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Novel non-nucleobase inhibitors of *Staphylococcus aureus* DNA polymerase IIIC

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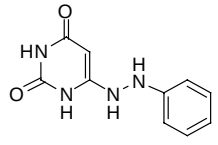
Abstract—The preparation and biological evaluation of 5-substituted-6-hydroxy-2-(anilino)pyrimidinones as a new class of DNA polymerase IIIC inhibitors, required for the replication of chromosomal DNA in Gram-positive bacteria, are described. These new dGTP competitive inhibitors displayed good levels of in vitro inhibition and antibacterial activity against *Staphylococcus aureus*. A new class of dATP competitive inhibitors, 6-substituted-2-amino-5-alkyl-pyrimidin-4-ones, whose antibacterial activity was unaffected by serum, were identified.

The emergence of bacterial resistance to antibiotics is an alarming problem in the treatment of infectious diseases. Numerous multi-resistant strains of Gram-positive (Gr+) pathogens have been identified, most notably vancomycin[1,2] and methicillin resistant[3] forms of *Staphylococcus aureus*. With this prospect, new antibiotics with novel modes of action have become a priority. Our current efforts to establish new targets based on bacteriophage inhibitory mechanisms have identified several components of the DNA replication machinery as attractive possibilities in this respect.[4] Within this machinery, DNA polymerase IIIC (DNA Pol IIIC), encoded by the gene *pol*C, has long been recognized as essential for the replication of the host chromosome of the low G+C content Gr+ bacteria.[5,6] When the enzyme is inhibited, the chromosomal DNA fails to replicate resulting in bacterial death.[7,8] Furthermore, it has little homology with its mammalian counterpart.

To date, most inhibitors of DNA Pol IIIC were uracil derivatives **1**–**3**.[9–13] These molecules inhibit Pol IIIC of *Bacillus subtilis* by promoting the formation of a catalytically inactive ternary complex between DNA and the enzyme, as demonstrated by Cozzarelli[8] and Wright and co-workers.[11] The molecular recognition of these inhibitors is believed to result from hydrogen-bonding with a corresponding cytosine (dGTP analogs) on the DNA template and the hydrophobic interaction of 6-aminoaryl of the nucleobase with a pocket near the enzyme active site. From this rationalization, a new series of inhibitors based on 6-hydroxy-2-(anilino)pyrimidinone scaffold **4** were designed. Much like the 6-anilinouracils, a single position in these pyrimidinediones (position 5) is the primary position to be optimized in terms of substitution. In this letter, efforts toward this achievement are detailed.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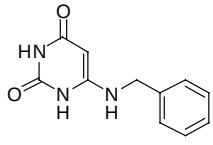
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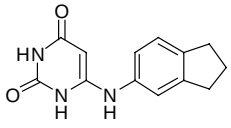
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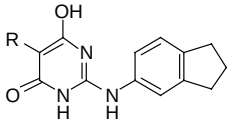
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A small library of 5-substituted-6-hydroxy-2-(arylamino)-pyrimidin-4(3*H*)-ones was initially prepared by condensation of an arylguanine hydrochloride **6** with a substituted malonate **5** to yield the hydroxypyrimidinone

Keywords: Antibacterial; Antimicrobial; DNA polymerase IIIC; In vivo; Protein binding.
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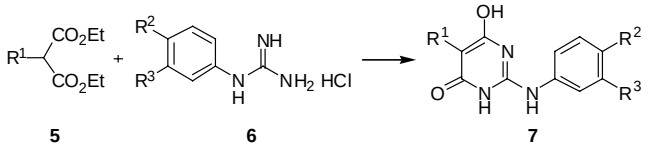
7 (Scheme 1).[14] The results for percent inhibition of *S. aureus* DNA Pol IIIC are shown in Table 1.[15] Taken from this small library, compounds **8** and **9** having an allyl or a benzyl substituents, respectively, displayed IC_{50}s from 31 to 37 μM and exhibited reasonable antimicrobial activity with MIC values lower than 16 μg/mL.

