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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
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Communication

Sulfur(VI) Fluoride Exchange (SuFEx)-Enabled High-Throughput Medicinal Chemistry

Seiya Kitamura,[#] Qinheng Zheng,[#] Jordan L. Woehl, Angelo Solania, Emily Chen, Nicholas Dillon, Mitchell V. Hull, Miyako Kotaniguchi, John R. Cappiello, Shinichi Kitamura, Victor Nizet, K. Barry Sharpless,* and Dennis W. Wolan*

Cite This: https://dx.doi.org/10.1021/jacs.9b13652
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ABSTRACT: Optimization of small-molecule probes or drugs is a synthetically lengthy, challenging, and resource-intensive process. Lack of automation and reliance on skilled medicinal chemists is cumbersome in both academic and industrial settings. Here, we demonstrate a high-throughput hit-to-lead process based on the biocompatible sulfur(VI) fluoride exchange (SuFEx) click chemistry. A high-throughput screening hit benzyl (cyanomethyl)carbamate (K_i = 8 μM) against a bacterial cysteine protease SpeB was modified with a SuFExable iminosulfur oxydifluoride [RN═S(O)F_2] motif, rapidly diversified into 460 analogs in overnight reactions, and the products were directly screened to yield drug-like inhibitors with 480-fold higher potency (K_i = 18 nM). We showed that the improved molecule is active in a bacteria-host coculture. Since this SuFEx linkage reaction succeeds on picomole scale for direct screening, we anticipate our methodology can accelerate the development of robust biological probes and drug candidates.

The introduction of high-throughput screening (HTS) robotics, liquid handler systems, and assay miniaturization have revolutionized screening of bioactive molecules. Relatively inexpensive HTS processes are now routinely used in cell-based and *in vitro* assays against biomedically relevant targets. Nevertheless, compound optimization is typically necessary to improve target specificity, potency, and stability. Lead optimization relies heavily on medicinal chemists, and extensive time and labor costs remain significant hurdles for probe and drug development.

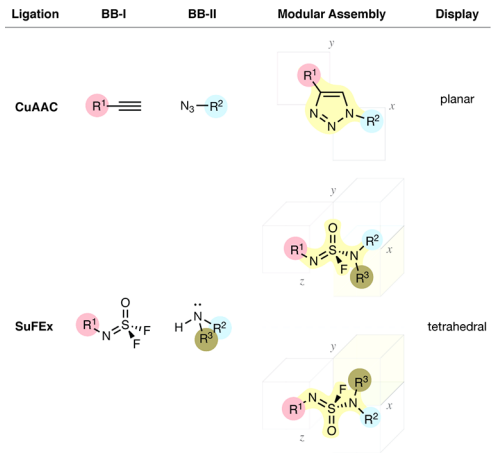

Figure 1. Comparison of CuAAC- and SuFEx-based medicinal chemistry campaigns. Lead molecules can be assembled through an iminosulfur oxydifluoride hub (building block I, BB-I) and by reactions with a collection of primary and secondary amines (BB-II) to generate a diversified library.

Click chemistry has found broad applications in materials chemistry, chemical biology, and drug development since the concept was first introduced in 1999.[1,2] The prototypic copper(I)-catalyzed azide−alkyne cycloaddition (CuAAC) reaction has been used in proof-of-concept studies on lead optimization, including the direct evaluation of biological potency;[3−8] a recent breakthrough on generating 1000-mer modular libraries of organic azides from primary amines has greatly boosted its potential in drug discovery.[9]

The sulfur(VI) fluoride exchange (SuFEx) represents the most recent set of ideal click chemistry transformations.[10] Specifically, aryl fluorosulfates ($ArOSO_2F$) and iminosulfur oxydifluorides (RN═S(O)F_2) are readily synthesized using two connective oxyfluoride gases, sulfuryl fluoride (SO_2F_2) and thionyl tetrafluoride ($O{=}SF_4$), respectively.[11] These two S^{VI}−F motifs have been successfully used as connective linkers in polymer synthesis and for construction of various functional molecules.[12−14] Sulfonyl fluoride (RSO_2F) and aryl fluorosulfate moieties have been successfully introduced into many bioactive molecules in chemical biology and drug discovery,[15−18] especially as covalently binding warheads.[19] In addition, these moieties have been used in protein research.[20,21] However, the potential of SuFEx to unite diverse modules using an $O{=}SF_4$ hub has not been explored in medicinal chemistry. Unlike the planar 1,4-disubstituted triazole formed in CuAAC,

