SI Materials and Methods

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Strains and Growth Conditions. All *A. baumannii* strains were derived from ATCC 19606 and grown in Luria-Bertani (LB) broth-Miller (BD) at 37 °C unless otherwise specified. LB plates contained 1.5% agar (BD) unless otherwise specified. Antibiotic concentrations used for *A. baumannii* were 100 μg ml^{-1} carbenicillin, 50 μg ml^{-1} apramycin and 25 μg ml^{-1} kanamycin unless otherwise specified. All *A. baylyi* strains were derived from ADP1 and grown in LB-Miller (EMD Millipore) at 30 °C. Antibiotic concentrations used for *A. baylyi* and *E. coli* were 50 μg ml^{-1} unless otherwise specified. Strains and plasmids used in this study are summarized in **Dataset S1**.

General materials and methods. PCR reagents were purchased from EMD Millipore and primers were ordered from Integrated DNA Technologies (IDT) or Eton Biosciences. PCR was performed with the addition of 5X Combinatorial Enhancer Solution (CES; contains 2.7 M betaine, 6.7 mM DTT, 6.7% (v/v) DMSO, and 55 μg ml^{-1} BSA) for *A. baumannii* strains. Restriction enzymes and Gibson Assembly master mix were purchased from NEB. Dansyl-PMBN was reported previously (1). Primers used in this study are summarized in **Dataset S2**.

Preparation of cell cultures used in high-throughput screening. Overnight cultures of Δ5 and Δ5 Δ*lpxA* were diluted 20-fold into LB and grown for ~3 hours to reach an OD_{600} of 0.7-0.9. Cells were harvested by centrifugation (4,000 x g, 5 min), washed once with LB, and resuspended in a solution of ~15% glycerol in LB to an OD_{600} of ~1.0. 1-ml aliquots were prepared and stored at -80 °C.

High-throughput chemical screening and hit follow-up. Stocks of Δ5 and Δ5 Δ*lpxA* were diluted 7-fold and 9-fold, respectively, with LB (MilliporeSigma). Cultures were incubated for 1 hour at 30 °C (~230 rpm, final OD_{600} of 0.2-0.3). Library compounds (final concentration 5 μM) and colistin (30 μg ml^{-1}) were pre-dispensed into 384-well plates by Labcyte Echo 555 at Calibr. 40-μl LB containing approximately 1 x 10^6 CFU ml^{-1} (OD 0.002) of Δ5 were then dispensed into the plates. Plates were incubated at 37 °C for 24 hours and OD_{600} was read on PerkinElmer Envision. Top 1100 hits that inhibited Δ5 were retested at 5 μM against Δ5 Δ*lpxA*. Those hits that had a larger inhibitory effect on Δ5 than Δ5 Δ*lpxA* were retested in dose response against both strains.

Minimal inhibitory concentration (MIC) measurements. Cell cultures at an OD_{600} of 0.0001 were dispensed into 96-well plates (100 μl per well). Compounds were added either by a multichannel pipette (2 μl in aqueous solution) or by D300 across a concentration range. Following 24-hour incubation at 37 °C, MICs were determined as the lowest concentrations at which OD_{600} remained at baseline. For drug-drug interactions, optical density (OD_{600}) of 100 μl Δ5 at OD_{600} 0.002 in LB (MilliporeSigma) was measured after 1.25-hour preincubation with PF-5081090 (Sigma-Aldrich) at 37 °C followed by 24-hour incubation with compound **1** or colistin at 37 °C.

Characterization of *A. baylyi* strains. Overnight cultures of bacterial strains were normalized to an OD_{600} of 0.0001; 200 μl was added into a 96-well plate. Optical density was measured every 10 minutes. The plate was shaken continuously between each

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measurement. Microscopy was carried out on a Nikon Ti microscope. Images were obtained using a DIC Plan Apo 100 x 1.4NA objective with a polarizer, DIC H condenser, DIC prism, and Hamamatsu ORCA-R2 cooled CCD camera.

Electroporation of *A. baumannii.* Electrocompetent cells were prepared following a published protocol with modifications (2). Cell cultures grown from 20-fold diluted overnight cultures in 20 ml LB were harvested at OD_{600} 0.7-0.9 by centrifugation (4,000 x g; 5 min). Cells were washed twice with ice-cold water and once with ice-cold 10% glycerol, pelleted at 12,000 rpm for 1 min, and finally resuspended in 150-µl ice-cold 10% glycerol. Into the cell suspension was added either a linear DNA fragment (5-8 µg) or a purified plasmid; the resulting mixture was electroporated in a pre-chilled 2-mm cuvette at 2.5 kV, 200 Ω, 25 µF, or a pre-chilled 1-mm cuvette at 1.8 kV, 200 Ω, 25 µF. Immediately following electroporation, cells were recovered for ~1 hour in 1 ml LB at 37 °C and spread onto LB agar with the appropriate antibiotics and incubated overnight at 37 °C.

Isolation and whole genome sequencing of *lpx*-null and *msbA* mutants. To isolate *lpxA*/*C*/*D* mutants, 100-µl overnight cultures of Δ5 were spread onto LB agarose (Ultrapure from Invitrogen) containing 3.84 µM compound **1**. Agarose was used because LPS-null mutants do not form colonies efficiently on agar. After incubation for two days, colonies were streaked onto plates containing apramycin, incubated overnight, and restreaked onto unsupplemented plates. Isolated colonies were grown in LB overnight, and their genomic DNA was extracted using the Promega Wizard kit. A genomic library was then prepared using the Nextera XT kit and sequenced by MiSeq (Illumina) in paired-end 75 nucleotide runs (v3). To isolate *msbA* mutants, 2- or 5-ml overnight cultures of Δ5 were centrifuged at 4,000 x g for 5 min and resuspended in 300 µl LB. The resulting suspension was spread onto LB agarose containing 4 µM compound **1** and incubated 22 hours. Colonies were transferred onto an LB agar plate containing 16 µg ml^{-1} colistin through replica plating. Both plates were incubated overnight. Colonies that were compound **1**-resistant and colistin-sensitive were identified by visual inspection of the two plates. A subset of these colonies was restreaked onto apramycin-containing LB agar. Isolated colonies were grown in LB overnight. Their genomic DNA was extracted using the ThermoPurelinkPro 96 Genomic DNA Purification kit. Genomic library was then prepared and processed by the Nextera XT kit and sequenced by HiSeq (Illumina) in paired-end 125 nucleotide runs (v4).

