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Inhibitors of *Bacillus subtilis* DNA Polymerase III. Structure–Activity Relationships of 6-(Phenylhydrazino)uracils

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Received March 16, 1977

6-(Phenylhydrazino)uracils inhibit the replication-specific enzyme DNA polymerase III of *Bacillus subtilis* by forming a strong, reversible complex with template–primer DNA and enzyme. The phenyl ring interacts with a hydrophobic enzyme site which, on the basis of structure–activity relationships of substituted analogues, appears to possess the following characteristics: (1) planarity or near-planarity; (2) a finite capacity to accommodate bulky substituents; and (3) location near the domain of the enzyme active site. A mutant DNA polymerase III, derived from a mutant strain of *B. subtilis* selected for resistance to 6-(*p*-hydroxyphenylazo)pyrimidines, is resistant only to inhibitors bearing *p*-hydroxy or amino groups and is hypersensitive to inhibitors containing nonpolar substituents; these results suggest the existence of mutable, secondary regions of the binding site which interact with para substituents and, thus, influence the strength of the primary phenyl–enzyme interaction.

6-(Arylazo)pyrimidine antimicrobials selectively inhibit replicative DNA synthesis in gram-positive bacteria by inhibiting, specifically, the replication-specific enzyme, DNA polymerase III (pol III).[1-3] The azo compounds are not active per se but are activated in vivo by reduction to hydrazino (H_2) forms.[4] The arylhydrazinopyrimidines act, in part, as analogues of purine deoxyribonucleoside triphosphates by pairing with pyrimidine bases in template DNA. The specific purine character of an inhibitor is dictated by the substitution pattern of its pyrimidine ring; for example, 6-(*p*-hydroxyphenylhydrazino)uracil (H_2-HPUra, 1) is competitive with deoxyguanosine 5′-triphosphate (dGTP), pairing specifically with cytosine in the template, and 6-(*p*-hydroxyphenylhydrazino)-2-amino-4-pyrimidone (H_2-HPiCyt, 2) is competitive with deoxyadenosine 5′-triphosphate (dATP), pairing specifically with thymine in the template[4] (see Figure 1). Although H_2-HPUra and H_2-HPiCyt mimic dGTP and dATP, they do not act simply as conventional substrate analogues. They actually inhibit by reacting reversibly with an inhibitor-specific binding site on the enzyme, linking template *and* enzyme in a strong, catalytically inactive ternary complex.[5,6] A model for the ternary complex is depicted in Figure 2.

Examination of the properties of drug-resistant enzymes[6,7] isolated from spontaneous *Bacillus subtilis* mutants resistant to HPUra and HPiCyt has yielded valuable information on the nature of the inhibitor binding

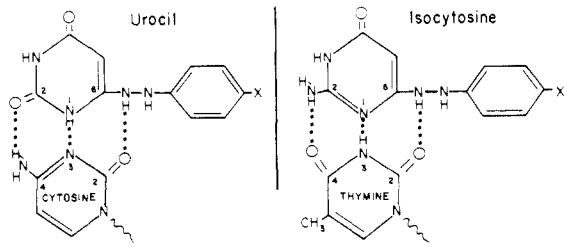


Figure 1. Hydrogen bonding between inhibitors and template pyrimidines. Left panel: H_2-HPUra (1, X = OH) and cytosine. Right panel: H_2-HPiCyt (2, X = OH) and thymine.

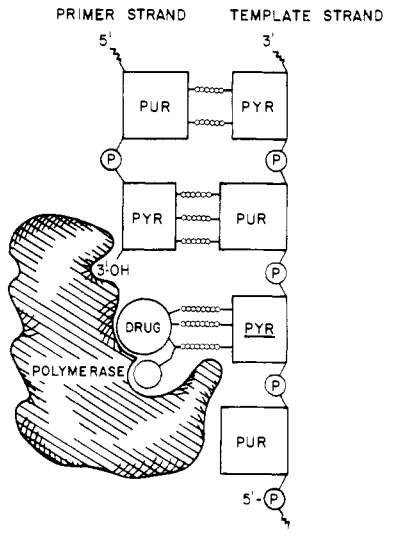


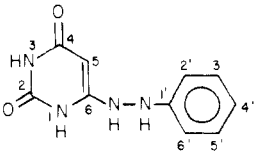
Figure 2. Schematic model of a drug–template:primer–enzyme complex: PUR, adenine, guanine; PYR, cytosine, thymine; DRUG, a 6-(phenylhydrazino)uracil; PYR, cytosine; POLYMERASE, *B. subtilis* DNA polymerase III.

