Supporting Information

A fluorescent probe distinguishes between inhibition of early and late steps of lipopolysaccharide biogenesis in whole cells

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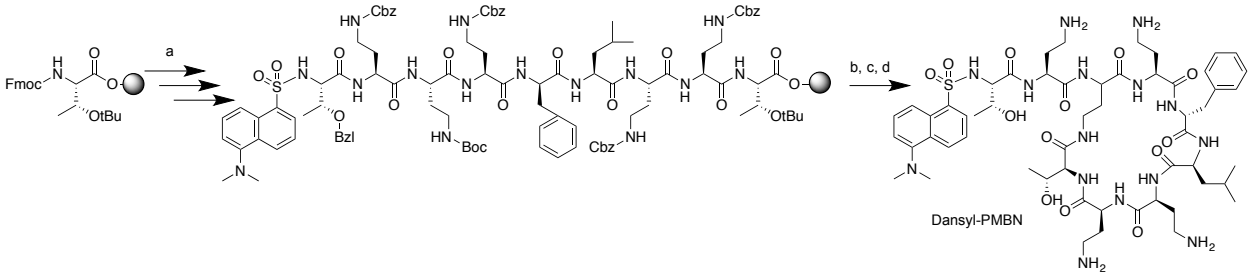
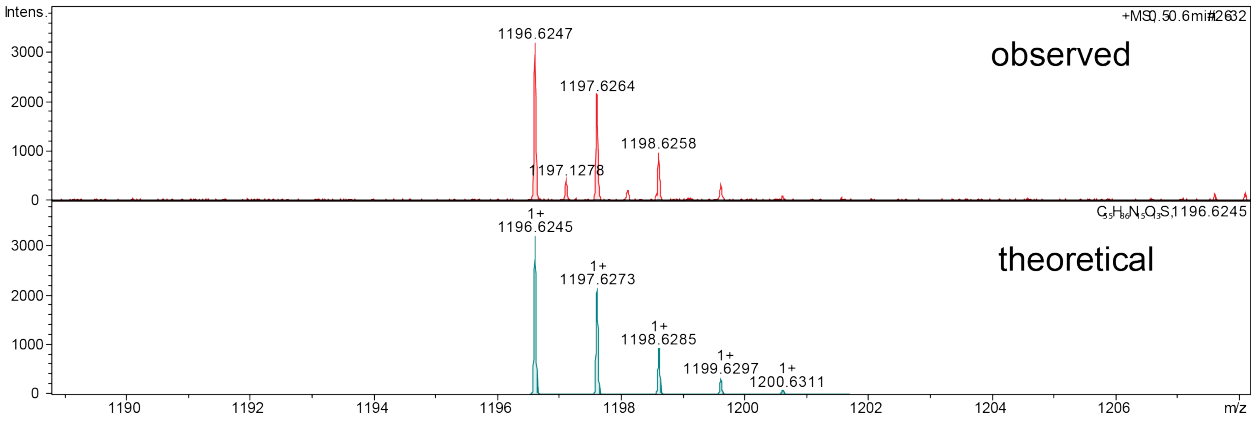
Scheme S1. Synthesis of fluorescent probe Dansyl-PMBN. (a) Fmoc solid phase synthesis using pre-loaded Wang resin; (b) 95 % TFA in H_2O; (c) HOBt (4 eq.), PyBOP (4 eq.), *N,N*-diisopropylethylamine (8 eq.) in DMF; (d) TMS-Cl (80 eq), NaI (80 eq.) in acetonitrile**.**



[M+H]+



Measured m/z	#	Ion Formula	m/z	Err [ppm]	mSigma	# mSigma	Score	rdb	E-Conf	N-Rule
1196.6247	1	C55H86N15O13S	1196.625	-0.2	44.7	1	100	20.5	even	ok

Figure S1. High resolution mass spectrum of Dansyl-PMBN. The compound was analyzed by ESI-MS: $C_{55}H_{85}N_{15}O_{13}S$ *m/z* calculated, $[M+H]^+$ 1196.6245; observed, $[M+H]^+$ 1196.647.

