Supporting Information

Discovery of Antibiotic (E)-3-(3-Carboxyphenyl)-2-(4-cyanostyryl)quinazolin-4(3H)-one

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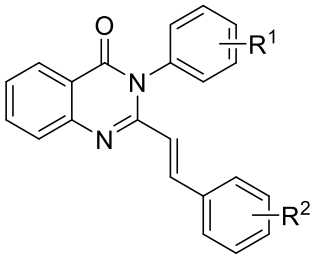
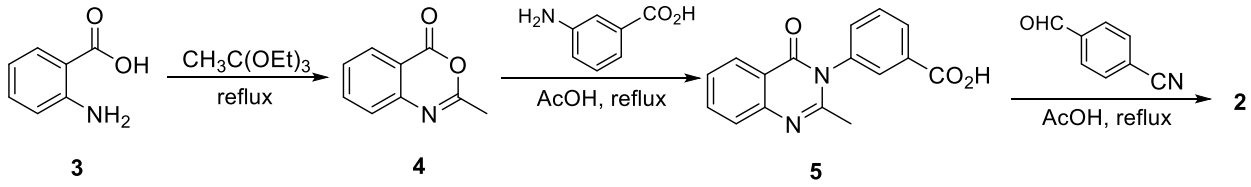
EXPERIMENTAL:

Chemistry. Organic reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). ^{1}H and ^{13}C NMR spectra were recorded on a Varian INOVA-500. Compound **1** was purchased from ChemDiv (San Diego, CA, USA). High-resolution mass spectra were obtained using a Bruker micrOTOF/Q2 mass spectrometer.



Substitutions in the quinazolinone core, at the *para* position for R^1 substituents and at *meta* and *ortho* positions for R^2 were generally not tolerated. The R^1 favored polar groups. Small electron-withdrawing groups were generally favored for R^2. The carboxylic acid group as R^1 in the *meta* position proved important for good pharmacokinetic properties. These observations led to compound **2**, which is featured in this report.



2-Methyl-4*H*-benzo[*d*][1,3]oxazin-4-one (4). Anthranilic acid (**3,** 20 g, 146 mmol) was dissolved in triethyl orthoacetate (45 mL, 245 mmol) and refluxed for 2 h. The reaction mixture was cooled on ice for 4 h to crystallize the intermediate. The resulting crystals were filtered and washed with hexanes to give **4** (17 g, 72% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.47 (s, 3H), 7.50 (t, J = 7.38 Hz, 1H), 7.54 (d, J = 7.98 Hz, 1H), 7.80 (t, J = 7.18 Hz, 1H), 8.18 (d, J = 7.78

Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 21.59, 116.84, 126.59, 128.42, 128.66, 136.77, 146.61, 159.89, 160.45. HRMS (*m/z*): $[M + H]^+$, calcd for $C_9H_8NO_2$, 162.0550; found , 162.0555.

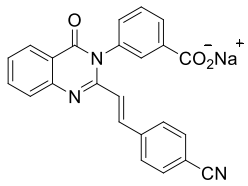
2-Methyl-3-(3-carboxyphenyl)-quinazolin-4(3*H*)-one (5). Compound **4** (2 g, 12.4 mmol) and 3-aminobenzoic acid (1.7 g, 12.4 mmol) were suspended in glacial acetic acid (8 mL, 140 mmol), and dissolved upon heating. The reaction was refluxed for 5 h, at which point 5 mL water was added to the cooled reaction mixture. The resulting precipitate was filtered and washed with water, followed by cold ethanol and hexane to give **5** (3.19 g, 92% yield). ^{1}H (500 MHz, DMSO-d_6) δ 2.87 (s, 3H), 7.52 (t, *J* = 7.38 Hz, 1H), 7.66-7.73 (m, 3H), 7.84 (t, *J* = 7.38 Hz, 1H), 8.01 (s, 1H), 8.09 (t, *J* = 7.58 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 24.13, 120.48, 126.32, 126.47, 126.72, 129.52, 129.83, 130.01, 132.40, 133.07, 134.67, 138.18, 147.37, 154.13, 161.44, 166.58. HRMS (*m/z*): $[M + H]^+$, calcd for $C_{16}H_{13}N_2O_3$, 281.0921; found, 281.0917.

Sodium (*E*)-3-(3-carboxyphenyl)-2-(4-cyanostyryl)quinazolin-4(3*H*)-one (2). Compound **5** (1.0 g, 3.6 mmol) and 4-formylbenzonitrile (0.56 g, 4.3 mmol) were suspended in glacial acetic acid (5 mL, 87 mmol), a suspension that dissolved upon heating. The reaction was refluxed for 18 h and 5 mL water was added to the cooled reaction mixture. The resulting precipitate was filtered and washed with water, followed by cold ethanol and hexanes to afford the carboxylic acid (0.77g, 75% yield). HRMS (*m/z*): $[M + H]^+$, calcd for $C_{24}H_{16}N_3O_3$, 394.1186; found 394.1214. The carboxylic acid (0.45 g, 1.1 mmol) was dissolved in hot ethanol, to which sodium 2-ethylhexanoate (0.28 g, 1.7 mmol) was added. The reaction mixture was stirred on ice for 2 h. The precipitate was filtered and washed with cold ethanol. The product was obtained by dissolving the precipitate in about 5 mL of water and subsequent lyophilization of the solution to give **2** as the sodium salt (0.4 g, 85% yield). ^{1}H NMR (600 MHz, DMSO-d_6) δ 6.47 (d, *J* = 15.55 Hz, 1H), 7.56 (t, *J* = 7.34 Hz, 1H), 7.59 (d, *J* = 8.22 Hz, 2H), 7.73 (m, 2H), 7.79 (m, 3H), 7.90

(m, 2H), 8.05 (s, 1H), 8.12 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 111.56, 118.61, 120.76, 123.42, 126.50, 127.01, 127.35, 128.26, 129.99, 130.06, 130.12, 132.33, 132.83, 133.46, 134.89, 136.95, 137.03, 139.25, 147.21, 150.74, 161.25, 166.52. HRMS (*m/z*): $[M + H]^+$, calcd for $C_{24}H_{15}N_3NaO_3$, 416.1006; found, 416.0987.