site. The resistant enzymes are 30–50-fold less sensitive to H_2-HPUra and H_2-HPiCyt than wild-type pol III but are unaltered with respect to K_m for deoxyribonucleotide substrates, suggesting that the inhibitor binding site is not identical with the enzyme active site.

We have previously exploited synthesis of arylhydrazinopyrimidines to examine the effects of substituents in the phenyl ring[8] and in the pyrimidine ring[9] on inhibitor activity; these results have been useful to the dissection of drug mechanism and have prompted us to continue synthesis and examination of the activity of a variety of novel inhibitor derivatives. This effort has three general objectives: first, to discern in more detail the nature of the immediate environment of the inhibitor binding site of pol III; second, to prepare irreversible inhibitors with which to identify and isolate the active site region of the enzyme, and, third, to obtain structure–activity relationships which will facilitate the design of therapeutically useful antibacterial compounds. This paper addresses the first objective and reports substituent effects on the inhibitory activity of 6-(phenylhydrazino)uracils; subsequent papers will deal with the effects of alterations in the hydrazino group and in the pyrimidine ring.

Inhibitor Assays. The polymerase assay used in this study (cf. Experimental Section for details) is a truncated reaction in which dGTP is deleted; it employs a DNAase-treated DNA which is rich in short regions of unpaired template (5–50 nucleotide residues) and, therefore, in sufficient concentration, allows pol III to catalyze the incorporation of considerable amounts of [^{3}H]-TMP despite the absence of one or more of the other three deoxyribonucleotides. This "truncated" assay has

Table I. Inhibitory Activity of 6-(Phenylhydrazino)uracils on *B. subtilis* DNA Polymerase III



Compd	Substituents	K_i, μM[a]
1	4'-OH	0.4
3		1.4
4	4'-Me	0.5
5	4'-Et	1.6
6	4'-*n*-Bu	2300
7	4'-F	3.0
8	4'-Cl	0.9
9	4'-Br	0.7
10	4'-NH_2	7.1
11	4'-NHAc	560
12	4'-NO_2	26.3
13	4'-CO_2H	98
14	4'-SO_2Me	209
15	3'-Me	2.5
16	3'-F	0.7
17	3'-Cl	1.1
18	3'-Br	1.4
19	2'-F	7.2
20	2'-Me	234
21	2'-Et	562
22	3',4'-Me_2	1.4
23	3',4'-Cl_2	0.6
24	3'-Cl-4'-Me	0.8
25	3'-Me-4'-OH	51.2
26	3'-Cl-4'-OH	8.8
27	3'-Cl-4'-OH	24.2
28	3',5'-Me_2-4'-OH	124
29	1'-NMe	480
30	1-Me	Inactive[b]
31	3-Me	1.6
32	3-Me-4'-OH	0.5
6-Aminouracil		Inactive
6-Hydrazinouracil		Inactive

[a] K_i is the concentration of inhibitor giving 50% inhibition of enzyme activity in truncated DNA synthesis, i.e., –dGTP (see Experimental Section and ref 6 for details). [b] Compounds which produce less than 10% inhibition at 1 mM are considered inactive.

two important advantages for the examination of arylhydrazinopyrimidine action. First, it is devoid of competitor nucleotide and, therefore, provides a very sensitive method for screening the activity of weak inhibitors, and, second, it permits convenient, direct determination of K_i, the concentration of inhibitor required to achieve 50% inhibition.

