Supporting Information

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SI Materials and Methods

Bacterial Strains and Growth Conditions. *Staphylococcus aureus* strains Newman, Δ*hssR*, Δ*hssS*, Δ*hssRS*, Δ*menB* and Δ*isdG* have been described previously (1–5). *S. aureus hemB::ermC* (Δ*hemB*) has been described previously and was transduced into Newman using bacteriophage Φ-85 (4, 6). The Δ*pfk*A deletion construct (pBT2-ΔpfkA) was made by cloning the flanking regions of the *S. aureus pfkA* gene (primers: pfkA-5.1A, pfkA-5.1B, pfkA-3.1A, and pfkA-3.1B) into the *S. aureus*/*Escherichia coli* shuttle vector pBT2ts (Table S2). This construct was then used to make an in-frame deletion of the *pfkA* gene in the wild-type *S. aureus* Newman background as previously described using tryptic soy broth (TSB) media without dextrose supplemented with 1% sodium pyruvate (TSB without dextrose purchased from Becton, Dickinson and Company) (7). *Corynebacterium diphtheriae* strain HCL2 and *Staphylococcus haemolyticus* strain NRS9 were used.

All *S. aureus* strains were grown on tryptic soy agar (TSA) containing 10 μg/mL chloramphenicol or 10 μg/mL erythromycin when appropriate and grown at 37 °C for 20–30 h. All overnights were grown in 5 mL of TSB at 37 °C with shaking at 180 rpm unless otherwise noted. All aerobic growth conditions were at 37 °C with shaking at 180 rpm in TSB unless otherwise stated.

Anaerobic growth was obtained by growing cultures in 5 mL of TSB in aeration tubes at 37 °C with shaking at 180 rpm for 15 h. Cultures were diluted to a theoretical OD_{600} of 0.0001, and 200 μl were added to 5 μl of additive in a 96-well plate. Cultures were placed in an anaerobic jar and incubated at 37 °C for 18 h.

Unless stated otherwise, vehicle treatment refers to a volume of DMSO comparable with the volume added containing the described compound (‘882 or ‘373).

Small Molecule Library Screen with Luciferase Reporter. An overnight of Newman carrying the *hrtAB* promoter-*lux* fusion reporter plasmid (p*hrt.lux*) was subcultured into 500 mL in a 1.5-L flask. The cells were grown for 1.5 h to an OD_{600} between 0.60 and 0.65. Seventy-five microliters of culture were transferred into each well of a 384-well, flat-bottom plate containing synthetic compounds that resulted in a final concentration of 6.7 μM. DMSO and heme were used as negative and positive controls, respectively. The plates were incubated for 3 h. Luminescence values were measured using either a Synergy HT Multi-Mode Microplate Reader (Biotek) or TopCount NXT Microplate Scintillation and Luminescence Counter (PerkinElmer). A compound was considered a hit if the raw luminescence value exceeded the luminescence of cells grown in the presence of 81.25 nM heme.

XylE Reporter Assays. Catechol oxygenase (XylE) assays were performed as described previously with modifications in growth conditions (*2*). For the Δ*hemB* experiments, overnights were subcultured 1:50 into 0.5 mL of media for 4 h. For all other reporter assays, the bacteria were grown for 9 h and diluted 1:100 into 0.5 mL of media for 15 h.

Chemical Libraries. The synthetic compounds were obtained from the Vanderbilt Institute of Chemical Biology (VICB) collection, which is a high-diversity, synthetic small-molecule library consisting of ~160,000 compounds. Select compounds were repurchased from either ChemBridge or ChemDiv.

Growth Curve Analyses. Overnight cultures of Newman were diluted 1:100 into 100 μL of media. Bacterial cells were incubated, and the OD_{600} was measured at the specified time points. For all Staphylococcal adaptations, cultures were inoculated in 0.5 mL of TSB for 1 h at 37 °C, 180 rpm and subcultured 1:100 in 100 μL of media containing the indicated additive. Cultures were pre-adapted overnight and analyzed as described above for a typical growth curve.

C. diphtheriae was inoculated into TSB containing vehicle, 5 μM heme, or 50 μM ‘882 and grown for 22–24 h. Cultures were diluted 1:50 into 5 mL of media containing 15 μM heme and grown with shaking at 180 rpm and 37 °C. cfus were enumerated on TSA.

Transposon Library Generation and Screen. A transposon library was generated using Tn*917* (8). The pTV1 transposition vector was transformed into *S. aureus* Newman and selected for on TSA containing erythromycin (10 μg/mL) and chloramphenicol (10 μg/mL). Tn*917* transposition was induced on TSA-erythromycin (10 μg/mL) at 43 °C for 24 h, and mutants were screened for chloramphenicol sensitivity and erythromycin resistance in TSB at 37 °C. Transposon mutants were arrayed in 96-well plates and stored at −80 °C.

Mutants were revived in 150 μL of TSB and grown for 7 h before being subcultured into 100 μL of 40 μM ‘882 in TSB and incubated overnight. Cultures were diluted 1:100 into 100 μL of TSB containing 20 μM heme. Bacterial cells were incubated, and the OD_{600} was measured at 5 h and 7 h. Mutants with an OD_{600} more than 2 SDs below the plate average were isolated for single colonies on TSA-erythromycin (10 μg/mL) and confirmed in a second adaptation in triplicate.

Transposon Integration Site Identification. Transposon mutants were isolated on TSA-erythromycin and grown overnight in TSB-erythromycin. Genomic DNA was isolated from 4 mL of the overnight using a Wizard Genomic purification kit (Promega). Genomic DNA (2 μg) was digested with DraI at 37 °C overnight. DraI was heat inactivated, and 200 μg of the digested DNA was ligated at room temperature for 2 h. Ligated DNA (20 ng) was PCR amplified using DLS479 and DLS480 (Table S2). PCR products were treated with exonuclease I (New England BioLabs) and shrimp alkaline phosphatase (Promega) according to the manufacturer’s instructions. PCR-amplified DNA was column purified using a PCR Purification Kit (Qiagen) and sequenced using DLS493 or DLS494 (Table S2). The sequence flanking the *tn917* inverted repeat was used to interrogate the *S. aureus* Newman genome sequence to determine the *tn917* integration site. Some integration sites were identified using previously published methods (9).

Metabolite Detection. An overnight of Newman was diluted 1:100 in 125 mL of TSB containing either vehicle or 40 μM ‘882. Each hour, 5 mL of culture were sampled and pelleted by centrifugation at 3,200 × *g*. The pH of the supernatant was measured using a SevenEasy pH meter (Mettler Toledo). Glucose and lactate concentrations were quantified according to the manual (R-Biopharm) with the assays scaled to a final volume of 300 μl.

For the characterization of Δ*pfkA*, cultures were grown overnight at 37 °C, in TSB containing 1% NaPyruvate (TSB+P), with shaking at 250 rpm. Cultures were diluted to an OD_{660} of 0.05 in 8 mL of TSB+P and incubated at 37 °C with shaking at 250 rpm (18 × 150 mm culture tube). Each hour, 350 μl of culture were sampled and analyzed for absorbance (OD_{660}). Two hundred microliters were pelleted at 16,000 × *g* for 1 min, and the

supernatants frozen at −20 °C. Samples were thawed on ice. The pH was measured using Sigma pH Tests Strips (P-3536), and glucose levels were quantified using the R-Biopharm D-Glucose Kit (10 716 251 035).

