimethylamino)-1-(β-D-ribofuranosyl)-v-triazolo[4,5-*d*]pyridazine (8b). A solution containing **5b** (0.40 g, 9.5 mmol), absolute ethanol (5 mL), and 40% aqueous dimethylamine (5 mL) was stirred at room temperature overnight as described for the preparation of **7b**. The usual workup followed by flash column chromatography using ethyl acetate/MeOH (9:1) as the eluant afforded **8b** (0.25 g, 80% yield) as a white solid: mp 196.5–197.5 °C; $[\alpha]^{25}_D$ −54.5° (*c* = 0.54, H_2O); ^1H NMR (300 MHz, DMSO-d_6) δ 3.52 (s, 6H, CH_3), 3.50–3.65 (m, 2H, H-5′, H-5″), 4.07 (m, 1H, H-4′), 4.25 (dd, 1H, H-3′), 4.65 (dd, 1H, $J_{2',1'}$ = 5.0 Hz, H-2′), 5.01 (b s, 1H, D_2O exchangeable, O*H*), 5.33 (b s, 1H, D_2O exchangeable, O*H*), 5.64 (b s, 1H, D_2O exchangeable, O*H*), 6.32 (d, 1H, $J_{1',2'}$ = 5.17 Hz, H-1′), 9.38 (s, 1H, H-7); ^{13}C NMR (75 MHz, DMSO-d_6) δ 41.2, 61.3, 70.3, 74.0, 86.4, 91.8, 129.7, 131.1, 133.0, 152.0. Anal. ($C_{11}H_{16}N_6O_4$) C, H, N.

4-(Benzylamino)-1-(β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazine (9a). A solution containing **5a** (0.50 g, 1.21 mmol), benzylamine (0.85 g, 6.05 mmol), and absolute ethanol (5 mL) was heated to 110 °C for 15 h as described for the preparation of **7a**. The usual workup followed by flash column chromatography using CH_2Cl_2/MeOH (9:1) as the eluant and then crystallization of the product from CH_2Cl_2/MeOH (1:1) afforded pure **9a** (0.43 g, 75% yield) as a white solid: mp 180 °C; $[\alpha]^{25}_D$ −49.4° (*c* = 0.84, MeOH); ^1H NMR (DMSO-d_6) δ 3.52–3.82 (m, 2H, H-5′, H-5″), 3.92–4.36 (m, 3H, H-4′, H-3′, H-2′), 4.77 (s, 2H, $CH_2C_6H_5$), 5.88 (d, 1H, $J_{1',2'}$ = 6.0 Hz, H-1′), 7.08–7.46 (m, 6H, C_6H_5, H-2), 8.52 (s, 1H, H-7), 9.08 (t, 1H, D_2O exchangeable, N*H*). Anal. ($C_{17}H_{19}N_5O_4$) C, H, N.

4-(Benzylamino)-1-(β-D-ribofuranosyl)-v-triazolo[4,5-*d*]pyridazine (9b). A solution of **5b** (0.43 g, 1.04 mmol) in absolute ethanol (5 mL) and benzylamine (0.57 mL, 5 mmol) was heated to 70 °C overnight. Filtration of the reaction mixture yielded a white crystalline product which was subsequently washed with CH_2Cl_2 and then recrystallized from CH_2Cl_2/EtOH (9:1) to give pure **9b** (0.25 g, 71% yield): mp 137–138 °C; $[\alpha]^{25}_D$ −48.0° (*c* = 0.85, EtOH); ^1H NMR (300 MHz, DMSO-d_6) δ 3.55–3.67 (dq, 2H, $J_{5',5''}$ = 12.0, $J_{5',4'}$ = 3.3, $J_{5'',4'}$ = 4.1 Hz, H-5′, H-5″), 4.07–4.13 (m, 1H, H-4′), 4.27 (dd, 1H, *J* = 4.51 Hz, H-3′), 4.65 (dd, 1H, *J* = 5.17 Hz, H-2′), 4.86 (b s, 2H, $CH_2C_6H_5$), 6.35 (d, 1H, $J_{1',2'}$ = 5.36 Hz, H-1′), 7.18–7.53 (m, 5H, C_6H_5), 8.71 (t, 1H, D_2O exchangeable, N*H*), 9.40 (s, 1H, H-7); ^{13}C NMR ($CDCl_3$) δ 42.2, 61.3, 70.4, 74.2, 86.6, 92.1, 126.6, 128.2, 128.3, 130.2, 130.8, 132.5, 134.1, 151.1. Anal. ($C_{16}H_{16}N_6O_4$) C, H, N.