Results

The values of K_i presented in Table I demonstrate a wide range of inhibitory activities of substituted 6-(phenylhydrazino)uracils. The unsubstituted analogue, **3**, is itself a potent pol III inhibitor, only slightly weaker than the most potent compound, **1**. The relatively narrow range of K_i for inhibitors with small, nonpolar meta or para substituents indicates that most of the inhibitor–enzyme binding energy results from interaction of the enzyme with the phenyl ring itself. The phenyl group is essential because 6-aminouracil and 6-hydrazinouracil have no inhibitory activity.

Previously, it was demonstrated[6,9] by direct experimentation involving gel filtration of mixtures of DNA and enzyme that K_i values of inhibitors are essentially identical with K_{cx}, a constant describing the drug concentration required to induce half-maximal DNA–enzyme complex

formation. Because the limiting reaction component under assay conditions is the enzyme, the reciprocal of K_i can be used as a binding constant to calculate the free energy of inhibitor–enzyme binding, $\Delta G = -RT \ln (1/K_i)$. The free energy of binding for **3** is −8100 cal/mol, a value considerably larger than −4600 cal/mol, the maximum contribution of a benzene ring to hydrophobic binding.[10] The difference may reflect the contribution from binding of the phenyl ring with the enzyme, although the contributions of other inhibitor moieties to binding cannot yet be excluded.

Para-Substituted Compounds. The *p*-Me group of **4** contributes ca. −620 cal/mol to the binding free energy relative to **3**; this value is commensurate with that established[11] for hydrophobic binding of a CH_2 group (−700 cal/mol). Halogens (Cl, Br) contribute less to binding than methyl, whereas F (compound **7**) decreases binding twofold compared to **3**. These data suggest that hydrophobic binding of para substituents increases inhibitory activity while inductive withdrawal of electrons from the phenyl ring decreases it. Large substituents rapidly decrease activity, probably by a steric effect. Thus, the *p*-*n*-Bu (**6**) and *p*-NHCOMe (**11**) analogues are rather weak inhibitors.

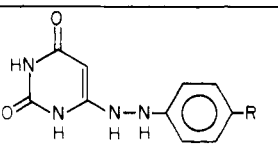
Inhibitors with electron-withdrawing groups in the para position (compounds **12–14**) show decreased activity compared to **3**, probably resulting from both steric and electronic effects. The negative effects of these groups on activity may result largely from resonance interaction with the 1′-NH of the hydrazino group, which could increase rotational energy barriers of the phenyl ring[8] (see Discussion).

The polar, hydrogen-bonding hydroxyl and amino groups impart anomalous inhibitory activities. The *p*-OH derivative, **1**, is the most potent inhibitor in this series whereas the *p*-NH_2 analogue, **10**, is considerably weaker. The potency of **1** is not in accord with observed contributions of hydrophobic substituents but could result, in part, from resonance release of an electron pair from OH to the phenyl ring. We believe, however, that the uniqueness of the *p*-OH group is derived mainly from its ability to hydrogen bond with an enzyme moiety or "secondary" binding site which is distinct from the primary site of binding with the phenyl ring, but which can also accommodate hydrophobic substituents. The relative weakness of **10** can be explained by assuming that the NH_2 group in a rigidly fixed drug–enzyme complex cannot hydrogen bond to this secondary binding site as efficiently as the OH group and may be exposed to a hydrophobic enzyme region. Limited access of the *p*-NHCOMe group of **11** to hydrogen bonding with this site may also account, in part, for its weak activity. Further evidence for the existence of a "secondary" binding site is derived from inhibitor studies with a "drug resistant" enzyme (see below).

Meta-Substituted Compounds. The meta substituents of compounds **15–18** contribute little to inhibitor–enzyme binding. Interestingly, the order of increasing activity in this series is Me < Br < Cl < F, opposite to that in the para-substituted series. Resonance donation of an electron pair by F may explain the enhanced binding observed for **16**.

Meta,Para-Disubstituted Compounds. Hydrophobic groups in the 3′,4′ positions of compounds **22–24** exert additive effects which slightly enhance inhibitor–enzyme binding. In contrast, substituted *p*-OH compounds (**25–27**) and a disubstituted *p*-OH compound (**28**) show marked decreases in activity relative to that of the *p*-OH inhibitor itself (**1**). These substituents ortho to the OH group may interfere with hydrogen bonding of OH with the secondary binding site, exposing it to a hydrophobic enzyme region.