Construction of mutants. For *A. baylyi* single deletion and multiple deletion strains, linear DNA antibiotic resistance cassettes for generating gene knockouts were constructed using PCR assembly and contained 1 kb of sequence homology both upstream and downstream of each of the genes being inactivated. To generate knockouts, overnight cultures of *A. baylyi* were diluted 1:15 into LB containing knockout DNA cassettes, shaken overnight, and plated with the appropriate antibiotics for selection. Single colonies were isolated. Knockouts were initially checked using agarose electrophoresis of PCR products in the region of the knockout, and subsequently verified using whole-genome sequencing.

For Δ5 and Δ5 Δ*lpxA A. baumannii* strains, biparental conjugation was used to construct the screening strain background, in which markerless deletions were made in the order of *pmrC*, *adeFGH*, *adeAB* and 0431, followed by marked deletion of *adeIJK*

replaced by an apramycin resistance cassette, aac(3)-IV (from plasmid pSET152). A published protocol was followed with modifications (3). Specifically, ~1-kb regions flanking the gene of interest were PCR amplified and cloned through Gibson assembly into pEX18Ap (carb^R, sacB) that was pre-digested with HindIII and KpnI. The resulting construct, after confirmed by Sanger sequencing, was introduced into competent cells of an *E. coli* helper strain (pRK2013; kan^R) through electroporation in a 1-mm cuvette at 1.8 kV, 200 Ω and 25 µF. The resulting transformants were plated onto LB agar containing kanamycin and carbenicillin. The plate was incubated overnight at 30 °C. On the day of *E. coli* electroporation, an *A. baumannii* recipient strain with the proper background was streaked onto LB agar and incubated at 30 °C. Following overnight incubation, *E. coli* transformants and the aforementioned *A. baumannii* were scraped from their respective plates and resuspended in LB; OD_{600} of the two strains were normalized to 2.0. 100 µl of each strain was mixed in 600 µl LB. The mixture was then washed twice with LB and pelleted (7,000 x g; 2 min). The pellet was resuspended in 50 µl LB and spotted on a pre-dried LB agar plate and incubated overnight at 30 °C. Post-incubation, half of the 50-µl spot was then scraped from the plate, resuspended in 500 µl LB and pelleted (7,000 x g; 2 min). The cell pellet was washed once with LB and resuspended in 500 µl LB. Ten-fold diluted cells were spread onto carbenicillin-containing Simmons Citrate agar (BD) and incubated at 37 °C for two days to select against *E. coli*. Colonies were validated by PCR. The validated colonies were inoculated into LB, serially passaged, and plated in serial 10-fold dilutions onto either LB agar, carbenicillin-containing LB agar, or 10% sucrose-containing LB agar to assess the progress of plasmid (pEX18Ap) curing. Mutants that lacked the gene of interest were isolated from the 10% sucrose plate, identified by agarose electrophoresis of PCR products, and streaked onto LB agar (30 °C). Colonies were reconfirmed by PCR and tested for carbenicillin sensitivity (30 °C). On this penta-knockout background, Δ5 Δ*lpxA* was constructed by allelic exchange, resulting in a kanamycin resistance cassette incorporated into the *lpxA* locus in the chromosome. Briefly, ~700-bp flanking regions were PCR amplified and ligated to a kanamycin resistance cassette, aph(3')-IIIa, through Gibson assembly. The ligation product, after PCR amplified and purified, was introduced into the penta-knockout strain by electroporation. Following selection on LB agar containing 40 µg ml^{-1} kanamycin, single colonies were isolated and confirmed by PCR. The resulting *lpxA* deletion mutant was serially passaged in LB broth for 12 days to restore its growth profile and isolated into single colonies. To construct Δ5, the wild-type allele of *lpxA* was PCR amplified from the chromosome, purified and subsequently introduced into Δ5 Δ*lpxA* by electroporation. Following 2.5-hour recovery in 1 ml LB at 37 °C, approximately 50 µl cell cultures were transferred into 1 ml LB for overnight recovery. The recovered cells were spread onto LB agar containing 0.01 µg ml^{-1} rifampicin and incubated overnight at 37 °C. Single colonies were isolated and tested by PCR; those containing wild-type *lpxA* were then restreaked to test sensitivity to apramycin and kanamycin, and revalidated by PCR.

For *A. baumannii* single deletion strains, BLAST searches using a late-stage secondary acyltransferase (LpxL; NP_415572.1) of *E. coli* MG1655 against the fully sequenced genome *A. baumannii* ATCC 17978 (CP000521.1) returned three hits: 0431 (ABO10886.2), 1255 (ABO11683.2) and 2609 (ABO13026.2). Each of the three candidate genes were knocked out in wild-type *A. baumannii* ATCC 19606 to generate markerless deletions using biparental conjugation as described above. The *lpxC*::kan^R

single deletion strain was reported previously (1). The *adeIJK*::apr^R single deletion strain was constructed similarly as the *lpxC*::kan^R strain except cation-adjusted Mueller Hinton Broth (Fluka Analytical) or LB (no NaCl) was used instead of LB. Single colonies were isolated from LB agar containing 150 µg ml^{-1} apramycin and confirmed by PCR.

Construction of plasmids and transformation into respective strains. For *A. baylyi*. pBAD-HisA was modified to generate a plasmid with *A. baylyi lpxC*. A pWH1266 origin of replication was inserted adjacent to the native origin, the ampicillin resistance gene of pBAD-HisA was exchanged for the tetracycline resistance gene from pBR322, and the coding sequence of *A. baylyi* LpxC was cloned into the vector under the control of the promoter and RBS of pBAD-HisA. Transformation was performed as described above for *A. baylyi* knockouts, except cultures were plated on LB agar containing 0.1 µg ml^{-1} tetracycline after 2 hours instead of overnight.

