Supporting Information

Richter et al. 10.1073/pnas.1217337110

SI Experimental Procedures

High-Throughput Screen. The antibiotic-sensitive strain *Staphylococcus aureus* RN4220 was used to screen for small-molecule growth inhibitors in a high-throughput screening assay using 384-well microplates. Cation-adjusted Mueller Hinton II broth supplemented with 0.005% Tween-80 was inoculated with a single colony of *S. aureus* RN4220. Following overnight incubation, the culture was diluted 1:100 into fresh medium, and incubation was continued until the bacterial suspension reached an optical density at 600 nm (OD_{600}) of 0.6, corresponding to a bacterial density of 5×10^8 colony forming units (CFU)/mL. An aliquot of this culture was diluted 1:400 with fresh medium and stored on ice until it could be dispensed in 384-well plates. Small-scale experiments were conducted to determine the Z′-factor $\{1 - [(3SD^-) + (3SD^+)/(Ave^-) - (Ave^+)]\}$ used commonly for quality assessment of raw data sets generated in high-throughput screening. In this formula, $(SD^{+/-})$ and $(Ave^{+/-})$ represent SD and average of positive and negative controls. Four 384-well plates were loaded with 25 μL medium per well. Half of the wells were supplemented with 4 nmol chloramphenicol each (positive controls), whereas the remaining wells were not (negative controls). Each well was inoculated with a 25-μL aliquot of the culture kept on ice ($\sim 3.125 \times 10^4$ CFU). The OD_{600} in each well was recorded after a 20-h incubation. Z′ values varied between 0.72 and 0.84. For the high-throughput screen (HTS) experiment, microplates were preloaded with medium and then processed by a Seiko pin-transfer robot with a Caliper Twister II robotic arm. The robot was programmed to add 2–3 nmol chemical library compound solubilized in 0.3 μL DMSO to each assay well. In additon to assay wells, every microplate included 16 positive and 16 negative control wells supplemented or not with 4 nmol chloramphenicol, respectively. Aliquots (25 μL) of the starter culture kept on ice were added to both assay and control wells and microplates were incubated at 42 °C in humidified chambers (humidity > 85%). After 20 h, the OD_{600} was measured using a plate reader in absorbance mode. The screen was carried out in duplicate. Compounds that reduced bacterial growth by 90% or more were defined as screen positives and retested once for validation using the HTS protocol. Small molecule libraries from different vendors (Asinex, ChemBridge, ChemDiv, Enamine, LifeChemicals, Maybridge, TimTec) were tested in duplicate at the National Screening Laboratory for Regional Centers of Excellence in Biodefense and Emerging Infectious Disease (NSRB) at Harvard Medical School (Boston, MA).

The screen included a total of 167,405 compounds and yielded 595 screen positives corresponding to a primary hit rate of 0.36% (Table S1). Hits were subjected to computational analysis (Molinspiration: www.molinspiration.com) of molecular properties (i.e., molecular weight, polar surface area, number of hydrogen bond donors and acceptors, and bond rotation) to determine violations of Lipinski's rule-of-five (1–3). Compounds displaying drug-like properties were submitted to the NSRB informatics group for analysis of promiscuous inhibitory activity. This analysis extracted 130 compounds from the original 595 hits. These 130 compounds were subjected to a validation experiment whereby growth inhibition was tested once more using a 384-well plate format. Seventy-three compounds displayed inhibition greater than 90%. Thirty-one compounds of this set were commercially available and purchased for secondary screening from the manufacturers of the respective libraries (ChemBridge, ChemDiv, Enamine, Maybridge, TimTec). A 98.9% (wt/wt) pure preparation of 2-oxo-2-(5-phenyl-1,3,4-oxadiazol-2-ylamino)ethyl 2-naphtho[2,1-b]furan-1-ylacetate (screen hit 1771) was obtained from Enamine (catalog no. T5526252).

