Supplementary information

SI Experimental Procedures

Bacterial strains and plasmids. The bacterial strains and plasmids used or constructed in this study are listed in Tables S3 and S4. Luria broth (LB) and LB agar plates were used throughout for growth of *Escherichia coli*, and BHI broth and BHI agar plates were used for *S. aureus*. Chloramphenicol was used at 10 μg/ml, and ampicillin was used at 100 μg/ml for plasmid selection. Unless otherwise stated, all of the cultures were grown aerobically at 37°C with shaking, and growth was monitored at 600 nm with a spectrophotometer (Hitachi U-2800).

Disk diffusion and lux assays. A single colony of bioluminescent *S. aureus* from BHI agar was resuspended in 200 μl of sterile water, diluted to 75 ml 0.7% (w/v) soft agar (375-fold dilution), and overlaid onto BHI plates. Paper disks (Advantec) were placed on the overlay, and the plates were incubated at 37°C. After 20 h, inhibition zones were measured, and luminescence was detected with an IVIS Spectrum In Vivo Imaging System (PerkinElmer).

Secondary screening. After primary screening, the primary hits were first examined by disk diffusion assay against *hla* promoter activity in USA300-pGLhla. Compounds were diluted to 500 μM, and 5 μl of each compound, in dimethyl sulfoxide (DMSO), was added onto a 6-mm paper disk. At the same time, 5 μl of DMSO on a paper disk was used as a negative control. Each compound was tested in duplicate.

Selected compounds were also screened with 14 promoters: USA300-pGLagrP2, USA300-pGLsaeP1, USA300-pGLsrtA, USA300-pGLhla, USA300-pGLspa, USA300-pGLpsm, USA300-pGLpvl, USA300-pGLfnbA, USA300-pGLclfA, COL-pGLcap5, AE052-pGLcap8, USA300-pGLfnbB, USA300-pGLagrP3, and USA300-pGLsaeP3.

Bacteria at 1E7 CFU were added to 100 μl of BHI medium in 96-well black plates with clear bottoms, and 2 μl of compounds were added to each well. The plates were incubated at 37°C for different time intervals, and data were collected using

a DTX multimode plate reader. The compounds were screened in triplicate, similar to our published study (1).

The repression ratio was calculated using the following equation:

$$\text{repression ratio} = \frac{readings\ of\ samples\ - readings\ of\ negative\ control}{readings\ of\ positive\ control - readings\ of\ negative\ control}$$

A low value for the repression ratio signified great suppressive activity of the compound. We selected compounds with repression ratios of 0.2 or lower as primary hits. Compounds that showed a repression effect on more than half of the selected promoters were selected as hits.

Tertiary screening. After the conditions of the ClpP enzyme assay and screening parameters were optimized, tertiary screening was commenced to screen for ClpP inhibitors among the selected hits (670 compounds) targeting the repression of the hla promoter.

Based on the fluorescence difference between the positive and negative controls, the inhibition ratio was calculated. A cut-off of the inhibition ratio at 0.2 resulted in 17 positive hits. Hit validation of the 17 compounds was conducted with two concentrations of compounds (10 μM and 100 μM). Three compounds (including M21) used at 100 μM showed larger than 50% inhibition on the protease activity of ClpP.

MIC. The MIC was determined by inoculating 5×10^4 *S. aureus* cells in 100 μl of BHI media in 96-well plates with serial dilution of antibiotics. The MIC was defined as the minimum concentration resulting in a cell density less than 0.01 OD at 620 nm (2, 3), which corresponded to no visible growth, after incubation at 37°C for 18 h.

Quantitative polymerase chain reaction (qPCR). The preparation of total RNA from *S. aureus* was performed with RNA protection reagent according to the manufacturer's instructions (Qiagen). Briefly, total RNA was prepared by

lysostaphin extraction using 5 × 10^8 CFU of bacteria at each time point, followed by further purification with an RNeasy kit (Qiagen) according to the manufacturer's instructions. The quality and quantity of total RNA were confirmed by agarose electrophoresis and ultraviolet spectrophotometry, respectively. Contaminating chromosomal DNA was removed by DNase treatment (Thermo Scientific). Purified *S. aureus* RNA was reverse transcribed onto cDNA with a Transcriptor First-Strand cDNA Synthesis Kit (Roche) and subjected to realtime PCR analysis using an ABI 7500 thermocycler (Thermo Scientific) using FastStart Universal SYBR Green Master (Rox) (Roche). The relative quantification of *S. aureus* transcripts was determined by the ratio of expression of the target transcripts relative to gyrB (i.e., a housekeeping or calibration gene). The sequences of primers for real-time PCR experiments are provided in Table S5.

