Supporting Information

Reconstitution of *S. aureus* lipoteichoic acid synthase activity identifies Congo red as a selective inhibitor

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General Information

All chemicals used were ordered from Sigma-Aldrich at the highest purity available unless otherwise noted. Compound 1771 was ordered from Enamine (compound number T5526252). aLL *S. aureus* strains were grown in tryptic soy broth and grown at 30°C. All MIC and IC_{50} calculations were performed with Graphpad Prism, and all western blot images were analyzed for signal intensity in ImageJ. Hi-resolution mass spectrometry was performed on a Thermo Fischer Scientific Q Exactive Plus Orbitrap Mass Spectrometer by Sunia Trauger and Jennifer Wang at the Harvard Small Molecule Mass Spectrometry core. NMR data was obtained on a Bruker 400 MHz NMR in deuterated DMSO and processed with TopSpin under the guidance of Charles Sheahan and Anne Rachupka at the HMS East Quad NMR Facility.

In cellulo detection and inhibition of LTA biosynthesis

An overnight culture of *S. aureus* RN4220 was diluted 1:100 into 1 mL tryptic soy broth containing either DMSO or Congo Red in concentrations ranging from 0.125 µg/mL to 16 µg/mL in two-fold increments. DMSO concentrations were normalized to 1% of the total culture volume. Cells were grown to an O.D. 600 of approximately 0.6, pelleted, and analyzed based on a previously described procedure.[1] Cells were resuspended in 2% SDS and 0.5 M Tris pH 8.0 in volumes normalized to O.D. This suspension was boiled at 95°C for 30 minutes and centrifuged at 16,000xg for 10 minutes. The supernatant was mixed with 2x SDS-PAGE loading dye and run on a 4-20% Tris-glycine gel (Bio-Rad). The gel was then transferred onto a PVDF membrane. The membrane was blocked with 5% milk, incubated first with an α-LTA antibody (Hycult), and then an α-mouse-HRP secondary antibody (Cell Signaling Technology), and finally visualized with ECL (Pierce).

Cloning of LtaS

The *ltaS* gene, SAOUHSC_00728, was cloned from *S. aureus* RN4220 genomic DNA using the forward primer LtaS_F and the reverse primer LtaS_R (Table S2). A pET28b vector was cut with NcoI and XhoI (New England Biolabs) and combined with the purified LtaS PCR fragment using the InFusion cloning system (Clontech) to afford a *C*-terminally His_6-tagged enzyme with a short linker of Leucine-Glutamate between the final amino acid of LtaS and the His_6-tag. Assembled fragments were transformed into Stellar cells (Clontech) and colonies were screened by PCR and sequence confirmed by Sanger sequencing. The T300A mutant was made by site-directed mutagenesis using the forward primer LtaST300A_F and the reverse primer LtaST300A_R with pET28b-LtaS as a template (Table S2). The SDM reaction was treated with DpnI and transformed into Stellar cells and confirmed by Sanger sequencing.

Expression and purification of LtaS

KRX cells (Promega) were transformed with pET28b-LtaS-His and grown to an O.D. 600 of 0.8 in terrific broth (Anova). Cells were induced with 1 mM IPTG and 0.05% rhamnose and grown overnight at 18°C shaking at 230 RPM. Cells were pelleted and resuspended in a buffer containing 25 mM HEPES pH 7.5 and 200 mM NaCl. Lysozyme and DNAse were added to concentrations of 1 mg/mL and 0.25 mg/mL, respectively, and stirred at 4°C for 30 minutes. This mixture was passed through an EmulsiFlex-C5 cell disruptor (Avestin) three times and centrifuged for 10 minutes at 5,000xg to remove cell debris. This supernatant was centrifuged at approximately 100,000xg for 50 minutes. The supernatant was removed and the pellet was

homogenized in a Dounce homogenizer in a buffer containing 25 mM HEPES pH 7.5, 200 mM NaCl, and 1% *n*-Dodecyl β-D-maltoside (DDM). The resulting homogenate was diluted to a protein concentration of approximately 5 mg/mL using the same buffer and tumbled at 4°C for 2 hours. This solution was then centrifuged at 100,000xg for 50 minutes. The supernatant was batch-bound with His60 Superflow Ni^{2+} IMAC resin (Clontech) at 4°C for 30 minutes. The resin was then transferred to a plastic column and the flow through was collected. The column was first washed with 10 column volumes of 25 mM HEPES 7.5, 200 mM NaCl, 0.25% DDM, and 10 mM imidazole, then washed with 10 column volumes of 25 mM HEPES 7.5, 200 mM NaCl, 0.1% DDM, and 50 mM imidazole, and finally eluted with 25 mM HEPES 7.5, 200 mM NaCl, 0.05% DDM, and 200 mM imidazole. The elution fraction was concentrated to 2 mL and loaded onto a Superdex 200 Increase 10/300 GL (GE Life Sciences) equilibrated with 25 mM HEPES 7.5, 200 mM NaCl, and 0.05% DDM. Fractions containing full-length LtaS as judged by a gel blotted with an α-His_6-HRP antibody were pooled, concentrated, and exchanged into 50 mM Tris pH 7.9, 0.05% DDM *via* spin filtration. The purified LtaS contained a small portion of eLtaS (although a majority of eLtaS was removed after gel fitration, Figure S1) Accumulation of an LTA polymer, produced while LtaS was expressed in the *E. coli* cells as reported previously,[1] carried over though purification as observed with an α-LTA antibody (Hycult). The purified LtaS was loaded onto 1 mL DEAE-Sepharose (Sigma) equilibrated with 50 mM Tris pH 7.9, 0.05% DDM and washed with 10 column volumes of 50 mM Tris pH 7.9, 0.05% DDM. LtaS was eluted with 50 mM Tris pH 7.9, 0.05% DDM, and 200 mM NaCl (residual eLtaS was eluted during the wash and LTA remained on the column until concentrations of NaCl of 300 mM were used, Figure S2). The resulting LtaS was confirmed to be LTA-free by western blot and concentrated to approximately 100 µM, flash frozen, and stored at -80°C. LtaS-His_6 T300A was purified as described above.

Purification of SpsB

The *S. aureus* SpsB protein was cloned, heterologously expressed in BL21 (DE3), and purified using affinity chromatography. First, the gene for SpsB in *S. aureus* NCTC 8325, SAOUHSC_00903, is misannotated as it begins at Adenine108 of the full-length gene. Use of that downstream methionine as start codon would remove the first 36 amino acids from the protein including the active site Ser36. The full-length gene was amplified from genomic DNA using the forward primer SpsB_F and the reverse primer SpsB_R (Table S2). The product was ligating into NdeI and BamHI digested pET15b using the Infusion kit (Clontech). The resulting plasmid encodes an N-terminal hexahistidine fusion on the translated SpsB protein. The plasmid was transformed into BL21 (DE3).

His_6-SpsB (hereafter termed SpsB) was expressed from 4 L of culture. The expressing culture was grown at 20°C for 40 hrs with 0.5 mM isopropylthiogalactosidase (IPTG) added after 16 hrs of growth. The cell paste was frozen at -80°C before thawing on ice and resuspending in 40 mL bind buffer (0.5 M NaCl, 20 mM Tris, pH 7.9, and 5 mM imidazole) to which 100 µL 10 mg/mL lysozyme and 5 mg DNase were added. The cell suspension was passed through an EmulsiFlex-C5 cell disruptor (Avestin) continuously for 5 min for lysis, and the cell debris was cleared twice at 5,000 x g. The supernatant was further centrifuged at 100,000 x g for 45 min, and the resulting pellet was resuspended in 20 mL of bind buffer with 0.1 % Triton X-100 detergent using a Dounce homogenizer. This suspension was stirred at 4°C for 1 hr before ultracentrifugation was repeated. The lysate was then applied to a Ni-Sepharose column (5mL) pre-charged with Ni-sulfate and bind buffer with 0.1% Triton X-100. SpsB was eluted with a 21 mL step-wise gradient of imidazole in 3 mL steps: 50, 100, 150, 200, 250, 300, 500, and 1000mM imidazole in bind buffer with 0.05% n-Dodecyl β-D-maltoside (DDM). Fractions containing SpsB

were pooled and applied to a Superdex75 column pre-equilibrated and run with SEC buffer, 20 mM Tris, pH 7.8, 150 mM NaCl, and 0.05% DDM. Fractions containing SpsB were pooled and exchanged by centrifugal filtration into SEC buffer containing 20% glycerol. The final, concentrated preparation was flash frozen in liquid nitrogen. The final yield was 1.5 mg per L of culture.