Received: December 18, 2019
Published: June 1, 2020

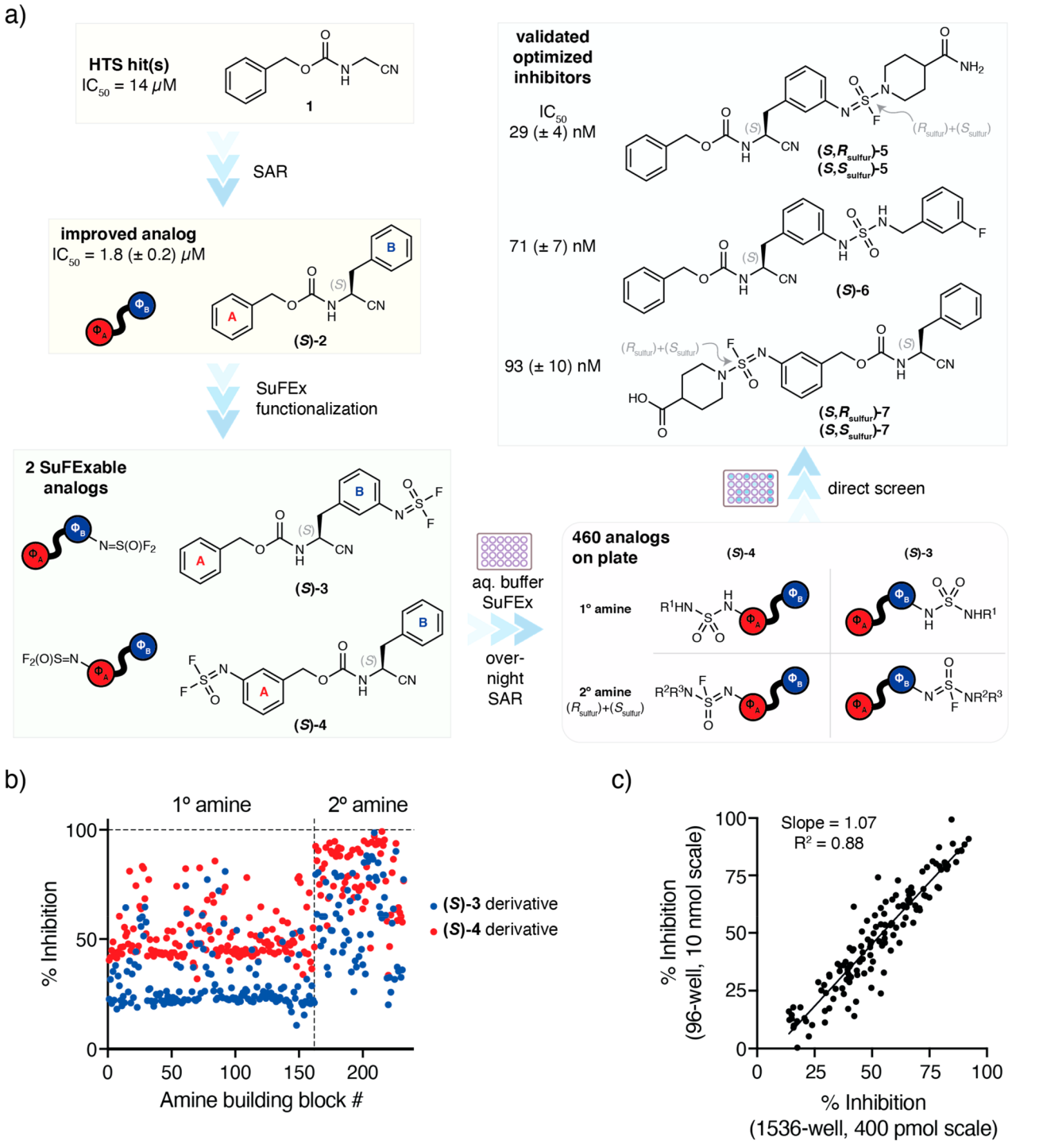

Figure 2. SuFEx-enabled high-throughput medicinal chemistry. (a) SuFEx-enabled SAR from HTS hit **1** identified several potent SpeB inhibitors. (b) Scatter plot of % inhibition of reaction crudes at 250 nM (derivatives of **3**, blue) or 2 μM (derivatives of **4**, red). (c) Correlation of SpeB inhibitory activity of reaction crudes in picomole and nanomole scale reaction.

the sulfur(VI)-center projects its ligands outward into three-dimensional space along the vertexes of a tetrahedron. In addition, the S^{VI}—N bonds formed in this embodiment of SuFEx reactivity are common in medicines; for example, more than 150 sulfonamide drugs are available on the market.[22] Here, we present a one-step, overnight reaction, high-throughput hit-to-lead optimization process based on iminosulfur oxydifluoride SuFEx click chemistry that can be performed on picomole scale.

We rely on the SuFEx reaction between iminosulfur oxydifluoride (RN═$S(O)F_2$) cores and primary or secondary amines to create a focused library of compounds containing a sulfamide or sulfuramidimidoyl fluoride linkage (Figure 1). This series of robust and near-perfect reactions was recently described for bioconjugation and DNA-encoded library construction,[23] and we posited that the biocompatible reaction conditions would enable us to measure the activity of products directly using *in vitro* enzyme assays to prioritize the molecules.

https://dx.doi.org/10.1021/jacs.9b13652
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Additional benefits with respect to medicinal chemistry include the following: (1) rapid and diverse compound synthesis from chemistry's most available and useful connective functional groups (i.e., primary and secondary amines); (2) three-dimensional substitutable sulfur center hubs (Figure 1); (3) display of multiple hydrogen-bond donors/acceptors; (4) tunable lipophilicity; and (5) stability in aqueous buffers.[23]