Pyridine Hemochromogen Heme Quantification Assay. Bacteria were grown for 8 h. The cultures were diluted 1:100 and grown aerobically for 15–17 h. Pellets were washed in 0.5 mL of 20 mM K_2HPO_4/KH_2PO_4 (pH 7.6) and resuspended in 0.5 mL of 20 mM K_2HPO_4/KH_2PO_4 (pH 7.6) containing 30 μg of lysostaphin and incubated at 37 °C for 20 min. Each sample was lysed by sonication, and the protein concentration was determined using a Pierce BCA Protein Assay Kit according to the manual (Thermo Scientific). Heme was extracted by adding an equal volume of 0.4 M NaOH and 40% pyridine and quantified as previously described using an extinction coefficient of 324 $mM^{-1}{\cdot}cm^{-1}$ (10).

Heme Quantification by LC-MS. *S. aureus* was grown in 5 mL of TSB for 8 h and subcultured 1:250 into 300 mL of TSB containing DMSO or 40 μM '882. Cells were grown at 37 °C, 200 rpm for 15 h, and cfus were enumerated. Cells were pelleted at 10,500 × *g* for 15 min, and the pellets were washed in 30 mL of 20 mM potassium phosphate (pH 7.6). Cells were pelleted at 3200 × *g* for 20 min, and the pellets were resuspended in 40 mL of TSM (100 mM Tris·HCl, pH 7, 500 mM sucrose, 10 mM $MgCl_2$) containing 25 μg/mL lysostaphin. Cells walls were digested at 37 °C for 20 min, and the samples were pelleted at 10,500 × *g* for 15 min. The protoplast pellets were flash frozen in liquid N_2 and maintained at −80 °C. Protoplast pellets were weighed and thawed in 7 mL of 0.1 M potassium phosphate buffer, pH 7. WT and '882-treated protoplast samples were sonicated (3 s on, 2 s rest, 50% amplitude, Branson 4c-15) on ice: four 5-min rounds, 5-min rest. The samples were then centrifuged for 50 min at 6,400 × *g*. The cleared lysates were syringe filtered (0.45 μm, Millipore) and diluted as needed to allow the measured extracted ion chromatogram (EIC) peak area to fit on the standard curve.

Porphyrin standards were purchased from Frontier Scientific. Methanol, water, acetonitrile, DMSO, and formic acid solvents were all HPLC or Trace Metal grade and were purchased from Fisher Scientific. Samples were stored in Supelco slit top 2-mL vials before and during analysis (Fisher Scientific). These vials were stored at −20 °C when not in use and kept at 5 °C while in the autosampler tray immediately before analysis.

uHPLC separations were carried out on a Dionex Ultimate 3000 uHPLC system using a BDS Hypersil C18 column (150 × 2.1 mm) with a 2.4-μM particle size (Thermo-Scientific; cat no. 28102–152130). Separations were performed at 2–4 kPa. Mass detection and analyte quantification were carried out using a microTOF-Q11 electrospray ionization time-of-flight quadrupole mass spectrometer equipped with a heated-electrospray ion source (Bruker).

Separations were achieved by linear gradient elution transitioning from 100% solvent A (aqueous) to 100% solvent B (organic) over 20 min, followed by a 3-min run of 100% solvent B and then a return to 100% solvent A in a final 3-min washing. A flow rate of 0.4 mL/min with a column temperature of 50 °C was determined to be optimal for separation using the above method. Solvent A: ultrapure water with 0.1% formic acid; solvent B: MeOH with 0.1% formic acid. UV spectra were measured over 390–420 nm using the HyStar software package.

The uHPLC was coupled to an electrospray mass analyzer operating in positive ion mode. The spectrometer used a capillary voltage of 4500 V and capillary temperature of 180 °C. The nebulizing gas was set at 6.0 L/min. The software used for data analysis was Bruker Compass Data Analysis. The microTOF-Q11 instrument is capable of resolving ±0.001 atomic mass unit (amu) differences. Due to the use of such high resolution, metabolites can be monitored via detection of their exact mass (heme parent ion mass: 616.1794 amu). The amounts of metabolites are quantified via integration of the corresponding extracted ion chromatogram (EIC) peak and comparison of the peak area to standard curves generated for known concentrations of pure hemin and internal standard (0.1–7 μM) measured at the same time as the analytes. Standard curves were generated in both potassium phosphate buffer. An internal standard (2-vinyl-4-hydroxymethyl-deuteroporphyrin IX) was added to each sample to a final concentration of 0.05 μM to compensate for run-to-run variability and instrument drift, neither of which proved to perturb the data substantially.

Intracellular Heme Quantification Assay Using the Iron-Regulated Surface Determinant System Heme Oxygenase, IsdG. *S. aureus* Δ*isdG* and Δ*hemB* bearing a plasmid constitutively expressing IsdG (p*lgt.isdG*) were grown overnight (11). Based on stationary phase culture density, Δ*isdG* and Δ*hemB* pellets were resuspended with 500 μL and 50 μL of lysostaphin in TSM buffer (6 μg/mL lysostaphin), respectively. Samples were incubated at 37 °C for 25 min and pelleted by centrifugation at 16,300 × g. Δ*isdG* and Δ*hemB* were resuspended in 250 μL and 200 μL, respectively, of 100 μM PMSF in SoluLyse (Genlantis). Samples were lysed by sonication and normalized by BCA (ThermoScientific), and IsdG quantities were assessed as described previously (11). Band density was quantified using the Odyssey System software (LI-COR), and arbitrary units were converted to protein concentration based on an IsdG standard.

Gentamicin Resistance Assay. An overnight of Newman grown on a Cel-Gro Tissue Culture Rotator (Lab-Line) was subcultured 1:100 into 5 mL of TSB containing the indicated additive (gentamicin, 10 μg/mL; '882, 20 μM), and growth on the rotator was continued for 24 h. cfus were enumerated on TSA and TSA containing 5 μg/mL gentamicin.

Neutrophil Killing Assay. All mice were maintained in compliance with Vanderbilt's Institutional Animal Care and Use Committee regulations. PMNs were elicited using casein and harvested from 13- to 16-wk-old male C57BL/6 mice (The Jackson Laboratory) as previously described (12). Bacteria were prepared and assayed as previously described with modifications (12). An overnight of Newman grown on a Cel-Gro Tissue Culture Rotator (Lab-Line) was subcultured 1:100 and grown in the rotator to midlog. Following growth, the bacteria were resuspended to an OD_{600} of 0.4 in PBS, and 1 mL of culture was pelleted. The pellet was incubated in 50% normal mouse serum, 50% RPMI/H [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in RPMI], 10 μM $MnCl_2$, and either vehicle or 40 μM '882 for 30 min. The pellet was resuspended in 1 mL RPMI/H with 20 μM $MnCl_2$ and 2× vehicle or '882.

NO· Growth Curve. One milliliter of an overnight of Newman grown at 37 °C, 250 rpm was washed with PNG (PN media with 0.5% glucose) (13). The cultures were diluted to an OD_{660} of 0.01 in PNG, and 200 μl were aliquoted into a 96-well plate. The plate was incubated at 37 °C with shaking (14 min 30 s 1 mm Orbital, 30 s 1 mm Linear) on a Tecan Infinite F200Pro. When the cultures reached an OD_{660} of 0.15, NO· was added with a final concentration of 10 mM 3-ethyl-3-(ethylaminoethyl)-1-hydroxy-2-oxo-1-triazene (NOC-12) and 1 mM diethylamine NONOate (DEANO).

***C. diphtheriae* Growth Curve.** *C. diphtheriae* was grown aerobically in brain heart infusion (BHI) medium for 20 h at 37 °C, 180 rpm. The cultures were back-diluted in BHI to a theoretical OD_{600} of 0.0001, and 150 μl of bacterial culture were added to 5 μl of '882 stock. Cultures were grown at 37 °C, 180 rpm for 18 h, and the OD_{600} was measured.

