# $N^2$-Phenyldeoxyguanosine: A Novel Selective Inhibitor of Herpes Simplex Thymidine Kinase

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A series of $N^2$-substituted guanine derivatives was screened against mammalian thymidine kinase and the thymidine kinase encoded by type I herpes simplex virus to examine their capacity to selectivity inhibit the viral enzyme. Several bases, nucleosides, and nucleotides displayed selective activity. The mechanism of action of the most potent derivative, $N^2$-phenyl-2′-deoxyguanosine (PhdG) was studied in detail. PhdG (a) inhibited the viral enzyme competitively with respect to the substrates thymidine and deoxycytidine, (b) was completely resistant to phosphorylation, (c) displayed limited toxicity for the HeLa cell lines employed as hosts for viral infection, and (d) selectively inhibited viral thymidine kinase function in intact cultured cells. The results indicate that the PhdG drug prototype has potential as a selective anti-herpes agent and as a novel molecular probe of the structure and function of herpes simplex thymidine kinase.

Herpes simplex virus type I (HSVI) encodes a unique thymidine kinase (TK), which is expressed in productively and latently infected cells in vivo and in culture.[1-4] HSVI-specific TK differs significantly from mammalian TK with respect to structure and catalytic properties.[4,5] One of the unique properties of the HSV-specific TK, a property that has been exploited in the development of selective antiviral nucleoside analogues, is the "plasticity" of its active site.[6] Whereas mammalian TK can phosphorylate only thymidine (TdR), HSV TK can phosphorylate thymidylic acid (dTMP), a variety of pyrimidine and purine deoxyribonucleosides, and nucleoside analogues such as acycloguanosine[7] and bromovinyldeoxyuridine.[8]

The results of studies of HSV pathogenesis and replication in vivo[9,10] strongly suggest that virus-specific TK function is required for virus replication and latent infection of nondividing cells in which host-specific pathways to dTTP are depressed or absent. For example, TK-deficient ($TK^-$) HSV mutants replicate poorly, if at all, in $TK^-$ and TMP synthetase deficient tissues such as the trigeminal ganglion[9] or adult brain.[10]

Considering the latter findings and the unique catalytic properties of the HSV TK, we have sought to develop a specific inhibitor of this enzyme, which could serve as a selective inhibitor of viral replication in the cells of nondividing tissues—particularly those of the nervous system, where HSV infection is significant clinically.[10] Our first step in this effort has been to screen a series of $N^2$-substituted guanine derivatives against HSVI TK in vitro and in vivo. One of the derivatives, $N^2$-phenyl-2′-deoxyguanosine (PhdG, **7**), appears to be a promising prototype for a TK-specific anti-herpes agent. The characterization of the TK inhibitory and preliminary biologic properties of **7** and related compounds is presented below.

## Chemistry

Methods developed for the preparation of $N^2$-substituted guanines and their nucleoside derivatives were used to prepare new compounds for this work, and are summarized in Scheme I. The $N^2$-phenylguanines **1**, **2**, and **4** were synthesized from 2-bromohypoxanthine and the aniline in refluxing 2-methoxyethanol, as previously reported for **3**[11] and **5**.[12] The 9-β-(2-deoxyribofuranosyl) derivative of **1**, compound **7**, was prepared in a multistep sequence, taking advantage of the regio- and stereoselectivity of the sodium salt glycosylation technique,[13] as recently applied by us for the synthesis of the related compound **8**.[12] Compound **1** was converted to 2-anilino-6-chloropurine in 74% yield with thionyl chloride in DMF. Reaction of the sodium salt

**Scheme I**

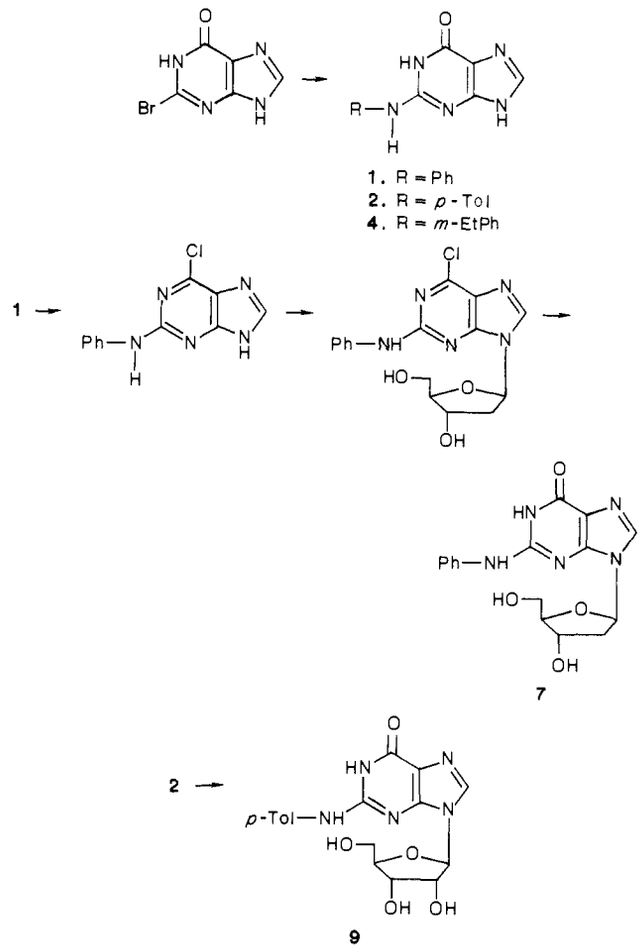


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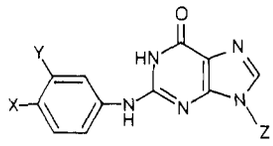
**Table I.** Effect of Selected $N^2$-Phenylguanine Derivatives on the Activity of HSVI TK



