Novel Pyrazolo[3,4-*d*]pyrimidine-Based Inhibitors of *Staphlococcus aureus* DNA Polymerase III: Design, Synthesis, and Biological Evaluation

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6-Anilinopyrazolo[3,4-*d*]pyrimidin-4-ones are novel dGTP analogues that inhibit the replication-specific enzyme DNA polymerase III (DNA pol III) of *Staphlococcus aureus* and other Gram-positive (Gr+) bacteria. To enhance the potential of these inhibitors as antimicrobial agents, a structure−activity relationship was developed involving substitutions at the 2, 4, and pyrazolo NH positions. All of the new inhibitors were tested for their ability to inhibit *S. aureus* DNA pol III and the growth of several other Gr+ bacteria in culture. 2-Anilino groups with small hydrophobic groups in the meta or para position enhanced both antipolymerase and antimicrobial activity. 2-Benzyl-substituted inhibitors were substantially less active. Substitution in the 4-position by oxygen gave the optimal activity, whereas substitution at the pyrazolo NH was not tolerated. These pyrazolo[3,4-*d*]pyrimidine derivatives represent a novel class of antimicrobials with promising activities against Gr+ bacteria.

Introduction

The management of life-threatening infectious disease caused by multiply resistant Gram-positive (Gr+) bacteria continues to present a formidable challenge to modern medicine.[1−3] One approach toward solving this problem involves the development of chemotherapeutic agents capable of selectively attacking new bacterial targets. One such target that has received considerable interest is DNA polymerase III (DNA pol III), an enzyme essential for the replication of Gram-positive bacterial DNA.[4] Inhibitors of DNA pol III first came to prominence in the early 1970s.[5−7] The prototype inhibitors were simple substituted uracil derivatives, e.g., **1**−**3**. The systematic study of the mechanism of inhibition of DNA pol III by these early inhibitors[8,9] has demonstrated two major structural features required for inhibitory action. These are (I) a substituted pyrimidine ring that permits base pairing to a pyrimidine or purine in the DNA template (e.g., Figure 1 for binding of 6-aminouracil to cytosine) and (II) a planar aryl ring situated at or near the 6-NH group. Figure 2 shows a cartoon representation of the interactions between the inhibitor, the DNA template, and the enzyme, resulting in an unproductive ternary complex.

We have recently disclosed a novel series of 2-(anilino)pyrimidine-based inhibitors of DNA pol III, e.g., **4**.[10] These prototypic inhibitors have shown good affinity and selectivity for the DNA pol III target but have suffered from either weak antibacterial activity or unacceptably low aqueous solubility. These deficiencies have prompted us to explore alternative inhibitor platforms. We report herein that the 2-(anilino)pyrimidine subunit can be replaced by the pyrazolo[3,4-*d*]pyrimidin-4-one moiety without negatively affecting inhibitor binding to DNA pol III. Indeed, our structure−activity relationships, which are described below, have indicated that the pyrazolo[3,4-*d*]pyrimidin-4-one subunit is a significant antimicrobial activity enhancing group. We postulated that the mechanism of inhibitory action of the pyrazolo[3,4-*d*]pyrimidin-4-ones would be similar to those of previously reported DNA pol III inhibitors. As shown in Figure 3, the inhibitor molecule consists of two domains: a base-pairing domain, equiva-

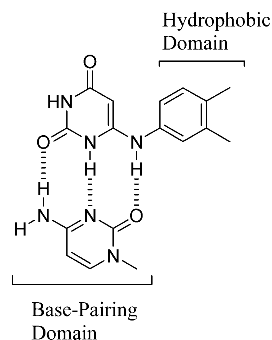
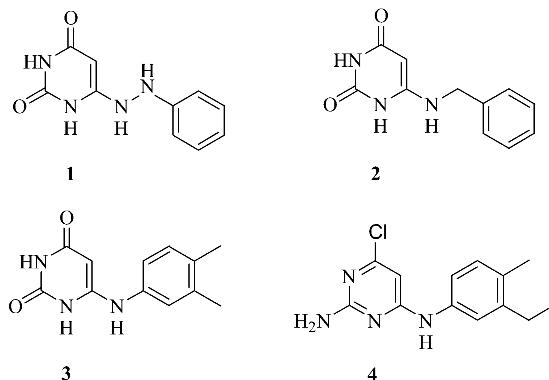


Figure 1. Hydrogen bonding of a 6-aminouracil with cytosine.



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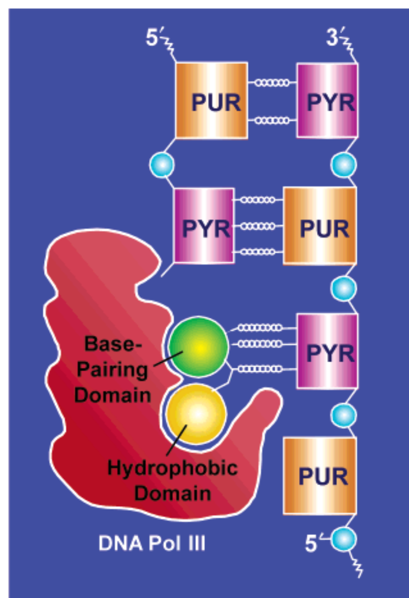


Figure 2. Cartoon representation showing the immobilization of DNA pol III in an unproductive ternary complex involving the inhibitor base-pairing and hydrophobic domains, the DNA template, and the enzyme.

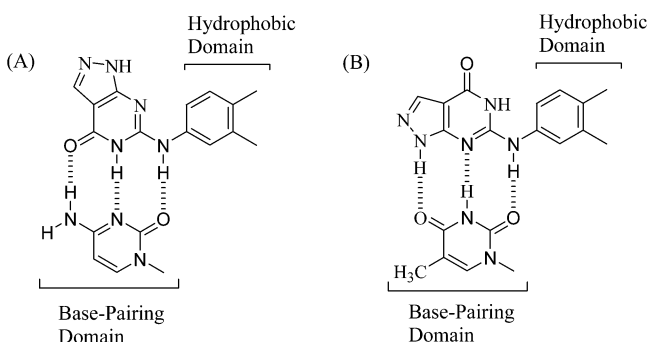


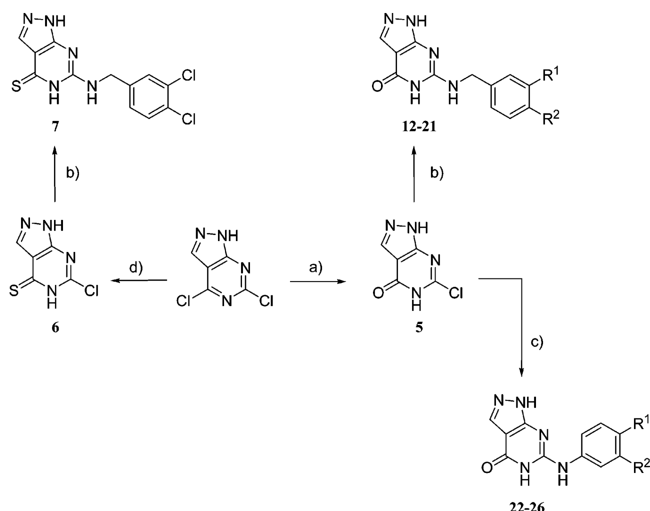
Figure 3. (A) dGTP mimic: hydrogen bonding of a 2-anilinopyrazolo[3,4-*d*]pyrimidin-4-one with cytosine. (B) dATP mimic: hydrogen bonding of a 2-anilinopyrazolo[3,4-*d*]pyrimidin-4-one with thymine.

lent to that of either guanine (panel A) or adenine (panel B), and an aryl domain from which it would derive its reactivity and selectivity for the enzyme target.