Western blot. After sodium dodecyl sulfate polyacrylamide gel electrophoresis, α-toxin was detected with rabbit anti-staphylococcal α-toxin antibody (1:20,000) (Sigma-Aldrich) and goat horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:5,000) (Sigma-Aldrich). Protein A was visualized with HRP-conjugated rabbit anti-staphylococcal Spa antibody (1:20,000) (Abcam), and PVL was visualized with rabbit anti-staphylococcal PVL antibody (1:20,000) (IBT). The secondary antibody goat anti-rabbit immunoglobulin G (H+L) – HRP (Thermo Scientific) was applied after standard blotting procedures were performed(4). For comparison of USA300 and clpP deletion strains, the total protein concentration of each sample was 250 μg/ml and 2.5 μl was added to each well. For 30-fold concentrated lanes, samples were concentrated with 10 kDa concentrator for 5-fold. Total protein concentration was around 1.25 mg/ml and 15 μlwas added to each well.

Adherence assay and invasion assay (5). Overnight bacterial culture with or without ampicillin and/or compound treatment were washed 3 times with PBS (pH 7.4) and then diluted to 10^7 CFU/ml with MEM medium before inoculation

(defined as the original bacterial CFU). A549 cells were seeded onto a 24-well tissue culture plate (Greiner) at a concentration of 2×10^5/ml in MEM for counting bacterial adherence and invasion ratio. Briefly, A549 cells were grown overnight at 37°C in 5% CO_2 to form confluent monolayers. The medium was removed in the following morning and A549 cells were washed twice with 1 ml of PBS, followed by infection with 1 ml of the prepared bacterial inoculum. For invasion assay, after infection of A549 cells at 37°C for 2 h, the supernatants from the wells were collected for total bacterial count (defined as the total bacterial CFU). A549 cells were then washed twice with PBS followed by incubation with MEM containing gentamicin (100 μg/ml; Sigma) and lysostaphin (10 μg/ml; Sigma) for 1 h at 37°C; all wells were then washed 3 times with 1 ml PBS. Subsequently, wells were trypsinized with 150 μl of 0.25% trypsin-EDTA for 5 min, the cells in each well were carefully collected into tubes, and then 400 μl of ice-cold 0.025% Triton X-100 was added to the tubes and put on ice. The numbers of bacterial CFU released from the lysed epithelial cells were determined by plating lysates on BHI agar plates (defined as the invaded bacterial CFU). For adherence assay, after infection of A549 cells at 37°C for 1 h, the medium was removed and A549 cells washed 3 times with 1 ml PBS. Subsequently, the total number of adhered and/or invaded bacteria that were released from the lysed epithelial cells was defined as the adhered bacterial CFU.

$$\text{Relative invasion} = \frac{\text{Internalized bacteria CFU of sample/Total CFU of sample}}{\text{Internalized bacteria CFU of control/Total CFU of control}}$$

The bacterial adhesion in each well was determined as the CFU that adhered to and invaded into the cells and is expressed as a percentage of the CFU in the inoculum. The controls were wells pretreated with medium alone (MEM) considered to have 100% adhesion. Adhesion and invasion were then normalized against controls according to the equations.

$$\text{Relative adherence} = \frac{\text{Adhered \& Internalized bacteria CFU of sample /Original CFU of sample}}{\text{Adhered \& Internalized bacteria CFU of control /Original CFU of control}}$$

Each experiment was repeated three times, and all of the relative adhesion and invasion values were calculated and statistically analyzed by Student's *t*-test, using SigmaPlot software 11.0. *P* values of <0.05 were considered significant (3).

Cytotoxicity assay. The cytotoxicity of compound M21 in Vero and Hep2 cells was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay according to manufacturer's instructions and performed according to our previous study(1). The highest concentration of M21 used was 500 μM due to solubility limitations. SigmaPlot 11.0 (Systat Software) was used for graph plotting. Experiments were carried out in triplicate and repeated twice for confirmation.

Construction of ClpP and ClpX expression vectors. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (NEB). Accuprime polymerase was purchased from Thermo Scientific and used in accordance with the manufacturer's instructions. Full-length *clpP* was amplified by PCR from *S. aureus* genomic DNA using the primers *clpP*-O-f and *clpP*-O-r. The complete coding sequence of *clpP* was cloned into the *Nco*I and *Xho*I sites of the vector pET28a.

clpX was amplified by PCR from *S. aureus* genomic DNA using the primers *clpX*-O-f and *clpX*-O-r. The complete coding sequence of *clpP* was cloned into the *Nco*I and *Xho*I sites of pET28a.

Expression and purification ClpP and ClpX. *E. coli* Rosetta (DE3) cells that harbored ClpP or ClpX expression vectors were grown at 37°C with shaking at 220 rpm in LB media supplemented with 50 μg/ml kanamycin to an OD_{600} value of 0.6. Protein expression was induced with 0.5-mM IPTG at 20°C overnight. The cells were harvested by centrifugation and stored at −80°C until use. The cell pellet was suspended in lysis buffer (40-mM Tris pH 8.5, 20-mM imidazole, 5-mM EDTA) and lysed at 4°C by sonication. After centrifugation at 18,000 g for 40 min to remove the insoluble materials, the supernatant was loaded onto a 5-ml His-

trap column (GE Healthcare), and the protein was eluted with a linear gradient of 50 to 400 mM imidazole. The protein was desalted with a PD-10 column equilibrated with 100 mM HEPES pH 7.0 and 100 mM NaCl for enzyme activity testing and DSF measurements.

