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Novel inhibitors of *Staphylococcus aureus* RnpA that synergize with mupirocin

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ABSTRACT

We recently discovered RnpA as a promising new drug discovery target for methicillin-resistant *S. aureus* (MRSA). RnpA is an essential protein that is thought to perform two required cellular processes. As part of the RNA degrasome Rnpa mediates RNA degradation. In combination with *rnpB* it forms RNase P haloenzymes which are required for tRNA maturation. A high throughput screen identified RNPA2000 as an inhibitor of both RnpA-associated activities that displayed antibacterial activity against clinically relevant strains of *S. aureus*, including MRSA. Structure-activity studies aimed at improving potency and replacing the potentially metabotoxic furan moiety led to the identification of a number of more potent analogs. Many of these new analogs possessed overt cellular toxicity that precluded their use as antibiotics but two derivatives, including compound **5o**, displayed an impressive synergy with mupirocin, an antibiotic used for decolonizing MSRA whose effectiveness has recently been jeopardized by bacterial resistance. Based on our results, compounds like **5o** may ultimately find use in resensitizing mupirocin-resistant bacteria to mupirocin.

Staphylococcus aureus infections are often associated with high rates of morbidity and mortality.[1] First described in 1961,[2,3] the emergence of methicillin-resistant strains of *S. aureus* (collectively referred to as MRSA) has become a significant health issue. On a positive note, a recent study by the U.S. Center for Disease Control found that the prevalence of community-acquired and hospital-acquired MRSA in the U.S. has declined somewhat since 2005 due, in part, to enhanced awareness and more careful healthcare practices.[4] However, there has been an increase in the number of multidrug resistant strains of *S. aureus* that respond poorly to treatment by multiple classes of antibiotics, including the current standard of care for MRSA, vancomycin. Taken together with the observation that antibiotic drug discovery research has declined significantly since the 1990s, it has become obvious that there is a real need for the development of new classes of antibiotics.[5]

Today's antibiotics act through a number of drug targets to exert their antimicrobial effects.[6] Resistant strains of *S. aureus* have acquired mechanisms that reduce the effectiveness of many of the known classes of antimicrobial agents. As a result, recent antibiotic drug candidates that act through familiar pathways have not been able to differentiate themselves from existing agents and have not received regulatory approval. What is needed is new classes of antibacterial drugs.

We recently disclosed our discovery of RnpA as a novel drug discovery target for *S. aureus*, including MRSA.[7] *S. aureus* RnpA is an essential protein that is thought to perform two required cellular processes. As a component of the RNA degradosome, it mediates mRNA degradation, providing substrates for new RNA synthesis. Together with *rnpB*, it forms RNase P holoenzymes, which are required for tRNA maturation. Thus, small molecule RnpA inhibitors have the potential to pose as dual threat antimicrobial agents, acting as multi-target ligands that eliminate the organism's ability to catalyze both mRNA decay and tRNA maturation. This, coupled with the facts that the enzyme is expressed in disease-associated *S. aureus* (including MRSA), is highly conserved across other emerging and re-emerging pathogens, and has no significant similarity to any human protein, make RnpA an attractive antibacterial drug target.

No direct inhibitors of RnpA have been reported in the literature, although four classes of small molecule RNase P inhibitors have been described (Fig. 1).[8] Puromycin mimics the 3′-terminal

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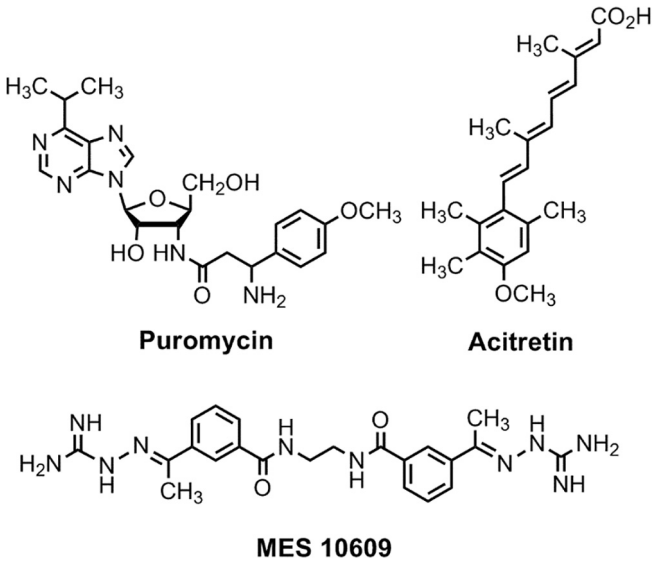


Fig. 1. Known RNase P inhibitors.