Synthesis

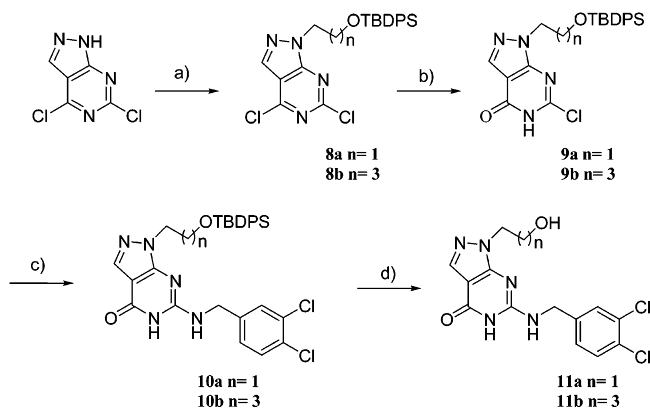
All of the pyrazolo[3,4-*d*]pyrimidine-based DNA pol III inhibitors were synthesized from commercially available 2,4-dicholorpyrazolo[3,4-*d*]pyrimidine. Substituted benzyl- and anilinopyrazolo[3,4-*d*]pyrimidin-4-ones were synthesized by the two-step sequence summarized in Scheme 1 (paths a,b and a,c, respectively). Reaction of 2,4-dichloropyrazolo[3,4-*d*]pyrimidine with aqueous 2 N KOH led to selective hydrolysis to yield 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one **5**.[11] Subsequent reaction with the appropriately substituted aniline or benzylamine in refluxing 2-methoxyethanol yielded the desired target compounds in good overall yields. Alternatively, reaction of 2,4-dichloropyrazolo[3,4-*d*]-pyrimidine with hydrogen sulfide in 0.5 N aqueous NaOH afforded 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]-pyrimidin-4-thione **6**[12] (Scheme 1, path d), which was further reacted with 3,4-dichlorobenzylamine to yield the pyrazolo[3,4-*d*]pyrimidin-4-thione-based DNA pol III inhibitor **7**. *N*-Hydroxyalkyl-substituted pyrazole inhibitors were synthesized by the four-step sequence summarized in Scheme 2. 2,4-Dichloropyrazolo[3,4-*d*]pyrimidine underwent smooth Mitsonobu reaction to yield the silyl-protected *N*-hydroxyalkyl 4,6-dichloropyrazolo-[3,4-*d*]pyrimidines **8a** and **8b** (Scheme 2).[13] Selective hydrolysis was achieved with 2 N KOH to yield the corresponding 6-chloropyrazolo[3,4-*d*]pyrimidin-4-ones **9a** and **9b**. Further reaction with 3,4-dichlorobenzylamine gave the desired *tert*-butyldiphenyl silyl ethers **10a** and **10b**, which were converted to their respective hydroxyalkyl forms **11a** and **11b** using tetrabutylammonium fluoride in THF at room temperature. Yields and properties of these compounds are provided in the Experimental Section.

Scheme 1[a]



[a] Reagents: (a) 2 N KOH, reflux, 20 min; (b) substituted benzylamine, 2-methoxyethanol, H_2O, reflux; (c) substituted aniline, 2-methoxyethanol, H_2O, reflux; (d) 0.5 N NaOH, hydrogen sulfide, 10 °C.

Scheme 2[a]

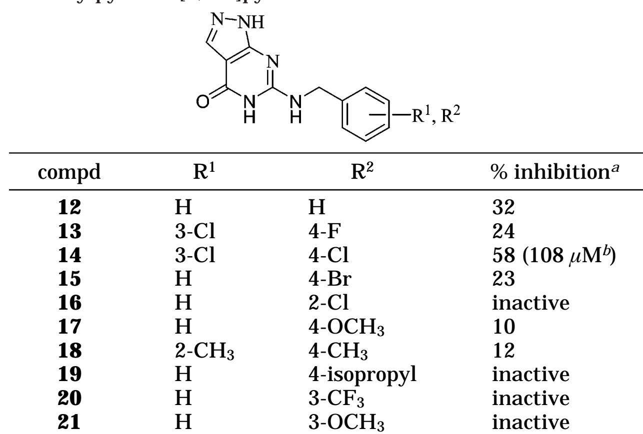


[a] Reagents: (a) TBDPSO(CH_2)$_n$OH, Ph_3P, DEAD, THF, room temp, 8 h; (b) 2 N KOH, reflux, 20 min; (c) 3,4-dichlorobenzylamine, $CH_3OCH_2CH_2OH$, H_2O, reflux, 3–8 h; (d) TBAF, THF, room temp, 16 h.

Results

2-Substituted Benzyl Derivatives. We began our investigations by exploring the 2-benzyl-substituted pyrazolo[3,4-*d*]primidin-4-one platform. The benzyl group represented a convenient group to introduce synthetically and a good probe, based on contemporaneous

Table 1. Anti-DNA pol III Activity of a Series of 2-Benzylpyrazolo[3,4-*d*]pyrimidin-4-ones