4-(Furfurylamino)-1-(β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazine (10a). A solution containing **5a** (0.50 g, 1.21 mmol), furfurylamine (1 mL, 10.3 mmol), and absolute ethanol (5 mL) was heated to 70 °C for 15 h as described for the preparation of

7a. The usual workup followed by flash column chromatography using ethyl acetate/MeOH (9:1) as the eluant afforded **10a** (0.30 g, 72% yield) as a tan solid: mp 160–162 °C; ^{1}H NMR (DMSO-d_6) δ 3.50–3.73 (m, 2H, H-5′, H-5″), 3.90–4.37 (m, 3H, H-4′, H-3′, H-2′), 4.60–4.83 (b d, 2H, CH_2C$_4$H$_3$O), 5.03–5.60 (m, 3H, D$_2$O exchangeable, 3 OH), 5.83 (d, 1H, $J_{1',2'}$ = 6.0 Hz, H-1′), 6.10–6.37 (m, 2H, C$_4H_3$O), 7.30 (t, 1H, D$_2$O exchangeable, NH), 7.43 (b s, 1H, C$_4H_3$O), 8.49 (s, 1H, H-2), 9.07 (s, 1H, H-7). Anal. (C$_{15}$H$_{17}$N$_5$O$_5$) C, H, N.

4-(Furfurylamino)-1-(β-D-ribofuranosyl)-*v*-triazolo[4,5-*d*]pyridazine (10b). A solution containing **5b** (0.54 g, 1.3 mmol), absolute ethanol (5 mL), and furfurylamine (5 mL) was heated to 70 °C for 12 h. The usual workup followed by flash column chromatography using ethyl acetate/MeOH (9:1) as the eluant afforded **10b** (0.33 g, 72% yield) as a white solid: mp 136–137 °C; [α]$^{25}_D$ −65.0° (*c* = 0.72, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.59–3.67 (m, 2H, H-5′, H-5″), 4.10 (m, 1H, H-4′), 4.26 (dd, 1H, H-3′), 4.66 (dd, 1H, H-2′), 4.86 (m, 2H, CH_2C$_4$H$_3$O), 5.13 (t, 1H, *J* = 5.12 Hz, D$_2$O exchangeable, OH), 5.43 (d, 1H, *J* = 5.18 Hz, D$_2$O exchangeable, OH), 5.72 (d, 1H, *J* = 6.01 Hz, D$_2$O exchangeable, OH), 6.30 (*A*BX, 1H, $J_{A,B}$ = 3.2, J_{AX} = 0.8 Hz, C$_4H_3$O), 6.36 (d, 1H, $J_{1',2'}$ = 5.34 Hz, H-1′), 6.39 (AB*X*, 1H, J_{BA} = 3.2, J_{BX} = 1.8 Hz, C$_4H_3$O), 7.57 (AB*X*, 1H, $J_{X,A}$ = 0.8, $J_{X,B}$ = 1.8 Hz, C$_4H_3$O), 8.61 (t, 1H, D$_2$O exchangeable, NH), 9.44 (s, 1H, H-7); ^{13}C NMR (DMSO-d_6) δ 37.3, 61.3, 70.4, 74.2, 86.5, 92.0, 106.8, 110.4, 130.3, 131.0, 132.5, 141.8, 150.9, 152.6. Anal. (C$_{14}$H$_{16}$N$_6$O$_5$) C, H, N.

4-(Cyclohexylamino)-1-(β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazine (11a). A solution containing **5a** (0.50 g, 1.21 mmol) and cyclohexylamine (5 mL) was stirred at reflux for 6 h. Concentration of the reaction mixture and subsequent purification by flash column chromatography using ethyl acetate/MeOH (9:1) as the eluant afforded **11a** (0.32 g, 75% yield) as a tan solid: mp 115–117 °C; [α]$^{25}_D$ −43.3° (*c* = 0.93, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.15–1.95 (m, 11H, C$_6H_{11}$), 3.60–3.73 (m, 2H, H-5′, H-5″), 4.01 (m, 1H, H-4′), 4.12 (m, 1H, H-3′), 4.29 (dd, 1H, H-2′), 5.20–5.39 (b d, 2H, D$_2$O exchangeable, OH), 5.55–5.65 (b s, 1H, D$_2$O exchangeable, OH), 5.88 (d, 1H, $J_{1',2'}$ = 5.34 Hz, H-1′), 6.60 (d, 1H, *J* = 8.07 Hz, D$_2$O exchangeable, NH), 8.53 (s, 1H, H-2), 9.08 (s, 1H, H-7); ^{13}C NMR (DMSO-d_6) δ 25.0, 25.4, 32.5, 48.9, 61.2, 70.1, 74.6, 86.2, 89.3, 129.5, 130.1, 131.5, 142.5, 152.3. Anal. (C$_{16}$H$_{23}$N$_5$O$_4$) C, H, N.