For *A. baumannii*, the appropriate insert (wild-type *lpxA/C/D*, wild-type *msbA* or mutant *msbA* (L150V)) was PCR amplified from respective strains and gel purified. The resulting fragment was cloned into pWH1266 that was pre-digested with BamHI and SalI by Gibson assembly. The ligated product was transformed into commercially available stellar *E. coli* competent cells (Takara) and confirmed by Sanger sequencing. Mutations were introduced into the native ribosome binding site of wild-type MsbA or mutant MsbA (L150V) to lower protein expression levels by site-directed mutagenesis. The resulting constructs were transformed into stellar *E. coli* and confirmed by Sanger sequencing. The purified plasmids were electroporated into either Δ5 or **1**-resistant mutants as described above.

For plasmids for reconstitution, N-His$_{10}$-MsbA (WT or L150V), wild-type and mutant *msbA* (L150V) was PCR amplified from the genomic DNA of the corresponding strains and cloned into pET-19b using Gibson assembly with a N-terminal His-tag. Site-directed mutagenesis was employed to change the enterokinase cleavage sequence to a thrombin cleavage sequence. The sequence of the constructs was confirmed using Sanger sequencing and they were transformed into BL-21 (DE3) for expression.

Comparison of cellular effect caused by compounds 1 and 2. Overnight cultures of Δ5 and **1**^{R} MsbA (L150V) were diluted 100-fold and either left untreated or were treated with 2 µM compound **1** or **2**. Cells were incubated on a roller drum for 14 hours at 37 °C and prepared for fluorescence microscopy and TEM as described below.

Fluorescence labeling using Dansyl-PMBN and fluorescence microscopy. Cells were incubated for 30 min with 12 µM Dansyl-PMBN and analyzed by fluorescence microscopy (dansyl channel, λ_{ex}= 405/15 nm, λ_{em}=535/50 nm). Microscopy was carried out on a Nikon Ti fluorescence microscope equipped with a Prior Proscan III linear-encoded motorized stage. Images were obtained using a Plan Apo 100 x 1.4NA objective with a polarizer, DIC H condenser, DIC prism, and Hamamatsu ORCA-R2 cooled CCD camera. A Prior LumenPro fluorescence light source was used. Images were processed in ImageJ by adjusting contrast identically for compared image sets in the fluorescence channel.

Silver staining analysis of LPS and whole cell lysates. Samples were normalized to OD_{600} 0.2. Silver staining was performed following a published protocol (1).

Transmission electron microscopy. 500-µL cell cultures (prepared as described above) were mixed 1:1 with 500 µL of 2 x fixative solution. Final concentration of fixative was 2.5% Glutaraldehyde 1.25% Paraformaldehyde and 0.03% picric acid in 0.1 M sodium cacodylate buffer (pH 7.4). The cell mixture was centrifuged (8000 rpm, 5 min) and left to fix as a pellet overnight at 4 °C. The next day the pellet was washed in 0.1 M cacodylate buffer and post-fixed with 1% osmium tetroxide (OsO4)/1.5% potassium ferrocyanide (KFeCN6) for 1 hour, washed 2 x in water, 1 x in 50 mM maleate buffer pH 5.15 (MB) 1x and incubated in 1% uranyl acetate in MB for 1 hour followed by 2 washes in water and subsequent dehydration in grades of alcohol (10 minutes each; 50%, 70%, 90%, 2x10min 100%). The samples were then put in propyleneoxide for 1 hour and infiltrated overnight in a 1:1 mixture of propyleneoxide and Spurr's low viscosity resin (Electron Microscopy Sciences, Hatfield, PA). The following day the samples were embedded in Spurr's resin and polymerized at 60 °C for 48 hours. Ultrathin sections (about 60 nm) were cut on a Reichert Ultracut-S microtome, picked up on to copper grids stained with lead citrate and examined in a JEOL 1200EX transmission electron microscope and images were recorded with an AMT 2k CCD camera. Images were processed in ImageJ by adjusting contrast independently for each image.

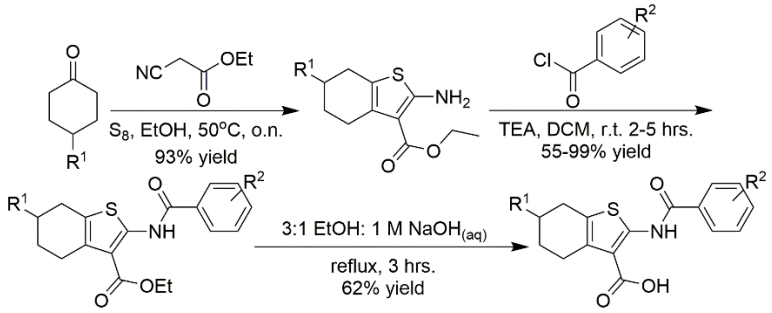
Overexpression and purification of N-His$_{10}$-MsbA (WT or L150V). A single carbenicillin resistant colony was inoculated into 7 mL LB broth containing 50 µg ml^{-1} carbenicillin and grown overnight. The culture was diluted 1,000-fold and grown until OD_{600} reached 0.8 (approximately 5 hours). The flasks were cooled to 18 °C, and isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.2 mM. Cells were grown at 18 °C overnight, and then harvested by centrifugation (5020 x g; 30 minutes). All subsequent steps were carried out at 0-4 °C. Cell pellets were washed with 50 mM Tris pH 8 containing 300 mM NaCl, flash-frozen with liquid nitrogen, thawed and resuspended in 50 mM Tris pH 8 containing 300 mM NaCl, 0.1 mM PMSF, 10 µg ml^{-1} lysosyme and 10 µg ml^{-1} DNAse. Cells were broken up by three passages through a French press cell disruptor at 15,000 psi. Debris was cleared from the crude lysate by centrifugation (5000 x g; 15 minutes). Membrane was harvested by ultracentrifugation (37,000 x g; 30 minutes). The pellet was homogenized in 50 mM Tris pH 8 containing 300 mM NaCl, 0.1 mM PMSF, and 10 µg ml^{-1} lysozyme. DDM was added (final concentration 1% w/v) and the resulting solution was incubated with rotation for 2 hours. Insoluble material was removed by ultracentrifugation (37,000 x g; 30 minutes). The soluble fraction was loaded onto a pre-equilibrated nickel-agarose column. The column was washed with 5 column volumes of 50 mM Tris pH 8 containing 300 mM NaCl, 0.05% w/v DDM and 20 mM imidazole and protein was eluted with 3 column volumes of 50 mM Tris pH 8 containing 300 mM NaCl 0.05% w/v DDM and 200 mM imidazole. Protein was 20-fold concentrated using a 50 kDa molecular weight cutoff concentrator, and purified by size-exclusion chromatography on a Superdex 200 increase column in buffer containing 25 mM Tris pH 8.0, 150 mM NaCl, 0.03% w/v DDM and 5% v/v glycerol. Purity was examined by SDS-PAGE and purified MsbA was concentrated and stored at - 80 °C.