Systemic Murine Model of Infection. All mice were maintained in compliance with Vanderbilt's Institutional Animal Care and Use Committee regulations. Seven-week-old C57BL/6 (The Jackson Laboratory) female mice were infected retro-orbitally with 2×10^7 cfus of Newman as described previously (14). '373 was prepared in 10% (vol/vol) Tween 80 and brought to a neutral pH with 0.1 M sodium hydroxide. IP treatments were given 2 h before infection and every subsequent 24 h until the mice were euthanized with CO_2 96 h postinfection. Liver abscesses and cfus were enumerated.

Imaging MALDI-MS/MS. Livers from mice infected and treated as described above were harvested 24 h post infection (1 h after the second treatment) and flash frozen on hexane and dry ice. Ten-micrometer sections were prepared using a cryostat and thaw-mounted directly to a gold-coated stainless steel MALDI target plate (Applied Biosystems). Thirty mg/mL of 2,5-dihydroxybenzoic acid (DHB) was prepared in 1:1 acetonitrile:water with 0.1% trifluoroacetic acid. Matrix was applied using a TLC glass reagent sprayer (Kontes Glass Company).

Mass spectra were acquired in positive-ion MS/MS mode using a MALDI LTQ XL linear ion trap mass spectrometer (Thermo Scientific) equipped with 337-nm nitrogen laser operating at 60 Hz. Two in-line neutral density filters were used. Protonated '373 molecules (*m/z* 255.2*)* were isolated and fragmented with Helium gas serving as the trapping and collisional gas. Ion density images were extracted from the raw data using ImageQuest version 1.0.1 (ThermoScientific).

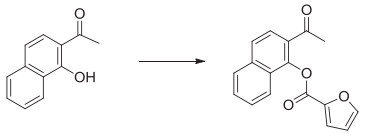
'373 Quantitation via HPLC. Levels of '373 in liver tissue analyzed via MALDI IMS were quantified using HPLC-MS/MS. Tissues were homogenized in 1:1 acetonitrile:methanol manually using a pestle and a Model 100 Sonic Dismembrator (Fisher Scientific). Homogenate was diluted to a final concentration of 20 mg/mL, and cellular debris was removed by centrifugation at $1{,}932 \times g$ for 5 min. Infected, untreated control liver was homogenized to serve as a blank and to prepare calibration standards.

Deferasirox (AK Scientific) was used as an internal standard and was spiked at a concentration of 100 pg/mL into the supernatant solution of all samples analyzed. A calibration series of '373 was spiked into control tissue supernatant at concentrations of 0, 7, 50, 100, 150, and 200 ng/mL.

Separations were performed on a 1260 Infinity HPLC system (Agilent Technologies) with a 6430 Triple Quadrupole Mass Spectrometer (Agilent Technologies) using a ZORBAX Rapid Resolution High Definition SB-C18 column (2.1 mm id × 50 mm, 1.8 μm). Analytes were eluted over 15 min using the following gradient: initial flow 50% solvent A (0.1% formic acid in water) and 50% solvent B (0.1% formic acid in 1:1 acetonitrile:methanol) for 30 s, ramped to 80% solvent B over 10 min, held for an additional minute, ramped back to 50% B over 3 min, and held for an additional minute.

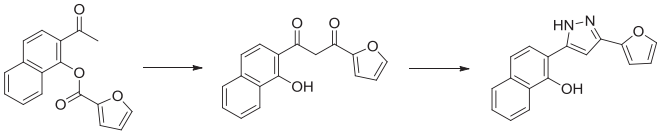
Optimal parameters for transitions were determined using Mass Hunter optimizer version B.04.01 (B4114). Four transitions were monitored for both the target of interest and the internal standard. For '373, a precursor [M+H]+ of 255 was monitored at a retention time of 4.7 min for transitions to 122 for quantitation and 65, 102, and 190 as qualifiers. For Deferasirox, a precursor [M+H]+ of 374 was monitored at a retention time of 5.0 min for transitions to 120 for quantitation and 65, 108, and 182 as qualifiers. Data were analyzed using Mass Hunter workstation software Quantitative Analysis version B.04.00/build 4.0.225.19. Calibration curves were generated as response ratios of '373 relative to the internal standard and had an R^2 value of 0.996.

'882 and '373 Synthesis and Preparation. ***General methods.*** All solvents and chemicals were purchased from Sigma-Aldrich with the exception of 4-dimethylaminopyridine (Acros) and 2-fluorobenzoyl chloride (TCI America). Dry dichloromethane was collected from an MBraun MB-SPS solvent system. *N,N*-dimethylformamide (DMF) was used as received in a bottle with a Sure/Seal. Triethylamine was distilled from calcium hydride and stored over KOH. All glassware was flame dried before use. Reactions were conducted at room temperature (~23 °C) and maintained under argon unless otherwise noted. Analytical TLC was performed on E. Merck silica gel 60 F254 plates and visualized using UV light. Melting points were determined using a Stanford Research Systems OpitMelt system. NMR spectra were recorded on a 400 MHz Bruker spectrometer. Data for ^{1}H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity (s, singlet; d, doublet; t, triplet; dd, doublet of doublets; q, quartet; m, multiplet; br, broad; app, apparent), coupling constants (Hz), and integration. All chemical shifts were reported relative to residual solvent peaks. LC/MS was conducted and recorded on an Agilent Technologies 6130 Quadrupole instrument.



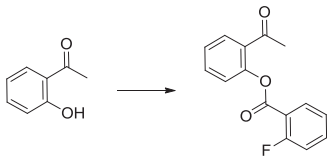
Molecule 1 **Molecule 2**

2-acetylnaphthalen-1-yl furan-2-carboxylate (Molecule 2). A solution of 1′-hydroxy-2′-acetonaphthone (**Molecule 1**) (2.06 g, 11.1 mmol), triethylamine (1.7 mL, 12 mmol), and 4-dimethylaminopyridine (67 mg, 0.55 mmol) in dichloromethane (60 mL) was cooled to 0 °C. To this solution was added neat 2-furoyl chloride (1.2 mL, 12 mmol) dropwise, and the reaction mixture was allowed to warm to room temperature. The reaction was judged complete by TLC after 1 h and washed sequentially with saturated aqueous sodium bicarbonate (30 mL) and brine (2 × 15 mL). The organic layer was dried ($MgSO_4$) and concentrated to give 2.79 g (90%) of crude **Molecule 2** as a light-yellow solid. The product was carried to the next step without further purification.



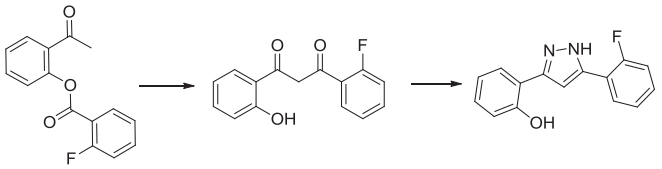
Molecule 2 **Molecule 3** **VU0038882**

2-(3-(furan-2-yl)-1H-pyrazol-5-yl)naphthalen-1-ol (VU0038882). Potassium *tert*-butoxide (2.02 g, 18.0 mmol) was dissolved in DMF (10 mL), and the solution was cooled to 0 °C. To the cold solution was added **Molecule 2** (2.79 g, 10.0 mmol) dissolved in DMF (8.0 mL) dropwise. The reaction mixture was stirred at 0 °C for 1 h and quenched with 1 M HCl (50 mL), resulting in the formation of a yellow precipitate. The precipitate was collected, dissolved in ethyl acetate (300 mL), washed with 0.25 M HCl (5 × 30 mL), brine (2 × 50 mL), dried ($MgSO_4$), and concentrated to provide 2.50 g (90%) of crude **Molecule 3**. The crude diketone was suspended in ethanol (40 mL) and heated to reflux, and neat hydrazine (300 μL, 9.5 mmol) was added. The reaction was maintained at reflux for 3 h and judged complete by TLC. The solution was concentrated, and the residue was recrystallized from hexane/ethyl acetate (50:50) to provide 1.36 g (44% over three steps) of **VU0038882** as dark-orange crystals: melting point (mp) 195–197 °C; ^{1}H NMR (Acetone-d6) δ 12.99 (br, 1H), 11.85 (s, 1H), 8.35–8.33 (m, 1H), 7.86 (d, J = 8.6, 1H), 7.84–7.81 (m, 1H), 7.73 (s, 1H), 7.56–7.45 (m, 3H), 7.20 (s, 1H), 6.89 (d, J = 3.4, 1H), 6.64 (dd, J = 3.4, 1.8, 1H); ^{13}C NMR (Acetone-d6) δ 154.1, 153.2, 145.7, 144.5, 136.6, 135.5, 128.6, 127.7, 126.6, 126.3, 125.3, 123.8, 120.0, 113.0, 110.9, 109.0, 99.6; low resolution mass spectrometry (LRMS) calculated for $C_{17}H_{12}N_2O_2$ $(M+H)^+$ *m/z*: 277.1, measured 277.1.