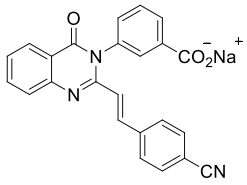
NMR SPECTRA:

^{1}H NMR (126 MHz, DMSO-d$_6$)



Compound **2**

^{13}C NMR (600 MHz, DMSO-d$_6$)



Compound **2**

In silico screening. A library of 1.2 million drug-like compounds from the ChemDiv subset of the ZINC database[1] was prepared for high-throughput virtual screening against the X-ray structure of PBP2a (PDB ID: 1VQQ).[2] The top scoring 10% of the compounds were cross-docked with Glide-SP,[3] Autodock,[4] Gold-chemscore, Gold-goldscore, and Gold-PLP.[5] The top scoring 2,000 poses from each were extracted and refined using Glide-XP mode. Finally, the best 2,500 were clustered according to structural similarity using hierarchical clustering. From these, 118 compounds were selected and purchased from ChemDiv for *in vitro* activity experiments.

Strains. *Staphylococcus aureus* strains NRS70 (also designated N315), NRS123 (also designated MW2, C1999000459, USA400, and 99065), NRS128 (also designated NCTC8325 and RN0031), NRS100 (also designated COL), NRS119 (also designated SA LinR #12), NRS120 (also designated SA LinR #13), VRS1 (also designated HIP11714), and VRS2 (also designated HIP11983) were obtained through the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA). *S. aureus* strains ATCC 29213, ATCC 27660, MRSA252 (ATCC BAA-1720), *S. epidermidis* ATCC 35547, *S. haemolyticus* ATCC 29970, *E. faecium* NCTC 7171 (ATCC 19734), *K. pneumoniae* ATCC 700603, *A. baumannii* ATCC 17961*, P. aeruginosa* ATCC 27853*, E. aerogenes* ATCC 35029 and *E. coli* ATCC 25922 were purchased from the American Type Culture Collection (ATCC).

MIC determination. MICs were evaluated following the CLSI microdilution method[6] in BBL™ Mueller-Hinton II broth as described previously.[7] Briefly, two-fold serial dilutions of compound were prepared in triplicate in 96-well plates and inoculated with 5×10^5 CFU/mL of the bacterial suspension. Plates were incubated at 37 ˚C for 16-20 h.

Cytotoxicity. HepG2 cells (ATCC HB-8065) were maintained in monolayer culture at 37 ˚C and 5% CO_2 in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, non-essential amino acids, 2 mM L-glutamine, and 1% penicillin-streptomycin. After overnight incubation, the cells were treated with compound **2** for 16 h at concentrations from 2 μg/mL to 128 μg/mL. The cells were washed with PBS twice, 150 μL of XTT working solution was added to each well, followed by 3-h incubation. Absorbance at 475 nm (test wavelength) and 660 nm (reference wavelength) was read with a microplate reader. Experiments were performed in triplicate and repeated twice. IC_{50} values were calculated using GraphPad Prism 5.

Hemolysis. Fresh heparinized human blood was washed three times by centrifugation at 1,200 *g* for 10 min in 100 mM PBS, pH 7.4. A 10% red-blood cell (RBC) suspension was prepared in PBS. Antibiotic **2** at 0.5, 5, and 50 μg/mL was added to aliquots of the 10% RBC suspension and incubated at 37 ˚C. A positive control of 0.2% Triton was used. Samples were centrifuged at 1,200 *g* for 10 min and the supernatant measured for absorbance at 541 nm.

Plasma-protein binding. Plasma was centrifuged at 1,200 *g* and 200 μL and was added to the sample chamber of a rapid equilibrium dialysis device, and 350 μL 0.1 M PBS, pH 7.4 supplemented with 0.15 mM NaCl was added to the adjacent chamber. Antibiotic **2** was added to the sample chamber to a final concentration of 10 μM and dialyzed in an orbital shaker for 6 h at 37 ˚C. Aliquots from both chambers were quenched with 1:2 v/v acetonitrile containing an internal standard. The samples were concentrated to dryness on a miVac concentrator and the residue resuspended in 50:50 acetonitrile/water. Samples were analyzed by reverse-phase ultraperformance liquid chromatography (UPLC) with UV/Vis detection.

UPLC analysis. A Waters Acquity UPLC system (Milford, MA, USA) was used, which was equipped with a binary solvent manager, an autosampler, a column heater, and a photodiode array detector. The chromatographic conditions consisted of elution at 0.4 mL/min with 10% acetonitrile/90% water for 2 min, followed by a 10-min linear gradient to 80% acetonitrile/20% water, and a 3-min linear gradient to 100% acetonitrile and UV/Vis detection at 290 nm. The column used was an Acquity UPLC HSS C18 1.8 μm, 2.1 x 100mm.

Plasma stability. Compound **2** (20 μM) was incubated in blank mouse plasma at 37 ˚C and aliquots were taken at specific time points and quenched with two volumes of acetonitrile containing internal standard.[8] Samples were centrifuged and analyzed by reverse-phase UPLC.

