Guanosine Analogues. Synthesis of Nucleosides of Certain 3-Substituted 6-Aminopyrazolo[3,4-*d*]pyrimidin-4(5*H*)-ones as Potential Immunotherapeutic Agents

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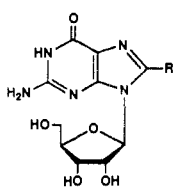
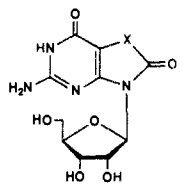
Several guanosine analogues were synthesized in the pyrazolo[3,4-*d*]pyrimidine ring system with various substituents at the 3-position. The new analogues prepared here include the CH_3 (2-amino-3-methyl-1-β-D-ribofuranosylpyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one, **13a**), the phenyl (2-amino-3-phenyl-1-β-D-ribofuranosylpyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one, **13b**), and the NH_2 (3,6-diamino-1-β-D-ribofuranosylpyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one, **17**) substituted derivatives. These new agents, as well as several other 3-substituted derivatives including H, Br, OCH_3, COOH, and oxo, were evaluated for their ability to potentiate certain murine immune functions relative to the known active agent 5-amino-3-β-D-ribofuranosylthiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione (**4**, 7-thia-8-oxoguanosine). The biological evaluation included the (1) ex vivo determination of increased natural killer cell function and (2) in vivo antiviral protection against a lethal challenge of Semliki Forest virus. The 3-unsubstituted (**5a**) and the 3-bromo (**5c**) derivatives were found to be the most active immunopotentiators in this series.

A number of ribonucleosides of guanine substituted at the 7- and/or 8-positions have been shown to stimulate the immune system and have been extensively studied primarily as modulators of B cell activation.[1] Such derivatives include 8-bromoguanosine (**1**), 8-mercaptoguanosine (**2**), and 7-methyl-8-oxoguanosine (**3**), the



1, R = Br
2, R = SH



3, X = NCH_3
4, X = S

syntheses of which were reported by Robins and coworkers[2,3] over 10 years before their immunostimulatory properties were discovered. These low molecular weight nucleosides have been shown to act as intracellular mitogens in murine splenic B lymphocytes[4] and appear to be relatively T-cell-independent activators of a subset of human B cells committed to the expression of IgM, IgG, or IgA isotypes.[5] In addition, these guanosines function as adjuvants for proliferation and differentiation of murine T cells in the presence of certain other stimulating signals.[6,7] More recently, 8-bromoguanosine was shown to activate murine natural killer cells and macrophages by inducing the production of interferon.[8]

Recently, we reported[9] the synthesis and immunological activity of a novel guanosine analogue 5-amino-3-β-D-ribofuranosylthiazolo[4,5-*d*]pyrimidine-2,7(3*H*,6*H*)-dione (**4**). This nucleoside was shown to provide excellent protection (92% survivors compared to 0% for placebo controls) against a lethal Semliki Forest virus infection in mice.[9] That protection was conferred by immunopotentiation rather than by direct antiviral properties was supported by the observation that **4** was devoid of significant direct in vitro antiviral activity[9] against this virus. Indeed, two properties of **4** include the induction of interferon and the activation of macrophages and natural killer cells.[9-11] The broad spectrum in vivo antiviral activity of this unique guanosine analogue has very recently been reported from our laboratory.[12]

The observed activity of **4**, a guanosine analogue modified at the 7- and 8-positions of the purine ring, prompted us to investigate other heterocyclic ring analogues of guanosine. In particular, we examined analogues in the pyrazolo[3,4-*d*]pyrimidine ring system, substituted at the 3-position (7-position in purine numbering). The 3-substituents described here were chosen to enable a qualitative structure–activity relationship to be developed in which a variety of functional groups could be investigated on the basis of size, resonance and field effects, hydrogen-bonding properties, etc., and those substituents thus selected included H, NH_2, Br, CH_3, OCH_3, phenyl, oxo, and carboxylic acid. These nucleosides were evaluated for immunomodulatory activity in systems designed to measure their effects on murine natural killer cell cytotoxic function and to measure the degree of protection provided against a lethal Semliki Forest virus infection in mice.