Making proteoliposomes containing LtaS

Liposomes were prepared based on a previously published method.[2] 3.2 mg of *E. coli* polar lipid extract (67% phosphatidylethanolamine, 23.2% phosphatidylglycerol, 9.8% cardiolipin, average molecular weight: 798 Da; Avanti Polar Lipids) was suspended in 25 mM HEPES pH 7.5, 200 mM NaCl, and 100 µM EDTA, and extruded with a lipid extruder (Avanti Polar Lipids) through a 100 µM membrane. These empty liposomes were diluted 1:2 with 25 mM HEPES, 200 mM NaCl, 100 µM EDTA, and 0.4% DDM and incubated on ice for 10 minutes. LtaS was added to a final concentration of 1 µM and tumbled at 4°C for 1 hour. This solution was then mixed with an equal volume of Biobeads (Bio-Rad) equilibrated in 25 mM HEPES, 200 mM NaCl, and 100 µM EDTA and tumbled at 4°C for two hours. The Biobeads were exchanged for fresh beads, tumbled overnight at 4°C, and then exchanged again and tumbled for 2 hours at 4°C. The Biobeads were removed by gentle centrifugation and the supernatant was stored at 4°C until use. Liposomes could be stored at 4°C for up to a month without loss of activity.

In vitro activity of LtaS in proteoliposomes

Conditions for *in vitro* reactions were adapted from Karatsa-Dodgson et. al.[3] In general, to a solution containing 20 mM succinate pH 6.0, 50 mM NaCl, 5% DMSO, and 1 mM $MnCl_2$ was added 2.5 µL of proteoliposomes containing 1 µM LtaS, resulting in a final LtaS concentration of 100 nM. This reaction was incubated at 30°C for 2 hours. The reaction was quenched with the addition of 2x SDS-PAGE loading dye and run on a 4-20% Tris-Glycine gel (Bio-Rad). The gel was then transferred onto a PVDF membrane. The membrane was blocked, incubated first with an α-LTA antibody (Hycult), and then an α-mouse-HRP secondary antibody (Cell Signal Technology), and visualized with ECL reagent (Pierce). Samples were also visualized *via* dot blot by spotting 3 µL of the quenched proteoliposome reaction on to a semi-wet PVDF membrane and performing a blot as described above. EDTA treated reactions contained 20 mM EDTA, which was added prior to proteoliposomes. For the boiled samples, the assay was prepared without manganese and was boiled at 95°C for 20 minutes. Manganese was then added to this solution to initiate the reaction and was treated as described above. For samples treated with SpsB, 1 µL of 5 µM SpsB was added to a reaction to which manganese was not yet added. This was incubated at 30°C for 1 hour, and manganese was then added to initiate the reaction and was treated as previously described.

DAG detection in proteoliposomes

100 µL reactions were prepared as described above, but were incubated at 30°C for 4 hours. To each reaction was added 150 µL Methanol, 225 µL 1M NaCl, and 250 µL chloroform. Reactions were vortexed and centrifuged at 1,500xg for 5 minutes to separate phases. The aqueous layer was removed and the organic layer was mixed with 200 µL pre-equilibrated upper phase (the top phase of a 1:1:0.95 mixture of Methanol:Chloroform:1M NaCl). This was vortexed and again centrifuged to separate layers. The aqueous phase was removed and the organic layer was evaporated in a speedvac. The dried residue was resuspended in 1x DAG assay buffer (provided by kit), and samples were treated as outlined by the DAG assay manufacturer (Cell Biolabs Inc.) (Figure S3). In short, extracts dissolved in 1x buffer were first mixed with a

kinase solution and incubated at 37°C for 2 hours. This was then added to a Lipase solution and incubated at 37°C for 30 minutes. To this was added the final enzyme mix containing the fluorophore and incubated at 25°C for 10 minutes. The plates were then read in a GloMax Explorer plate reader (Promega) with an excitation of 520 nm and emission of 580-620 nm. Concentrations of of 2 mM to 31 µM DAG were used to establish a standard curve. A proteoliposome reaction to which no kinase solution was added was used to establish the background signal.

In vitro inhibition of LtaS measured by blotting and DAG assay

Generally, proteoliposome reactions were set up as described above, but increasing concentrations of dyes **1-4** or 1771 were added to the reaction. DMSO concentration was normalized to 5% for all reactions. Activity was assessed as previously described on both SDS-PAGE gel and PVDF dot blots or *via* DAG quantification. Concentrations of 312 nM to 20 µM were used.

Measuring the MIC of inhibitors and dyes against *S. aureus* strains

Each strain of *S. aureus* for which the MIC was measured was grown overnight in TSB at 30°C, and diluted 1:100 the next day. After the O.D. 600 reached approximately 1, the cells were diluted 1:1000 in TSB and mixed with varying concentrations of each inhibitor, normalized for a DMSO concentration of 1% using a D300 liquid dispenser (Tecan) in a clear 96-well plate. Plates were grown for approximately 17 hours at 30°C, and then the O.D. 600 was measured. MIC's were calculated using GraphPad Prism with the non-linear regression dose-response equation. An MIC of ~1 µg/mL was calculated for Congo red, and MICs of ~8 µg/mL were calculated for all strains treated with 1771. At high dye concentrations, the MIC could not be calculated except for Congo red at concentrations up to 128 µg/mL.

Measurement of IC_{50} of inhibition by Congo Red

Triplicate titrations of proteoliposome reactions with Congo red ranging from 312 nM to 20 µM were incubated at 30°C for one hour and quenched with SDS-PAGE loading dye, and loaded on a 4-20% gel (Bio-Rad). The resulting α-LTA western blots were analyzed by densitometry using imageJ. Each lane was integrated and converted to percent activity with respect to a DMSO only control proteoliposome reaction. The results were graphed and analyzed *via* non-linear regression with the normalized dose-response equation in GraphPad Prism (Figure S4).

Additive screening of Congo red polymerization

LtaS proteoliposome reactions were performed as described above. Each additive was added to the reaction from a 10x stock solution prior to proteoliposome addition. Additives assayed included 0.005% Tween-20, 0.01% Triton X-100, 100, 300, and 500 mM NaCl (in addition to the 50 mM already in the assay), and 100 nM and 1 µM BSA (dissolved in 1x liposome reaction buffer). All additive screens were run in parallel with a control containing no additive. After 1h incubation at 30°C, reactions were spotted on either PVDF or nitrocellulose and subjected to an α-LTA blot.