Microsomal stability. Compound **2** (2 μM) was incubated with pooled rat or human S9 (1 mg/mL), containing 1 mM NADPH and 3.3 mM $MgCl_2$ in 100 mM potassium phosphate buffer, pH 7.4 at 37 ˚C. Aliquots were taken at 0, 5, 10, 20, 30, 40, and 60 min, and mixed with two volumes of acetonitrile containing internal standard. The precipitated protein was centrifuged at 20,000 *g* for 15 min, and the supernatant was analyzed by reversed-phase UPLC.

Alkylating-agent assay. The potential for compound **2** to serve as an alkylating agent was investigated using the 4-(*p*-nitrobenzyl)pyridine (NBP) assay.[9] NBP reacts with alkylating agents, such as acrylonitrile, to form a blue-colored adduct. Antibiotic **2** (1 mM) was incubated with NBP (0.2 mM) in 70% water/30% dioxane at 37 °C for up to 17 h. A 2.4-mL aliquot was taken and mixed with 0.6 mL of 99% triethylamine. Absorbance was read on a BioTek Epoch UV spectrophotometer. Acrylonitrile was used as positive control. The NBP assay showed that quinazolinone **2** is not an alkylating agent (Figure S1).

Glutathione assay. To assess the potential for antibiotic **2** to form glutathione adducts, antibiotic **2** (100 μM) was incubated with human liver S9 (4 mg/mL), NADPH (1 mM), glutathione (5 mM), and $MgCl_2$ in 0.1 M phosphate buffer (pH 7.4) at 37 °C for 40 min. The reaction was terminated by the addition of two volumes of ice-cold acetonitrile. The sample was vortexed and centrifuged. The supernatant was dried in a speed vacuum concentrator, the residue was reconstituted in 200 μL of 10% acetonitrile/90% water. An aliquot was analyzed by LC/MS operated in full scan and MS/MS modes. No glutathione adducts were detected.

Macromolecular synthesis assays. The incorporation of radiolabeled precursors—[methyl-^{3}H]-thymidine, [5,6-^{3}H]-uridine, L-[4,5-^{3}H]-leucine, or D-[2,3-^{3}H]-alanine—into DNA, RNA, protein, or peptidoglycan, respectively, in logarithmically growing *S. aureus* (ATCC 29213) was measured using a previously published method, with aliquots taken every 20 min over a 120-min incubation period.[7,10] Radiolabeled precursors were purchased from Perkin Elmer (Waltham, MA, USA). Known antibiotics for each pathway were used as positive controls: ciprofloxacin (0.5 μg/mL), rifampicin (8 ng/mL), tetracycline (31 ng/mL), fosfomycin (16 μg/mL), and meropenem (0.06 μg/mL), respectively. Maximum inhibition observed for compound **2** is reported in Figure 1, corresponding to 120 min in all cases except for DNA synthesis, for which 80 min is reported. The percent inhibition was calculated by comparison to a negative control containing no antibiotic.

***In vitro* transcription and translation assays.** For *in vitro* translation, the *E. coli* S30 Extract System for Circular DNA (Promega, Madison, WI, USA) was used to set up the reactions using plasmid pUC19 containing the *lacZ* gene for β-galactosidase. Reaction mixtures were supplemented with two-fold dilutions of antibiotic **2** and the β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega, Madison, WI, USA) was used to quantify the

amount of β-galactosidase translated by measuring absorbance at 420 nm. For *in vitro* transcription, a TranscriptAid T7 High Yield Transcription kit (Thermo Scientific, Waltham, MA, USA) was used with a pET24a/*dacB* DNA construct under the T7 promoter. The purified plasmid was linearized using the restriction endonuclease *XhoI* and then purified according to the manufacturer's instructions. A series of samples were prepared and supplemented with two-fold dilutions of antibiotic **2**. Samples were analyzed by running on a denaturing 1% formaldehyde agarose gel and stained with ethidium bromide to visualize RNA. Intensities of the bands were quantified and compared to the control to determine the amount of transcription occurring in the presence of **2**.

Membrane isolation. *S. aureus* (ATCC 29213), a methicillin-sensitive strain, was grown in Difco Lysogeny Broth (LB) at 37 ˚C until an OD_{625} ~0.8. Cells were centrifuged at 3,200 *g* for 30 min at 4 ˚C and washed once with cold 100 mM NaH_2PO_4, 50 mM $NaHCO_3$, pH 7.5 buffer. The cells were resuspended in 10 mL cold buffer containing complete EDTA-free protease inhibitor, 200 μg/mL lysostaphin, 15 μg/mL DNase I, 10 mM $MgCl_2$, and 1 mM EDTA and incubated for 30 min at 37 ˚C. Cells were sonicated using a Branson Sonifer for 5 x 1 min cycles, with 2 min of rest in between each cycle, and the lysate was centrifuged at 3,200 *g* for 20 min at 4 ˚C. The supernatant was then ultracentrifuged at 32,000 rpm for 1 h at 4 ˚C and the pellet was washed once with cold buffer. The resulting membrane was resuspended in buffer, quantified using the BCA Protein Assay Kit (Piece Biotechnology, Rockford, IL, USA), and the concentration was adjusted to 9 mg/mL.