Chemistry

Reports from this laboratory have described the synthesis of a number of ribonucleosides of pyrazolo[3,4-*d*]-

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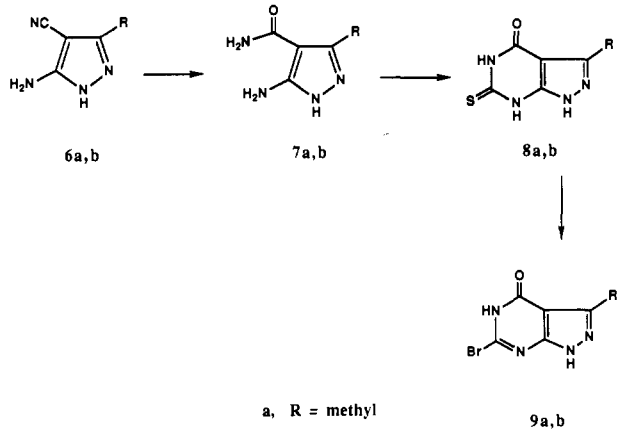
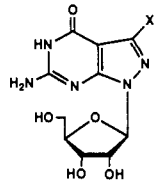
Scheme I



pyrimidines substituted at the 3-, 4-, and 6-positions.[13-20] The synthesis of new guanosine analogues, including the 3-methyl (**13a**), the 3-phenyl (**13b**), and the 3-amino (**17**) derivatives of 6-amino-1-β-D-ribofuranosylpyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one are reported here. The other nucleosides selected for biological evaluation were prepared as reported previously and include the 3-unsubstituted[13] (**5a**), the 3-carboxy[17] (**5b**), the 3-bromo[17] (**5c**), the 3-methoxy[18] (**5d**), and the 3-oxo[20] (**5e**) derivatives.



5a, X = H
5b, X = COOH
5c, X = Br
5d, X = OCH_3
5e, X = OH

The preparation of the 3-methyl (**13a**) and 3-phenyl (**13b**) derivatives is outlined in Schemes I and II (series **a** and **b**, respectively). Treatment of 5-amino-3-methyl-1*H*-pyrazole-4-carbonitrile[21] (**6a**) with concentrated sulfuric acid gave an excellent yield of 5-amino-3-methyl-1*H*-pyrazole-4-carboxamide (**7a**). Ring closure of **7a** with potassium ethyl xanthate provided 3-methyl-6-thioxo-1,5,6,7-tetrahydropyrazolo[3,4-*d*]pyrimidin-4-one (**8a**) in 89% yield. Reaction of **8a** with bromine in aqueous HBr resulted in replacement of the thioxo function by a bromine atom and gave 6-bromo-3-methyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (**9a**). This type of oxidative displacement has been previously reported from our laboratory.[17,22] Glycosylation of **9a** by a high-temperature procedure developed in our laboratory[15,17] using 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-ribofuranose (**10**) and BF_3·OEt_2 in refluxing nitromethane provided 6-bromo-3-methyl-1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)pyrazolo[3,4-*d*]-pyrimidin-4(5*H*)-one (**11a**) in 62% yield after chromatographic purification. A small amount (14%) of a byproduct was isolated in the purification process and was tentatively identified by proton NMR as the xanthosine analogue, 3-methyl-1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-4,6(5*H*,7*H*)-dione (**12**), undoubtedly produced from hydrolysis of the 6-bromo function in either **9a** or **11a** during the reaction. Treatment of **11a** with methanolic ammonia at 120 °C yielded the deblocked guanosine analogue, 6-amino-3-methyl-1-β-D-ribofuranosylpyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (**13a**). Compound **12** was similarly deblocked to yield 3-methyl-1-β-D-ribofuranosylpyrazolo[3,4-*d*]pyrimidine-4,6(5*H*,7*H*)-dione (**14**). The structural assignment of **13a** as the N-1-β anomer was made by comparison of the UV spectral characteristics of **13a** to several other 3-substituted derivatives in this ring system which have previously been characterized, i.e. the 3-bromo, the 3-carboxy, and the 3-ethoxy derivatives. The close similarity observed in the absorption maxima among these N-1-β nucleosides lends support to the assignment of N-1 as the site of glycosylation for **13a**. Thus, the absorption maxima for the n→π* transition (at pH 11) are 259 nm for **13a** and 261, 254, and 255 nm for the Br, methoxy, and ethoxy derivatives, respectively. N-2 glycosides, such as the Br derivative[17] prepared previously, absorb at much longer wavelengths (i.e. 285 nm at pH 11). The anomeric assignment of **13a** as β was made on the basis of the proton NMR comparison of the coupling constant for the anomeric proton of **13a** (4.2 Hz) with those of Br (4.7 Hz), OEt (4.5 Hz), and COOH (4.5 Hz). The α nucleosides would be expected to have larger constants than their respective β anomers, as predicted by the Karplus equation.[23] Nevertheless, it was considered important to verify the assignment of configuration by preparing the standard 2,3-*O*-isopropylidene derivative of **13a**. Thus, this derivative was prepared by the acid-catalyzed reaction of **13a** with 2,2-dimethoxypropane in acetone. The chemical shift difference between the isopropylidene methyl proton signals was observed to