Molecule 4 **Molecule 5**

2-acetylphenyl 2-fluorobenzoate (Molecule 5). A solution of 2′-hydroxyacetophenone (**Molecule 4**) (3.5 mL, 29 mmol), triethylamine (4.5 mL, 32 mmol), and 4-dimethylaminopyridine (203 mg, 1.66 mmol) in dichloromethane (150 mL) was cooled to 0 °C. To this solution was added neat 2-fluorobenzoyl chloride (3.9 mL, 33 mmol) dropwise, and the reaction mixture was allowed to warm to room temperature. The reaction was judged complete by TLC after 1 h and was washed sequentially with saturated aqueous sodium bicarbonate (50 mL) and brine (2 × 30 mL). The organic layer was dried ($MgSO_4$), and concentrated to give 5.12 g (68%) of crude **Molecule 5** as a white solid. The product was carried to the next step without further purification.



Molecule 5 **Molecule 6** **VU0420373**

2-(5-(2-fluorophenyl)-1H-pyrazol-3-yl)phenol (VU0420373). Potassium *tert*-butoxide (4.44 g, 39.6 mmol) was dissolved in DMF (20 mL), and the solution was cooled to 0 °C. To the cold solution was added **Molecule 5** (5.12 g, 19.8 mmol) dissolved in DMF (12 mL) dropwise. The reaction was stirred at 0 °C for 1 h and quenched with 1 M HCl (50 mL), resulting in the formation of a white precipitate. The precipitate was collected, dissolved in ethyl acetate (300 mL), washed with 0.25 M HCl (5 × 30 mL), brine (2 × 30 mL), dried ($MgSO_4$), and concentrated to provide 4.46 g (87%) of crude **Molecule 6**. The crude diketone was suspended in ethanol (80 mL) and heated to reflux, and neat hydrazine (570 μL, 9.5 mmol) was added. The reaction was maintained at reflux for 2 h and judged complete by TLC. The reaction mixture was concentrated and then dissolved in EtOAc (100 mL), washed with brine (2 × 20 mL), dried ($MgSO_4$), and concentrated to give a white solid. The product was recrystallized from hexane/ethyl acetate to give 2.30 g (31% over three steps) of **VU0420373** as small white crystals: mp 174–176 °C [literature (lit) 170–173 °C (15)]; ^{1}H NMR (Acetone-d6) δ 12.77 (br, 1H) 10.89 (br, 1H), 7.94 (t, J = 7.28, 1H), 7.78 (d, J = 7.7, 1H), 7.47 (q, J = 7.2, 1H), 7.33 (q, J = 8.2, 2H), 7.27 (s, 1H), 7.21 (t, J = 7.7, 1H), 6.93 (q, J = 7.9, 2H); ^{13}C NMR (Acetone-d6) δ 161.6, 159.1, 157.2, 131.7, 130.2, 129.50, 129.47, 127.8, 126.11, 126.09, 120.4, 117.8, 117.7, 117.6, 117.3, 102.8; LRMS calculated for $C_{15}H_{11}FN_2O$ $(M+H)^+$ *m/z*: 255.1, measured 255.2.

Stocks of ‘882 were prepared at a concentration of 50 mM in DMSO and stored at −20 °C.

Statistical Analysis. Where indicated, Student *t* tests were calculated using either Microsoft Excel 2007 or GraphPad Prism 5. Tests of skewness, kurtosis, and D’Agostino and Pearson omnibus normality test were calculated using GraphPad Prism 5. When sample sizes were too small to apply standard tests for normality, samples were considered to be normally distributed if their skewness and kurtosis fell approximately within ±1 and ±3, respectively. Results were considered significant if the *P* value was less than or equal to 0.05.

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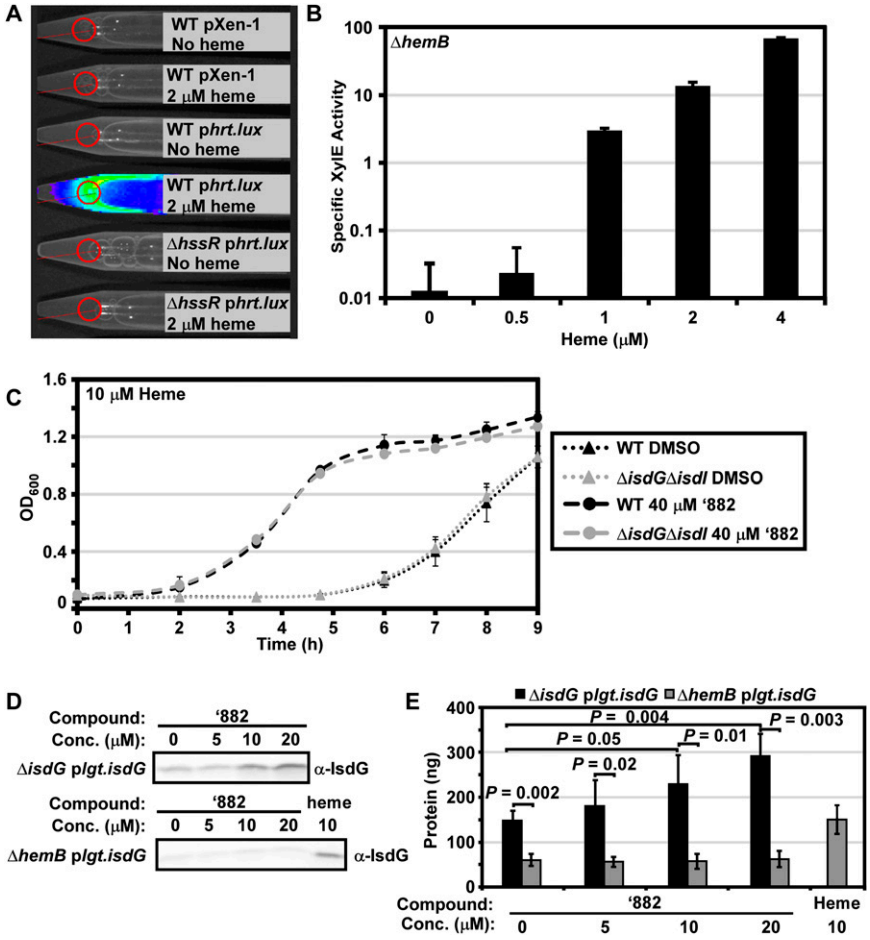