CCA sequence of tRNA and serves as a competitive inhibitor of tRNA precursor cleavage by RNase P.[9] Retinoids such as acitretin inhibit the action of both human[10] and *D. discoideum*[11] RNase P, although the exact mechanism of inhibition is not known. Aminoglycosides, including neomycin B and kanamycin A and B (structures not shown), have been shown to inhibit RNase P *in vitro* activity by interfering with the binding of active site magnesium ions that are essential for activity.[12] Most recently, a series of benzamide-based RNase P inhibitors, exemplified by MES 10609, was disclosed in a patent awarded to Message Pharmaceuticals.[13] All of these various series displayed potencies that were in the low micromolar to low millimolar range.

A high throughput screen of over 29,000 small molecules identified a number of compounds that inhibited mRNA turnover by recombinant *S. aureus* RnpA, including RNPA1000 (**1**) and RNPA2000 (**2**) (Fig. 2).[7] Compounds **1** and **2** demonstrated moderate antibacterial activity *in vitro* against the predominant methicillin-susceptible and MRSA lineages circulating throughout the U.S., were effective against *S. aureus* biofilm associated cells, and showed selectivity for RnpA over a number of other ribonucleases evaluated, including *E. coli* RNase H, RNase A, RNase I and *S. aureus* RNase J1. *In vivo*, **1** reduced *S. aureus* pathogenesis in a murine acute lethal model of infection. However, the presence of a reactive Michael acceptor group in compound **1** drove us to select compound **2** for initial hit-to-lead activities. Key tasks for this scaffold included eliminating the thiourea[14–16] hydrazide[17] and furan moieties,[18] which can be metabolized to give reactive species that cause significant toxicity. In the present report we describe our efforts to identify suitable bioisosteric replacements for the furan group.

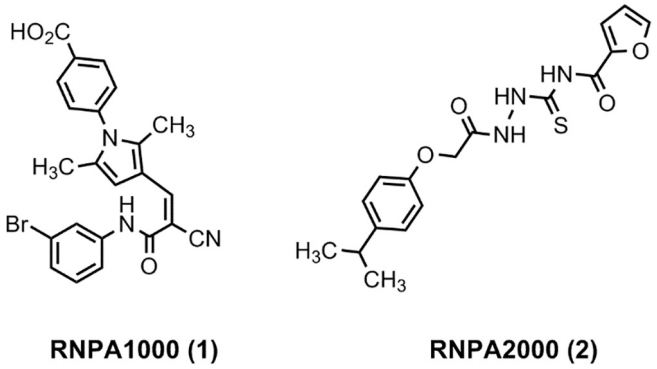
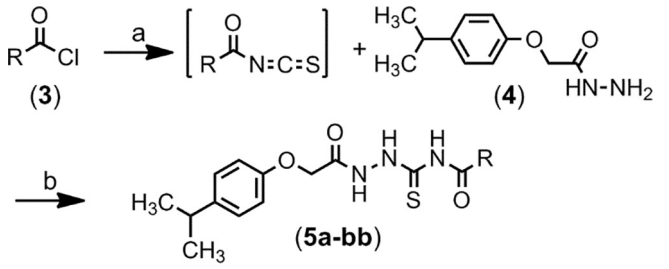


Fig. 2. Structures of RNPA1000 and RNPA2000.