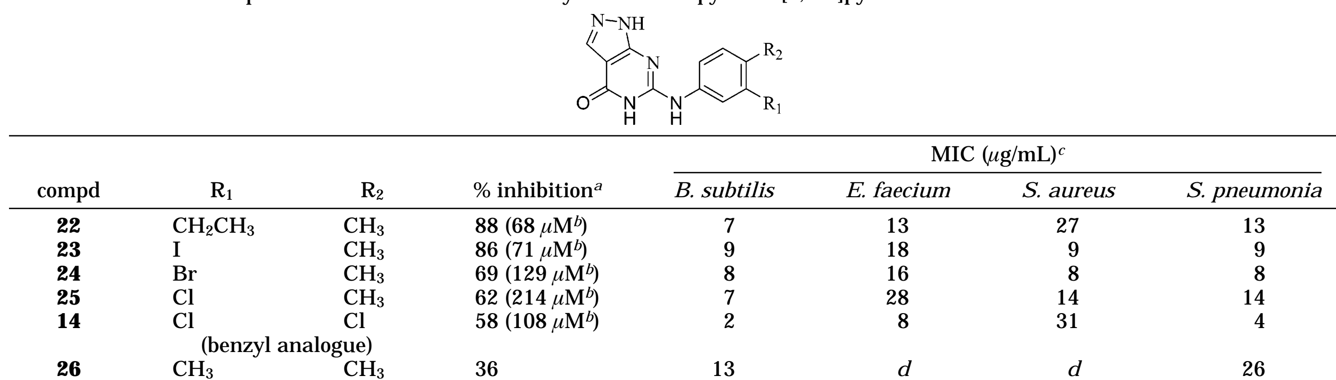
compd	R^1	R^2	% inhibition[a]
12	H	H	32
13	3-Cl	4-F	24
14	3-Cl	4-Cl	58 (108 μM[b])
15	H	4-Br	23
16	H	2-Cl	inactive
17	H	4-OCH_3	10
18	2-CH_3	4-CH_3	12
19	H	4-isopropyl	inactive
20	H	3-CF_3	inactive
21	H	3-OCH_3	inactive

[a] % inhibition against pol III of *S. aureus* at 500 μM inhibitor. [b] IC_{50} value.

studies from the literature,[14] for determining the potential of the pyrazolo[3,4-*d*]pyrimidin-4-one subunit as a viable inhibitor platform. Anti-DNA-pol-III activities of these derivatives are presented in Table 1. While 6-(benzylamino)-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one **12** itself was only weakly active, substituents on the benzyl ring generated interesting results. In general, sterically bulky substituents and ortho-substituted derivatives were inactive as inhibitors, whereas compounds with a single meta or para substituent were only weakly active, e.g., **15**. The simultaneous presence of substituents in the para and meta positions, especially when these were both hydrophobic groups, e.g., **14**, yielded the best inhibitors in this series. Polar substituents in any position on the benzyl ring conferred weak activity on the inhibitors. Such substitution of the benzyl ring also affected antibacterial potency in a manner, quite gratifyingly, consistent with that seen for enzyme inhibition. Thus, the most potent DNA pol III inhibitor in this series, i.e., **14**, also possessed the most potent antimicrobial potency in this series with MIC values of 2.0, 8.0, 31.0, and 4.0 μg/mL against *B. subtilis, E. faecium, S.aureus*, and *S pneumonia*, respectively. However, in general, only those inhibitors with inhibitory activity of >50% at 100 μM inhibitor led to measurable antimicrobial activity. In this regard, the 3,4-dichlorobenzyl derivative **14** was the optimum substituent enhancing both the antipolymerase and antibacterial potency. To determine whether the pyrazolo[3,4-*d*]pyrimidin-4-ones were acting as dGTP or dATP mimics, truncated versions of the DNA polymerase assay were run in which one of the competing nucleotides (dGTP or dATP) was at subsaturating levels and the other at saturating levels (and vice versa). The inhibitory activity of the pyrazolo[3,4-*d*]pyrimidin-4-ones, e.g., **14** and **23**, was ca. 10-fold lower in the presence of saturating levels of dGTP and unaffected by saturating levels of dATP, thereby confirming that the action of these inhibitors was derived in part by their capacity to mimic and compete with dGTP (Figure 3A) and not dATP (Figure 3B). Variation of the substituents in the 4-position generally led to loss in activity; both the 4-amino[15] and 4-thio analogues, e.g., **7**, were essentially inactive. This is in line with our proposed hydrogen-bonding paradigm described in Figure 3A. Thus, any substituent at the 4-position would interfere with the capacity of that compound to form hydrogen bonds with a cytosine base in the DNA template. Substitution at the pyrazole NH also led to a drastic loss in activity, with the *N*-hydroxyalkyl derivatives **11a** and **11b** devoid of any biological activity.

2-Substituted Anilino Derivatives. The 2-anilinopyrazolo[3,4-*d*]pyrimidin-4-ones showed improved activity as DNA pol III inhibitors. The antipolymerase and antimicrobial potency for a series of 2-anilinopyrazolo-[3,4-*d*]pyrimidin-4-ones are described in Table 2. As the data indicates, relatively small hydrophobic substituents such as alkyl and halogen groups in the meta and para positions produced the best inhibitors. Indeed, maximum inhibitor potency was achieved with the combination of a large hydrophobic meta substituent, e.g., I or CH_2CH_3, and a small hydrophobic para substituent, e.g., CH_3.[16] Much to our delight, a corresponding improvement in the antimicrobial potency was also observed with these 2-anilinopyrazolo[3,4-*d*]pyrimidin-4-ones. Thus, the best DNA pol III inhibitor, e.g., **23**, also possessed the most potent antibacterial activity against our panel of Gram-positive bacteria (Table 2) and were uniformly inactive against an outer-membrane permeable strain of *Escherichia coli*, a Gram-negative Pol III deficient organism.[17] The ability of the analogues to selectively inhibit the DNA synthesis of Gr+ bacteria was investigated by whole-cell dose−response labeling. The results are summarized in Figure 4. Each compound was tested against five labeled precursors to investigate the effect on DNA synthesis (6-[^{3}H]thymidine), RNA synthesis (5,6-[^{3}H]uracil), protein synthesis

Table 2. In Vitro Anti-pol-III and Antibacterial Activity of 2-Anilinopyrazolo[3,4-*d*]pyrimidin-4-ones



				MIC (μg/mL)[c]			
compd	R_1	R_2	% inhibition[a]	*B. subtilis*	*E. faecium*	*S. aureus*	*S. pneumonia*
22	CH_2CH_3	CH_3	88 (68 μM[b])	7	13	27	13
23	I	CH_3	86 (71 μM[b])	9	18	9	9
24	Br	CH_3	69 (129 μM[b])	8	16	8	8
25	Cl	CH_3	62 (214 μM[b])	7	28	14	14
14	Cl (benzyl analogue)	Cl	58 (108 μM[b])	2	8	31	4
26	CH_3	CH_3	36	13	*d*	*d*	26

[a] % inhibition against pol III of *S. aureus* at 500 μM. [b] IC_{50} value. [c] Minimum inhibitory concentration (μg/mL). [d] Growth affected but incomplete inhibition.