Table II. K_i of Inhibitors against Wild-Type Pol III and Pol III/azp-12



Compd	R	K_i, μM: Wild-type pol III	K_i, μM: Pol III/azp-12
1	OH	0.4	20
3	H	1.4	2.1
4	Me	0.5	0.4
10	NH_2	7.1	76.4

Table III. Inhibitory Activity of 6-(Phenylhydrazino)uracils against Wild-Type Pol III and Pol III/azp-12

Compd	Substituent[a]	% inhibn at 20 μM drug: Wild-type pol III	% inhibn at 20 μM drug: Pol III/azp-12
7	4′-F	82	93
8	4′-Cl	91	95
9	4′-Br	82	95
15	3′-Me	80	85

[a] Refer to the structure of Table I for numbering system.

Ortho-Substituted and 1′-NMe Compounds. Substituents ortho to the hydrazino group (compounds **19–21**) decrease inhibitory activity; with the exception of the *o*-F analogue, **19**, this decrease is dramatic. The negative effect of *o*-alkyl groups may result from repulsion of these groups by the enzyme or from elevation of the energy barrier to rotation of the phenyl ring about the phenyl–N bond.

Methylation of the N atom adjacent to the phenyl ring (cf. compound **29**) also causes a marked decrease in activity; this cannot be due to loss of enzyme binding to the 1′-NH group, because this group can be replaced by a CH_2 group (see following paper) without changing inhibitory activity.

On the basis of these results and the observed effects of electron-withdrawing groups, we believe that relatively free rotation about the phenyl–N and N–N bonds is essential to the formation of an active, inhibitory conformation of drug molecules (see Discussion for further details).

1- and 3-Methyl Derivatives. The 1-Me analogue, **30**, does not inhibit pol III, thereby supporting the requirement of the model of Figure 1 that the 1-H atom participate in pairing with cytosine. The observation that 3-Me analogues, e.g., **31** and **32**, are fully potent inhibitors indicates that the 3-H atom is *not* involved in the pairing interaction with template DNA, nor is it involved in binding with the enzyme.

Inhibition of the "Drug-Resistant", Mutant Polymerase, Pol III/azp-12. Pol III/azp-12 was isolated[6] from a *B. subtilis* mutant selected for spontaneous resistance to the *p*-OH inhibitors, HPUra and HPiCyt. The data of Table II indicate that pol III/azp-12 is 50 times less sensitive to **1** than the wild-type enzyme, *equi*sensitive to the unsubstituted inhibitor, **3**, and *hyper*sensitive to the *p*-Me derivative, **4**. Hypersensitivity of pol III/azp-12 is not restricted to **4**; the data of Table III show this to be the response to all inhibitors which bear meta or para

hydrophobic substituents. These observations suggest that mutation effects a relatively specific repulsion of the *p*-OH group, leaving the primary binding capacity of the phenyl ring unaffected. Indeed, the mutation apparently affects, with considerable specificity, a portion of the secondary binding site, which we postulated above to interact with the *p*-OH group. Mutation of a hydrogen-bonding group in this site to a hydrophobic group would be commensurate with hypersensitivity of pol III/azp-12 to hydrophobically substituted inhibitors. This mutation also would be expected to confer resistance to inhibitors bearing polar groups other than hydroxyl; this prediction seems sound, because pol III/azp-12 is tenfold less sensitive to the *p*-NH_2 inhibitor, **10**, than the wild-type enzyme (see Table II).

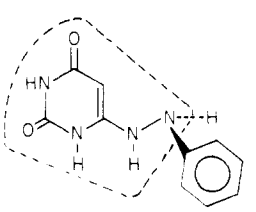
Discussion

Several features of the inhibitor-binding site of pol III are suggested on the basis of the structure–activity relationships described above. First, the binding site interacts with the phenyl ring of the inhibitors. Second, this site is hydrophobic, probably planar, and possesses a strictly limited capacity. Third, the location and orientation of the binding site is complementary to an "active conformation" of the inhibitor phenyl ring. Finally, an additional enzyme moiety, the "secondary" binding site, interacts weakly with substituents in the phenyl ring and may represent the locus of mutations in drug-resistant enzymes.