ClpP peptidase assay. The peptidase activity of ClpP was measured by fluorescence-based substrate cleavage assay. In a typical experiment, 10 µl of diluted ClpP solution (0.25 mg/ml) was added to 40 µl of Suc-LeuTyr-AMC (Suc-LY-AMC) (Chemsky) concentrations ranging from 25 to 400 µM in ClpP buffer (100-mM HEPES pH 7.0, 100-mM NaCl) in black, 96-well, flat-bottom plates (Thermo Scientific). To optimize the concentration of protein in this assay, 10-µl serial dilutions of protein, starting from 25 µM, were added to 40 µl of Suc-LY-AMC (100 µM in ClpP buffer). Fluorescence was measured with a DTX multimode plate reader at 32°C (excitation, 360 nm; emission, 465 nm).

ClpP peptidase activity was determined using a fluorescence-based assay with Suc-LY-AMC as the substrate with 1-µM ClpP, as described previously. A signal less than 5% of the respective DMSO control was considered full inhibition. ClpP (10 µM) was incubated with different inhibitors (100 µM) for 15 min at 32°C. The samples were then diluted into ClpP buffer containing 100-µM Suc-LY-AMC, and the activity was recorded as detailed above.

Non-competitive inhibition was conducted by using ClpP (20 µM) with different concentrations of M21. The same volume of DMSO was added to the control group. After incubation at room temperature for 1 h, the serially diluted substrate (500, 400, 300, 200, 100, 75, 50, and 25 µM) was added into each well of the 96-well plate. Fluorescence was measured using a DTX multimode plate reader at 32°C (excitation, 360 nm; emission, 465 nm) for 30 min.

DSF. The thermal shift assay was conducted as previously described(6). The fluorescent dye SYPRO orange (Sigma-Aldrich), an environmentally sensitive fluorophore, was used to monitor the unfolding of ClpP. This assay was performed using the Vii 7 Real Time PCR Detection System (Bio-Rad).

All of the components were diluted in DSF buffer. Solutions of ClpP (final concentration, 2 μM), 5X SYPRO orange, serial dilution of M21 (DMSO<1%), and DSF buffer (150-mM NaCl and 10-mM HEPES [pH 7.5]) to 50 μl were added to the wells of a 96-well, 0.2-ml optical PCR plate. The plate was heated from 25°C to 90°C at a rate of 1°C/min. The fluorescence intensity was measured with Ex/Em: 490 nm/530 nm. The data were processed as previously described (6).

ClpP proteolytic assay. To further investigate and compare the proteolytic activity of the ClpP proteins, we conducted protein degradation assays using β-casein as a substrate according to previously published protocols(7). Briefly, assays were typically conducted by pre-incubation of a degradation mixture consisting of 4-μM ClpP, DMSO, or compound in buffer (25-mM HEPES, 5-mM $MgCl_2$, 5-mM KCl, 0.03% Tween-20, and 10% glycerol) at room temperature for 1 h. A mixture with 4-μM ClpX protein, 1.5 units of creatine kinase, 16-mM creatine phosphate, 3-mM ATP, and 4.5-μM β-casein was then added to the reaction and incubated at 37°C. Samples were taken at different time points, and the reaction was stopped by the addition of 4× Laemmli buffer and boiling. The proteins were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Construction of *S. aureus clpP* deletion mutant. Plasmid pKOR1-*clpP* was transformed into *S. aureus* USA300. The resulting transformant was used to delete *clpP* in *S. aureus* via a previously reported procedure (8). For mutation in *S. aureus*, the site mutation of A118G and T146A of *clpP* in chromosome was done in a manner similar to the introduction of *clpP* deletion. We transformed plasmids pKOR1-*clpP*-A118G or pKOR1-*clpP*-T146A into USA300-Δ*clpP*.

Urease activity. Bacteria were inoculated into urease agar base (Oxoid) containing the indicator phenol red. The indicator turns red when urease catalyzes the hydrolysis of urea to form two molecules of ammonia. Different compounds were added and incubated at 37°C for 1 day.

Molecular modeling. For molecular docking simulations, the ClpP structure from *S. aureus* (Protein Data Bank [PDB] entry 3v5e) was used. For preparation of the

compounds and ClpP protein structures for docking, the three-dimensional structure of M21 was obtained with wincoot 0.8.1. Polar hydrogen atoms and Gasteiger charges were then added using autodock tools. Docking experiments with compound M21 were performed using a grid box covering the entire tetradecamer consisting of ClpP chains A to N with a grid size of 102 × 98 by 100 points and 1.0-Å resolution using Autodock vina software(9). For flexible docking, flexible sidechains included amino acids 116 to 122, 135 to 148, and 170 to 177. After docking, the conformation of the compound was analyzed, and the (H bond and hydrophobic) interactions and three-dimensional models were generated with UCSF Chimera(10), LIGPLOT(11), or PyMol (Schrodinger LLC).