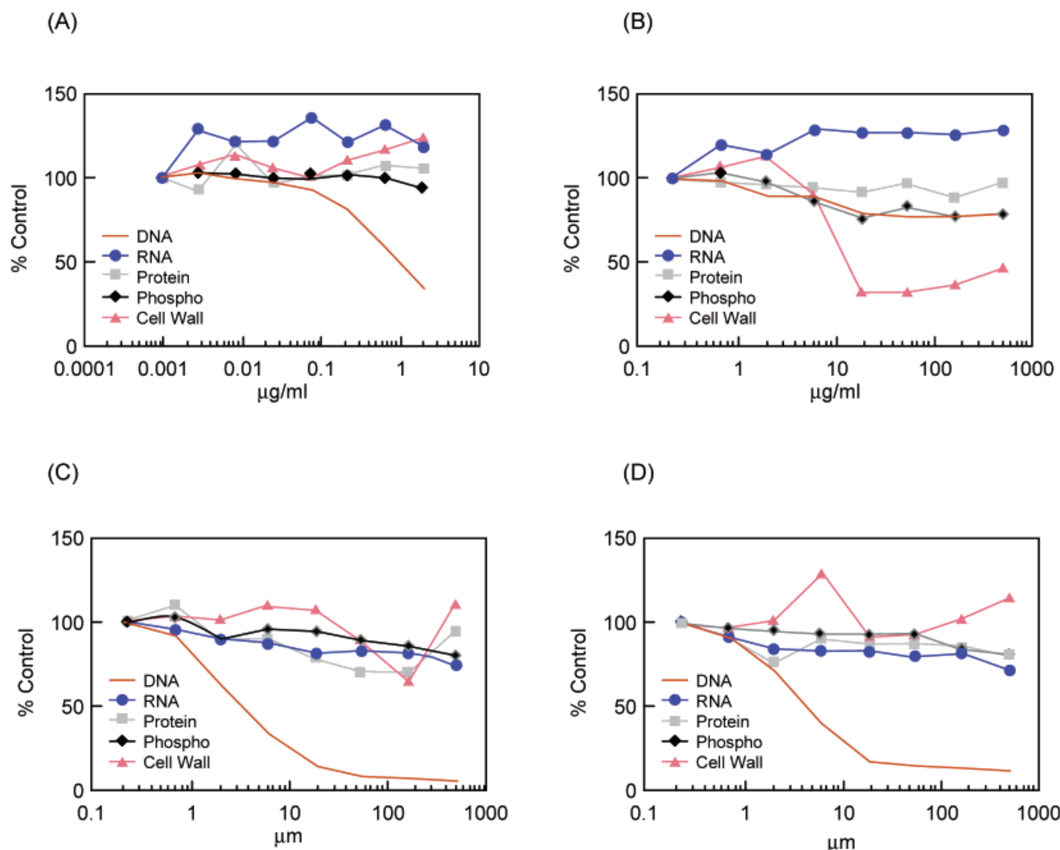


Figure 4. Mode of action study by whole-cell dose–response labeling: (A) ciprofloxacin; (B) vancomycin; (C) 2-(3-iodo-4-methyl)-anilinopyrazolo[3,4-*d*]pyrimidin-4-one, **23**; (D) 2-(3-bromo-4-methyl)anilinopyrazole[3,4-*d*]pyrimidin-4-one, **24**.

(4,5-[^{3}H]leucine), phospholipid synthesis (2-[^{3}H[glycerol), and cell wall synthesis (2,3-[^{3}H]alanine). Ciprofloxacin and vancomycin, which selectively inhibit DNA synthesis and bacterial cell wall, respectively, were run as control compounds. As shown in Figure 4C, the 2-(3-iodo-4-methyl)anilinopyrazolo[3,4-*d*]pyrimidin-4-one derivative **23** selectively inhibited DNA synthesis of *S. aureus* without affecting any of the other macromolecules investigated. Panel D confirmed this finding for 2-(3-bromo-4-methyl)anilinopyrazolo[3,4-*d*]pyrimidin-4-one **24**. In general, compounds that were active in the MIC assay were tested for the mode of action (MOA) by the method described above, and without exception, all the active analogues were shown to be selective DNA synthesis inhibitors.

Discussion

The main purpose of this work was to determine whether the pyrazolo[3,4-*d*]pyrimidine subunit was a viable platform for inhibitor design. As the results in Tables 1 and 2 indicate, the pyrazolo[3,4-*d*]pyrimidin-4-one platform has shown considerable promise as an inhibitor nucleus. Collectively, we can draw the following conclusions from our results. First, our results have shown that a fused heterocycle, in this case a pyrazolo [3,4-*d*]pyrimidin-4-one, can replace the simpler uracil and 2-aminopyrimidine platforms that have been described for all previous DNA pol III inhibitors.[18,19] This design platform offers several advantages such as improved physical properties and enhanced opportunities for SAR development. The pyrazolo[3,4-*d*]pyrimidin-4-one also lacks objectionable functionalities, such as the hydrazino group, which have plagued some of the earlier inhibitor designs.[7,20] Second, consistent with our observation of improved physical properties, we have shown enhanced antimicrobial potency across a broad panel of Gram-positive bacteria. We believe this is due in part to improved aqueous solubility, which facilitates inhibitor transport through the bacterial cell wall and membrane. Third, our macromolecular labeling mode of action studies have shown these inhibitors selectively inhibit DNA synthesis. In summary, we have discovered a novel series of selective DNA pol III inhibitors. Our results demonstrate the potential of the pyrazolo[3,4-*d*]pyrimidin-4-one design as model antibacterial agents and confirm the general hypothesis that bacterial DNA pol III is a valid target for antimicrobial drug development.

Experimental Procedures

Chemistry. ^{1}H NMR spectra were recorded on a Varian InNova 500 MHz instrument in $CDCl_3$ or DMSO-d_6 solutions, unless otherwise stated, using TMS as an internal standard. Column chromatography was performed on Merck silica gel 60 (0.06–0.200 mm). Analytical thin-layer chromatography (TLC) was carried out using Merck silica gel 60 F_{254} plates. All compounds were detected as single spots on TLC plates and visualized using UV light and phosphomolybdic acid stain. Low-resolution mass spectra (MS) were determined on a Micromass Platform LC by electrospray positive ionization. High-resolution mass spectra (HRMS) were determined on a 3 T Finnigan NewStar Fourier transform ion cyclotron resonance mass spectrometer (FTICR-/MS) by electrospray ionization in positive ion mode. Elemental analyses were performed by Robertson Microlit Laboratories, Inc., Madison, NJ, and are within ±0.4% of the calculated values unless otherwise stated. Reagents were purchased commercially and used without

further purification unless otherwise stated. 6-Chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one **5** and 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-thione **6** were synthesized according to known procedures.[11-12]

6-[(3,4-Dichlorobenzyl)amino]1,5-dihydro-4*H*-pyrazolo-[3,4-*d*]pyrimidine-4-thione, 7. A mixture of 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-thione (0.05 g, 0.27 mmol) and 3,4-dichlorobenzylamine (0.143 g, 0.81 mmol) in 2-methoxyethanol (4 mL)/H_2O (1.5 mL) was heated at reflux for 8 h. The reaction was diluted with H_2O (10 mL), and the product was collected by filtration. The solid was washed with MeOH (2 × 5 mL) and recrystallized from MeOH/H_2O (9:1) to afford **7** as a white powder. ^{1}H NMR (DMSO): δ 4.52 (d, J = 5 Hz, 2 H), 7.16 (bs, 1 H), 7.31 (d, J = 8.2 Hz, 1 H), 7.56−7.59 (m, 2 H), 7.89 (bs, 1 H), 11.86 (bs, 1 H), 13.12 (bs, 1 H). MS *m*/*z*: 327 (M^+ + 1). Anal. ($C_{12}H_9Cl_2N_5O$) C, H. N: calcd, 21.47; found, 20.70. HRMS: (M^+) calcd, 326.0029; found, 326.0032.