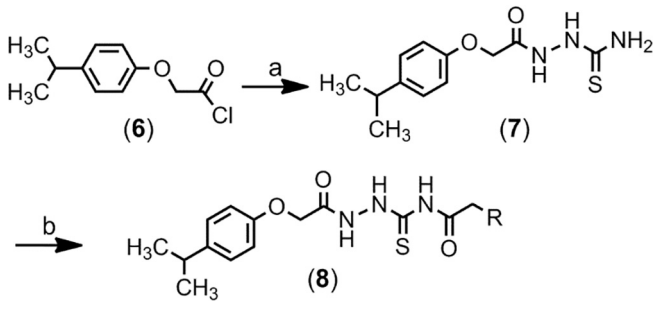
The general method of Li et al. was used to synthesize most of the required thiahydrazide analogs (Scheme 1).[19] Acid chlorides (**3**) were sequentially treated in acetonitrile with potassium thiocyanate followed by 2-(4-isopropylphenoxy)acetohydrazide (**4**) to give the desired products (**5**) in 15–80% yield.[20] Final products that did not precipitate from the reaction were obtained by reversed-phase chromatography. In our hands, the use of potassium thiocyanate and acetonitrile provided better yields than the original conditions described by Li et al. (ammonium thiocyanate, CH_2Cl_2/PEG400), although the final products tended to precipitate out of the reaction mixture more often when the original conditions were used.

The synthetic sequence shown in Scheme 1 worked well when the acid chloride component (**3**) was either an aryl acid chloride (e.g., 2-furoyl chloride) or a cinnamoyl chloride (e.g., 2-(2-furoyl)-acryloyl chloride). However, when benzylic hydrogens were present in the acid chloride component no reaction with thiocyanate was realized. The final product in these reactions resulted from direct combination between the acid chloride (**3**) and the acylhydrazide (**4**). An alternate synthetic sequence was devised to prepare the desired homologated thiahydrazide analogs (Scheme 2).[21] 2-(4-Isopropylphenoxy)acetyl chloride (**6**) was treated with semicarbathiazide to give the resulting carbothioamide (**7**), which was subsequently reacted with acid chloride (**3**) to provide the desired final product (**8**) in low yield.

The compounds were tested for their ability to inhibit recombinant *in vitro S. aureus* RnpA-mediated mRNA degradation activity (Table 1; ≥97% protein purity), as previously described.[7,22] Likewise, *in vitro* inhibitory effects on tRNA maturation activity were measured using RNase P reconstituted with equimolar amounts of *S. aureus* RnpA and *rnpB*, as previously described.[22] Antibacterial susceptibility testing was performed using *S. aureus* strain UAMS-1, a well-characterized methacillin-sensitive osteomyelitis clinical

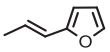
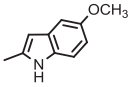
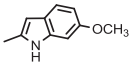
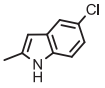
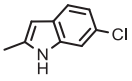
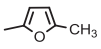
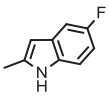
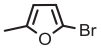
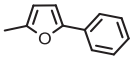
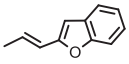


Scheme 1. Synthesis of thiahydrazide inhibitors of RnpA. *Reagents and conditions*: (a) Potassium thiocyanate, CN_3CN, rt; (b) add 2-(4-isopropyl-phenoxy)acetohydrazide (**4**), stir at room temperature.



Scheme 2. Synthesis of homologated thiahydrazides. *Reagents and conditions*: (a) Semicarbathiazide, CN_3CN, rt; (b) R-CH_2-C(O)Cl (**3**), THF, 60 °C.

Table 1
RnpA inhibitory activity, MIC and cytotoxicity of compounds **2**, **5a**–**5bb** and **8**.