A systematic study of the elongation of the alkyl linker between the phenyl group and the pharmacophore was undertaken and the results are listed in Table 1. Elongation of the alkyl chain from the benzyl group of **9** to the phenylethyl **10** and phenylpropyl **11** showed an increase in activity of 2- and 10-fold, respectively, against DNA Pol IIIC, and a concurrent antibacterial activity with an MIC of 8 μg/mL. In contrast, the phenylbutyl **12** showed a drastic decrease in its enzymatic activity compared to the phenylpropyl **11** and its antimicrobial potency was eliminated. The maximum length of the carbon chain was determined to be three carbons between the phenyl group and the pharmacophore. As we demonstrated, the length of the side chain of the pyrimidinone is very important. In order to determine the optimal length in the series derived from **8**, several compounds possessing different alkyl chain lengths were evaluated as shown in Table 1. It was observed that as the chain length increases, up to a 5-carbon chain (**15**), the DNA Pol IIIC inhibition also increased.



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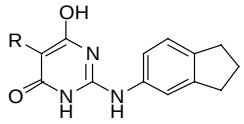
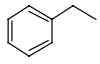
Scheme 1. Reagents and condition: MeONa, MeOH, 125 °C.

Table 1. In vitro DNA Pol IIIC inhibition and *Staphylococcus aureus* MIC of 5-alkyl-6-hydroxy-2-(2,3-dihydro-1*H*-inden-6-ylamino)pyrimidin-4(3*H*)-ones



Compound	R	% inhibition[a]	MIC[b]
8		65 (31)	16
9		90 (37)	8
10	Phenylethyl	68 (18)	8
11	Phenylpropyl	75 (3)	8
12	Phenylbutyl	35	>128
13	CH_2=CH–CH_2–	65 (30.9)	8–16
14	CH_2=CH$(CH_2)_2$–	87 (17.9)	8
15	CH_2=CH$(CH_2)_3$–	89	16
16	CH_2=CH$(CH_2)_4$–	81	16
17	Phenoxyethyl	74	16
18	Phenoxypropyl	74 (4)	8
19	Phenoxybutyl	67 (14)	8
20	3-Aminopropyl	40	>128
21	3-Hydroxypropyl	74	128
22	*N*-Propyl-piperidine	69	128

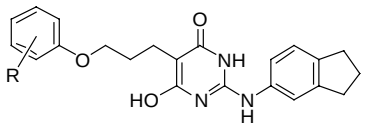
[a] % inhibition against Pol IIIC of *S. aureus* at 100 μM concentration of inhibitor. Values in parentheses correspond to the IC_{50}.
[b] Minimum inhibitory concentration (μg/mL) in *S. aureus* RN4220.

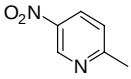
Unfortunately, the insolubility of the compounds in aqueous media mirrored the increase in chain length. To circumvent this problem, an oxygen atom was introduced in the chain. The phenyl in the previous set of molecules was replaced for a phenoxy group (Table 1). The three modified compounds **17**, **18**, and **19** retained both their in vitro and antimicrobial activities. The optimum number of carbon atoms tolerated with the phenoxy was again determined to be three. The phenoxypropyl **18** was the most potent compound in vitro with an IC_{50} of 4 μM and an MIC of 8 μg/mL. The same avenue explored by Wright and co-workers was attempted to increase the aqueous solubility of the compounds by introducing amino or hydroxyl groups on their side chains[16,17] as shown with compounds **20**, **21**, and **22**. Their affinity toward the enzyme was not affected by the introduction of these polar functionalities, but we observed total loss of their antibacterial activity, unlike that reported for the 6-anilinouracil series.[16,17]

Having these results in hand, we decided to focus on derivatives from compound **18**. Electron-donating groups were first introduced on the phenol ether. Although compounds **23** and **24** were slightly more active than **18** (respectively, 83%, 81%, and 80% inhibition at 100 μM compared to 74% for **2**), the presence of an electron-donating group (at least in the para position) was detrimental in terms of antibacterial activity. On the other hand, the presence of a simple electron-withdrawing group appeared to be promising (**27** inhibits DNA Pol IIIC to 91% at 100 μM (IC_{50} = 5.6 μM)). Other derivatives were then prepared as shown in Table 2. We were pleased to see that a close analog of our lead structure showed more potent inhibition but unfortunately it resulted in less antibacterial activity (Table 2, compound **28**, IC_{50} = 3 μM, MIC = 16 μg/ml). Early in the study, it was found that this series of compounds was difficult to solubilize in aqueous media. To solve this problem and to maintain the antibacterial activity, we prepared compounds possessing a more polar electron withdrawing group.