4-(Cyclohexylamino)-1-(β-D-ribofuranosyl)-*v*-triazolo-[4,5-*d*]pyridazine (11b). A solution containing **5b** (0.31 g, 0.75 mmol), absolute ethanol (5 mL), and cyclohexylamine (5 mL) was stirred at room temperature overnight. The usual workup followed by flash column chromatography using ethyl acetate/MeOH (9:1) as the eluant afforded **11b** (0.19 g, 71% yield) as white crystalline needles: mp 110–112 °C; [α]$^{25}_D$ −0.83° (*c* = 0.96, EtOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.12–1.98 (m, 11H, C$_6H_{11}$), 3.52–3.67 (m, 2H, H-5′, H-5″), 4.06 (m, 1H, H-4′), 4.22 (dd, 1H, H-3′), 4.62 (dd, 1H, $J_{2',3'}$ = 10.4, $J_{2',1'}$ = 5.2 Hz, H-2′), 5.08 (t, 1H, *J* = 4.59 Hz, D$_2$O exchangeable, OH), 5.36 (d, 1H, *J* = 4.98 Hz, D$_2$O exchangeable, OH), 5.65 (d, 1H, *J* = 5.85 Hz, D$_2$O exchangeable, OH), 6.29 (d, 1H, *J* = 5.36 Hz, H-1′), 7.86 (d, 1H, *J* = 8.1 Hz, D$_2$O exchangeable, NH), 9.31 (s, 1H, H-7); ^{13}C NMR (DMSO-d_6) δ 25.0, 25.3, 32.2, 49.6, 61.3, 70.4, 74.2, 86.5, 92.0, 130.1, 130.3, 132.4, 150.7. Anal. (C$_{15}$H$_{22}$N$_6$O$_4$) C, H, N.

4-(((*R*)-1-Phenyl-2-propyl)amino)-1-(β-D-ribofuranosyl)-imidazo[4,5-*d*]pyridazine (12a). A solution of **13** (0.44 g, 1.08 mmol), D-amphetamine (0.22 g, 1.62 mmol), triethylamine (0.5 mL), and absolute ethanol (15 mL) was heated to reflux for 12 h. [D-Amphetamine sulfate, purchased from Sigma Chemical Co., was dissolved in H$_2$O (10 mL), and the pH of the solution was adjusted (pH = 10) using concentrated aqueous NH$_4$OH. Extraction using CH$_2$Cl$_2$ (2 × 25 mL) and combination of the organic layers followed by drying over MgSO$_4$, filtration, and concentration afforded pure D-amphetamine.] The reaction mixture was stirred at 80 °C for 15 h and then concentrated under vacuum, and the resulting residue was recrystallized using ethyl acetate/methanol (98:2) to afford **12a** (0.25 g, 60% yield) as a yellow solid: mp 82–84 °C; [α]$^{25}_D$ −44.4° (*c* = 0.93, MeOH); ^{1}H NMR (MeOH-d_4) δ 1.00 (d, 3H, *J* = 6.0 Hz, CH_3), 2.52–2.72 (m, 2H, CH_2C$_6$H$_5$), 3.76–3.89 (m, 2H, H-5′, H-5″), 4.09–4.49 (m, 3H, H-4′, H-3′, H-2′), 6.02 (d, 1H, $J_{1',2'}$ = 6.0 Hz, H-1′), 7.02–7.26 (m, 5H, C$_6H_5$), 8.79 (s, 1H, H-2), 9.78 (s, 1H, H-7). Anal. (C$_{18}$H$_{23}$N$_5$O$_4$) N; C calcd 59.21, found 59.72; H calcd 6.01, found, 6.50.