Scheme II

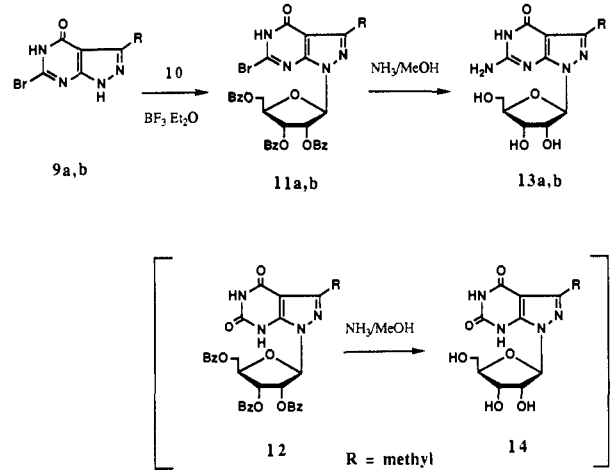


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Table I. In Vivo Effect of Guanosines on Murine NK Cell Activity[a]

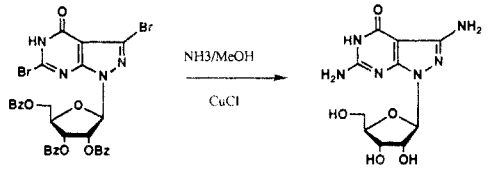
compound[d]	3-substituent	% cyclotoxicity;[b] E:T ratio[c] 50:1	100:1
placebo[f]		3.6 ± 0.6[e]	9.3 ± 3.6
5a	H	26.9 ± 1.2	41.1 ± 0.7
5c	Br	24.4 ± 9.0	33.2 ± 0.4
placebo		12.5 ± 1.0	17.1 ± 0.4
17	NH_2	16.8 ± 4.0	25.0 ± 4.0
placebo		6.1 ± 2.5	11.9 ± 4.0
13a	CH_3	17.0 ± 4.0	29.7 ± 3.5

[a] CBA/CaJ mice (two or three per group, female, 6–8 weeks old). [b] Spleen cells were isolated and cytotoxicity was determined against YAC-1 cells in a 4 h ^{51}Cr release assay. Assays were performed in triplicate. [c] Effector to target ratio. [d] Mice were injected intraperitoneally with compound (150 mg/kg) in 2% aqueous sodium bicarbonate in half-daily doses (75 mg/kg b.i.d., 1-day treatment). [e] Standard deviation. [f] 2% aqueous sodium bicarbonate (0.1 mL/injection) used as placebo control.

be 0.174 ppm, a value in agreement with the β assignment according to Imbach's rule[24] for the determination of anomeric configuration of N-nucleosides.

The 3-phenyl series was completed in essentially the same manner as described above for the 3-methyl series starting with pyrazole **6b**, which was prepared in two steps by the general method of Kobayashi.[25] First, reaction of triethyl orthobenzoate, malononitrile and acetic anhydride provided (phenylethoxymethylene)malonitrile (**15**) in 63% yield. Treatment of **15** with hydrazine hydrate gave the desired 5-amino-3-phenyl-1*H*-pyrazole-4-carbonitrile[25] (**6b**) in 92% yield. The preparation of the guanosine analogue (**13b**) in the 3-phenyl series was then accomplished by the same five-step procedure as that used to prepare the corresponding 3-methyl analogue described above. The configuration of **13b** was confirmed as β by preparation of the isopropylidene derivative, as in the case of compound **13a**. The chemical shift difference between the isopropylidene methyl proton signals for this derivative was found to be 0.176 ppm.

Finally, the 3-amino derivative **17** was prepared from the 3-bromo precursor (**16**)[17] by treatment with ammonia in a copper-catalyzed nucleophilic aromatic substitution similar to that described for 3-substituted allopurinol riboside.[15] The structural assignment of **17** as N-1-β was based on the known structure of **16**, previously established to be N-1-β.[17]



16 17

Immunological Studies

All deprotected pyrazolo[3,4-*d*]pyrimidine guanosine analogues were evaluated for their effects on certain immune functions in vivo in mice. The results reported here reflect representative findings of several repeat experiments.

First, the ability of selected compounds to augment murine natural killer (NK) cell activity against T cell lymphoma (YAC-1) target cells was determined in an ex vivo system; that is, animals were treated with the test agents and then spleen cells were removed and assayed in vitro for cytotoxicity against the target cells labeled previously with ^{51}Cr. The results shown in Table I indicate that at a dose of 150 mg/kg, the 3-unsubstituted (**5a**) and the 3-bromo (**5c**) derivatives manifested the best adjuvant effect with respect to the control groups. Both **5a** and **5c** augmented NK cytotoxicity by about 7-fold at the lower effector/target ratio (50:1). The 3-methyl derivative (**13a**) showed a moderate adjuvant effect, while the 3-amino compound (**17**) exhibited only a very weak effect.