Reconstitution of Purified N-His$_{10}$-MsbA. We found that *A. baumannii* MsbA complements the temperature-sensitive *E. coli msbA* mutant, meaning that the *A. baumannii* protein can flip *E. coli* LPS and function in *E. coli* lipids. Reconstitutions were therefore carried out using commercially available *E. coli* phospholipids (polar lipid extract, Avanti) and rough lipopolysaccharide (LPS) from Ra mutant *E. coli* EH100 (Sigma Aldrich). All steps were carried out at 4 °C. 30 mg ml^{-1} aqueous *E. coli* phospholipids were subjected to sonic irradiation in a bath sonicator for 5 minutes and then homogenized by seven passages through a 27-guage needle. 2 mg ml^{-1} aqueous LPS was also sonicated for 2 minutes (10 seconds on, 10 seconds off). These two solutions were mixed with buffer to form 770 µl of a mixed micelle solution containing 7.8 mg ml^{-1} *E. coli* phospholipids, 0.52 mg mL^{-1} LPS, 0.2% w/v DDM, 52 mM pH 7.5 HEPES, 52 mM NaCl, 5.2 mM $MgCl_2$, and 2 mM β-mercaptoethanol. The mixed micelles incubated for 10 minutes, then 30.08 µl 1.9 mg ml^{-1} of the purified *N*-His$_{10}$-MsbA solution was added. The resulting mixture was kept for 20 minutes, and then diluted 100-fold with cold 50 mM HEPES pH 7.5 containing 50 mM NaCl, 5 mM $MgCl_2$ and 2 mM β-mercaptoethanol (buffer A). Liposomes were pelleted by ultracentrifugation (64,000 rpm; 2 hours) at 4 °C. The liposomes were resuspended in 80 mL buffer A, and pelleted by ultracentrifugation (64,000 rpm; 2 hours). The pellets were resuspended in 2 mL 50 mM HEPES pH 7.5 containing 50 mM NaCl, 5 mM $MgCl_2$ and 1 mM dithiothreitol. Proteoliposomes were stored at -80 °C, and all ATPase assays were carried out using proteoliposomes that had undergone a freeze-thaw step and then sonicated for several minutes until homogenous (10 seconds on, 10 seconds off).

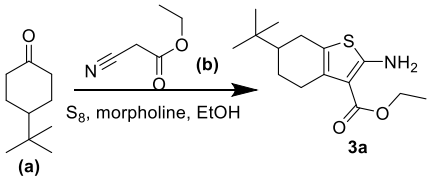
ATPase Assay. Liposomes containing MsbA were assayed for ATPase activity at a protein concentration of 6.4 µg ml^{-1} in a 25-µL reaction mixture containing 50 mM HEPES pH 7.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM NaCl, 2 mM ATP and 0-10 µM of a small molecule as appropriate. All the components were pre-incubated on ice for 30 minutes before the addition of ATP. Samples were incubated for 0, 15, 30 and 45 minutes at 37 °C after the addition of ATP, and then the reactions were stopped by the addition of 25 µl 12% SDS. The amounts of P_i released were determined by a published colorimetric method, and potassium phosphate was used as a standard (4). After the addition of SDS, 50 µl of a solution containing equal volumes of 60 mg ml^{-1} ascorbic acid in 1 N HCl and 10 mg ml^{-1} ammonium molybdate in 12% SDS was added. The samples were incubated at room temperature for 7 min and 75 µl of an aqueous solution containing 20 mg mL^{-1} sodium citrate tribasic dihydrate, 2 mg mL^{-1} sodium arsenite, and 2% v/v acetic acid was added. The absorbance at 850 nm was measured after 20 minutes. The assay was linear in the range of 0.05-1.2 mM P_i.

Chemical synthesis of compounds 1-4. All reactions were performed in oven-dried glassware equipped with PTFE-coated magnetic stir bars, fit with rubber septa, and under N_2 atmosphere unless otherwise specified. Commercial reagents were purchased at the highest purity available from Oakwood Scientific, Sigma Aldrich, TCI, Combi-blocks, and Enamine and used without further purification. Dichloromethane and triethylamine were dried by passing through columns of activated alumina. 0.25-mm pre-coated silica gel

plates from Silicycle Inc. with fluorescent indicator were used for thin layer chromatography. Column chromatography was performed using a Combiflash and RediSep Rf disposable columns filled with 40 to 63 µm silica gel. NMR spectra were collected using Joel 400 (400 MHz), Varian Unity/Inova 500 (500 MHz) or 600 (600 MHz) spectrometers at the Harvard University NMR facility and chemical shifts are reported relative to the major deuterated solvent peak as reported in literature (5). The splitting patterns of the peaks are described as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). High resolution mass spectrometric data were obtained on an Agilent 6210 time-of-flight HPLC/MS spectrometer (ESI-TOF) at the Harvard University NMR facility. Overall scheme and each step of the synthesis is detailed below.



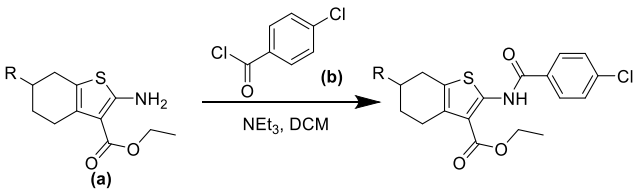
Step 1: General procedure for amino-tetrahydrobenzothiophene formation



Morpholine (0.84 mL) was added dropwise to a stirred solution of **(a)** (6.5 mmols), **(b)** (7.1 mmols), and S_8 (7.1 mmols) in EtOH (19 mL) at 0 °C. The solution was warmed to 50 °C and stirred overnight. The reaction mixture was concentrated *in vacuo* and purified by flash column chromatography (hexanes: ethyl acetate) affording 1.6991 g (93% yield) product.