4-(((*R*)-1-Phenyl-2-propyl)amino)-1-(β-D-ribofuranosyl)-*v*-triazolo[4,5-*d*]pyridazine (12b). A solution of **5b** (0.25 g, 0.61 mmol) and D-amphetamine (1.34 g, 0.36 mmol) in absolute ethanol (5 mL) was stirred at 80 °C overnight. The usual workup followed by flash column chromatography using ethyl acetate/MeOH (9:1) as the eluant afforded **12b** (0.27 g, 75% yield) as a yellow solid: mp 71–73 °C; [α]$^{25}_D$ +3.01° (*c* = 0.83, EtOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.21 (d, 3H, *J* = 6.48 Hz, CH_3), 2.77 (dAB$_q$, 1H, *J* = 6.8 Hz, CH_2C$_6$H$_5$), 3.06 (dAB$_q$, 1H, *J* = 7.1 Hz, CH_2C$_6$H$_5$), 3.50–3.64 (m, 2H, H-5′, H-5″), 4.04 (m, 1H, H-4′), 4.20 (dd, 1H, *J* = 4.3 Hz, H-3′), 4.60 (dd, 1H, *J* = 5.2 Hz, H-2′), 4.74 (m, 1H, CHCH$_3$), 5.05 (b s, 1H, D$_2$O exchangeable, OH), 5.34 (b s, 1H, D$_2$O exchangeable, OH), 5.64 (b s, 1H, D$_2$O exchangeable, OH), 6.28 (d, 1H, $J_{1',2'}$ = 5.43 Hz, H-1′), 7.11–7.27 (m, 5H, C$_6H_5$), 7.97 (d, 1H, *J* = 8.4 Hz, D$_2$O exchangeable, NH), 9.30 (s, 1H, H-7); ^{13}C NMR (DMSO-d_6) δ 20.0, 41.7, 47.9, 61.5, 70.6, 74.4, 86.7, 92.2, 126.4, 128.6, 128.7, 129.6, 130.6, 132.7, 139.9, 151.5. Anal. (C$_{16}$H$_{22}$N$_6$O$_4$) C, H, N.

4-Chloro-1-(β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazine (13). A solution of **5a** (0.23, 0.56 mmol) and a saturated methanolic ammonia solution (8 mL, saturated at −5 °C) were stirred in a sealed vessel for 12 h as described for the preparation of **7a**. The reaction mixture was vented and concentrated. The residue was recrystallized from 95% ethanol to give **13** (0.15 g, 93% yield) as a white solid: mp 156–157 °C; [α]$^{25}_D$ −46.0° (*c* = 1.00, MeOH); ^{1}H NMR (300 Mhz, DMSO-d_6) δ 3.65–3.75 (dq, 2H, H-5′, H-5″), 4.04–4.09 (m, 1H, H-4′), 4.14 (dd, 1H, $J_{3',4'}$ = 7.53, $J_{3',2'}$ = 4.62 Hz, H-3′), 4.32 (dd, 1H, $J_{2',1'}$ = 6.15 Hz, H-2′), 5.29–5.34 (m, 2H, D$_2$O exchangeable, 2 OH), 5.61 (d, 1H, *J* = 6.21 Hz, D$_2$O exchangeable, OH), 6.05 (d, 1H, $J_{1',2'}$ = 6.3 Hz, H-1′), 8.94 (s, 1H, H-2), 9.89 (s, 1H, H-7); ^{13}C NMR (DMSO-d_6) δ 61.0, 70.2, 75.0, 86.7, 90.1, 132.2, 139.0, 139.5, 146.8, 147.6. Anal. (C$_{10}$H$_{11}$N$_4$O$_4$Cl) C, N; H: calcd, 3.87; found, 4.33.

Methods. Biological Evaluation. Adenosine Deaminase. Inhibition of calf intestinal mucosa ADA (type III, Sigma Chemical Co., St. Louis, MO) was measured directly from the spectral change at 265 nm[36,37] on a Gilford Model 240 spectrophotometer. The 1-mL reaction volumes contained 0.003 unit of enzyme, 25 μM adenosine (approximately the K_m concentration), and 50 mM potassium phosphate buffer, pH 7.4, at 30 °C. For assessment of the substrate activity, duplicate sterile solutions of **6a** or **6b** in 50 mM potassium phosphate were incubated in the presence and absence of ADA. The solutions were boiled for 1 min and analyzed on a Varian 5000 liquid chromatograph linked to a Kratos Spectroflow 757 UV detector. A Waters μBondapak C$_{18}$ column was eluted with water at 1 mL/min. Compound **6a** and the corresponding hydroxy analogue **3a**, monitored at 253 nm, emerged at 14.9 and 8.8 min; **6b** and **3b**, monitored at 263 nm, emerged at 10.2 and 7.7 min, respectively.

Purine Nucleoside Phosphorylase (PNP). Inhibition of human erythrocytic PNP activity by **3a** and **3b** was measured at 293 nm by a coupled xanthine oxidase assay[38] based on the method of Kalckar.[39] The 1-mL reaction mixtures contained 0.006 unit of partially purified[38] PNP (specific activity 2), 50 μM inosine, 50 mM potassium phosphate, pH 7.5, and 0.02 unit of xanthine oxidase, at 30 °C. One unit of PNP catalyzes the phosphorolysis of 1 μmol of inosine/min under standard assay conditions.[38] In assays which tested for substrate activity, sterile solutions of the analogues in 50 mM potassium phosphate were incubated in the presence of PNP. The samples were deproteinated in Centricon 30 microconcentrators (Amicon, Beverly, MA) and analyzed by HPLC as described above, except that the peaks were eluted with a 20-min linear gradient of 0–50% methanol in water and detected at 254 nm. Retention times for **3a**, its aglycone, and **3b** were approximately 13.6, 11.4, and 13.2 min, respectively.