The inactivity of 6-amino- and 6-hydrazinouracils, each of which can potentially hydrogen bond to template cytosine, indicates that the major source of inhibitor–enzyme binding is the phenyl ring. Hydrophobic substituents provide little additional inhibitor potency; minor gains are offset by group size which, when excessive, rapidly diminishes enzyme binding. These observations and the results of calculation of binding free energies (see above) suggest that phenyl–enzyme binding may involve van der Waals or possibly charge-transfer interactions with an amino acid side chain.

Substituents which would be expected to alter ground-state conformation and to elevate barriers to rotation about the hydrazino N–N and/or N–phenyl bonds have profound negative effects on inhibitor activity. When an inhibitor molecule is hydrogen bonded with cytosine in the DNA template, all of the atoms depicted within the dotted lines of structure **33** must be coplanar to allow maximum "Watson–Crick" pairing. Consideration of inhibitor chemistry and direct examination[12] of the crystal structure of **1** show this to be the case. In the solid state, the hydrazino substituents adopt an extended conformation[12] as depicted in structure **33**. We do not believe this to be the active conformation of inhibitors in solution. We proposed previously[8] that inhibitors adopt a conformation in which the pyrimidine and phenyl rings are more nearly cis to each other (cf. structure **34**). Several fragments of evidence strongly support the latter proposal. First are the negative effects of para electron-withdrawing groups, *o*-alkyl groups and a 1′-NMe group on inhibitor–enzyme binding. Second is the observation that uracil 6-hydrazones such as **35**, which cannot adopt the conformation of structure **34**, are inactive as pol III inhibitors (N. C. Brown, unpublished results). Third and most convincing is our recent observation that substituted 6-anilinouracils, **36**, which can be considered rigid analogues of structure **34**, are active inhibitors of pol III (see following paper).

The results shown in Tables II and III clearly demonstrate that the mutation generating pol III/azp-12 affects *only* the interaction of the *p*-OH group of **1** and the



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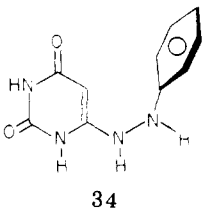

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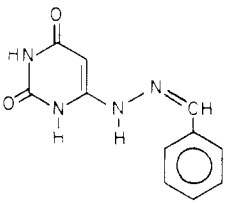

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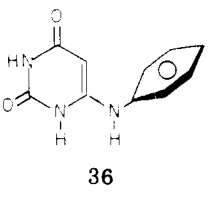

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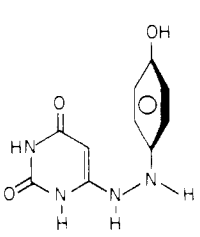
p-NH_2 group of **10** with the enzyme. Because mutation does not affect binding of the phenyl group (cf. activities of compound **3**, Table II), we have postulated that it affects a secondary binding site. This is probably the same enzyme region independently suggested on the basis of structure–activity relationships of para-substituted inhibitors. A simple mechanism explaining this selective resistance is the change from a polar, hydrogen-bonding amino acid moiety in wild-type enzyme to a hydrophobic moiety in pol III/azp-12.

The existence of a secondary mutable, hydrogen-bonding moiety in the wild-type enzyme, coupled with the probability that the *p*-OH group of **1** in its active conformation (**37**) is located near space normally occupied by the deoxyribose ring of dGTP (**38**), suggests that this moiety may comprise part of the enzyme active site. It was recently found[13] that a mutant pol III of the azp-12 type is also resistant to the inhibitory action of the arabinonucleotides, *ara*-ATP (**39**) and *ara*-CTP. These compounds differ



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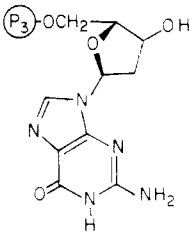

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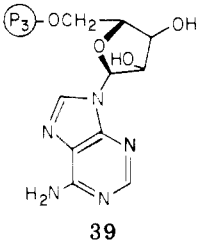

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from deoxyribonucleotide substrates in the presence of a β-OH group at C-2′ of the sugar ring. Thus, a mutation in polymerase which simultaneously repels an arabinonucleotide and the *p*-OH of **1** conceivably affects a region of the enzyme which normally encounters the deoxyribose ring of substrates. This might appear unlikely in view of the lack of effect of the mutation on substrate binding;[6] however, it is possible that a mutation causing an increase in substrate K_m would not be expressed, because such an enzyme could not satisfactorily perform its essential role in vivo in replicative DNA synthesis.