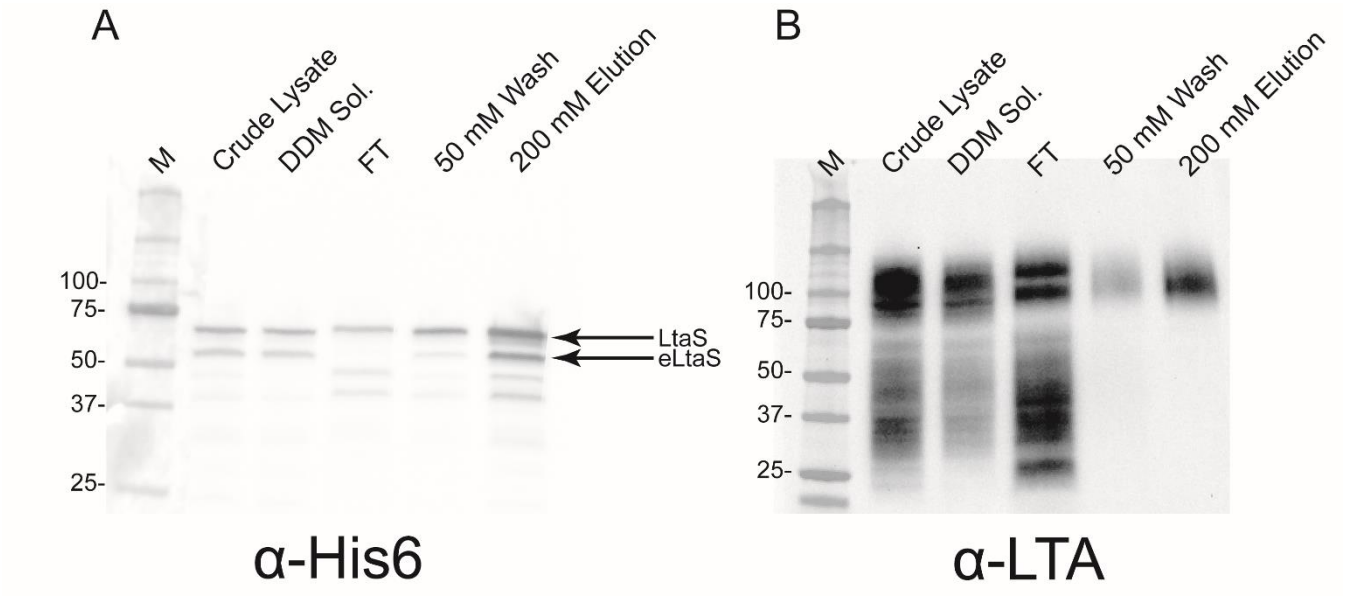


Figure S1. Purification of LtaS-his$_6$ from *E. coli*. (A) Full-length LtaS measured approximately 65 kDa on SDS-PAGE. eLtaS, the soluble C-terminal domain, was detected after Ni^{2+} purification DDM sol. refers to the solubilization step during purification, and FT and the washes indicate affinity purification steps. (B) An LTA-like polymer was detected in the crude extract of cells expressing LtaS, and this LTA band co-purified with LtaS through Ni^{2+} purification.

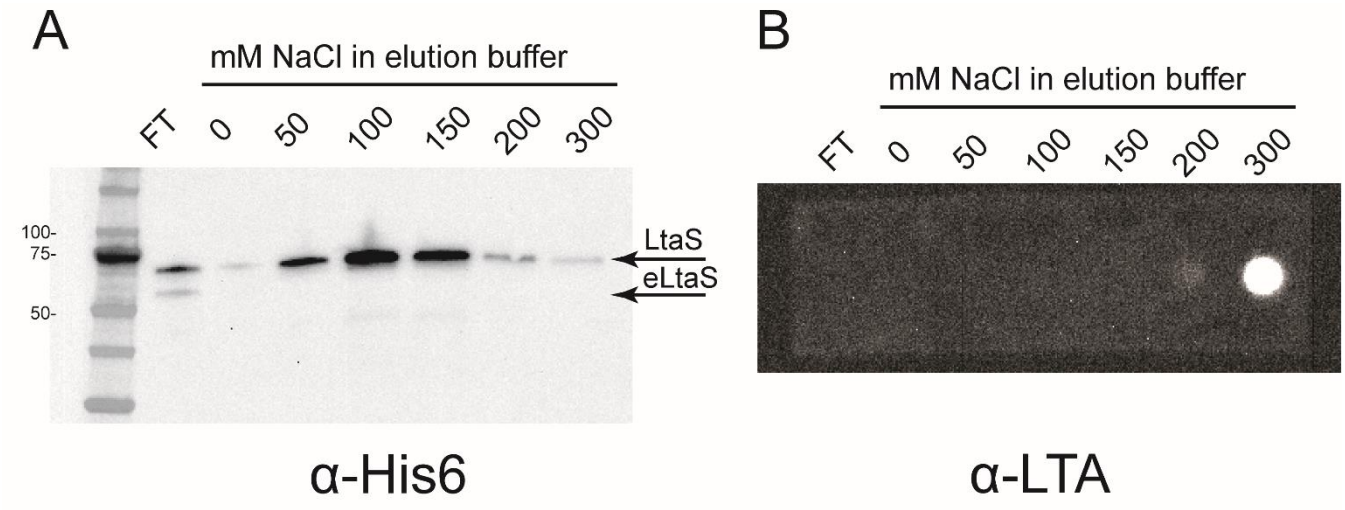


Figure S2. Removal of co-purified LTA from LtaS *via* DEAE-Sepharose ion exchange. (A)Some LtaS and eLtaS did not bind to the column and was detected in the flowthrough, while pure LtaS was eluted between 50 mM and 150 mM NaCl when probed with an α-His_6 antibody. (B) Dot blot analysis of the same column fractions and probed with an α-LTA antibody showed that the LtaS-bound LTA was successfully separated, and remained on the ion exchange column until a concentration of 300 mM NaCl.

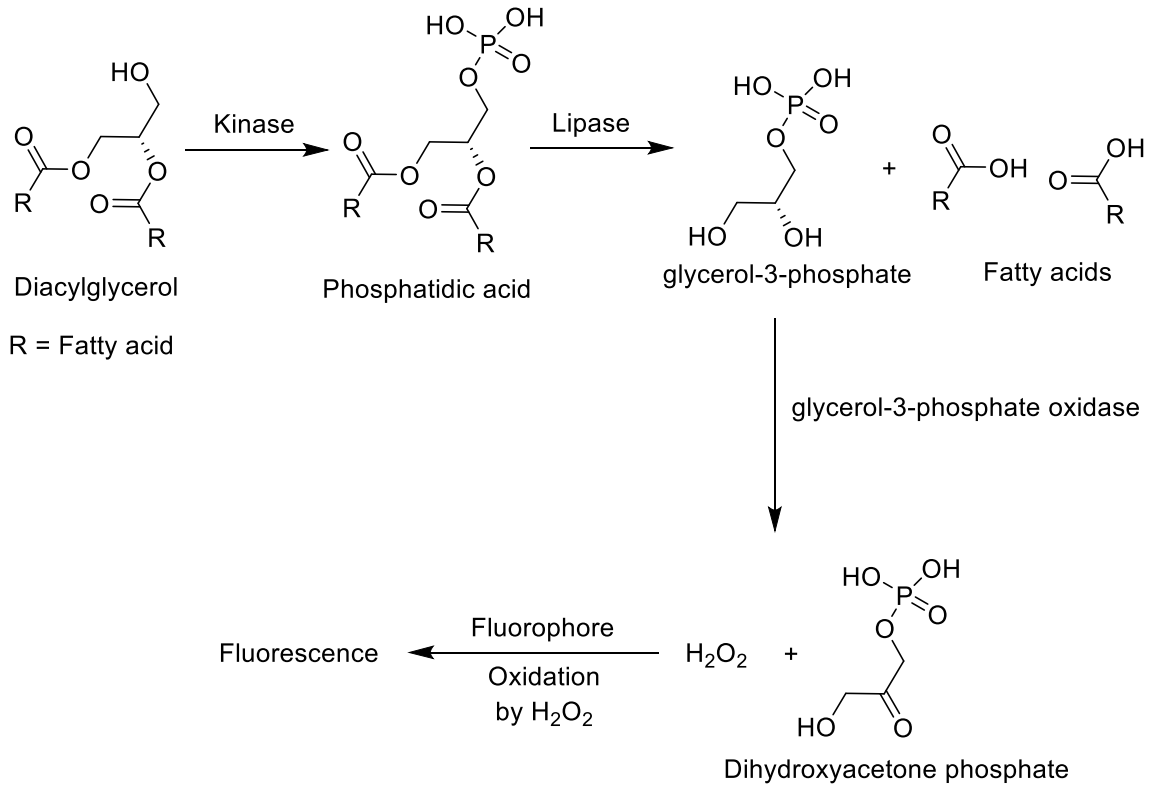


Figure S3. Workflow for DAG detection assay. DAG is first converted to phosphatidic acid (PA) and then hydrolyzed to glycerol-3-phosphate (G3P) and free fatty acids. G3P oxidase then converts G3P to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide oxidizes a fluorophore that then fluoresces when exposed to 520 nm light.

