Supporting Information

Zhang et al. 10.1073/pnas.1408601111

SI Materials and Methods

Virtual Screening for Sortase Inhibitors. Scaffold hopping and molecular docking were combined for the virtual screening of sortase A inhibitors. The molecular similarity searching method SHAFTS was used in the scaffold hopping of template ligands LPAT* and Topsentin A (1). Using the ADMET calculation module (Discovery Studio2.5), identified molecules were analyzed for physical and chemical drug-like properties; 2,380 of such compounds were selected from the drug-like SPECS database. Subsequent molecular docking based on the known sortase A-LPAT* solution structure [Protein Data Bank (PDB) ID code 2KID] and the binding site of LPAT* was defined as a docking target (2). Glide and DOCK4.0 were used in molecular docking to assess molecules by both experience-based and force field-based scoring function. According to predicted binding free energy and geometrical matching quality, the top 300 compounds were selected and classified by scaffold diversity in Pipeline Pilot 5.0. Finally, the 105 compounds that best met our criteria were purchased and subjected to experimental evaluation.

Proteins Expression and Purification. The DNA sequence encoding *Staphylococcus aureus* sortase A residues Ala-25–Lys-206 (*S. aureus* $SrtA_{\Delta N24}$) and *Staphylococcus pyogenes* sortase A residues Val-82–Thr-249 (*S. pyogenes* $SrtA_{\Delta N81}$) were cloned into the NdeI–XhoI restriction sites of the pET28a vector (Novagen). The *Escherichia coli* BL21 (DE3) strain was transformed with the recombinant plasmids and grown in lysogeny broth medium containing 30 μg/mL kanamycin at 37 °C. When the A_{600} reached 0.6, sortase expression was induced by isopropyl β-D-thiogalactopyranose with a final concentration of 1 mM, and growth continued for an additional 4 h at 30 °C. The cells were harvested by centrifugation at 5,000 × *g* for 10 min and stored at −80 °C until use. For protein purification, the cells were suspended in 35 mL lysis buffer (50 mM Tris·HCl, pH 7.5, 200 mM NaCl, and 40 mM imidazole), lysed by sonification, and then centrifuged at 15,000 × *g* for 30 min at 4 °C. The supernatant was then passed through 0.22-mm syringe filters and loaded onto a HisTrap HP 5-mL column (GE Healthcare), washed with four column volumes of 40 mM imidazole, and eluted with 300 mM imidazole. The protein was concentrated via centrifugal filtration (Millipore) with a molecular weight cutoff of 10 kDa and then loaded onto a HiLoad 16/60 Superdex 75 column (GE Healthcare). The eluted protein was analyzed by 12% (wt/vol) SDS/PAGE, and purity was estimated at >95%. Protein was concentrated to 10 mg/mL in 20 mM Tris·HCl (pH 8.0), 50 mM NaCl, and 30% (vol/vol) glycerol and stored at −80 °C.

The DNA sequence encoding the surface protein *S. aureus* SasX (Ala-30–Ser-178) or *S. aureus* IsdA (Ala-64–Asn-323) with the conserved LPXTG motif was cloned into the NcoI–XhoI restriction enzymatic site in the pET28b vector (Novagen); a His-tag was added to the C terminus of each surface protein, $SasX_{30–178}$ and $IsdA_{64–323}$, via the insertion of appropriate coding sequence. As described above for sortase, $SasX_{30–178}$ and $IsdA_{64–323}$ were expressed in *E. coli* BL21 (DE3) and purified via a HisTrap HP 5-mL column (GE Healthcare) and HiLoad 16/60 Superdex 75 column (GE Healthcare) chromatography.

Enzymatic Activity of Sortase A. Sortase A activity assay was performed in 96-well black plates (PerkinElmer) in a final volume of 200 μL. Fluorescent-quenched peptide substrate abz-LPATG-dnp (purchased from Shanghai GL Biochem) was used for the K_m determination (3). Substrate, at eight different concentrations, ranging from 2.5 to 200 μM, was incubated at 25 °C with 1 μM sortase A diluted in assay buffer containing 50 mM Tris·HCl, 150 mM NaCl, 5 mM $CaCl_2$, and 0.1% Triton X-100, pH 7.5. The increase in fluorescence intensity was monitored every 2 min for 20 min using Flex Station 3 (Molecular Devices) at an excitation wavelength of 309 nm and emission wavelength of 420 nm. The initial velocity (V_0) was determined from the linear portion of the progress curve, and K_m was calculated from the Michaelis–Menten equation using GraphPad Prism software (San Diego), where $V = V_{max}[S]/([S] + K_m)$. Triplicate measurements were taken for each data point, and the data are reported as mean ± SE.

Inhibition of Sortase A. All compounds were dissolved in DMSO as stock solution at a concentration of 40 mM. For the first screening, compounds were mixed with the assay buffer to give a final concentration of 100 μM, and then the sortase A protein (final concentration at 1 μM) was added and preincubated for 10 min at 25 °C. Catalysis was initiated by adding peptide substrate to a final concentration of 10 μM. The V_0 obtained in the absence of inhibitor was taken as 100% and designated as the control value, and the initial inhibitory velocity (V_i) was obtained in the presence of compounds. Percent inhibition was calculated according to the following equation: inhibitory rate = $(1 - V_i/V_0) \times 100\%$. Compounds with inhibitory rates against sortase A greater than 50% were used for IC_{50} determination. For IC_{50} calculation, inhibitors at 10 concentrations (ranging from 0.8 to 400 μM) were mixed with sortase A (final concentration at 1 μM) in assay buffer and preincubated at 25 °C for 10 min. Catalysis was again initiated by adding substrate to a final concentration of 10 μM. IC_{50} values were determined using sigmoidal dose–response by GraphPad Prism software. Triplicate measurements were taken for each data point. The data are reported as mean ± SE.

