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Article

Inhibition of the *Yersinia pestis* Methylerythritol Phosphate Pathway of Isoprenoid Biosynthesis by α-Phenyl-Substituted Reverse Fosmidomycin Analogues

Haley S. Ball,* Misgina Girma, Mosufa Zainab, Honoria Riley, Christoph T. Behrendt, Claudia Lienau, Sarah Konzuch, Leandro A. A. Avelar, Beate Lungerich, Iswarduth Soojhawon, Schroeder M. Noble, Thomas Kurz, and Robin D. Couch

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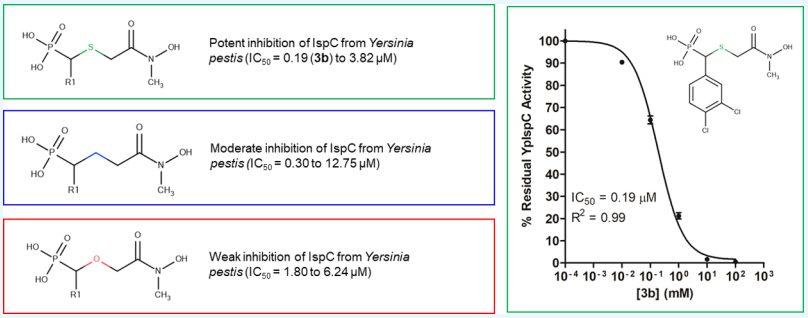

ABSTRACT: Fosmidomycin inhibits IspC (1-deoxy-D-xylulose 5-phosphate reductoisomerase), the first committed enzyme in the methylerythritol phosphate (MEP) pathway of isoprenoid biosynthesis. The MEP pathway of isoprenoid biosynthesis is essential to the causative agent of the plague, *Yersinia pestis*, and is entirely distinct from the corresponding mammalian pathway. To further drug development, we established structure−activity relationships of fosmidomycin analogues by assessing a suite of 17 α-phenyl-substituted reverse derivatives of fosmidomycin against *Y. pestis* IspC. Several of these compounds showed increased potency over fosmidomycin with IC_{50} values in the nanomolar range. Additionally, we performed antimicrobial susceptibility testing with *Y. pestis* A1122 (*Yp*A1122). The bacteria were susceptible to several compounds with minimal inhibitory concentration (MIC) values ranging from 128 to 512 μg/mL; a correlation between the IC_{50} and MIC values was observed.

INTRODUCTION

The plague, also known as the "Black Death," is caused by the bacterium *Yersinia pestis*.[1] The discovery of antibiotics, beginning with the introduction of penicillin in the 1940s,[2] was anticipated to be the end of bacterial diseases.[3] However, the evolution of antibiotic resistance has since resolved this notion. In fact, the increase in drug resistance has coincided with a decline in antibiotic discovery.[3] Compounding the issue of antibiotic resistance is the threat of bioterrorism, which became a reality with the 2001 anthrax attacks.[3,4]

In 2000, the US Centers for Disease Control and Prevention (CDC) released a response plan for bioterrorism, wherein they categorized several infectious agents according to their likelihood to be used as biothreats.[5] According to the CDC, *Y. pestis* is presently classified as a "category A" bioterrorism agent, that is, a high-priority agent.[5] High-priority agents are classified as such by their ease of transmission, high mortality rate, and likelihood to require a specialized public health response.[5]

Antibiotic treatment is effective against plague bacteria;[6] however, a 2017 plague outbreak in Madagascar resulted in approximately 2417 cases and 209 deaths within a period of 4 months.[7] The plague takes two main clinical forms: bubonic and pneumonic.[6] Thirty to sixty percent of cases of bubonic plague result in fatality,[6] and, if left untreated, pneumonic plague is always fatal.[1] Given the nature of antibiotic resistance, the need for preventative biothreat countermeasures, and the recent plague epidemics in Madagscar,[7] continued development of antibiotics is necessary for the prevention of widespread outbreaks and deaths.