| compd | X | Y | Z | $EC_{50}$, $\mu$M, in HSVI TK assay[a] |
|---|---|---|---|---|
| 1 | H | H | H | 8 |
| 2 | $CH_3$ | H | H | 50 |
| 3 | $n$-$C_4H_9$ | H | H | 50 |
| 4 | H | $C_2H_5$ | H | 3 |
| 5 | $CH_3$ | $C_2H_5$ | H | 7 |
| 6 | $CH_2CH_2CH_2$ | | H | 15 |
| 7 | H | H | 2-deoxy-$\beta$-D-ribofuranosyl | 0.3 |
| 8 | $n$-$C_4H_9$ | H | 2-deoxy-$\beta$-D-ribofuranosyl | 25 |
| 9 | $CH_3$ | H | $\beta$-D-ribofuranosyl | 30 |
| 10 | $n$-$C_4H_9$ | H | $\beta$-D-ribofuranosyl | 75 |
| 11 | H | H | 2-deoxy-5-phospho-$\beta$-D-ribofuranosyl | 3 |
| 12 | $n$-$C_4H_9$ | H | 2-deoxy-5-phospho-$\beta$-D-ribofuranosyl | 400 |
| 13 | $n$-$C_4H_9$ | H | $CH_2OCH_2CH_2OH$ | 140 |
| 14 | [2-($p$-$n$-butylanilino)-2′-deoxyadenosine] | | | inactive[b] |

[a] Concentration causing 50% inhibition of TdR phosphorylation; the TK assay included TdR at a concentration of 1 $\mu$M and was performed as described in the Experimental Section. [b] No inhibition detected at a concentration of 1 mM.

of this base with 2-deoxy-3,5-di-$p$-toluoyl-$\beta$-D-ribofuranosyl chloride in dry acetonitrile gave, as the predominant product, 2-anilino-6-chloro-9-(2-deoxy-3,5-di-$p$-toluoyl-$\beta$-D-ribofuranosyl)purine in 43% yield. After removal of $p$-toluoyl groups with sodium methoxide in methanol, the 6-chloro nucleoside was treated with 2-mercaptoethanol and sodium methoxide in refluxing methanol for 72 h to give $N^2$-phenyl-2′-deoxyguanosine, PhdG (7), in 62% yield. The identity of all nucleosides as 9-$\beta$-(2-deoxyribofuranosyl) derivatives was based on the similarity of $^1$H NMR spectra of sugar ring protons to those of deoxyguanosine and 8.[11] The characteristic pseudotriplet for the 1′-H resonance of $\beta$-anomers, and the difference between 8-H and sugar ring proton chemical shifts between 7 and 9 regioisomers[11] assured the assignment of 9-$\beta$ to the major product of glycosylation above and, eventually, to 7.

The 9-$\beta$-ribofuranosyl derivative of 2, compound 9, was prepared by direct glycosylation of 2 with tetra-$O$-acetylribose and trimethylsilyl trifluoromethanesulfonate as catalyst.[11] The conditions of the reaction, selected to maximize conversion to the more stable 9-$\beta$ isomer,[14] gave the desired product after deacetylation in 63% yield. The characteristic $^1$H NMR spectrum[11] was used to identify the product as 9.

## Results

**Screening of $N^2$-Substituted Purines for Anti-TK Activity.** Table I summarizes the results of examination of several (arylamino)purines and their nucleoside derivatives on the activity of HSVI-specific TK. Identical assays of the host-specific HeLa TK showed that all compounds listed in the table were inactive at concentrations as high as 1 mM. The results give a preliminary structure–activity relationship for inhibition of the viral enzyme and allow several generalizations. $N^2$-Arylguanines (1–6), their 9-$\beta$-D-ribofuranosyl (9, 10) and 2-deoxyribofuranosyl (7, 8) derivatives, and 5′-monophosphates of the latter (11, 12) display significant inhibitory activity toward HSV TK at concentrations in which they exert no inhibitory effect on the host TK, i.e. they are selective for HSV TK. Among the guanine analogues, the substituents in the $N^2$-phenyl group strongly influenced potency; the unsubstituted compound 1 was sixfold more active than two $p$-alkyl derivatives, 2 and 3, but a meta-substituted derivative, 4, was more potent than 1. Two disubstituted compounds, 5 and 6, had activity similar to that of 1. A 2′-deoxyribonucleoside derivative, compound 7 (PhdG), displayed the highest potency, exceeding those of the simple base, the ribonucleoside, the more complex nucleotide forms, and even the acycloguanosine-like derivative, *acyclo*-BuPGR (13). The anti-HSV TK activity may require a purine nucleus of the guanine (6-oxo) type; in one case the equivalent adenine (6-$NH_2$) derivative, i.e. 14, was inactive.