The inhibition of DNA Pol IIIC was retained in compounds **29** and **31**, but unfortunately, the introduction of these functional groups led to a complete loss of antibacterial activity (>128 μg/mL).

Table 2. Effect of substitution on the 2-amino-5-alkene-6-hydroxy-pyrimidine side chain



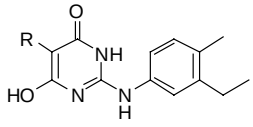
Compound	R	% inhibition[a]	MIC[b]
18	H	74 (4.1)	8
23	*p*-NH_2	83 (9)	>64
24	*p*-OMe	81 (11.6)	16
25	*p*-NO_2	70 (2.6–7.7)	4–8
26	*m*-OMe, *p*-NO_2	72	8
27	*p*-CN	91 (5.6)	8
28	*o*-OMe, *p*-CN	95 (3)	16
29	*p*-COOH	90	>128
30	p-COOMe	74	64
31	*p*-Tetrazole	93	>128
32		85 (5.1)	64

[a] % inhibition against Pol IIIC of *S. aureus* at 100 μM concentration of inhibitor. Values in parentheses correspond to the IC_{50}.
[b] Minimum inhibitory concentration (μg/mL) in *S. aureus* RN4220.

In the case of 6-anilinouracils, Wright and co-workers[11b,11c] had determined the 3-ethyl-4-methylphenyl group to be the optimum substituent, closely followed by the indanyl, on the 6-amino nitrogen. To confirm if this tendency could be extrapolated to this series, the most active compounds were synthesized as their 3-ethyl-4-methylphenyl derivatives (Table 3). Compounds **33**, **34**, and **35** with the 2-(3-ethyl-4-methylphenylamino) substituent displayed a 4- to 9-fold increase in affinity in vitro with respect to their counterparts **8**, **9**, and **28**, respectively. Unfortunately this increase in affinity did not improve the antibacterial activity.

In order to determine the mode of action of this newly synthesized class of inhibitors, the cellular levels of DNA synthesis in Gr+ bacteria were investigated. The results are summarized in Figure 1. The compounds were tested against three different metabolism pathways: DNA synthesis (methyl-[^{3}H]thymidine), RNA synthesis (5-[^{3}H]uridine), and protein synthesis ([^{35}S]-methionine).[18] Panels A and B demonstrate that compounds **8** and **9** inhibit DNA synthesis of *S. aureus* without affecting the production of either of the other macromolecules investigated, which is consistent with DNA Pol IIIC inhibition.

Table 3. Effect of the 2-(3-ethyl-4-methylanilino) substituent of different inhibitors



Compound	R	% inhibition[a]	MIC[b]
33	Benzyl	94 (4)	8
34	Allyl	90 (7.7)	8
35	(4-Cyano-2-methoxy phenyl)propyl	81	16

[a] % inhibition against Pol IIIC of *S. aureus* at 100 μM concentration of inhibitor. Values in parentheses correspond to the IC_{50}.
[b] Minimum inhibitory concentration (μg/mL) in *S. aureus* RN4220.

To ensure the exclusion of grossly toxic compounds, they were also tested for their ability to inhibit the growth of mammalian cells so as to ascertain potential cytotoxicity in the host. This was monitored through both ATP and MTS assays performed in 96-well microtiter plates.[19] In the ATP assay, the production of ATP was used as the indicator of cell viability and in the MTS assay the production of reducing equivalents as the indicator of cell viability. None of the tested compounds showed any cytotoxicity in the MTS assay (not shown here).

As was determined by ATP production (Fig. 2), most of the pyrimidinones did not exhibit excessive toxicity (a few selected compounds appear in this figure).

In the case of **8**, **28**, and **33**, the ATP production is unaffected even at 100 μM. For compounds **11** and **34** the ATP production decreases only at a concentration of 100 μM, whereas compound **18** appears more cytotoxic as the ATP production is affected at 50 μM. The graph in Figure 2 illustrates clearly that most of these compounds are not cytotoxic at the levels used for the in vivo test. Finally, before completing the in vivo test, we performed an acute toxicity tolerability (ATT) test on three mice (two doses of 50 mg of compound at a 4 h interval were injected intraperitoneally in 40% HPCD or 10% DMSO, 90% peanut oil). In all cases, we observed a 100% mouse survival.