Inhibition of Nucleoside Transport. Nucleoside transport into human erythrocytes was measured at room temperature by an inhibitor-stop assay,[40] with [8-^{14}C]adenosine (specific activity, 56 mCi/mmol; Moravek Biochemicals, LaBrea, CA) used as the labeled permeant. Analogues were preincubated with triplicate 100-μL aliquots of cells for 10 min before the addition of 100 μL of adenosine (final concentration, 10 μM). Transport into the cells (10% suspension, v/v) was terminated after 0.5 s by the

addition of an equal volume of ice-cold "stopping solution" containing 750 μM dilazep (a transport inhibitor; gift of Dr. M. Gudenzi, Asta-Werke, AG, Frankfurt, FDR) and centrifugation through oil. Radioactivity was measured after solubilizing and decolorizing the cells. Corrections for extracellular trapping of the permeant in the cell pellets were determined from samples to which stopping solution was added before the permeant. The K_m for adenosine transport is 25 ± 14 μM at 22 °C.[41]

Cyclic AMP Assays. Adenosine stimulates the adenylate cyclase system of HL-60 cells *via* A_2-like receptors. A_1 effects (inhibition of adenylate cyclase activity) are not demonstrable.[42] A highly responsive culture of HL-60 cells, which had been subcultured 169–172 times (biweekly schedule), was used as the test system. HL-60 cells (American Type Culture Collection, Rockville, MD) were grown in RPMI-1640 medium supplemented with penicillin, streptomycin (GIBCO, Grand Island, NY), and 10% heat-inactivated calf bovine serum (Hyclone Labs, Logan, UT). Cells were harvested by centrifugation and suspended in Hank's balanced salt solution without bicarbonate, containing 20 mM Hepes, pH 7.2. Duplicate cell suspensions ((2–4) × 10^6 cells) were preincubated at 37 °C for 5 min with the phosphodiesterase inhibitor, RO 20-1724 (final concentration, 100 μM; gift of Dr. Milan Uskokovic of Hoffmann-LaRoche, Nutley, NJ). In experiments which tested for inhibition, the analogue was present during preincubation. Stimulant (adenosine or an analogue) was added to a final volume of 400 μL. Reactions were stopped and cyclic AMP was quantitated essentially according to the method of Birnbaum and Fain,[43] which uses a competitive binding assay[44] and charcoal separation of free [2,8-^{3}H]-3′,5′-cyclic AMP (specific activity, 20 Ci/mmol; Moravek Biochemicals) from the radioligand complexed with bovine adrenal binding protein.[45] Samples were diluted to fall inside a 0–5-pmol range and assayed in triplicate. In experiments that included adenosine as the agonist, cells were preincubated for 5 min with 0.2 μM 5-iodotubercidin, 1 μM 2′-deoxycoformycin (from the Drug Development Branch, National Cancer Institute, Bethesda, MD), and 10 μM dilazep, inhibitors of adenosine kinase, adenosine deaminase and nucleoside transport, respectively. These additions had no significant effect on cyclic AMP accumulation in response to a nonmetabolizable agonist, 5′-*N*-ethylcarboxamidoadenosine. Concentration-response curves were analyzed by a nonlinear least-squares fit of the Michaelis–Menten equation with the computer program Enzfitter (Elsevier-BIOSOFT, Cambridge, U.K.). EC_{50} values, estimated by assuming a single binding site, were 2–3 μM for adenosine. Cyclic AMP accumulation during a 15-min stimulation with 2 or 250 μM adenosine was 131 ± 48 (n = 4) or 275 (n = 2) pmols/10^6 cells, respectively. Control (unstimulated) cyclic AMP levels in these experiments are typically 3–4 pmols/10^6 cells.