Bocillin FL PBP binding assays. The Bocillin FL competition assays were performed with purified PBP2a and membrane extracts. Bocillin FL was purchased from Life Technologies (Grand Island, NY, USA). PBP2a was purified using a previously described protocol.[11] For

purified PBP2a, 1 μM protein in 25 mM HEPES, pH 7, 1 M NaCl buffer was incubated at 37 ˚C in the presence of varying concentrations of compound **2** for 10 min. For membrane extracts, 150 μg of the extract in 100 mM NaH_2PO_4, pH 7.5, 50 mM $NaHCO_3$ buffer was incubated at 37 ˚C for 10 min in the presence of varying concentrations of compound **2**. Bocillin FL (20 μM for purified protein and 30 μM for membranes) was added, and the reactions were incubated a further 10 min, then quenched by the addition of Laemmli sample buffer (2× stock solution) and boiling for 5 min.[12] Samples were centrifuged, loaded to SDS-PAGE, the gels were visualized immediately using a Storm840 Scanner, and fluorescence was quantified using ImageQuant software. IC_{50} values were calculated using GraphPad Prism 5, using the previously published equation.[12]

Intrinsic fluorescence-quenching assay. This assay was performed using the method previously developed.[13] Briefly, purified PBP2a was allowed to react with excess oxacillin to occupy the active site. Antibiotic **2** was titrated in and the fluorescence measured for three scans over 1 min cycles and then averaged for each titration. The change in the maximum fluorescence intensity was calculated as the difference compared to the intensity in the absence of compound. Any dilution effect was subtracted out using a buffer titration control. Compound **2** extinction coefficients for 280 nm and 320 nm are 8248 $M^{-1}cm^{-1}$ and 10612 $M^{-1}cm^{-1}$, respectively. The λ_{max} of emission is 490 nm when excited at 280 nm, and there is no emission below 400 nm for compound **2**.

Animals. Outbred mice (ICR female, 6-8 weeks old, 17-20 g body weight, Harlan Laboratories, Indianapolis, IN, USA) providing a heterogeneous population were used. Mice were maintained on a 12:12 light/dark cycle at 72 ± 2 ˚F and provided with Teklad 2019 Extruded Rodent Diet

and water *ad libitum*. All procedures were performed in accordance with the University of Notre Dame Institutional Animal Care and Use Committee.

***In vivo* efficacy.** Groups (n = 6/group) of mice were infected with MRSA (0.5 mL of ATCC 27660 at a final concentration of $5x10^7$ CFU/mL in 5% porcine mucin) intraperitoneally (ip).[14] Following infection, mice were given iv doses by tail vein injection of compound **2**, vancomycin (5 mg/kg, positive control), or vehicle (negative control) at 30 min and 4.5 h after infection. The number of surviving mice was monitored for 48 h. The negative control (vehicle) invariably resulted in the death of all mice and the positive control (vancomycin, 5 mg/kg) rescued all mice. Quinazolinone **2** was dissolved in saline, sterile-filtered, and administered at doses of 2.5, 5, 7.5, 10, 20, and 30 mg/kg. The ED_{50} value was calculated using a Probit analysis as shown in Figure S2.

Pharmacokinetics (PK) studies. A single dose of compound **2** was administered to mice (n = 3 per time point) by tail vein injection or by oral gavage at 10 mg/kg. Compound **2** was well tolerated in mice. Blood was collected by cardiac puncture in heparinized syringes at 2, 5, 20, and 40 min and at 1, 2, 3, 4, 8, 18, and 24 h after iv dosing and at 0.5, 1, 2, 3, 4, 6, 9, 24, and 30 h after po dosing. Blood was centrifuged to obtain plasma. A 50-μL aliquot of plasma was mixed with 100 μL of acetonitrile containing internal standard (5 μM final concentration), followed by centrifugation at 20,000 *g* for 15 min. The supernatant was analyzed by reverse-phase UPLC (Figure S3). The method was linear from 0.05 to 100 μg/mL, with a coefficient of determination R^2 of 0.9998. The lower limit of detection was 0.02 μg/mL and the limit of quantification was 0.05 μg/mL. The interday and intraday precisions were within ± 11% at the low, medium, and high quality control levels; accuracy ranged from 94% to 99% and the relative error was within 5%. PK parameters were calculated using Phoenix WinNonlin 6.3 noncompartmental analysis

and uniform weighing for lambda z calculations (Table S1). Terminal half-lives were estimated to be longer than >20 h.

Anti-sense MRSA strain sensitization. Overnight cultures were prepared by streaking glycerol stocks onto LB agar supplemented with 34 μg/mL chloramphenicol. Fresh LB agar was cooled to 48 ˚C, supplemented with chloramphenicol and 0 or 50 mM xylose, and was seeded with 1 × 10^7 CFU/mL and allowed to set and dry. Antibiotics were diluted in water and spotted onto the agar and the plates incubated at 36 ˚C for 18-20 h.

Structural determination of the PBP2a:2 complex. Wild-type PBP2a crystals were grown following the procedure previously published.[15] Wild-type PBP2a crystals were soaked in the precipitation solution containing 1 mM antibiotic **2** for 24 h at 4 ˚C. Crystals were then soaked briefly in a cryo-protectant (70:30 v/v mixture of paratone/paraffin oil) prior to flash cooling at 100K. Diffraction data sets were collected at synchrotron beamline PX1 at the SLS facility (Switzerland) at 0.9999 Å wavelength. Data sets resulting from three separate soaking experiments were merged and then solved by molecular replacement and refined as detailed below**.** The crystallographic statistics for the resulting 1.95-Å resolution complex are shown in Table S2. Diffraction data sets were processed using XDS[16], scaled with SCALA from the CCP4 package[17], and the structure was solved by molecular replacement using PHASER[18] with the PBP2a structure as the initial model (PDB ID: 1VQQ).[2] The models were refined with several cycles using PHENIX[19] and BUSTER[20]. Considering the high resolution for the diffraction data, a restrained individual isotropic B-factor refinement procedure was used and the occupancy for the ligand was also refined (refined occupancy 0.82). Water molecules were added with PHENIX. As also observed for the PBP2a:ceftaroline complex (PDB code 3ZG0), a muramic acid molecule was found close to the allosteric site; this molecule occupies the same location that

the muropeptide observed in the PBP2a:muropeptide complex (PDB code 3ZG5) occupies.[15] The electron density for the muramic acid is exceptionally good. Final electron-density maps for the ligands were recalculated using FEM (featured-enhanced map) in PHENIX[19] in order to reduce model bias and errors associated with mobility of flexible loops. Ramachandran statistics are as follows: 97.27% residues in most favored regions, 2.50% residues in allowed regions, and 0.23% in disallowed regions. The PDB ID for the deposited coordinates is 4CJN.