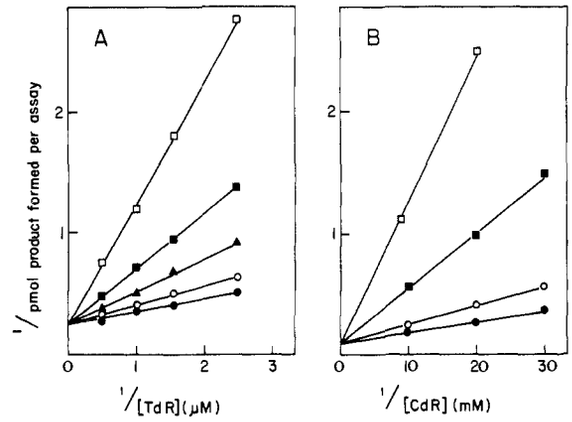



**Figure 1.** Lineweaver–Burk plots of the effect of PhdG (7) on the activity of HSV TK in the presence of the pyrimidine nucleoside substrates TdR and CdR. The enzyme was assayed as described in the Experimental Section with variation of the concentration of nucleoside substrate. Panel A, TdR phosphorylation; Panel B, CdR phoshorylation. (●), no 7; (○), 40 nM 7; (▲), 80 nM 7; (■), 400 nM 7; (□), 1 $\mu$M 7. For TdR phosphorylation, 0.3 milliunit of TK was used per assay; for CdR phosphorylation 0.6 milliunit of TK was used per assay.

**The Inhibitors Are Competitive with TK Substrates.** Two derivatives, the deoxyribonucleosides of 1 and 3, PhdG (7) and BuPdG (8), were selected for kinetic analysis of their inhibitory activity. The Lineweaver–Burk plot derived from experiments with 7 against the two pyrimidine substrates, TdR and CdR, are shown in panels A and B, respectively, of Figure 1. Against either substrate the kinetics of inhibition were consistent with a competitive mechanism. Inhibition was clearly prevented by sufficient substrate, and the $K_i$ determined with CdR as substrate, 0.3 $\mu$M, was the same as that determined with TdR. The results of the corresponding set of experiments with 8 were qualitatively identical with those obtained with 7 and, therefore, are not shown graphically. BuPdG (8) was competitive with both TdR and CdR, and in both cases displayed an apparent $K_i$ value of 25 $\mu$M.

**Active 2′-Deoxyribonucleosides Are Not Converted to Their 5′-Phosphates.** We exploited 7 and 8 to address the possibility that they, like GdR, TdR, CdR, acycloguanosine,[7] and bromovinyldeoxyuridine,[8] are bona fide

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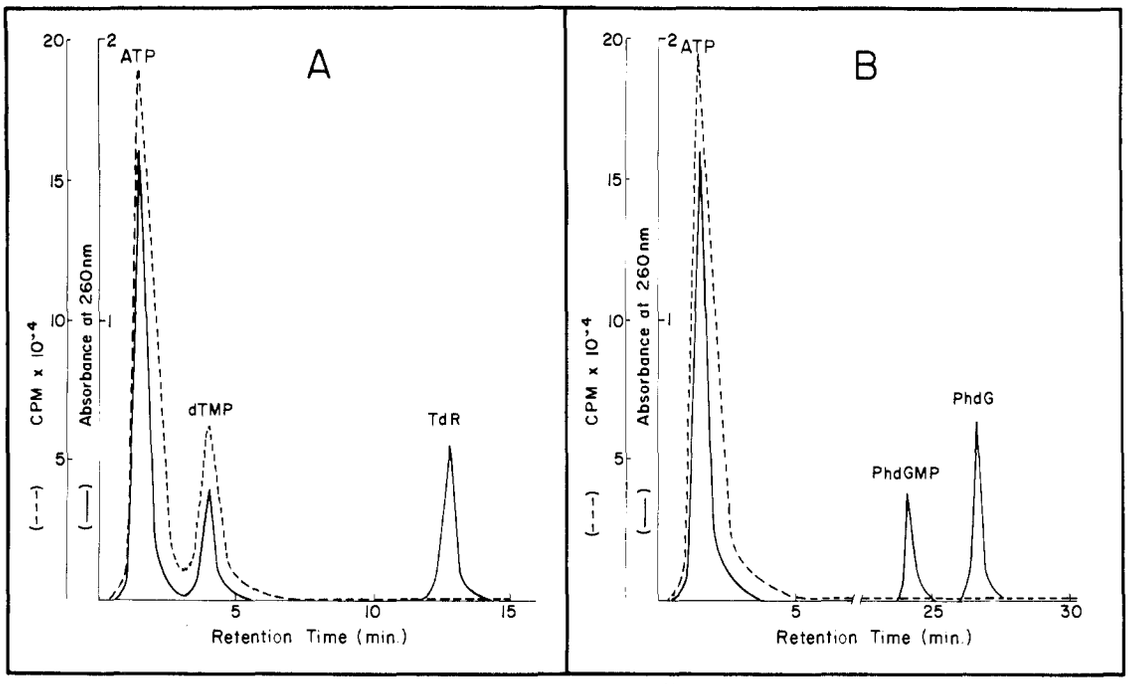


