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Elucidating the catalytic power of glutamate racemase by investigating a series of covalent inhibitors

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Abstract

The application of covalent inhibitors has experienced a renaissance within drug discovery programs in the last decade. To leverage the superior potency and drug target residence time of covalent inhibitors, there have been extensive efforts to develop highly specific covalent modifications to reduce off-target liabilities. Herein, we present a series of covalent inhibitors of an antimicrobial drug target, glutamate racemase, discovered through structure-based virtual screening. A combination of enzyme kinetics, mass spectrometry, and surface-plasmon resonance experiments details a highly specific 1,4-conjugate addition of a small molecule inhibitor with a catalytic cysteine of glutamate racemase. Molecular dynamics simulations and quantum mechanics-molecular mechanics geometry optimizations reveal the chemistry of the conjugate addition. Two compounds from this series of inhibitors display antimicrobial potency comparable to β -lactam antibiotics, with significant activity against methicillin-resistant *S. aureus* strains. This study elucidates a detailed chemical rationale for covalent inhibition and provides a platform for the development of antimicrobials with a novel mechanism of action against a target in the cell wall biosynthesis pathway.

Entry for the Table of Contents

Author Contributions

N.R.V., K.R.W., and M.A.S. conceived the experiments. N.R.V. and P.W.R. were involved in the design and execution of biochemical and cellular experiments, while N.R.V., M.P., and Y.L. were responsible for the design and execution of mass spectrometry experiments. K.R.W. and M.A.S. were involved in docking, MD, and QM/MM calculations. The manuscript was written and edited by all authors. Correspondence and requests for materials should be addressed to M.A.S.

Supporting information for this article is given via a link at the end of the document.

Through structure-based virtual screening, we present a series of covalent inhibitors of glutamate racemase, an antimicrobial drug target. Using experimental and computational techniques, we provide a chemical rationale for the conjugate addition of a small molecule inhibitor with a catalytic cysteine of glutamate racemase. Additionally, each compound was tested against a panel of opportunistic pathogens to determine their antimicrobial potency.

Keywords

drug discovery; enzymes; glutamate racemase; inhibitors; computational chemistry

Introduction

Glutamate racemases (GR, EC 5.1.1.3) are a family of cofactor independent racemases that are responsible for the stereoinversion of D- and L-glutamate.^[1] The ability of these racemases to abstract the α -carbon proton of glutamate ($pK_a \sim 29$) in a cofactor independent fashion has been the topic of extensive research.^[2–10] A pair of strictly conserved cysteine residues (Cys74/Cys185) participate in a two-base mechanism to abstract the α -carbon proton from glutamate, and donate a proton to the opposite side of the stabilized enolate intermediate in a 1,1-proton transfer.^[11] This unique chemistry allows for the sole production of an essential cell wall component, D-glutamate.

Incorporation of D-glutamate into the cell wall precursor, UDP-N-acetylmuramic acid-pentapeptide, is required in the first committed step of cell wall biosynthesis.^[1] Consequently, D-glutamate is essential for the viability of virtually all Gram-positive and Gram-negative bacteria. Crosslinking of the peptidoglycan is required to maintain structural integrity of the cell wall. A defective cell wall causes the cell to collapse under osmotic pressure, resulting in cell lysis. Due to a concerning rise in the number of antibiotic resistant bacterial infections^[12], GRs have been exploited for the development of novel antimicrobials.

The essentiality of these racemases for viability of many bacteria, along with the wealth of structural and mechanistic information, has spurred numerous drug discovery efforts. High throughput biochemical assays, virtual screening efforts, and substrate analogs have resulted in the development of numerous competitive and non-competitive inhibitors.^[13–15] Unfortunately, they have all fallen short of clinical success due to various reasons: lack of *in*

in vivo efficacy, poor pharmacokinetic/dynamic profiles, and narrow spectrum of activity. There remains one unexplored avenue for targeting GR, the development of covalent inhibitors. Recent literature suggests there has been a resurgence of interest in covalent inhibitors in the last decade.^[16–18] Historically, some of the most successful drugs rely on the formation of covalent bonds to inactivate their targets, e.g. penicillin, aspirin, and omeprazole. Covalent inhibitors are afforded the benefits of longer residence time and greater potency, both of which are thought to improve *in vivo* efficacy.^[19]