Isoprenoids are one of the largest and most diverse group of natural products, enumerating over 30,000 known products.[8,9]

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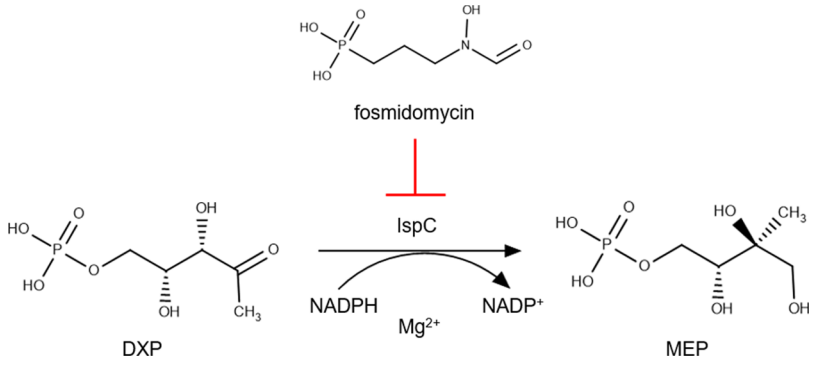

Figure 1. Inhibition of IspC by fosmidomycin.

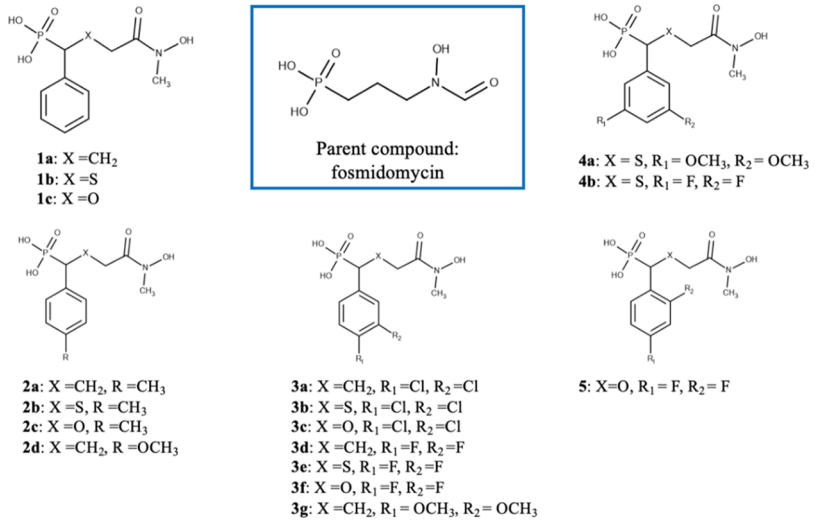

Figure 2. α-Phenyl-substituted β-carba, thia, oxa reverse fosmidomycin analogues. The β position, X, is either carbon (β-carba), sulfur (β-thia), or oxygen (β-oxa). R, R_1, and R_2 are either CH_3, OCH_3, Cl, or F.

They are fundamental biomolecules involved in vital biological functions such as electron transport and peptidoglycan biosynthesis in bacteria.[8,10,11] Bacteria synthesize isoprenoids via the methylerythritol phosphate (MEP) pathway of isoprenoid biosynthesis.[12,13] The MEP pathway is entirely distinct from the corresponding mammalian pathway, the mevalonic acid pathway, making it an attractive target for antibiotic development.[12,13]

Previously, we cloned, expressed, and characterized the first committed enzyme of the MEP pathway, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR/IspC) from *Y. pestis*.[14] IspC catalyzes the reduction and isomerization of 1-deoxy-D-xylulose 5-phosphate (DXP) to yield 2-*C*-methylerythritol 4-phosphate (MEP) (Figure 1).[15] Furthermore, we demonstrated the effectiveness of inhibiting both the purified *Y. pestis* enzyme (YpIspC) and liquid cultures of *Y. pestis* using the known IspC inhibitor, fosmidomycin (Figure 1).[14]

Previous studies have shown that α-phenyl substitutions of reverse derivatives of fosmidomycin are efficacious.[16−18] Furthermore, crystal structures of IspC- and α-phenyl-substituted reverse derivatives of fosmidomycin have been resolved.[19−21] However, no crystal structures of YpIspC in the presence of fosmidomycin or one of its analogues have been resolved. Therefore, to elucidate the structure−activity relationships (SARs) of α-phenyl-substituted reverse derivatives of fosmidomycin, we systematically assessed the potency of 17 compounds against YpIspC and performed antimicrobial susceptibility assays with *Yp*A1122. Specifically, we assessed the effect of methylene, oxygen, or sulfur at the β-position, the electron-donating or -withdrawing effects of the substituents on the α-phenyl ring, and the substitution pattern of the α-phenyl ring (Figure 2).