Reversed-Phase HPLC Assay. Purified sortase A and abz-LPATG-dnp were used for reversed-phase HPLC analysis following a published assay (4). In 1 mL assay buffer (50 mM Tris·HCl, 150 mM NaCl, and 5 mM $CaCl_2$, pH 7.5), sortase A was added to a final concentration of 2 μM, and compound **6e** was added at concentrations ranging from 3.125 to 50 μM. The reactions were initiated by adding substrate peptide and Gly_3 to a final concentration of 100 μM and 3 mM, respectively, and then incubated for 2 h at 37 °C. After the reaction was quenched by the addition of 200 μL of 6 N HCl, a 40-μL sample was loaded on a reversed-phase HPLC (Agilent 1100) and separated on a C-18 column (150 × 4.6 mm; Agilent) equilibrated with water containing 0.1% trifluoroacetic acid (TFA). The peptides were separated by a linear gradient of 0–60% (vol/vol) acetonitrile/0.1% TFA (1 mL/min) and analyzed by UV detection at 350-nm wavelength. The extent of reaction was calculated from the decrease of the area of the substrate peak. Peak detection was quantified by 100 μM of standard substrate peptide. Integrated areas of peaks corresponding to the fluorescence-containing substrate and products were used to calculate the percent of substrate converted to product.

Inhibition of Sortase Transpeptidation. Compound **6e** (25–200 μM) was added to 50 μL assay buffer (50 mM Tris·HCl, 150 mM NaCl, and 5 mM $CaCl_2$, pH 7.5) with or without 10 μg $SrtA_{\Delta N24}$, 10 μg $IsdA_{64–323}$, or 10 μg $SasX_{30–178}$, and 3 mM Gly_3 and incubated for 2 h at 37 °C. Reactions were quenched with 50 μL 2× SDS loading buffer. Samples were subjected to 12% (wt/vol) Tris-glycine SDS/PAGE, and transpeptidation was analyzed by quantifying precursor and mature transpeptidation products.

Reversible Inhibition. To examine the reversible inhibition of sortase by isolated compounds, sortase A inhibitor complexes were rapidly diluted, and activity was determined (5). Briefly, 100 μL purified sortase A (150 μM) was mixed with compound **6e**; at the final concentration (93.1 μM sortase A), the concentration of the inhibitor was 10-fold higher than its IC_{50}. Following incubation at room temperature for 1 h, 9.9 mL reaction buffer was added. Then 190 μL of the diluted mixture was plated, and 10 μL of substrates was added to a final concentration of 10 μM. The increase in fluorescence intensity was monitored every 2 min for 20 min using Flex Station 3 (Molecular Devices) at an excitation wavelength of 309 nm and emission wavelength of 420 nm. Recovery of enzymatic activity after rapid dilution (100-fold) was calculated by comparing the V_i obtained in the presence of compound with the V_0 obtained in the absence of inhibitor. Triplicate measurements were taken for each data point. The data are reported as mean ± SE.

Surface Plasmon Resonance. Surface plasmon resonance (SPR) technology-based binding assays were performed with a Biacore T200 instrument (GE Healthcare) and running buffer HBS-EP [10 mM Hepes (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.05% (vol/vol) surfactant P20)] at 25 °C. Sortase A was covalently immobilized onto sensor CM5 chips by a standard amine-coupling procedure in 10 mM sodium acetate (pH 4.5), thereby retaining 3,108 response units (RUs) of sortase A on the chip. Compound **6e** was serially diluted and injected onto a sensor chip at a flow rate of 30 μL/min for 120 s (contact phase), followed by 120 s of buffer flow (dissociation phase). The K_d value was derived using Biacore T200 Evaluation software Version 1.0 (GE Healthcare) and steady state analysis of data at equilibrium.

Circular Dichroism Experiments. Circular dichroism (CD) spectra were acquired at wavelength range from 180 to 260 nm using a Jasco J-810 spectropolarimeter at 25 °C. Baseline was calibrated by phosphate buffer (20 mM sodium phosphate, pH 8.0) before each measurement series. Measurements were taken with 2 μM sortase A in 0.1-cm path length quartz cuvettes. Compound **6e** was variably diluted to generate different concentrations and incubated with sortase A for 1 h at 25 °C before CD analyses. Three consecutive scans were averaged, and spectra were acquired for data display.

Ig Binding to Staphylococci. Overnight cultures of *S. aureus* were 1:100 diluted into fresh tryptic soy broth (TSB) with or without inhibitor supplement and grown at 37 °C with rotation to A_{600} 0.5. Aliquots (600 μL) were removed, and staphylococci were sedimented by centrifugation (12,000 × *g* for 5 min). Bacteria were suspended in 600 μL PBS, FITC-labeled IgG (2 μL; eBioscience) was added, and samples were incubated at room temperature for 1 h. Staphylococci were again sedimented by centrifugation (12,000 × *g* for 5 min) and washed twice with PBS. Bacterial fluorescence intensity was monitored using the Flex Station 3 (Molecular Devices) at 495-nm excitation and 520-nm emission. Sample aliquots were spread on agar plates, and colony-forming units were enumerated to derive relative fluorescence units per A_{600} unit. Triplicate measurements were taken for each reaction, and average and SEMs were calculated.

Assembly of SpA into the Cell Wall. Overnight cultures of *S. aureus* were diluted into fresh TSB and grown at 37 °C with rotation in the presence or absence of compound **6e**. When A_{600} reached 0.5, two 1-mL aliquots were removed, and bacteria were sedimented by centrifugation (12,000 × *g* for 5 min), washed with PBS, and suspended in 500 μL lysis buffer (50 mM Tris·HCl, 0.5 M sucrose, and 10 mM $MgCl_2$, pH 7.5) containing 10 μg/mL lysostaphin (6). Samples were incubated at 37 °C for 30 min, protoplasts were sedimented by centrifugation (7,000 × *g* for 20 min), and the supernatants (cell wall anchored proteins) were removed. Sample aliquots were mixed with an equal volume of 2× SDS/PAGE loading buffer and analyzed by immunoblotting for SpA.