Growth Inhibition Assay. Bacterial cultures were grown at 37 °C to an OD_{600} of 0.6 and diluted 100-fold with ice-cold medium. The growth medium was brain heart infusion (BHI) broth for staphylococci, enterococci, and bacilli. *Clostridium perfringens* was grown in BHI supplemented with 0.5% yeast extract and 0.1% L-cysteine in a nitrogen atmosphere. *Escherichia coli* BL21 was cultured in cation-adjusted Mueller-Hinton II medium supplemented with 0.005% Tween-80. Growth inhibition was carried out in triplicate using 96-well microplates. Assay plates were preloaded with 100-μL aliquots of twofold dilution series of compound prepared in growth medium, and 10 μL of ice-cold starter culture was added to every assay well. The plates were incubated at 37 °C for 18–22 h, and the density of cultures was recorded with a plate reader. Normalized OD_{600} measurements were used to derive the minimum inhibitory concentration (MIC) for growth inhibition of each compound. The software package GraphPad Prism 5 was used to calculate the 50% growth inhibitory concentration (IC_{50}) values.

Cytotoxicity Assay. Human promyelocytic leukemia HL-60 cells (CCL-240; American Type Culture Collection) were maintained in RPMI 1640 (CellGro) supplemented with 10% (vol/vol) heat-inactivated FBS (HyClone), 2 mM L-glutamine, penicillin (50 U/mL), and streptomycin (50 μg/mL). Cell cultures were grown at 37 °C in 5% CO_2 until cell density reached 10^6 cells/mL. Cells were washed three times and suspended in DMEM (Invitrogen). Microtiter plates (96 wells) were loaded with 10^4 cells per well suspended in 100 μL DMEM containing increasing concentrations of compound. The plates were incubated for 4 h at 37 °C in 5% CO_2. The cytotoxicity of compounds was assessed by measuring the activity of lactate dehydrogenase (LDH) released from cells that were damaged during the incubation. The LDH assay was performed in triplicate using the Cytotoxicity Detection Kit according to the manufacturer's instructions (Roche). LDH measurements were normalized as the percentage of the total LDH activity in a cell lysate.

Inhibition of LTA Synthesis by Small Molecules. An overnight culture of *S. aureus* RN4220 was diluted 100-fold in BHI medium supplemented or not with a subinhibitory concentration of hit compound. *E. coli* strains producing either lipoteichoic acid synthase $(LtaS)_{SA}$ or $LtaS2_{BA}$ for polyglycerol phosphate synthesis were grown as described earlier (4, 5). The cultures were incubated at 37 °C, and bacterial growth was monitored over time until the control culture without compound reached OD_{600} 1.0. Aliquots of 1 mL were removed from each culture and mixed with 0.5-mL glass beads (0.1 mm diameter). Bacteria were lysed in a bead beater, and glass beads were removed by centrifugation (1 min at 200 × *g*). The supernatant was again centrifuged for 10 min at 16,000 × *g* to sediment cell debris containing cell-associated LTA. The pellet was suspended with 0.5 M Tris-HCl (pH 8.0)/2% (wt/vol) SDS buffer in a volume normalized according OD_{600} values. Samples were heat-treated at 95 °C for 30 min and cleared by centrifugation. Supernatants were separated by SDS/PAGE and analyzed by immunoblotting using a monoclonal antibody to detect LTA/polyglycerol-phosphate and polyclonal antibodies for LtaS and sortase A (SrtA) for *S. aureus* extracts. Immune-reactive signals for LTA and LtaS were normalized against the envelope protein SrtA, which was unaffected by LTA synthesis inhibitors.

Electron Microscopy. Bacterial cultures were washed in water before fixation with 2% (vol/vol) glutaraldehyde in PBS. Sample processing was performed as described earlier (5). Examination of specimen was performed with a Fei Nova NanoSEM 200 scanning electron microscope (SEM: FEI). The SEM was operated with an acceleration voltage of 5 kV, and samples were viewed at a distance of 5 mm. Thin sectioning of samples was performed as described (5), and images were recorded using a Tecnai F30 (Philips/FEI) transmission electron microscope (field emission gun operating with a 300-kV accelerating voltage, using a magnification of 15,000–30,000×) and a high-performance CCD camera with a 4 k × 4 k resolution. Images were captured using Gatan DigitalMicrograph software and processed using Adobe Photoshop (Adobe). The thickness of the cell wall envelope was determined by examining at least 15 thin-sectioned cell images captured on micrographs during transmission electron microscopy (5). Data were plotted in Graphpad Prism 5.0, and the Student *t* test (unpaired, two-tailed) was used for statistical analyses.

