New Adenosine Kinase Inhibitors with Oral Antiinflammatory Activity: Synthesis and Biological Evaluation

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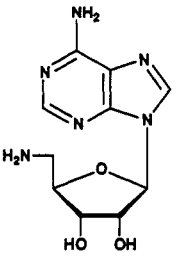
Several 5-iodotubercidin analogues in the pyrazolo[3,4-*d*]pyrimidine ring system were synthesized as potential inhibitors of adenosine kinase by a direct Lewis acid-catalyzed glycosylation procedure using both the preformed carbohydrate and the heterocyclic base as starting materials. The 5′-hydroxyl, -chloro, -azido, -deoxy, -amino, and -fluoro derivatives were prepared and evaluated in three systems for biological activity relative to adenosine, the true substrate, and 5-iodotubercidin, a known inhibitor. First, each compound was studied kinetically for inhibition of purified human placental adenosine kinase activity. The order of potency was: iodotubercidin > hydroxyl > amino ≥deoxy > fluoro> chloro >> azido. The K_i values for the 5′-hydroxyl and 5′-amino compounds, the two most potent inhibitors, were 80 and 150 nM, respectively. The inhibition appeared to be essentially competitive in nature, although a noncompetitive component of significance for the more potent inhibitors cannot be ruled out. Second, a bioassay was conducted in which the toxicity of 6-methylmercaptopurine riboside toward human CEM lymphoblasts was reversed by varying concentrations of the compounds. The order of effectiveness of the compounds in this system, representing a functional inhibition of adenosine kinase in cultured cells, was about the same as that with the purified enzyme, except that the 5′-chloro and 5′-fluoro compounds were ineffective. Third, the 5′-hydroxyl derivative was evaluated in vivo in a rat pleurisy inflammation model and displayed biological activity at a dose of 30 mg/kg given orally. Finally, the in vitro toxicity of each compound was assessed in CEM lymphoblasts. Results indicated that the two most potent inhibitors in the pyrazolo[3,4-*d*]pyrimidine ring system, the 5′-hydroxyl (**7**) and the 5′-amino (**20**), were 15-fold and 75-fold, respectively, less growth inhibitory than 5-iodotubercidin.

Introduction

Adenosine is a ubiquitous modulator of a variety of physiological and cellular functions. It is an endogenous feedback inhibitor of neutrophil activation that is produced in increased quantities after hypoxia, metabolic stress, and DNA damage.[1,2] When released from cells, adenosine blocks neutrophil adherence, activation, and phagocytosis, probably by binding to adenosine A2 receptors on the cell surface. Increasing the adenosine concentration at hypoxic sites, therefore, would likely result in significant antiinflammatory activity. Adenosine, however, is rapidly metabolized by several enzymes including adenosine kinase (ATP: adenosine 5′-phosphotransferase, EC 2.7.1.20) and adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4). One mechanism by which it should be possible to increase local concentrations of free adenosine at sites of inflammation would be to inhibit adenosine kinase. The concept that one might inhibit this enzyme, while maintaining some pharmacological selectivity vis-à-vis avoiding systemic adenosine effects, provides a basis for the design of new therapeutic agents for the treatment of inflammatory disease.

Compounds which are known to be good inhibitors of adenosine kinase include 5′-deoxy-5′-aminoadenosine[3] (**1**), 5-iodotubercidin[4] (**2a**), and 5′-deoxy-5-iodotubercidin[5] (**2b**). While the adenosine derivative **1** shows only moderate activity,[6] the iodotubercidins are much more potent inhibitors,[7,8] but are also considerably more difficult and expensive to synthesize. Another ring system which is isosteric with respect to purine is the pyrazolo[3,4-*d*]-pyrimidine system, the same class of heterocycle represented by the drug allopurinol, which is the hypoxanthine analogue. The synthesis of several 5′-modified iodotubercidin analogues in this allopurinol ring system, and their evaluation as inhibitors of adenosine kinase are the subject of this report.



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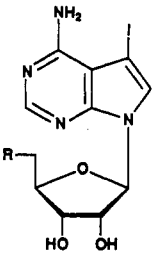

2a, R = OH
2b, R = H

Chemistry

The preparation of the 5-iodotubercidin analogues in the pyrazolo[3,4-*d*]pyrimidine system was facilitated by earlier studies[9] with bromo derivatives in this ring system, indicating that a direct high-temperature glycosylation procedure was most suitable for this heterocycle, in that the desired N-1 β isomer is produced as the predominant isomer under these conditions. Thus, these studies were extended to the iodo series reported here. The preformed heterocycle, 4-amino-3-iodo-1*H*-pyrazolo[3,4-*d*]pyrimidine (**4**) was first prepared as shown in Scheme I by

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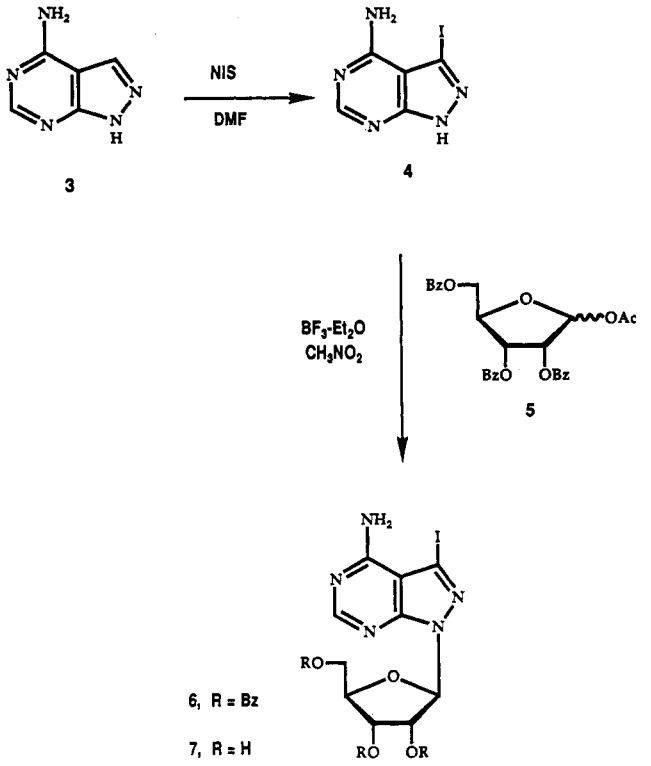
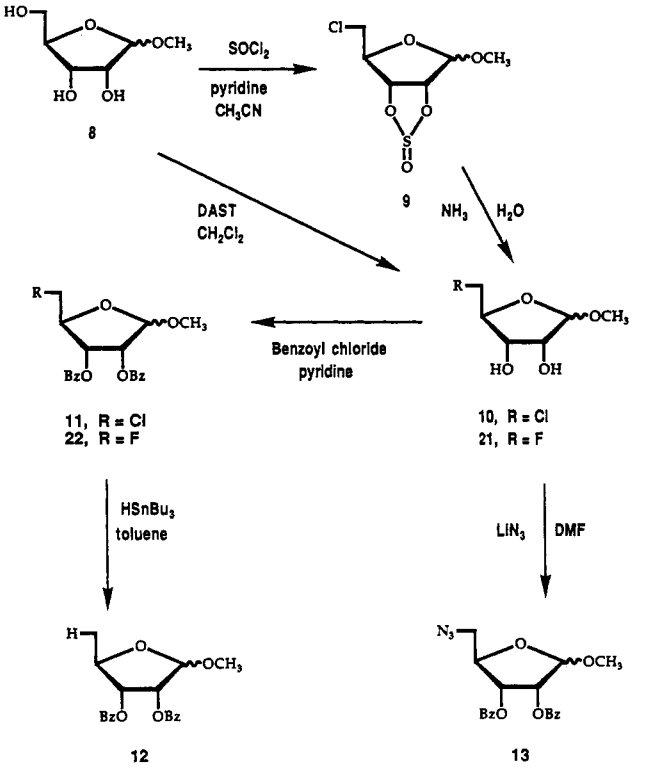
Scheme I