As a proof-of-concept, we started with a moderate inhibitor (**1**, IC_{50} = 14 μM) of the cysteine protease SpeB, a virulence factor secreted from the bacterial pathogen *Streptococcus pyogenes*, previously identified in our HTS campaign (Figure 2a).[24,25] Although peptidic SpeB inhibitors were reported, such as E-64,[26,27] potent small molecule inhibitors have not been found against SpeB. In preliminary SAR studies, introduction of an (*S*)-benzyl moiety (**2**, Figure 2a) improved the potency to 1.8 μM. The SpeB:**1** cocomplex X-ray structure[25] and the initial SAR campaign (Table S1) suggested that additional surface sites on SpeB were accessible for compound optimization via extension of **2** from the meta positions of both benzyl substituents. An iminosulfur oxydifluoride SuFEx handle was therefore introduced at the meta positions of each benzyl group in **2**, generating **3** and **4**, respectively. The installed and still reactive SuFEx hubs in **3** and **4** were then coupled with a panel of 230 amines to generate 460 analogs using DMSO:PBS = 1:1 as solvent and overnight incubation at 37 °C. Representative reactions were monitored using LC-CAD-MS^2 and are shown in Table S2 and Supplementary Data. It should be noted that the reactions between iminosulfur oxydifluoride containing molecules with the amines showed improved rates and yield when PBS, pH 7.4 was added to DMSO (DMSO/PBS = 1:1, Table S3). Primary amines and secondary, cyclic amines generated the desired product in good yields, while acyclic secondary amines gave poorer conversion in our reaction conditions (Table S2). The reactions to generate SpeB inhibitor analog libraries were performed at relatively low concentrations (i.e., 50 μM compound **3** or **4** and 250 μM amine), which likely accounts for the lower product yields than our previously reported reactions on the millimolar scale.[23]