SUPPORTING RESULTS:

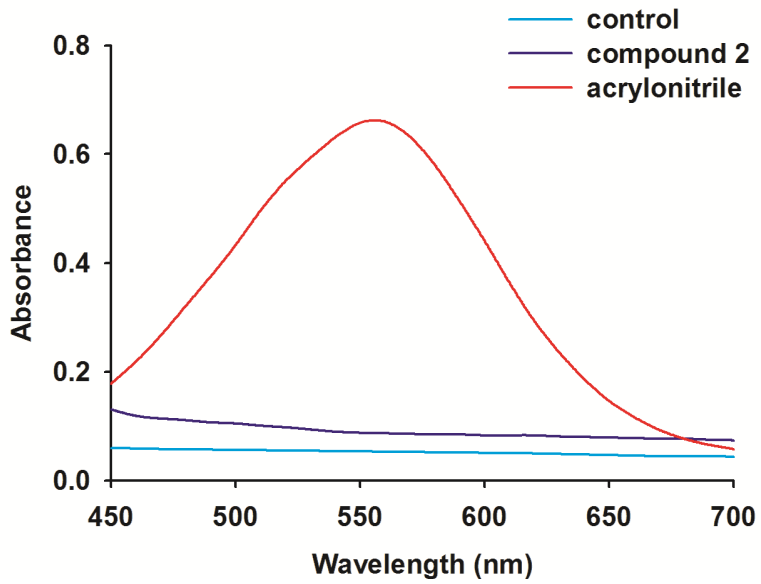


Figure S1. NBP assay for quinazolinone 2. Antibiotic **2** (1 mM or 393 μg/mL) was incubated with NBP at 37 °C for 17 h. Acrylonitrile was used as positive control and 70% water/30% dioxane as negative control.

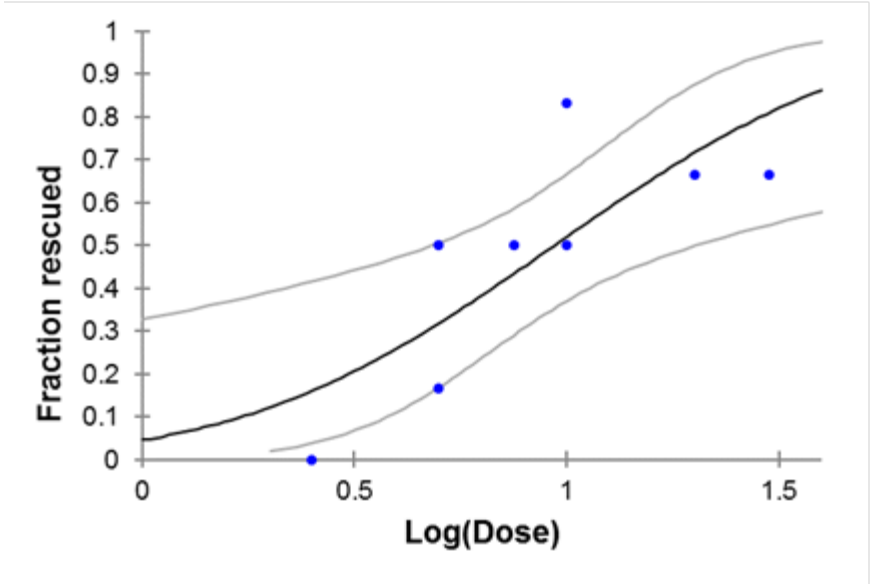


Figure S2. *In vivo* efficacy of quinazolinone 2 in the mouse peritonitis model. Mice (groups of 6) were infected intraperitoneally with MRSA ATCC 27660 and given iv doses of antibiotic **2** at 30 min and 4.5 h after infection. The number of surviving mice was recorded after 48 h and the percentage of rescued mice was plotted against log(dose). Data points are shown as blue dots and the logistic regression fit as a black line. The 95% confidence intervals are given as gray lines. The ED_{50} (of 9.4 mg/kg) was calculated using the Probit analysis[21] and the R^2 of the fit was 0.875, with the minimum constrained to 0 and the maximum constrained to 1.

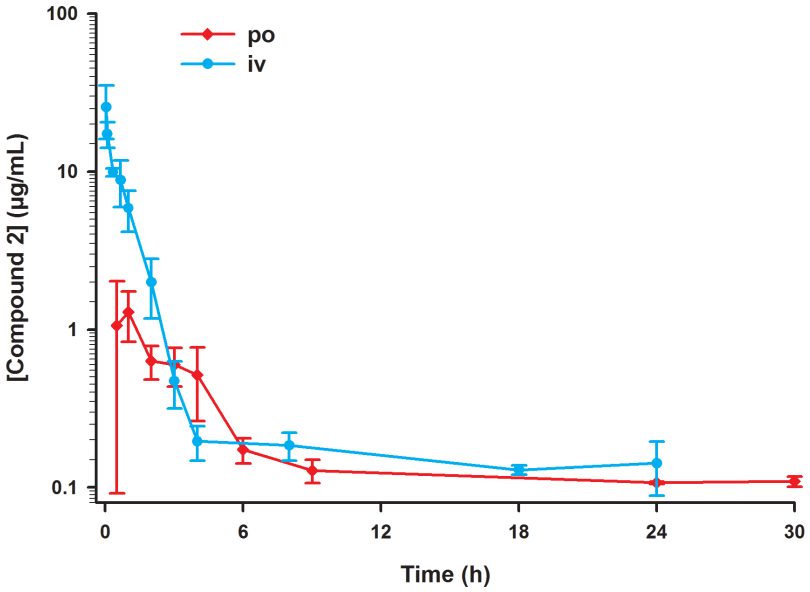


Figure S3. Plasma concentrations of quinazolinone **2** in mice after single dose administration. Female ICR mice (n = 3 per time point per route of administration). Concentrations were determined using a bioanalytical method that included a calibration curve of known concentrations of **2** in blank mouse plasma relative to internal standard.