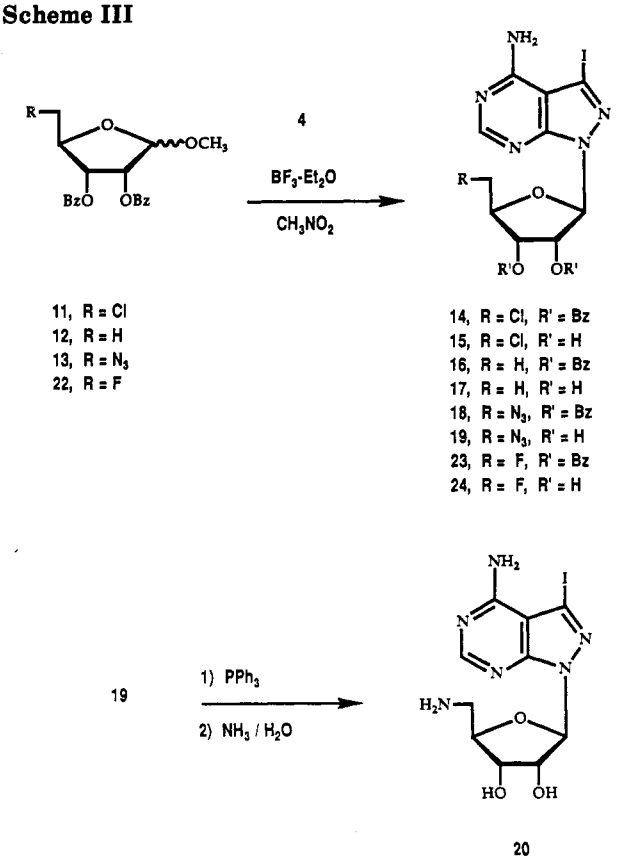
Scheme II



electrophilic iodination of 4-amino-1*H*-pyrazolo[3,4-*d*]-pyrimidine[10] (**3**) using *N*-iodosuccinimide in dimethylformamide at 80 °C. This heterocycle was then used in all of the glycosylation reactions described in this report, all of which involve a direct SN-1 type, Lewis acid-catalyzed procedure. This procedure, as illustrated in Scheme I for the preparation of the simple 5′-OH compound **7**, utilizes nonsilylated **4** and the appropriate ester-blocked carbohydrate in boiling nitromethane in the presence of boron trifluoride etherate. Thus, treatment of **4** with the commercially available 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-ribofuranose under these conditions gave 4-amino-3-iodo-1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)pyrazolo[3,4-*d*]-pyrimidine (**6**) in 86% yield after purification. Removal of the ester blocking groups with sodium methoxide provided the 5-iodotubercidin analogue **7** in 81% yield. The site of glycosylation and anomeric configuration of **7** was assigned on the basis of both proton NMR and ultraviolet spectra. The UV spectrum of the ribonucleoside **7** is strikingly similar to that of the free base **4**, an observation which supports the N-1 assignment for the site of sugar attachment. An N-2 isomer in this ring system would be expected to have a UV absorption maximum at longer wavelengths than that of the free base.[11,12] The anomeric assignment of **7** as β is supported by a comparison of the proton NMR coupling constants for the anomeric proton of the previously established 3-bromo derivative[9] (4.5 Hz) vs the 3-iodo derivative (5.0 Hz), **7**. The corresponding α nucleosides would be expected to have larger H1′–2′ coupling constants as predicted by the Karplus equation.[13]

In order to prepare the 5′-modified ribonucleosides, a classical approach could be pursued wherein the preformed nucleoside **7** is substituted selectively at the 5′-position with a functional group (i.e. halogen or a tosylate, triflate, or mesylate ester) which then could be displaced with an appropriate nucleophile to obtain the desired 5′-substituted compound. However, the successful synthesis of these compounds by this route is complicated by the tendency of an intramolecular cyclization to occur between the N-7 of the pyrazolo[3,4-*d*]pyrimidine heterocycle and the 5′ carbon of the carbohydrate to form a so-called cyclonucleoside. While this type of cyclization has been useful in assigning the anomeric configuration of purine and related nucleosides, the cyclonucleoside product has not been found useful as a versatile intermediate from which 5′-substituted nucleosides could be prepared and is therefore to be avoided when substitution is the desired outcome. To avoid this complication, we elected to preform the carbohydrate moiety such that the desired 5′ substituent is introduced prior to glycosylation, and thus, no subsequent nucleophilic displacement would be required. Accordingly, several 5-modified D-ribofuranoses were prepared by a simple and convenient procedure starting with the readily available 1-*O*-methyl D-ribofuranoside[14] (**8**) as shown in Scheme II. Treatment of **8** with excess thionyl chloride in the presence of pyridine provided a good yield of methyl 5-deoxy-5-chloro-2,3-*O*-sulfinyl-D-ribofuranoside (**9**) as a mixture of anomers and *S*-epimers. The preparation of the versatile crystalline benzoyl-blocked ribofuranose **11** was accomplished by reaction of **9** with aqueous ammonia followed by benzoyl chloride in pyridine. Two other 5-modified sugars were synthesized from **11**. Dehalogenation of **11** using tributyltin hydride in the presence of AIBN provided methyl 5-deoxy-2,3-di-*O*-benzoyl-β-D-ribofuranoside (**12**) in 88% yield after purification. Formation of the corresponding 5-deoxy-5-azido derivative **13** was accomplished by treatment of **11** with lithium azide in DMF at 90 °C. The three carbohydrates were then used for glycosylation studies using the preformed heterocycle **4** to provide the blocked 5′-chloro (**14**), the 5′-deoxy (**16**), and the 5′-azido (**18**) nucleosides under the same conditions as were used to prepare compound **6**. The yields of each of these glycosylation reaction products were, in general, somewhat lower than that of the initial reaction of the 1-*O*-acetyl-blocked sugar to produce compound **6**. This may be due to the reduced reactivity of the 1-*O*-methyl-protected