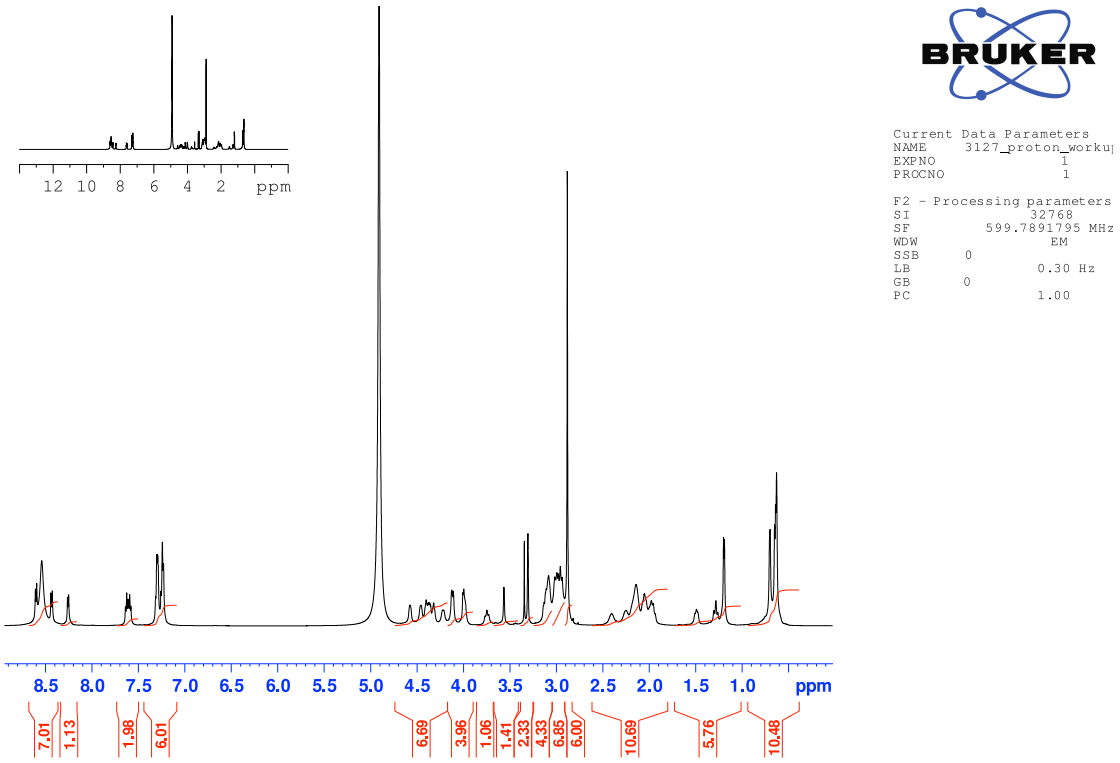


Figure S2. ^{1}H NMR of Dansyl-PMBN. The ^{1}H NMR was acquired using an Agilent DD2-600 NMR.

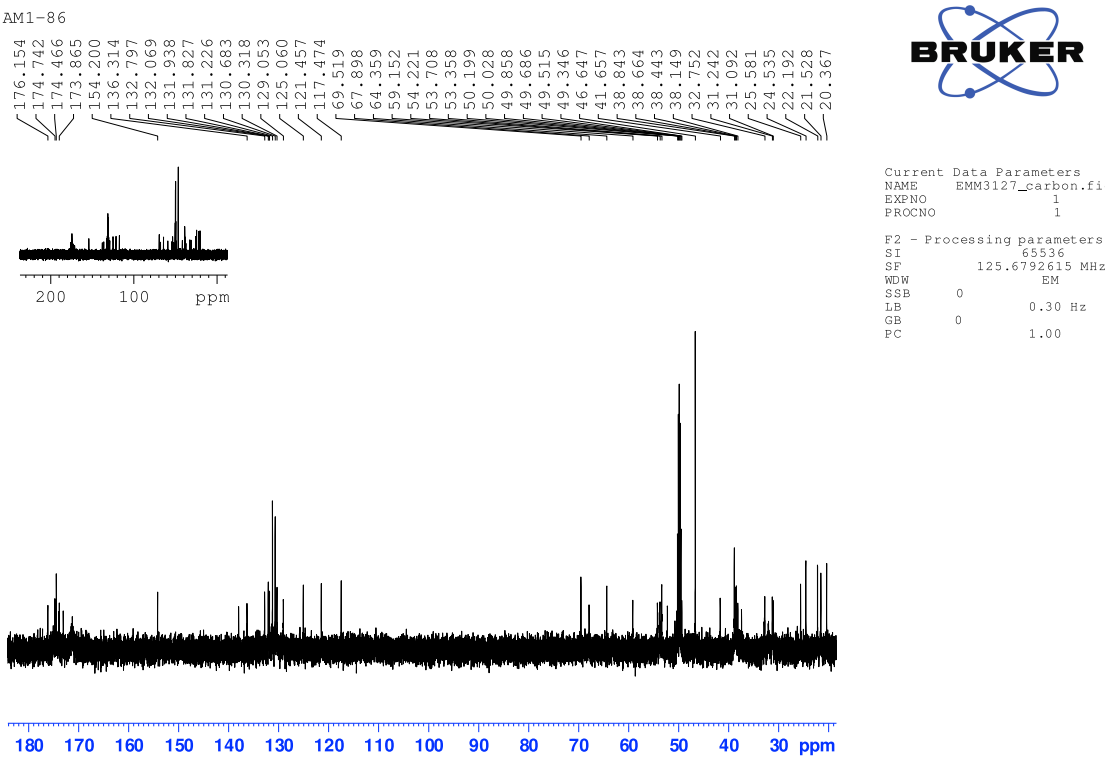


Figure S3. ^{13}C NMR of Dansyl-PMBN. The ^{13}C NMR was acquired using an Agilent DD2-600 NMR.