Given the potency of their antibacterial effects against the Smith strain of *S. aureus*, we selected compounds **8**, **28**, and **33** for testing in an in vivo infection model employing the same organism. Specifically, the in vivo mouse peritonitis model was performed according to the protocol described by Tarantino (*S. aureus* ATCC13709 in PBS was used for the infection).[16] The results, expressed as the number of mice surviving at 3 days after infection, are summarized in Table 4. As expected, all the animals treated with vancomycin survived through the 3 day period, while the 10 mice in the control group (treated with vehicle alone) died.

Concerning compound **8**, 5 out of 10 mice survived the infection, after two injections at 50 mg/kg, which is promising given its relatively high IC_{50} (31 μM). When the dose was decreased to a single injection at 50 mg/kg, 4 out of 10 mice survived the infection. We were pleased to see that all animals treated with **28** at a dose of 2×50 mg/kg survived. Surprisingly, compound **33**, which showed good in vitro potency, was not effective. Even though **28** and **33** had similar IC_{50}s and MICs, compound **28**,which was more water soluble than **33**, due to the substituents on its aromatic moiety, displayed better in vivo efficacy against *S. aureus* than **33**. These encouraging results suggest that the 2-amino-6-hydroxy-pyrimidin-4-one possesses potential as a new class of anti-infective compounds.

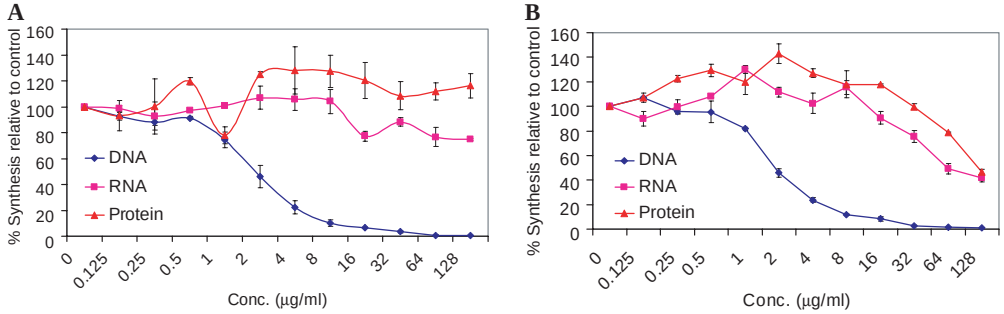


Figure 1. Mode of action study by whole-cell dose–response labeling: (A) 2-(2,3-dihydro-1*H*-inden-6-ylamino)-5-allyl-6-hydroxypyrimidin-4(3*H*)-one, **8**; (B) 2-(2,3-dihydro-1*H*-inden-6-ylamino)-5-benzyl-6-hydroxypyrimidin-4(3*H*)-one, **9**.

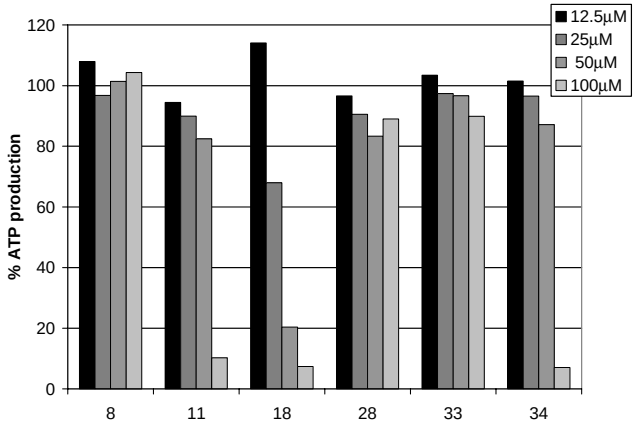


Figure 2. ATP production in the presence of increasing concentration of the compound.

The so-called 'furry test tube' serves as a less stringent infection model in antibiotic testing. In this assay, each animal is infected and treated at the same peritoneal compartment. This model allows bypassing the issues related with intravenous doses. Indeed, the problem associated with the 2-amino-6-hydroxypyrimidinone class is a strong in vitro tendency to bind to serum albumin that severely decreased in antibacterial activity. This resulted in MIC values $\geqslant$128 μg/mL for most of the compounds when measured in the presence of 50% human or mouse serum. A potential reason is the presence of the 6-hydroxy functionality which can be seen as a vinylogous acid and, that would favor binding to albumin. This issue needed to be addressed by the selection of a proper bioisostere, which would play the same role in the mechanism of inhibition (hydrogen donor or acceptor) but would lack the acid character which is probably the origin of the strong serum binding.