Scheme III



sugars. Nevertheless, the ease and simplicity of the preparation of the sugar intermediates renders this approach very attractive, even without conversion of the 1-*O*-methyl group to the more reactive 1-*O*-acetyl function. Deprotection of each of these blocked nucleosides with sodium methoxide provided the 5′-chloro (**15**), the 5′-deoxy, (**17**) and the 5′-azido (**19**) compounds.

In order to explore the effects on the adenosine kinase inhibitory activity of similar compounds whose 5′ substituents are capable of hydrogen bonding, the 5′-deoxy-5′-amino derivative **20** was prepared by reduction of the azido function of **19** to an amino group by the Staudinger reaction[15] followed by aqueous ammonia workup. In addition to the amino function, the 5′-deoxy-5′-fluoro derivative **24** was prepared by first synthesizing the appropriate protected sugar as depicted in Scheme III. Thus, treatment of **8** with excess (diethylamino)sulfur trifluoride (DAST) in dichloromethane for 2 h gave a 58% yield of methyl 5-deoxy-5-fluoro-D-ribofuranoside (**21**). Benzoylation of **21** provided the fully protected 5-fluoro derivative **22**, which was then used for the glycosylation reaction in the same manner as described above for the other sugars. The assignments of site of glycosylation and anomeric configuration of all deprotected nucleosides were again based upon UV spectra which were virtually qualitatively identical to one another, and the proton NMR anomeric coupling constants, which ranged from 3.5 to 5.0 Hz.

Biological Evaluation and Discussion

Inhibition kinetics with purified human adenosine kinase were determined for the target compounds, and compared to 5-iodotubercidin, a known inhibitor (Figure 1). Lineweaver–Burk plots indicated competitive inhibition; the K_i values are shown in Table I. The most potent

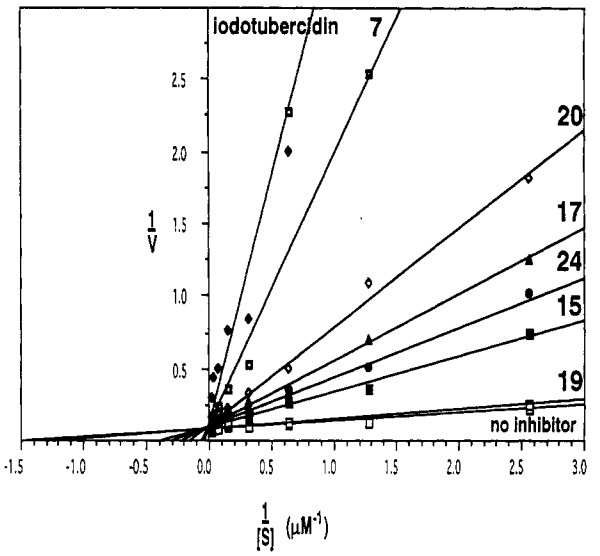


Figure 1. Lineweaver–Burk plot of inhibition of human adenosine kinase. The inhibition of adenosine to AMP by the target compounds is shown by the plot of $1/V$ vs $1/[S]$ in the presence or absence of 1 μM inhibitor. Reaction velocity is in units of pmol/min/μg protein. Each point is the average of three measurements.

Table I. Comparison of Effectiveness of Target Compounds

compound	5′-group	K_i[a]	ED_{50}[b]	ID_{50}[c]	ID_{50}/ED_{50}
iodotubercidin	OH	0.03	0.008	0.9	115
adenosine	OH		10	10	1
7	OH	0.04	0.012	15	1250
15	Cl	0.30	>10	4	<0.4
17	H	0.17	1.1	8	7.3
19	N_3	8.5	2.1	5.5	2.6
20	NH_2	0.15	0.013	75	5769
24	F	0.16	0.3	>100	>333

[a] K_i values, in μM, for inhibition of human placenta adenosine kinase are derived from data in Figure 1. [b] ED_{50} values are the concentrations, in μM, that reversed MMPR toxicity by 50%. [c] ID_{50} values are the concentrations, in μM, that inhibit CEM growth by 50%.

inhibitor in the pyrazolo[3,4-*d*]pyrimidine ring system is the 5′-hydroxyl derivative, which is nearly as potent an inhibitor as 5-iodotubercidin, and which has a K_i value (40 nM) 17.5-fold lower than the K_m of adenosine kinase for adenosine (700 nM).

Each compound was also tested for functional inhibition of adenosine kinase in human CEM lymphoblasts. This was accomplished by culturing the cells with 1 μM 6-methylmercaptopurine riboside (MMPR) in the presence of varying concentrations of the enzyme inhibitors. MMPR is phosphorylated by adenosine kinase to form a toxic metabolite. At a concentration of 1 μM it inhibited the growth of wild type CEM cells by 95% (Figure 2). CEM cells deficient in adenosine kinase are resistant to 100 μM MMPR. The effective doses of the adenosine kinase inhibitors that prevented MMPR toxicity by 50% (the ED_{50} values) are listed in Table I. In the pyrazolo-[3,4-*d*]pyrimidine series, the 5′-hydroxyl (**7**) and the 5′-amino (**20**) compounds were most effective, inhibiting MMPR toxicity by 50% at concentrations of 0.012 and 0.013 μM, respectively. The apparent lack of correlation between the activity in the purified enzyme assay and the MMPR experiment (K_i versus ED_{50} values) for some of the compounds such as **15** and **20** might be due to a variety of factors such as transport, metabolism once inside the cells, etc. Compound **15** appears to be toxic below its apparent ED_{50} for the MMPR experiment, and thus a value of >10 μM is suspect in this type of experiment.