Mutagenesis. Thirty sets of primers were used to amplify the entire plasmid pET28a-*clpP* for generation of site-specific mutations. The resulting PCR products were extracted with Purelink PCR purification kits and transformed into Rosetta (DE3) competent cells. The mutated sequences of the *clpP* gene were verified and confirmed by DNA sequencing (BGI). Variant ClpP proteins were expressed in *E. coli* for further enzymatic cleavage assays using Suc-LY-AMC as the substrate.

CETSA. Rosetta (DE3) cells that harbored pET28a-clpP or the mutated plasmid were cultured with the same methods for protein expression. After bacteria harvest, sonication, and centrifugation, the total protein concentration was determined, and the samples were normalized to the same amount of total protein. The samples were then treated with 400-μM M21 or the same amount of DMSO. The samples were aliquoted into PCR tubes and heated at the designated temperatures ranging from 37°C to 90°C for 3 min in a 96-well thermal cycler. The tubes were cooled immediately on ice for another 3 min after heating. The samples were vortexed gently and centrifuged at 16,000 *g* for 10 min to obtain the supernatant. All samples were subjected to western blot analysis for detection and quantification of ClpP content. Diluted ClpP polyclonal

antibody serum (1:1000) and the secondary antibody goat anti-mouse immunoglobulin G (H+L) – HRP (Thermo Scientific) were applied after the standard blotting procedures were performed.

Mice infection models. *S. aureus* strain USA300 were cultured to the early exponential phase of growth, washed twice with sterile phosphate-buffered saline solution (PBS), and resuspended in PBS at 5×10^6 CFU/100 µl for bacteremia model and 5×10^7 CFU/100 µl for lethal infection model.
The female Balb/c mice, 6-8weeks, were infected intravenously (i.v.) through the tail vein with *S. aureus* and randomized into three groups (7 to 8 mice per group). Another group with 7 mice received the same number of *S. aureus* USA300-Δ*clpP* as control. One hour after infection, mice received intraperitoneal injections of either the designated concentrations of compound M21 (0.076 mg or 0.038 mg per mouse per dose) or an injection of buffer (PBS with 5% DMSO and 2% Tween-80) as the control. For delayed treatment, the same treatment regimen started 3 days after infection. The M21 and injection buffer treatments were performed twice per day at 12-h intervals. On day 7, 7 to 8 animals from each group were euthanized. Their kidneys were harvested, homogenized in PBS, and plated on BHIA to obtain bacterial counts.

Histology. Kidney samples from i.v. infection lethal model were stored in 10% formalin for 48h and then rinsed in 70% ethanol. Tissues were embedded in paraffin, thin-sectioned, stained with hematoxylin and eosin (H&E) or Gram stain and examined by microscopy.

Pharmacokinetic study. Sample collection for testing and calibration curve construction were done according to methods described by Zhou and colleagues (12). Briefly, 0.38 mg of M21 in injection buffer was applied to mice by i.p. injection. After injection, blood from mice were collected with heparin coated tubes at different time points, including 10 min, 20 min, 30 min, 45 min, 1 h, 1.5 h, 2 h and 4 h.

For Sample preparation, we used M21's analog ((5-{4-[(4-chlorobenzyl)oxy]benzylidene}-4-oxo-2-thioxo-1,3-thiazolidin-3-yl)acetic acid) with chloride on benzyl group as internal control. 10 µl internal control, 150 µl plasma and 600 µl of acetylnitrile (CAN) were mixed, centrifuged, dried, and dissolved in ACN for MS analysis

For UPLC-ESI-Q-TOF-MS analysis, the plasma samples was analyzed using an Acquity UPLC system coupled to a Synapt G2-HDMS mass spectrometer system (Waters Corp., MA, USA). The chromatography was performed on a Waters ACQUITY BEH C18 column (1.7µm, 2.1×100 mm, I.D., 1.7 mm, Waters, Milford, MA, USA). The mobile phase consisted of (A) 0.1% acetic acid in water and (B) acetonitrile. The UPLC gradient program was applied as follows: 40% B to 90%B (0 to 5 min), 90 to 100% B (5 to 5.1 min), 100% B (5.1 to 7.5 min), 100 to 40% B (7.5 to 7.6 min), 40% B (7.6 to 10 min). The column and autosampler temperature were maintained at 45°C and 10°C, respectively. The injection volume was 5 µl.
The mass spectral data were acquired in negative mode and MRM method was used to perform quantify the M21 concentration in plasma. The capillary voltage, sampling cone voltage and source offset were maintained at 2.5 kV, 60 V, and 60 V, respectively. The Nitrogen was used as desolvation gas at a flow rate of 800 L/h. The source and desolvation temperatures were maintained at 120 °C and 400 °C, respectively.