3a (Ethyl 2-amino-6-(tert-butyl)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate): ^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.07 (s, 2H), 4.36 – 4.16 (m, 2H), 3.08 – 2.89 (m, 1H), 2.66 – 2.40 (m, 2H), 2.28 (ddt, *J* = 17.2, 11.4, 2.4 Hz, 1H), 2.03 – 1.87 (m, 1H), 1.46 (tdd, *J* = 11.7, 4.9, 2.1 Hz, 1H), 1.37 – 1.18 (m, 4H), 1.03 – 0.82 (m, 9H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 166.10, 162.18, 132.32, 117.95, 59.31, 45.16, 32.38, 28.06, 27.27, 26.02, 24.41, 14.47. HRMS (ES+) m/z=282.1522 ([M+H]$^+$, calcd:282.1527).

Step 2: General procedure for acylation

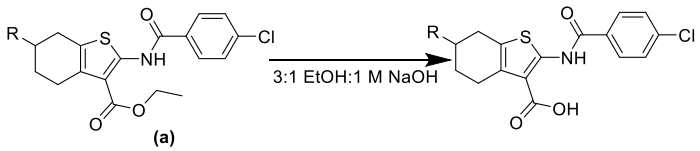


Triethylamine (1.5 equiv.) was added dropwise to a stirred solution of **(a)** (0.25 g, 1 equiv.) in dichloromethane (7 mL) at 0 °C. **(b)** (1.1 equiv.) was then added dropwise. The solution was warmed to room temperature and stirred overnight. The reaction was diluted (dichloromethane), washed (10 mL 1 N HCl x 1, 10 mL $NaHCO_3$ x 1), dried (Na_2SO_4), filtered, concentrated *in vacuo*, and purified by flash column chromatography (dichloromethane: methanol).

2 (Ethyl 2-(4-chlorobenzamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate): 0.4527 g (56% yield) obtained. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 12.33 (s, 1H), 7.97 – 7.90 (m, 2H), 7.48 (d, J = 8.5 Hz, 2H), 4.36 (q, J = 7.1 Hz, 2H), 2.78 (d, J = 5.1 Hz, 2H), 2.68 (t, J = 5.3 Hz, 2H), 1.81 (h, J = 5.8 Hz, 4H), 1.40 (t, J = 7.2 Hz, 3H). ^{13}C NMR (126 MHz, Chloroform-d) δ 167.07, 162.21, 147.79, 138.75, 131.84, 131.08, 130.91, 129.15, 128.81, 127.25, 112.10, 77.32, 77.06, 76.81, 60.68, 26.39, 24.42, 22.97, 22.82, 14.33. HRMS (ES+) m/z=364.0769 ([M+H]$^+$, calcd:364.0774).

3b (Ethyl 6-(tert-butyl)-2-(4-chlorobenzamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate): 0.3684 g (99% yield) obtained. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 12.27 (s, 1H), 8.11 – 7.96 (m, 1H), 7.82 (ddd, J = 7.8, 1.8, 1.1 Hz, 1H), 7.66 – 7.37 (m, 2H), 4.35 (q, J = 7.1 Hz, 2H), 3.03 (ddt, J = 17.2, 5.2, 1.7 Hz, 1H), 2.70 (ddt, J = 15.8, 4.9, 1.6 Hz, 1H), 2.64 – 2.49 (m, 1H), 2.40 (dddd, J = 16.2, 11.6, 3.3, 1.7 Hz, 1H), 2.00 (ddd, J = 12.8, 5.6, 2.3 Hz, 1H), 1.54 – 1.41 (m, 1H), 1.45 – 1.29 (m, 3H), 1.31 – 1.19 (m, 1H), 0.93 (s, 9H). ^{13}C NMR (100 MHz, Chloroform-d) δ 166.89, 161.84, 147.66, 135.17, 134.70, 134.26, 132.35, 131.12, 130.42, 130.24, 130.10, 128.62, 128.00, 127.98, 125.08, 111.91, 77.37, 77.05, 76.74, 60.69, 44.90, 32.42, 27.48, 27.28, 25.85, 24.39, 14.32, 9.91. HRMS (ES+) m/z=420.1395 ([M+H]$^+$, calcd:420.1400).

Step 3: General procedure for saponification



1 M NaOH (7 mL) was added to a solution of **(a)** (200 mg) in EtOH (21 mL). The solution was warmed to reflux for 3 hours. The reaction was then cooled to room temperature, diluted (25 mL 1 N HCl), extracted (EtOAc x 3), dried (Na_2SO_4), filtered, concentrated *in vacuo*, and purified by flash column chromatography (dichloromethane: methanol).