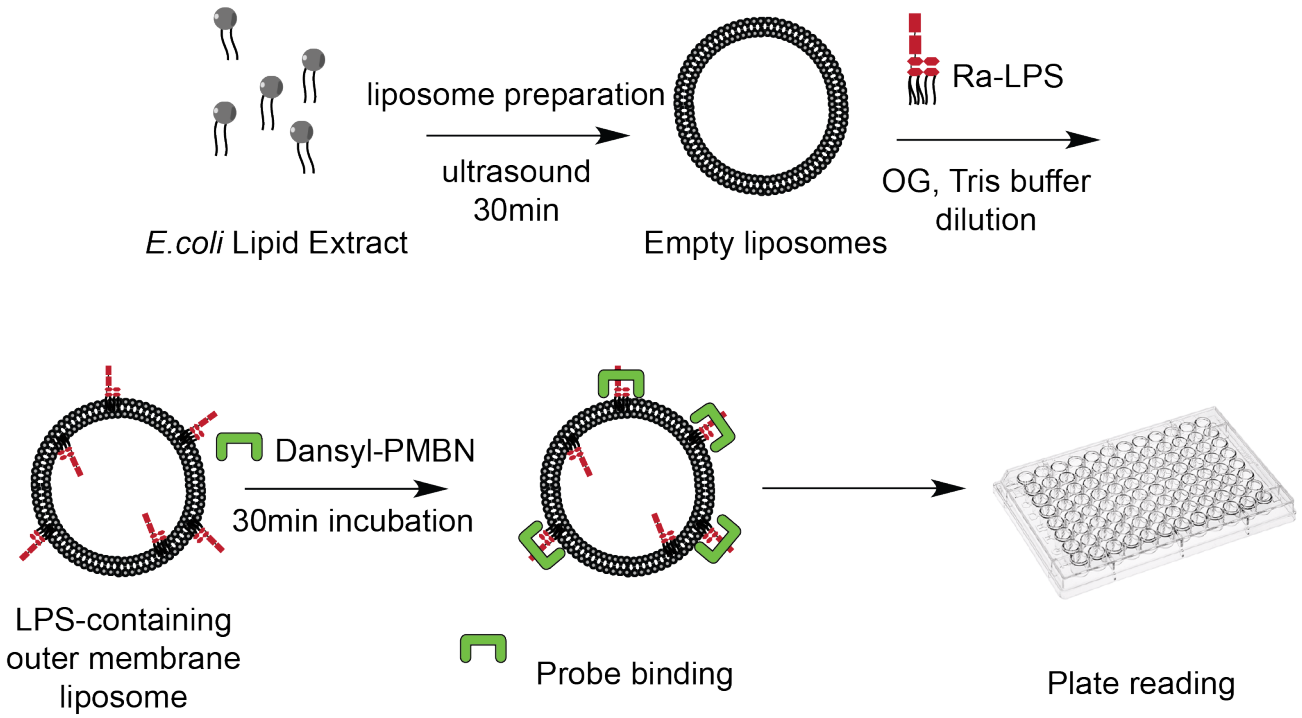


Figure S4: Schematic of the liposome binding assay. Lipid extract from *E. coli* was dissolved in water and sonicated to prepare liposomes, and Ra-LPS was next incorporated into the lipid bilayer using a detergent-dilution method. The as-prepared liposomes were then incubated with Dansyl-PMBN, and the fluorescence was measured using a 96-well plate reader.

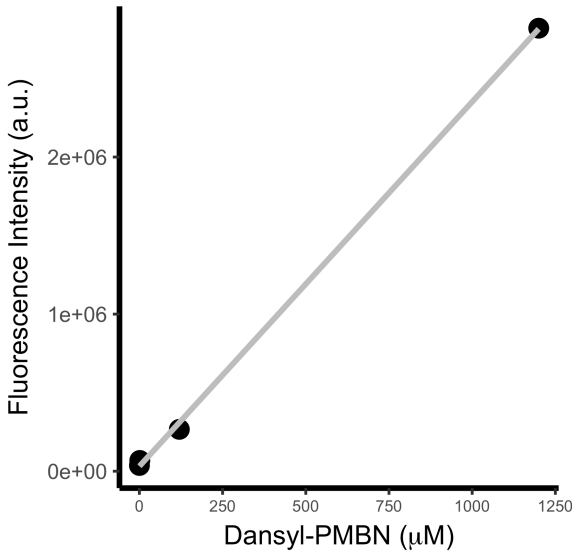


Figure S5. Standard curve of Dansyl-PMBN. Dansyl-PMBN at concentrations ranging from 0.12 μM to 1200 μM were prepared in TBS (pH=8.0). Samples were read on a fluorescent plate reader using E_x/E_m=340/520nm.