Table S1: M21 serum concentration by i.p. injection after 30 min

Dose/kg	Dose	Serum concentrations
19.25 mg/kg	0.38mg	175.7±24.9 μM
3.85 mg/kg	0.076mg	25.8±6.26 μM
1.925 mg/kg	0.038mg	8.51±1.0 μM

Table S2: Small molecule screening data

Category	Parameter	Description
Assay	Type of assay	Phenotypic, whole-cell screen
	Target	*S. aureus* USA300-pGLhla
	Primary measurement	Detection of bioluminescence and absorbance
	Assay protocol	
Library	Library size	50,240
	Library composition	Compounds synthesized using diversity-oriented synthesis strategy
	Source	ChemBridge Corporation
Screen	Format	384-well plates
	Concentration(s) tested	20 μg/ml
	Plate controls	DMSO (negative control), USA300-pGL (positive control)
	Reagent/ compound dispensing system	Beckman coulter HTS system
	Detection instrument and software	DTX880 (Molecular Devices), Multimode detection software 2.0
	Assay validation/QC	Plate corrected individual Z-scores
	Correction factors	
	Normalization	Normalized triplicated data with median and normalized one plate with positive and negative controls
	Additional comments	
Post-HTS analysis	Hit criteria	Growth inhibition <0.7, decreased luminescence> 65%
	Hit rate	1.9%
	Additional assay(s)	Hits confirmed using secondary screening
	Confirmation of hit purity and structure	Re-synthesis of the hit compound; characterization by proton and carbon NMR and high resolution mass spectrometry
	Additional comments	

Table S3: Strains used in this study

Strain	Phenotype	Source
USA300 FPR 3757	MRSA, Agr+	ATCC ABB1776
Mu3	VISA	ATCC 700698
Top10	*E. coli*	Thermo Scientific
Rosetta (DE3)	*E. coli*	Lab source
Rosetta-pET28a – *clpP*	For expressing ClpP	This study
Rosetta-pET28a – *clpX*	For expressing ClpX	This study
USA300-Δ*clpP*	USA300 with *clpP* replaced by kanR	This study
USA300-ClpP (A118G)	USA300 with site mutation on ClpP A118G	This study
USA300-ClpP (T146A)	USA300 with site mutation on ClpP T146A	This study
USA300-pGL	USA300 harbouring plasmid pGL	(13)
USA300-pGLspa	USA300 harbouring plasmid pGLspa	(13)
USA300-pGLhla	USA300 harbouring plasmid pGLhla	(13)
USA300-pGLsaeP1	USA300 harbouring plasmid pGLsaeP1	(13)
USA300-pGLsaeP3	USA300 harbouring plasmid pGLsaeP3	(13)
USA300-pGLagrP2	USA300 harbouring plasmid pGLagrP2	(13)
USA300-pGLagrP3	USA300 harbouring plasmid pGLagrP3	(13)
USA300-pGLpvl	USA300 harbouring plasmid pGLpvl	(13)
USA300-pGLeap	USA300 harbouring plasmid pGLeap	(13)
USA300-pGLpsm	USA300 harbouring plasmid pGLpsm	(13)
USA300-pGLfnbA	USA300 harbouring plasmid pGLfnbA	(13)
USA300-pGLsrtA	USA300 harbouring plasmid pGLsrtA	(13)
USA300-pGLclfA	USA300 harbouring plasmid pGLclfA	(13)
COL-pGLcap5	COL harbouring plasmid pGLcap5	(13)
AE052-pGLcap8	AE052 harbouring plasmid pGLcap8	(13)

Table S4: Plasmids used in this study

Plasmid	Feature	Reference
pET28a(+)		Lab stock
pET28a-*clpP*	For expressing ClpP protein	This study
pET28a-*clpX*	For expressing ClpX protein	This study
pKOR1	Shutter plasmid in *E. coli* and *S. aureus* for gene deletion	(8)
pKOR1-*clpP*	For deletion of *clpP* gene pKOR1-clpP:kan	This study
pKOR1-*clpP*-A118G	For replacement of *kan* gene back to ClpP-A118G	This study
pKOR1-*clpP*-T146A	For replacement of *kan* gene back to ClpP-T146A	This study
pOS1hrtAB	Shutter plasmid in *E. coli* and *S. aureus* for gene expression	(14)
pOS1hrtAB-*clpP*	For overexpression of ClpP in *S. aureus*	This study