1-(2-{[*tert*-Butyl(diphenyl)silyl]oxy}ethyl)-4,6-dichloro-1*H*-pyrazolo[3,4-*d*]pyrimidine, 8a. Diethyl azodicarboxylate (0.23 g, 1.30 mmol) was added dropwise over 5 min at room temperature to a stirred solution of 4,6-dichloro-1*H*-pyrazolo-[3,4-*d*]pyrimidine (0.16 g, 0.87 mmol), triphenylphosphine (0.34 g, 1.30 mmol), and alcohol (0.52 g, 1.70 mmol) in THF (10 mL). The mixture was stirred for 4 h and evaporated to dryness. The crude residue was poured into Et_2O, and the insoluble materials were filtered off. The filtrate was concentrated, and the residue was purified by flash chromatography, eluting with EtOAc/hexanes (1:4) to provide 0.058 g (14%) of **8a**. ^{1}H NMR ($CDCl_3$): δ 0.92 (bs, 6 H), 1.09 (bs, 3 H), 4.14 (bs, 2 H), 4.60 (bs, 2 H), 7.30−7.48 (m, 10 H), 7.73 (bs, 1 H). MS *m*/*z*: 472 (M^+ + 1).

1-(4-{[*tert*-Butyl(diphenyl)silyl]oxy}butyl)-4,6-dichloro-1*H*-pyrazolo[3,4-*d*]pyrimidine, 8b. Diethyl azodicarboxylate (0.28 g, 1.61 mmol) was added dropwise over 5 min at room temperature to a stirred solution of 4,6-dichloro-1*H*-pyrazolo-[3,4-*d*]pyrimidine (0.20 g, 1.06 mmol), triphenylphosphine (0.421 g, 1.61 mmol), and alcohol (0.697 g, 2.12 mmol) in THF (10 mL). The mixture was stirred for 4 h and evaporated to dryness. The crude residue was poured into Et_2O, and the insoluble materials were filtered off. The filtrate was concentrated, and the residue was purified by flash chromatography, eluting with EtOAc/hexanes (1:4) to provide 0.418 g (79%) of **8b**. ^{1}H NMR ($CDCl_3$): δ 1.05 (s, 9 H), 1.54−1.60 (m, 2 H), 2.05−2.11 (m, 2 H), 3.70 (t, J = 6.2 Hz, 2 H), 4.48 (t, J = 7.1 Hz, 2 H), 7.37−7.45 (m, 6 H), 7.65 (dd, J = 6.6 Hz, J = 1.4 Hz, 4 H), 8.14 (s, 1 H). MS *m*/*z*: 500 (M^+ + 1).

1-(2-{[*tert*-Butyl(diphenyl)silyl]oxy}ethyl)-6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one, 9a. A solution of **8a** (0.05 g, 0.11 mmol) and KOH (0.03 g, 0.53 mmol) in 1,4-dioxane/H_2O (5:1) (1.2 mL) was heated 155 °C for 8 h. The mixture was acidified to pH ~3 using 1 N HCl, and the residue was treated with EtOAc and water. The organic layer was separated and dried ($MgSO_4$), and the solvent was evaporated. The crude residue was purified by flash chromatography, eluting with MeOH/$CHCl_3$ (1:9) to afford **9a** (0.041 g, 89%) as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 0.94 (s, 6 H), 1.09 (s, 3 H), 4.09 (t, J = 5.3 Hz, 2 H), 4.49 (t, J = 5.3 Hz, 2 H), 7.33−7.44 (m, 8 H), 7.51−7.54 (m, 2 H), 7.72−7.75 (m, 1H), 10.52 (bs, 1 H). MS *m*/*z*: 454 (M^+ + 1).

1-(4-{[*tert*-Butyl(diphenyl)silyl]oxy}butyl)-6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one, 9b. A solution of **8b** (0.050 g, 0.1 mmol) and KOH (0.028 g, 0.5 mmol) in 1,4-dioxane/H_2O (5:1) (1.2 mL) was heated at 155 °C for 8 h. The mixture was acidified to pH ~3 using 1 N HCl, and the residue was treated with EtOAc and water. The organic layer was separated and dried ($MgSO_4$), and the solvent was evaporated. The crude residue was purified by flash chromatography, eluting with MeOH/$CHCl_3$ (1:9) to afford **9b** (0.046 g, 96%) as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 1.05 (s, 9 H), 1.54−1.60 (m, 2 H), 2.01−2.07 (m, 2 H), 3.69 (t, J = 6.2 Hz, 2 H), 4.35 (t, J = 7.1 Hz, 2 H), 7.36−7.45 (m, 6 H), 7.66 (d, J = 7.1 Hz, 4 H), 8.09 (s, 1 H). MS *m*/*z*: 482 (M^+ + 1).

1-(2-{[*tert*-Butyl(diphenyl)silyl]oxy}ethyl)-6-[(3,4-dichlorobenzyl)amino]1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one, 10a. A solution of **9a** (0.04 g, 0.09 mmol) and 3,4-dichlorobenzylamine (0.045 g, 0.23 mmol) in 2-methoxyethanol (4 mL)/H_2O (1.5 mL) was heated at reflux for 8 h. The reaction mixture was treated with EtOAc and water. The organic layer was separated and dried ($MgSO_4$), and the solvent was evaporated. The crude residue was purified by flash chromatography, eluting with MeOH/$CHCl_3$ (1:9) to afford **10a** (0.05 g, 99%) as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 0.96 (s, 6 H), 1.09 (s, 3 H), 4.07 (t, J = 5.8 Hz, 2 H), 4.38 (t, J = 5.9 Hz, 2 H), 4.55 (d, J = 5.7 Hz, 2 H), 7.15−7.20 (m, 1 H), 7.30−7.47 (m, 10 H), 7.54−7.57 (m, 1 H), 7.69−7.75 (m, 2 H). MS *m*/*z*: 594 (M^+ + 1).

1-(4-{[*tert*-Butyl(diphenyl)silyl]oxy}butyl)-6-[(3,4-dichlorobenzyl)amino]-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one, 10b. A solution of **9b** (0.050 g, 0.1 mmol) and 3,4-dichlorobenzylamine (0.055 g, 0.31 mmol) in 2-methoxyethanol (4 mL)/H_2O (1.5 mL) was heated at reflux for 8 h. The reaction mixture was treated with EtOAc and water. The organic layer was separated and dried ($MgSO_4$), and the solvent was evaporated. The crude residue was purified by flash chromatography, eluting with MeOH/$CHCl_3$ (1:9) to afford **10b** (0.060 g, 97%) as a yellow oil, which was carried on without further purification. MS *m*/*z*: 621 (M^+ + 1).