We performed a high-throughput virtual screen against an isoform of GR from *B. subtilis* (RacE), which resulted in the identification of a novel series of covalent inhibitors. This series of molecules contains a privileged rhodanine scaffold, commonly found in pan-assay interference compounds (PAINS), which takes advantage of the highly polar active site through a large number of H-bond donor/acceptors.^[20] With a combination of biochemical and computational experiments, we show that the catalytic Cys74 of RacE participates in a 1,4-conjugate addition with the α,β -unsaturated carbonyl in this series of small molecules. Furthermore, *in vivo* experiments against a panel of opportunistic pathogens, including methicillin-resistant *S. aureus* (MRSA), showed potent antimicrobial activity, demonstrating the potential use of these compounds for the development of innovative antimicrobials.

Results and Discussion

Enzymatic assays indicate covalent mechanism of inhibition.

A high-throughput virtual screen against the active site of a GR from *B. subtilis*, henceforth referred to as RacE, using a molecular dynamics (MD)/docking approach previously described^[9, 11], resulted in the identification of **1** (Figure 1A/B). The rhodanine functional group was docked between the pair of catalytic cysteines, in a fashion analogous to the substrate D-glutamate. The potency of **1** appeared to increase the longer the compounds were pre-incubated with RacE, which is highly indicative of a covalent mechanism of inhibition (Figure 1C). Covalent inhibition requires at least two-steps, after the molecule reversibly binds the protein target (governed by the inhibition constant, K_i), and once the geometric and energetic requirements are met, covalent bond formation ensues to form the final complex, the rate of which is dictated by k_{inact} (Scheme S1 and S2).^[16] The first-order decay in activity of RacE was fit for different concentrations of **1** to determine k_{obs} , and values of k_{obs} vs. concentration of **1** were fit with a straight line to determine the $(k_{\text{inact}}/K_i) \times 10^{-3}$, to yield a value of $0.544 \pm 0.014 \text{ min}^{-1} \mu\text{M}^{-1}$ (Figure 1D).

Since initial docking studies placed the rhodanine head group into the active site, further derivatization focused on probing structure-activity relationships (SAR) around the furan ring (Figure 1B). The potent activity of **1** (the original hit) prompted the synthesis of **2–4** for further investigation (Table 1). Due to the placement of the rhodanine head group in the active site, our synthetic efforts focused on modifications of the benzene ring. Derivatization around the furan ring had a significant impact on the K_i and k_{inact} . Compound **2** displayed the most potent K_i of $38.6 \pm 6.5 \mu\text{M}$, while **4** has the largest k_{inact} of $0.297 \pm 0.018 \text{ min}^{-1}$ (Table 1). Interestingly, the addition of an ortho-substituted nitro group in **4** improved the k_{inact} for this compound, likely due to electron withdrawing effects, as seen previously.^[21] Overall, the ratio of k_{inact}/K_i is the second-order rate constant that describes the rate of target

protein inactivation. Compound **4** had the largest k_{inact}/K_i by far, $3.75 \pm 0.44 \text{ min}^{-1} \mu\text{M}^{-1}$, which was 6–10 times larger than the other compounds.

Covalent mechanism of inactivation confirmed by SPR and LC/MS.

Surface plasmon resonance (SPR) was used to probe the interaction of this compound series with RacE. Briefly, RacE is immobilized on the surface of a biosensor and compounds are injected across this flow cell, where real time protein-ligand binding is observed. A reversible, competitive inhibitor discovered in previous screening efforts, chloro-benzimidazole-2-sulfonic acid (chloro-BISA), was tested as a positive control in the SPR assay.^[14] Chloro-BISA binding to RacE displayed a purely reversible binding profile (Figure 2A). The measured dissociation constant (K_d) of $2.0 \pm 1.0 \mu\text{M}$ for chloro-BISA was similar to the previously determined K_i of $13 \pm 2.4 \mu\text{M}$ (Table S1).^[14]