The reaction products (see Supporting Information) were directly screened for SpeB inhibition with an established kinetic fluorogenic substrate assay.[25,26] Scatter plots of the screening results are shown in Figure 2b. Notably, molecules derived from secondary amines give higher potency compared to those derived from primary amines. All 460 amine structures and their corresponding SpeB inhibition are provided in Tables S4 and S5. Additionally, the panel of amines alone (absence of **3** or **4** in the reactions), the reaction conditions, and the fluoride ion byproduct were assessed for inhibition of SpeB hydrolysis, with no appreciable effect on proteolysis or the assay (Figures S1 and S2). Molecules selected based on potency, lipophilicity, and molecular weight were resynthesized on milligram scale (Table S6), and their potencies in the original screen were fully confirmed (Figure S3). Structures of representative SpeB inhibitors with improved IC_{50} values are shown in Figure 2a. Notably, no correlations between SpeB inhibitor potency and the physiochemical properties of the molecules (i.e., LogP, MW, number of hydrogen bond donors/acceptors) are observed (Figure S4).

With significantly improved SpeB inhibitors in hand, we also determined that miniaturization of the SuFEx reaction was feasible using an Echo Acoustic liquid handler.[28,29] A strong correlation in inhibitory potency was observed between the picomole-scale (1536-well, 2 μL, 200 μM of iminosulfur oxydifluoride compound, 400 pmol) and nanomole-scale (96-well, 50 μL, 10 nmol) syntheses, thereby demonstrating the successful miniaturization of the library construction via the crucial sulfamide-forming linkage reaction between modules (Figures 2c and S5). These direct-screen, condensation linked libraries thus promise to join those CuAAC triazole fusion libraries.[9] Importantly, unlike previously reported nanoscale medicinal chemistry attempts,[29] our subnanoscale SuFEx-based library synthesis does not require specialized equipment, such as dryboxed liquid handlers and highly sensitive mass spectrometry for the biological assay. This SuFEx-based format can be readily adapted in screening facilities with standard HTS robotics and liquid handler systems.

Enzyme kinetics established that **5** is a reversible, competitive inhibitor with K_i = 18 ± 1 nM (Figures S6 and S7). Its binding affinity was further validated by differential scanning fluorometry (Figure S8). The X-ray crystal structure of SpeB in complex with **5** sheds light on its improved potency (Figures 3a and S9, Table S7). Interestingly, **5** binds SpeB in a U-shaped conformation with an intramolecular CH−π interaction[30,31] between the benzyl moiety and a hydrogen on the piperidyl group that likely contributes to the bent binding confirmation (Figure S10). **5** binds within the SpeB active site whereby the carbonyl oxygen is oriented toward the SpeB oxyanion hole created by the main-chain H−N amide nitrogens of residues