Fibrinogen-Binding Assay. Overnight cultures of *S. aureus* were diluted 1:100 into fresh TSB, inhibitors were added at variable concentration, and cultures were grown at 37 °C to A_{600} reaching to 0.5. Every 0.5 h for 2.5 h following the A_{600} reaching 0.5, 800-μL cell suspensions were removed and sedimented by centrifugation (12,000 × *g* for 5 min). Cells suspended in 200 μL PBS were added to the wells of fibrinogen-coated 96-well microtiter plates (Cell Biolabs) and incubated for 2 h at 37 °C. Liquid was removed, wells were washed twice with 200 μL PBS, and samples were fixed with 100 μL 4% (vol/vol) glutaraldehyde. Staphylococci bound to fibrinogen were stained for 1 h with 100 μL crystal violet dye, and wells were washed extensively with PBS. Plates were then dried, and 200 μL extraction solution (10% acetic acid) was added to each well and incubated for 10 min on an orbital shaker. The absorbance at 570 nm was subsequently measured using Flex Station 3 (Molecular Devices). Triplicate measurements were taken for each data point.

***S. aureus* Growth in the Presence of Compound 6e.** Briefly, overnight cultures of *S. aureus* Newman strain in TSB were diluted by 1:1,000 into fresh TSB medium containing variable amounts of compound **6e** (0, 50, and 200 μM). To measure the bacterial growth, *S. aureus* Newman was incubated at 37 °C with shaking at 230 rpm for 12 h. Absorbance at 600 nm was measured per hour using GeneQuant (GE Healthcare). Data are presented as the means of triplicate measurements.

Chemistry. ***General.*** ^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian-MERCURY Plus-400 NMR spectrometer. Chemical shifts are reported in ppm (δ scale) as referenced to tetramethylsilane, and coupling constant (*J*) values are reported in Hertz. Data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant in Hertz, and integration. Low-resolution electrospray ionization MS (HRESI) was recorded on a Finnigan LCQ/DECA spectrometer, and high-resolution electrospray ionization MS (HRESI) was recorded on a Micromass Ultra Q-TOF spectrometer. Low-resolution and high-resolution MS (LRMS/HRMS) was recorded on a Thermo-DFS spectrometer. Flash and column chromatography were performed using silica gel (230–400 mesh). Analytical TLC was performed silica gel plates and visualized under UV light (254 nm). All solvents and reagents were purchased from commercial sources and used as received.

Structure–activity relationship of sortase inhibitors. We optimized the chemical structure of compound **6a** to improve the inhibitory activity (Scheme S1) (7). Starting with the aromatic carboxylic ester **1**, compound **2** was prepared after hydrazine hydrate treatment. Condensation of compound **2** with CS_2 in the presence of KOH generated product 3. Hydrazine hydrate promoted cyclization of compound **3** to generate intermediate **4**. Cyclization between compound **4** and acyl chloride **5** achieved second ring closure to yield target compound **6**. A SAR dataset for 3,6-disubstituted triazolothiadiazoles based on 14 synthetic derivatives is summarized in Table S1. At position 3, replacement of 4-pyridyl substituent with other residues, such as 3-pyridyl, 3,4,5-trimethylhydroxyl, or 2-indolyl, decreased the inhibitory activity (compare compounds **6a**, **6c–6e**, and **6g–6j** with **6k**, **6m**, and **6n**, respectively). Compound **6l** with 3-pyridyl at position 3 represented an exception, as this molecule exhibited also good inhibitory activity, indicating that combinations of two suitable substitutions are important to increase the inhibitory activity. At position 6, the aromatic heterocyclic substituent bearing hydrogen-bond donors contributed to the inhibitory activity (compare compounds

6g, **6h**, and **6j** with **6f** and **6i**). The substituent pattern on the phenyl ring of the 3-(4-pyridyl)-triazolothiadiazole also affected the inhibitory activity, as evidenced from the SAR of compounds **6a**, **6c**, and **6d**. Specifically, the *o*-iodo-substituted compound **6b** did not display inhibitory activity. Introducing a sulfonic group to the *m*-position on the 6-phenyl afforded the best optimized compound **6e** {3-(4-pyridinyl)-6-(2-sodiumsulfonatephenyl)[1,2,4] triazolo[3,4-b][1,3,4]thiadiazole} with an IC_{50} of 9.3 μM, which represents a fourfold improvement over the screening hit, compound **6a** (Table S1).

General procedure of the synthesis of 3-(4-pyridinyl)-6-substituted-1,2, 4-triazolo [3,4-b][1,3,4] thiadiazole.

General procedure of the synthesis of hydrazide 2. To a stirred solution of ester **1** (10 mmol) in ethanol (20 mL) was added hydrazine hydrate (5 mmol), and the solution was refluxed for 12 h. The solvent was removed under reduced pressure. The solid was recrystallized from ethanol to afford the pure product **2**.

Isonicotinic acid hydrazide: ^{1}H NMR (DMSO-d_6): δ 4.61 (s, 2H), 7.71 (dd, J = 4.5 and 1.7 Hz, 2H), 8.69 (dd, J = 4.5 and 1.7 Hz, 2H), 10.09 (s, 1H).

Nicotinic hydrazide: ^{1}H NMR (DMSO-d_6): δ 4.56 (s, 2H), 7.49 (m, 1H), 8.14 (m, 1H), 8.69 (m, 1H), 8.95 (m, 1H), 9.97 (s, 1H).

2-Indolecarbohydrazide: ^{1}H NMR (DMSO-d_6): δ 4.50 (s, 2H), 7.02 (m, 1H), 7.08 (m, 1H), 7.15 (m, 1H), 7.42 (m, 1H), 7.58 (d, J = 8 Hz, 1H), 9.78 (s, 1H), 11.60 (s, 1H).

3,4,5-Trimethoxybenzhydrazide: ^{1}H NMR (DMSO-d_6): δ 3.69 (s, 3H), 3.81 (s, 6H), 4.46 (s, 2H), 7.16 (s, 2H), 9.71 (s, 1H).

General procedure of the synthesis of potassium dithiocarbazate 3. To an ice-cooled solution of hydrazide **2** (10 mol) in anhydrous ethanol (50 mL) was added anhydrous KOH (10 mmol), followed by CS_2 (13 mmol). The reaction mixture was stirred at 0 °C for 1 h, and then warmed slowly to room temperature and stirred overnight. The precipitate was filtered and washed thoroughly with ethanol. The precipitate was dried under reduced pressure to afford the solid intermediate **3**. It was used directly for the next step without further purification.