Table S5: Primers used in this study

Gene/promoter[a]	Primer for promoter cloning	Enzyme site
rt-*hla*-f	AAAAAACTGCTAGTTATTAGAACGAAAGG	
rt-*hla*-r	GGCCAGGCTAAACCACTTTTG	
rt-*spa*-f	CAGCAAACCATGCAGATGCTA	
rt-*spa*-r	GCTAATGATAATCCACCAAATACAGTTG	
rt-*agrB*-f	GCCCATTCCTGTGCGACTTA	
rt-*agrB*-r	GGGCAAATGGCTCTTTGATG	
rt-*lukS*-f	GAGGTGGCCTTTCCAATACAAT	
rt-*lukS*-r	CCTCCTGTTGATGGACCACTATTA	
rt-*psm*-f	TATCAAAAGCTTAATCGAACAATTC	
rt-*psm*-r	CCCCTTCAAATAAGATGTTCATATC	
rt-*srtA*-f	AAGTGTTACGGACAAT	
rt-*srtA*-r	CATTACTTTAGATTGCAT	
clpP-O-f	GTGTACAccatggggAATTTAATTCCTACAGTTATTG	*Nco* I
clpP-O-r	GTAGGCACTCGAGTTTTGTTTCAGGTACCATCACT	*Xho* I
clpX-O-f	GTGTACAccatggggTTTAAATTCAATGAAGATGAAG	*Nco* I
clpX-O-r	GTAGGCACTCGAGAGCTGATGTTTTACTATTATTA	*Xho* I
ClpP-m-f	CTTTGTACAAAAAAGCAGGCTGATAGGTGGCTATCAAGCG	
ClpP-m-r	CTTTGTACAAGAAAGCTGGGTCGAGTCAGCTAGTGGTCCT	
ClpP-u-r	GCTAGCGTACTGCAGGGAGTCGACCCAAAAGTAGATTTAACTTAGTG	*KpnI*
ClpP-d-f	GTCGACTCCCTGCAGTACGCTAGCTATCTATGAAATGGTTATCACG	*KpnI*
Kan-f	CGCCGGTACCATGATTGAACAAGATGGATTGC	*KpnI*
Kan-r	CGCCGGTACCTAATAATTCAGAAGAACTCGTC	*KpnI*

P116A-f	GTTTCGCGTTAGCAAATGCAGAAGTAATGA
N117A-f	GCGTTACCAGCTGCAGAAGTAATGATTCAC
A118G-f	GTTACCAAATGGAGAAGTAATGATTCACCA
E119A-f	ACCAAATGCAGCAGTAATGATTCACCAACC
V120A-f	AAATGCAGAAGCAATGATTCACCAACCATT
M121A-f	TGCAGAAGTAGCGATTCACCAACCATTAGG
I122A-f	AGAAGTAATGGCTCACCAACCATTAGGTGG
H123A-f	AGAAGTAATGATTGCCCAACCATTAGGTGG
E135A-f	CAAGCAACTGCAATCGAAATTGCTGCAAAT
I136A-f	GCAACTGAAGCCGAAATTGCTGCAAATCAC
E137A-f	ACTGAAATCGCAATTGCTGCAAATCACATT
I138A-f	GAAATCGAAGCTGCTGCAAATCACATTTTA
A139G-f	ATCGAAATTGGTGCAAATCACATTTTAAAA
A140G-f	GAAATTGCTGGAAATCACATTTTAAAAACA
N141A-f	ATTGCTGCAGCTCACATTTTAAAAACACGT
H142A-f	GCTGCAAATGCCATTTTAAAAACACGTGAA
I143a-f	GCAAATCACGCTTTAAAAACACGTGAAAAA
L144A-f	AATCACATTGCAAAAACACGTGAAAAATTA
K145A-f	CACATTTTAGCAACACGTGAAAAATTAAAC
T146A-f	ATTTTAAAAGCACGTGAAAAATTAAACCGC
R147A-f	TTAAAAACAGCTGAAAAATTAAACCGCATT
E148A-f	AAAACACGTGCAAAATTAAACCGCATTTTA
D170A-f	AAAGACACAGCTCGTGATAACTTCTTAACT
R171A-f	GACACAGATGCTGATAACTTCTTAACTGCA

D172A-f	ACAGATCGTGCTAACTTCTTAACTGCAGAA
N173A-f	GATCGTGATGCCTTCTTAACTGCAGAAGAA
F174A-f	CGTGATAACGCCTTAACTGCAGAAGAAGCT
L175A-f	GATAACTTCGCAACTGCAGAAGAAGCTAAA
T176A-f	AACTTCTTAGCTGCAGAAGAAGCTAAAGAA
A177G-f	TTCTTAACTGGAGAAGAAGCTAAAGAATAT
P116A-r	TGCATTTGCTAACGCGAAACGTTTACCTTT
N117A-r	TACTTCTGCAGCTGGTAACGCGAAACGTTT
A118G-r	CATTACTTCTCCATTTGGTAACGCGAAACG
E119A-r	AATCATTACTGCTGCATTTGGTAACGCGAA
V120A-r	GTGAATCATTGCTTCTGCATTTGGTAACGC
M121A-r	TTGGTGAATCGCTACTTCTGCATTTGGTAA
I122A-r	TGGTTGGTGAGCCATTACTTCTGCATTTGG
H123A-r	TGGTTGGGCAATCATTACTTCTGCATTTGG
E135A-r	AATTTCGATTGCAGTTGCTTGTCCTTGAGC
I136A-r	AGCAATTTCGGCTTCAGTTGCTTGTCCTTG
E137A-r	TGCAGCAATTGCGATTTCAGTTGCTTGTCC
I138A-r	ATTTGCAGCAGCTTCGATTTCAGTTGCTTG
A139G-r	GTGATTTGCACCAATTTCGATTTCAGTTGC
A140G-r	AATGTGATTTCCAGCAATTTCGATTTCAGT
N141A-r	TAAAATGTGAGCTGCAGCAATTTCGATTTC
H142A-r	TTTTAAAATGGCATTTGCAGCAATTTCGAT
I143a-r	TGTTTTTAAAGCGTGATTTGCAGCAATTTC
L144A-r	ACGTGTTTTTGCAATGTGATTTGCAGCAAT