Fig. S1. Identification of a small molecule activator of heme biosynthesis. (*A*) *S. aureus* wild type (WT) and Δ*hssR* were transformed with either promoterless *luxABCDE* (pXen1) or *luxABCDE* reporter gene fused to the *hrtAB* promoter (p*hrt.lux*). Robust luminescence of WT containing p*hrt.lux* was observed upon exposure to heme. Luminescence is dependent on HssRS as Δ*hssR* containing p*hrt.lux* did not produce light in the presence of heme. Compounds (160,000) from the Vanderbilt Institute for Chemical Biology (VICB) small molecule library were screened using p*hrt.lux*. (*B*) Triplicate cultures of the *S. aureus* heme auxotroph *hemB*::*ermC* (Δ*hemB*) carrying p*hrt.xylE* were grown in medium containing the indicated concentration of heme. XylE reporter activity was measured in cell lysates and normalized to lysate protein concentration. Error bars correspond to 1 SD from the mean. (*C*) Triplicate cultures of Δ*isdG*Δ*isdI S. aureus* were grown overnight in medium containing the indicated additive and subcultured into medium containing 10 μM heme. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) over time. Error bars represent 1 SD from the mean. (*D* and *E*) IsdG stabilization was used as a reporter for intracellular heme levels. *S. aureus* Δ*isdG* (black bars) and Δ*hemB* (gray bars) were transformed with plasmids that constitutively express IsdG (p*lgt.isdG*). These strains were grown in the presence of the indicated additive. Whole cell lysates, along with an IsdG standard, were blotted with anti-IsdG polyclonal antisera. Intracellular levels of IsdG were quantified using densitometry. Shown is the average of at least four replicates. Error bars correspond to 1 SD from the mean. Statistics were calculated using a two-tailed, Student's *t* test. (*D*) Representative blots of the Δ*isdG* (*Upper*) and Δ*hemB* (*Lower*) data shown in *E*.

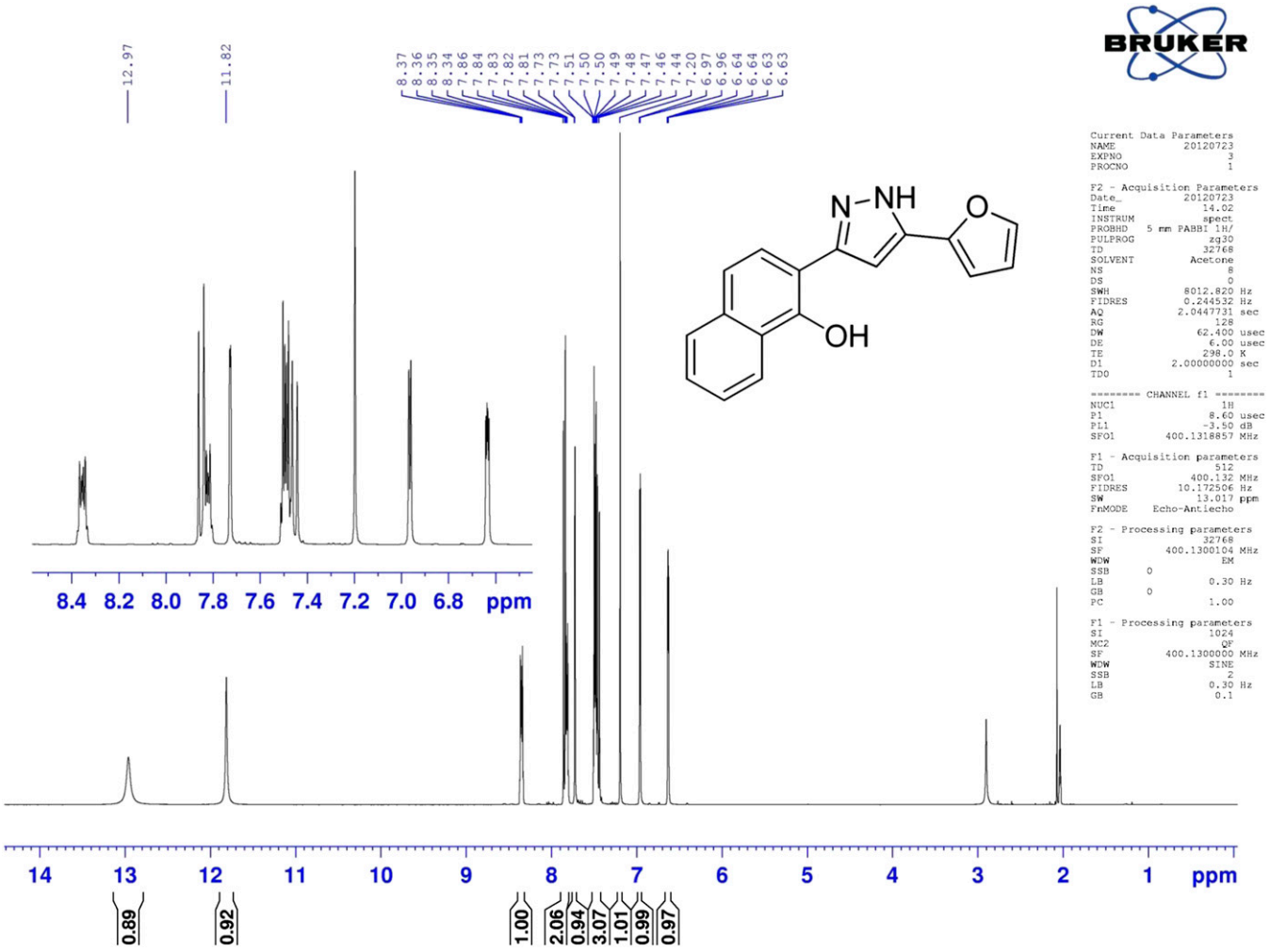

Fig. S2. ^{1}H NMR spectrum of VU0038882.