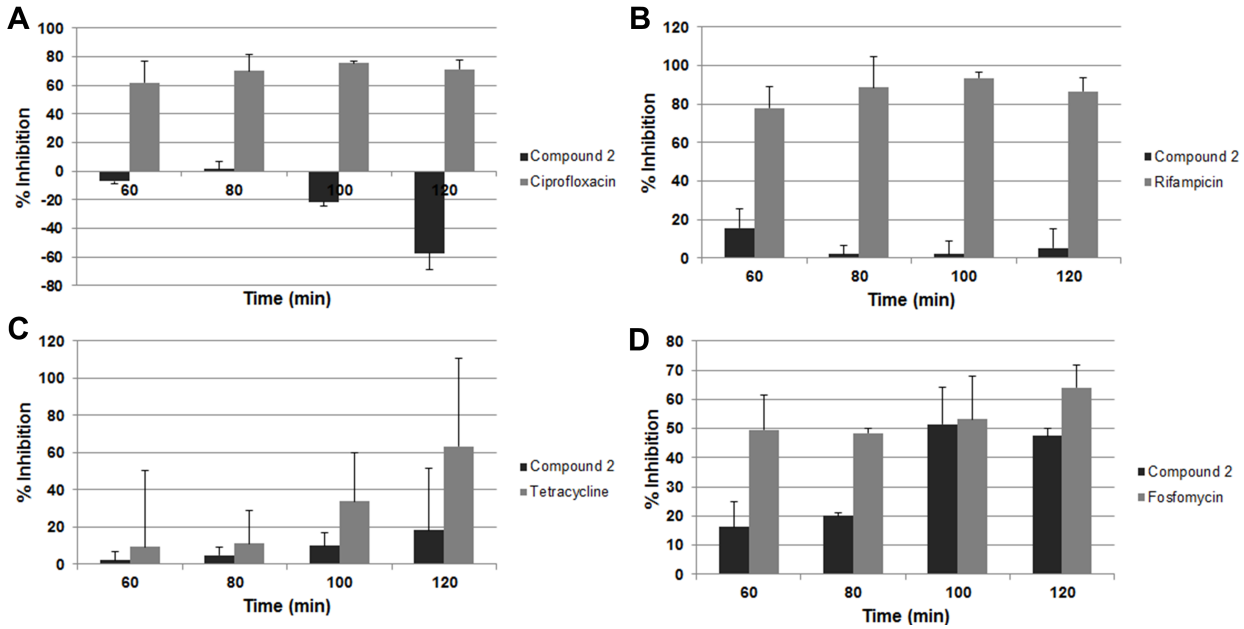


Figure S4. Macromolecular synthesis assays. Compound **2** in all cases was studied at 1 µg/mL, or ½ the MIC. Sufficent bacterial growth for this analysis in the presence of the antibiotic was achieved after 60 min of growth. (A) DNA synthesis assay, ciprofloxacin used at 0.5 µg/mL; the increased DNA synthesis in the presence of sub-MIC levels of compound **2** might be due to a stress response.[22] (B) RNA synthesis assay, rifampicin used at 8 ng/mL. (C) Protein synthesis asssay, tetracycline used at 31 ng/mL. (D) Peptidogylcan synthesis, fosfomycin used at 16 µg/mL.

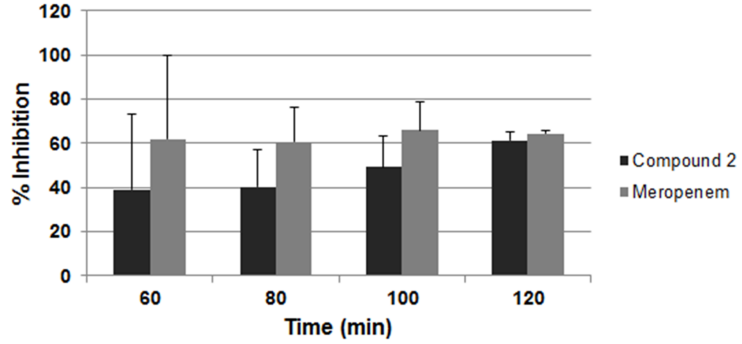


Figure S5. Peptidoglycan synthesis assay using meropenem (0.06 μg/mL) as the positive control. Compound **2** (1 μg/mL) maximum inhibition of peptidoglycan synthesis was 61 ± 4%, compared to meropenem at 64 ± 2%. Sufficent bacterial growth for this analysis in the presence of the antibiotic was achielved after 60 min of growth.