Entry	R Group	RnpA[a] IC_{50}, μM	RNase P[b] IC_{50}, μM	MIC, UAMS-1 μg/mL[c]	HEPG2 μg/mL[d]	Entry	R Group	RnpA[a] IC_{50}, μM	RNase P[b] IC_{50}, μM	MIC, UAMS-1 μg/mL[c]	HEPG2 μg/mL[d]
2		275	140	16	>128	**5n**		100	32	64	>256
5a		250	150	16	16	**5o**		60	1	32	>128
5b		250	25	64	>256	**5p**		>250	25	128	128
5c		>500	395	64	>256	**5q**		50	100	>256	512
5d		>500	>500	32	>128	**5r**		50	0.5	16	>256
5e		>500	50	64	64	**5s**		40	90	32	>128
5f		>250	25	16	16	**5t**		240	50	64	>256
5g		100	1	32	>128	**5u**		50	50	32	32
5h		>500	160	64	>256	**5v**		50	50	>256	>512
5i		>500	150	128	>512	**5w**		>500	100	256	256
5j		20	150	32	>128	**5x**		250	< 50	16	32
5k		30	105	32	>128	**5y**		<50	<50	64	256
5l		30	40	32	>128	**5z**		150	90	128	512
5m		40	50	64	64	**5aa**		30	26	>256	256
8		25	25	16	32	**5bb**		>500	350	64	512
	Mupirocin[e]	>0.25[f]	>0.25[f]	0.125	>512 [g]	**1**	RNPA1000[e]	100	175	32	25

[a] Inhibition of mRNA degradation by recombinant *S. aureus* RnpA.
[b] Inhibtion of tRNA cleavage by a 1:1 mixture of *S. aureus* RnpA and RnpB.
[c] Minimum inhibitory concentration toward *S. aureus* strain UAMS-1.
[d] Lowest concentration causing cytotoxicity in HepG2 cells.
[e] Data taken from Eidem et al.[22]
[f] Highest concentration tested (2X MIC for *S. aureus* strain UAMS-1).
[g] Rabbit corneal endothelial cells.[26]

isolate,[23] according to Clinical and Laboratory standards guidelines (CLSI)[24] in 96-well format (Table 1). Minimum inhibitory concentrations (MICs) were defined as the lowest concentrations of test compounds at which there was no visible bacterial growth. Overt cytotoxicity was assessed in human HepG2 hepatocytes using the MTT method.[25]

The goal of this initial SAR campaign was to identify suitable replacements for the furan moiety of compound **2**, which is known to undergo oxidative metabolism in the 5-position to give reactive electrophilic species that can cause toxicity.[18] The presence of a heteroatom in this ring appeared to be necessary since substitution of the furan by a phenyl ring (**5bb**) essentially abolished the RnpA mRNA degradation inhibitory activity and greatly reduced RNase P inhibitory potency. Substitution of the furan with a thiophene group (compound **5b**, Table 1) resulted in a compound with similar inhibitory potency against both mRNA degradation and

RNase P activity as that seen for **2**. Having the heteroatom in the 2-position appeared to be favorable for furans, thiophenes and pyridines (compounds **5a**–**5i**). While structural information on the binding site of these molecules is not available, the pocket harboring the furan seems to be fairly lipophilic since oxazole (**5e**) and isoxazole (**5d**) were not tolerated, especially when measuring RnpA-mediated mRNA degradation activity. That lipophilic pocket also seems to be somewhat longer than originally hypothesized from the activity of compound **2**, as suggested by the enhanced potency of derivatives possessing longer lipophilic groups in place of furan. For example, analogs possessing lipophilic substitutions on the 5-position of the furan moiety (**5k**–**5m**) as well as benzofuran, benzothiophene and indole (**5o**–**5s**) all demonstrated superior activity against the enzyme's mRNA degradation and RNase P functions compared to **2**. Homologation also enhanced potency, as evidenced by the results seen with compounds **5n**, **5aa** and **8**.

For the indole group, substitution on the nitrogen (**5t**) was detrimental while substitution in the 5- and 6-positions was tolerated (**5u**–**5y**). Structural changes did not always affect the two activities similarly (i.e., changes that affected mRNA degradation activity did not always impose a similar effect on tRNA processing). The reason for this is not clear at present, although it may indicate that the molecules elicit their effects on mRNA turnover and tRNA processing by binding to two distinct sites on the protein.