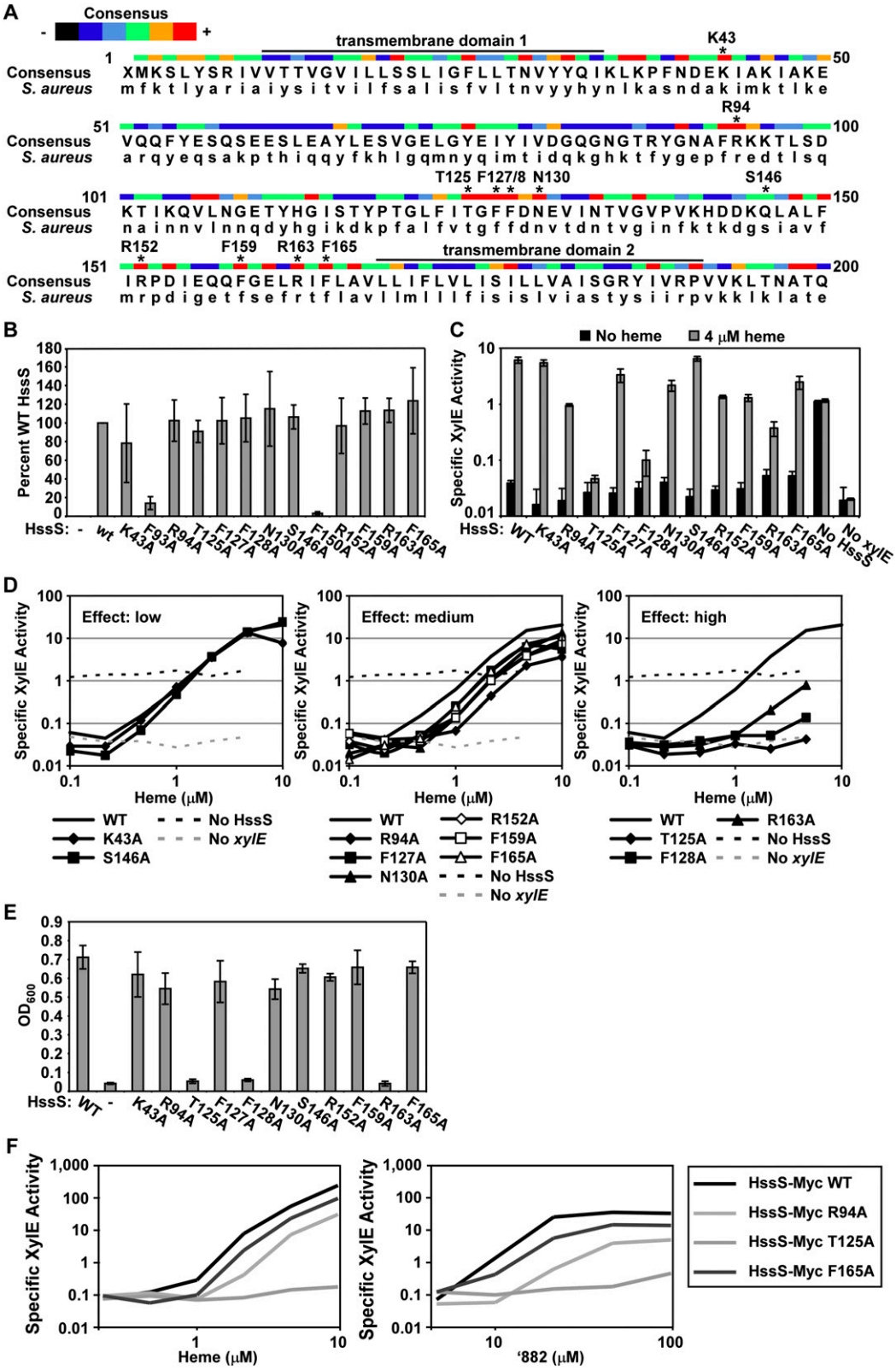

Fig. S3. HssS extracytoplasmic domain residues required for heme and '882 sensing. (*A*) HssS extracytoplasmic domain residues are conserved across *Firmicutes* (*1*). Shown is the conservation of residues (colored bars), HssS consensus sequence (capitalized letters), and the *S. aureus* COL HssS sequence (lowercase letters). Red bars represent absolutely conserved residues; asterisks denote residues selected for mutagenesis studies. (*B*) Expression levels of each HssS-Myc mutant were assessed by immunoblot. Membrane-containing fractions were prepared from Δ*hssS* expressing no HssS (–), c-Myc-tagged wildtype HssS (WT), and c-Myc-tagged HssS variants containing the indicated mutations. The level of each point mutant is expressed as the percent abundance of WT HssS-Myc. (*C*) Effect of HssS extracytoplasmic domain mutations on heme sensing activity. Plasmids were constructed that contain an *hrtAB* promoter–*xylE* fusion and express the

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indicated HssS-Myc variant. These strains were grown in the absence of heme or in 4 μM heme, and XylE activity was determined. Included in this analysis was a strain containing the *hrtAB* promoter reporter without HssS-Myc (no HssS) and a strain containing a promoterless *xylE* (no *xylE*). (*D*) Dose-dependent activity of HssS-Myc point mutants. HssS-Myc mutants were grouped according to the magnitude to which the mutation affects heme-dependent induction of XylE activity according to the data shown in *C* (low effect: no reduction; medium effect: between WT and 1 unit of XylE activity; high effect: between 1 unit of XylE activity and background). Included in this analysis was a strain containing the *hrtAB* promoter reporter and no copy of HssS-Myc (no HssS) and a strain containing a promoterless *xylE* (no *xylE*). The strains were grown in the presence of the indicated heme concentration, and XylE reporter activity was determined. (*E*) Rescue of *S. aureus* Δ*hssS* heme sensitivity by HssS-Myc point mutants. *S. aureus* Δ*hssS* (–) or Δ*hssS* expressing the indicated HssS-Myc variants were grown for 24 h in the presence of 30 μM heme, and culture density was determined. (*F*) *S. aureus* Δ*hssS* was transformed with plasmids containing an *hrtAB* promoter–*xylE* fusion and encoding Myc-tagged, WT HssS (HssS-Myc), or HssS-Myc mutated at the indicated extracytoplasmic domain residue (R94A, T125A, or F165A). The resulting strains were grown in the presence of the indicated concentration of heme (*Left*) or '882 (*Right*), and XylE activity was quantified. In all cases, triplicate experiments were performed and averaged; error bars represent 1 SD from the mean.

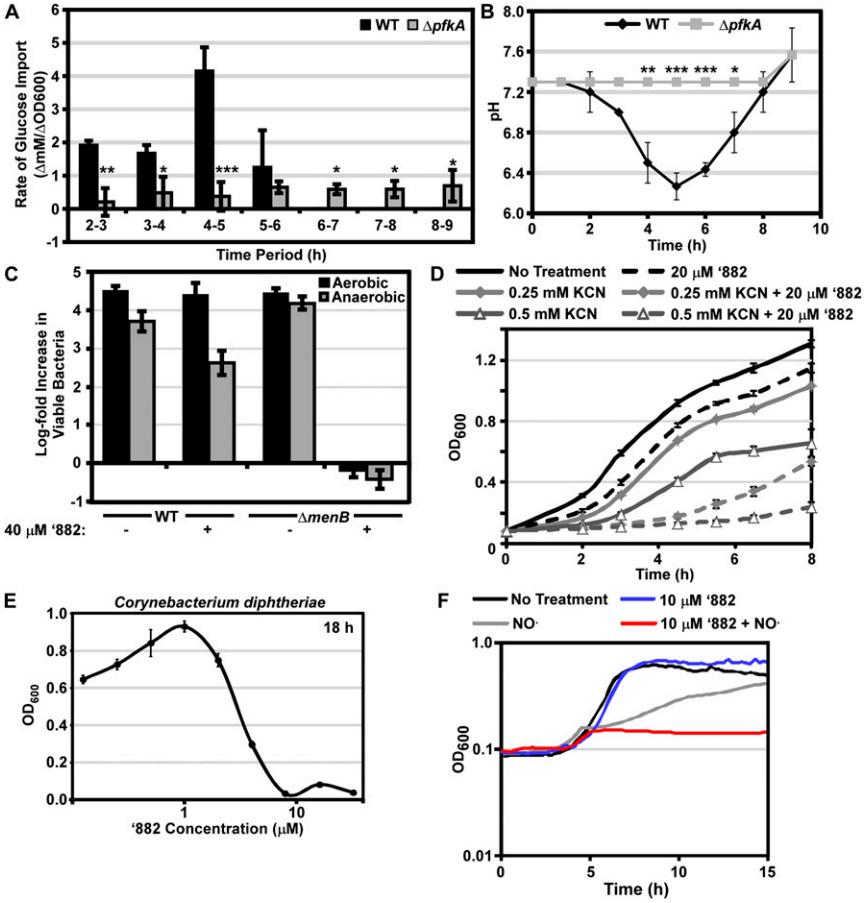

Fig. S4. Central metabolism and '882 toxicity. Wild-type (WT) and Δ*pfkA* Newman cultures were sampled during growth, and the absorbance at 660 nm measured. (*A*) Glucose levels in supernatants were quantified and used to calculate the rate of glucose import [Δglucose (mM/h)/Δabsorbance (OD_{660}/h)]. (*B*) The pH of the supernatants was measured. (*A* and *B*) Data collected in three independent experiments are shown. Error bars represent twice the SEM ($n = 3$). Compared with WT, $*P \leq 0.05$, $**P \leq 0.001$, and $***P \leq 0.0001$. (*C*) cfus from Fig. 5*A* were enumerated at the beginning and end of the time course for bacteria treated with vehicle and 40 μM '882. The fold increase over the input of bacteria was determined. (*D*) Triplicate cultures of *S. aureus* were grown in the presence of vehicle (DMSO), 1 mM potassium cyanide (KCN), 40 μM '882, or a combination of 1 mM KCN and 40 μM '882. Growth was monitored by measuring the absorbance at 600 nm (OD_{600}) at the indicated time points. (*E*) Triplicate cultures of *C. diphtheriae* were grown in the presence of the indicated concentration of '882. Growth was measured by OD_{600} at 18 h. Shown is the average of three independent experiments. (*C*–*E*) Error bars represent 1 SD from the mean. (*F*) *S. aureus* was grown in the presence or absence of 10 μM '882. When the cultures reached an OD_{660} of 0.15, nitric oxide ($NO\cdot$) was added to the indicated cultures with a final concentration of 10 mM 3-ethyl-3-(ethylaminoethyl)-1-hydroxy-2-oxo-1-triazene (NOC-12) and 1 mM diethylamine NONOate (DEANO).