Light Microscopy. Cells were fixed using 4% (vol/vol) buffered formalin and observed. Images were obtained with a CCD camera on an Olympus IX81 microscope using 100× or 40× objectives. The lengths of bacilli were measured from acquired DIC images with ImageJ and converted to lengths in micrometers using reference images with an objective micrometer. Data were displayed in a box and whisker plot. The Student *t* test (unpaired, two-tailed) was used for statistical analyses.

Biochemical Assays Using Recombinant eLtaS. Purified recombinant extracellular catalytic domain of LtaS (eLtaS) and SrtA were used to examine protein interactions with a synthetic phosphatidylglycerol modified with nitro-benzoxadiazole (NBD-PG) (6, 7). For this study, we used 1-palmitoyl-2-{12-[7-nitro-2–1,3-benzoxadiazole-4-yl) amino]dodecanoyl]-*sn*-glycerol-3-[phospho-*rac*-(1-glycerol) purchased from Avanti Polar Lipids. eLtaS was expressed from a recombinant insert in vector pProEX (Invitrogen, Life Technologies) as described by Lu et al. (6). SrtA was purified using plasmid pHTT27 as described by Ton-That et al. (8). Both proteins were purified via nickel-nitrilotriacetic acid resin (8). To study the interaction with NBD-PG_{C16}, size-exclusion HPLC was performed with a BioBasic SEC300 column equilibrated in a 50 mM Hepes-KOH buffer (pH 7.5) containing 10 μM $MnCl_2$. Chromatograms were recorded by measuring absorbance at 460 nm to detect the presence of NBD-PG_{C16}. To monitor hydrolytic activity of eLtaS, the less hydrophobic 1-hexanoyl-2-{6-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (Avanti Polar Lipids) containing chains of only six carbon atoms was used as substrate (NBD-PG_{C6}). In a reaction volume of 200 μL, 2 nmol eLtaS and 2 nmol NBD-PG_{C6} were incubated ±20 nmol compound 1771 in 10 mM sodium succinate and 10 mM $MnCl_2$, pH 6.5, at 37 °C for 6 h. A control sample did not contain eLtaS. After incubation, samples were extracted with 440 μL 50% (vol/vol) chloroform and 50% (vol/vol) methanol and centrifuged for phase separation. Aqueous and organic phases were dried and suspended in 100 μL HPLC sample buffer [49.5% hexane, 49.5% isopropanol, 1% acetic acid (vol/vol/vol), supplemented with 0.8 mL/L triethylamine]. Samples were subjected to normal-phase HPLC on an Astec Diol column (SUPELCO). After sample injection, the column was equilibrated for 5 min with mobile phase containing 82% hexane, 17% isopropanol, and 1% acetic acid (vol/vol/vol), supplemented with 0.8 mL/L triethylamine. A 0–100% gradient was applied for 15 min using elution buffer consisting of 85% isopropanol and 1% acetic acid (vol/vol/vol), supplemented with 0.8 mL/L triethylamine. Elution profiles were monitored by fluorescence (excitation at 460 nm, emission at 534 nm), and representative peak fractions were collected for mass spectrometry analysis using a MALDI-TOF instrument (AB SCIEX Voyager-DE PRO) in positive reflectron mode.