**Figure 2.** HPLC analysis of the products of incubation of HSV TK with [$\gamma$-$^{32}$P]ATP and either TdR or PhdG (7). The TK reaction was performed as described in the Experimental Section, except that 200 $\mu$M [$\gamma$-$^{32}$P]ATP ($3 \times 10^5$ cpm/nmol) was used as the labeled substrate in lieu of [$^3$H]TdR. The preparation and HPLC analyses of the samples were performed as described in the Experimental Section. In the experiment of panel A, unlabeled TdR was present at 80 $\mu$M and 7 was absent during enzyme incubation; after 60 min of incubation, TdR and dTMP were added as markers in a final concentration of 200 $\mu$M each. In the experiment summarized by panel B, TdR was absent during incubation and was replaced by 200 $\mu$M 7; after 2 h of incubation, 11 (PhdGMP) was added to a final concentration of 200 $\mu$M, and the mixture was heated for 5 min at 80 °C prior to analysis.

substrates subject to phosphorylation. The experimental approach involved incubation of HSV TK with [$\gamma$-$^{32}$P]ATP and inhibitor in conditions promoting formation of 5′-phosphorylated product and HPLC analysis of the mixtures to detect such a product. Because the results with both inhibitors were identical, only those obtained with the more potent inhibitor, **7**, are presented. As shown in panel B of Figure 2, which displays the chromatogram of the mixture containing **7**, no $^{32}$P was detectable in the position of the marker, PhdGMP (**11**). In contrast, in the chromatogram of the control mixture shown in panel A, label was clearly transferred from ATP to TdR, a natural substrate, to form dTMP. Considering that the analyses displayed in Figure 2 are capable of detecting 0.005% conversion of the nucleoside to its 5′-monophosphate, we conclude that **7** and **8** interact with the active site of HSV TK without significant conversion to their respective 5′-monophosphate forms.

**PhdG Is Not Highly Toxic for the Viral Host Cell.** Three lines of HeLa cells were employed for these experiments: wild type TK$^+$, its TK$^-$ mutant, and 5a, the TK$^-$ mutant transformed to the TK$^+$ phenotype by transfection with the HSV TK gene. Subconfluent, duplicate cultures of each were exposed to PhdG (**7**) at various concentrations and subjected every 6 h (for a total of 72 h) to phase microscopic examination and analysis of cell number. The results, which are not shown, indicated that **7** at a concentration as high as 3 $\mu$M had no effect on cell morphology or replication for at least 72 h. However, replication was inhibited by about 50% in all three cell lines after exposure of cells to 10 $\mu$M **7** for 72 h.

**PhdG Selectively Inhibits TdR Anabolism in Cells in Which Anabolism Is Dependent on HSV TK.** We next asked if the selective effect of **7** on HSV TK in vitro was also obtained in vivo, in the environment of the intact cell. To answer this question we exploited the wild type TK$^+$ HeLa line, its TK$^-$ mutant derivative, and 5a, a derivative of the latter line converted to the TK$^+$ phenotype by transfection with the HSVI TK gene. The approach examined the effect of **7** on the short-term incorporation of [$^3$H]TdR into cellular DNA. The results, which are shown in Figure 3, indicate that the compound inhibited incorporation only in the cell line in which it depended on HSV TK function (panel C). The results strongly suggest that **7** exerts a selective inhibitory effect on HSV TK, leaving the host TK to function essentially normally.

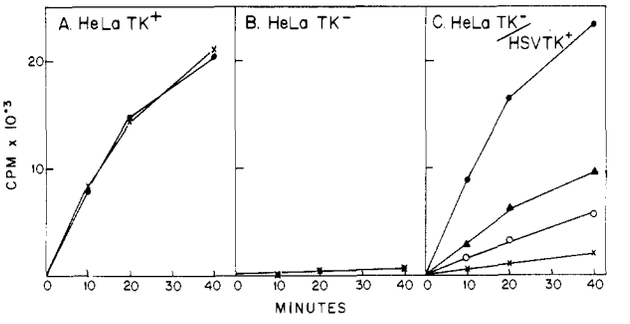


**Figure 3.** Effect of **7** on the incorporation of [$^3$H]TdR into DNA of HeLa TK$^+$, HeLa TK$^-$, and the TK$^+$ HSV TK-specific transfectant of HeLa TK$^-$. Incorporation of [$^3$H]TdR into subconfluent cultures was assayed as described in the Experimental Section. (●), control, no **7**; (▲), 5 $\mu$M **7**; (○), 10 $\mu$M **7**; (X), 50 $\mu$M **7**.