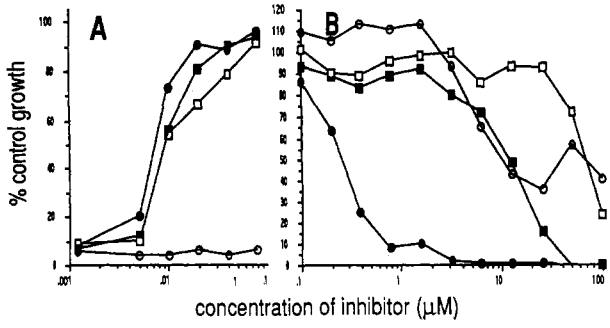


Figure 2. Functional inhibition of adenosine kinase in cultured cells. CEM cells were grown in the presence of 1 μM MMPR (the ID_{95}) (panel A) with indicated concentrations of inhibitors. The two best inhibitors, 7 (■) and 20 (□), are shown here, compared to iodotubercidin (●) and adenosine (○). In parallel experiments (panel B), CEM cells were grown without MMPR, with higher concentrations of inhibitor to determine the toxicity of the compounds. Each point is the average of three determinations.

Table II. In Vivo Oral Antiinflammatory Activity of 7 vs Iodotubercidin in the Rat Carrageenan-Induced Pleurisy Model

treatment[a]	dose[b] (mg/kg)	exudate[c] (mL)	% change	total cells[c] (×10⁶)	% change
vehicle		1.67 ± 0.05		177 ± 9.9	
iodotubercidin	2.5	1.00 ± 0.06	−40*	101 ± 6.0	−43*
7	30	1.11 ± 0.05	−34*	136 ± 4.2	−23*

* $p < 0.01$. [a] Experiments were performed with seven animals per group. [b] Dosage administered po 1 h before intrapleural injection of carrageenan. [c] Measured 4 h after administration of carrageenan.

Compound **20** may be preferentially concentrated to some extent inside these cells. In this regard, it should be noted that the 5′-amino function of **20** is protonated, for the most part, at physiological pH.

Although the compounds in this series are not better inhibitors of adenosine kinase than iodotubercidin, they are much less toxic to human lymphoblasts. Figure 2 (panel B) shows the effects of the inhibitors on the growth of CEM cells, in medium without MMPR. The doses that inhibited growth by 50% (ID_{50}) are listed in Table I. The ID_{50}/ED_{50} ratio provides a measure of the in vitro "therapeutic index" for each compound. As indicated in Table I, **7** and **20** have "therapeutic indexes" approximately 20- and 50-fold higher than iodotubercidin, respectively. In addition, compound **24** is remarkably nontoxic.

Compound **7** was then tested for oral antiinflammatory activity in a rat carrageenan-induced pleurisy model as described previously.[16] When given by gavage at a dosage of 30 mg/kg 1 h before intrapleural injection of carrageenan, compound **7** reduced exudate volume by 34% and total exudate cell density by 23% (Table II) compared to controls, as measured 4 h after administration. The activity of **7**, like that of iodotubercidin, is presumed to be due to the fuctional inhibition of adenosine kinase in vivo in this model of acute inflammation.

In summary, various ribonucleosides in the pyrazolo[3,4-*d*]pyrimidine series modified at the 5′-position are nearly as potent inhibitors of human adenosine kinase as iodotubercidin but are much less toxic to cultured cells and are much easier to synthesize by conventional methods. The 5′-hydroxyl derivative (**7**) also displayed modest oral antiinflammatory activity in rats. Antiinflammatory agents which appear to act by virtue of increasing extracellular adenosine concentrations may have promising therapeutic potential. However, the therapeutic usefulness of compounds such as those described here remains to be determined.

Experimental Section

Chemistry. Melting points were obtained on a Mel-temp II capillary melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra were obtained on a Varian XL400 spectrometer at 400 MHz or on a Varian Unity 500 at 499.8 MHz. The chemical shifts are expressed in δ values (parts per million) relative to tetramethylsilane as internal standard. Ultraviolet spectra were obtained using a Uvikon 930 (Kontron Instruments) spectrophotometer. Infrared spectra were obtained on a Nicolet Model 205 spectrophotometer. Mass spectra were obtained using a Finnigan MAT spectrometer in the FAB (positive) mode. Elemental analyses were performed by Robertson Microlit Laboratories, Madison, NJ. Thin-layer chromatography was performed on silica gel 60 F-254 plates (EM Reagents), and 10% sulfuric acid in methanol followed by heat was used to detect nucleosides and carbohydrates. E. Merck silica gel (230–400 mesh) was used for flash column chromatography.

Biological Materials. [2,8-^{3}H]Adenosine (1.2 TBq/mmol) was purchased from Moravek Biochemicals; PEI-cellulose sheets came from Whatman. Adenosine and 6-methylmercaptopurine riboside were purchased from Sigma, and 5-iodotubercidin was obtained from Research Biologicals Incorporated.

Enzyme Assays. Adenosine kinase was purified 600-fold from human placenta by the method of Spychala and Fox.[17] The enzyme was used at a concentration of 20 units/mL, where 1 unit is the amount of enzyme that phosphorylates 1 μmol of adenosine per minute. Assays were done in a total volume of 50 μL containing 5 mM ATP, 2.5 mM $MgCl_2$, 50 mM Tris-HCl pH 7.4, 15 mM NaF, and 5 μM deoxycoformycin to inhibit any remaining adenosine deaminase. Reactions were started by addition of [^{3}H]-adenosine, used at 37 kBq per reaction, with unlabeled adenosine added to give concentrations from 0.4 to 50 μM. After 20 min at 37 °C, the reactions were terminated by boiling for 2 min. Aliquots of 15 μL were spotted on PEI-cellulose TLC sheets and developed in 1:1 methanol–water. The origin was cut out and counted by liquid scintillation spectrophotometry.

Cell Culture Toxicity Tests. Human CEM lymphoblasts were obtained from The American Type Culture Collection and were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Hyclone) and 2 mM glutamine. CEM cells were plated in 96-well U-bottom plates at 2×10^5 cells/mL with or without 1 μM MMPR and varying concentrations of the compounds under investigation. After 96 h, cell number was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction as described elsewhere.[18]

In Vivo Animal Studies. Compound **7** was evaluated in the rat pleurisy model in which pleurisy was induced in Sprague–Dawley rats (Charles River, Wilmington, MA) by the intrapleural injection of λ-carrageenan (Marine Colloids, Springfield, NJ) by methods described previously.[16]

4-Amino-3-iodo-1*H*-pyrazolo[3,4-*d*]pyrimidine (4). 4-Amino-1*H*-pyrazolo[3,4-*d*]pyrimidine[11] (**3**, 2.9 g, 21.7 mmol) was suspended in dry DMF (75 mL), and *N*-iodosuccinimide (5.9 g, 26 mmol) was added. The mixture was heated with stirring at 80 °C for 6 h, evaporated to dryness in vacuo, and triturated with ethanol (50 mL) to yield 5.0 g (88%) of crude product. This material was purified by suspending in 50% aqueous ethanol (100 mL), heating to near boiling, and slowly adding concentrated ammonium hydroxide (50 mL) to effect solution. The resulting solution was decolorized with activated charcoal, filtered, and boiled to remove most of the ammonia. The resulting white solid was collected and dried to yield 4.1 g (71%) of pure product. mp >280 °C dec; UV (pH 1) λ_{max} 233 nm (ε 13 800); UV (pH 7) λ_{max} 282 nm (ε 8100), 262 (8400), 242 (7 900); UV (pH 11) λ_{max} 294 nm (ε 5400), 262 (7100), 237 (13 100), 214 (28 300); ^{1}H NMR (DMSO-d_6) δ 13.80 (s, 1H, NH), 8.17 (s, 1H, H_6), 7.50 (br s, 2H, NH_2). Anal. $C_5H_4IN_5$ (C, H, N).