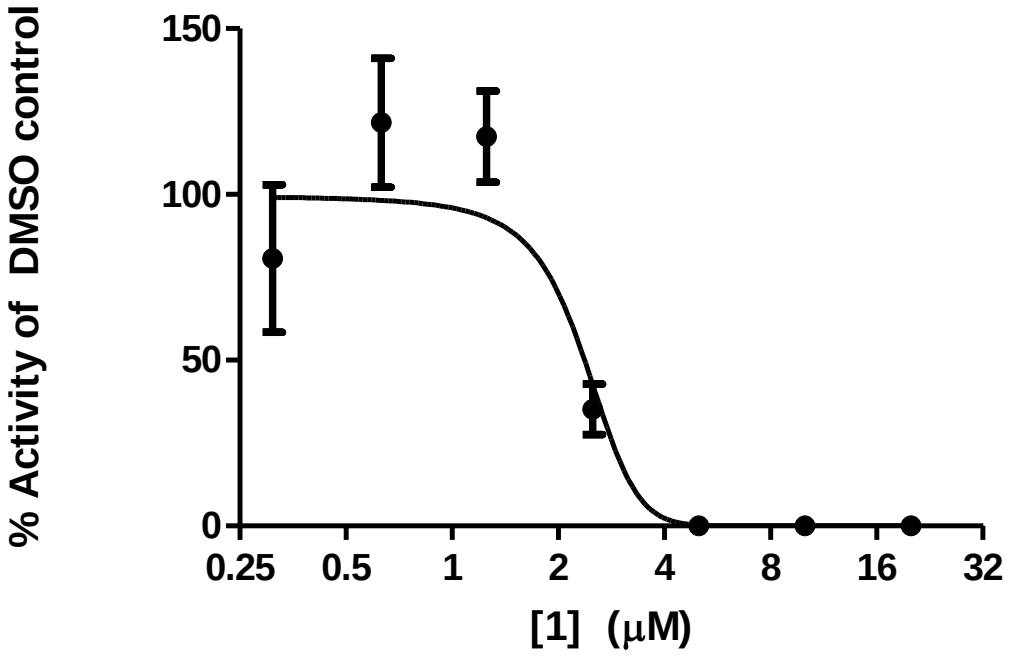


Figure S4. Inhibition of LTA polymerization by 1. Triplicate reactions of LTA over the indicated concentration range were run on SDS-PAGE, and the signal resulting from an α-LTA western blot were integrated in ImageJ and fit with a dose-response curve in Graphpad Prism, resulting in a calculated IC_{50} of 2.3 ± 0.2.

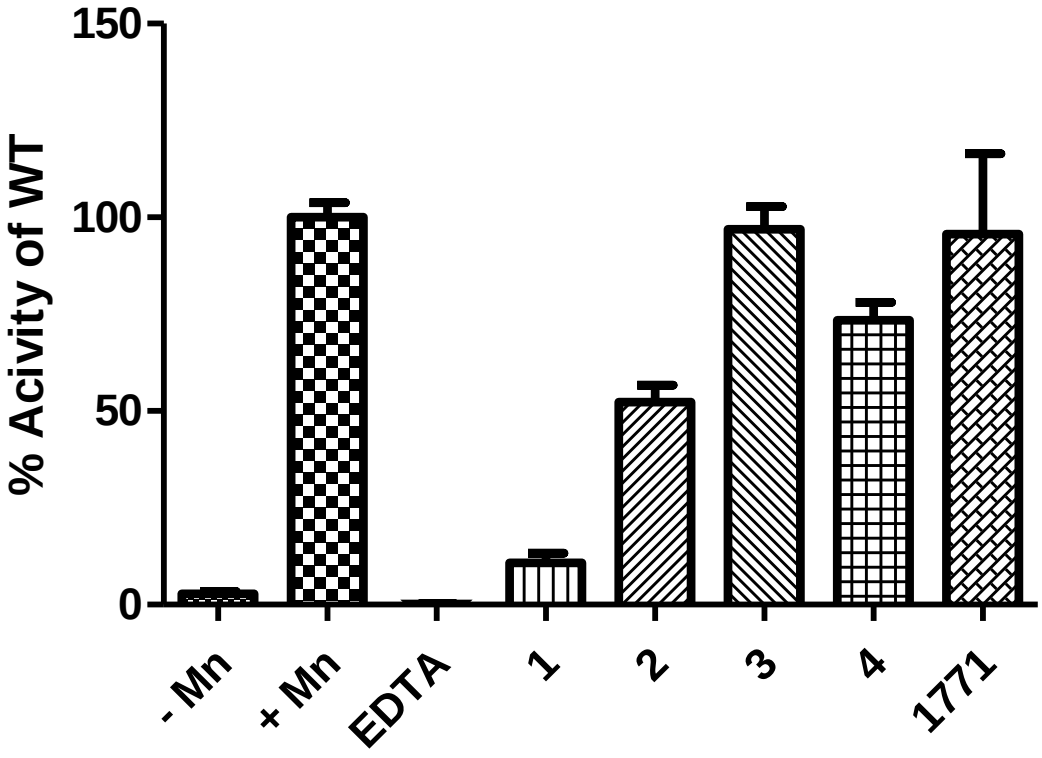


Figure S5. DAG production of LtaS in liposomes with 20 μM of 1-4, and 1771. EDTA treatment reduced activity to background levels, and treatment with 20 μM **1** reduced DAG production to approximately 10% of the positive control. No other compound decreased DAG levels below 50%.

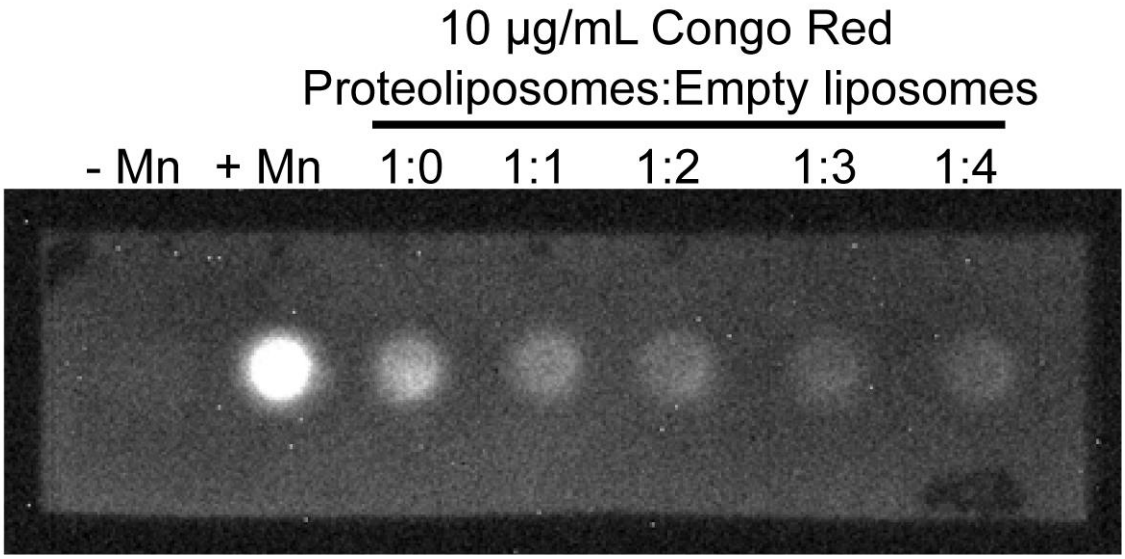


Figure S6. Dot blot showing that adding empty liposomes to the assay does not decrease inhibition by Congo red.