Antimicrobial efficacy could not be accurately assessed for many of these more potent analogs due to the presence of overt cytotoxicity as demonstrated in human HepG2 cells (Table 1). Only the analogs possessing unsubstituted 2-furanyl groups (**5a**, **8**) demonstrated antimicrobial potency similar to that seen with **2** (MIC = 16 μg/mL) that could not be explained by overt cytotoxicity. However, in the other compounds tested, a trend toward greater antibacterial potency against *S. aureus* UAMS-1 (MIC = 32 μg/mL) was seen with the compounds that demonstrated good activity against both RnpA and RNase P functions. Compounds that were potent at only one of these targets (e.g., **5i**, **5w**) provided relatively weaker antibacterial activity. Another factor that might be influencing the antimicrobial activity of these analogs is lipophilicity. Almost all of the structural changes that resulted in improved RnpA inhibitory activity resulted in increased lipophilicity compared to compound **2** (Table S1). In general, effective antibiotics that penetrate bacterial cell walls and act intracellularly tend to be more hydrophilic compared to other drug classes and often possess a logP "sweet spot" that is characteristic of the structural class.[27] It is possible that the increased inhibitory activity against the target, as seen in many of our improved molecules is not being realized in the antibacterial assay due to reduced cellular penetration.

Antibiotics that function within the same biochemical pathway often display synergy. A classic example is the synergy seen between sulfonamides and trimethoprim which both function within the folic acid biosynthesis pathway.[28] Compound **2** was previously shown to synergize with mupirocin, an inhibitor of isoleucyl-tRNA synthetase used topically for the treatment of impetigo and *S. aureus* decolonization. However, the effectiveness of this approach has come into question due to the emergence of bacterial resistance to mupirocin.[29] Since a synergistic approach represents a potential means of re-sensitizing resistant bacteria to mupirocin we explored the potential synergy of the present series with mupirocin.

Compounds that displayed RnpA inhibitory activity, reasonable antimicrobial activity (MIC $\leq$ 32 μg/mL) and a high therapeutic window over general cytotoxicity were examined for their ability to synergize with mupirocin as previously described[22] by determining their fractional inhibitory concentration[30] index (FICI) using standard checkerboard assays.[31] Individual wells of a 96-well microtiter plate were inoculated with 1×10^5 CFU ml^{-1} of *S. aureus* UAMS-1. Each row contained increasing concentrations of the test compound in 2-fold increments (0, 0.5, 1, 2, 4, 8, 16, 32 μg/mL). Each column contained increasing increments of mupirocin (0, 0.03125, 0.0625, 0.125 μg/mL). The plates were incubated for 18 h at 37 °C and growth was measured. The FICIs were determined using the formula: FICI = (MIC of test compound in combination/MIC of test compound alone) + (MIC of mupirocin in combination/MIC of mupirocin alone). A potential synergistic combination was defined as showing an FICI $\leq$ 0.5.[31] Results are summarized in Table 2 and detailed data are provided in the Supplementary Data Section.

Table 2
FICIs of selected compounds.

Compound	Conc. μg/mL	Mupirocin μg/mL	FICI*
2	4	0.0625	0.49
5g	8	0.0625	0.5
5k	4	0.03125	0.5
5l	2	0.0625	0.3125
5o	8	0.03125	0.25
5r	1	0.0625	0.56
5s	8	0.0625	0.5

* FICI = Fractional Inhibitory Concentration Index. MIC for mupirocin alone = 0.125 μg/mL.

Many of the compounds tested showed an additive effect (FICI between 0.5 and 1.0) with mupirocin but compounds **5l** and **5o** were shown to be synergistic based on the recommended guidelines,[31] exhibiting FICI values of <0.5 at multiple sub-MIC dose combinations. The superior synergy seen with these compounds compared to **2** and the other analogs tested correlated most closely with their ability to more potently inhibit both RnpA-associated activities. This hypothesis is supported by two observations. First, previously reported data shows that compound **1**, which inhibits RnpA-mediated mRNA degradation but does not prevent RNase P-associated tRNA maturation, does not synergize with mupirocin.[22] Secondly, compounds like **5g** and **5r**, which showed potent RNase P inhibitory activity (IC_{50} = 1 μM and 0.5 μM, respectively) but weak RnpA mRNA degradation inhibitory activity, also failed to show definitive synergistic effects (Table 2 and Checkerboard Synergy Charts, pS17 supplementary data). Taken together, these data suggest that both RnpA and RNase P inhibition must be present for these analogs to synergize with mupirocin.