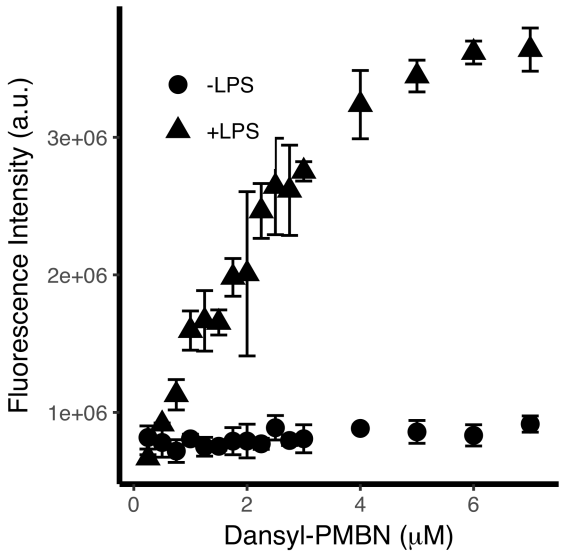


Figure S6. Dansyl-PMBN fluorescence intensity is LPS dependent. Dansyl-PMBN was titrated into a cuvette containing 1 mL of 5 mM TBS buffer (pH=8.0) and 3 μg of Ra-LPS (+LPS) or TBS buffer alone (-LPS). Samples were read on a fluorescent plate reader using E_x/E_m=340/520nm. Error bars represent the standard deviation from three replicate experiments.

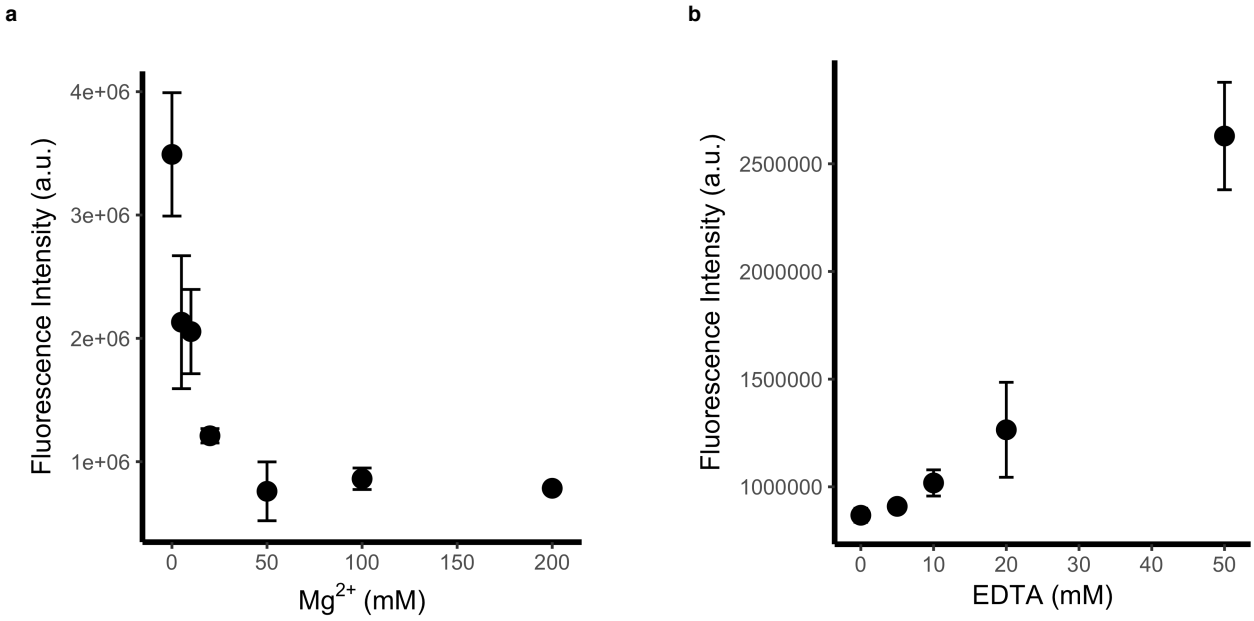


Figure S7. Dansyl-PMBN binding mechanism is similar to PMB. (a) Inhibition of Dansyl-PMBN binding to LPS by Mg^{2+}. Ra-LPS was present in a cuvette at a concentration of 3 μM in 5 mM TBS buffer (pH=8.0). $MgCl_2$ was added to the same cuvette in portions, and the fluorescence emission at 520 nm was determined after each addition. (b) Reversal of inhibition by EDTA. When final concentration of Mg^{2+} was 50 mM, EDTA was titrated into the cuvette to the final concentrations indicated above. Error bars represent the standard deviation from three replicate experiments.