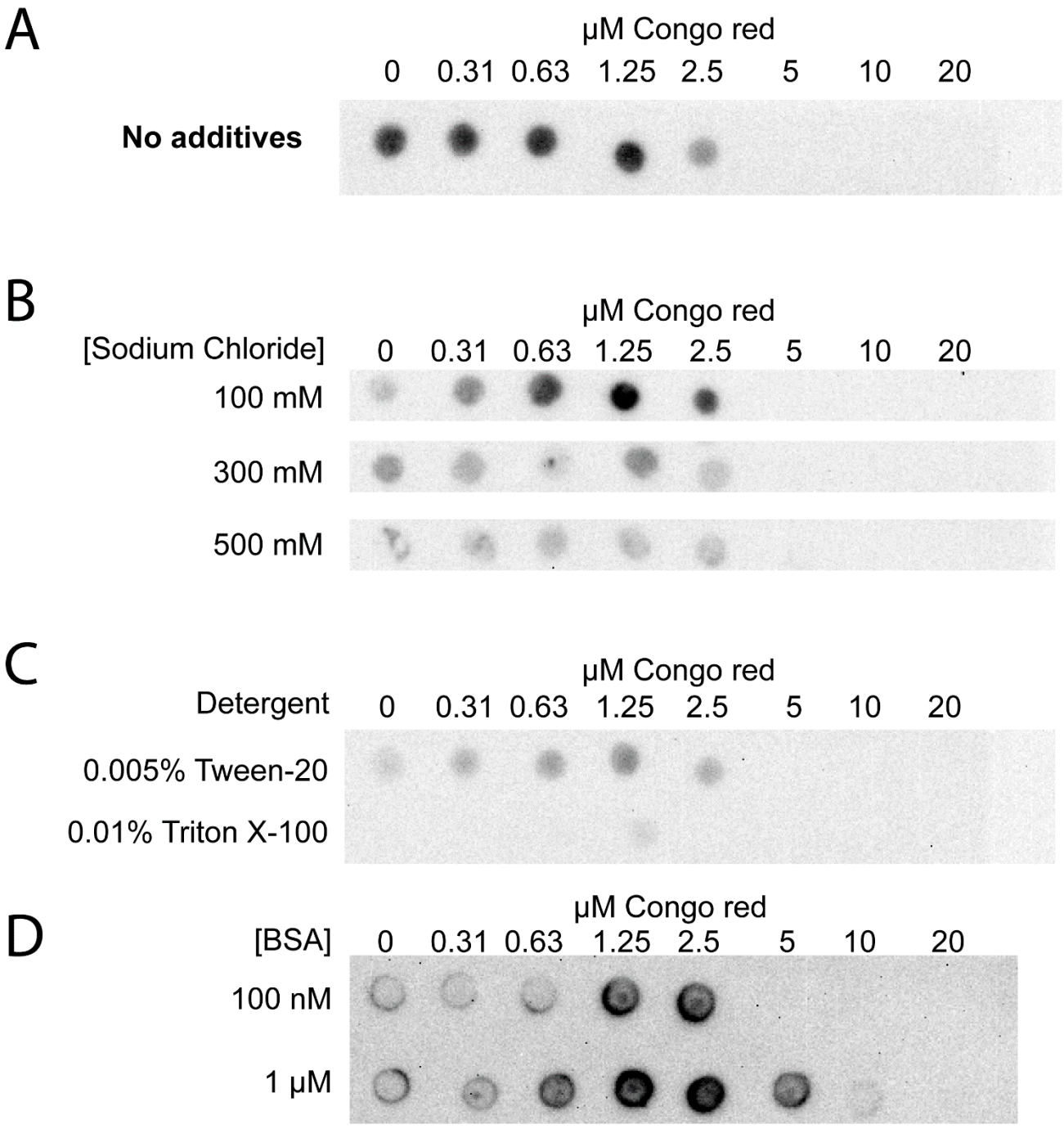


Figure S7. Additives to proteoliposome assay do not significantly alter kinetics of LtaS. Compared to no additives (A), adding sodium chloride (B), or detergent (C), did not affect the apparent IC_{50} of Congo red against LtaS. However, adding BSA at a 10:1 ratio (D) increased the IC_{50} by approximately 2-fold.

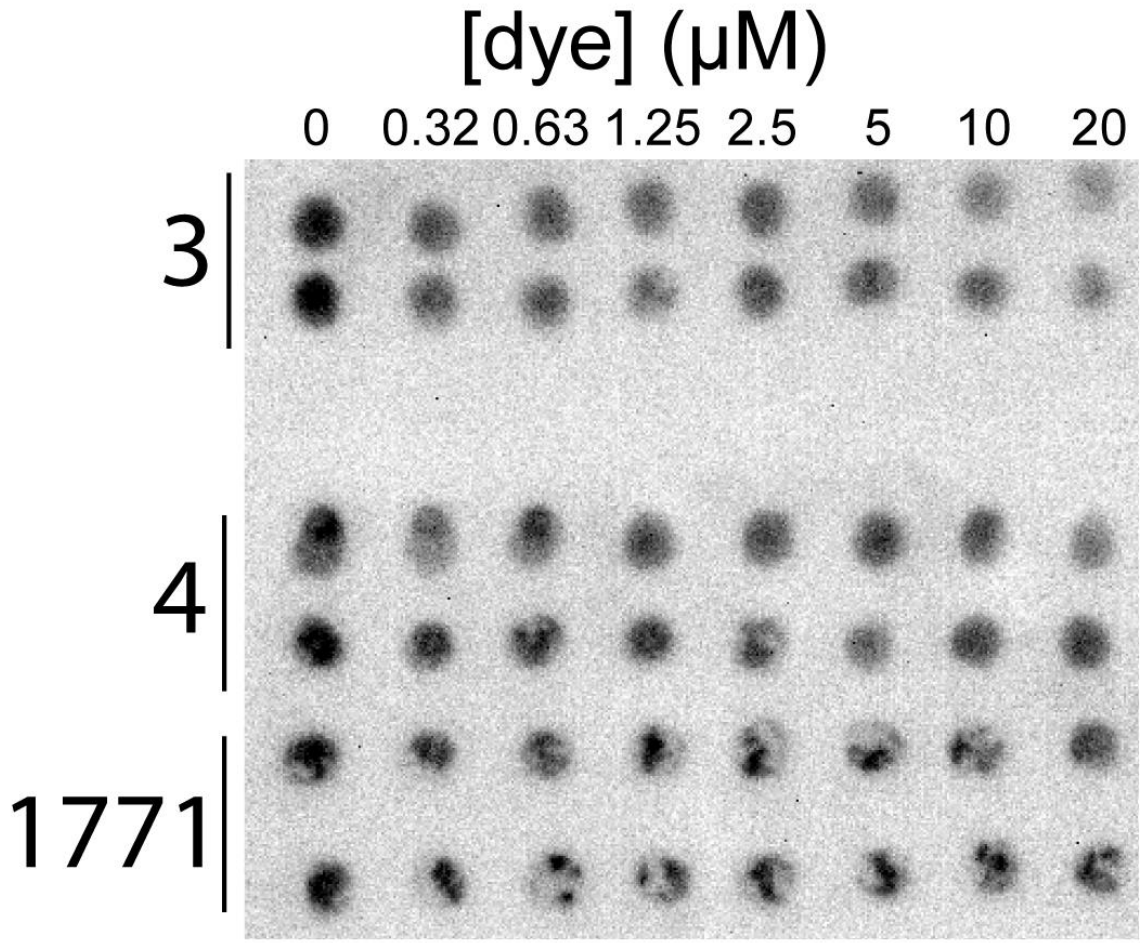


Figure S8. treatment of LtaS proteoliposomes with dyes 3 and 4 and the inhibitor 1771. No compound exhibits significant inhibition up to 20 μM as detected by Dot Blot.