RESULTS AND DISCUSSION

Biological Evaluation of IspC Inhibitors. All assayed compounds were synthesized according to previously described procedures.[19−24] The addition of differently substituted phenyl rings at the α-position of fosmidomycin analogues can increase their potency against IspC enzymes from bacteria and the protozoan, *Plasmodium falciparum*.[17,19−21,24] However, to our knowledge, no inhibitors from this class have been tested against YpIspC. Furthermore, no crystal structures of YpIspC bound to fosmidomycin or one of its analogues have been resolved. To expand upon our understanding of SARs between YpIspC and fosmidomycin analogues, we screened a comprehensive library of our α-phenyl substituted reverse fosmidomycin analogues (Table 1). The determined half-maximal inhibitory concentrations (IC_{50} values) and the determined minimal inhibitory concentrations (MICs) for each compound are presented in Table 1. *Yp*A1122 is indicated as being either resistant (R), or susceptible (S), to each compound.

The most potent compounds in Table 1 (β-thia and β-carba analogues **3b**, **3d**, **3e**, and **4b**) are twice as potent as

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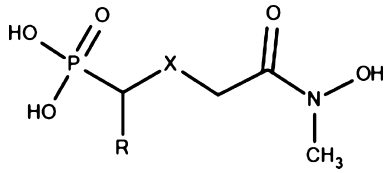
Table 1. Inhibition of *Y. pestis* A1122 and *Y. pestis* IspC by α-Phenyl-Substituted Reverse Fosmidomycin Analogues



compound	X	R	*Yp*IspC IC_{50} (μM)[a]	95% confidence interval of *Yp*IspC IC_{50} (μM)	*Yp*A1122 MIC (μg/mL)[b]
1a	CH_2	Ph	1.06	0.88−1.27	512 (S)
1b	S	Ph	0.39	0.29−0.51	128 (S)
1c	O	Ph	4.03	2.84−5.74	>512 (R)
2a	CH_2	4-CH_3-Ph	3.42	2.43−4.81	>512 (R)
2b	S	4-CH_3-Ph	0.72	0.54−0.97	256 (S)
2c	O	4-CH_3-Ph	6.21	3.93−9.80	>512 (R)
2d	CH_2	4-OCH_3-Ph	2.38	1.47−3.86	>512 (R)
3a	CH_2	3,4-Cl-Ph	1.36	1.06−1.74	>512 (R)
3b	S	3,4-Cl-Ph	0.19	0.15−0.23	128 (S)
3c	O	3,4-Cl-Ph	1.80	1.25−2.61	>512 (R)
3d	CH_2	3,4-F-Ph	0.30	0.24−0.38	128 (S)
3e	S	3,4-F-Ph	0.25	0.18−0.36	128 (S)
3f	O	3,4-F-Ph	1.90	1.34−2.70	>512 (R)
3g	CH_2	3,4-OCH_3-Ph	12.75	9.75−16.66	>512 (R)
4a	S	3,5-OCH_3-Ph	3.82	2.22−6.55	>512 (R)
4b	S	3,5-F-Ph	0.32	0.23−0.46	256 (S)
5	O	2,4-F-Ph	6.24	3.94−9.89	>512 (R)
fosmidomycin			0.71[c]		128 (S)

[a]*Yp*IspC = IspC from *Y. pestis*, IC_{50} = half-maximal inhibitory concentration. All assays were performed in duplicate. [b]*Yp*A1122 = *Y. pestis* strain A1122, MIC = minimal inhibitory concentration, R = resistant, S = susceptible. Six replicates were performed for each compound. [c]The IC_{50} value for this compound has been published previously.[14] IC_{50} values of fosmidomycin against IspC from *Escherichia coli*, *Francisella tularensis*, *Mycobacterium tuberculosis*, and *P. falciparum* are 0.035 μM (0.22 μM has also been reported), 0.247, 0.080, and 0.14 μM respectively.[14,19]

fosmidomycin, with IC_{50} values in the nanomolar range. To assess the effect of replacing the β-methylene group with either a sulfur or oxygen atom, the potency of four isosteric sets of β-carba, β-thia, and β-oxa analogues (**1a−1c**, **2a−2c**, **3a−3c**, and **3d−3f**) was determined. For each set of isosteres, the β-thia analogues showed increased inhibition over their β-carba and β-oxa counterparts, with the β-oxa analogues showing the lowest levels of inhibition among the three. This pattern of inhibition was observed for both the enzymes and the bacteria. These results are consistent with our previous findings with IspC orthologs from *Escherichia coli* and *Mycobacterium tuberculosis*.[20] Taken together, these results further establish that replacement of the β-methylene group with a sulfur atom is an effective strategy for the development of reverse fosmidomycin analogues for IspC proteins of bacterial origins.