Table II. Effects of Pyrazolopyrimidine Nucleosides and **4** on a Semliki Forest Virus Infection in Mice

3-substit	compound	dose,[a] mg/kg	survivors/ total (%)	mean survival time,[b] days
		Experiment 1		
	placebo[c]		1/12 (8)	8.3 ± 3.0[d]
	4	100	10/12 (83)[e]	11.5 ± 3.5
	4	200	12/12 (100)[e]	>21
H	**5a**	100	9/12 (75)[e]	9.0 ± 2.0
	5a	200	12/12 (100)[e]	>21
Br	**5c**	100	11/12 (92)[e]	9.0 ± 0.0
	5c	200	11/12 (92)[e]	9.0 ± 0.0
OCH_3	**5d**	100	2/12 (17)	7.8 ± 2.5
	5d	200	2/12 (17)	8.6 ± 2.3
CH_3	**13a**	100	8/12 (67)[e]	8.0 ± 1.4
	13a	200	6/11 (55)	6.4 ± 1.3
		Experiment 2		
	placebo[c]		2/12 (17)	5.6 ± 0.7
	4	100	10/12 (83)[e]	8.0 ± 1.4[f]
	4	200	10/12 (83)[e]	8.5 ± 2.1[f]
NH_2	17	100	1/12 (8)	6.2 ± 1.3
	17	200	3/12 (25)	6.8 ± 1.1[f]
COOH	**5b**	100	1/12 (8)	6.0 ± 1.1
	5b	200	3/12 (25)	6.3 ± 1.5
Ph	**13b**	100	1/12 (8)	6.0 ± 0.9
	13b	200	0/12 (0)	7.2 ± 1.7[f]
OH	**5e**	100	1/12 (8)	7.0 ± 2.0[f]
	5e	200	0/12 (0)	6.9 ± 1.1[f]

[a] Half-daily doses administered 24 and 18 h before virus inoculation. [b] Of mice that died. Survivors lived through 21 days. [c] A 2% sodium bicarbonate solution served as the placebo and as diluent for the compounds. [d] Standard deviation. [e] Statistically significant ($p < 0.02$), determined by the two-tailed Fisher exact test. [f] Statistically significant ($p < 0.05$), by two-tailed t test.

Second, all deprotected guanosine analogues were tested in vivo for their ability to provide protection against a lethal Semliki Forest virus infection in mice. The results of this study shown in Table II indicate that, at doses of 100 and 200 mg/kg per day given prophylactically, compounds **5a** and **5c** provided excellent protection and showed about the same degree of protection and potency as compound **4**, which was used as a positive control. Here, as in the case of the NK test system, compounds **5a** and **5c** were found to be the most active of those agents tested. Compound **13a** showed moderate protection while **5b**, **5d**, **5e**, **13b**, and **17** provided no significant protection against this virus. It is interesting to note that those compounds bearing groups capable of hydrogen bonding (amino, oxo, methoxy, and carboxy) at the 3-position were devoid of biological activity in these systems. All compounds studied showed no significant in vitro antiviral activity against this virus, which suggests that **5a**, **5c**, and **13a** provide protection via immunopotentiation rather than via direct antiviral properties. The induction of interferon is likely the principal factor responsible for the NK cell stimulation and the antiviral properties in these animal models.

The exact mode of action by which these guanosines activate the various cells of the immune system is still unclear. However, recent studies in our laboratories[11,26]

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and others[27] suggest the involvement of guanine nucleotide (G) binding proteins of the phosphatidylinositol–protein kinase C pathway as the site of action of these low molecular weight compounds. The difference observed in the relative activity of the various substituted guanosine analogues in the pyrazolopyrimidine ring system may thus be a function of their binding properties vis-à-vis the cellular G-proteins, with the 3-substituents apparently playing an important role in determining those binding properties.

Experimental Section

Melting points were taken on a Thomas-Hoover capillary melting point apparatus or on a Haake-Buchler digital melting point apparatus and are uncorrected. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 300.1 MHz with an IBM NR300AF spectrometer. The chemical shifts are expressed in δ values (parts per million) relative to the residual proton in the deuterated solvents as reference (i.e. DMSO set at 2.50 ppm, etc.). Ultraviolet spectra (UV: sh = shoulder) were recorded on a Beckman DU-50 spectrophotometer. Elemental analyses were performed by Robertson Laboratory, Madison, NJ. Evaporations were carried out under reduced pressure with the bath temperature below 40 °C. Thin-layer chromatography (TLC) was run on silica gel 60 F-254 plates (EM reagents). E. Merck silica gel (230–400 mesh) was used for flash column chromatography. HPLC purity determinations were done on a Waters 600 solvent delivery system equipped with a Waters 990 photodiode array detector and a Beckman ultrasphere 5 μm reversed-phase column (4.6 × 250 mm).