RacE binding to **2** exhibited significantly slower association and dissociation phases, which was fit with a two-state kinetic model to account for covalent bond formation (Figure 2B). The measured K_d of $22 \pm 1 \mu\text{M}$ agreed well with the K_i of $38.6 \pm 6.5 \mu\text{M}$ determined from the coupled enzyme assay (Table S1). The k_{d1} for **2** was approximately 1,500-fold lower than chloro-BISA, while the k_{a1} was approximately 15,000-fold smaller (Table S1). The long target residence time is consistent with our covalent mechanism of inhibition, and is thought to improve *in vivo* efficacy.^[19] Importantly, these data are well described by a covalent inhibition model; K_d here reflects the dissociation constant of the Michaelis complex on the SPR chip, not that of the final covalently bound product, which is defined as irreversible in the SPR model.

Mass spectrometry (MS) was used to identify the presence of a site-specific covalent adduct. All attempts at identifying tryptic peptides of wild-type RacE (RacE-WT) labeled with **1–4** by LC/MS were not fruitful, most likely due to the loss of the covalent adduct upon protein denaturation (Figure S3). X-ray crystal structures of HCV RNA polymerase (NS5b) bound to various inhibitors have shown similar rhodanine scaffolds containing α,β -unsaturated carbonyls can undergo 1,4-conjugate additions with cysteines (Figure S3).^[21] Furthermore, they demonstrated that the covalent bond formation was completely reversible. Therefore, we hypothesized that one of the catalytic cysteines (Cys74/Cys185) employed by GR is undergoing a similar conjugate addition, imparting the covalent nature of inhibition (Figure 3A). We hypothesized that our RacE covalent adducts were labile, and in the absence of a properly folded protein scaffold the covalent adduct would dissociate, as demonstrated previously.^[21–22] As such, we modified our protocol to keep the GR protein intact for MS experiments.

By using a top-down proteomics approach, we examined the adduction of RacE-WT and the C74A mutant by **1** since docking simulations suggested that Cys74 was in the best position to act as a nucleophile. LC/MS of RacE-WT with **1** displayed adducts with the expected mass-to-charge ratio of $365.7 \pm 0.2 \text{ Da}$ (Figure 3B). The mass addition observed is consistent with the proposed 1,4-conjugate addition chemistry, and we observed a single adduct of **1** with a final percent conjugation of 28% (Figure S5). Importantly, RacE-C74A was not able to form adducts with **1** (Figure 3C). This result indicates that the covalent adduction of RacE-WT utilizes Cys74 as a nucleophile. While this does not rule out the

possibility that Cys185 is participating in the reaction, the essentiality of Cys74 together with the docking results led us to focus our computational investigations on the conjugation between Cys74 and **1**.

Compounds containing a rhodanine functional group with an exocyclic alkene are known to be thiol reactive, which could lead to off target protein modification.^[23–24] Using a simple NMR spectroscopic method designed to identify thiol-trapping agents, we attempted to determine the gross thiol reactivity of **1** with a biologically relevant nucleophile, β -mercaptoethanol (β -ME).^[25] Surprisingly, we could not detect any conjugate addition, even with high concentrations of β -ME (100 mM) under basic conditions (Figure S6). This result implies that there is specificity to the conjugate addition we are observing with RacE-WT, in contrast to other α,β -unsaturated carbonyls that readily react with β -ME, cysteamine and glutathione.^[22, 25] We hypothesize that the inherent reactivity of the catalytic cysteines in glutamate racemase facilitates the 1,4-conjugate addition observed.

Docking and MD simulations of original hit.