Procedure of synthesis of 4-amino-5-substituted-3-mercapto-1,2,4-triazole 4. To a stirred solution of potassium dithiocarbazate **3** (10 mmol) in water (20 mL) was added hydrazine hydrate (30 mmol), and the mixture was refluxed for 12 h. The color of the solution changed to green with the evolution of hydrogen sulfide gas. The solution was cooled at room temperature and acidized with concentrated HCl to pH 1. The crude product was precipitated and washed thoroughly with cooled water. The precipitate was recrystallized from ethanol to afford the pure product **4**.

4-amino-5-(4-pyridinyl)-3-mercapto-1,2,4-triazole: ^{1}H NMR (DMSO-d_6): δ 4.62 (s, 2H), 7.72 (dd, J = 4.4 and 1.6 Hz, 2H), 8.70 (dd, J = 4.4 and 1.6 Hz, 2H), 10.11 (s, 1H).

4-amino-5-(3-pyridinyl)-3-mercapto-1,2,4-triazole: ^{1}H NMR (DMSO-d_6): δ 5.81 (s, 2H), 7.59 (m, 1H), 8.38 (m, 1H), 8.72 (m, 1H), 9.15 (m, 1H), 14.07 (s, 1H).

4-amino-5-(2-indolyl)-3-mercapto-1,2,4-triazole: ^{1}H NMR (DMSO-d_6): δ 5.93 (s, 2H), 7.05 (m, 1H), 7.08 (m, 1H), 7.20 (m, 1H), 7.44–7.47 (m, 2H), 7.65 (d, J = 8 Hz, 1H), 11.76 (s, 1H), 13.99 (s, 1H).

3,4,5-trimethoxybenzhydrazide: ^{1}H NMR (DMSO-d_6): δ 3.72 (s, 3H), 3.82 (s, 6H), 5.83 (s, 2H), 7.34 (s, 2H), 13.94 (s, 1H).

Synthesis of 3,6-disubstituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazoles 6. The acid (1 mmol) was added to $S(O)Cl_2$ (2 mL), and the mixture was refluxed for 6 h. $S(O)Cl_2$ was removed under reduced pressure to give intermediate **5**. Two milliliters of $P(O)Cl_3$ was added to the crude product, followed by 4-amino-5-substituted-3-mercapto-1,2,4-triazole **4**. Then the mixture was refluxed for 18 h. $P(O)Cl_3$ was removed under reduced pressure. The ice was added, the mixture was neutralized with NaOH, the and crude was filtered. The precipitate was recrystallized from ethanol and DMSO to afford target compound **6**.

3-(4-Pyridinyl)-6-(2-iodophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (6b). ^{1}H NMR (DMSO-d_6): δ 7.41 (m, 1 H), 7.65 (m, 1 H), 7.85 (m, 1 H), 8.16 (m, 1H), 8.21 (dd, J = 4.8 and 1.2 Hz, 2 H), 8.82 (dd, J = 4.8 and 1.2 Hz, 2H); ^{13}C NMR (TFA-d_1): δ 93.96, 124.51, 128.87, 130.38, 131.51, 134.46, 139.91, 140.98, 141.82, 142.80, 156.61, 174.75; LRMS m/z (% relative abundance) 405 (M^+, 100); HRMS m/z calcd for $C_{14}H_8IN_5S$ 404.9545, found 404.9542.

3-(4-Pyridinyl)-6-phenyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (6c). ^{1}H NMR (DMSO-d_6): δ 7.65–7.74 (m, 3H), 8.12 (m, 2H), 8.27 (dd, J = 1.6 and 4.8 Hz, 2H), 8.85 (dd, J = 1.6 and 4.8 Hz, 2H); ^{13}C NMR (TFA-d_1): δ 124.51, 125.80, 127.36, 129.76, 135.31, 139.95, 140.96, 142.80, 156.26, 174.90; LRESI m/z (% relative abundance) 280.2 ($[M+H]^+$, 100); HRESI m/z calcd for $C_{14}H_{10}N_5S$ $[M+H]^+$ 280.0657, found 280.0665.

3-(4-Pyridinyl)-6-(3,4,5-trihydroxyphenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (6d). ^{1}H NMR (DMSO-d_6): δ 7.01 (s, 2H), 8.21 (d, J = 4 Hz, 2H), 8.85 (d, J = 4 Hz, 2H); ^{13}C NMR (DMSO-d_6): δ 106.72, 119.05, 119.87, 133.05, 139.06, 143.94, 147.14, 151.15, 155.45, 168.44; LRMS m/z (% relative abundance) 326.1 (M^+, 100); HRESI m/z calcd for $C_{14}H_8N_5O_3S$ $[M-H]^-$ 326.0348, found 326.0355.

3-(4-Pyridinyl)-6-(2-sodium sulfonatephenyl)[1,2,4]triazolo[3,4-b][1,3,4] thiadiazole (6e). ^{1}H NMR (DMSO-d_6): δ 7.65 (t, J = 7.7 Hz, 1H), 7.92 (dm, J = 7.7 Hz, 1H), 8.07 (dm, J = 7.7 Hz, 1H), 8.19 (m, 1H), 8.25 (d, J = 4.5 Hz, 2H), 8.87 (d, J = 4.5 Hz, 2H,); ^{13}C NMR (TFA-d_1): δ 124.87, 125.42, 127.15, 130.77, 131.11, 132.10, 139.92, 141.28, 142.89, 143.11, 156.55, 173.04; LRESI m/z (% relative abundance) 358 ($[M-Na]^-$, 100); HRESI m/z calcd for $C_{14}H_8N_5O_3S_2$ $[M-Na]^-$ 358.0069, found 358.0073.