It is not clear how a mutation in the active site region of DNA polymerase III could repel certain inhibitors without affecting substrate binding. Our further studies on inhibitor–enzyme binding and the successful design and use of irreversible inhibitors may help to resolve these problems.

Experimental Section

Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Elemental analyses (C, H, and N) were done by HetChem Co., Harrisonville, Mo.; all agreed to ±0.4% of

Table IV. 6-(Phenylhydrazino)uracils

Compd	Yield, %	Purifn procedure[a]	Mp, °C	Formula[b]
6	44	A	>300	$C_{14}H_{18}N_4O_2 \cdot H_2O$
11	73	B	>300	$C_{12}H_{13}N_5O_3 \cdot H_2O$
13	83	C	>300	$C_{11}H_{10}N_4O_4 \cdot H_2O$
15	84	C	290 dec	$C_{11}H_{12}N_4O_2 \cdot 0.5H_2O$
16	65	C	>300	$C_{10}H_9N_4O_2F$
17	80	C	275 dec	$C_{10}H_9N_4O_2Cl \cdot 0.5H_2O$
18	73	C	>300	$C_{10}H_9N_4O_2Br \cdot 0.5H_2O$
19	75	C	255 dec	$C_{10}H_9N_4O_2F \cdot 0.5H_2O$
20	51	C	>300	$C_{11}H_{12}N_4O_2$
21	51	C	249 dec	$C_{12}H_{14}N_4O_2 \cdot 2H_2O$
23	44	C	>300	$C_{10}H_8N_4O_2Cl_2$
24	71	C	>300	$C_{11}H_{11}N_4O_2Cl \cdot 0.5H_2O$

[a] A, crystallized from 50% acetic acid–water; B, crystallized from water containing sodium dithionite; C, precipitated by acetic acid from a solution of compound in 1 N sodium hydroxide containing sodium dithionite. [b] C, H, and N analyses.

calculated values. Nuclear magnetic resonance spectra were obtained with a Perkin-Elmer R-12B instrument (60 MHz) equipped with a Nicolet TT7 Fourier transform accessory; proton spectra were consistent with the proposed structures. Most starting materials were commercially available, although *o*-ethylphenylhydrazine[14] and *p*-acetamidophenylhydrazine hydrochloride[15] were prepared by reduction of the diazotized anilines with stannous chloride.[16]

p-n-Butylphenylhydrazine Hydrochloride. A solution of sodium nitrite (4.8 g, 0.069 mol) in water (20 mL) was added during 30 min to a stirred, cold (0 °C) suspension of *p*-*n*-butylaniline (10.0 g, 0.067 mol) in 6 N hydrochloric acid (80 mL). After an additional 15 min, a solution of stannous chloride (44.5 g, 0.23 mol) in 6 N hydrochloric acid (80 mL) was added slowly, and the resulting suspension was stirred for 4 h at 0 °C. The solid was filtered with suction and dissolved in 40% potassium hydroxide solution (100 mL) covered with ethyl acetate (100 mL). The organic layer was separated and the aqueous layer was extracted with ethyl acetate (3 × 100 mL). The combined organic extracts were shaken with 10% hydrochloric acid (50 mL), and a colorless solid developed in the organic layer. The aqueous layer was separated and chilled, yielding 1.0 g of product. The organic suspension was filtered with suction to produce 3.1 g of product (total yield 31%). *p*-*n*-Butylphenylhydrazine hydrochloride crystallized from ethanol as colorless plates, mp 232–234 °C. Anal. ($C_{10}H_{17}N_2Cl$) C, H, N.