5-Amino-3-methyl-1*H*-pyrazole-4-carboxamide (7a). Finely powdered 5-amino-3-methyl-1*H*-pyrazole-4-carbonitrile[21] (**6a**, 14.3 g, 0.12 mol) was added slowly to cold (ice bath) concentrated sulfuric acid (30 mL). Complete conversion to amide **7a** required heating in an oil bath (50 °C) for 6 h. The reaction mixture was added slowly to cold concentrated ammonium hydroxide (200 mL) while the temperature was maintained below 10 °C. The resultant precipitate was filtered, washed with cold water, and dried to give 15.2 g (93%). Recrystallization from aqueous methanol gave small plates melting at 199–201 °C: UV λ_{max} (pH 1) 226 nm (ϵ 7300), 245 (5700); UV λ_{max} (pH 7) 222 nm (ϵ 6300), 242 (sh 4800); UV λ_{max} (pH 11) 220 nm (ϵ sh 6300), 245 (4800); ^{1}H NMR (DMSO-d_6) δ 2.25 (s, 3 H, CH_3), 5.46 and 6.58 (br s, 4 H, 2-NH_2), 11.4 (br s, 1 H, NH). Anal. ($C_5H_8N_4O$) C, H, N.

3-Methyl-6-thioxo-1,5,6,7-tetrahydropyrazolo[3,4-*d*]pyrimidin-4-one (8a). To a solution of the pyrazole **7a** (20.8 g, 0.15 mol) in dimethylformamide (1 L) was added with stirring potassium ethyl xanthogenate (70.0 g, 0.44 mol), and the mixture was heated in an oil bath (120 °C). After 6 h the dimethylformamide was evaporated and the residue was dissolved in water (300 mL). The solution was acidified with glacial acetic acid and cooled. The resultant precipitate was filtered, washed with water, and dried to give 24.1 g (89%). An analytical sample was prepared by recrystallization from water: mp >300 °C; UV λ_{max} (pH 1) 212 nm (ϵ 7800), 252 (8900), 288 (11 800); UV λ_{max} (pH 7) 238 nm (ϵ 8400), 283 (12900); UV λ_{max} (pH 11) 237 nm (ϵ 10 200), 282 (13 700); ^{1}H NMR (DMSO-d_6) δ 2.45 (s, 3 H, CH_3), 11.75, 12.88, and 13.29 (3 s, 3 H, NH). Anal. ($C_6H_6N_4OS$) C, H, N, S.

6-Bromo-3-methylpyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (9a). A suspension of thiopyrazolopyrimidinone **8a** (6.58 g, 36.2 mmol) in 48% aqueous hydrobromic acid (60 mL) was cooled in an ice bath. Bromine (5.6 mL, 0.11 mol) was added dropwise over a period of 1 h. The reaction was allowed to stand for 14 h at −10 °C. The resulting solid was filtered, washed with acetone, slurried in ice water, and refiltered to give 3.7 g of **9a** contaminated with starting material (**8a**). The original filtrate was neutralized with 50% aqueous sodium hydroxide (temperature below 10 °C) and acidified to pH 4. After cooling, the precipitate was collected by filtration and dried, resulting in pure **9a** (1.6 g). A portion was recrystallized from methanol: mp >300 °C; UV λ_{max} (pH 1) 215 nm (ϵ 26 000), 254 (8700); UV λ_{max} (pH 7) 208 nm (ϵ 27 000), 256 (9200); UV λ_{max} (pH 11) 207 nm (ϵ 40 000), 258 (9700); ^{1}H NMR (DMSO-d_6) δ 2.41 (s, 3 H, CH_3), 12.6 and 13.5 (2 br s, 2 H, NH). Anal. ($C_6H_5BrN_4O$) C, H, N, Br.