To determine possible orientations of **1** inside the active site of RacE, docking methods were employed on previously modeled RacE structures. In earlier studies, MD simulations of RacE structures without substrate, and bound to the reversible, competitive inhibitor, BISA, had been performed.^[14, 26] These simulations revealed that the BISA-RacE structures (RacE-closed) displayed a less solvated, more compact active site, in direct contrast to the substrate free RacE structures (RacE-open) which have greater solvent exposure in the active site (Figure S7). In the current study, docking to the RacE-closed (Figure 4A) structures from the MD trajectory resulted in complexes with Cys74 in close proximity, 3.86–4.66 Å to the β -carbon of the α,β -unsaturated carbonyl of **1** (Figure 4B). The orientation in the RacE-closed structures is ideal for a potential 1,4-conjugate addition with Cys74, while other catalytic cysteine (Cys185) proved to be too far away from **1** (Figure 4C). The docking poses of **1** from the RacE-open (Figure 4D) structures did not form near-attack complexes with either catalytic cysteine (Figure 4E/F). The RacE-closed + **1** near attack complexes were further subjected to atomistic MD simulations to generate potential starting geometries for quantum mechanics-molecular mechanics (QM/MM) geometry optimizations (see SI for full computational details).

QM/MM demonstrates a 1,4-conjugate addition with C74.

QM/MM geometry optimizations were performed using QSite from the Schrödinger's suite.^[27–30] Snapshots from the MD simulation were used as the starting structures for the QM/MM geometry optimizations. These starting geometries were selected from the MD ensemble based on promising distances and angles between C74 and **1**, where the 1,4-conjugate addition was more probable. Key distances and angles were tabulated for every snapshot and sixteen structures were selected to pursue QM/MM geometry optimizations based on optimal distances and angles between C74 and **1**. The QM portion of the calculation employed the *ab initio* density functional theory/Becke, three-parameter, Lee-Yang-Parr (DFT/B3LYP) with the 6–31G* basis set, while the OPLS_2005 force field was employed for the MM portion.^[31–34] After QM/MM geometry optimizations, there was a convergence of a 1,4-conjugate addition between the thiolate of Cys74 and the β -carbon of

1. The QM region contained **1**, Asp10, Cys74, Cys185, His187, and water molecules less than 4.5 Å from the atoms of **1** (Figure 5A).

The QM/MM geometry optimizations revealed that C74 can perform a 1,4-conjugate addition with **1**. Upon attack, the bond distance between the sulfur of Cys74 and **1** was 1.95 ± 0.02 Å and the β -carbon adopted the expected sp^3 hybridization (angles around the β -carbon range between 103.2–113.3°, Figure 5B). The simulation also revealed that the location of the water molecules around the atoms of **1** significantly impacted the ability of the Cys74 thiolate to covalently attack the β -carbon of **1**. The snapshots where the α,β -unsaturated carbonyl was more solvated did not result in a 1,4-conjugate addition between the S(Cys74) and the β -carbon of **1**. This critical role of desolvation in this cofactor independent class of racemases has been observed in other studies.^[9, 35] A ligand interaction map for RacE-closed covalently bound to **1** (Figure 5C) demonstrates the desolvation around the catalytic Cys74 and the respective substrates.

Compounds possess significant antimicrobial activity against panel of opportunistic pathogens, including MRSA.

Antimicrobial activity of the compounds was tested against a series of opportunistic Gram-positive and Gram-negative bacteria (Table 2). These opportunistic pathogens typically infect people with weakened immune systems. These compounds displayed similar potency to positive control antibiotics ampicillin and chloramphenicol, which have different mechanisms of action, namely, cell wall and protein synthesis. The original virtual screening hit, **1**, was ineffective in the cell-kill assay, this is possibly caused in part by lower membrane permeability due to the charged carboxylate group. Compounds **3** and **4** displayed the most potent activity *in vivo*, and notably, performed well against *S. aureus*, with MIC values ranging from 1.6–5.9 µg/mL (Table 2, top).

The panel of compounds were also tested against four strains of community associated methicillin-resistant/susceptible *S. aureus* (CA-MRSA/MSSA). The MIC values for **2–4** were 10–20-fold higher against the CA-MRSA/MSSA strains tested (Table 2, bottom). This lower susceptibility could be caused by numerous factors, including expression of drug efflux pumps and altered membrane permeability.^[36] All rhodanine compounds tested against *E. coli* had no detectable antimicrobial activity, future studies will focus on determining the breadth of the spectrum of activity for these novel antimicrobial agents.