Synthesis of Inhibitors. 6-(Phenylhydrazino)uracils were prepared by reaction of 6-aminouracil with the appropriate phenylhydrazine or phenylhydrazine hydrochloride (see ref 8 for details of synthesis and purification). Properties of compounds not previously reported are summarized in Table IV; preparations of compounds **3–5**, **7–9**, **12**, **14**, and **29** are reported in ref 8, and those of **22**, **30**, and **31** are given in ref 17.

Inhibitors containing a *p*-OH group were made as the azo compounds by condensation of 6-hydrazinouracil with a *p*-benzoquinone; preparation and properties of compounds **24–28** are given in ref 8.

6-(p-Aminophenylazo)uracil (10). The acetamido compound, 11 (300 mg, 1.1 mmol), was heated at reflux in 2 N sodium hydroxide solution (8 mL) for 1.5 h. The dark solution was neutralized with concentrated hydrochloric acid and chilled overnight to yield 106 mg (42%) of **10** as a deep red powder, mp >300 °C. The product was purified by precipitation from 1 N sodium hydroxide solution with concentrated hydrochloric acid, centrifugation of the fine suspension, and repeated (five times) washing with water and centrifugation of the pellet: NMR (Me_2SO-d_6, internal Me_4Si) δ 11.02 (br s, 3-H), 10.66 (br s, 1-H), 7.72 (d, J = 9.0 Hz, 2′, 6′-H), 6.86 (br s, NH_2), 6.69 (d, J = 9.0 Hz, 3′,5′-H), 5.74 (s, 5-H). Anal. ($C_{10}H_9N_5O_2$) C, H, N.

3-Methyl-6-(p-hydroxyphenylazo)uracil (32). A solution of *p*-benzoquinone (94 mg, 0.87 mmol) in warm ethanol (1 mL) was added in one portion to a stirred suspension of 3-methyl-6-hydrazinouracil (122 mg, 0.8 mmol) in 10% hydrochloric acid (4 mL). After 0.5 h the suspension was filtered and the orange product was crystallized from 50% dimethylformamide–water giving 164 mg (85%) of **32**: mp 275 °C dec; NMR (Me_2SO-d_6, internal Me_4Si) δ 11.1 (br s, 1-H), 7.88 (d, J = 9.0 Hz, 2′,6′-H), 6.98 (d, J = 9.0 Hz, 3′,5′-H), 6.13 (s, 5-H), 3.19 (s, CH_3). Anal. ($C_{11}H_{10}N_4O_3$) C, H, N.

DNA Polymerase III. Preparation and Assay Conditions. DNA polymerase III was prepared as described previously;[6] the DNA cellulose fraction V was used in all experiments. The assay for polymerase was performed at 30 °C for 5 min in 0.05 mL of an incubation mixture containing 20% glycerol, 50 mM Tris HCl (pH 7.6), 10 mM magnesium chloride, 30 mM sodium chloride, 1.2 mM DNAase 1-activated, calf thymus DNA (Worthington; see ref 6 for details of preparation), 5 mM dithiothreitol, 0.1 mg/mL of bovine serum albumin (Armour, crystallized), dATP, dCTP, and [^{3}H]-TTP (100–200 cpm/pmol), 10 μM each, and 0.005–0.1 unit of enzyme. Enzyme units and the preparation of the sample for the determination of acid-insoluble radioactivity are described elsewhere.[4]

Inhibitor Reduction. 6-(Phenylhydrazino)uracils are unstable in air, particularly in alkaline aqueous solution, and undergo oxidation[8] to the inactive azo compounds. To maintain the inhibitors in the reduced state and to effect quantitative reduction[8] of azo compounds, 5 mM solutions of all inhibitors in 100 mM sodium hydroxide were mixed at 30 °C with equal volumes of 100 mM aqueous sodium dithionite and diluted into assay mixtures; the presence of dithiothreitol in assay mixtures protects the compounds from significant oxidation during assay.

Acknowledgment. The authors are grateful to Joseph Gambino, Joseph D'Ambrosio, and Susan Kohle for technical assistance. This work was supported by Grants GM21747 (G.E.W.) and CA15915 (N.C.B.) from the National Institutes of Health.

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