Animal Experiments. Inhibitor solution was prepared immediately before injection into animals. Briefly, a frozen stock of compound 1771 was prewarmed at 37 °C for 5 min and suspended into compound buffer [20 mM Hepes-KOH (pH 7.5), 100 mM NaCl]. The clinical isolate *S. aureus* Newman was used for infection because it has been extensively characterized in to study the therapeutic effects of small molecule inhibitors (9). In vitro, *S. aureus* Newman displayed similar sensitivity toward compound 1771 as *S. aureus* USA300. An overnight culture of strain Newman was diluted 1:100 into fresh tryptic soy broth and grown to OD_{600} 0.4. Bacteria were centrifuged at 7,000 × *g*, washed, and suspended in 1/10th volume of PBS. Six-week-old female BALB/c mice ($n = 15$; Charles River) were injected retroorbitally with 1×10^8 CFU suspended in 100 μL PBS. Mice were monitored for survival over 10 d. Animals received either two injections of inhibitor (32 mg/kg) or compound buffer (Mock) before infection and an additional six doses after infection, all separated by 12-h intervals. Animal experiments were performed in accordance with the institutional guidelines following experimental protocol reviewed and approved by the Institutional Biosafety Committee (IBC) and the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago. The log-rank test was performed to analyze the statistical significance by Prism (GraphPad Software), and $P < 0.05$ was deemed significant.

Selecting for *S. aureus* Mutants with Increased Resistance to Compound 1771. Initial experiments sought to isolate antibiotic-resistant variants of *S. aureus* RNA4220 on Luria–Bertani (LB) agar plates with the Kirby-Bauer disk diffusion assay. Staphylococci (2.4×10^7 CFU) were inoculated per plate, which allowed for the isolation of streptomycin-resistant variants (filter disk with 50 μmol streptomycin) but not for the isolation of compound 1771–resistant variants (filter disk with 200 μmol streptomycin). The mutation frequency for compound 1771 resistance was then analyzed with Mueller-Hinton agar plates containing a concentration gradient from 10 to 200 μM compound 1771. Large square plates (225-mm side length) were inoculated with 2×10^9 CFU *S. aureus* RN4220 per plate and incubated at either 37 °C or 42 °C. Small, slow-growing colonies were observed only after 3–4 d of incubation. Three isolates were subjected to susceptibility testing against the compound 1771 inhibitor.

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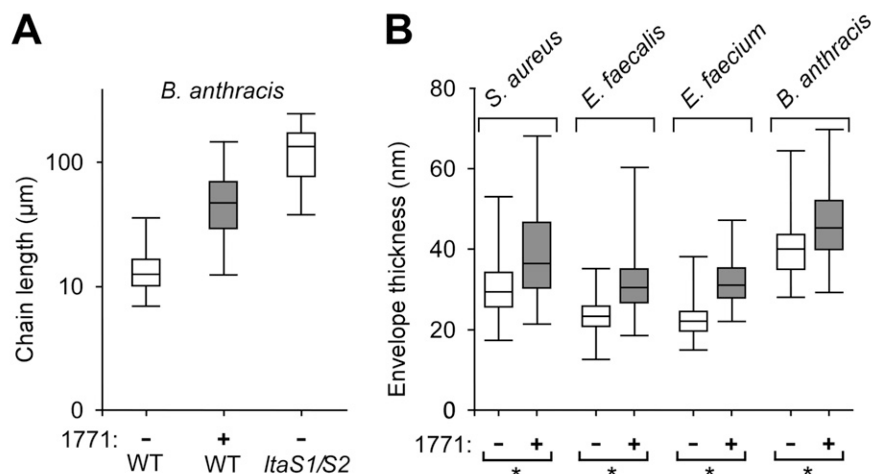