4-Amino-3-iodo-1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-pyrazolo[3,4-*d*]pyrimidine (6). 1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-D-ribofuranose (**5**, 5.4 g, 10.7 mmol) was dissolved in dry nitromethane (50 mL), and 4-amino-3-iodopyrazolo[3,4-*d*]pyrimidine (**3**, 2.0 g, 7.1 mmol) was added. The resulting suspension was brought to reflux temperature, and boron trifluoride etherate (1.32 mL, 1.52 g, 10.7 mmol) was added through the condenser. The suspension became clear immediately and began to darken slowly. After 2 h at reflux, the mixture was cooled and evaporated

in vacuo to a thick, dark oil. The oil was dissolved in ethyl acetate (200 mL) and poured with stirring into aqueous saturated sodium bicarbonate (150 mL), the layers were separated, and the water layer was extracted with ethyl acetate (2 × 100 mL). The organic layers were combined and washed once with water (100 mL), and the organic layer was dried over sodium sulfate and evaporated to provide a dry foam which was purified by flash column chromatography on silica gel using acetone–dichloromethane (5:95). Yield of pure **6** was 4.30 g (86%) as a dry foam: ^{1}H NMR ($CDCl_3$) δ 8.35 (s, 1H, H_6), 8.12 (d, J = 7.0 Hz, 2H, benzoyl), 7.97 (t, 4H, benzoyl), 7.55, 7.45 and 7.38 (3 m, 9H, benzoyl), 6.78 (d, J = 3.7 Hz, 1H, $H_{1'}$), 6.42 (dd, J = 3.6 Hz, 1H, $H_{2'}$), 6.25 (t, peak width 11.3 Hz, 1H, $H_{3'}$), 5.97 (br s, 2H, NH_2), 4.83 (q, 1H, $H_{4'}$), 4.75 and 4.64 (2 m, 2H, $H_{5',5''}$). Anal. $C_{31}H_{24}IN_5O_7$ (C, H, N).

4-Amino-3-iodo-1-β-D-ribofuranosylpyrazolo[3,4-*d*]pyrimidine (7). Compound **6** (3.5 g, 5 mmol) was suspended in dry methanol (200 mL), and solid sodium methoxide was added to a pH > 10. The mixture was warmed briefly to help dissolve the remaining starting material. After 10 min a white solid separated, which was a mixture of fully deblocked product as well as a significant amount of 5'-benzoyl-protected product. THF (100 mL) was added, and the suspension was warmed at 60 °C for 30 min to complete the deblocking process. The mixture was cooled and filtered to yield a total of 1.59 g (81%) in three crops: mp > 247 °C dec; MS (FAB) m/z 394 (M + H)$^+$; UV (pH 1) λ_{max} 233 nm (ϵ 19 000); UV (pH 7) λ_{max} 284 nm (ϵ 12 300), 266 (7900), 243 (7600); UV (pH 11) λ_{max} 286 nm (ϵ 9800), 262 (8300); ^{1}H NMR (DMSO-d_6) δ 8.21 (s, 1H, H_6), 6.04 (d, J = 5.0 Hz, 1H, $H_{1'}$), 5.37 and 5.14 (2 br s, 2H, 2' and 3' OH's), 4.82 (t, 1H, 5'OH), 4.57 (t, peak width 10 Hz, 1H, $H_{2'}$), 416 (t, peak width 8.8 Hz, 1H, $H_{3'}$), 3.89 (q, 1H, $H_{4'}$), 3.55 and 3.43 (2 m, 2H, $H_{5',5''}$). Anal. $C_{10}H_{12}IN_5O_4$ (C, H, N).

Methyl 5-Deoxy-5-chloro-2,3-*O*-sulfinyl-D-ribofuranoside (9). Methyl D-ribofuranoside (**8**, 12.6 g, 77 mmol), prepared by the procedure of Barker and Fletcher,[14] was combined with dry acetonitrile (100 mL), and dry pyridine (25 mL) was added. Thionyl chloride (16.4 mL, 0.23 mol) was added dropwise over a period of 40 min, maintaining the temperature at 50–55 °C, during which time the mixture became clear and darkened slowly. After 1h at room temperature the mixture was heated at 60 °C for 90 min and then stirred at room temperature overnight. The mixture was evaporated to half volume in vacuo, poured with stirring into ice/water (150 mL), and extracted with ethyl acetate (2 × 200 mL). The organic layer was washed with 1 N HCl (2 × 150 mL), dried over sodium sulfate, and evaporated in vacuo to provide a thick syrup: crude yield 16.4 g (93%) as a mixture of *S* epimers and anomers. Purification for analytical purposes was accomplished by passing through a short silica gel column eluting with 2% methanol in dichloromethane; the first fractions contained pure **9**: MS (FAB) m/z 251 (M + Na)$^+$; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.59 (d, J = 6.5 Hz, 1H, H_2), 5.30 (m, 1H, H_3), 5.10 (s, $H_{1-\beta}$), 4.50 and 4.40 (2 m, 1H, $H_{4-\alpha}$ and β), 3.75 and 3.58 (2 m, 2H, $H_{5,5'-\alpha}$ and β), 3.39 and 3.38 (2 s, 3H, $OCH_{3-\alpha}$ and β). Anal. $C_6H_9ClO_5S$ (C, H).