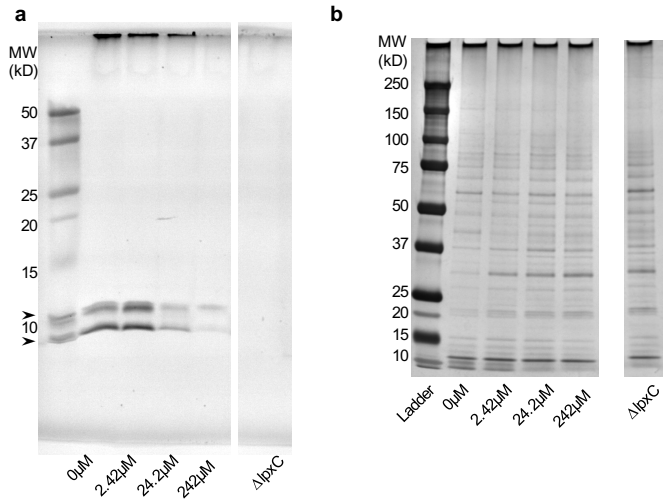


Figure S8. LPS and cell lysate gels for Figure 3. (a) Full gel including molecular weight markers for LPS levels as monitored by silver stain (Figure 3b). ➤ refers to bands that are not present in pre-stained molecular weight marker but are stained during LPS silver stain protocol. (b) Cell lysate loading controls for the respective samples in Figure 3b and part (a).

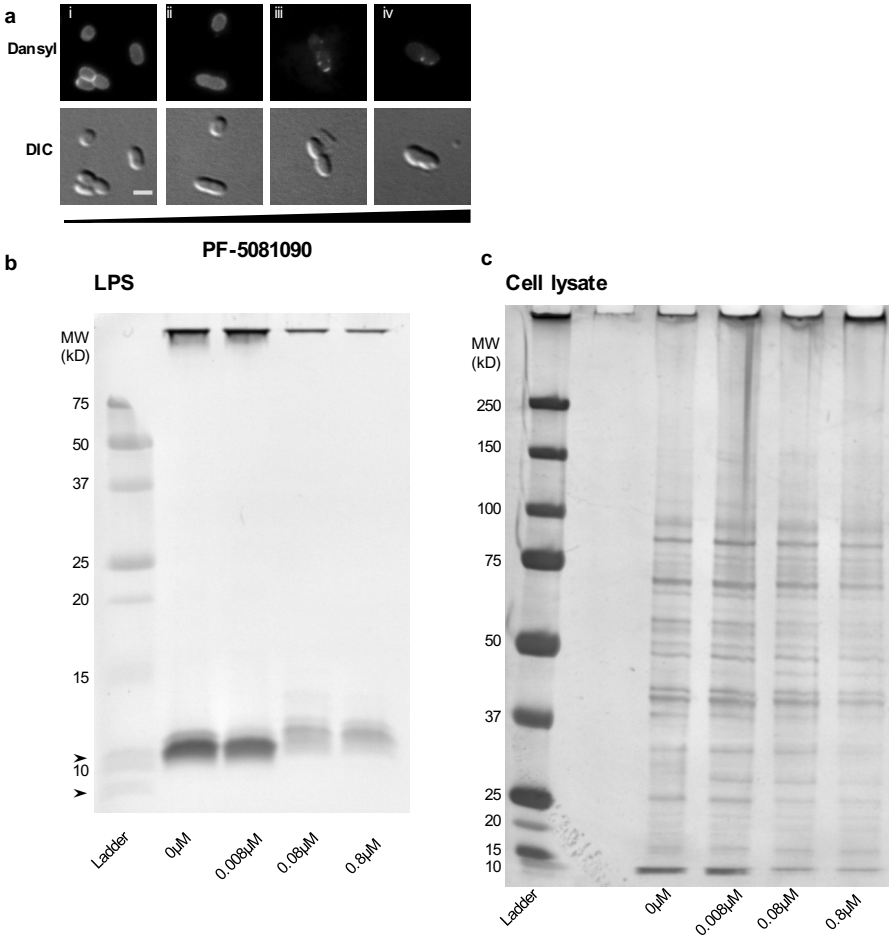


Figure S9: Decreases in LPS levels can be measured in *E. coli.* *E. coli* NR754 was treated with LpxC inhibitor PF-5081090 for 2 hours and samples were saved for imaging and silver stain analysis. (a) Images show a decreased fluorescence. (i) 0 μM (ii) 0.008 μM (iii) 0.08 μM (1x MIC) (iv) 0.8 μM. Scale bar: 2 μm. Punctae in panels 3 and 4 were assumed to be due to induced permeability defects upon exposure to inhibitory concentrations of PF-5081090. (b) LPS levels by silver stain show a decrease upon PF-5081090 treatment. The presence of higher molecular weight LPS structures is likely due to increased modification of the small amounts of LPS remaining in cells. ➢ refers to bands that are not present in pre-stained molecular weight marker but are stained during LPS silver stain protocol. (c) Cell lysate loading controls for the respective samples in (b).