## Discussion

Our long-range goal with respect to HSV TK is to develop an agent that will prevent HSV replication in tissues—particularly nervous tissues—in which TK is required to supply dTTP for viral replication.[9,10] The agent we envision as ideal would have several specific properties to complement its action on viral TK; the most important include (a) resistance to metabolism—especially to cytotoxic nucleotide forms, (b) lack of toxicity for viral host

cells at virotoxic concentrations, (c) capacity to penetrate tissue and cells freely, and (d) capacity to inhibit both viral wild-type and mutant (for example, acycloguanosine-resistant) TKs.

The results presented in this paper indicate that $N^2$-phenyl-2′-deoxyguanosine, **7** (PhdG), is an acceptable structural starting point for the synthesis of the ideal agent. The compound is clearly selective for the viral TK, both in vitro (cf. table) and in its "natural" cellular biophase (Figure 3). PhdG, at a concentration above those capable of inhibiting HSV TK, displays some toxicity for the mammalian host cell upon prolonged exposure to the drug. This result is not surprising, because members of this class of compounds, especially **3** and **8**, are inhibitors of mammalian cell growth and DNA synthesis.[12] One approach to improve the capacity of the PhdG prototype to selectively inhibit HSV TKs is to increase the potency of the drug for isolated HSVI- and HSVII-specific TK, via manipulation of the structure of the $N^2$-substituent; this effort has already yielded simple *base* forms, which are as potent as the prototype nucleoside (results not shown).

Regardless of their ultimate applicability as clinically useful anti-herpes agents, **7** and related members of this $N^2$-substituted guanine family offer considerable promise as novel molecular probes of HSV TK structure and function, and they may complement studies with the 5′-ethynyl derivative of TdR, which has recently been shown[15] to be a potent and selective inhibitor of HSVI and HSVII TK. For example, the competitive action of PhdG (cf. Figure 1) clearly recommends it and its derivatives as novel tools with which to probe the structure and function of the active site of HSV TK. And because these purine-based agents can inhibit HSV TK in its normal intracellular environment (cf. Figure 3), they may, like 5′-ethynyl-TdR, be useful as TK-specific reagents with which to isolate novel mutant forms of HSV TK and to dissect its role in HSV virogenesis and pathogenesis.

## Experimental Section

The following compounds listed in the table were synthesized as described previously: **3**, **8**, **10**, and 11 (ref 11); **5**, **13**, and **14** (ref 12); **6** (ref 16). All new compounds were fully characterized by $^1$H NMR and elemental analyses (C, H, N, P); analyses were done by the Microanalysis Laboratory, University of Massachusetts, Amherst, MA, and agree to ±0.4% of calculated values. NMR spectra were determined with a Bruker WM 250 instrument, and UV analyses were done with a Gilford Response spectrophotometer. Melting points were determined with a Mel-temp apparatus and are uncorrected. Preparative HPLC was done with a Waters Model 6000 pump and differential refractometer detector. Unlabeled nucleotides were obtained from Boehringer. [$^3$H-CH$_3$]TdR, [5-$^3$H]CdR, and [$^{32}$P]ATP were from New England Nuclear. All other chemicals were at least of reagent grade.

**$N^2$-Phenylguanines** were prepared from 2-bromohypoxanthine and the appropriate amine in refluxing 2-methoxyethanol as described.[11] $N^2$-Phenylguanine (**1**) was obtained in 60% yield after crystallization from methanol: mp 342–344 °C; $^1$H NMR ($Me_2SO$-$d_6$) $\delta$ 12.78 (s, 9-H), 10.53 (s, 1-H), 8.64 (s, 2-NH), 7.70 (s, 8-H), 7.34 (m, Ph). Anal. ($C_{11}H_9N_5O\cdot{}^1/_6H_2O$) C, H, N. $N^2$-*p*-Tolylguanine (**2**) was obtained in 69% yield by crystallization from dimethylformamide (DMF)/water: mp 332–335 °C; $^1$H NMR ($Me_2SO$-$d_6$) $\delta$ 12.73 (s, 9-H), 10.45 (s, 1 -H), 8.51 (s, 2-NH), 7.81 (s, 8-H), 7.51 (d, $J$ = 7.8 Hz, 2′,6′-H), 7.13 (d, $J$ = 7.8 Hz, 3′,5′-H), 2.27 (s, $CH_3$). Anal. ($C_{12}H_{11}N_5O\cdot{}^1/_3$DMF) C, H, N. $N^2$-(*m*-Ethylphenyl)guanine (**4**) was isolated in 59% yield after crystallization from 25% aqueous DMF: mp 310–312 °C; $^1$H NMR ($Me_2SO$-$d_6$) $\delta$ 12.76 (s, 9-H), 10.48 (s, 1-H), 8.56 (s, 2-NH), 7.81 (s, 8-H), 7.1–7.5 (m, 2′,5′,6′-H), 6.87 (d, $J$ = 7 Hz, 4′-H), 2.62 (q, $CH_2$), 1.20 (t, $CH_3$). Anal. ($C_{13}H_{13}N_5O$) C, H, N.