3-(4-Pyridinyl)-6-(4-pyridinyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (6f). ^{1}H NMR (DMSO-d_6): δ 8.10 (dd, J = 1.2 and 4.8 Hz, 2H), 8.28 (dd, J = 1.2 and 4.8 Hz, 2H), 8.86 (dd, J = 1.2 and 4.8 Hz, 2H), 8.90 (dd, J = 1.2 and 4.8 Hz, 2H); ^{13}C NMR (TFA-d_1): δ 123.90, 125.36, 140.52, 141.97, 142.55, 143.17, 144.92, 157.61, 166.16; LRMS m/z (% relative abundance) 279 (M^+, 100); HRMS m/z calcd for $C_{13}H_8N_6S$ 280.0531, found 280.0532.

3-(4-Pyridinyl)-6-(2-indolyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (6g). ^{1}H NMR (DMSO-d_6): δ 7.15 (m, 1 H), 7.34 (m, 1 H), 7.49 (m, 1 H), 7.56 (m, 1 H), 7.71 (m, 1 H), 8.35 (m, 2 H), 8.88 (m, 2 H), 12.43 (s, 1 H); ^{13}C NMR (TFA-d_1): δ 111.62, 122.07, 122.18, 124.54, 127.41, 128.22, 139.23, 140.06, 140.81, 142.83, 155.81, 165.20; LRMS m/z (% relative abundance) 318 (M^+, 100). HRMS m/z calcd for $C_{16}H_{10}N_6S$ 318.0688, found 318.0688.

3-(4-Pyridinyl)-6-(6-benzimidazolyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (6h). ^{1}H NMR (DMSO-d_6): δ 7.80 (d, J = 8 Hz, 1H), 7.92 (d, J = 8 Hz, 1H), 8.25 (d, J = 4 Hz, 2H), 8.32 (s, 1H), 8.45 (s, 1H), 8.83 (d, J = 4 Hz, 2H); ^{13}C NMR (TFA-$d1$): δ 114.81. 116.46, 124.42, 126.17, 126.53, 130.23, 133.34, 140.12, 141.29, 141.68, 142.73, 156.75, 172.16; LRESI m/z (% relative abundance) 342.4 ($[M+Na]^+$, 100); HRESI m/z calcd for $C_{15}H_9N_7SNa$ $[M+Na]^+$ 342.0538, found 342.0537.

3-(4-Pyridinyl)-6-(2-furyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (6i). ^{1}H NMR (DMSO-d_6): δ 6.89 (dd, J = 3.6, 1.8 Hz, 1H), 7.65 (d, J = 3.6 Hz, 1H), 8.16 (d, J = 1.8 Hz, 1H), 8.18 (dd, J = 4.5, 1.7 Hz, 2H), 8.83 (dd, J = 4.6, 1.7 Hz, 2H); ^{13}C NMR (TFA-d_1): δ 113.74, 118.10, 124.44, 139.91, 140.89, 142.73, 149.42, 156.07, 163.10; LRMS m/z (% relative abundance) 269 (M^+, 100); HRMS m/z calcd for $C_{12}H_7N_5OS$ 269.0371, found 269.0380.

3-(4-Pyridinyl)-6-(2-pyrrolyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (6j). ^{1}H NMR (DMSO-d_6): δ 6.34 (m, 1H), 7.06 (m, 1H), 7.28 (m, 1H), 8.31 (dd, J = 1.6 and 4.8 Hz, 2H), 8.84 (dd, J = 1.6 and 4.8 Hz, 2H), 12.46 (s, 1H); ^{13}C NMR (TFA-d_1): δ 117.60, 119.09, 124.56, 128.29, 140.03, 140.56, 142.72, 155.24, 164.39; LRESI m/z (% relative abundance) 267 ($[M-H]^-$, 100); HRESI m/z calcd for $C_{12}H_7N_6S$ $[M-H]^-$ 267.0453, found 267.0449.

3-(3-Pyridinyl)-6-(2-indolyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (6k). ^{1}H NMR (DMSO-d_6): δ 7.14 (m, 2H), 7.33 (t, J = 8 Hz, 1H), 7.46 (m, 1H), 7.54 (d, J = 8 Hz, 1H), 7.68–7.71 (m, 2H), 8.71 (m, 1H), 8.80 (d, J = 4 Hz, 1H), 9.59 (s, 1H), 12.43 (s, 1H); ^{13}C NMR (TFA-d_1): δ 111.66, 121.99, 123.89, 127.34, 128.22, 128.50, 139.28, 139.94, 140.08, 144.10, 144.59, 154.78, 165.03; LRMS m/z (% relative abundance) 318 (M^+, 100); HRMS m/z calcd for $C_{16}H_{10}N_6S$ 318.0688, found 318.0691.

3-(3-Pyridinyl)-6-(6-benzimidazolyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (6l). ^{1}H NMR (DMSO-d_6): δ 7.70 (m, 1H), 7.82 (s, 1H), 7.93 (s, 1 H), 8.47 (m, 1H), 8.68 (m, 1H), 8.78 (d, J = 8 Hz, 1H), 9.48 (s, 2 H), 12.92 (s, 1H); ^{13}C NMR (TFA-d_1): δ 114.90, 116.50, 123.91, 125.87, 126.54, 128.66, 130.22, 133.40, 139.96, 140.56, 141.68, 143.96, 144.81, 155.61, 172.23; LRESI m/z (% relative abundance) 318 ([M-H]$^-$, 100); HRESI m/z calcd for $C_{15}H_8N_7S$ [M-H]$^-$ 318.0562, found 318.0568.

3-(3,4,5-Trimethoxyphenyl)-6-(2-indolyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (6m). ^{1}H NMR (DMSO-d_6): δ 3.76 (s, 3H), 3.94 (s, 6H), 7.12 (m, 1 H), 7.31 (m, 1H), 7.43 (m, 1H), 7.52 (d, J = 8 Hz, 1H), 7.57 (s, 2 H), 7.69 (d, J = 8 Hz, 1H), 12.30 (s, 1H); ^{13}C NMR (TFA-d_1): δ 56.34, 60.78, 106.90, 111.53, 122.00, 122.89, 123.29, 127.13, 127.40, 127.89, 139.01, 142.25, 145.02, 153.43, 153.58, 154.38, 163.97; LRESI m/z (% relative abundance) 406.1 ([M-H]$^-$, 100); HRESI m/z calcd for $C_{20}H_{16}N_5O_3S$ [M-H]$^-$ 406.0974, found 406.0984.