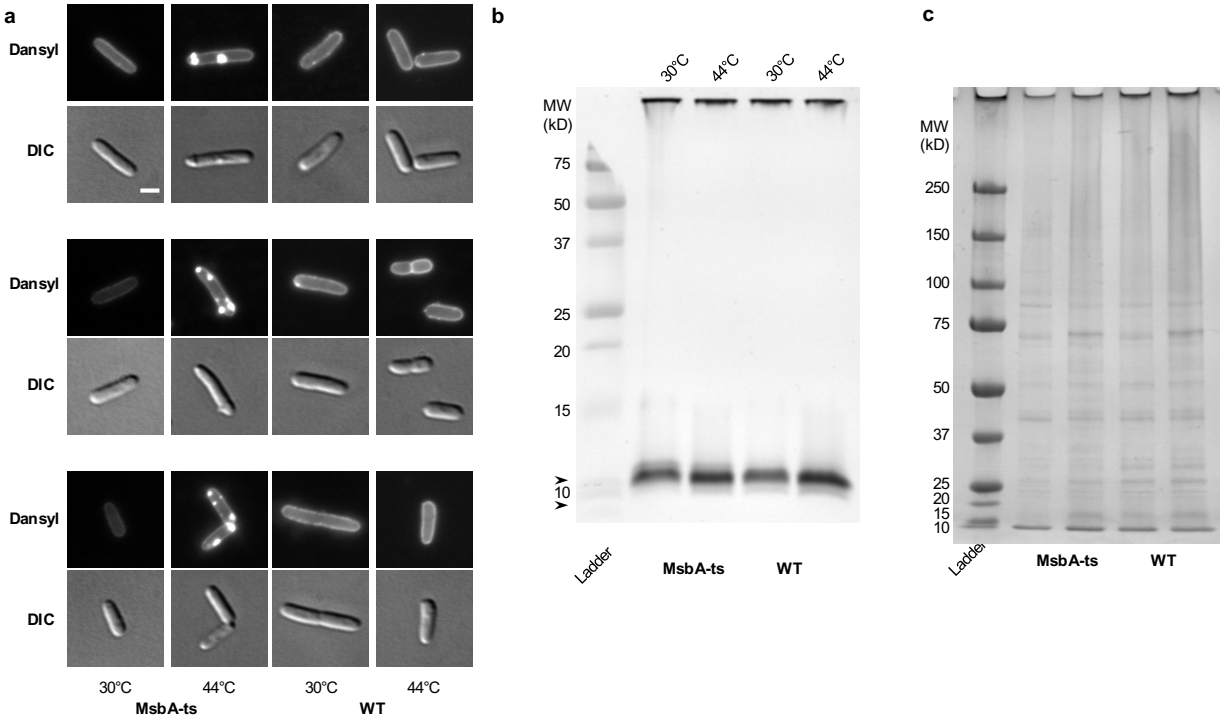


Figure S10. MsbA inactivation in *E. coli.* (a) Additional fluorescence images for the samples presented in Figure 4. Scale bar: 2 μm. (b) Full gel including molecular weight markers for LPS levels as monitored by silver stain (Figure 4b). ➤ refers to bands that are not present in pre-stained molecular weight marker but are stained during LPS silver stain protocol. (c) Cell lysate silver stain for the samples presented in Figure 4.

Table S1. MIC values of PMB, PMBN, and Dansyl-PMBN.

MIC	*A. baumannii* 19606	*E. coli* NR754	*E. coli* WD2	*E. coli* LCB273	*B. subtilis*
PMBN	>40μM	>40μM	>40μM	>40μM	>40μM
PMB	0.312μM	0.078μM	0.039μM	0.078μM	20μM
Dansyl-PMBN	>40μM	0.312μM	0.312μM	0.312μM	20μM

Table S2: Dynamic light scattering characterization of liposomes. Error bars represent the standard deviation from three replicate experiments. Diameters were shown in Z-average.

Diameter (nm) Z-average	Before addition of Dansyl-PBMN	After addition of Dansyl-PMBN
PL liposome with LPS	175.4 ± 3.4	174.2 ± 1.5
PL liposome without LPS	168.9 ± 5.7	172.1 ± 8.4

Table S3: Primers used for the construction of *A. baumannii* mutants

Primer	Sequence (5'→3')	Application
C1	GAAGGTGAAATCTTCTACGG	PCR amplification for *lpxC* upstream flanking region
C2[a]	CGAATTCGCGGCCGCTTCTAACCT CCATCCACGGTATGTG	PCR amplification for *lpxC* upstream flanking region
C3[b]	GAGCTCGCTTGGACTCCTGTGCTA TAAATCAGGACATGCC	PCR amplification for *lpxC* downstream flanking region
C4	CTTATTGCCCCAACCGGAAC	PCR amplification for *lpxC* downstream flanking region
C5	TAACCAGATTGTTGACCTCG	PCR amplification to confirm *lpxC*::Kan^R deletion mutant
C6	CGCAATGGCAAAGACTTACG	PCR amplification to confirm *lpxC*::Kan^R deletion mutant
K1	TAGAAGCGGCCGCGAATTCG	PCR amplification for kanamycin resistance gene
K2	ACAGGAGTCCAAGCGAGCTC	PCR amplification for kanamycin resistance gene

[a] The first 20 bp (underlined) are specific for the 5' part of aph(3')-IIIa.
[b] The first 20 bp (underlined) are specific for the 3' part of aph(3')-IIIa.