6-[(3,4-Dichlorobenzyl)amino]-1-(2-hydroxyethyl)-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one, 11a. A solution of **10a** (0.05 g, 0.08 mmol) and tetrabutylammonium fluoride (1 M in THF, 0.13 mL, 0.13 mmol) in THF (8.0 mL) was stirred overnight at room temperature. THF was evaporated in vacuo, and the residue was treated with EtOAc and water. The organic layer was separated and dried ($MgSO_4$), and the solvent was evaporated. The crude residue was purified by flash chromatography, eluting with MeOH/$CHCl_3$ (1:9) to afford **11a** (0.017 g, 61%) as a yellow oil. ^{1}H NMR (DMSO): δ 3.70 (dt, J = 6.2 Hz, J = 5.7 Hz, 2 H), 4.09 (t, J = 6.1 Hz, 2 H), 4.49 (d, J = 5.9 Hz, 2 H), 4.77 (t, J = 5.8 Hz, 1 H), 7.04−7.07 (m, 1 H), 7.38 (dd, J = 8.3 Hz, J = 2.1 Hz, 1 H), 7.57 (d, J = 8.3 Hz, 1 H), 7.64 (d, J = 1.8 Hz, 1 H), 7.73 (s, 1H). MS *m*/*z*: 355 (M^+ + 1). HRMS for $C_{14}H_{13}Cl_2N_5O_2$ (M^+): calcd, 354.0519; found, 354.0522.

6-[(3,4-Dichlorobenzyl)amino]-1-(4-hydroxybutyl)-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one, 11b. A solution of **10b** (0.060 g, 0.10 mmol) and tetrabutylammonium fluoride (1 M in THF, 0.16 mL, 0.16 mmol) in THF (8.0 mL) was stirred overnight at room temperature. THF was evaporated in vacuo, and the residue was treated with EtOAc and water. The organic layer was separated and dried ($MgSO_4$), and the solvent was evaporated. The crude residue was purified by flash chromatography, eluting with MeOH/$CHCl_3$ (1:9) to afford **11b** (0.060 g, 97%) as a yellow oil. ^{1}H NMR (CD_3OD): δ 1.37−1.43 (m, 2 H), 1.81−1.87 (m, 2 H), 3.50 (t, J = 6.5 Hz, 2 H), 4.17 (t, J = 6.8 Hz, 2 H), 4.57 (s, 2 H), 7.32 (d, J = 8.2 Hz, 1 H), 7.48 (d, J = 8.4 Hz, 1 H), 7.56 (bs, 1 H), 7.82 (s, 1H). MS *m*/*z*: 383 (M^+ + 1). HRMS for $C_{16}H_{17}Cl_2N_5O_2$ (M^+): calcd, 382.0832; found, 382.0826.

General Procedure for Preparation of Compounds 12−26. 6-Benzylamino-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one, 12. A mixture of 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one (100 mg, 0.59 mmol) and benzylamine (0.192 g, 1.80 mmol) in 2-methoxyethanol (4 mL)/H_2O (1.5 mL) was heated at reflux for 8 h. The reaction mixture was diluted with H_2O (10 mL), and the product was collected by filtration. The solid was washed with MeOH (2 × 5 mL) and recrystallized from MeOH/H_2O (9:1) to afford **12** as a white powder. ^{1}H NMR (DMSO): δ 4.49 (d, J = 5.7 Hz, 2 H), 6.88 (bs, 1 H), 7.23−7.26 (m, 1 H), 7.31−7.34 (m, 4 H), 7.74 (bs, 1 H), 10.3 (bs, 1 H), 12.9 (bs, 1 H). MS *m*/*z*: 242 (M^+ + 1). Anal. ($C_{12}H_{11}N_5O$) C, H, N.

6-[(3-Chloro-4-fluorobenzyl)amino]-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one, 13. Starting from 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one and 3-chloro-4-fluorobenzylamine, compound **13** was obtained according to the procedure described for **12**. ^{1}H NMR (DMSO): δ 4.47 (d, J = 5.9 Hz, 2 H), 6.95 (bs, 1 H), 7.31−7.38 (m, 2 H), 7.53 (dd, J = 7.1 Hz, J = 1.8 Hz, 1 H), 7.73 (s, 1 H), 10.48 (bs, 1 H), 12.88

(s, 1 H). MS *m/z*: 295 ($M^+ + 1$). HRMS for $C_{12}H_9ClFN_5O$ (M^+): calcd, 294.0552; found, 294.0543.

6-[(3,4-Dichlorobenzyl)amino]-1,5-dihydro-4*H*-pyrazolo-[3,4-*d*]pyrimidin-4-one, 14. Starting from 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one and 3,4-dichlorobenzylamine, compound **14** was obtained according to the procedure described for **12**. ^{1}H NMR (DMSO): δ 4.49 (d, J = 5.9 Hz, 2 H), 6.99 (bs, 1 H), 7.31 (dd, J = 8.4 Hz, J = 1.7 Hz, 1 H), 7.56−7.59 (m, 2 H), 7.74 (bs, 1 H), 10.39 (bs, 1 H), 12.88 (bs, 1 H). MS *m/z*: 311 ($M^+ + 1$). Anal. ($C_{12}H_9Cl_2N_5O$) C, H. N: calcd, 22.58; found, 22.14.

6-[(4-Bromobenzyl)amino]-1,5-dihydro-4*H*-pyrazolo-[3,4-*d*]pyrimidin-4-one, 15. Starting from 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one and 4-bromobenzylamine, compound **15** was obtained according to the procedure described for **12**. ^{1}H NMR (DMSO): δ 4.46 (d, J = 5.9 Hz, 2 H), 6.93 (bs, 1 H), 7.27 (d, J = 8.5 Hz, 1 H), 7.51 (d, J = 8.5 Hz, 1 H), 7.72 (s, 1 H), 10.50 (bs, 1 H), 12.86 (s, 1 H). MS *m/z*: 321 ($M^+ + 1$). HRMS for $C_{12}H_{10}BrN_5O$ (M^+): calcd, 320.0142; found, 320.0140.

6-[(2-Chlorobenzyl)amino]-1,5-dihydro-4*H*-pyrazolo-[3,4-*d*]pyrimidin-4-one, 16. Starting from 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one and 2-chlorobenzylamine, compound **16** was obtained according to the procedure described for **12**. ^{1}H NMR (DMSO): δ 4.56 (d, J = 6.0 Hz, 2 H), 6.94 (bs, 1 H), 7.27−7.38 (m, 3 H), 7.45 (dd, J = 7.3 Hz, J = 1.8 Hz, 1 H), 7.73 (bs, 1 H), 12.89 (bs, 1 H). MS *m/z*: 276 ($M^+ + 1$). Anal. ($C_{12}H_{10}ClN_5O$) C, H, N.