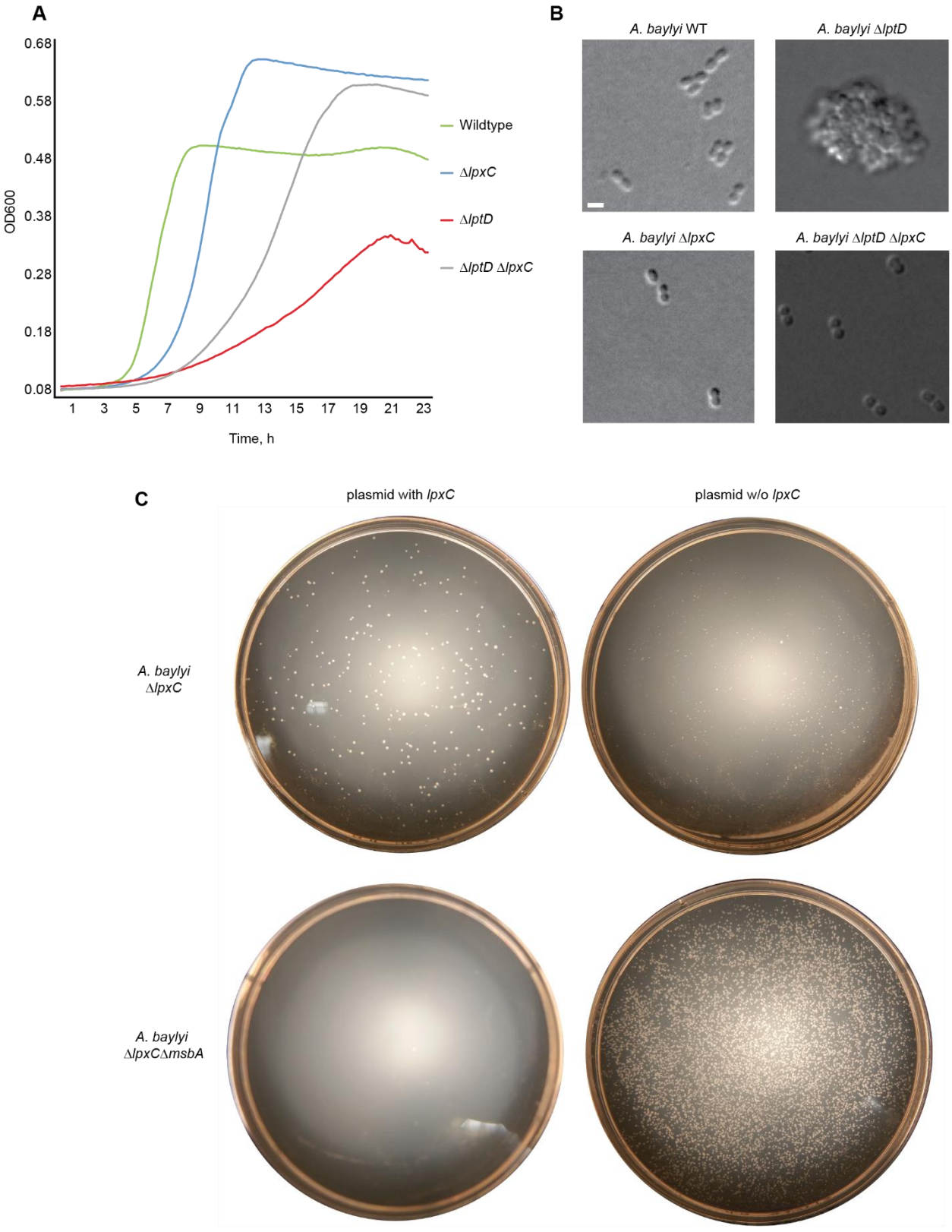
1 (2-(4-chlorobenzamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid): 0.1145 g (62% yield) obtained. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.28 (s, 1H), 12.39 (s, 1H), 7.89 (d, *J* = 7.6 Hz, 2H), 7.68 (d, *J* = 7.6 Hz, 2H), 2.73 (s, 2H), 2.62 (s, 2H), 1.72 (s, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.16, 146.79, 138.11, 131.81, 131.51, 129.88, 129.48, 126.85, 113.41, 40.68, 40.48, 40.27, 40.06, 39.85, 39.64, 39.43, 26.39, 24.33, 23.09, 22.79. HRMS (ES+): 336.0456 ([M+H]$^+$, calcd:336.0461).

3 (6-(tert-butyl)-2-(4-chlorobenzamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid)**:** 0.1158 g (62% yield) obtained. ^{1}H NMR (500 MHz, DMSO-d_6) δ 13.35 (s, 1H), 12.39 (s, 1H), 7.98 – 7.58 (m, 4H), 3.05 (dd, *J* = 17.2, 4.8 Hz, 1H), 2.70 (dd, *J* =

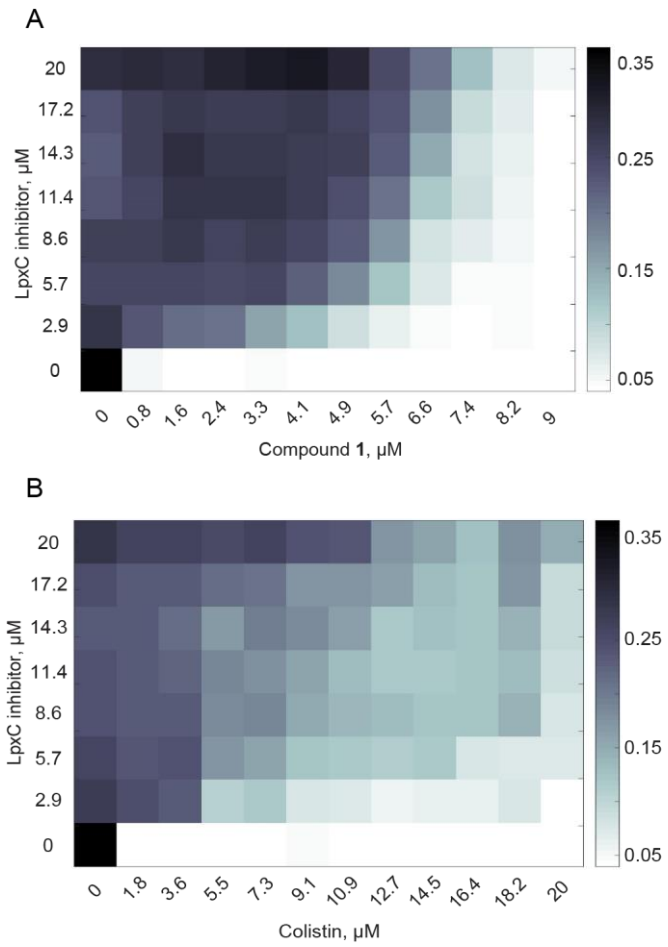
15.9, 4.7 Hz, 1H), 2.51 (s, 1H), 2.39 (t, *J* = 14.0 Hz, 1H), 1.98 (d, *J* = 10.9 Hz, 1H), 1.44 (tdd, *J* = 11.9, 4.6, 2.0 Hz, 1H), 1.23 (tt, *J* = 12.6, 6.3 Hz, 1H), 0.92 (s, 9H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.10, 146.80, 134.87, 134.47, 133.02, 131.86, 131.75, 127.58, 126.11, 113.31, 44.96, 40.69, 40.48, 40.27, 40.06, 39.85, 39.64, 39.43, 32.73, 27.66, 27.43, 25.88, 24.45. HRMS (ES+) m/z=414.0901 ([M+Na]$^+$, calcd:414.0906).