Concomitantly, the electron-donating or -withdrawing effects of the substituents on the α-phenyl ring were assessed. The compounds contained either electron-donating groups (CH_3, OCH_3, compounds **2a−d**, **3g**, **4a**) or electron-withdrawing groups (Cl, F, compounds **3a−f**, **4b**, **5**). Compounds without substituents on the α-phenyl ring were assessed for comparison (compounds **1a−c**). Compounds containing electron-donating groups showed decreased potency over analogues without substitutions on the α-phenyl ring. Compound **1a** is three times more potent than its methylated analog, **2a**, and 12 times more potent than its 3,4-dimethoxyphenyl analog **3g**. Furthermore, compounds **2a** and **3g** have no activity against the bacteria at 512 μg/mL. Similarly, *Yp*A1122 is susceptible to **1b** at 128 μg/mL, whereas *Yp*A1122 is susceptible to its methylated analog, **2b**, at 256 μg/mL, and resistant to its 3,5-dimethoxyphenyl analog, **4a**, at 512 μg/mL.

Conversely, compounds containing electron-withdrawing groups showed increased potency over analogues without substitutions or electron-donating groups on the α-phenyl ring. 3,4-Difluorophenyl analog, **3d**, is three times more potent than **1a**, and 42 times more potent than **3g**. Similarly, 3,4-dichlorophenyl analog, **3b**, is 20 times more potent than 3,5-dimethoxyphenyl analog, **4a**. Four compounds were further examined to probe the effect of the substitution pattern of the α-phenyl ring. 3,4-Difluorophenyl compound, **3e**, was more potent than its 3,5-difluorophenyl isostere, **4b**. This correlation can also be observed with the *Y. pestis* bacterium. *Yp*A1122 is susceptible to **3e** at 128 μg/mL, whereas *Yp*A1122 is susceptible to **4b** at 256 μg/mL. Additionally, 3,4-difluorophenyl compound, **3f**, is three times more potent than its 2,4-difluorophenyl isostere, **5**. Altogether, these results demonstrate that compounds containing electron-withdrawing groups (Cl, F) show promise for inhibitor design and suggest that 3,4-substitution patterns are more promising than 3,5- or 2,4-substitution patterns. Further study is warranted to confirm the SARs of the substitution pattern conclusively.

In *E. coli*, fosmidomycin is known to enter bacterial cells via the glycerol-3-phosphate transporter (GlpT).[25−27] Conversely, fosmidomycin uptake is limited in other organisms, such as *M. tuberculosis*, by the lack of a GlpT.[26] Previously, using a BLAST search with the *E. coli* K12 GlpT sequence (accession no. P08194), we identified a homologous transport protein (accession no. YP_002347496) in the *Y. pestis* CO92 proteome.[14] A BLAST search with this transport protein (accession no. YP_002347496) identifies a homologous

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transporter (accession no. AEL73320, 100% identity) in the *Y. pestis* A1122 proteome (taxonomy ID: 1035377).

It is possible that uptake of fosmidomycin and/or fosmidomycin analogues **1a**, **1b**, **2b**, **3b**, **3d**, **3e**, and **4b** is fully or partially dependent on the *Y. pestis* A1122 transporter. Previous studies have also shown that uptake of fosmidomycin analog, FR900098, is only partially dependent on GlpT, and that uptake of lipophilic phosphonate prodrugs of fosmidomycin analogues is not dependent on GlpT.[28] In these studies, the more hydrophobic nature of these compounds was attributed as a source of their partial-dependence or independence on GlpT.[28] Future study is warranted to confirm the mechanism of uptake of these analogues and to assess their dependence on a transporter protein to enter the cell. Nonetheless, the enzyme SARs offer insight for the direction of future synthesis and possibly a lipophilic phosphonate prodrug strategy may yield better bacterial growth inhibition.