6-[(4-Methoxybenzyl)amino]-1,5-dihydro-4*H*-pyrazolo-[3,4-*d*]pyrimidin-4-one, 17. Starting from 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one and 4-methoxybenzylamine, compound **17** was obtained according to the procedure described for **12**. ^{1}H NMR (DMSO): δ 3.71 (s, 3 H), 4.40 (d, J = 5.7 Hz, 2 H), 6.80 (bs, 1 H), 6.89 (d, J = 8.7 Hz, 1 H), 7.25 (d, J = 8.7 Hz, 1 H), 7.73 (bs, 1 H), 12.88 (bs, 1 H). MS *m/z*: 272 ($M^+ + 1$). Anal. ($C_{13}H_{13}N_5O_2$) C, H, N.

6-[(2,4-Dimethylbenzyl)amino]-1,5-dihydro-4*H*-pyrazolo-[3,4-*d*]pyrimidin-4-one, 18. Starting from 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one and 2,4-dimethylbenzylamine, compound **18** was obtained according to the procedure described for **12**. ^{1}H NMR (DMSO): δ 2.23 (s, 3H), 2.25 (s, 3 H), 4.40 (d, J = 5.5 Hz, 2 H), 6.71 (bs, 1 H), 6.96 (d, J = 7.8 Hz, 1 H), 7.00 (s, 1 H), 7.13 (d, J = 7.8 Hz, 1 H), 7.73 (s, 1 H), 10.24 (bs, 1 H), 12.87 (bs, 1 H). MS *m/z*: 270 ($M^+ + 1$). Anal. ($C_{14}H_{15}N_5O$) C, H, N.

6-[(4-Isopropylbenzyl)amino]-1,5-dihydro-4*H*-pyrazolo-[3,4-*d*]pyrimidin-4-one, 19. Starting from 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one and 4-isopropylbenzylamine, compound **19** was obtained according to the procedure described for **12**. ^{1}H NMR (DMSO): δ 1.16 (s, 3 H), 1.17 (s, 3 H), 2.81−2.88 (m, 1 H), 4.43 (d, J = 5.7 Hz, 2 H), 6.83 (bs, 1 H), 7.18−7.25 (m, 4 H), 7.72 (bs, 1 H), 10.35 (bs, 1 H), 12.87 (bs, 1 H). MS *m/z*: 284 ($M^+ + 1$). Anal. ($C_{15}H_{17}N_5O$) C, H, N.

6-{[(3-(Trifluoromethyl)benzyl]amino}-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one, 20. Starting from 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one and 3-trifluoromethylbenzylamine, compound **20** was obtained according to the procedure described for **12**. ^{1}H NMR (DMSO): δ 4.58 (d, J = 6.0 Hz, 2 H), 7.01 (bs, 1 H), 7.54−7.64 (m, 3 H), 7.67 (s, 1 H), 7.74 (bs, 1 H), 12.87 (bs, 1 H). MS *m/z*: 310 ($M^+ + 1$). Anal. ($C_{13}H_{10}F_3N_5O$) C, H, N.

6-[(3-Methoxybenzyl)amino]-1,5-dihydro-4*H*-pyrazolo-[3,4-*d*]pyrimidin-4-one, 21. Starting from 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one and 3-methoxybenzylamine, compound **21** was obtained according to the procedure described for **12**. ^{1}H NMR (DMSO): δ 3.72 (s, 3 H), 4.46 (d, J = 5.9 Hz, 2 H), 6.81 (dd, J = 8.3 Hz, J = 1.9 Hz, 1 H), 6.88−6.90 (m, 3 H), 7.24 (t, J = 8.2 Hz, 1 H), 7.73 (bs, 1 H), 12.87 (bs, 1 H). MS *m/z*: 272 ($M^+ + 1$). Anal. ($C_{13}H_{13}N_5O_2$) C, H, N.

6-[(3-Ethyl-4-methylphenyl)amino]-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one, 22. Starting from 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one and 3-ethyl-4-methylaniline, compound **22** was obtained according to the procedure described for **12**. ^{1}H NMR (DMSO): δ 1.15 (t, J = 7.6 Hz, 3 H), 2.21 (s, 3 H), 2.56 (q, J = 7.6 Hz, 2 H), 7.08 (d, J = 8.3 Hz, 1 H), 7.25 (s, 1 H), 7.42 (m, 1 H), 7.80 (s, 1 H), 8.62 (bs, 1 H), 10.29 (bs, 1 H), 13.05 (bs, 1 H). MS *m/z*: 270 ($M^+ + 1$). HRMS for $C_{14}H_{15}N_5O$ (M^+): calcd, 270.1349; found, 270.1336.

6-[(3-Iodo-4-methylphenyl)amino]-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one, 23. Starting from 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one and 3-iodo-4-methylaniline, compound **23** was obtained according to the procedure described for **12**. ^{1}H NMR (DMSO): δ 2.32 (s, 3 H), 7.26 (d, J = 8.5 Hz, 1 H), 7.44 (dd, J = 8.3 Hz, J = 2.0 Hz, 1 H), 7.87 (s, 1 H), 8.16 (d, J = 1.8 Hz, 1 H), 8.82 (s, 1 H), 10.41 (s, 1 H). MS *m/z*: 368 ($M^+ + 1$). Anal. ($C_{12}H_{10}IN_5O$) H, N. C: calcd, 39.26; found, 38.80. HRMS (M^+): calcd, 368.0003; found, 368.0002.

6-[(3-Bromo-4-methylphenyl)amino]-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one, 24. Starting from 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one and 3-bromo-4-methylaniline, compound **24** was obtained according to the procedure described for **12**. ^{1}H NMR (DMSO): δ 2.29 (s, 3 H), 7.29 (d, J = 8.0 Hz, 1 H), 7.36 (dd, J = 8.3 Hz, J = 2.2 Hz, 1 H), 7.82 (s, 1 H), 8.01 (d, J = 2.3 Hz, 1 H), 8.84 (s, 1 H), 10.42 (s, 1 H), 13.17 (s, 1 H). MS *m/z*: 321 ($M^+ + 1$). HRMS for $C_{12}H_{10}BrN_5O$ (M^+): calcd, 320.0412; found, 320.0136.

6-[(3-Chloro-4-methylphenyl)amino]-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one, 25. Starting from 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one and 3-chloro-4-methylaniline, compound **25** was obtained according to the procedure described for **12**. ^{1}H NMR (DMSO): δ 2.27 (s, 3 H), 7.28−7.31 (m, 2 H), 7.90 (s, 1 H), 8.95 (s, 1 H), 10.44 (s, 1 H). MS *m/z*: 276 ($M^+ + 1$). HRMS for $C_{12}H_{10}ClN_5O$ (M^+): calcd, 276.0647; found, 276.0634.