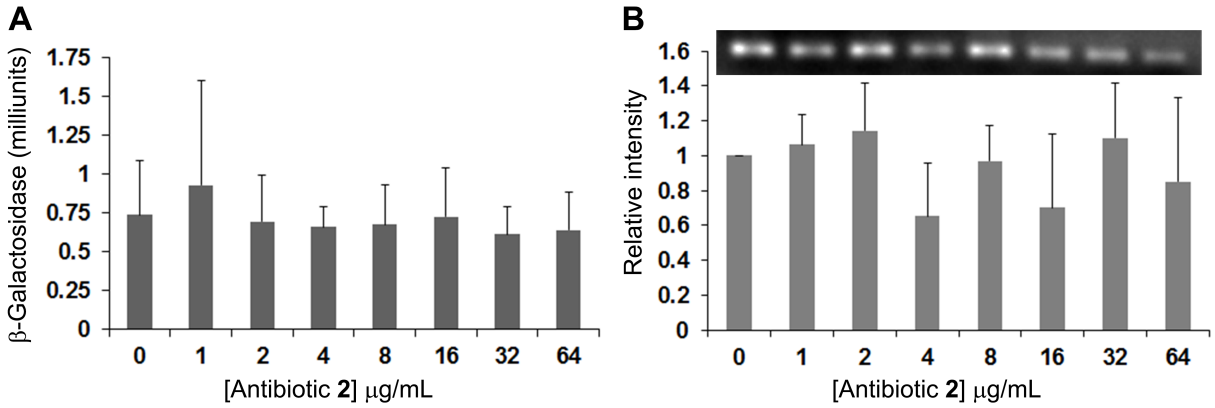


Figure S6. Effect of quinazolinone **2** on i*n vitro* transcription and translation. (A) A coupled transcription/translation assay using *E. coli* S30 extract system for circular DNA was performed in the presence of various amounts of **2**. The amount of β-galactosidase was quantified in duplicate using the β-galactosidase enzyme assay system kit. A standard curve using known concentrations of β-galactosidase was prepared to convert absorbance readings to milliunits of β-galactosidase. No inhibition of *in vitro* translation was observed in the presence of up to 64 μg/mL antibiotic **2**. (B) A transcription assay using a TranscriptAid T7 high yield transcription kit was performed in the presence of antibiotic **2**. Samples were loaded in triplicate onto a denaturing 1% formaldehyde agarose gel and the band intensities analyzed and compared to the control (0 μg/mL antibiotic **2**).

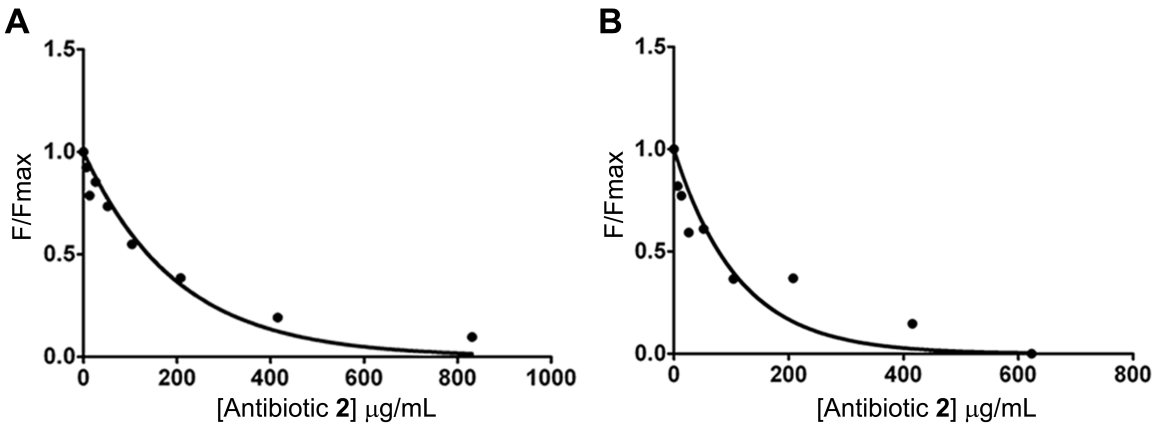


Figure S7. Regression curves for Bocillin FL assay. Nonlinear regression curves from Bocillin FL assays. (A) Relative fluorescence intensity of the PBP2a band (where F is the measured fluorescence and Fmax is maximum fluorescence in the absence of antibiotic) is plotted against the concentration of antibiotic **2** (μg/mL). (B) Fluorescence labeling of *S. aureus* membrane preparation (150 μg) by Bocillin FL (30 μM) in the presence of increasing amounts of antibiotic **2** (expressed in μg/mL). Relative fluorescence intensity of the PBP1 band is plotted against the concentration of antibiotic **2** (μg/mL). Data were fit by nonlinear regression using the published equation,[12] with R^2 values of 0.95 and 0.87 for panels A and B, respectively.