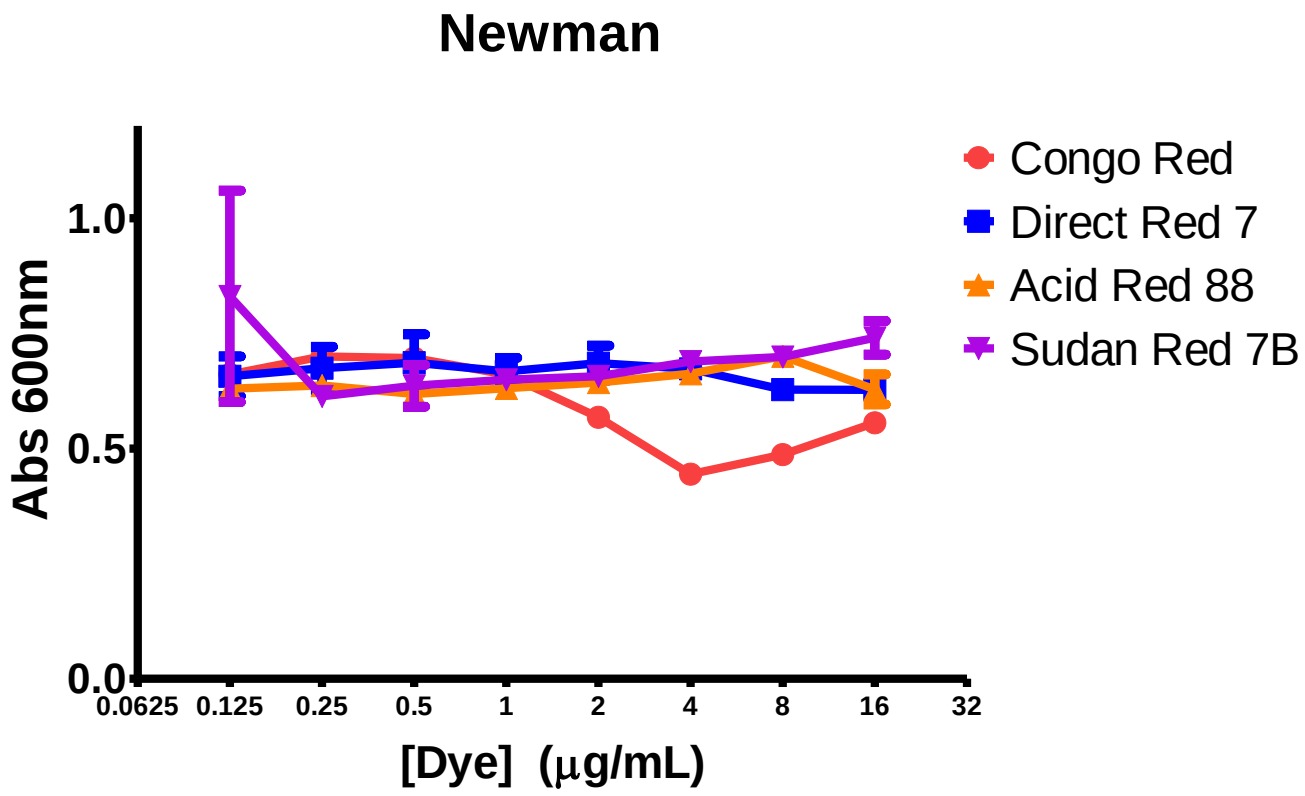


Figure S9. Growth of WT Newman *S. aureus* was unaffected by any azo dye.

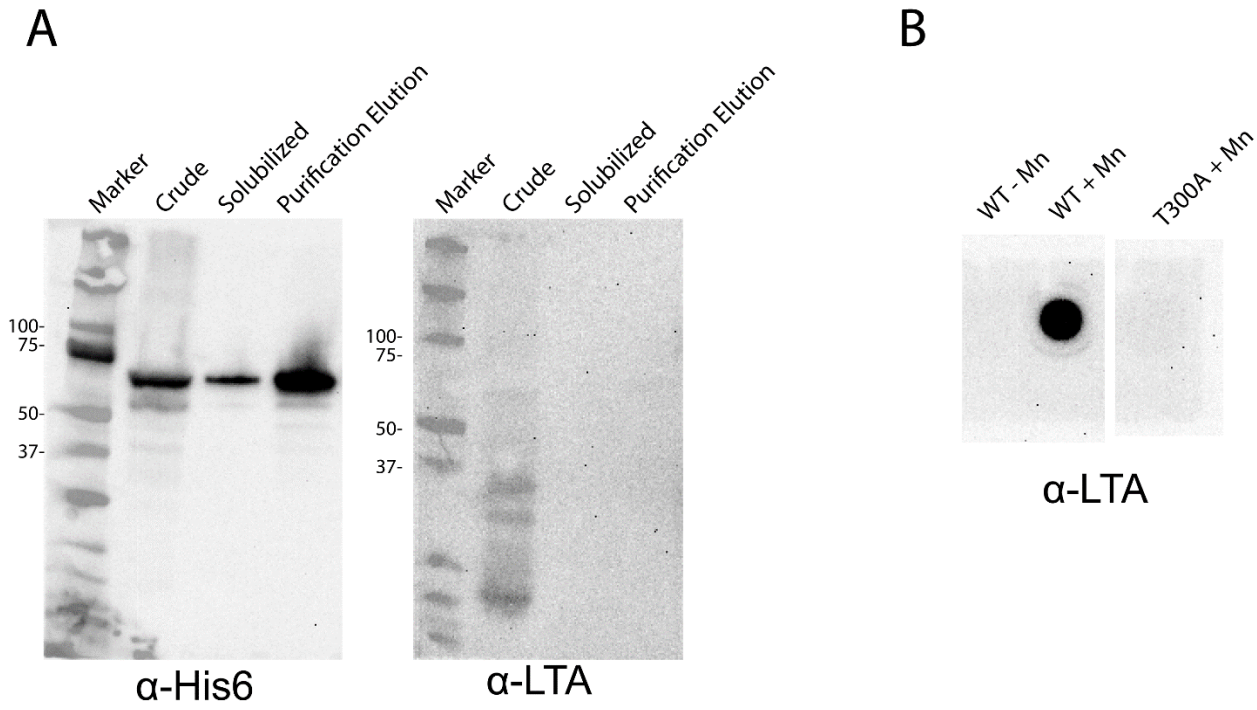


Figure S10. Purification of T300A and lack of LTA biosynthesis. (A) After purification from *E. coli*, T300A did not retain any LTA, and a blot for LTA of the same purification fractions did not reflect those observed when full-length LtaS was expressed and purified from *E. coli*. (B) Proteoliposomes containing T300A, in addition to not producing DAG (Figure 2), did not produce a detectable signal when LTA production reactions with proteoliposomes containing T300A were blotted with an α-LTA antibody.

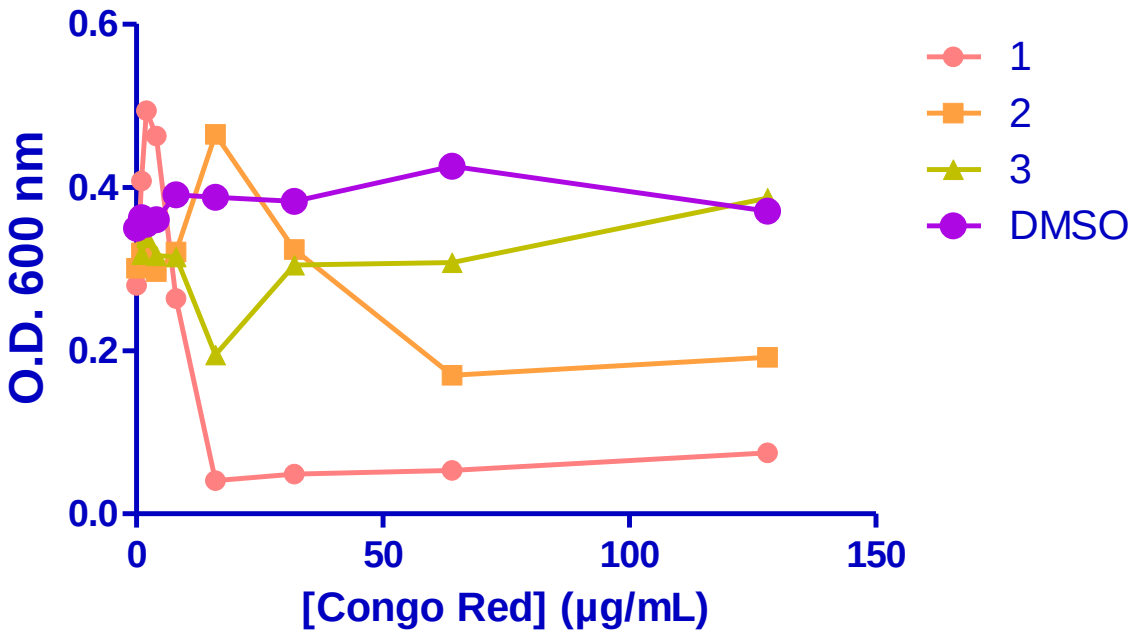


Figure S11. Only Congo red kills Δ*tarO* at high concentrations. Sudan Red 7B (**4**) is not shown due to precipitation and aggregation at high concentrations, leading to high O.D. measurements.