Table 4. Effect of some compounds on the lethal infection of mice by *Staphyloccocus aureus*[a]

Compound	No. of survivors at 3 days postinfection (total no. treated)
Vehicle[b] (40%HPCD)	0 (10)
Vancomycin (20 mg/kg)	10 (10)
8[c]	5 (10)
8[d]	4 (10)
28[c]	10 (10)
33[d]	1 (10)

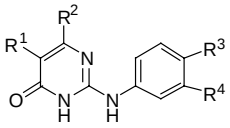
[a]Intraperitoneal infection followed by a single or double injection of drug or drug vehicle intraperitoneally.
[b] Saline for vancomycin.
[c] Injection of 2 × 50 mg/kg of drug at 15 and 60 min postinfection.
[d] Injection of 1 × 50 mg/kg of drug at 15 min postinfection.

We then explored a series of molecules where the 6-position was substituted by different isosteres. For the first set of modifications, the 6-hydroxyl group of **8** was replaced by functionalities that lacked acidic hydrogen. Compound **36**, having methyl ether at position 6, was prepared by simple Fisher esterification in methanol catalyzed by sulfuric acid of the corresponding **8**. At this position, the introduction of a hydrogen bond acceptor group, for instance a methoxy group (**36**), was ineffective. The ether group might be sterically too large to fit into the active site of the enzyme.

We then designed another series of inhibitors where position 6 was substituted by a chloride. Compounds **37** and **38** were prepared from the corresponding 6-hydroxypyrimidinones by refluxing them overnight in the presence of phosphorus oxychloride and diethylaniline in acetonitrile.[20] The resulting 4,6-dichloropyrimidines were then heated in a sealed tube with a mixture of 50% aqueous NaOH and dioxane to furnish the desired 6-chloropyrimidinones.[21] The 6-chloroderivatives **37** and **38**, substituted with 5-allyl and 5-phenylpropyl groups, respectively, displayed fair in vitro inhibitions and antimicrobial activities as shown in Table 5. Compound **37** was very promising (IC_{50} of 2.3 μM and a MIC of 1 μg/mL). The 6-chloroderivatives provided a 16-fold increase in potency as compared with **8** (IC_{50} of 31 μM and a MIC of 16 μg/mL). Unfortunately, in the presence of 50% human serum, these inhibitors lost all antimicrobial activity, a result suggestive of protein binding (Table 5).

Compound **39**, being substituted by a 6-hydrogen and a 5-propyl, was obtained by simple catalytic reduction of the compounds **37** with 10% palladium on charcoal under a hydrogen atmosphere in a mixture of ethanol and concentrated ammonium hydroxide.[22] This compound showed no activity. The presence of a substituent other than hydrogen at position 5 seemed mandatory to maintain some DNA Pol IIIC inhibition.

Table 5. In vitro DNA Pol IIIC inhibition and *Staphylococcus aureus* MIC of 2-(2,3-dihydro-1*H*-inden-6-ylamino)pyrimidin-4(3*H*)-ones



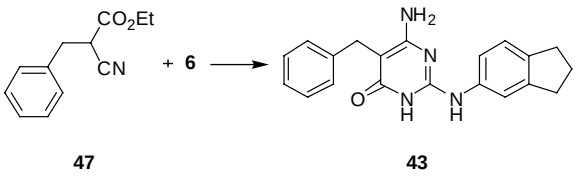
Compound	R^1	R^2	R^3	R^4	% dGTP inh[a]	dATP IC_{50}[b]	MIC[c]	MIC HS[d]
8	Allyl	OH	–CH_2–CH_2–CH_2		65 (31)	—	16	>128
36	Allyl	MeO	–CH_2–CH_2–CH_2		33	—	128	>128
37	Allyl	Cl	–CH_2–CH_2–CH_2		53 (2.3)	—	1	>128
38	Phenylpropyl	Cl	–CH_2–CH_2–CH_2		46	—	16	>128
39	Propyl	H	–CH_2–CH_2–CH_2		7	—	>128	>128
40	H	NH_2	–CH_2–CH_2–CH_2–		5	—	>128	>128
41	H	NH_2	Methyl	Ethyl	36 (104)	28	16	32
42	Allyl	NH_2	Methyl	Ethyl	32 (164)	7.7	8	32
43	Phenylpropyl	NH_2	–CH_2–CH_2–CH_2–		0	>100	>128	>128
44	Phenylpropyl	NH_2	Methyl	Ethyl	12	7.9	>128	>128
45	Benzyl	NH_2	–CH_2–CH_2–CH_2–		17	>100	>128	>128
46	Benzyl	NH_2	Methyl	Ethyl	3	9.7	>128	>128