6-[(3,4-Dimethylphenyl)amino]-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one, 26. Starting from 6-chloro-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one and 3,4-dimethylaniline, compound **26** was obtained according to the procedure described for **12**. ^{1}H NMR (DMSO): δ 2.17 (s, 3 H), 2.20 (s, 3 H), 7.08 (d, J = 8.2 Hz, 1 H), 7.28 (s, 1 H), 7.38 (m, 1 H), 7.80 (s, 1 H), 8.62 (bs, 1 H), 10.28 (bs, 1 H), 13.05 (s, 1 H). MS *m/z*: 256 ($M^+ + 1$). Anal. ($C_{13}H_{13}N_5O$) C, H, N.

Cloning, Expression, and Purification of DNA Polymerase III from *Staphylococcus aureus*. DNA polymerase III (pol III) was cloned by PCR from *S. aureus* genomic DNA. The pol III gene was cloned into pET15. The pol III/pET15 was transformed into BL21 cells. The cells were grown at 29 °C in M9ZB media containing ampicillin and chloramphenicol to an OD of 1.0. The cells were induced by adding 1 mM IPTG and grown for 3 h. The cells were centrifuged at 2500*g* for 10 min. The cell pellet was resuspended in 50 mM Tris, pH 7.4, 0.1% Triton X100, 1 mM EDTA, 20% glycerol, and complete protease inhibitor cocktail (Boehringer Mannheim) and were lysed by freeze−thawing three times in a dry ice and methanol bath. The lysate was centrifuged at 10000*g* for 1 h.

The supernatant was loaded onto a HiTrapQ column that was equilibrated in 50 mM Tris, pH 7.4, 20% glycerol, and 1 mM EDTA. Following loading, the column was washed with 20 column volumes of buffer. The pol III was eluted using a linear NaCl gradient from 0 to 0.5 M. The pol III activity eluted between 0.3 and 0.4 M NaCl. The peak of activity was loaded onto a HiTrap Blue column equilibrated in 50 mM Tris, pH 7.4, 20% glycerol, 1 mM EDTA, and 1 mg/mL BSA. The column was washed with 10 column volumes of equilibration buffer, followed by a wash with 20 column volumes of equilibration buffer containing 0.5 M NaCl. The pol III was eluted by washing the column with equilibration buffer containing 3 M NaCl.

DNA Polymerase III Enzyme Inhibition Assay. The reaction mix consisted of 30 mM Tris-HCl, pH 7.5, 20% glycerol, 4 mM DTT, 10 mM MgOAc, 0.003 mM dATP, 0.003 mM dGTP, 0.001 mM dCTP, 0.001 mM [^{3}H]dTTP, and 0.35 mg/mL "activated" calf thymus DNA (Worthington Enzymes) in a final volume of 0.1 mL. The assay was initiated by the addition of enzyme and incubated for 30 min at 30 °C. The assay was stopped by the addition of cold 10% TCA/0.1%

NaPPi. The incorporation of [^{3}H]dTTP into the calf thymus DNA was monitored by capturing the precipitated DNA onto a GF/C filter, followed by counting in a scintillation counter.

Whole-Cell-Based Assays, MIC Protocol. Five organisms were employed in the MIC assay. These were *B. subtilis* (F2 strain), *E. faecium* (RLA-1 strain), *S. aureus* (MB2865 strain), *S. pneumonia* (MCL5350 strain), and *E. coli envA* (MB4926 strain). Standard microbiological techniques were used in the routine maintenance of the cultures. Samples were tested initially in three concentrations, 100, 10, and 1 μM final. Samples were resuspended at a concentration of 2 or 10 mM in DMSO (if required). Then serial 2-fold dilutions of this stock were prepared from a final initial high concentration of 100 μM. Samples were diluted either by hand or using a Denley−Wellpro liquid handling system (manufactured by Labsystems). An amount of 5 μL of each concentration was transferred from the dilution plate to replica assay plates either by hand or using a Quadra-96 model 320 liquid handling device (manufactured by Tomtec). The medium was added either by hand or using the Denley−Wellpro to a final assay volume of 100 μL to give a final DMSO concentration of 5%. DMSO was run as the vehicle control. Assay plates were inoculated using an MIC-2000 (manufactured by Dynex Technologies) from cultures diluted into phosphate-buffered saline (made by Gibco-BRL). Assay plates were incubated overnight at 37 °C and then examined for the presence/absence of growth of the bacteria in the wells. Results are expressed as the MIC, that is, the minimum inhibitory concentration that gives inhibition of the growth of the indicated organism (i.e., >100, 100, 50, 25, 12.5, etc.).

Mode of Action Assay, Whole-Cell Dose−Response Labeling. Compounds that were active in the MIC assay were tested for the mode of action (MOA) by whole-cell dose−response labeling. Each compound was tested versus five different labeled precursors to investigate the effect on DNA synthesis (6-[^{3}H]thymidine, 1 μCi/mL final, plus uridine at 50 μg/mL final concentration), RNA synthesis (5,6-[^{3}H]uracil, 1 μCi/mL final), protein synthesis (4,5-[^{3}H]leucine, 5 μCi/mL final), phospholipid synthesis (2-[^{3}H]glycerol, 1 μCi/mL final), and cell wall synthesis (2,3-[^{3}H]alanine, 5 μCi/mL final, plus chloramphenicol at 100 μg/mL final). All labeled precursors were from either Amersham or New England Nuclear. In most cases, MB2865 was the assay organism, but in cases where compounds had activity against the bacillus strain and not MB2865, then that strain was used. Overnight cultures grown in Muller−Hinton medium were diluted 1:100 (3 mL) into 300 mL (prewarmed) of MH broth containing 50 μg/mL uridine, incubated at 37 °C with shaking to an OD of 0.1 (mid-log), and harvested by centrifugation. The cells were resuspended to an OD of 0.5 in prewarmed MH medium. Test and control compounds were serially diluted either 2-fold or 3-fold (depending on potency) in the appropriate diluent for the compound (water or DMSO), and then 5 μL of each was transferred to each of five assay plates. A final assay volume of 100 μL also included precursor diluted in MH medium to obtain the final concentration indicated above and 70 μL of cells at OD 0.5. Addition of cells started the reaction, which proceeded for 20 min and was stopped by addition of 50% TCA to a final concentration of 10%. TCA precipitable counts were collected onto glass fiber filters (Wallac) using a Skatron Micro96 harvester. Filter mats were placed in bags with 10 mL of scintillation fluid and loaded into cassettes for measurement in an LKB 1205 Betaplate counter (Wallac). Results are expressed as the percentage of control counts (vehicle alone, no drug) present in each sample well plotted as a function of percent of control versus concentration of compound on a semilog plot.

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Note Added after ASAP Posting. This manuscript was released ASAP on 3/29/2003 with errors in the experimental details for **8a**, **9a**, **10a**, and **11a**. The correct version was posted on 4/9/2003.

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