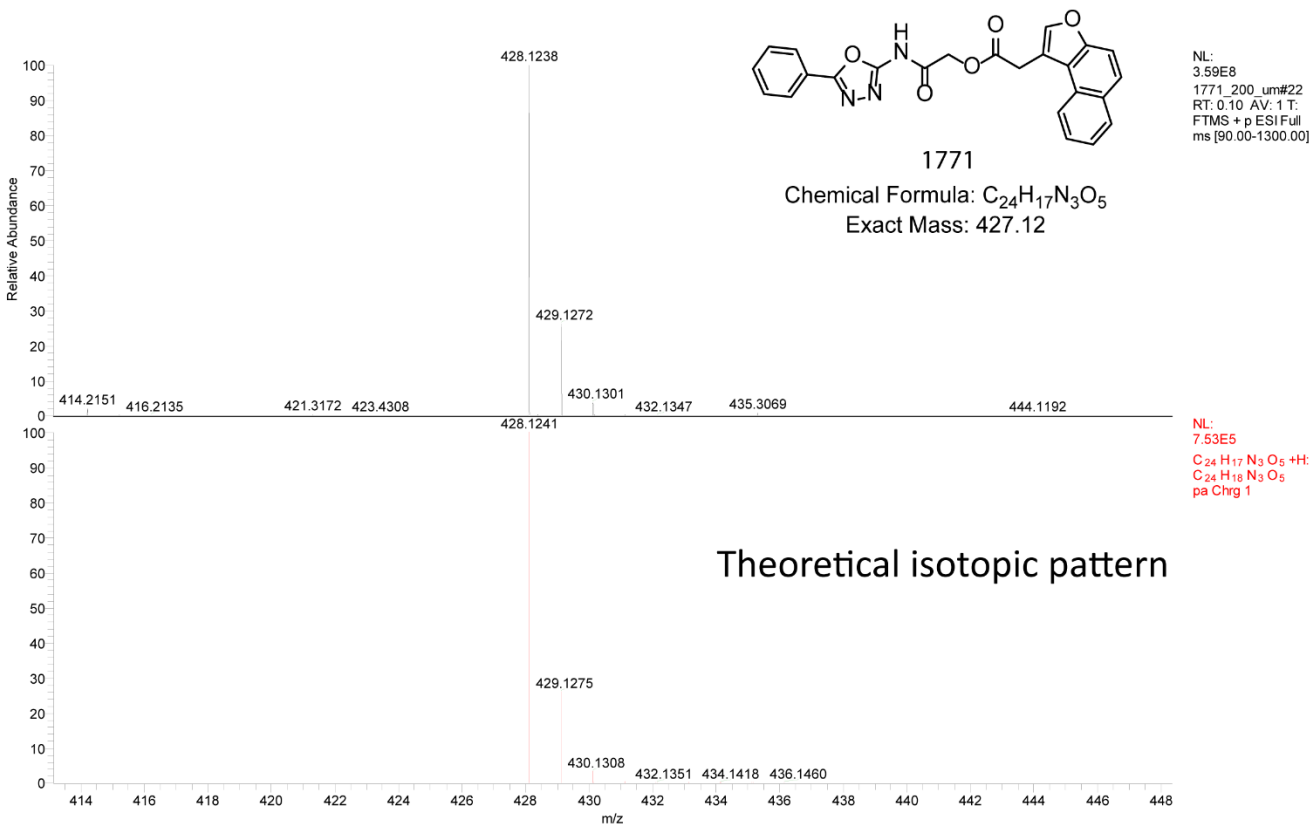


Figure S12. High-resolution MS data confirming that compound 1771 has the correct atomic composition.

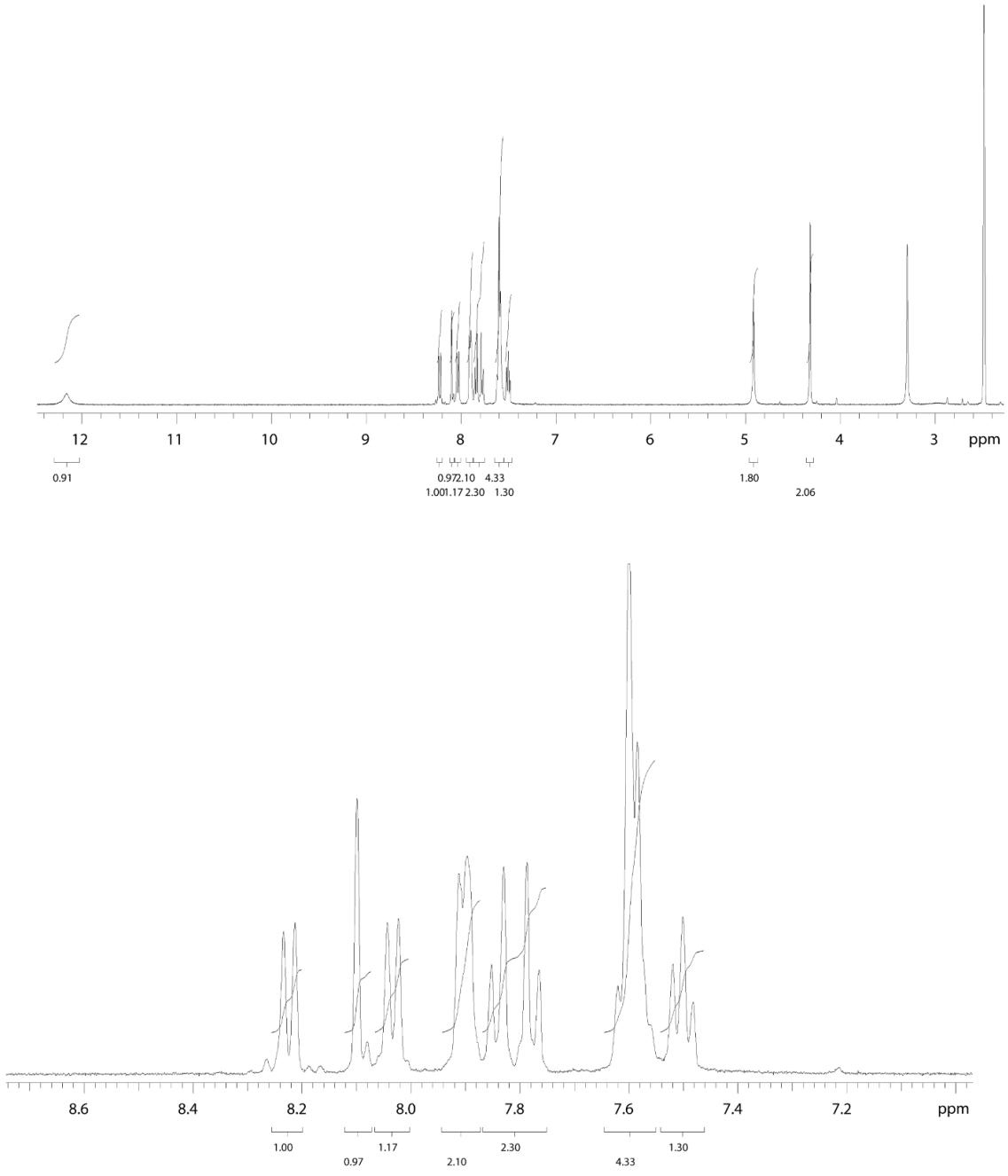
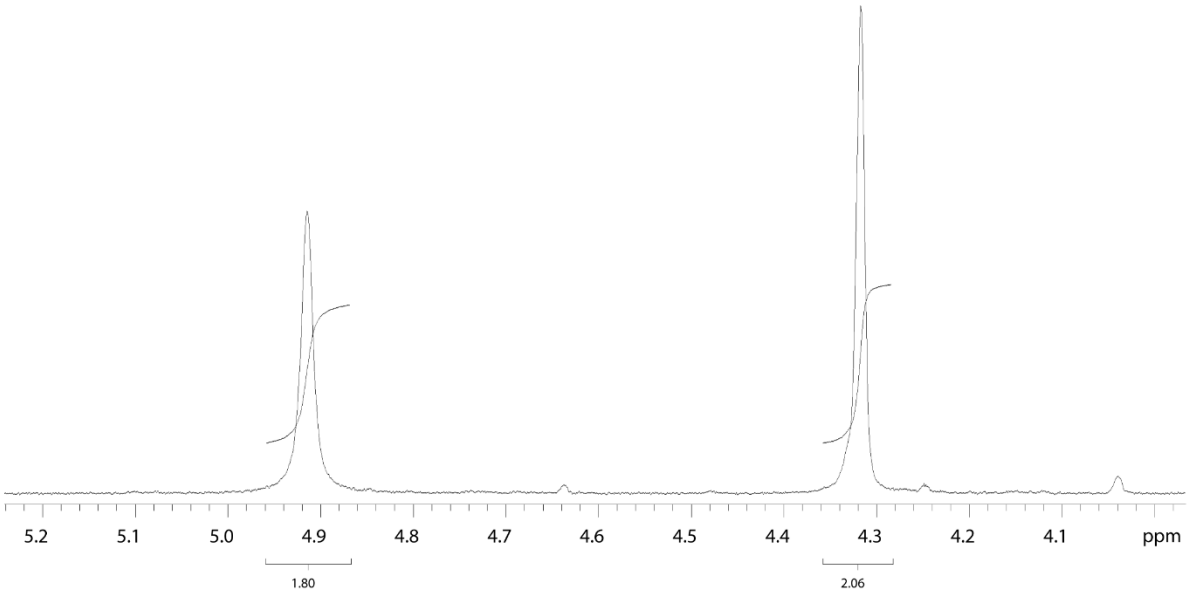


Figure S13. Compound 1771, ordered from enamine, exhibits the correct shifts and high purity in a proton NMR spectrum. All protons are accounted for, and display the correct integration values. NMR was performed in deuterated DMSO.