3-(2-Indolyl)-6-(6-benzimidazolyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (6n). ^{1}H NMR (DMSO-d_6): δ 7.10 (m, 1H), 7.24 (m, 1H), 7.50 (m, 2H), 7.74 (d, J = 8 Hz, 1H), 7.88 (d, J = 8 Hz, 1H), 8.02 (m, 1H), 8.40 (s, 1H), 8.61 (s, 1H), 12.14 (s, 1H); ^{13}C NMR (TFA-d_1): δ 111.78, 114.45, 114.97, 116.56, 122.19, 122.31, 126.60, 126.71, 127.18, 127.99, 130.43, 133.48, 139.12, 140.20, 141.93, 154.03, 170.91; LRESI m/z (% relative abundance) 358.2 ([M+H]$^+$, 100); HRESI m/z calcd for $C_{18}H_{12}N_7S$ [M+H]$^+$ 358.0875, found 358.0875.

1. Liu X, Jiang H, Li H (2011) SHAFTS: A hybrid approach for 3D molecular similarity calculation. 1. Method and assessment of virtual screening. *J Chem Inf Model* 51(9): 2372–2385.
2. Suree N, et al. (2009) The structure of the Staphylococcus aureus sortase-substrate complex reveals how the universally conserved LPXTG sorting signal is recognized. *J Biol Chem* 284(36):24465–24477.
3. Huang X, et al. (2003) Kinetic mechanism of Staphylococcus aureus sortase SrtA. *Biochemistry* 42(38):11307–11315.
4. Kruger RG, Dostal P, McCafferty DG (2004) Development of a high-performance liquid chromatography assay and revision of kinetic parameters for the Staphylococcus aureus sortase transpeptidase SrtA. *Anal Biochem* 326(1):42–48.
5. Copeland RA (2005) *Evaluation of Enzyme Inhibitors in Drug Discovery: A Guide for Medicinal Chemists and Pharmacologists* (Wiley, New York).
6. DeDent AC, McAdow M, Schneewind O (2007) Distribution of protein A on the surface of Staphylococcus aureus. *J Bacteriol* 189(12):4473–4484.
7. Mathew V, Keshavayya J, Vaidya VP, Giles D (2007) Studies on synthesis and pharmacological activities of 3,6-disubstituted-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazoles and their dihydro analogues. *Eur J Med Chem* 42(6):823–840.

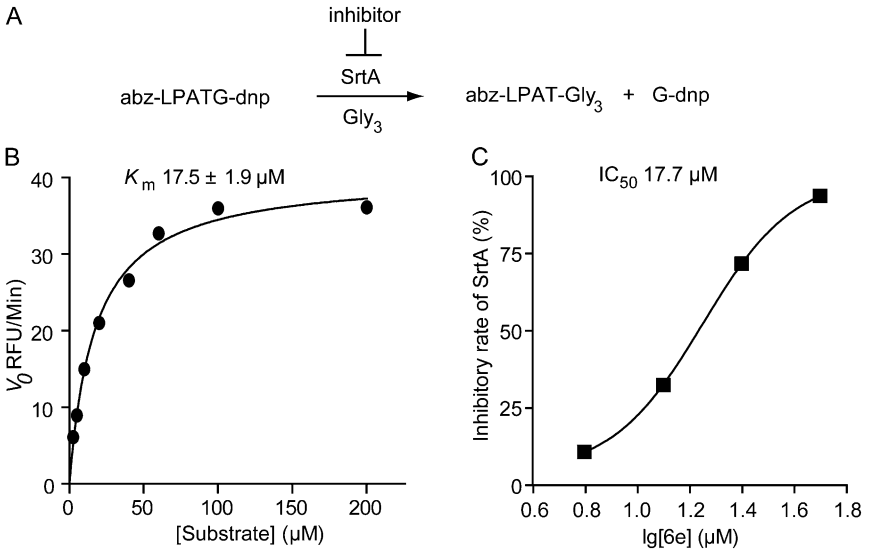


Fig. S1. Inhibition of sortase function in vitro. (*A*) Sortase catalyzed transpeptidation with abz-LPATG-dnp and Gly_3 generates abz-LPAT-Gly_3 and G-dnp, which was perturbed with the presence of inhibitors. (*B*) K_m calculation for sortase A-mediated transpeptidation in vitro. The initial velocity (V_0) was determined from the linear portion of the progress curve, and K_m was calculated from the Michaelis–Menten equation using GraphPad Prism software, where $V = V_{max}[S]/([S] + K_m)$. Triplicate measurements were taken for each data point, and the data are reported as mean ± SE. (*C*) IC_{50} calculation of compound **6e** for sortase A inhibition in the HPLC-based assay.