In conclusion, SAR within the RNPA2000 series of molecules to find suitable replacements for the undesirable furanyl moiety identified a number of bioisosteric heterocyclic groups. In particular, the 2-benzofuranyl and 2-indolyl groups provided molecules with good RnpA inhibitory activity and potent RNase P inhibitory activity. Most of the analogs tested demonstrated somewhat less potent antibacterial activity and higher overt cytotoxicity than the prototype molecule **2** but two analogs, **5l** and **5o**, were shown to be synergistic with mupirocin against a contemporary *S. aureus* clinical isolate and may find use as combination agents in mupirocin decolonization. Studies to identify suitable replacements for the acylhydrazide and thiourea moieties in the scaffold are currently underway and will be reported at a later date.

Acknowledgements

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A. Supplementary data

Supplementary data (synthetic procedures and spectroscopic characterization, logP calculation method, logP values for compounds **2**, **5a**–**5bb**, **8**, biological procedures, detailed checkerboard synergy charts) associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.01.022.

References

1. Schorr AF, Tabak YP, Killian AD, Gupta V, Liu LZ, Kollef MH. Healthcare-associated bloodstream infection: a distinct entity? Insights from a large U.S. database. *Crit Care Med*. 2006;34:2588.
2. Jevons MP. "Celbenin"-resistant Staphylococci. *Br Med J*. 1961;1:124.
3. Barber M. Methicillin-resistant Stapylococci. *J. Clin. Path.*. 1961;14:385.
4. Dantes R, Mu Y, Bellflower R, et al. National burden of invasive methicillin-resistant Staphylococcus aureus infections, United States, 2011. *JAMA Int Med*. 2011;173:1970.
5. Spellberg B, Guidos R, Gilbert D, et al. The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. *Clin Infect Dis*. 2008;46:155.
6. Walsh C, ed. *Antibiotics, Actions, Origins, Resistance*. Washington, D.C.: American Society for Microbiology; 2003.
7. Olsen PD, Kuechenmeister LJ, Anderson KL, et al. Small molecule inhibitors of Staphylococcus aureus RnpA alter cellular mRNA turnover, exhibit antimicrobial activity and attenuate pathogenesis. *PLoS Pathog*. 2011;7:e1001287.
8. Eder PS, Hatfield D, Vioque A, Gopalan V. Bacterial RNase P as a potential target for novel anti-infectives. *Curr Opin Invest Drugs*. 2003;4:937.
9. Vioque A. Protein synthesis inhibitors and catalytic RNA. Effects of puromycin in tRNA precursor processing by the RNA component of Escherichia coli RNase P. *FEBS Lett*. 1989;246:137.
10. Pavlidou D, Vourekas A, Monastirli A, Kalavrizioti D, Tsambaos D, Drainas D. Isolation of ribonuclease P activity from human epidermis and its regulation by retinoids in vitro. *Acta Derm Venerol*. 2006;86:114.
11. Papadimou E, Beorgiou S, Tsambaos D, et al. Inhibition of ribonuclease P activity by retinoids. *J Biol Chem*. 1998;273:24375.
12. Mikkelsen NE, Brannvall M, Virtanen A, Kirsebom LA. Inhibition of RNase P RNA cleavage by aminoglycosides. *PNAS*. 1999;96:6155.
13. Giordano T, Sturgess MA, Rao SJ. Inhibitors of RNase P proteins as antibacterial compounds. *U.S. Patent 7,001,924 B2*. Awarded February 21, 2006.
14. Miller AE, Bishchoff JJ, Pae K. Chemistry of aminoiminomethanesulfinic and –sulfonic acids related to the toxicity of thioureas. *Chem Res Toxicol*. 1988;1:169.
15. Sarubu R, Bizzoarro FT, Corbett WL, et al. Discovery of piragliatin- first glucokinase activator studied in type 2 diabetic patients. *J Med Chem*. 2012;55:7021.