Conclusions

We report a series of covalent inhibitors targeting the family of glutamate racemase enzymes with significant antimicrobial properties. There has been a renewed interest in covalent inhibitors in drug discovery campaigns due to their exemplary ligand efficiency and target-residence time. High-throughput virtual screening resulted in the identification of a novel GR inhibitor, with an unprecedented covalent mechanism of inhibition for GR. Orthogonal biochemical and computational approaches were employed to thoroughly characterize the nature of this covalent inhibition. This compound series appears to be selectively reactive with a powerful catalytic cysteine in GR, as no reaction was observed with the surrogate

cysteine, β -ME. This attenuated electrophilicity may limit off target reactivity and improve the compound's pharmacological profile.

Mass spectrometry results are consistent with a 1,4-conjugate addition, which has been observed with these rhodanine containing α,β -unsaturated carbonyls in other systems. A point mutant of the RacE catalytic cysteine demonstrated that **1** utilizes Cys74 for adduction. This result is significant because this family of small molecules may only be reactive selectively towards cysteine thiolates with a high degree of nucleophilicity, with strict geometrical requirements. QM/MM geometry optimizations reveal that desolvation of the GR complex facilitated the reaction between the Cys74 thiolate and the β -carbon of **1**. Desolvation of the active site is a critical step to ensure the proper ionization of the catalytic Cys74. The *in silico* model of the covalent attack will prove invaluable in the development of future derivatives and alternative families of covalent inhibitors. Finally, using a broth microdilution method, **3** and **4** show potent and comparable activity to the β -lactam family of antibiotics against a panel of bacteria, including MRSA. Due to the compounds binding to a catalytic cysteine, it is unlikely pathogens will be able to rescue GR activity, as active site mutations are not well tolerated. This family of covalent inhibitors represents a new avenue for the development of novel inhibitors targeting GRs.

Experimental Section

Protein Expression/Purification and Enzymatic Assays.

The glutamate racemase (GR) gene from *B. subtilis* was cloned into a pET-15b vector (Novagen) and expressed in *E. coli* BL21 (DE3) pLysS with an N-terminal 6x-his tag.^[9] Activity of GR was measured using a coupled-enzyme assay.^[45] Full method details are listed in the Supporting Information.

Compound Synthesis and Characterization.

Compound **1** was purchased from ChemBridge and compounds **2**, **3**, and **4** were synthesized by ENAMINE Ltd. The products were isolated and purified until it was more than 95% pure as determined by LC/MS and ¹H NMR. Full details in the Supporting Information.

Surface Plasmon Resonance.

Hexa-histidine tagged GR was fixed to the surface of a HisCap biosensor chip (SensiQ) using a his-capture couple method. Binding interactions were measured using OneStep injections, which involve inline dilution of analyte during injection on a SensiQ Pioneer AE instrument. Further details on SPR methods and data analysis are provided in the Supporting Information.

Mass Spectrometry.

All protein samples were treated with DMSO (Sigma-Aldrich) solvated compounds or a DMSO vehicle control. Samples were buffer exchanged with an Amicon centrifugal concentrator (10kDa) into 25 mM ammonium acetate (Sigma-Aldrich), pH=8. Analysis of intact protein and tryptic peptides by ESI-MS or ESI-MS/MS was conducted using nanoelectrospray LC-MS/MS on a Q-TOF API-US instrument (Waters Corporation). A

PepMap dC18, 5 μ m, 0.075 $\times$ 100 mm column (LCPackings Corporation) was used for resolution of peptides. Mass calibration solutions were prepared from Michrom bovine serum albumin digests (Bruker). Fourteen peptides ranging in m/z from 927.495 (Tyr-Leu-Tyr-Glu-Ile-Ala-Arg) to 2615.123 (Val-His-Lys-Glu-Cys-Cys-His-Gly-Asp-Leu-Leu-Glu-Cys-Ala-Asp-Asp-Arg-Ala-Asp-Leu-Ala-Lys), with carboxymethylcysteine as a global modification, established a mass accuracy of 4 p.p.m.

MIC determinations.