4 (2-(4-chlorobenzamido)-6-methyl-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid): This compound was purchased and used as received. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.43 (s, 1H), 7.90 (d, *J* = 8.4 Hz, 2H), 7.69 (d, *J* = 8.3 Hz, 2H), 2.98 – 2.89 (m, 1H), 2.73 (dd, *J* = 16.0, 4.9 Hz, 1H), 2.63 (ddd, *J* = 17.3, 11.5, 6.2 Hz, 1H), 2.24 (dd, *J* = 16.0, 9.6 Hz, 1H), 1.87 – 1.78 (m, 2H), 1.39 – 1.26 (m, 1H), 1.03 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.15, 162.12, 146.91, 138.11, 131.52, 131.49, 129.88, 129.47, 126.49, 113.26, 40.68, 40.47, 40.27, 40.06, 39.85, 39.64, 39.43, 32.36, 31.04, 29.31, 26.17, 21.74. HRMS (ES+): 372.0432 ([M+Na]$^+$, calcd:372.0437).

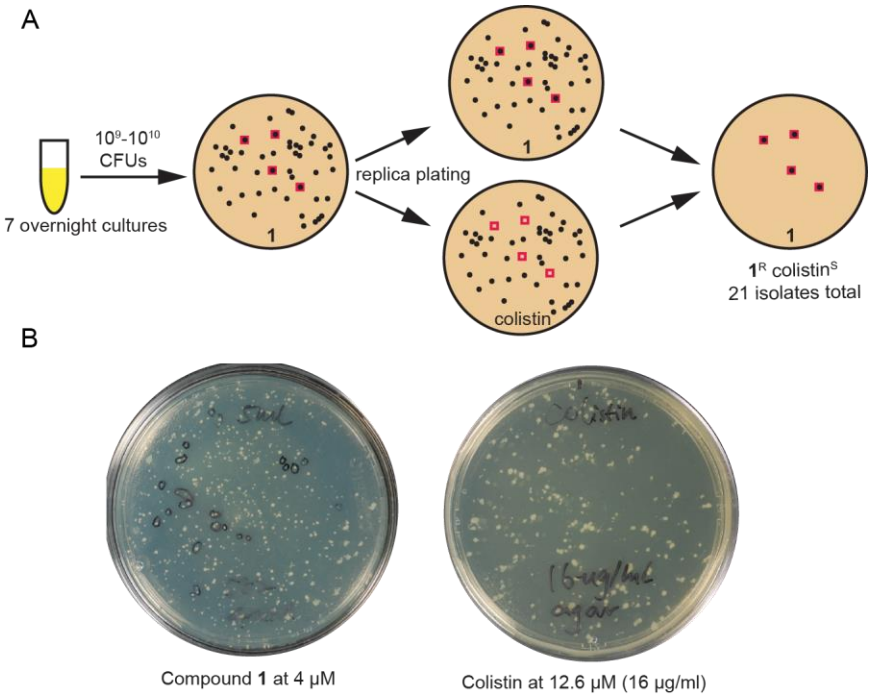
SI Figures and Tables



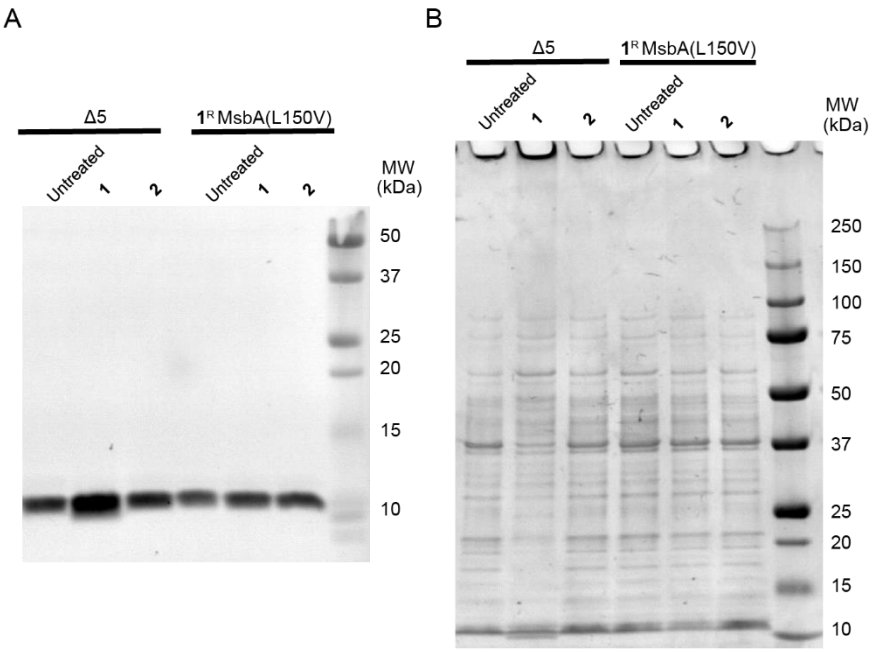
Supplementary Figure 1. **Active LPS biosynthesis underlies the conditional essentiality of the LPS biogenesis genes in *A. baylyi.*** (*A*) Complete removal of LPS by knocking out *lpxC* does not negatively affect the growth rate of *A. baylyi*. Inactivation of the terminal steps of LPS biogenesis causes growth defects. The growth of the Δ*lptD* strain is slow and stunted. This defect is remedied by removal of LPS, indicating that it is caused by improper LPS targeting. The double knockout (Δ*lptD* Δ*lpxC*) grows at a somewhat slower rate than the WT likely due to the presence of multiple antibiotic resistance cassettes. (*B*) Wild-type *A. baylyi* and a Δ*lpxC* mutant display the normal well-separated coccobacillus morphology typical of *Acinetobacter*. In contrast, Δ*lptD A. baylyi* cells form clumps. Δ*lptD* Δ*lpxC A. baylyi* does not display this phenotype, indicating that the inability to carry out LPS biogenesis to completion underlies the clumping phenotype. Scale bar: 2 μm. (*C*) A plasmid expressing LpxC cannot be transformed into Δ*lpxC* Δ*msbA A. baylyi*, while it is readily transformed into Δ*lpxC A. baylyi*. The same plasmid without LpxC can be transformed into either of the two strains, indicating that active LPS biosynthesis is incompatible with the loss of MsbA.