Methyl 5-Deoxy-5-chloro-D-ribofuranoside (10). Compound **9** (15.0 g, 65.8 mmol) was dissolved in methanol (150 mL), concentrated ammonium hydroxide (15 mL) was added slowly with stirring, and after 15 min a solid separated. Reaction was complete after 3 h. The mixture was filtered to remove inorganic material, and the filtrate was evaporated to yield a thick oil (crude yield 14 g). The oil was dissolved in water (250 mL) and extracted with ethyl acetate (4 × 200 mL); the organic layer was dried over sodium sulfate and evaporated to provide a thick syrup which solidified under prolonged exposure to high vacuum atmosphere: yield 9.7 g (81%); MS (FAB) m/z 205/207 (M + Na and M + 2 + Na)$^+$; ^{1}H NMR (DMSO-d_6) δ 5.17 and 5.05 (2 br s, 2H, 2 and 3 OH), 4.80 (d, J = 6.8 Hz, $H_{1-\alpha}$), 4.63 (s, $H_{1-\beta}$), 3.95, 3.65, 3.58 (3 m, 5H, H_2, H_3, H_4, $H_{5,5'}$), 3.29 and 3.23 (2 s, 3H, OCH_3 α and β). Anal. $C_6H_{11}ClO_4$ (C, H).

Methyl 5-Deoxy-5-chloro-2,3-di-*O*-benzoyl-β-D-ribofuranoside (11). Compound **10** (4.5 g, 24.7 mmol) was dissolved in dry pyridine (50 mL), and benzoyl chloride (15 mL, 130 mmol) was added slowly with stirring. The mixture was stirred at room temperature overnight, and water (50 mL) was added. The mixture was stirred for 30 min and then evaporated to a semisolid paste. The residue was dissolved in chloroform (250 mL), extracted with 1 N HCl (2 × 100 mL) and saturated aqueous sodium bicarbonate (1 × 200 mL), dried over sodium sulfate, evaporated to a thick syrup, and purified by flash silica gel column chromatography using hexanes–ethyl acetate (12:1) to yield 5.1 g (53%) after crystallization from hexanes–ether: mp 55–56 °C; MS (FAB) m/z 413/415 (M + Na and M + 2 + Na)$^+$; ^{1}H NMR ($CDCl_3$) δ 7.89 and 8.00 (2 d, J = 7.3 Hz, 4H, ortho benzoyl H's), 7.53 and 7.58 (2 t, 2H, para benzoyl H's), 7.41 and 7.34 (2 t, 4H, meta benzoyl H's), 5.70 (t, 1H, H_3), 5.64 (d, $J_{2,3}$ = 5.0 Hz, 1H, H_2), 5.15 (s, 1H, H_1), 4.59 (q, 1H, H_4), 3.86 and 3.76 (2 m, 2H, $H_{5,5'}$), 3.49 (s, 3H, OCH_3). Anal. $C_{20}H_{19}ClO_6$ (C, H).

Methyl 5-Deoxy-2,3-di-*O*-benzoyl-β-D-ribofuranoside (12). The 5-chloro compound **11** (500 mg, 1.28 mmol) was dissolved in dry toluene (15 mL), and azobisisobutyronitrile (AIBN, 40 mg, 0.24 mmol) was added. To the reaction mixture was added tributyltin hydride (1.38 mL, 1.49 g, 5.1 mmol) by syringe, and the mixture was heated under argon at 110 °C for 2 h. The mixture was evaporated to a syrup and partitioned between acetonitrile and hexanes, and the acetonitrile layer was evaporated to provide a syrup which contained residual tributyltin hydride and was therefore purified by flash column chromatography on silica gel eluting with hexanes followed by hexanes–ethyl acetate (7:1) to yield 400 mg (88%) of **12** as a colorless syrup: ^{1}H NMR ($CDCl_3$) δ 7.89 and 8.00 (2 d, J = 7.3 Hz, 4H, ortho benzoyl H's), 7.53 and 7.58 (2 t, 2H, para benzoyl H's), 7.44 and 7.33 (2 t, 4H, meta benzoyl H's), 5.60 (d, J = 5.2 Hz,1H, H_2), 5.46 (t, 1H, H_3), 5.10 (s, 1H, H_1), 4.48 (m, 1H, H_4), 3.47 (s, 3H, OCH_3), 1.50 (d, J = 6.4 Hz, 3H, $5CH_3$). Anal. $C_{20}H_{20}O_6$ (C, H).

Methyl 5-Deoxy-5-azido-2,3-di-*O*-benzoyl-β-D-ribofuranoside (13). Crystalline **11** (5.0 g, 12.8 mmol) was combined with dry DMF (50 mL), and lithium azide (3.1 g, 64 mmol) was added. The azide salt dissolved slowly as the mixture was heated to 90 °C. After about 15 min, a precipitate of lithium chloride began to form, and heating was continued for a total of 5 h. The mixture was cooled, poured into 350 mL of water, and extracted with ethyl acetate (2 × 300 mL), and the organic layer was washed (1 × 200 mL) with water and dried (sodium sulfate), followed by evaporation in vacuo to yield 4.3 g (85%) of **13** as a thick syrup. IR ν (cm^{-1}) 2098 (N_3); MS (FAB) m/z 420 (M + Na)$^+$; ^{1}H NMR ($CDCl_3$) δ 7.89 and 8.03 (2 d, J = 7.3 Hz, 4H, ortho benzoyl H's), 7.53 and 7.58 (2 t, 2H, para benzoyl H's), 7.44 and 7.33 (2 t, 4H, meta benzoyl H's), 5.63 and 5.62 (overlapping t and d, 2H, H_3 and H_2), 5.16 (s, 1H, H_1), 4.52 (m, 1H, H_4), 3.63 and 3.55 (2 m, 2H, $H_{5,5'}$), 3.52 (s, 3H, OCH_3). Anal. $C_{20}H_{19}N_3O_6$ (C, H, N).

4-Amino-3-iodo-1-(2,3-di-*O*-benzoyl-5-deoxy-5-chloro-β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (14). The iodo heterocycle (**4**, 335 mg, 1.28 mmol) was glycosylated using compound **11** (500 mg, 1.28 mmol) in the same manner as described for the preparation of **6**. After 2 h the reaction mixture was worked up as described and purified by column chromatography on silica (3.8 × 17 cm), eluting with methanol in dichloromethane (4%) to yield 610 mg (82%) of **14** as a dry foam: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.36 (s, 1H, H_6), 7.95, 7.54 and 7.36 (3 m, 10H, benzoyls), 6.73 (d, J = 3.5 Hz, 1H, $H_{1'}$), 6.30 (m, 1H, $H_{2'}$), 6.13 (t, peak width 11.0 Hz, 1H, $H_{3'}$), 6.08 (br s, 2H, NH_2), 4.67 (m, 1H, $H_{4'}$), 3.91 and 3.86 (2 m, 2H, $H_{5',5''}$). Anal. $C_{24}H_{19}ClIN_5O_5$ (C, H, N).