Fig. S1. Quantitative analyses of *Bacillus anthracis* chain length and envelope diameter in cultures with (+) or without (–) compound 1771. (*A*) Light microscopy images of vegetative forms of *B. anthracis* Sterne recovered from liquid cultures incubated without (–) or with 5 μM 1771 (+) were analyzed for chain length and compared with the *ltaS1*/*ltaS2* mutant of *B. anthracis* Sterne. Data are presented as a box and whiskers plot ($n = 100$). Statistical significance was analyzed with the Student *t* test (unpaired, two-tailed). Average chain lengths of *B. anthracis* Sterne without inhibitor [13.9 μm (±0.5)] vs. *B. anthracis* Sterne with the 1771 LTA synthesis inhibitor [51.9 μm (±2.9)] ($P < 0.0001$). Average chain lengths of *B. anthracis* Sterne WT vs. the *ltaS1*/*ltaS2* mutant [128.0 μm (±7.7)] ($P < 0.0001$). (*B*) Thin-sectioned samples of bacterial cultures were analyzed by transmission electron micrographs (see Fig. 2 for an example), and the diameter of the bacterial envelope was quantified. Measurements are presented as box and whiskers plot ($n = 124$). The line inside the box marks the median. Bars outside the box represent maximum and minimum values. *S. aureus, Enterococcus faecalis,* and *Enterococcus faecium* were isolated from colonies grown on BHI agar without (–) or with 30 μM 1771 (+) and *B. anthracis* from colonies grown on BHI agar without (–) or with 8 μM 1771 (+). The unpaired Student *t* test function of Prism 5 software was used to compare the means of cell wall envelope measurements of untreated (–) and compound-treated (+) bacteria. The asterisks (*) denotes $P < 0.0001$.

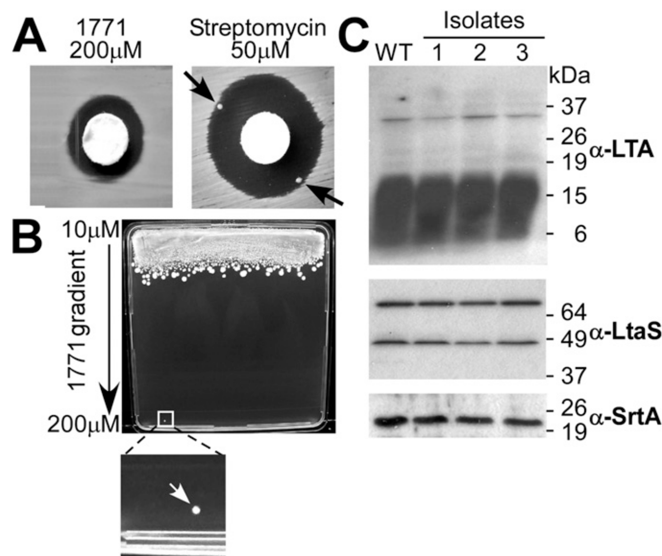

Fig. S2. Selecting for *S. aureus* variants with increased resistance to compound 1771. (*A*) *S. aureus* RN4220 (2.4×10^7 CFU) was spread on LB agar, and filter disks soaked with compound 1771 or streptomycin were placed on the agar surface before incubation for 16 h and photography. Black arrows identify large colonies formed from antibiotic-resistant variants. (*B*) The mutation frequency of *S. aureus* RN4220 was determined on Mueller-Hinton agar plates containing a concentration gradient from 10 to 200 μM compound 1771. Large square plates (225-mm side length) were inoculated with 2.0×10^9 CFU and incubated at either 37 °C or 42 °C (example shown). Small, slow-growing colonies were observed only after 3–4 d incubation (white arrow). Three of these isolates were analyzed for compound 1771 MIC and IC_{50} values (Table S7). (*C*) *S. aureus* RN4220 (WT) and three isolates from compound 1771 gradient plates were grown in LB and lysed in a bead beater, and cell extracts were subjected to SDS/PAGE and immunoblotting using a monoclonal antibody to detect LTA/polyglycerol-phosphate (α-LTA) and rabbit-polyclonal antibodies for LtaS (α-LtaS) and SrtA (α-StrA). The migratory positions of molecular weight markers (in kilodaltons) are indicated. The coding sequences for the *ltaS* gene from *S. aureus* RN4220 (WT) and the three isolates were amplified by PCR and subjected to DNA sequence analysis; mutational changes in the *ltaS* gene were not detected.