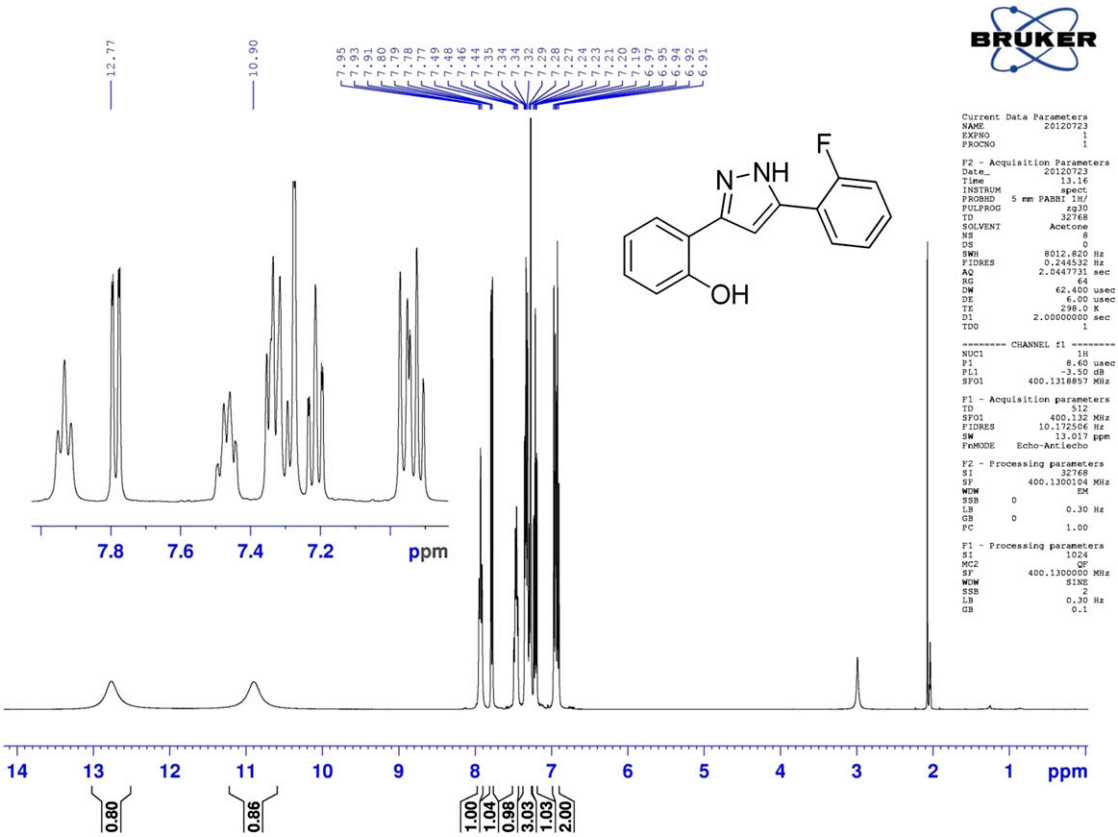

Fig. S5. ^{1}H NMR spectrum of VU0420373.

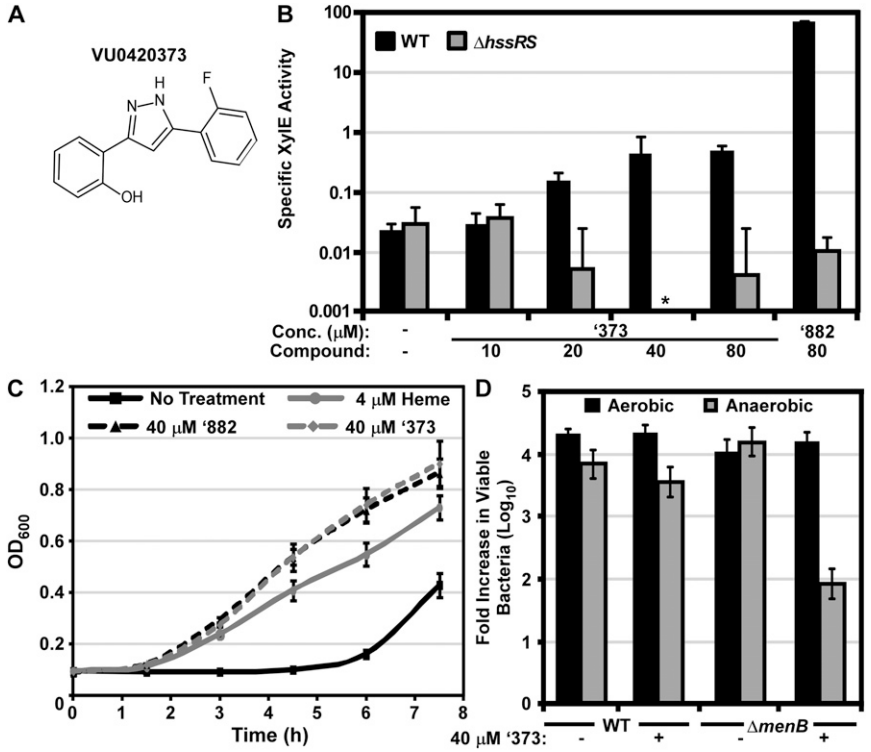

Fig. S6. '373 exhibits effects similar to '882. (*A*) Shown is the structure of derivative VU0420373 ('373). (*B*) Triplicate cultures of *S. aureus* wildtype (WT) and Δ*hssR* transformed with either a promoterless *xylE*-containing plasmid (p*xylE*) or an *hrtAB* promoter–*xylE* fusion-containing plasmid (p*hrt.xylE*) were grown in medium containing the indicated additive. Following growth to stationary phase, XylE activity was measured and normalized to lysate protein concentrations. An asterisk denotes that the value was below the limit of detection. (*C*) Triplicate cultures of *S. aureus* were grown overnight in medium containing the indicated additive. Cultures were back-diluted into medium containing 20 μM heme. The absorbance at 600 nm (OD_{600}) was taken at the indicated times after inoculation. (*D*) *S. aureus* WT and the menaquinone auxotroph (Δ*menB*) were grown in triplicate under aerobic and anaerobic conditions in the presence of vehicle (DMSO) or 40 μM '373. cfus were enumerated at the beginning and end of the time course. Based on these values, the fold increase over the input of bacteria was determined. (*B–D*) Error bars represent 1 SD from the mean.