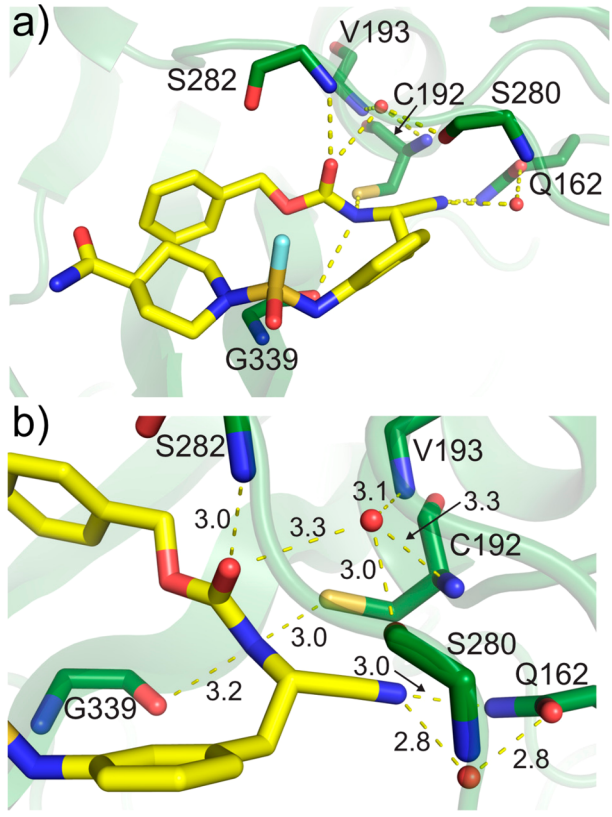

Figure 3. X-ray structure of SpeB-**5** (PDB ID 6UQD). (a) **5** (yellow carbon) in complex with the SpeB active site (green carbon) is shown as sticks (red, oxygen; blue, nitrogen; mustard, sulfur; teal, fluorine) with key interactions highlighted. (b) Zoomed view of the potential hydrogen bonds between SpeB and **5** near the catalytic Cys192 with distances (Å). The absolute configuration of sulfur could not be determined based on the electron density; thus, one (R_{sulfur}) of two configurations is depicted.

https://dx.doi.org/10.1021/jacs.9b13652
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Cys192 and Val193 (Figure 3b). It should be noted that **5** consists of two diastereomers, i.e., a fixed (*S*)-configured benzyl attached carbon center and mixture of (R_{sulfur})- and (S_{sulfur})-configurations at the stereogenic sulfur center.

Based on biological stability and solubility in PBS (Table S8), **7** was selected for further biological characterization. As shown in Figure 4a, **7** is stable against human liver microsomes *in vitro*,

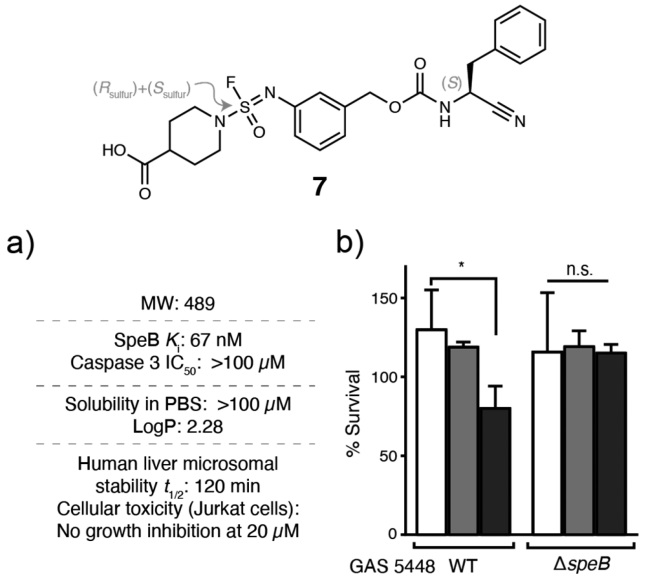

Figure 4. Improved SpeB inhibitor **7** is drug-like and biologically active in bacteria-neutrophil coculture. (a) Physicochemical properties and pharmacological parameters of **7**. Solubility in PBS,[34,35] caspase activity,[36] microsomal stability, and cellular toxicity were measured as described.[37] LogP was predicted with ChemDraw Ultra 17.1. (b) The inhibition of SpeB by **7** prevents *S. pyogenes* WT GAS5448 from neutrophil-induced killing; however, no effect is observed on the Δ*speB* strain. Vehicle (white bar), **7** (20 μM (light gray bar), or 40 μM (dark gray bar)). Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test, * $p \leq 0.05$.

soluble in PBS, selective for SpeB (over other cysteine proteases), and noncytotoxic to human Jurkat T lympohocytes. We tested the effect of inhibitor **7** in an established neutrophil killing assay, wherein SpeB activity boosts relative resistance of *S. pyogenes* against human neutrophils.[32,33] Wild-type (WT) *S. pyogenes* (M1 serotype strain 5448) and a corresponding isogenic mutant strain lacking the SpeB gene (ΔSpeB) were preincubated with **7** prior to introduction of freshly isolated neutrophils from human blood. The presence of **7** decreased the viability of WT *S. pyogenes* in a concentration-dependent manner, while no similar drug effect of **7** occurred in the Δ*speB* mutant strain (Figure 4b).