**$N^2$-Phenyl-9-(2-deoxy-β-D-ribofuranosyl)guanine (7)** was prepared by the following multistep sequence: (a) A cold solution of **1** (10 g, 44 mmol) in DMF (140 mL) was treated dropwise with thionyl chloride (10.3 mL, 88 mmol). After the solution was warmed for 1 h at 50 °C, the cooled reaction mixture was added slowly to a solution of sodium bicarbonate (15 g) in water (150 mL). Excess sodium bicarbonate was added to raise the pH to 6, and the solution was chilled for 24 h. The precipitate was collected and dissolved in 60 mL of concentrated ammonium hydroxide with warming. After the mixture stood at room temperature for 3 days, the yellow solid was collected and washed with water to give 8.0 g (74%) of 2-anilino-6-chloropurine. Crystallization from ethanol gave an analytical sample: mp 155–160 °C; $^1$H NMR ($Me_2SO$-$d_6$) $\delta$ 8.23 (s, 8-H). Anal. ($C_{11}H_8N_5Cl\cdot{}^1/_3H_2O$) C, H, N.

(b) A mixture of 2-anilino-6-chloropurine (6.75 g, 27.4 mmol) and sodium hydride (1.44 g, 30 mmol; 50% suspension in mineral oil) in dry acetonitrile (200 mL) was stirred at room temperature for 20 min. 2-Deoxy-3,5-di-*p*-toluoyl-β-D-ribofuranosyl chloride (10.5 g, 27.4 mmol) was added during 20 min, and the solution was stirred for 2 h. An equal volume of chloroform was added, and, after filtration through Celite, the solvents were removed, leaving a yellow semisolid. This residue, in several portions, was purified by HPLC (silica gel, 10 μm; 25 mm × 50 cm column) with 5% acetone in toluene as eluant at a flow rate of 11.25 mL/min. The major product, the 9-β isomer, was eluted in 50 min: 7.0 g (43%); mp 190–192 °C. Anal. ($C_{32}H_{28}N_5O_5Cl\cdot{}^1/_2H_2O$) C, H, N. The minor isomer, presumably 7-β, was eluted in 124 min (1.61 g, 10%).

(c) The major nucleoside product from step b above was deblocked by treatment with sodium methoxide (20 mmol) in methanol (350 mL). After being left to stand at room temperature for 2.5 h, the solution was neutralized with glacial acetic acid, and the solvent was removed. Chromatography of the residue on silica gel with 10% ethanol in chloroform and crystallization from concentrated ammonium hydroxide/ethanol gave 3.0 g (71%) of 2-anilino-6-chloro-9-(2-deoxy-β-D-ribofuranosyl)purine: mp 178–180 °C; $^1$H NMR ($Me_2SO$-$d_6$) $\delta$ 9.90 (s, 8-H), 6.34 (t, 1′-H, $J_{av}$ = 6.6 Hz), all other resonances as expected. Anal. ($C_{16}H_{16}N_5O_3Cl$) C, H, N.

(d) A solution of the 6-chloronucleoside in ethanol (150 mL) was treated with 2-mercaptoethanol (2.9 mL) and a solution of 1 N sodium methoxide in methanol (12.6 mL). After being heated at reflux for 72 h, the solution was diluted with an equal volume of water, adjusted to pH 5 with glacial acetic acid, and chilled for 72 h. The colorless precipitate was collected and washed with water to give 1.76 g (62%) of **7**. The product can be crystallized from methanol to give colorless crystals: mp 228–231 °C; $^1$H NMR ($Me_2SO$-$d_6$) $\delta$ 10.56 (s, 1-H), 8.75 (s, 2-NH), 8.03 (s, 8-H), 7.0–7.6 (m, Ph), 6.25 (t, $J_{av}$ = 6.7 Hz, 1′-H), 5.28 (d, 3′-OH), 4.88 (t, 5′-OH), 4.38 (m, 3′-H), 3.87 (t, 4′-H), 3.56 (m, 5′,5″-H), 2.55 (m, 2′-H), 2.32 (m, 2″-H); UV (pH 12.5) $\lambda_{max}$ 283 nm ($\epsilon$ 21 700). Anal. ($C_{16}H_{17}N_5O_4\cdot{}^1/_6H_2O$) C, H, N.

**$N^2$-*p*-Tolyl-9-β-D-ribofuranosylguanine (9)** was obtained by glycosylation of **2** with tetra-*O*-acetylribose as described.[11] Crystallization of the protected (acetylated) products from ethanol gave the 9-β isomer in 63% yield. Deblocking with saturated ammonia in methanol gave a quantitative yield of **9**: mp 244–246 °C; $^1$H NMR ($Me_2SO$-$d_6$) $\delta$ 9.1 (s, 2-NH), 8.02 (s, 8-H), 7.52 (d, $J$ = 7.5 Hz, Ph 2,6-H), 7.15 (d, $J$ = 7.5 Hz, Ph 3,5-H), 5.76 (d, $J$ = 5.7 Hz, 1′-H), 4.51 (2′-H), 4.10 (3′-H), 3.90 (4′-H), 3.57 (m, 5′,5″-H), 2.27 (s, $CH_3$); UV (pH 10) $\lambda_{max}$ 274.5 nm ($\epsilon$ 13 840). Anal. ($C_{17}H_{19}N_5O_5\cdot H_2O$) C, H, N.