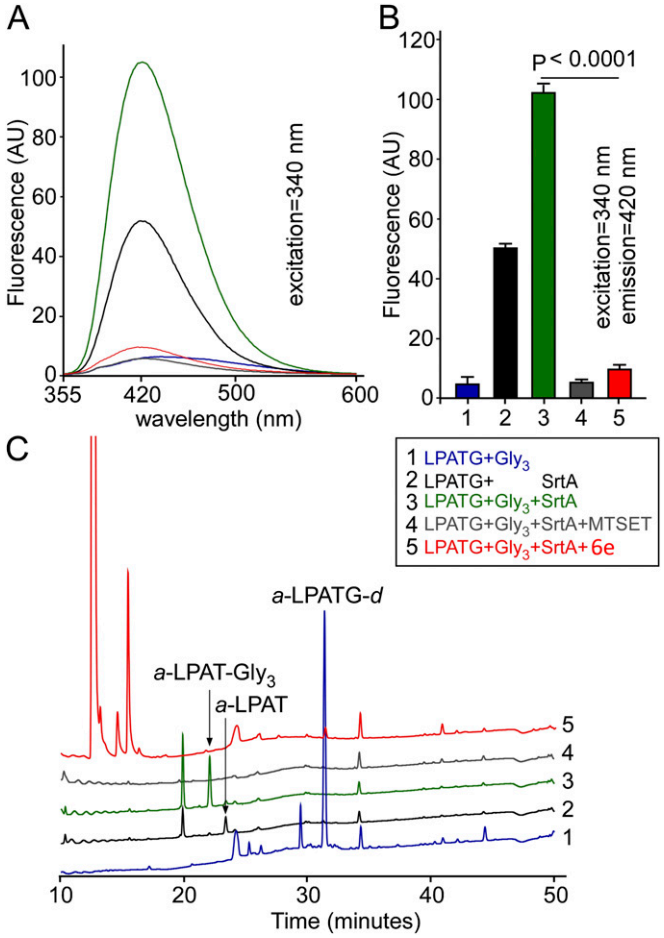


Fig. S2. Compound **6e** inhibits the in vitro transpeptidation reaction of recombinant $SrtA_{\Delta N}$ enzyme. (*A*) The in vitro transpeptidation reaction catalyzed by purified sortase ($SrtA_{\Delta N}$) was monitored using the abz-LPATG-dpn (*a*-LPATG-*d*) FRET assay. Purified $SrtA_{\Delta N}$ (0.5 μM) was incubated with 20 μM *a*-LPATG-*d* peptide at 37 °C for 18 h, and cleavage was monitored as an increase in fluorescence. Addition of 20 μM Gly_3 peptide accelerated cleavage, whereas the reaction was inhibited by the addition of *N*,*N*,*N*-trimethyl-2-(methylsulfonylthio)ethanaminium chloride (MTSET) or compound **6e**. Fluorescence emission was monitored as a function of emission wavelength over a spectrum of 355–600 nm. (*B*) Emission maximum at 420 nm revealed that compound **6e** inhibits $SrtA_{\Delta N}$ activity. (*C*) Reaction products were subjected to reversed-phase (RP)-HPLC analysis to compare cleavage and transpeptidation products. MALDI-MS of the RP-HPLC peaks confirmed the predicted peptide ions (abz-LPATG-dnp $[Na^+]$ m/z = 850.4057, abz-LPAT $[Na^+]$ 542.5354, abz-LPAT-Gly_3 $[Na^+]$ 713.5383).

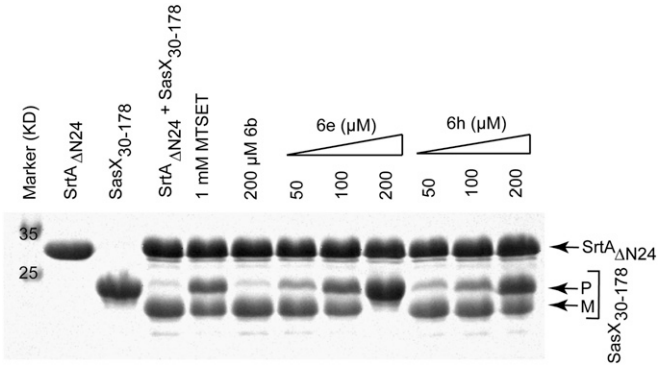


Fig. S3. SDS/PAGE analysis of transpeptidation reactions with 10 μg *S. aureus* $SrtA_{\Delta N24}$, 10 μg $SasX_{30–178}$, and 3 mM Gly_3 were incubated for 2 h at 37 °C with variable concentrations of compounds MTSET, **6b**, **6h**, or **6e**. Reactions were quenched with 50 μL 2× SDS loading buffer. Samples were subjected to 12% (wt/vol) Tris-glycine SDS/PAGE, and transpeptidation was analyzed by quantifying precursor (P) and mature (M) transpeptidation products.

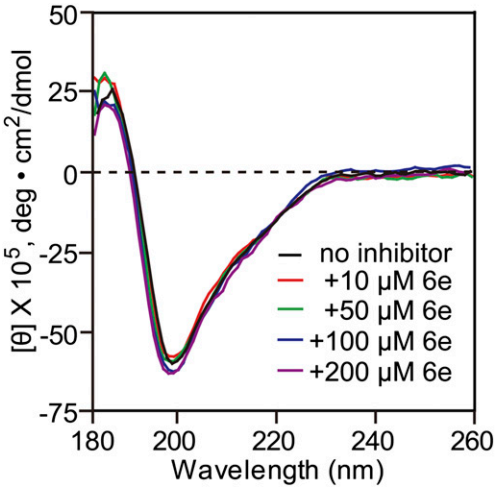
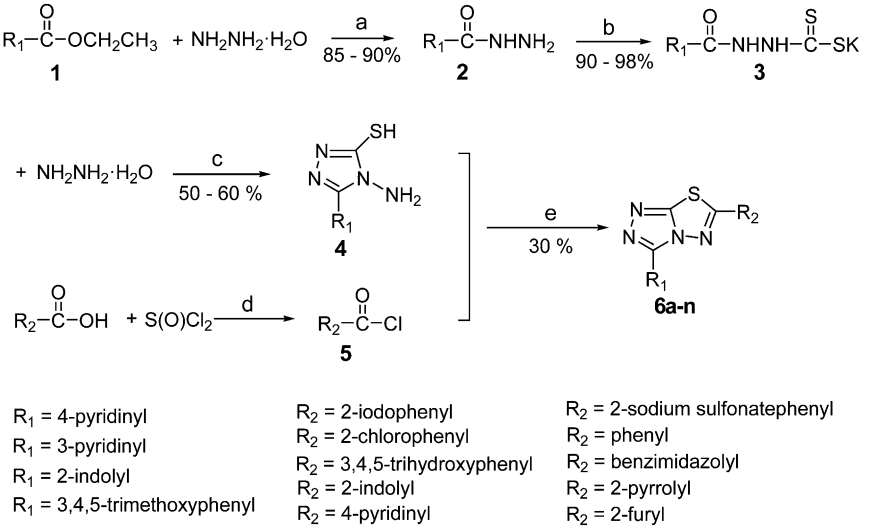


Fig. S4. Binding of compound **6e** to sortase A monitored by using far-UV CD spectroscopy. Addition of 200 μM compound **6e** to purified *S. aureus* $SrtA_{\Delta N24}$ (100-fold excess of inhibitor vs. enzyme) caused minor changes in secondary structure content, indicating that the inhibitor does not promote protein aggregation.