Antitumor Activity. L1210 cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated (56 °C, 30 min) fetal bovine serum and 50 μL of mercaptoethanol/L medium (RPMI-L). B16 cells were maintained in RPMI-1640 medium supplemented with 15% heat-inactivated fetal bovine serum and antibiotics (RPMI-C). The population doubling times for L1210 and B16 cells were 12 and 21 h, respectively. Exponentially growing L1210 cells (1×10^3 cells in 0.1 mL) and B16 cells (($2–3 \times 10^3$ cells in 0.1 mL) were seeded on day 0 in a Falcon 3072 96-well flat-bottom tissue culture plate. On day 1, 0.1 mL of medium containing graded concentrations of the test analogues was added to the initial volume. The cultures were incubated for 3 days in a humidified incubator maintained at 37 °C. The plates were centrifuged, and 100 μL of the growth medium was removed. Cell cultures were incubated with 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 1 mg/mL in Dulbecco's phosphate buffered saline) for 4 h at 37 °C. The colored formazan precipitate was solubilized in 200 μL of 0.04 N HCl in isopropyl alcohol. Absorbances were read on a Titertek Multiskan MCC scanning well spectrophotometer. ID_{50} values were determined by the computer program EZ-ED50 (Perrella Scientific, Inc., Conyers, GA) that fit all the data from eight determinations/concentration and 12 concentrations/analogue to the following equation: $Y = (A_m - A_o)/(1 + (X/ID_{50})^n) + A_o$ where A_m is the absorbance of the control cells, A_o is the absorbance of the cells in the presence of an infinite drug concentration or background, Y is the observed absorbance, X is the drug concentration, ID_{50} is the dose of drug that inhibits the growth of cells to one-half that of the control cells, and n is the slope factor of the fitted curve. The n (slope factor) represents the shape of the dose-response curve. The slope factor should have a value which is close to 1 for a simple mass-action dose response.

Acknowledgment. The authors wish to thank Prof. Elie Abushanab for many helpful discussions concerning the synthetic portion of this project. We also thank Dr. Hussein El Subbagh and Mrs. Rena Fullerton for technical assistance. J.D.S. is grateful to Shih-Ying Li and Rose Marie Smith for their fine technical assistance and to Robert E. Parks, Jr., for his support. This work was supported in part by the Roger Williams Cancer Center CORE Grant P30 CA13943, by Grants CA07340 and CA47268 awarded by the National Cancer Institute, N.I.H., and D.H.H.S., and by the American Cancer Society Grant CH-7.

References

(1) Srivastava, P. C.; Robins, R. K.; Meyer, R. C., Jr. *Synthesis and Properties of Purine Nucleosides and Nucleotides*; Townsend, L.B., Ed.; Plenum Press: New York, 1988; Vol. 1, pp 113–281.
(2) Ueland, P. M. Pharmacological and Biochemical Aspects of 5-Adenosylhomocysteine and S-Adenosylhomocysteine Hydrolase. *Pharmacol. Rev.* **1982**, *34*, 223–253.