4-Amino-3-iodo-1-(5-deoxy-5-chloro-β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (15). Compound **14** (0.54 g, 0.93 mmol) was suspended in dry methanol (20 mL), and solid sodium methoxide was added to a pH > 10. TLC after 30 min indicated that deprotection was complete. After 2 h Dowex H+ resin was added to neutralize, and a white, fluffy solid formed. The reaction mixture was decanted from the resin and filtered, and the solid was washed with methanol and then ether and dried to yield 200 mg (52%, first crop) of **15**: mp >215 °C; MS (FAB) m/z 434/436 (M + Na and M + 2 + Na)$^+$; UV (pH 1) λ_{max} 232 nm (ϵ 19 600); UV (pH 7) λ_{max} 284 nm (ϵ 9700), 265 (8100), 243 (7900); UV (pH 11) λ_{max} 284 nm (ϵ 9700), 266 (8200); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.24 (s, 1H, H_6), 6.09 (d, J = 4.0 Hz, 1H, $H_{1'}$), 5.55 and 5.39 (2 d, J = 5.6 and 5.9 Hz, 2H, 2' and 3' OH's, exchangeable), 4.58 (q, 1H, $H_{2'}$), 4.29 (q, 1H, $H_{3'}$), 4.07 (q, 1H, $H_{4'}$), 3.82 and 3.67 (2 m, 2H, $H_{5',5''}$). Anal. $C_{10}H_{11}ClIN_5O_3$ (C, H, N).

4-Amino-3-iodo-1-(2,3-di-*O*-benzoyl-5-deoxy-β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (16). The iodo heterocycle (**4**, 293 mg, 1.12 mmol) was glycosylated using compound **12** (400 mg, 1.12 mmol) in the same manner as described for the preparation of **6**. After 90 min the reaction mixture was worked

up as described and purified by column chromatography on silica (2.1 × 17 cm), eluting with methanol in dichloromethane (4%) to yield 280 mg (43%) of **16** as a dry foam: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.36 (s, 1H, H_6), 7.94, 7.52, and 7.36 (3 m, 10H, benzoyls), 6.66 (d, J = 4.0 Hz, 1H, $H_{1'}$), 6.33 (m, 1H, $H_{2'}$), 5.96 (br s, 2H, NH_2), 5.80 (t, peak width 11.5 Hz, 1H, $H_{3'}$), 4.67 (t, 1H, $H_{4'}$), 1.54 (d, J = 6.0 Hz, 3H, $5CH_3$). Anal. $C_{24}H_{20}IN_5O_5$ (C, H, N).

4-Amino-3-iodo-1-(5-deoxy-β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (17). Compound **16** (275 mg, 0.47 mmol) was suspended in dry methanol (20 mL), and solid sodium methoxide was added to a pH > 10. After 18 h Dowex H^+ resin was added to neutralize, and the reaction mixture was evaporated to dryness. The residual solid was washed with ether to remove methyl benzoate and dried to yield 135 mg (76%) of **17**. Recrystallization from water with a small amount of ethanol gave colorless microneedles: mp 224–226 °C; MS (FAB) m/z 400 $(M + Na)^+$; UV (pH 1) λ_{max} 233 nm (ε 19 700); UV (pH 7) λ_{max} 284 nm (ε 10 000), 262 (8300), 243 (8000); UV (pH 11) λ_{max} 284 nm (ε 10 000), 262 (8300), 242 (8300); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.23 (s, 1H, H_6), 6.02 (d, J = 3.8 Hz, 1H, $H_{1'}$), 5.56 and 5.11 (2 d, J = 5.6 and 5.7 Hz, 2H, 2′ and 3′ OH's, exchangeable), 4.51 (q, 1H, $H_{2'}$), 3.99 (q,1H, $H_{3'}$), 3.95 (t, 1H, $H_{4'}$), 1.22 (d, J = 6.2 Hz, 3H, $5CH_3$). Anal. $C_{24}H_9ClIN_5O_5$ (C, H, N).

4-Amino-3-iodo-1-(2,3-di-*O*-benzoyl-5-deoxy-5-azido-β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (18). The iodo heterocycle (4, 530 mg, 2 mmol) was glycosylated using compound **13** (800 mg, 2 mmol) in the same manner as described for the preparation of **6**. After 2.5 h the reaction mixture was worked up as described and purified by column chromatography on silica (2.1 × 17 cm), eluting with methanol in dichloromethane (4%) to yield 400 mg (32%) of **18** as a dry foam: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.36 (s, 1H, H_6), 7.95, 7.54, and 7.36 (3 m, 10H, benzoyls), 6.73 (d, J = 3.5 Hz, 1H, $H_{1'}$), 6.30 (m, 1H, $H_{2'}$), 6.13 (t, peak width 11.0 Hz, 1H, $H_{3'}$), 6.08 (br s, 2H, NH_2), 4.67 (m, 1H, $H_{4'}$), 3.91 and 3.86 (2 m, 2H, $H_{5',5''}$). Anal. $C_{24}H_{19}IN_8O_5$ (C, H, N).

4-Amino-3-iodo-1-(5-deoxy-5-azido-β-D-ribofuranosyl)-pyrazolo[3,4-*d*]pyrimidine (19). Compound **18** (0.38 g, 0.61 mmol) was suspended in dry methanol (30 mL), and solid sodium methoxide was added to a pH > 10. After 18 h, the reaction mixture was neutralized with Dowex H^+ resin and filtered, and the filtrate was evaporated to dryness. The crude solid product was washed with methanol and then ether and recrystallized from ethyl acetate–absolute ethanol to yield 100 mg (39%, first crop) of **19**: mp 107–110 °C; MS (FAB) m/z 441 $(M + Na)^+$; UV (pH 1) λ_{max} 233 nm (ε 17 600); UV (pH 7) λ_{max} 284 nm (ε 9000), 262 (7700), 242 (7500); UV (pH 11) λ_{max} 284 nm (ε 8600), 262 (7400); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.24 (s, 1H, H_6), 6.10 (d, J = 3.7 Hz, 1H, $H_{1'}$), 5.57 and 5.33 (2 d, J = 5.2 and 5.4 Hz, 2H, 2′ and 3′ OH's, exchangeable), 4.57 (q, 1H, $H_{2'}$), 4.33 (q, 1H, $H_{3'}$), 4.04 (q, 1H, $H_{4'}$), 3.54 and 3.39 (2 m, 2H, $H_{5',5''}$). Anal. $C_{10}H_{11}IN_8O_3$ (C, H, N).