Table S1. Summary of HTS for LTA synthesis inhibitors

Screening step	Assay description	Compound count
Primary screen*	Compound library screen: total	167,405
	Screen hits: identified[†]	595
	Compound validation test: total	130
	Screen hits: confirmed[†]	73
Secondary screen	Dose–response analysis: total[‡]	31
	MRSA inhibitor: *no E. coli* inhibitor, not cytotoxic	15
	MRSA inhibitor: *E. coli* inhibitor and/or cytotoxic	7
	Not active	9

MRSA, methicillin-resistant *S. aureus.*

*The primary screen was performed using *S. aureus* strain RN4220.

[†]Growth inhibition of *S. aureus* RN4220 was ≥90% using compound library stock solutions at a final concentration of ~40–60 μM.

[‡]Cytotoxicity of small molecules was measured using HL60 cells.

Table S2. Gram-positive bacteria used in this study and their *ltaS* homologs

Genome*	Accession[†]	Locus tag[‡]	AA[§]	LtaS[¶]
S. aureus RN4220	AFGU01000065	VBIStaAur106174_1432	646	Ib
S. aureus USA300	NC_007793	VBIStaAur129981_0779	646	Ib
E. faecalis V583	NC_004668	VBIEntFae7065_1183	702	IIc
E. faecalis V583	NC_004668	VBIEntFae7065_1703	686	IIb
E. faecium TX0082	NZ_GL455902	VBIEntFae156024_1065	706	IIc
E. faecium TX0082	NZ_GL455938	VBIEntFae156024_1931	697	IIb
C. perfringens SM101	NC_008262	VBICloPer122123_0554	543	IVb
C. perfringens SM101	NC_008262	VBICloPer122123_2172	627	IVb
C. perfringens SM101	NC_008262	VBICloPer122123_0549	602	IVb
B. anthracis Sterne	NC_005945	VBIBacAnt108183_1411	628	IIIa
B. anthracis Sterne	NC_005945	VBIBacAnt108183_2930	657	Ia
B. anthracis Sterne	NC_005945	VBIBacAnt108183_3865	639	IIIb
B. anthracis Sterne	NC_005945	VBIBacAnt108183_5426	642	IIIb
B. anthracis Ames	NC_003997	VBIBacAnt69550_2926	657	Ia
B. anthracis Ames	NC_003997	VBIBacAnt69550_3865	639	IIIb
B. anthracis Ames	NC_003997	VBIBacAnt69550_1410	628	IIIa
B. anthracis Ames	NC_003997	VBIBacAnt69550_5428	642	IIIb
B. cereus G9241	NZ_AAEK01000034	VBIBacCer116370_4313	657	Ia
B. cereus G9241	NZ_AAEK01000009	VBIBacCer116370_1936	628	IIIa
B. cereus G9241	NZ_AAEK01000119	VBIBacCer116370_6093	601	IIIb
B. cereus G9241	NZ_AAEK01000026	VBIBacCer116370_3821	639	IIIb

*Genome sequences of microbes under investigation in this study were analyzed with BLAST searches for homologs of *S. aureus ltaS* using the PATRIC database.

[†]GenBank accession number of the genome sequence.

[‡]Locus tag of the *ltaS* homolog.

[§]Amino acid residues of the predicted gene product.

[¶]LtaS type classification.

Table S3. Inhibitory activity of compound 1771 against Gram-positive pathogens

Microbe	*ltaS* genes	MIC (μM)	IC_{50} (μM)*	IC_{50} (μM) 95% CI*
S. aureus USA300	1	50	14.0	12.6–15.7
E. faecalis V583	2	50	27.3	24.4–30.4
E. faecium TX0016	2	50	23.3	20.1–27.1
C. perfringens SM101	3	6.2	2.5	2.0–3.1
B. anthracis Ames	4	12.5	7.6	6.8–8.5
B. anthracis Sterne	4	12.5	7.7	5.1–11.6
B. cereus G9241	4	25	10.7	Not calculated[†]

*IC_{50} values and 95% CIs were calculated by fitting data with variable slope sigmoidal dose–response function (GraphPad Prism 5).