CONCLUSIONS

In summary, we report kinetic data and antimicrobial susceptibility data to establish SARs of a suite of 17 α-phenyl substituted reverse derivatives of fosmidomycin for YpIspC. These compounds varied by substitution of the β-methylene with oxygen or sulfur, addition of electron-donating or -withdrawing substituents on the α-phenyl ring, and by the substitution pattern of the α-phenyl ring. Our results showed that replacement of the β-methylene group with a sulfur atom is a useful strategy for developing reverse fosmidomycin analogues for YpIspC. This result is consistent with other IspC proteins of bacterial origins. We also found that attaching electron-donating substituents to the α-phenyl ring is not a useful strategy for developing reverse fosmidomycin analogues for YpIspC. Lastly, our results show that 3,4-difluorophenyl substitutions are more potent than 3,5- or 2,4-difluorophenyl substitutions; however, further study is needed to unequivocally establish the relationship between potency and the substitution pattern. These results provide useful information for the future development of novel IspC inhibitors.

EXPERIMENTAL SECTION

Bacterial Cell Culture. Recombinant proteins were expressed in *E. coli* BL21 CodonPlus (DE3)-RIL cells (Stratagene, La Jolla, CA). *E. coli* was cultured at 37 °C in Luria−Bertani (LB) media supplemented with 100 μg/mL ampicillin and 50 μg/mL chloramphenicol with constant shaking at 250 rpm. Agar (1.5% wt/vol) was added to prepare solid media. *Y. pestis* strain A1122 was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH. The *Yp*A1122 bacterial isolates were subcultured on blood agar plates (tryptic soy agar with 5% sheep blood) 48 h prior to antimicrobial susceptibility testing.

Expression and Purification of *Y. pestis* IspC. *Y. pestis* IspC was cloned, expressed, and purified as described previously.[14] The *Y. pestis* ispC gene was synthesized (GenScript USA Inc, Piscataway, NJ) and cloned into a pET101/D-TOPO vector to facilitate the expression of a C-terminal His_6-tagged protein. The plasmid was transformed into chemically competent *E. coli* BL21 CodonPlus (DE3)-RIL cells (Stratagene, La Jolla, CA) for protein expression.

To express the His_6-tagged protein, 1 L of LB media was inoculated with a 10 mL overnight seed culture and incubated with shaking at 37 °C and 250 rpm. At an OD_{600} of 1.8, protein expression was induced by addition of isopropyl *b*-D-thiogalactopyranoside to 0.5 mM. After protein induction, the culture was incubated with shaking at 37 °C and 250 rpm for an additional 18 h. Cells were harvested via centrifugation (4648*g*, 20 min, 4 °C) and stored at −80 °C. Protein was subsequently isolated and purified from the cells via chemical lysis and affinity chromatography.

Cells were lysed with lysis buffer 1 (100 mM Tris pH 8.0, 0.032% lysozyme, 3 mL per gram cell pellet), followed by lysis buffer 2 (0.1 M $CaCl_2$, 0.1 M $MgCl_2$, 0.1 M NaCl, 0.020% DNase, 0.3 mL per gram cell pellet). The clarified cell lysate was collected after centrifugation (48,000*g*, 20 min, 4 °C) and passed through a TALON immobilized metal affinity column (Clontech Laboratories, Mountain View, CA).

The column was washed with 20 column volumes of equilibrium buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5, 300 mM NaCl), 10 column volumes of wash buffer 1 (50 mM HEPES pH 7.5, 300 mM NaCl, 10 mM imidazole), and 15 column volumes of wash buffer 2 (100 mM HEPES pH 7.5, 600 mM NaCl, 20 mM imidazole). The protein was eluted with 5 column volumes of elution buffer (150 mM imidazole pH 7.0, 300 mM NaCl), and then exchanged into storage buffer (0.1 M Tris pH 7.5, 1 mM NaCl, 5 mM dithiothreitol) during concentration by ultrafiltration. The protein concentration was determined using Advanced Protein Assay Reagent (Cytoskeleton, Denver CO) with γ-globulins (Sigma-Aldrich) as the standard. Purified protein was visualized via Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The yield of YpIspC averaged 30 mg per 1 L of culture.