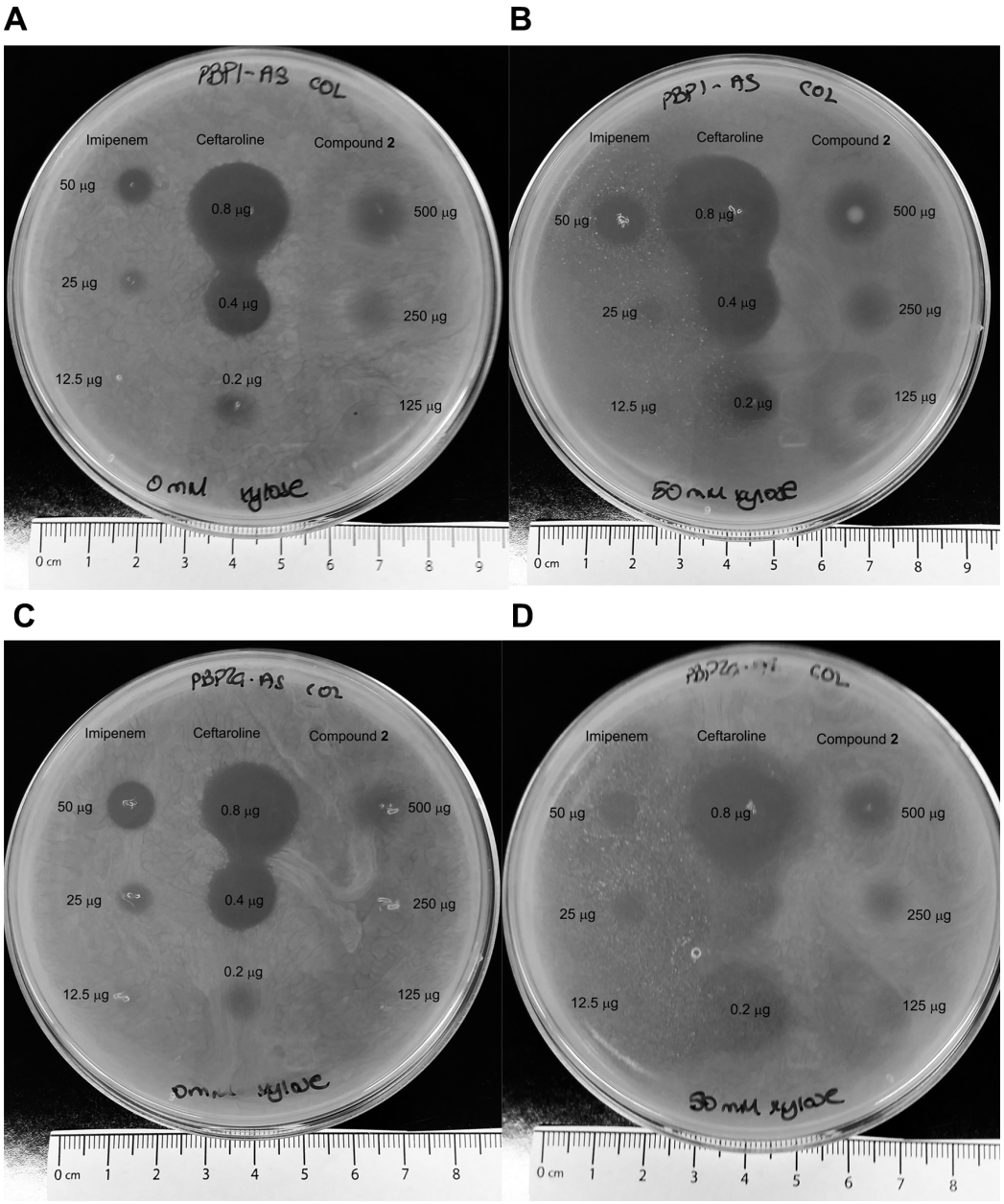


Figure S8. Sensitization of MRSA COL strains under anti-sense control. (A) COL *pbpA* anti-sense strain without xylose and (B) with 50 mM xylose. (C) COL *pbp2a* anti-sense strain without xylose and (D) with 50 mM xylose. Plates are spotted with imipenem (50, 25, and 12.5 µg), ceftaroline (0.8. 0.4, and 02 µg), and compound **2** (500, 250, and 125 µg). An increase in zone of inhibition is observed for all three antibiotics in the presence of xylose for the *pbpA* and *pbp2a* strains. The MIC of compound **2** against this COL strain was >128 µg/mL necessitating the high concentration used.

Table S1. Pharmacokinetic parameters of quinazolinone 2 in mice after single iv or po dose administration.

Parameter	po	iv
Dose (mg/kg)	10	10
$AUC_{0\text{-last}}$ (µg·min/mL)	408	1179
$AUC_{0\text{-}\infty}$ (µg·min/mL)	738	1456
Vd (mL/kg)	-	303
CL (mL/min/kg)	-	6.87
C_0 (µg/mL)	-	33.0
$t_{½elimination}$ (h)	>20 h	>20 h
C_{max} (µg/mL)	1.29	-
T_{max} (min)	60.0	-
F (%)	50	-

$AUC_{0\text{-last}}$ = area under the concentration-time curve from time zero to the last time point
$AUC_{0\text{-}\infty}$ = area under the concentration-time curve from time zero to infinity
Vd = volume of distribution, calculated by Dose/C_0
CL = clearance, calculated by Dose/$AUC_{0\text{-}\infty}$
C_0 = concentration at time zero
C_{max} = maximum concentration
T_{max} = time at maximum concentration
F = absolute oral bioavailability, calculated by $AUC_{0\text{-last po}}$/ $AUC_{0\text{-}\infty\text{ iv}}$

Table S2. Data collection and refinement statistics for PBP2a complex.

	PBP2a: **2** complex
Data collection	
Space group	$P2_12_12_1$
Cell dimensions	
a, *b*, *c* (Å)	81.20, 103.83,186.68
Resolution (Å)	63.96-(2.06-1.95)*
R_{sym} or R_{merge}	0.093 (1.61)
R_{pim}	0.018 (0.37)
CC(1/2)	1.00 (0.906)
Average $\langle I / \sigma I \rangle$	19.5 (1.9)
Completeness (%)	100.0 (100.0)
Redundancy	25.6 (18.4)
Refinement	
Resolution (Å)	63.88-1.95
Total No. reflections	2956712
No. Unique reflections	115623
R_{work} / R_{free}	0.198/0.247
No. atoms	
Protein	10302
Antibiotic **2**	30
Muramic acid	34
Water	247
B-factors	
Protein	72.20
Ligand/ion	83.30
Water	54.20
R.m.s. deviations	
Bond lengths (Å)	0.008
Bond angles (°)	1.07
PDB Code	4CJN

*Values in parentheses are for highest-resolution shell.

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