In conclusion, we provide a proof-of-concept for an expedited high-throughput SAR process to improve potency of an HTS hit molecule to develop biologically useful molecules. This study highlights the utility of SuFEx chemistry for rapidly generating diversified molecules for hit-to-lead applications and shows the potential of click chemistry's near-perfect linkage step(s), and then immediate biological testing. Efforts to improve and expand the method are underway to develop an HT medicinal chemistry platform applicable for routine use.[38] Molecules described here represent the first potent and selective small molecule SpeB inhibitors and can be used to address biological functions of this protease in cellular and animal models and establish it as a potential target for the development of treatments to combat streptococcal infections.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.9b13652.

Additional texts, figures and tables (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

K. Barry Sharpless − *Department of Chemistry, The Scripps Research Institute, La Jolla, California 92037, United States*; Email: sharples@scripps.edu

Dennis W. Wolan − *Department of Molecular Medicine and Department of Integrative Structural and Computational Biology, The Scripps Research Institute, La Jolla, California 92037, United States*; orcid.org/0000-0001-9879-8353; Email: wolan@scripps.edu

Authors

Seiya Kitamura − *Department of Molecular Medicine, The Scripps Research Institute, La Jolla, California 92037, United States*; orcid.org/0000-0003-2453-849X

Qinheng Zheng − *Department of Chemistry, The Scripps Research Institute, La Jolla, California 92037, United States*; orcid.org/0000-0002-8440-8673

Jordan L. Woehl − *Department of Molecular Medicine, The Scripps Research Institute, La Jolla, California 92037, United States*

Angelo Solania − *Department of Molecular Medicine, The Scripps Research Institute, La Jolla, California 92037, United States*; orcid.org/0000-0001-7872-4330

Emily Chen − *California Institute for Biomedical Research, The Scripps Research Institute, La Jolla, California 92037, United States*

Nicholas Dillon − *Department of Pediatrics and Skaggs School of Pharmacy and Pharmaceutical Sciences, UC San Diego, La Jolla, California 92093, United States*

Mitchell V. Hull − *California Institute for Biomedical Research, The Scripps Research Institute, La Jolla, California 92037, United States*

Miyako Kotaniguchi − *Laboratory of Advanced Food Process Engineering, Osaka Prefecture University, Sakai, Osaka 599-8570, Japan*

John R. Cappiello − *Department of Chemistry, The Scripps Research Institute, La Jolla, California 92037, United States*

Shinichi Kitamura − *Laboratory of Advanced Food Process Engineering, Osaka Prefecture University, Sakai, Osaka 599-8570, Japan*; orcid.org/0000-0001-7115-6561

Victor Nizet − *Department of Pediatrics, Collaborative to Halt Antibiotic-Resistant Microbes (CHARM), and Skaggs School of Pharmacy and Pharmaceutical Sciences, UC San Diego, La Jolla, California 92093, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/jacs.9b13652

Author Contributions

#S.K. and Q.Z. contributed equally to this work.

Notes

The authors declare no competing financial interest.

https://dx.doi.org/10.1021/jacs.9b13652
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S.K., Q.Z., K.B.S., and D.W.W. conceived the project. S.K. designed the experiments. S.K. designed, synthesized, and characterized molecules. Q.Z. and S.K. performed SOF_4 reaction. S.K., A.S., and J.W. performed the *in vitro* kinetics studies and analysis. S.K., E.C., and M.V.H. performed the high-throughput synthesis and assay. S.K. and D.W.W. performed the crystallography and structure analysis. A.S. performed the toxicity screen. N.D. and V.N. performed neutrophil assays. S.K. and M.K. performed LC-CAD analysis. J.R.C. proofread the manuscript.

■ ACKNOWLEDGMENTS

The authors gratefully acknowledge financial support from The Scripps Research Institute (to D.W.W.), NIH Grant R01 AI077780 (to V.N.), R01 GM117145 (to K.B.S.), R35 GM136286 (to D.W.W.), and the Ellen Browning Scripps Foundation (to Q.Z.). We thank I. Wilson, R. Stanfield, M. Elsliger, and X. Dai for computational assistance; H. Rosen and A. Ward for access to instrumentation; and the staff of beamline 12.2 at the Stanford Synchrotron Radiation Lightsource.

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https://dx.doi.org/10.1021/jacs.9b13652
J. Am. Chem. Soc. XXXX, XXX, XXX−XXX