[a] % inhibition against Pol IIIC of S. aureus at 100 μM concentration of inhibitor. Values in parentheses correspond to the IC_{50}.
[b] IC_{50} (μM) in the 'low dATP assay.'
[c] Minimum inhibitory concentration (μg/mL) against *S. aureus* RN4220.
[d] Minimum inhibitory concentration (μg/mL) against *S. aureus* RN4220 in the presence of 50% human serum.

We then explored a third series of molecules where the 6-position was substituted by a basic amino group presumably less likely to favor binding to serum proteins. Synthesis of the 6-aminopyrimidinones **43** was achieved by cyclocondensation of the appropriately substituted ethyl cyanoacetate **47** on the arylguanine **6** in acetonitrile at 125 °C as illustrated in Scheme 2. Unexpectedly, compound **40** substituted at position 2 by a 2,3-dihydro-1*H*-inden-5-amine did not display any in vitro DNA Pol IIIC inhibition or antibacterial activity like the other series. However, the 3-ethyl-4-methylaniline analog **41** exhibited an IC_{50} of 104 μM and an MIC of 16 μg/mL (Table 5). Introduction of an allyl side chain at position 5 of this inhibitor (compound **39**) increased the antibacterial activity (MIC 8 μg/mL) by 2-fold but decreased the inhibitory potency toward the enzyme (164 μM). Other 6-amino derivatives were prepared (**43**, **44**, **45**, and **46**) without improvement in the inhibitory or antibacterial activities (Table 5). Interestingly, the 2,6-diaminopyrimidinone derivatives **41** and **42** were tested in the presence of 50% human serum and retained their antibacterial activities (MIC of 32 μg/mL). Furthermore, they did not show cytotoxicity in ATP and MTS assays at 100 μM. The basic amino group at position 6 is believed to be partially positively charged at physiological pH and therefore resulted in reduced affinity for serum proteins.

Structural relationships between the 2,6-diaminopyrimidinone analogs and the active site model for DNA Pol IIIC enzyme led us to the hypothesis that these inhibitors are dATP competitors and interact preferably with thymidine residues as shown in Figure 3.

The IC_{50}s of DNA Pol IIIC inhibitors were determined in an in vitro DNA replication assay utilizing *S. aureus* replication proteins. The results are shown in Table 5. In this assay, duplication of singly primed circular ssM13 template by *pol*C was dependent on the presence of the processivity clamp β, the clamp loading complex proteins δδ′ and τ, and single-stranded binding protein (SSB). The assay conditions were adapted from those described by Bruck et al.[23] for use in Streptavidin-coated FlashPlates™ with [^{3}H]dTTP as the marker. The assay conditions were sensitized for dATP and dGTP analogs by using reduced dATP or dGTP concentrations, respectively.[24] Inhibitor **41** and its parent **42** resulted in IC_{50}s of 28 and 7.7 μM, respectively. Compounds **43** and **45** substituted with phenylpropyl and benzyl, respectively, retained their in vitro activities (IC_{50}s of 7.9 and 9.7 μM) but demonstrated no antibacterial activity. Conversely, parent compounds **44** and **46**, substituted with 2,3-dihydro-1*H*-inden-5-amine, completely lost their activities. In this series, a clear trend can be noted; the affinity and selectivity conferred by



Scheme 2. Reagents and condition: CH_3CN, 125 °C.