(3) Panzica, R. P.; Bhat, G. A.; Earl, R. A.; Montero, J.-L. G.; Roti Roti, L. W.; Townsend, L. B. The Chemistry and Biological Activity of Certain 4-Substituted and 3,4-Disubstituted Pyrazolo[3,4-*d*]pyrimidine Nucleosides. In *Chemistry and Biology of Nucleosides and Nucleotides*; Harmon, R. E., Robins, R. K., Townsend, L. B., Eds.; Academic Press: New York, 1978; pp 121–134.
(4) Nasr, M.; Litterst, C.; McGowan, J. Computer-Assisted Structure-Activity Correlations of Dideoxynucleoside Analogs as Potential Anti-HIV Drugs. *Antiviral Res.* **1990**, *14*, 125–148.
(5) el Kouni, M. H.; Cha, S. Metabolism of Adenosine Analogues by Schistosoma Mansoni and the Effect of Nucleoside Transport Inhibitors. *Biochem. Pharmacol.* **1987**, *36*, 1099–1106.
(6) Stone, T. W. Purine Receptors and Their Pharmacological Roles. In *Advances in Drug Research*; Testa, B., Ed.; Academic Press: New York, 1989; Vol. 18, pp 291–429.
(7) Ikehara, M.; Fukui, T. Deamination of Adenosine Analogs with Calf Intestine Adenosine Deaminase. *Biochim. Biophys. Acta* **1974**, *338*, 512–519.
(8) Wilson, D. K.; Rudolph, F. B.; Quiocho, F. A. Atomic Structure of Adenosine Deaminase Complexed with a Transition-State Analog: Understanding Catalysis and Immunodeficiency Mutations. *Science* **1991**, *252*, 1278–1284.
(9) Carbon, J. Synthesis of Some Imidazo[4,5-*d*]pyridazines and Imidazo[4,5-*d*]triazolo[4,3-*b*]pyridazines. *J. Org. Chem.* **1960**, *25*, 579–582.
(10) Cook, P. D.; Dea, P.; Robins, R. K. Synthesis of Imidazo[4,5-*d*]-pyridazine Nucleosides Related to Inosine. *J. Heterocycl. Chem.* **1978**, *15*, 1–8.
(11) Gagnier, R. P.; Halat, M. J.; Otter, B. A. Synthesis and NMR Studies of Some Imidazo[4,5-*d*]pyridazine Nucleosides. *J. Heterocycl. Chem.* **1984**, *21*, 481–489.
(12) Ramesh, K.; Panzica, R. P. A Convenient Synthesis of 1-(β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazin-4(5*H*)-one (2-Aza-3-deazainosine) and Its 2′-Deoxy Counterpart by Ring Closure of Imidazole Nucleosides. *J. Chem. Soc., Perkin Trans. 1* **1989**, 1769–1774.
(13) Ramesh, K.; Panzica, R. P. Synthesis and Biological Activity of Certain 4-Substituted Imidazo[4,5-*d*]pyridazine Nucleosides. *Nucleosides Nucleotides* **1989**, *8*, 1141–1142.
(14) Robins, M. J.; Uznanski, B. Conversions of Adenosine and Guanosine to 2,6-Dichloro, 2-Amino-6-Chloro, and Derived Purine Nucleosides. *Can. J. Chem.* **1981**, *59*, 2601–2607.
(15) Castle, R. N.; Seese, W. S. Imidazo[4,5-*d*]pyridazines. I. Synthesis of 4,7-Disubstituted Derivatives. *J. Org. Chem.* **1958**, *23*, 1534–1538.
(16) Chen, S.-F.; Panzica, R. P. S-Demethylation of Nitrogen Heterocycles. *J. Heterocycl. Chem.* **1981**, *18*, 303–307.
(17) Bhat, G. A.; Montero, J.-L. G.; Panzica, R. P.; Wotring, L. L.; Townsend, L. B. Synthesis and Biological Activity of Certain Pyrazolo[3,4-*d*]pyrimidine Nucleosides Related to Adenosine. *J. Med. Chem.* **1981**, *24*, 1165–1172.
(18) Fleysher, M. H.; Bernacki, R. J.; Bullard, G. A. Some Short-Chain N^6-Substituted Adenosine Analogues with Antitumor Properties. *J. Med. Chem.* **1980**, *23*, 1448–1452.
(19) Adamson, R. H.; Zaharevitz, D. W.; Johns, D. G. Enhancement of the Biological Activity of Adenosine Analogs by the Adenosine Deaminase Inhibitor 2′-Deoxycoformycin. *Pharmacology* **1977**, *15*, 84–89.