6-Bromo-3-methyl-1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (11a). A suspension of finely divided pyrazolopyrimidinone **9a** (0.60 g, 2.62 mmol), 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-ribofuranose (**10**, 2.64 g, 5.24 mmol), and dry nitromethane (25 mL) was heated to reflux with stirring under argon. Boron trifluoride etherate (0.55 mL, 5.54 mmol) was added by syringe. Immediately the reaction became homogeneous and turned light brown. After 5 min[28] the volatiles were removed by rotary evaporation, and the residue was dissolved in ethyl acetate (100 mL) and poured into cold saturated aqueous sodium bicarbonate (50 mL). The organic phase was washed with water (50 mL), dried, and evaporated. Purification by silica gel flash chromatography (1% methanol in dichloromethane) gave 1.25 g (62%) of **11a** and 0.30 g (14%) of the putative dioxo isomer **12**. For **11a** as an oil: UV λ_{max} (CH_3OH) 230 nm, 271; ^{1}H NMR ($CDCl_3$) δ 2.50 (s, 3 H, CH_3), 4.60 (ABC, 2 H, J = 3.5, 4.1, 7.4 Hz, $C_{5'}H$), 4.81–4.85 (m, 1 H, $C_{4'}H$), 6.22–6.32 (m, 2 H, $C_{2',3'}H$), 6.61 (d, 1 H, J = 2.3 Hz, $C_{1'}H$), 7.28–8.10 (3 m, 15 H, benzoyls).

For **12** as an amorphous powder: UV λ_{max} (CH_3OH) 231 nm, sh 255; ^{1}H NMR ($CDCl_3$) δ 2.67 (s, 3 H, CH_3), 4.72 (ABC, 2 H, J = 3.2, 3.9, 12.1 Hz, $C_{5'}H$), 4.80–4.85 (m, 1 H, $C_{4'}H$), 6.08 (d, 1 H, J = 1.7 Hz, $C_{1'}H$), 6.17 (dd, 1 H, J = 1.3, 5.1 Hz, $C_{2'}H$), 6.27 (dd, 1 H, J = 5.2, 7.5 Hz, $C_{3'}H$), 7.34–8.05 (3 m, 15 H, benzoyls).

6-Amino-3-methyl-1-β-D-ribofuranosylpyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (13a). The blocked nucleoside **11a** (1.2 g, 2.0 mmol) was placed in a steel bomb and cooled in a dry ice acetone bath and methanolic ammonia (100 mL, saturated at 0 °C) was added. After heating for 20 h at 120 °C, the bomb contents were evaporated to dryness, and the residue was flash chromatographed with ethyl acetate–methanol (92:8, v/v) as eluent. Recrystallization from aqueous ethanol gave **13a** as fine white needles: mp 280–281 °C dec; UV λ_{max} (pH 1) 252 nm (ϵ 13 400), 215 (26 800); UV λ_{max} (pH 7) 253 nm (ϵ 13 800), 215 (26 300); UV λ_{max} (pH 11) 259 nm (ϵ 11 700), 206 (35 500); ^{1}H NMR (DMSO-d_6) δ 2.30 (s, 3 H, CH_3), 5.80 (d, J = 4.5 Hz, $C_{1'}H$), 6.65 (br s, 2 H, NH_2), 10.54 (br s, 1 H, NH) and other sugar protons. Anal. ($C_{11}H_{15}N_5O_5 \cdot {}^1/_2H_2O$) C, H, N.

For **14**: UV λ_{max} (pH 1) 211 nm (ϵ 10 200), 250 (6300); UV λ_{max} (pH 7) 210 nm (ϵ 11 100), 248 (6400); UV λ_{max} (pH 11) 222 nm (ϵ 18 700), 279 (3500); ^{1}H NMR (DMSO-d_6) δ 2.51 (s, 3 H, CH_3), 5.66 (d, J = 4.2 Hz, $C_{1'}H$), 10.67 and 11.31 (2 s, 2 H, NH) and other sugar protons. Anal. ($C_{11}H_{14}N_4O_6$) C, H, N.

(Phenylethoxymethylene)malononitrile (15). Triethyl orthobenzoate (50.0 g, 0.22 mol), malononitrile (14.7 g, 0.22 mol), and acetic anhydride (425 mL) were heated under reflux for 2 h. The volatiles were then removed by vacuum distillation, and the residue was crystallized from aqueous ethanol to give 27.8 g (63%): mp 84–85 °C; UV λ_{max} (MeOH) 273 nm (ϵ 12 400); ^{1}H NMR ($CDCl_3$) δ 1.38 (t, 3 H, J = 7.1 Hz, CH_3), 4.20 (q, 2 H, J = 7.1 Hz, CH_2), 7.26–7.66 (m, 5 H, phenyl). Anal. ($C_{12}H_{10}N_2O$) C, H, N.

5-Amino-3-phenyl-1*H*-pyrazole-4-carbonitrile (6b). A mixture of ethanol (10 mL), (phenylethoxymethylene)malononitrile (**15**, 23.5 g, 0.13 mol), and hydrazine hydrate (85%, 12.0 g) was heated on a stream bath for 1 h after which water (50 mL) was added and the flask was cooled. The resulting precipitate was filtered and dried to give 20.1 g (92%). A portion was recrystallized from aqueous methanol: mp 200–201 °C (lit.[25] mp 200 °C); ^{1}H NMR (DMSO-d_6) δ 7.37–7.48 (m, 3 H, phenyl meta and para protons), 7.78 (d, 2 H, J = 6.9 Hz, phenyl ortho protons), 8.44 (br s, 2 H, NH_2), 12.21 (br s, 1 H, NH).