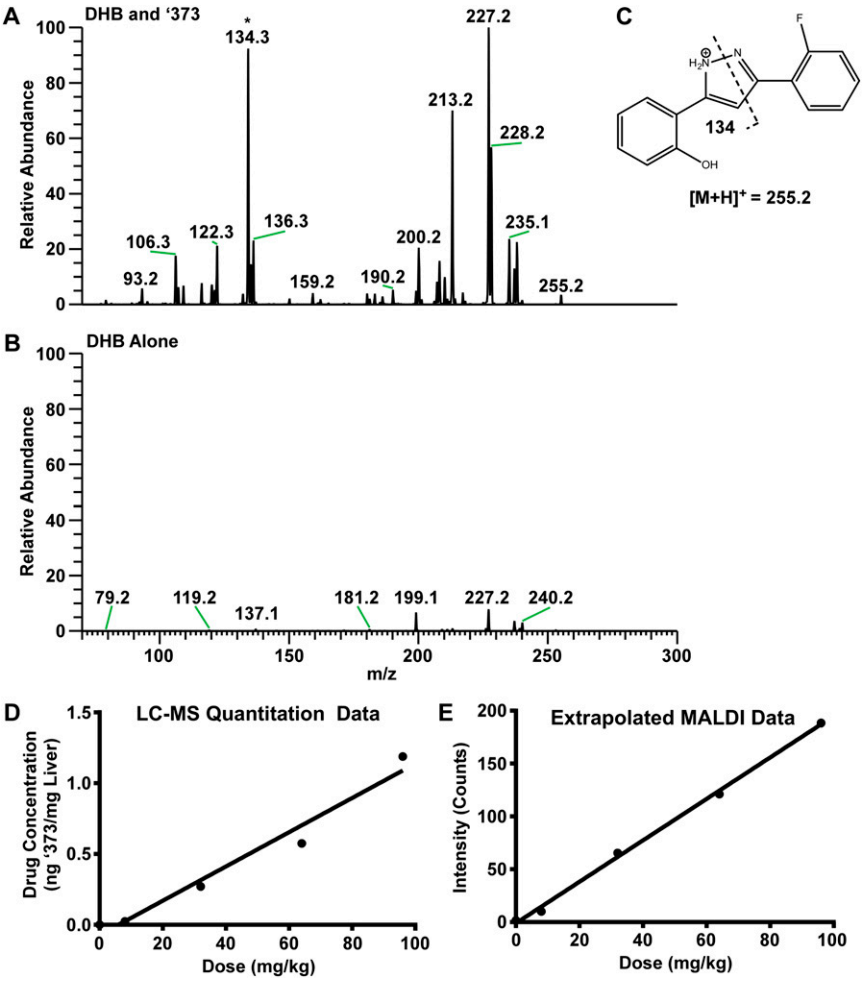

Fig. S7. MALDI-MS/MS and LC-MS analysis of '373. '373 was applied to a stainless steel MALDI target plate using DHB as a matrix and analyzed by MALDI-MS/MS (*A*). DHB matrix did not contribute to the observed *m/z* fragments of '373 (*B*). (*C*) The *m/z* 134 fragment was selected for measuring the accumulation of '373 in vivo. (*D*) Quantitative data from liver extract showing an increase in ng of '373 per mg liver tissue commensurate to dose. (*E*) Average spectral intensity extrapolated from MALDI Imaging Mass Spectrometry (IMS) data (Fig. 6*D*) for each liver showing a similar dose–response relationship as observed by HPLC-MS in *D*.

Table S1. Transposon mutants less sensitive to '882

Tranposon ID	Integration site	Newman	Gene name	Gene description
Cofactors				
11e11*	555437–8	NWMN_0482	*pdxT*	Pyridoxal 5′-phosphate biosynthesis
21D1	1728844–5	NWMN_1561	*hemL*	Glutamate-1-semialdehyde aminotransferase
Carbohydrates				
6E11	755114–5	NWMN_0672		Aldo/keto reductase family protein
33F2, (66E5, 68B5, 68E8)†, 67E11	1583073–4, 1584195–6, 1584189–90	NWMN_1414	*malA*	α-D-1,4-glucosidase
Cell wall				
52A8	1443696–7	NWMN_1310	*alr2*	Alanine racemase 2
51C3	1444887–8	NWMN_1311	*lysA*	Diaminopimelate decarboxylase
50 G5	1513348–9	NWMN_1349	*ald*	Alanine dehydrogenase
Amino acids and proteins				
(24B2, 27D3, 24H6)*	595672–3	NWMN_0516	*ilvE*	Branched-chain amino acid aminotransferase
75D6	1610144–5	NWMN_1439	*gcvPB*	Glycine cleavage system P protein, subunit 2
6F8	1678876–7	NWMN_1513		Peptidase U32 family protein
DNA and RNA				
8D8	1614867–8	NWMN_1446		Competence protein ComGC-like protein
6H6	1659859–60	NWMN_1490		DNA internalization-related competence protein ComEC/Rec2
4E5, 5B10, 14D7, 18G5, 51G4*	1628735–36, 1629189–90, 1629015–6, 1629152–3, 1628880–1	NWMN_1461		ATP dependent RNA helicase DEAD/DEAH box family protein
76C11	1660941–2	NWMN_1491		Competence protein ComEB required for DNA binding and uptake
17B7*	1681514–5	NWMN_1517		Conserved hypothetical protein
2F2*	2296289–90	NWMN_rRNA15		23S rRNA
Transporters				
11B6, 12F8*	982508–9, 982803–4	NWMN_0886		Hypothetical protein
(66C5, 69D11)	1670973–4	NWMN_1505		Hypothetical protein contains NRAMP domain
Regulators				
63C6	736803–4	NWMN_0655		MarR family protein
48F5*	756768–9	NWMN_0674	*saeS*	*S. aureus* accessory element histidine kinase
44C2*	1213708–9	NWMN_1109	*pryR*	Pyrimidine operon regulatory protein
10D6*	1569662–3	NWMN_1399	*srrB*	Staph respiratory response histidine kinase
32G6*	1461723–4	NWMN_1328		Response regulator
31H6*	1813162–3	NWMN_1629	*ccpA*	Catabolite control protein A
Phage				
33G8	1124401–2	NWMN_1026		Conserved hypothetical protein; identical to ORF040 of Bacteriophage 53
66F2	1990835–6	NWMN_1776		Conserved hypothetical protein
Hypothetical and intergenic				
69E5	780077–8	NWMN_0695/0696	Intergenic	Hypothetical protein (similar to MDR transporter) and di-/tripeptide ABC transporter
9F8*	846026–7	NWMN_0751	Promoter	Hypothetical protein
49G8	1060937–8	NWMN_0955/0956	Intergenic	Conserved hypothetical proteins
50F8	1624756–7	NWMN_1457/*sodA*	Intergenic	Zn specific metalloregulatory protein and superoxide dismutase Mn/Fe family protein
8G6	1668046–7	NWMN_1502/1503	Intergenic	Hypothetical protein and enterotoxin family protein
77D12	1670682–3	NWMN_1504	Promoter	Hypothetical protein
46B9	1690951–2	NWMN_1524		Aminotransferase, class V
52F2	1755385–6	NWMN_1584	Promoter	Hypothetical protein
2F9	1931846–7	NWMN_1732		Hypothetical protein

Table S1. Cont.

Tranposon ID	Integration site	Newman	Gene name	Gene description
52B8	2137622–3	NWMN_1930		Hypothetical protein
52G4	2270169–70	NWMN_2051/ 52	Intergenic	Lytic regulatory protein and truncated resolvase
32C7*	2384526–7	NWMN_2161		Conserved hypothetical protein

*An asterisk denotes mutants that are less sensitive to both heme and ‘882.
†Mutants grouped in parentheses reflect identical integration sites.

Table S2. Primer sequences

Primer name	Sequence
pfkA-5.1A	TGGTAGAATTCTGCAAGAAAGACCTACGACC
pfkA-5.1B	TCCACCACTAGTTAAAACTGCCAAGGTGAAACAGCTAAGGG
pfkA-3.1A	CCCTTAGCTGTTTCACCTTGGCAGTTTTAACTAGTGGTGGA
pfkA-3.1B	TGGTAGAATTCCCAGCAGCAGTTTCACCAG
DLS479	TACGGCGAAGGATCACTCATGG
DLS480	TATATATTTCTGCTTCGCTAGG
DLS493	TATGGAGCGCGATATTCGCCG
DLS494	ACTGTCGAATAAAATTGAAGC