R_1 = 4-pyridinyl
R_1 = 3-pyridinyl
R_1 = 2-indolyl
R_1 = 3,4,5-trimethoxyphenyl

R_2 = 2-iodophenyl
R_2 = 2-chlorophenyl
R_2 = 3,4,5-trihydroxyphenyl
R_2 = 2-indolyl
R_2 = 4-pyridinyl

R_2 = 2-sodium sulfonatephenyl
R_2 = phenyl
R_2 = benzimidazolyl
R_2 = 2-pyrrolyl
R_2 = 2-furyl

Scheme S1. Synthesis of 3,6-disubstituted-1,2,4-triazolo[3,4-b][1,3,4] thiadiazole. Reagents and conditions: (*a*) EtOH, reflux, 12 h; (*b*) CS_2/KOH, EtOH, 0 °C to rt, overnight; (*c*) H_2O, reflux; 12 h; (*d*) reflux, 6 h; and (*e*) $P(O)Cl_3$, reflux, 18 h.

Table S1. Summary of IC_{50} for inhibition of *S. aureus* sortase in vitro

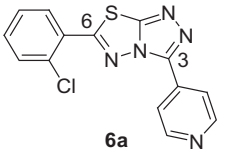
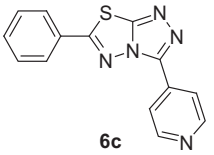
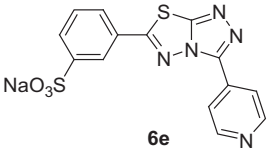
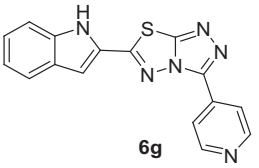
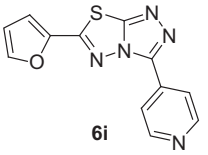
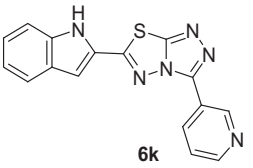
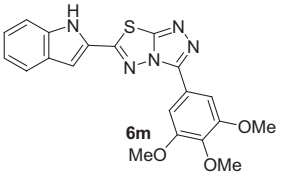
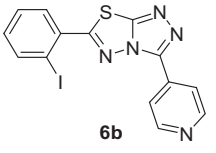
Compound	IC_{50} (μM)
 **6a**	37.7 ± 2.5
 **6c**	31.7 ± 4.3
 **6e**	9.3 ± 0.7
 **6g**	22.7 ± 3.6
 **6i**	39.3 ± 4.2
 **6k**	NA
 **6m**	87.6 ± 5.6
 **6b**	Not active (NA)

Table S1. Cont.

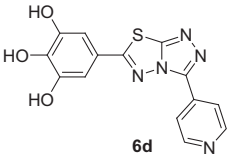
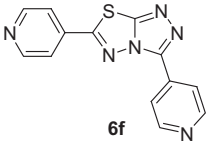
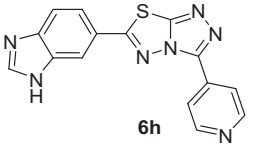
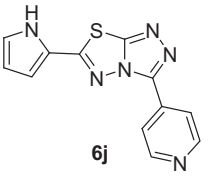
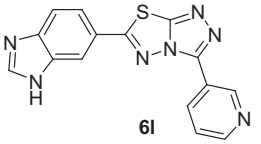
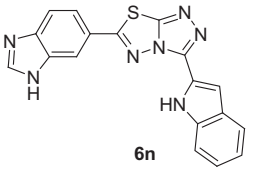
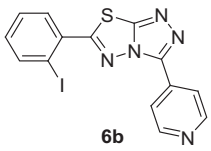
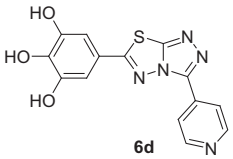
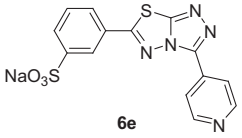
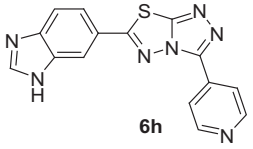
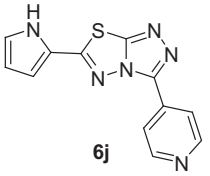
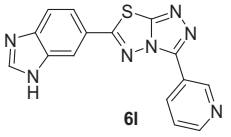
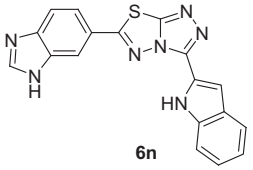
Compound	IC_{50} (μM)
 **6d**	42.7 ± 4.8
 **6f**	>100
 **6h**	17.0 ± 2.9
 **6j**	14.3 ± 1.2
 **6l**	28.7 ± 4.7
 **6n**	79.4 ± 5.2

Table S2. Summary of IC_{50} for inhibition of Gram-positive bacterial sortase in vitro

Compound	IC_{50} (μM) for sortase A			
	S. aureus	*S. pyogenes*	*S. pneumoniae*	*Bacillus anthracis*
 **6b**	NA	NA	NA	NA
 **6d**	42.7 ± 4.8	1.4 ± 0.6	51.9 ± 4.8	13.3 ± 2.9
 **6e**	9.3 ± 0.7	0.8 ± 0.1	36.9 ± 5.8	120.5 ± 13.8
 **6h**	17.0 ± 2.9	0.8 ± 0.2	43.2 ± 5.7	26.6 ± 3.2
 **6j**	14.3 ± 1.2	0.9 ± 0.1	18.1 ± 2.7	Not determined
 **6l**	28.7 ± 4.7	4.0 ± 0.1	21.5 ± 3.7	Not determined
 **6n**	79.4 ± 5.2	46.4 ± 3.9	22.3 ± 1.7	32.8 ± 6.4