5-Amino-3-phenyl-1*H*-pyrazole-4-carboxamide (7b). Finely powdered phenylpyrazole **6b** (20.0 g, 0.10 mol) was added slowly to concentrated sulfuric acid (150 mL). After the powder had

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(28) Longer reaction times increased byproduct (**12**) formation. One can also minimize this product by rigorously excluding water.

dissolved, complete reaction required heating in an oil bath at 50 °C for 60 h. The reaction mixture was added dropwise to cold (dry ice–acetone bath) concentrated ammonium hydroxide (500 mL) while the temperature was maintained near 0 °C. The precipitate that formed was filtered, suspended in cold water, collected, and dried to give 21.5 g (98%). A small portion was recrystallized from methanol: mp 206–207 °C (lit.[25] mp 202–203 °C); ^{1}H NMR (DMSO-d_6) δ 5.81 (br s, 4 H, NH_2, $CONH_2$), 7.41–7.52 (m, 5 H, phenyl protons).

3-Phenyl-6-thioxo-1,5,6,7-tetrahydropyrazolo[3,4-*d*]pyrimidin-4-one (8b). A mixture of **7b** (17.5 g, 0.083 mol), dimethylformamide (500 mL), and potassium ethyl xanthogenate (60.0 g, 0.38 mol) was heated under reflux for 6 h. The dimethylformamide was then evaporated under reduced pressure and the residue was treated with 5% aqueous citric acid (250 mL). After cooling, the fine precipitate was filtered, washed with water, and dried to give 20.9 g (91%): mp >300 °C (lit.[25] mp >300 °C); ^{1}H NMR (DMSO-d_6) δ 7.36–7.45 (m, 3 H, phenyl meta and para protons), 8.27 (d, 2 H, J = 7.4 Hz, phenyl ortho protons), 10.48, 10.75, and 12.90 (br s, 3 H, NH). Anal. ($C_{11}H_8N_4OS$) C, H, N, S.

6-Bromo-3-phenylpyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (9b). Finely powdered **8b** (10.0 g, 0.041 mol) was suspended in 48% hydrobromic acid (100 mL) and cooled to 0 °C. Bromine (20 mL) was added slowly while the reaction temperature was kept below 10 °C and the mixture was stirred overnight in the refrigerator. The suspension was filtered and the solid was rinsed with cold water. The solid was dissolved in 0.5 N sodium hydroxide, reprecipitated by adjusting to pH 4 with 10% aqueous hydrobromic acid, and cooled. The finely divided solid was filtered, rinsed with cold water, and dried to give 6.4 g (54%). The original filtrate was again cooled, neutralized with concentrated sodium hydroxide, and re-acidified to pH 4 with 10% aqueous hydrobromic acid. After cooling, filtration, and drying, the additional yield was 2.5 g. A portion was recrystallized from ethyl acetate: mp >300 °C; UV λ_{max} (pH 1) 250 nm (ε 12 600); UV λ_{max} (pH 7) 256 nm (ε 13 000); UV λ_{max} (pH 11) 253 nm (ε 15 200); ^{1}H NMR (DMSO-d_6) δ 7.48 (m, 3 H, phenyl meta and para protons), 8.28 (d, 2 H, J = 7.2 Hz, phenyl ortho protons), 13.1 and 14.0 (br s, 2 H, NH). Anal. ($C_{11}H_7BrN_4O$) C, H, N, Br.

6-Bromo-3-phenyl-1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (11b). Compound **9b** (1.07 g, 36.8 mmol) and hexamethyldisilazane (25 mL) were heated under reflux for 6 h. Excess silylating agent was removed by vacuum distillation and the residue was evacuated under high vacuum for an additional 1 h. The solid was dissolved in dry acetonitrile (50 mL) and 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (2.04 g, 4.0 mmol) was added. Trimethylsilyl trifluoromethanesulfonate (0.75 mL, 4.0 mmol) was added and the mixture was stirred with protection from moisture. After 16 h the reaction mixture was evaporated to dryness, redissolved in ethyl acetate (100 mL), and poured slowly into cold saturated aqueous sodium bicarbonate (50 mL). The organic phase was washed with water, dried, and evaporated to give a brown foam. Flash chromatography was performed with dichloromethane–methanol (99:1, v/v) as eluent to give 1.82 g (67%). Blocked phenylpyrazole **11b** was obtained as a light yellow powder from diethyl ether–hexanes: mp 155–156 °C; UV λ_{max} (MeOH) 230 nm (ε 56 400), 245 (sh, 23 700); ^{1}H NMR ($CDCl_3$) δ 6.74 (d, 1 H, J = 2.4 Hz, C_1'H), 7.11–7.59 (m, 15 H, benzoyls), 7.95–8.05 (m, 3 H, phenyl meta and para protons), 8.39 (d, 2 H, J = 7.8 Hz, phenyl ortho protons), 11.55 (br s, 1 H, NH), and other sugar protons.