Supplementary Figure 2. Drug-drug interaction landscapes indicate pathway engagement. (*A*) Treatment of Δ5 cells with a known LpxC inhibitor (PF-5081090) showed dose-dependent suppression against compound **1**. (*B*) A similar pattern was observed between the LpxC inhibitor and colistin. Color bar on the right indicates OD values.



Supplementary Figure 3. Compound 1-resistant, colistin-sensitive mutants were identified by counter selection with colistin. (*A*) Schematic of colony screening method to identify *msbA* mutants. Colonies that were resistant to compound **1** (4 µM) are counter screened by replica plating to assess their sensitivity to colistin (12.6 µM); those that are sensitive to colistin (red box) were non-*lpx* null mutants. (*B*) 5-ml overnight culture of Δ5 was spread onto a LB agarose plate containing 4 µM compound **1**. Compound **1**-resistant colonies appeared on the selection plate after overnight incubation (*left*). A subset of these colonies that appeared colistin-sensitive (circled, *left*) was identified by replica plating onto a LB agar plate containing 12.6 µM (16 µg/ml) colistin (*right*). Subsequent examination showed that these colonies contain mutations in *msbA*.



Supplementary Figure 4. Treatment with compound 1 causes increase in LPS levels as determined by silver stained SDS-PAGE gel. Samples were normalized by OD to 0.2. Δ5 and compound **1**-resistant cells, diluted 100-fold from the respective overnight cultures, were treated with compound **1** or **2** at 2 μM for 14 hours before being stained with Dansyl-PMBN (12 μM) for imaging; no-treatment control was included as a comparison. (*A*) Treating Δ5 cells with compound **1** led to increase in LPS levels compared to no treatment, **1**-treated resistant mutant (**1**^{R} MsbA (L150V)), or **2**-treated both strains. (*B*) Cell lysate loading control of the corresponding samples in (*A*) showed comparable levels of cell lysates, indicating that increase in LPS levels in compound **1**-treated Δ5 cells reflected changes of LPS amount, not due to uneven loading.

Supplementary Table 1. MIC values of three single knockouts in secondary acyltransferases of lipid A biosynthesis.

Strain	MIC (μM; μg/ml in parentheses)			
	Rifampicin	Bacitracin	Vancomycin	Colistin
WT	0.36 (0.3)	75 (110)	86 (128)	1.6 (2)
Δ0431	0.17 (0.14)	19 (27)	43 (64)	1.6 (2)
Δ1255	0.36 (0.3)	75 (110)	86 (128)	1.6 (2)
Δ2609	0.17 (0.14)	19 (27)	22 (32)	0.8 (1)

MICs are measured in micromolar or micrograms per milliliter (shown in parentheses).

Supplementary Table 2. Small molecule screening data

Category	Parameters	Description
Assay	Type of assay	Cell-based
	Target	LPS biosynthesis and transport
	Primary measurement	Optical density at 600 nm
	Reagents	*A.baumannii* 19606 Δ5, Δ5 Δ*lpxA*, colistin
	Assay protocol	37℃ incubation for 24 h in 40 µl LB
	Additional comments	The Δ5 Δ*lpxA* strain reaches a maximum OD_{600} that is slightly lower than Δ5
Library	Library size	5 µM final concentration in DMSO
	Library composition	Commercially available and bioactive compounds
	Source	Diversity sets I and II provided by California Institute for Biomedical Research (Calibr)
Screen	Format	384 well (Corning 3701)
	Plate controls	Positive control: colistin (30 µg/ml); negative control: DMSO or none
	Reagent and compound dispensing systems	Liquid media: Thermo Multidrop Combi; compounds: Labcyte Echo 555 Liquid Handler
	Detection instrument and software	PerkinElmer Envision; Genedata Screener
	Assay validation	Z' > 0.5 for Δ5
	Correction factor	None
	Normalization	None
Post-HTS analysis	Hit criteria	Top 500 hits having the lowest normalization score in Diversity set I; top 604 hits that inhibited the growth of the Δ5 strain (OD < 0.2) in Diversity set II
	Hit rate	0.007% (n = 11 of 151,781, including 150,000 library compounds and 1781 analogs)
	Additional assays	SAR by catalog, followed by counter screen at 5 µM against both Δ5 and Δ5 Δ*lpxA* in 384-well plates. Reconfirmed hits that preferentially inhibited Δ5 than Δ5 Δ*lpxA* and lowered the OD of Δ5 to 0.2 or below were retested for dose response in both 384- and 96-well plates. Assay conditions: LB, 37 °C, 24 h
	Confirmation of hit identity	Synthesized independently, ^{1}H-NMR, ^{13}C-NMR, LC/MS

Supplementary Table 3. The removal of the entire LPS pathway through loss-of-function mutations in either *lpxA, lpxC or lpxD* confers resistance to compound 1.

lpx gene affected	Mutation	Position	Protein mutation
lpxA	Deletion (G to –)	608	Frameshift to truncation
	Substitution (T to G)	200	V67 to G
lpxC	Substitution (C to T)	508	Q170 to STOP
	Deletion (T to –)	525	Frameshift to truncation
	Insertion of 1.1 kb fragment with target site duplication (TAAAAAT)	392	Disrupted
lpxD	Deletion (ATG to –)	689-691	D230 to –; in-frame deletion

Supplementary Table 4. A complete list of mutations in MsbA that confers resistance to compound 1. Resistant colonies were isolated from seven independent cultures, with three or four from each culture.

MsbA mutation	Number of isolates
L150V	10
L68F	3
E36A	1
V39F	1
V39G	1
L58P	1
P170L	1
T283M	1
*ΔVN298-299	2

Asterisk (*) indicates mutation that conferred moderate resistance to compound **1** (MIC 1.8 µM) and high sensitivity to rifampicin (MIC < 0.004 µM).

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