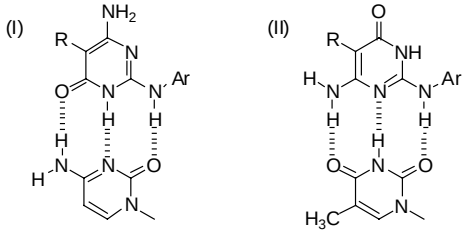


Figure 3. (I) Hydrogen bonding of aminopyrimidinone with cytosine; (II) hydrogen bonding of aminopyrimidinone with thymidine.

the 3′-ethyl-4′-methyl-aniline versus the 2,3-dihydro-1*H*-inden-5-amine are essential to produce *in vitro* DNA Pol IIIC inhibition as well as antimicrobial activity.

In summary, 6-hydroxy-2-(anilino)pyrimidinones as a novel non-nucleobase class of *S. aureus* DNA Pol IIIC inhibitors were developed. From the SAR study, it was determined that the activities within this series of compounds were sensitive to the variation in the chain length of the alkyl linker at position 5. These compounds were found to have good in vitro and in vivo antibacterial activities. Further structural modifications of the pyrimidinone core may afford improved in vivo properties leading to a compound suitable for the treatment of infections. We have also developed novel 2,6-diaminopyrimidinone inhibitors of *S. aureus* DNA Pol IIIC. From the SAR study, it was determined that the introduction of an amino group at position 6 of the 2,6-diaminopyrimidinone was beneficial for retaining antimicrobial activity in the presence of 50% human serum. This novel series of inhibitors does not exhibit cytotoxicity and selectively inhibits *S. aureus* DNA Pol IIIC by competing with dATP. Further SAR studies on this new series of inhibitors are currently under investigation.

Acknowledgments

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References and notes

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14. All new compounds showed purity >95% by HPLC at two wavelengths (254 nm and 220 nm) and are characterized by ^{1}H NMR and HPLC–MS spectroscopy.
15. For the DNA Pol IIIC inhibition assay, a double-stranded DNA oligonucleotide was used as the substrate. Assays were completed in triplicate in a 25 μL volume (20 mM Tris-HCl, pH 7.5, 4% glycerol, 0.1 mM EDTA, 5 mM DTT, 40 μg/ml IgG, 8 mM $MgCl_2$, 5 μM dATP, 5 μM dCTP, 0.2 μM dGTP, 2.5 μM [^{3}H]dTTP (1 Ci/mmol), and 0.4 μM PolC oligonucleotide substrate. Reactions were initiated by the addition of 125 ng of purified PolC and incubated at 37 °C for 30 min. The reactions were quenched by the addition of 20 mM EDTA (final concentration) followed by spotting of the reaction mixtures on DE81 filter paper. The filter papers were dried then washed 4× with ~200 ml 0.15 mM ammonium formate/0.01 mM sodium phosphate solution, followed by one wash in ~200 ml of H_2O. Filters were dried and then radioactivity was determined by liquid scintillation counting. The compounds were screened in triplicate at three initial concentrations; 10, 50, and 100 μM. Compounds with 70% inhibition or greater at 100 μM and/or MICs of 16 μg/mL or lower in *S. aureus* RN4220 were further analyzed by a 12-point serial dilution, spanning the IC_{50} by approximately 2-fold.
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18. Norfloxacin, which selectively inhibits DNA synthesis and Rifampicin, which inhibits both protein and RNA synthesis of bacteria, were used as control compounds.
19. Cryopreserved primary human hepatocytes were used in the ATP assay and cultured HeLa cells were used in the MTS assay. For the ATP assay, compounds at 100, 50, 25, and 12.5 μM were incubated with 1×10^4 primary human hepatocytes per well in Krebs–Henseleit buffer for 2 h at 37 °C under 5% CO_2. At the end of the incubation, the ATP content was determined by the addition of luciferin and luciferase, and monitored by luminescence. For the MTS assay, compounds at 100, 50, 25, and 12.5 μM were incubated with 2×10^4 HeLa cells per well in Dulbecco's modified Eagle's Medium containing 1% bovine growth serum for 18 h at 37 °C under 5% CO_2. At the end of the incubation, the amount of reducing equivalents was determined by the reduction of MTS reagent to a formazan product as revealed by absorbance at 490 nm.
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24. To validate our 'low dATP assay,' some of our previous inhibitors were tested in 'low dGTP assay' to determine their IC_{50}. The obtained values were coherent with the ones reported.