(20) Hutzenlaub, W.; Tolman, R. L.; Robins, R. K. Synthesis and Antitumor Activity of Certain 3-β-D-Ribofuranosyl- and 2'-Deoxy-β-D-ribofuranosyl-*v*-triazolo[4,5-*d*]pyrimidines. *J. Med. Chem.* **1972**, *15*, 879–883.
(21) Earl, R. A.; Townsend, L. B. Synthesis of 8-Aza-3-deazaguanosine [6-Amino-1-(β-D-Ribofuranosyl)-*v*-triazolo[4,5-*c*]pyridin-4-one] via a Novel 1,3-Dipolar Cycloaddition Reaction. *Can. J. Chem.* **1980**, *58*, 2550–2561.
(22) Bennett, L. L., Jr.; Vail, M. H.; Chumley, S.; Montgomery, J. A. Activity of Adenosine Analogs against a Cell Culture, Line Resistant to 2-Fluoroadenine. *Biochem. Pharmacol.* **1966**, *15*, 1719–1728.
(23) Guranowski, A.; Montgomery, J. A.; Cantoni, G. L.; Chiang, P. K. Adenosine Analogues as Substrates and Inhibitors of *S*-Adenosylhomocysteine Hydrolase. *Biochemistry* **1981**, *20*, 110–115.
(24) Agarwal, R. P.; Sagar, S. M.; Parks, R. E., Jr. Adenosine Deaminase from Human Erythrocytes: Purification and Effects of Adenosine Analogs. *Biochem. Pharmacol.* **1975**, *24*, 693–701 and references therein.
(25) Shewach, D. S.; Krawczyk, S. H.; Acevedo, O. L.; Townsend, L. B. Inhibition of Adenosine Deaminase by Azapurine Ribonucleosides. *Biochem. Pharmacol.* **1992**, *44*, 1697–1700.
(26) (a) Niedzwicki, J. G. Unpublished results. (b) Niedzwicki, J. G.; Abernethy, D. R. Structure-Activity Relationship of Ligands of Human Plasma Adenosine Deaminase$_2$. *Biochem. Pharmacol.* **1991**, *41*, 1615–1624.
(27) Stoeckler, J. D. Purine Nucleoside Phosphorylase: a Target for Chemotherapy. In *Developments in Cancer Chemotherapy*; Glazer, R. E., Ed.; CRC Press: Boca Raton, FL, 1984; pp 35–60.
(28) Ealick, S. E.; Rule, S. A.; Carter, D. C.; Greenhough, T. J.; Babu, Y. S.; Cook, W. J.; Habash, J.; Helliwell, J. R.; Stoeckler, J. D.; Parks, R. E., Jr.; Chen, S.-F.; Bugg, C. E. Three-Dimensional Structure of Human Erythrocytic Purine Nucleoside Phosphorylase at 3.2 Å Resolution. *J. Biol. Chem.* **1990**, *265*, 1812–1820.
(29) Erion, M. D.; Guida, W. C.; Stoeckler, J. D.; Ealick, S. E. Manuscript in preparation.
(30) Paul, B.; Chen, M. F.; Paterson, A. R. P. Inhibitors of Nucleoside Transport. A Structure-activity Study Using Human Erythrocytes. *J. Med. Chem.* **1975**, *18*, 968–973.
(31) Stoeckler, J. D.; Rosenfield, C. G.; Chu, S.-H.; Li, S.-Y.; Acton, E. M.; Ryan, K. J.; Parks, R. E., Jr. Inhibition of Nucleoside Transport by Nitrobenzylthioformycin Analogs. *Biochem. Pharmacol.* **1990**, *40*, 615–619.
(32) Stoeckler, J. D.; Bussolari, J. C.; Panzica, R. P. Unpublished results.
(33) Born, G. V. R.; Haslam, R. J.; Goldman, M. Comparative Effectiveness of Adenosine Analogues as Inhibitors of Blood Platelet Aggregation and as Vasodilators in Man. *Nature* **1965**, *205*, 678–680.
(34) Bruns, R. F. Adenosine Receptor Activation in Human Fibroblasts: Nucleoside Agonists and Antagonists. *Can. J. Physiol. Pharmacol.* **1980**, *58*, 673–691.
(35) The poor yield is probably the result of the relative nucleophilicity of NH_3 coupled with the lack of leaving efficiency of the chloro group. With the exception of one run in which a 60% yield was obtained, all others were in the range of 42–46%. No products arising from glycosyl cleavage were detected.
(36) Kalckar, H. M. Differential Spectrophotometry of Purine Compounds by Means of Specific Enzymes III. Studies of the Enzymes of Purine Metabolism. *J. Biol. Chem.* **1947**, *167*, 461–475.
(37) Agarwal, R. P.; Parks, R. E., Jr. Adenosine Deaminase from Human Erythrocytes. *Methods Enzymol.* **1978**, *51*, 502–507.
(38) Stoeckler, J. D.; Agarwal, R. P.; Agarwal, K. C.; Parks, R. E., Jr. Purine Nucleoside Phosphorylase from Human Erythrocytes. *Methods Enzymol.* **1978**, *51*, 530–538.
(39) Kalckar, H. M. Differential Spectrophotometry of Purine Compounds by Means of Specific Enzymes I. Determination of Hydroxypurine Compounds. *J. Biol. Chem.* **1947**, *167*, 429–443.
(40) Chen, S.-F.; Cleaveland, J. C.; Hollmann, A.; Weimann, M. C.; Parks, R. E., Jr.; Stoeckler, J. D. Changes in Nucleoside Transport of HL-60 Human Promyelocytic Cells During *N,N*-Dimethylformamide Induced Differentiation. *Cancer. Res.* **1986**, *46*, 3449–3455.
(41) Paterson, A. R. P.; Harley, E. R.; Cass, C. E. Inward Fluxes of Adenosine in Erythrocytes and Cultured Cells Measured by a Quenched-Flow Method. *Biochem. J.* **1984**, *224*, 1001–1008.
(42) Stoeckler, J. D.; Li, S. Y. Unpublished results.
(43) Birnbaum, M. J.; Fain, J. N. Activation of Protein Kinase and Glycogen Phosphorylase in Isolated Rat Liver Cells by Glucagon and Catecholamines. *J. Biol. Chem.* **1977**, *252*, 528–535.
(44) Gilman, A. G. A Protein Binding Assay for Adenosine 3':5'-Cyclic Monophosphate. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *67*, 305–312.
(45) Brown, B. L.; Albano, J. D. M.; Ekins, R. P.; Sgherzi, A. M. A Simple and Sensitive Saturation Assay Method for the Measurement of Adenosine 3':5'-Cyclic Monophosphate. *Biochem. J.* **1971**, *121*, 561–562.