6-Amino-3-phenyl-1-β-D-ribofuranosylpyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (13b). Blocked pyrazolopyrimidine **11b** (1.4 g, 1.9 mmol) was dissolved in ethanol (75 mL), placed in a steel bomb, and cooled in dry ice–acetone. Anhydrous ammonia was bubbled through until saturation. The bomb was sealed and heated in an oil bath for 18 h at 120 °C. The ethanol was then removed and the residue was flash chromatographed on silica gel (dichloromethane–methanol, 10%) to give 0.42 g (62%). Recrystallization from methanol gave fine needles: mp 220–222 °C; UV λ_{max} (pH 1) 247 nm (ε 21 400), 209 (sh 21 000); UV λ_{max} (pH 7) 247 nm (ε 20 800), 209 (ε 19 900) UV λ_{max} (pH 11) 247 nm (ε 17 600), 204 (39 200); ^{1}H NMR (DMSO-d_6) δ 5.97 (d, 1 H, J = 4.2 Hz, C_1'H), 6.75 (br s, 2 H, NH_2), 7.37–7.45 (m, 3 H, phenyl meta and para protons), 8.30 (d, 2 H, J = 7.8 Hz, phenyl ortho protons), 10.76 (s, 1 H, NH), and other sugar protons. Anal. ($C_{16}H_{17}N_5O_5$) C, H, N.

3,6-Diamino-1-β-D-ribofuranosylpyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (17). Crude 3,6-dibromo-1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one[17] (**16**; 8.0 g, 7.21 mmol) was heated in a steel bomb with copper wire (0.20 g), copper(I) chloride (0.15 g), and 200 mL of a saturated methanolic ammonia. After 16 h at 120 °C, the contents of the bomb were filtered and the filtrate was evaporated to dryness. The residue was flash chromatographed on silica gel with dichloromethane–methanol (75:15, v/v) to give 3.2 g of crude material. Further purification with preparative HPLC (reversed phase, C-18) with water–acetonitrile (3%) gave 1.6 g of **17**. Attempts to crystallize small portions failed: mp 180 °C dec; UV λ_{max} (pH 1) 229 nm (ε 25 600), 253 (sh 9900); UV λ_{max} (pH 7) 230 nm (ε 27 700), 260 nm (sh 6200); UV λ_{max} (pH 11) 227 nm (ε 23 600), 253 (7600); ^{1}H NMR (DMSO-d_6) 5.21 (br s, 2 H, NH_2) 5.70 (d, J = 4.8 Hz, C_1'H) 6.57 (br s, 2 H, NH_2), 10.37 (br s, 1 H, NH), and other sugar protons. Anal. ($C_{10}H_{14}N_6O_5 \cdot {}^1/_2H_2O$) C, H, N.

Immunomodulation Studies. NK Cell Cytotoxicity Assay. To determine the effect of test compounds on NK cell cytotoxicity, mice were treated ip with drug (150 mg/kg) dissolved in 2% sodium bicarbonate (or 2% sodium bicarbonate alone as placebo) in groups of two each. After 24 h mice were sacrificed and spleen cells from each group were assayed in triplicate at two effector-to-target cell ratios for cytotoxicity against the T cell lymphoma target YAC-1 in a standard 4 h ^{51}Cr release experiment as described by Welsh.[29] Optimum doses of test compounds and incubation times used here were selected on the basis of preliminary experiments (data not shown) and were found to be the same for all the guanosines.

Semliki Forest Virus Model. Swiss Webster female mice (Charles River Labs, Wilmington, MA) weighing about 20 g each at the beginning of the experiment were inoculated intraperitoneally with test compounds (or placebo) in aqueous 2% sodium bicarbonate solution at −24 and −18 h relative to virus inoculation. The optimal dose of compound 4 (the most active compound) was established in preliminary experiments, and all other guanosines were compared to **4** at these doses for relative potency. The dosing schedule indicated here was also found to be optimum for all guanosines tested. A lethal dose ($10 \times LD_{50}$) of the Semliki Forest virus (Original strain) was administered by ip injection to groups of 12 mice.

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