[†]Hill slope value of dose–response graph is >15.

Table S4. Compound 1771 prevents cell cluster formation in staphylococci and enterococci

Microbe	Cell clusters/all cocci*	
	Mock	Compound 1771
S. aureus RN4220	53/636	3/387
E. faecalis V583	34/390	2/496
E. faecium TX0016	41/255	1/295

*Bacterial growth in the presence of the LTA synthesis inhibitor (compound 1771) or in its absence (mock) was analyzed by scanning electron microscopy to reveal clusters of associated cocci (≥8 cells) compared with the sum of all individual cocci, diplococci, or coccal clusters. Compound 1771 inhibition of *S. aureus* cluster formation was analyzed in multiple independent trials for statistical significance with the unpaired two-tailed Student *t* test ($P = 0.0079$).

Table S5. Treatment of *S. aureus*, *E. faecalis*, *E. faecium*, and *B. anthracis* with compound 1771 increases the diameter of the bacterial cell wall envelope

Microbe	Cell envelope diameter (nm)*			Significance† (*P* value)
	Mock	Compound 1771	Δ	
S. aureus RN4220	31.2 ± 0.6	38.8 ± 0.9	7.5 ± 1.1	<0.0001
E. faecalis V583	23.8 ± 0.4	31.6 ± 0.6	7.8 ± 0.7	<0.0001
E. faecium TX0016	22.9 ± 0.3	32.4 ± 0.5	9.6 ± 0.6	<0.0001
B. anthracis Sterne	40.5 ± 0.6	46.8 ± 0.7	6.3 ± 1.0	<0.0001

*Bacteria were grown overnight on BHI agar in the absence (mock) or presence of the LTA synthesis inhibitor (1771) and were prepared for thin-section transmission electron microscopy to measure the envelope thickness, i.e., the diameter of the cell wall in nm. The mean ± SEM of 124 measurements is indicated. The differential of the diameter (Δ) was calculated by subtracting the mean of the compound 1771–treated bacteria from that of mock-treated controls.

†Data were analyzed with the two-tailed Student *t* test for statistical significance, and *P* values were recorded.

Table S6. Inhibitory activity of compound 1771 and its derivatives

Compound	*S. aureus*			*B. anthracis*		
	MIC (μM)	IC_{50} (μM)	IC_{50} (μM) 95% CI	MIC (μM)	IC_{50} (μM)	IC_{50} (μM) 95% CI
1771	12.5	7.48	6.40–8.74	12.5	6.53	6.37–6.70
1771–1	>400	>400	NA	>400	169.2	112.9–253.8
1771–2	>400	>400	NA	>400	>400	NA
1771–3	>400	>400	NA	>400	>400	NA
1771–4	>400	242.4	165.7–354.7	>400	223.6	142.9–349.8
1771–5	>400	85.6	66.4–110.3	200.0	88.1	75.3–03.0
1771–6	50.0	15.2	12.6–18.3	25.0	14.8	13.1–16.7
1771–7	50.0	27.7	25.2–30.5	50.0	24.1	20.1–29.0

S. aureus RN4220 and *B. anthracis* Sterne IC_{50} values and 95% confidence intervals were calculated by fitting data with variable slope sigmoidal dose–response function (GraphPad Prism 5). Corresponding graphs are shown in Fig. 4. NA, not applicable.

Table S7. Resistance of *S. aureus* RN4220 parent and three colony isolates from gradient plates with 10–200 μM compound 1771 (Fig. S2)

S. aureus strain	MIC (μM)	IC_{50} (μM)	IC_{50} (μM) 95% CI
Parent	12.5	7.3	5.9–9.1
Isolate 1	18.8	8.7	8.3–9.2
Isolate 2	18.8	9.2	8.8–9.6
Isolate 3	18.8	12.2	11.8–12.6

S. aureus RN4220 and three isolates picked from selective agar plates containing compound 1771 were compared for their susceptibility to compound 1771 in growth inhibition experiments. Observed MIC and calculated IC_{50} are presented.