Experimental Procedures

General materials and methods. All chemical reagents were of analytical grade, obtained from commercial suppliers, and used without further purification unless otherwise noted. The *E. coli* Polar Lipid Extract (100600P) was purchased from Avanti Polar Lipids Inc. Rough LPS from *E. coli* serotype EH100 (Ra mutant; Ra-LPS) and PF-5081090 were purchased from Sigma-Aldrich. Wang resin preloaded with Fmoc-Thr(tBu) was purchased from Bachem. Protected amino acids were purchased from Santa Cruz Biotechnology, Novabiochem and AAPPTec. Dansyl chloride was purchased from Calbiochem. *n*-Octyl-β-D-Glucopyranoside (OG) was purchased from Anatrace. Precision Plus Protein™ All Blue Prestained Protein Standard was purchased from BioRad.

Ultracentrifuge were performed on Beckman Coulter Optima XPN. Fluorescence was detected on Spectramax i3 - UV/VIS multimode microplate reader. High-performance liquid chromatography (HPLC) was conducted on an Agilent 1260 series Prep HPLC using a Phenomenex column (Luna C18, 5 µM, 100 Å, 250 x 21.2 mm). SDS-PAGE gels were imaged using an Azure c400 Imager (Azure Biosystems).

Chemical synthesis of Dansyl-polymyxin B nonapeptide (Dansyl-PMBN). Synthesis of protected linear dansylated peptide was performed manually using standard Fmoc solid phase peptide chemistry (Scheme S1). Synthesis was started using Wang resin preloaded with Fmoc-Thr(tBu)-OH (loading 0.58 mmol/g, 45 µM scale). Fmoc deprotection was carried out using a solution of 20% piperidine in dimethylformamide (DMF) for 30 minutes at room temperature. Coupling of the amino acids was performed using 2 molar equivalents of Fmoc amino acid and 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxid hexafluorophosphate (HATU) and 4 molar equivalents of diisopropylethylamine (DIPEA) in DMF. Coupling was carried out for 30-60 minutes at room temperature. Completion of coupling step was determined using the Kaiser test. Any remaining free amines were acetylated with 50 molar equivalents of acetic anhydride and pyridine in DMF for 30 minutes at room temperature. Deprotection, coupling, and acetylation were then repeated for the remainder of the peptide. After all amino acids were added, the dansyl functionality was coupled on resin using 4 molar equivalents of dansyl chloride and 8 molar equivalents of DIPEA. The dansylated peptide was cleaved from resin using 95% trifluoroacetic acid (TFA) over ice for 2 hours. The cleavage solution was added dropwise to ice cold diethyl ether to precipitate the peptide. The precipitate was collected by centrifugation and then dried to yield a light yellow powder. The peptide was then cyclized using 4 molar equivalents of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and hydroxybenzotriazole (HOBt) and 8 molar equivalents of DIPEA for 4 hours at room temperature. Reaction was concentrated to dryness and then washed with water to remove excess PyBOP. The cyclized peptide was then deprotected using 80 molar equivalents of chlorotrimethylsilane (TMS-Cl) and sodium iodide in acetonitrile for 3 hours. The deprotection was quenched by the addition of 0.05 mL methanol and then concentrated to dryness. The peptide was then extracted using 1:1 ether: 30% acetic acid. The peptide was next purified by HPLC, and eluted using the gradient method 90%-1% water/acetonitrile (+ 0.1% formic acid) over 20 minutes. The compound was analyzed by ESI-MS: $C_{55}H_{85}N_{15}O_{13}S$ *m/z* calculated, $[M+H]^+$ 1196.6245; observed, $[M+H]^+$ 1196.6247 (Figure S1). ^1H and ^{13}C NMR spectra were also obtained (Figures S2, S3).

Bacterial strains and growth conditions. *A. baumannii* 19606 and *B. subtilis* 3610 were purchased from ATCC. *E. coli* LCB273 was obtained from the Yale *E. coli* genetic stock center. *E. coli* WD2 was kindly provided by the Raetz laboratory. *E. coli* NR754 was kindly provided by the Ruiz laboratory (Ohio State University).

Construction of *lpxC*::Kan^r deletion strain of *A. baumannii.* The *lpxC*::Kan^r deletion strains was constructed by gene replacement through homologous recombination. This led to insertion of the kanamycin resistance cassette (Kan^r) into the genome. Flanking regions, consisting of ~500 bp at the 5' and 3' ends of the *lpxC* gene, were PCR amplified from the *A. baumannii* ATCC 19606 genome with primers C1/C2 and C3/C4, and purified by gel extraction. The first 20 bp of C2 and C3 are complementary to an aph(3')-IIIa kanamycin resistance cassette to allow for ligation using the Gibson Assembly master mix (NEB; E2611L) when mixed together. The aph(3')-IIIa gene was PCR amplified and purified from plasmid pIM1440 (Addgene; Plasmid #30501). The resulting linear sequences, *lpxC*::Kan^r was further PCR amplified and purified to generate sufficient amounts for transformation. To construct the *lpxC*::Kan^r deletion strain, ~7-10 µg of the *lpxC*::Kan^r linear fragment, were electroporated into *A. baumannii* ATCC 19606 based on a published protocol(1). Single colonies were then isolated and confirmed by PCR as *lpxC*::Kan^r mutants with primers C5/C6. Primer sequences are available in Table S3.