4-Amino-3-iodo-1-(5-deoxy-5-amino-β-D-ribofuranosyl)-pyrazolo[3,4-*d*]pyrimidineacetic Acid Salt (20). Compound **19** (400 mg, 0.96 mmol) was dissolved in dry pyridine (12 mL), and triphenylphosphine (0.5 g, 19 mmol) was added. The solution was stirred at room temperature under argon for 1 h before concentrated ammonium hydroxide (3 mL) was added. After 2 h the reaction mixture was evaporated to dryness in vacuo, and the residue was flash chromatographed on silica (3.8 × 19 cm) eluting with solvent E–acetic acid (5:1 v/v) (solvent E is ethyl acetate–acetone–methanol–water 7:1:1:1 by volume). The residue from the pooled fractions was triturated with ethyl ether and dried to yield 270 mg (62%) of the acetic acid salt of **20** as a light tan solid: mp >175 °C dec; UV (pH 1) λ_{max} 233 nm (ε 10 300); UV (pH 7) λ_{max} 284 nm (ε 5200), 265 (4400), 243 (4400); UV (pH 11) λ_{max} 284 nm (ε 5200), 262 (4400); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.23 (s, 1H, H_6), 6.04 (d, J = 5.04 Hz, 1H, $H_{1'}$), 4.57 (t, 1H, $H_{2'}$), 4.15 (t, 1H, $H_{3'}$), 3.84 (q, 1H, $H_{4'}$), 2.75 and 2.65 (2 m, 2H, $H_{5',5''}$). Anal. $C_{10}H_{13}IN6O_3{\cdot}CH_3COOH$ (C, H, N).

Methyl 5-deoxy-5-fluoro-D-ribofuranoside (21). Methyl D-ribofuranoside (8, 880 mg, 5.37 mmol) was combined with dry dichloromethane (10 mL), and the suspension was cooled to −40 °C. (Diethylamino)sulfur trifluoride (DAST, 4.2 mL, 32.2 mmol) was added over a period of 5 min by syringe under an argon atmosphere. The cooling bath was removed, and the pale yellow mixture was allowed to warm to room temperature and stirred for a total of 2 h. Methanol (3 mL) was slowly added with cooling to −10 °C. After being stirred for 1 h, the mixture was evaporated to dryness in vacuo. The residue was dissolved in a small amount of methanol (3 mL), and ammonium hydroxide (5 mL) was added slowly (exothermic), and the mixture was sirred for 2 h, filtered to remove inorganic material, and absorbed onto silica gel (10 mL). Flash column chromatography on silica (2.8 × 20 cm), eluting with dichloromethane–methanol (95:5), afforded 520 mg (58%) of **21** as a syrupy mixture of anomers. Anal. $C_6H_{11}FO_4$ (C, H).

Methyl 5-Deoxy-5-fluoro-2,3-di-*O*-benzoyl-D-ribofuranoside (22). Compound **21** (480 mg, 2.9 mmol) was dissolved in dry pyridine (3 mL), and benzoyl chloride (1.5 mL, 12.8 mmol) was added dropwise rapidly. A solid formed, and the mixture was stirred at room temperature for 18 h. Aqueous sodium bicarbonate (10 mL, saturated) was added to the reaction mixture, stirred for 2 h, and then extracted with ethyl acetate (2 × 35 mL). The organic layer was washed with 1 N HCl (2 × 15 mL) and again with bicarbonate, dried (sodium sulfate), and evaporated onto silica gel (15 mL). Flash column chromatography on silica (3.8 × 16 cm) eluting with hexanes–ethyl acetate (11:1) gave 750 mg (69%) of the title compound as a dry foam: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.89 and 8.10 (2 m, 4H, ortho benzoyl H's), 7.52 and 7.56 (2 m, 2H, para benzoyl H's), 7.41 and 7.34 (2 m, 4H, meta benzoyl H's), 5.69 (t, 1H, H_3), 5.62 (t, 1H, H_2), 5.16 (s, 1H, H_1), 4.77 (m, 0.5H, H_5), 4.56 (m, 1H, H_4), 4.11 (m, 0.5H, $H_{5'}$), 3.48 (s, 3H, OCH_3); ^{19}F (470 MHz, $CFCl_3$ in $CDCl_3$) δ −233.40 and −228.25 (2 m, α and β anomers). Anal. $C_{20}H_{19}FO_6$ (C, H, N).

4-Amino-3-iodo-1-(2,3-di-*O*-benzoyl-5-deoxy-5-fluoro-β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (23). The iodo heterocycle (4, 510 mg, 1.95 mmol) was glycosylated using compound **22** (730 mg, 1.95 mmol) in the same manner as described for the preparation of **6**. After 90 min the reaction mixture was worked up as described and purified by column chromatography on silica (3.8 × 12 cm), eluting with dichloromethane–acetone (9:1) to yield 610 mg (52%) of **23** as a dry foam: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.36 (s, 1H, H_6), 7.97, 7.55 and 7.37 (3 m, 10H, benzoyls), 6.75 (d, J = 3.7 Hz, 1H, $H_{1'}$), 6.30 (dd, J = 3.6 Hz, 1H, $H_{2'}$), 6.18 (br s, 2H, NH_2), 6.10 (t, peak width 11.3 Hz, 1H, $H_{3'}$), 4.88 (m, 1H, $H_{4'}$), 4.75 (2 m, 2H, $H_{5',5''}$). Anal. $C_{24}H_{19}FIN_5O_5$ (C, H, N).

4-Amino-3-iodo-1-(5-deoxy-5-fluoro-β-D-ribofuranosyl)-pyrazolo[3,4-*d*]pyrimidine (24). Deprotection of **23** (570 mg, 0.95 mmol) under the same conditions as described for compound **7** afforded a white solid before neutralization with ion-exchange resin. The solid was filtered from the reaction mixture, washed with methanol and ethyl ether, and dried to yield 240 mg (64%) of pure product (**24**). A small sample was recrystallized from water for analysis: mp > 255 °C dec; UV (pH 1) λ_{max} 232 nm (ε 18 400); UV (pH 7) λ_{max} 284 nm (ε 9300), 265 (7800), 242 (7500); UV (pH 11) λ_{max} 284 nm (ε 9700), 264 (8000), 242 (7800); ^{1}H NMR (DMSO-d_6) δ 8.22 (s, 1H, H_6), 6.09 (d, J = 3.5 Hz, 1H, $H_{1'}$), 5.54 and 5.33 (2 d, J = 5.5 and 6.0 Hz, 2H, 2′ and 3′ OH's, respectively), 4.61, 4.51 and 4.41 (3 m, 0.5H–1H–0.5H, $J_{F,H5'}$ = 47 Hz, $H_{5'}H_{5''}$) 4.46 (m, 1H, $H_{2'}$), 430 (m, 1H, $H_{3'}$), 4.10 and 4.06 (2 m, $J_{F,H4'}$ = 23 Hz,1H, $H_{4'}$); ^{19}F NMR (470 MHz, $CFCl_3$ in DMSO-d_6) δ −226.71 (m). Anal. $C_{10}H_{11}FIN_5O_3$ (C, H, N).

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