Enzyme Assays. *Y. pestis* IspC activity was assayed at 37 °C by spectrophotometrically monitoring the enzyme catalyzed oxidation of NADPH upon addition of 1-deoxy-D-xylulose 5-phosphate (DXP, Echelon Biosciences, Salt Lake City, UT) to the assay mixture, as described previously.[29] The oxidation of NADPH was monitored at 340 nm using an Agilent 8453 UV−visible Spectrophotometer equipped with a temperature-regulated cuvette holder. All assays were performed in duplicate.

Half-maximal inhibition (IC_{50}) of enzyme activity was determined using nonlinear regression of a plot of fractional enzyme activity as a function of inhibitor concentration (sigmoidal dose−response curve) using GraphPad Prism 5.0, wherein the top plateau of the curve was set to 100% residual enzyme activity. As fosmidomycin is a slow, tight-binding inhibitor,[15] the fosmidomycin analogues were preincubated with the enzyme at 37 °C for 10 min prior to the addition of the substrate, DXP.

Antimicrobial Susceptibility Assays. The MIC of each compound was determined using the broth microdilution method outlined by the Clinical and Laboratory Standards Institute (CLSI).[30] Briefly, the compounds were twofold diluted in cation-adjusted Mueller−Hinton Broth (CAMHB) in 96-well round-bottom, polystyrene microtiter plates to final concentrations ranging from 1 to 512 μg/mL. Wells containing broth only served as growth and sterility controls. *Yp*A1122 was cultured on blood agar plates (tryptic soy agar with 5% sheep blood) at 37 °C for 48 h. After 48 h, the colonies were directly suspended in phosphate buffered saline and diluted in CAMHB before inoculation into the wells of the 96-well plate to yield a final starting inoculum concentration of approximately 5×10^5 colony-forming units (CFUs)/mL. The plates were incubated at 37 °C for 24 h. The MIC was recorded as

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the lowest concentration of compound that inhibited visible bacterial growth after 24 h of incubation (i.e., no turbidity is observed and optical density at 600 nm is zero). Six replicates were performed for each compound.

Synthesis of Compounds. The synthesis of the carba analog **1a** was performed according to Behrendt et al., 2010 and the carba analogues **2a**, **2d**, **3a**, and **3d** according to Behrendt et al., 2011.[19,23] The thia analogues **1b**, **2b**, **3b−c** were synthesized following the same procedures described by Kunfermann et al., 2013 and the analogues **4a−b** according to Lienau et al., 2019.[20,24] The oxa analogues **1c**, **2c**, **3c**, **3f**, and **5** were synthesized according to Brücher et al., 2012 and the 3,4 dimethoxy analog, **3g**, was synthesized according to Konzuch et al., 2014.[21,22]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b04171.

Molecular formula strings (XLS)

Half-maximal inhibitory concentration (IC_{50}) plots and compound characterization (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Haley S. Ball − *Department of Chemistry and Biochemistry, George Mason University, Manassas, Virginia 20110, United States; Wound Infections Department, Bacterial Diseases Branch, Walter Reed Army Institute of Research, Silver Spring, Maryland 20910, United States;* orcid.org/0000-0002-8840-3861; Email: hball3@gmu.edu

Authors

Misgina Girma − *Department of Chemistry and Biochemistry, George Mason University, Manassas, Virginia 20110, United States*

Mosufa Zainab − *Department of Chemistry and Biochemistry, George Mason University, Manassas, Virginia 20110, United States*

Honoria Riley − *Department of Chemistry and Biochemistry, George Mason University, Manassas, Virginia 20110, United States*

Christoph T. Behrendt − *Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, 40225 Düsseldorf, Germany*

Claudia Lienau − *Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, 40225 Düsseldorf, Germany*

Sarah Konzuch − *Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, 40225 Düsseldorf, Germany*

Leandro A. A. Avelar − *Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, 40225 Düsseldorf, Germany*

Beate Lungerich − *Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, 40225 Düsseldorf, Germany*

Iswarduth Soojhawon − *Wound Infections Department, Bacterial Diseases Branch, Walter Reed Army Institute of Research, Silver Spring, Maryland 20910, United States*

Schroeder M. Noble − *Wound Infections Department, Bacterial Diseases Branch, Walter Reed Army Institute of Research, Silver Spring, Maryland 20910, United States*

Thomas Kurz − *Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, 40225 Düsseldorf, Germany;* orcid.org/0000-0002-9474-4224

Robin D. Couch − *Department of Chemistry and Biochemistry, George Mason University, Manassas, Virginia 20110, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.9b04171

Notes

The authors declare no competing financial interest.

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