**$N^2$-Phenyl-9-(2-deoxy-β-D-ribofuranosyl)guanine 5′-monophosphate (11)** was prepared by heating at reflux a solution of 2-bromo-2′-deoxyinosine 5′-phosphate (Freese, S.; Wright, G., to be published) with a 10-fold excess of aniline in 0.1 M phosphate buffer (pH 6.1) containing 17% methanol. After 3 h the cooled reaction mixture was applied to a Sephadex A-25 column (Pharmacia), and the product was eluted with a gradient of triethylammonium bicarbonate (0.2–1 M). Fractions containing the product were evaporated and lyophilized to give **11** as the mono(triethylammonium) salt in 50% yield: $^1$H NMR ($D_2O$) $\delta$ 8.09 (s, 8-H), 6.40 (t, 1′-H, $J_{av}$ = 6.86 Hz), all other resonances

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as expected; $^{31}P$ NMR ($D_2O$) $\delta$ 3.9. Anal. ($C_{22}H_{23}N_5O_7P$) P.

**Cells and Viruses.** The cells were *Mycoplasma*-free lines derived from HeLa S3. The wild-type HeLa S3 line, its TK-deficient ($TK^-$) derivative, and the HeLa $TK^-$/HSV $TK^+$ line, 5a, were obtained from Dr. G. Della Valle of the University of Pavia. HeLa 5a is a derivative of HeLa $TK^-$ stably transformed to the $TK^+$ phenotype with a functional copy of the HSVI TK gene. Herpes simplex virus HSVI (17 $sym^+$ strain) was provided by Dr. P. Pignatti, University of Verona.

**Culture of Cells for Preparation of TK.** Cells were grown in monolayer culture in 175-$cm^2$ plastic flasks in Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal calf serum; the serum was dialyzed and subsequently heated to 56 °C for 1 h prior to use. HSVI (17 $sym^+$)-infected HeLa $TK^-$ served as the source of viral TK. Confluent monolayers of $TK^-$ cells were incubated with virus for 1 h at 37 °C in DMEM in the absence of serum, with a multiplicity of 10 PFU per cell. Infected monolayers were rinsed with warm phosphate-buffered saline (PBS, 50 mM potassium phosphate, pH 7.4, 150 mM NaCl) to remove unadsorbed virus and returned to complete medium. After 20 h of incubation, the cells were rinsed and suspended in PBS with a rubber policeman and collected by centrifugation at 4 °C. HeLa $TK^+$, cells, which were used as the source of host TK, were grown to 80% confluence, rinsed with cold PBS, and harvested by the procedure used for virus infected cells.

**TK Assay.** The host- and virus-specific enzymes were assayed by a method adapted from that of Cheng and Ostrander;[4] 0.1–0.4 milliunits of enzyme were incubated at 37 °C for 30 min in 50 $\mu$L of a mixture containing 30 mM HEPES-$K^+$, pH 7.5, 6 mM $MgCl_2$, 6 mM ATP ($Mg^{2+}$ salt), 0.5 mM dithioerythritol (DTE), and 10 $\mu$M [$^3$H]TdR (sp act 25 Ci/mmol) or 100 $\mu$M [$^3$H]CdR (sp act 2.5 Ci/mmol). The reaction was terminated by spotting 45 $\mu$L of the incubation mixture on a 25 mm DEAE paper disk (DE81 paper; Whatman). The disk was immediately immersed and washed in an excess of 1 mM ammonium formate (pH 5.6) to remove unconverted nucleoside. The disk was washed with distilled water and finally ethanol. Radioactive TMP was estimated by scintillation counting of the disk in 5 mL of Omnifluor (New England Nuclear). One unit of TK is defined as the amount of TK that, under the above conditions, produced dTMP at the rate of 1 nanomol/min.

**Enzyme Purification.** Both the virus- and the host-specific TKs were purified from extracts of their respective sources by the procedure described below; an extract of uninfected HeLa $TK^-$ cells subjected to this procedure yielded no detectable TK activity. All procedures were carried out at 4 °C, and *all buffers* contained phenylmethanesulfonyl fluoride (PMSF) at 1 mM and DTE at 0.5 mM. Protein concentrations of extracts and enzyme fractions were estimated with the Bio-Rad assay reagent.