16. Scott AM, Powell GM, Upshall DG, Curtis CG. Pulmonary toxicity of thioureas in the rat. *Environ Health Perspect*. 1990;85:43.
17. Lee WM. Drug-induced hepatotoxicity. *New Eng J Med*. 1995;333:1118.
18. Burka L, Boyd M. In: Anders M, ed. *Bioactivation of Foreign Compounds*. Orlando, FL: Academic Press; 1985.
19. Li Z, Wang X, Da Y. Synthesis of 2-(5-(2-chlorophenyl)-2-furoylamido)-5-aryloxymethyl-1,3,4-thiadiazoles under microwave irradiaton. *Synth Commun*. 2001;31:1829.
20. The synthesis of (**2**) exemplifies a typical procedure: A mixture of 2-furoyl chloride (1 eq) and potassium thiocyanate (1.7 eq) in dry acetonitrile was stirred at room temperature for 1 hour. To the reaction was then added 2-(4-isopropylphenoxy)-acetohydrazide (0.97 eq) (**5**) and the resulting mixture was stirred for an additional 1 hour at room temperature. In the case of (**2**), the desired product precipitated as a white solid, which was collected by vacuum filtration, washed with acetonitrile and dried under vacuum to obtain a 76% yield of the desired product. In cases where products do not precipitate, the reaction mixtures are concentrated on a rotary evaporator and the crude reaction mixtures are purified on a Gilson 281 automated HPLC system using a Phenomenex 5u Gemini C-18 column and a gradient of acetonitrile in water with 0.1% trifluoroacetic acid.
21. In a typical procedure, a mixture of (4-(4-isopropylphenoxy)acetyl chloride (1 eq) and thiosemicarbazide (1.7 eq) were stirred in dry acetonitrile at room temperature overnight. The solution was concentrated and the solid residue was purified on a Gilson 281 automated HPLC system using a Phenomenex 5u Gemini C-18 column and a gradient of acetonitrile in water with 0.1% trifluoroacetic acid. The resulting crude intermediate (1 eq) was dissolved in dry tetrahydrofuran and sodium hydride (1 eq) was added. The reaction was stirred for 1 h. 2-Furanacetyl chloride (1 eq) was added and the reaction was stirred at 60 °C overnight. The solution was concentrated and the solid residue was purified by HPLC as described above to give the desired product as a white solid.
22. Eidem TM, Lounsbury N, Emery JF, et al. Small-molecule inhibitors of *Staphylococcus aureus* RnpA-mediated RNA turnover and tRNA processing. *Antimicrob Agents Chemother*. 2015;59:2016.
23. Sassi M, Sharma D, Rrinsmede SR, Felden B, Augagneur Y. Genome sequence of the clinical isolate *Staphylococcus aureus* subsp. *aureus* strain UAMS-1. *GenomeA*. 2015;3. e01584-14.
24. Garcia LS, ed. *Clinical Microbiology Procedures Handbook*. Third Edition. Washington, D.C.: Am. Soc. For Microbiology Press; 2010.
25. Mossman TJ. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983;65:55.
26. Cutarelli PE, Lazarus HM, Jacobs MR, Lass JH. Antimicrobial activity and in vitro corneal epithelial toxicity of antimicrobial agents for Gram positive corneal pathogens. *Curr Eye Res*. 1993;12:603.
27. Macielag MJ. Chemical properties of antimicrobials and their uniqueness. In: Dougherty TJ, Pucci MJ, eds. *Antibiotic Discovery and Development*. New York: Springer, US, New York; 2012:793–820.
28. Bushby SR. Synergy of trimethoprim-sulfamethoxazole. *Can Med Assoc J*. 1975;112:63.
29. Abad CL, Pulia MS, Safdar N. Does the nose know? An update on MRSA decolonization strategies. *Curr Infect Dis Rep*. 2013;15:455.
30. Elion GB, Singer S, Hitchings CH. Antagonists of nucleic acid derivatives VIII. Synergism in combinations of biologically related antimetabolites. *J Biol Chem*. 1954;208:477.
31. Olds FC. Synergy, antagonism and what the chequerboard puts between them. *J Antimicrob Chemother*. 2003;52:1.