K145A-r	TTCACGTGTTGCTAAAATGTGATTTGCAGC
T146A-r	TTTTTCACGTGCTTTTAAAATGTGATTTGC
R147A-r	TAATTTTTCAGCTGTTTTTAAAATGTGATT
E148A-r	GTTTAATTTTGCACGTGTTTTTAAAATGTG
D170A-r	GTTATCACGAGCTGTGTCTTTTTGTATTTT
R171A-r	GAAGTTATCAGCATCTGTGTCTTTTTGTAT
D172A-r	TAAGAAGTTAGCACGATCTGTGTCTTTTTG
N173A-r	AGTTAAGAAGGCATCACGATCTGTGTCTTT
F174A-r	TGCAGTTAAGGCGTTATCACGATCTGTGTC
L175A-r	TTCTGCAGTTGCGAAGTTATCACGATCTGT
T176A-r	TTCTTCTGCAGCTAAGAAGTTATCACGATC
A177G-r	AGCTTCTTCTCCAGTTAAGAAGTTATCACG

a, rt refers to RT PCR primers.

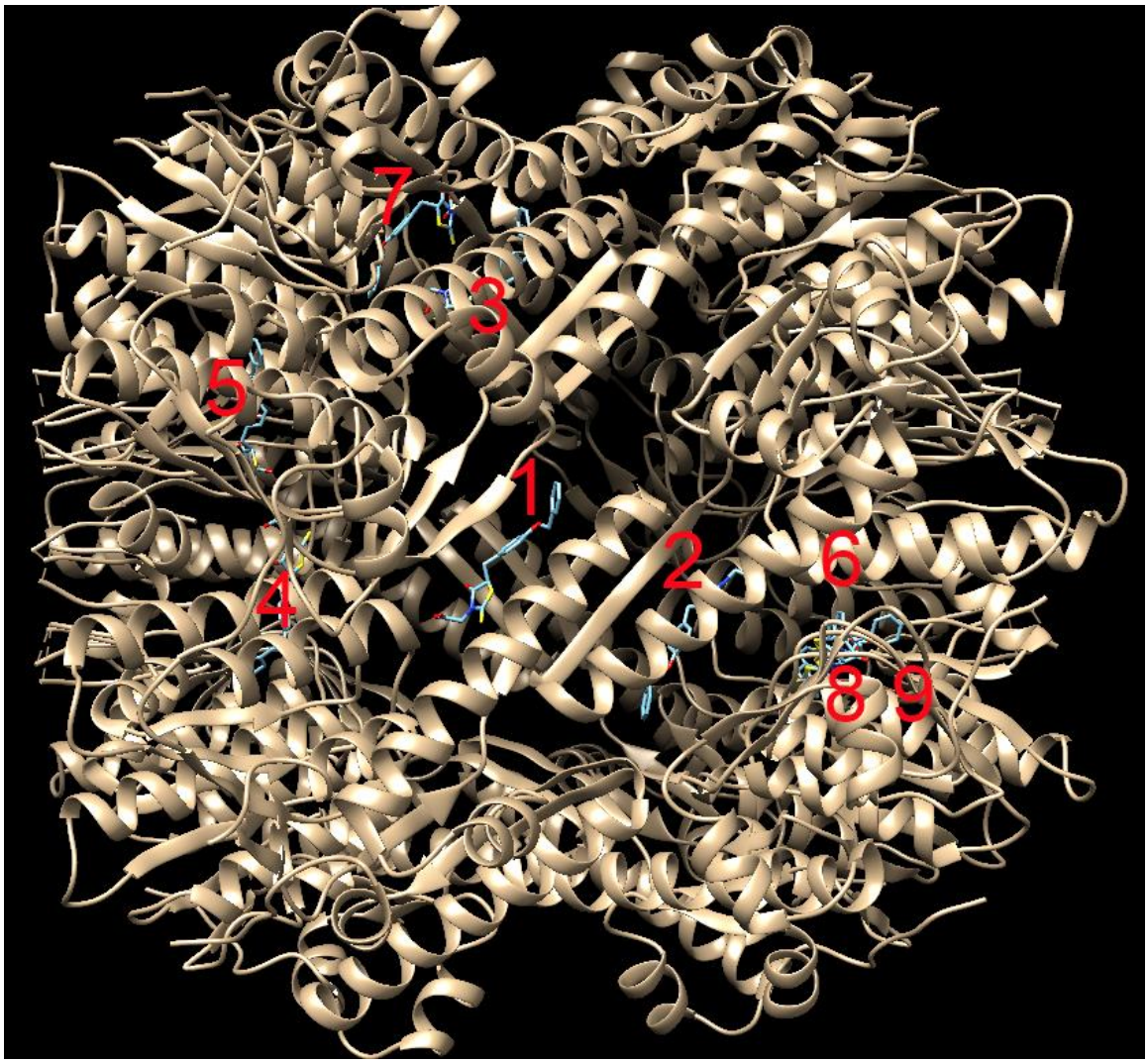

Figure S1 Molecular docking of M21 to ClpP with Autodock vina. A grid box covering the whole ClpP tetradecamer was applied as binding pocket and 9 best conformations of M21 are labeled with numbers based on the Autodock score. No. 1 with the highest score. Due to symmetry of ClpP, No. 