Cells were centrifuged from PBS to yield a firm pack of 0.5 g. The pack was suspended in 4 mL of buffer A (5 mM potassium phosphate, pH 7.0, and 1 mM $MgCl_2$). After 10 min on ice, the suspension was homogenized in a Dounce homogenizer with a loose fitting pestle. The homogenate was centrifuged at 1000*g* for 10 min to yield a nuclear pellet and a cloudy supernatant. The supernatant was saved, and the pellet was washed twice by centrifugation from 3 mL of buffer A containing 0.5% NP40. The two washes were combined with the supernatant, and the resulting fraction was centrifuged for 10 min at 2000*g*. The pellet was discarded, and the supernatant was applied to a 0.8 × 5 cm (2.5-mL bed volume) column of DEAE-cellulose (DE52, Whatman), which had been equilibrated with buffer B (20 mM potassium phosphate, pH 7.0). The column "flowthrough", which contained the enzyme, was immediately applied to a 1.5 mL (0.8 × 3 cm) column of phosphocellulose (P11, Whatman) equilibrated with buffer C (buffer B containing 20% glycerol). The column was washed with two volumes of buffer C and subjected to elution in the same buffer, with a linear gradient of potassium phosphate, pH 7.0 (20–600 mM; total volume 15 mL; 3 mL/h, 0.25-mL fractions). TK activity emerged in a single symmetrical peak at 250 mM phosphate. The peak fraction and the two flanking fractions were pooled and mixed with bovine serum albumin (BSA; Calbiochem, A grade; heated at 65 °C for 60 min) and glycerol to give concentrations of, respectively, 3 mg/mL and 50% (v/v). The latter preparation could be stored at −80 °C for at least 6 months without significant loss of activity. The specific activity of the host and viral enzymes was approximately 80 and 100 nmol/min per mg, respectively; the latter values corresponded to a purification of approximately 100-fold for each enzyme.

**High-Pressure Liquid Chromatography.** Nucleotides and nucleosides were separated by a reverse-phase method employing the Bio-Rad 100 MAPS preparative system. A 0.4 × 15 cm reverse-phase $C_{18}$ BioSil ODS-5S column was used in the following conditions: injection volume, 20 $\mu$L; detection, UV 260 nm; temperature, ambient; eluents, buffer A (20 mM $KH_2PO_4$, pH 5.6), buffer B (20 mM $KH_2PO_4$, pH 5.6, 60% MeOH). The specific conditions for separation of ATP from dTMP and TdR or ATP from **7** and its 5′-monophosphate (**11**) included: from 0–20 min a gradient from 100% buffer A to 77% buffer A/23% buffer B; from 20–30 min 100% buffer B. The flow rate was normally 1 mL/min; when [$^{32}$P]ATP was to be collected, flow was reduced to 0.5 mL/min.

**Incorporation of [$^3$H]TdR into DNA.** Cells were grown at 37 °C in suspension in spinner flasks to a density of $10^6$ cells/mL. [$^3$H]TdR (sp act 25 Ci/mmol) was added to a concentration of 33 $\mu$Ci/mL, and incubation was continued. At 10, 20, and 40 min, 0.08-mL samples of culture were spotted on 25-mm GF/C (Whatman) filters. The filters were immersed immediately after spotting in a large volume of ice-cold 5% trichloroacetic acid (TCA) containing 10 mM $Na_4P_2O_7$. The filters were washed successively in TCA and ethanol, dried, and counted in Omnifluor-based scintillation fluid. Incorporation of [$^3$H]TdR was inhibited more than 98% by the inclusion of 10 $\mu$g/mL aphidicolin during the labeling period. The radioactivity in cold TCA-insoluble material remained completely insoluble in cold TCA following digestion in 0.5 M NaOH for 1 h at 37 °C.

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**Registry No.** 1, 40769-49-1; **2**, 57338-66-6; **3**, 114300-69-5; **4**, 114300-70-8; **5**, 104715-65-3; **6**, 83173-13-1; **7**, 114300-71-9; **8**, 87781-96-2; **9**, 77976-95-5; **10**, 87781-99-5; **11**, 114300-72-0; **11** triethyl amine salt, 114300-73-1; **12**, 87782-03-4; **13**, 104715-80-2; **14**, 99304-82-2; 2-bromohypoxanthine, 87781-93-9; aniline, 62-53-3; *p*-toluidine, 106-49-0; *m*-ethylphenylamine, 587-02-0; 2-anilino-6-chloropurine, 114300-74-2; 2-anilino-6-chloropurine sodium salt, 114300-75-3; 2-deoxy-3,5-di(*p*-toluoyl)-β-D-ribofuranosyl chloride, 52304-86-6; 9-(2-deoxy-3,5-di(*p*-toluoyl)-β-D-ribofuranosyl)-2-anilino-6-chloropurine, 114300-76-4; 7-(2-deoxy-3,5-di(*p*-toluoyl)-β-D-ribofuranosyl)-2-anilino-6-chloropurine, 114300-77-5; 2-anilino-6-chloro-9-(2-deoxy-β-D-ribofuranosyl)purine, 114300-78-6; tetra-*O*-acetylribose, 30571-54-1; 9-(triacetyl)-β-D-ribofuranosyl)-$N^2$-(*p*-tolyl)guanosine, 77976-96-6; 2-bromo-2′-deoxyinosine 5′-phosphate, 114300-79-7; thymidine kinase, 9002-06-6.