Minimal inhibitory concentration (MIC) measurements. MIC measurements were obtained using a broth dilution method. Serial, two-fold antibiotic dilutions were made and dispensed into a 96-well plate in triplicate for each bacterial strain. Cells were seeded at a density of ~10^4 cells/mL. Plates were incubated at 37°C for 24 hours before the OD_{600} was measured using a plate reader. MIC was determined as the concentration at which OD_{600} fell to baseline values.

Fluorescence microscopy. Microscopy was carried out on a Nikon Ti fluorescence microscope. The microscope was equipped with a Prior Proscan III linear-encoded motorized stage. Images were obtained using a 1.5 x tube lens and a Plan Apo 100 x 1.4NA objective with a polarizer, DIC H condenser and DIC prism in the light path. A Prior LumenPro fluorescence light source was used for fluorescence imaging. The microscope had excitation and attenuation wheels, which were used to set λ_{ex}= 405/15 nm and λ_{em}=535/50 nm (dansyl channel). MetaMorph image acquisition software controlling a Hamamatsu ORCA-R2 cooled CCD camera was used to acquire images. Images were processed in ImageJ by adjusting contrast identically for compared image sets. For fluorescence quantification, background was subtracted using a rolling ball radius of 50 pixels. Cells were segmented and the normalized fluorescence for each cell was calculated. For each sample type, average and standard deviation of normalized cellular fluorescence was calculated (n=10 cells for 0, 2.42 and 24.2 µM, n=6 cells for 242 µM and n=7 cells for ΔlpxC samples).

Dynamic light scattering. Dynamic light scattering (DLS) characterization was performed on a Beckman NPF-1 Delsa Nano C particle size and zeta potential analyzer. The diameters of the prepared liposomes were measured using DLS and shown in Z-average diameter (nm).

Fluorescent property characterization of Dansyl-PMBN. All fluorescence measurements were obtained with E_x=334 nm and E_m=520 nm. The standard curve of Dansyl-PMBN probe was recorded on a fluorimeter by measuring Dansyl-PMBN (concentrations ranging from 0.12-1200 µM) in TBS buffer (pH=8.0). The LPS-dependent fluorescence of Dansyl-PMBN was measured by titrating the Dansyl-PMBN into a cuvette containing 1 mL of 5 mM TBS buffer (pH=8.0) and 3 µg of Ra-LPS. Buffer alone was tested as a negative control. The inhibition of Dansyl-PMBN binding to LPS was measured by adding $MgCl_2$ into a cuvette containing Ra-LPS at a concentration of 3 µM in 5 mM TBS (pH=8.0) and fluorescence was measured on a 96-well plate reader. Reversal of inhibition was tested in a cuvette containing Ra-LPS at a concentration of 3 µM in 5 mM TBS (pH=8.0) with 50 mM Mg^{2+}. EDTA was titrated into the cuvette to the final concentrations as indicated, and fluorescence was measured on a 96-well plate reader.

Silver Stain SDS-PAGE. For cell lysate silver stain, the gel was soaked in 100 mL of Fix 1 (30% ethanol and 10% acetic acid) for 15 minutes. It was then soaked in 100 mL of Fix 2 (30% ethanol, 0.5% acetic acid, 0.66 M sodium acetate, 0.5% glutaraldehyde, and 100 mg sodium sulfate) for 30 minutes. The gel was washed in 100 mL of water for 5 minutes (three times), and then stained in silver nitrate solution (100 mL water, 0.5 mL 29% silver nitrate and 25 µL 37% formaldehyde) for 35 minutes. The gel was rinsed briefly in water and developed in 200 mL developer (80 µL 37% formaldehyde and 0.24 M sodium carbonate) until bands were visible. Development was stopped in 100 mL 50 mM EDTA, pH=7. For LPS silver stain, gel was soaked overnight in 200 mL Wash (40% ethanol, 5% acetic acid). Periodic acid (0.7%) was then added and incubated for 5 minutes. The gel was rinsed three times over 2 hours with 200 mL of water. The gel was then soaked in 115 mL freshly prepared Stain solution (0.86 % silver nitrate, 0.13 M ammonium hydroxide, 24 mM sodium hydroxide) for 10 minutes. The gel was washed three times over 45 minutes with 200 mL of water. The gel was finally soaked in 200 mL Developer (0.26 mM citric acid and 80 µL 37% formaldehyde, 37°C) until bands were visible. Development was stopped in 100 mL 0.33% acetic acid for 1 hour.

Reference:

(1) Aranda, J.; Poza, M.; Pardo, B. G.; Rumbo, S.; Rumbo, C.; Parreira, J. R.; Rodríguez-Velo, P.; Bou, G. *BMC Microbiol.* **2010**, *10*, 279.