1, 2 and 3 are located in the similar position; No 6, 8 and 9 are in the similar position; No 4 and 5 are in similar position.

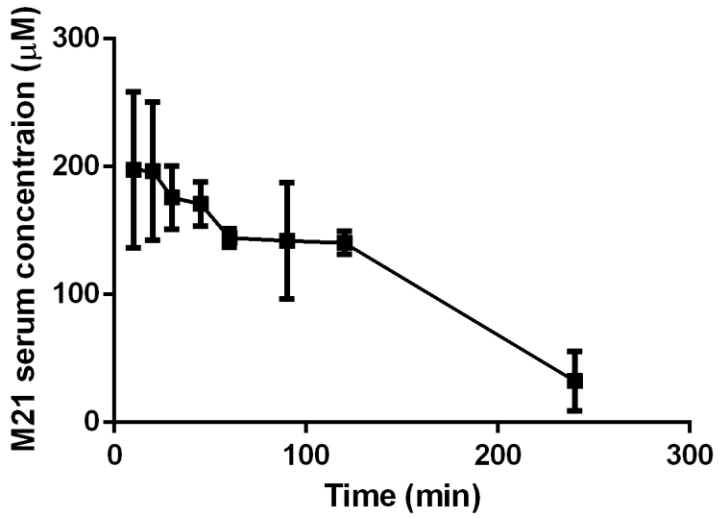

Figure S2 M21 serum concentrations. Plasma M21 concentrations after administration of single intraperitoneal doses of 19.25 mg/kg to balb/c mice (6-8w). Each symbol represents the mean ± standard levels in the plasma of six mice.

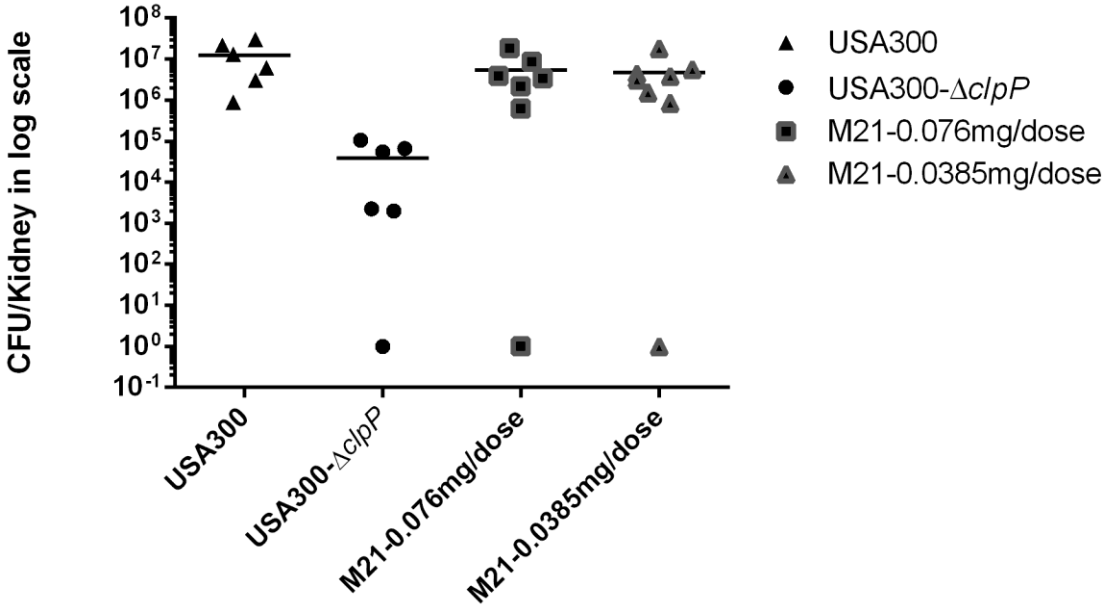

Figure S3 M21 failed in delayed treatment 3 days after infections. After intravascular infection of *S. aureus* USA300, 3 days after infection, mice were given treatment with vehicle or M21 at different dosages. 7 days later, mice were sacrificed and kidneys were collected for bacterial load analysis. All data represent mean values.

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