A broth microdilution method was utilized to evaluate the minimal inhibitory concentration (MIC) for the compound series against a panel of opportunistic hospital pathogens.^[46–47] Data for OD₆₀₀ growth versus log[concentration] of the various compounds were fitted with a modified Gompertz equation to determine the MIC in GraphPad Prism (5.0).^[48] Detailed information for the MIC protocol and bacterial strains is provided in the SI.

Computational Methods.

Full computational methods are detailed in the SI. To capture the covalent attack on **1**, a layered computational approach was employed. First, a high-throughput virtual screen was performed (as described in Whalen *et al.*^[11]), which resulted in the selection of **1**, and was purchased from ChemBridge. Next, more detailed docking simulations were performed with AutoDock Vina, where possible orientations of **1** in the pocket were examined.^[49] Third, MD simulations were performed using Yet Another Scientific Artificial Reality Application (YASARA) Structure v.16.9.23 and the distances and angles were calculated between the Cys74 thiolate and the β -carbon of **1** for each structure, ultimately, sixteen structures were selected as starting structures for QM/MM geometry optimizations. These sixteen structures were imported into Maestro (Schrödinger LLC).^[18] The Protein Preparation Wizard was used to verify and correct bond types and orders for all atoms.^[50–51] Lastly, QM/MM geometry optimizations were performed using QSite v.16.4 (Schrödinger LLC).^[27, 30] These optimizations resulted in a covalent attack between the Cys74 thiolate and the β -carbon of **1**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

Enzyme kinetics suggest a covalent mechanism of inhibition. (A) Crystal structure of RacE (PDB: 1ZUW) bound to D-glu shows the pair of catalytic cysteines flanking the α -carbon of the substrate (D-glu). D-glu, Cys74, Cys185, His187, and Asp10 are shown in cyan sticks and labeled. (B) The original hit, **1**, docked into RacE, where the compound and catalytic residues are labeled and shown in cyan sticks. (C) Loss in activity of RacE follows a first order exponential decay with respect to time in the presence of **1**. (D) The relationship

between k_{obs} and compound concentration can be used to determine k_{inact}/K_i for **1** (shown in a 2D representation), see Supporting Information for details.

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Figure 2.

SPR binding studies of RacE with OneStep analyte injections. (**A/B**) SPR shows reversible binding profile for the chloro-BISA (**A**) and **2** (**B**) binding to RacE-WT where raw data is shown in black and the fitted model in red for both sensorgrams. Sensorgram for **2** binding RacE-WT shows an unusually slow association and dissociation phase. Further details on OneStep injections and data fitting are provided in the Supporting Information.

Figure 3.

Mass spectrometry yields insight into mechanism of covalent modification of RacE. (A) Proposed 1,4-conjugate addition for covalent modification of RacE catalytic Cys74 by **1**. The catalytic Cys74 attacks the β -carbon of the α,β -unsaturated carbonyl (left), yielding the proposed final modification of Cys74 following the protonation of the enolate by Asp10 (right) (B) Mass spectra obtained by LC/MS for RacE-WT negative control treated with DMSO (top) and RacE-WT treated with **1** (bottom), mass of the adducts are consistent with our proposed 1,4-conjugate addition showing a single adduct formed per protein. (C) Mass

spectra obtained by LC/MS for RacE-C74A negative control with DMSO (top) and **1** (bottom). The mass spectrum of RacE-C74A treated with **1** shows the formation of no adducts. Raw MS data for all LC/MS data is provided in Figure S4.

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Figure 4.

MD geometries of RacE + **1** structures between Cys74/Cys185 and **1**. (a) Structure of BISA-RacE labeled as RacE-closed, which displayed a less solvated, more compact active site. (b,c) The distance between S(Cys74/185) and the β -carbon of **1** and the angle between S(Cys74/185)- β -carbon(**1**)-H(β -carbon of **1**) for all RacE-closed + **1** snapshots, b, c respectively. (d) Structure of the substrate free RacE structure labeled RacE-open. (e,f) The distance between S(Cys74/185) and the β -carbon of **1** and the angle between S(Cys74/185)- β -carbon(**1**)-H(β -carbon of **1**) for all RacE-open + **1** snapshots, e, f respectively. The three red dots in (b) indicate three RacE-closed + **1** snapshots that resulted in a 1,4-conjugate addition between Cys74 and **1**.