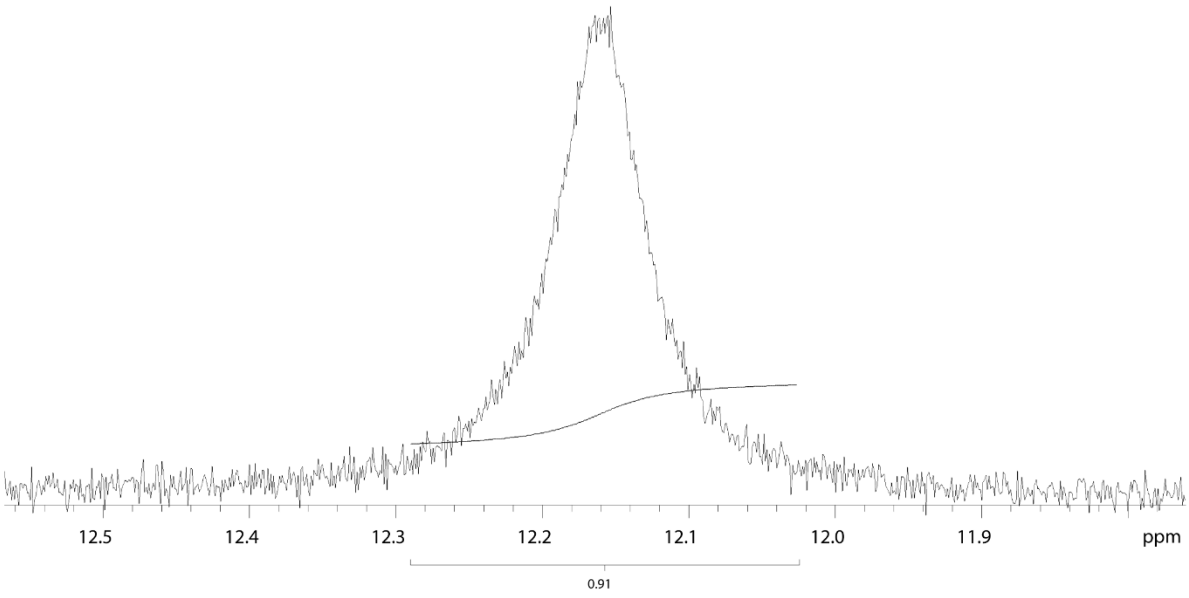


Figure S13 (cont'd).

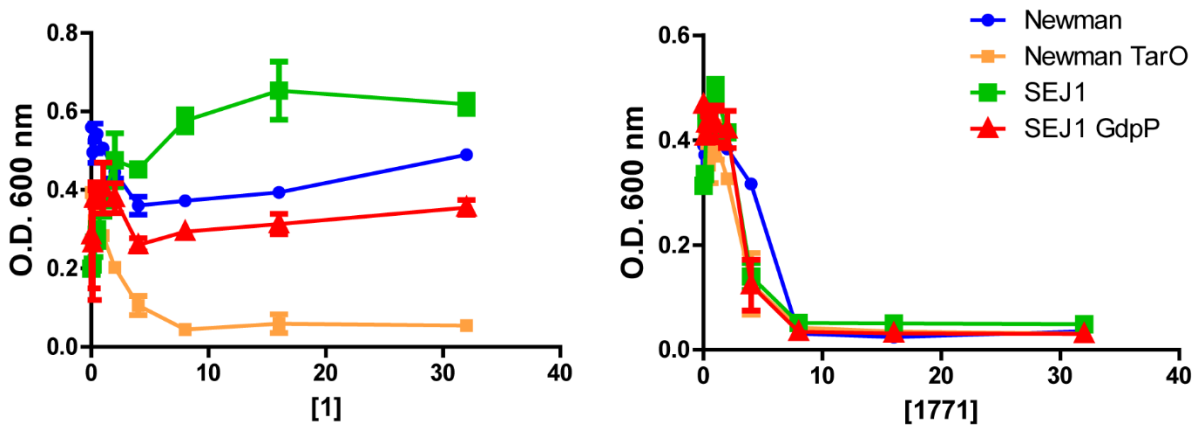


Figure S14. Growth inhibition of *S. aureus* strains by 1771 and Congo Red. (A) Congo Red was lethal only to a Δ*tarO* strain, consistent with inhibition of the LTA pathway. (B) 1771 efficiently killed all tested strains at 8 µg/mL.

Table S1. Genes that are synthetic lethal with WTA biosynthesis (Δ*tarO*)

Gene	Function (putative)	Reference	Locus
618	Unknown	6	SAOUHSC_00618
GraR	Cell stress response	5	SAOUHSC_00665
VraG	Cell stress response	5	SAOUHSC_00667
718	Unknown	6	SAOUHSC_00718
LtaS	Makes the glycerol phosphate polymer of LTA	4	SAOUHSC_00728
UgtP	Makes diglucosyl-DAG	6	SAOUHSC_00753
DltA	Attaches D-ala to DltC	5	SAOUHSC_00869
DltB	(Transfers D-Ala across the membrane)	5	SAOUHSC_00870
DltC	Interacts with DltB to transfer D-Ala across the membrane	5	SAOUHSC_00871
DltD	(O-acyltransferase required for D-Alanylation of LTA)	5	SAOUHSC_00872
948	Unknown	6	SAOUHSC_00948
965	Unknown	5	SAOUHSC_00965
Stk1	Cell stress response	6	SAOUHSC_01187
LyrA	Lysostaphin resistance	5	SAOUHSC_02611

Table S2. Primers used in this study

Primer name	5' - 3' sequence
LtaS_F	AGGAGATATACCATGAGTTCACAAAAAAAGAAAAT
LtaS_R	GGTGGTGGTGCTCGAGTTTTTTAGAGTTTGCTTTAG
SpsB_F	GCCGCGCGGCAGCCATAAAAAAGAAATATTGGAATGG
SpsB_R	GTTAGCAGCCGGATCCTTAATTTTTAGTATTTTCAGGATTGA AATTATGTTTAAATTC
LtaST300A_F	AGGTAAAGCATCTGACTCTGAATTTACAATGGATAACA
LtaST300A_R	TCAGATGCTTTACCTTGACCTGTTTGATGGAA

Strains used in this study

Organism	Strainr	Plasmid	Description	Reference/Source
E. coli	Stellar		Cloning strain	TaKaRa
E. coli	Stellar	pET28b-LtaS	Cloning strain bearing pET28b-LtaS	This Study
E. coli	Stellar	pET28b-LtaS T300A	Cloning strain bearing pET28b-LtaS T300A	This Study
E. coli	KRX		Expression strain	Promega
E. coli	KRX	pET28b-LtaS	Expression strain bearing pET28b-LtaS	This Study
E. coli	KRX	pET28b-LtaS T300A	Expression strain bearing pET28b-LtaS T300A	This Study
S. aureus	RN4220		RN450 partial agr defect ST8; CC8; codon in both hsdR and sauUSI	[7]
S. aureus	Newman		ST8; CC8 isolated in 1952 human clinical MSSA	[8]
S. aureus	Newman Δ*tarO*		unmarked deletion of *tarO*	Laboratory Stocks
S. aureus	SEJ1		RN4220Δspa	[9]
S. aureus	SEJ1 *gdpP*::Kan^R		*gdpP* replaced with Kanamycin resistance cassette	Laboratory Stocks

(1) Gründling, A.; Schneewind, O. *Proc. Natl. Acad. Sci.* **2007**, *104* (20), 8478.
(2) Hagan, C. L.; Kim, S.; Kahne, D. *Science* **2010**, *328* (5980), 890.
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