Figure 5.

QM/MM geometry optimizations demonstrate a 1,4-conjugate addition with Cys74. (A) A RacE-closed structure obtained from the MD simulation and shown in detail is the QM region displayed in cyan sticks. Active site water molecules are represented at the QM level, but are not shown for clarity. (B) QM/MM optimizations led to the thiolate of Cys74 attacking the β -carbon of **1** and the formation of a covalent bond, resulting in a final distance of 1.95 ± 0.02 Å and a tetrahedral orientation. Two different views are shown. Water molecules are shown by the red spheres. (C) A ligand interaction map of the optimized

structure produced by Maestro. This map shows an interaction between the β -carbon of **1** and sulfur of Cys74. The solvent exposure is shown by the gray shading around the atoms of **1**. For the sulfur of Cys74 to attack the β -carbon of **1** the α,β -unsaturated carbonyl could not have any solvent exposure.

Table 1.

Summary of covalent inactivation kinetics of RacE as measured by the coupled-enzyme assay for inhibitors **1**–**4** containing a 5-methylene- α -thioxothiazolin-4-one core (right). Raw data used to fit k_{obs} , K_i and k_{inact} for **2**–**4** are provided in the Supporting Information (Figure S1). Values reported in table represent mean and standard deviations.

Cmpd	R	k_{inact} [min ⁻¹]	K_i [μM]	k_{inact}/K_i [min ⁻¹ μM ⁻¹]
1		-	-	0.543 ± 0.014
2		0.021 ± 0.002	38.6 ± 6.5	0.544 ± 0.095
3		-	-	0.091 ± 0.063
4		0.297 ± 0.018	79.2 ± 8.0	3.75 ± 0.44

Table 2.

Testing of rhodanine compounds against a panel of opportunistic pathogens. Ranges of minimal inhibitory concentration (MIC) data is shown for positive controls ampicillin (AMP) and chloramphenicol (CHL), and compounds **1–4**. The MIC ($\mu\text{g/mL}$) was determined for the series of compounds against a panel of bacteria using a broth microdilution method. Four clinically isolated strains of community associated methicillin-resistant/sensitive *Staphylococcus aureus* (CA-MRSA/MSSA) were also tested against the compound series and the MIC ($\mu\text{g/mL}$) are provided. Values reported in table represent mean and standard deviations.

Compound	<i>Bacillus cereus</i>	<i>Bacillus subtilis</i>	<i>Micrococcus flavus</i>	<i>Streptococcus dysgalactiae</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>
AMP	0.016 – 32 ^[37]	0.03 to > 8 ^[38]	0.031 – 4 ^[39]	0.06 – 4 ^[40]	0.02 – 25 ^[41]	1 to > 32 ^[42]
CHL	1 – 4 ^[37]	1 – 8 ^[43]	7.0 $\pm$ 1.0	2 to > 16 ^[44]	3.1 ^[41]	1 to > 32 ^[42]
1	640 $\pm$ 1	> 910	> 910	> 910	> 910	> 910
2	0.7 $\pm$ 1.1	> 72	> 72	> 72	1.6 $\pm$ 1.3	> 72
3	4.6 $\pm$ 1.1	21 $\pm$ 1	9.2 $\pm$ 1.0	53 $\pm$ 1	5.9 $\pm$ 1.1	> 160
4	5.5 $\pm$ 1.3	7.5 $\pm$ 2.9	5.2 $\pm$ 1.6	18 $\pm$ 1	5.0 $\pm$ 1.1	> 1500

Compound	CA-MRSA MW2(USA 400)	CA-MRSA LAC (USA 300)	CA-MRSA MN PARK T ⁺ (USA 200)	CA-MSSA MN PAT. (USA200)
1	> 910	> 910	> 910	> 910
2	> 72	13 $\pm$ 1	13 $\pm$ 1	13 $\pm$ 1
3	70 $\pm$ 1	10 $\pm$ 2	25 $\pm$ 1	24 $\pm$ 1
4	38 $\pm$ 1	9.7 $\pm$ 1.2	16 $\pm$ 1	32 $\pm$ 1