Supporting Information

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SI Materials and Methods

Bacterial Growth Conditions. *Pseudomonas aeruginosa* 10145, *Acinetobacter baumannii* 19606 and *Escherichia coli* 25922 were obtained from ATCC. Broth microdilution assays were performed at 37 °C in CA-MHB. Biofilm inhibition assays were performed in LB or RPMI medium 1640 containing 10% (vol/vol) FBS (1).

Susceptibility Assays. Antimicrobial susceptibility testing was performed as recommended by the Clinical and Laboratory Standards Institute (CLSI) (M100-S19 CLSI) (2). Minimum inhibitory concentration (MIC) was determined after 18 h of incubation at 37 °C.

Time-Kill Kinetics. Overnight bacterial cultures were diluted to $OD_{600} = 0.07$ in fresh medium and incubated at 37 °C with shaking for 1 h. Increasing concentrations of $_D(KLAKLAK)_2$ or controls were added to the cultures and incubated at 37 °C with shaking for up to 24 h. To determine the number of viable bacteria, aliquots were taken at 4, 10, and 24 h after treatment, and dilutions were plated in duplicate onto LB agar. The plates were incubated at 37 °C, and the colony-forming units (CFU) were counted after 24 h (3). The synergistic time-kill kinetics was performed as described above. Briefly, bacterial cultures were treated with $_D(KLAKLAK)_2$ and piperacillin (Sigma) at one-half the MIC each, as determined by broth microdilution (2).

Biofilm Assays. *P. aeruginosa* PAO1 biofilm was grown as described (1). Briefly, a PAO1 overnight culture was diluted 1:100 in LB or RPMI medium 1640 containing 10% (vol/vol) FBS into 96-well ultra-low attachment sterile plates (Sigma) and incubated for 24 h at 37 °C. To assess the effect of treatment on the biofilm, increasing concentrations of $_D(KLAKLAK)_2$ or controls diluted in LB or RPMI medium 1640 containing 10% (vol/vol) FBS were added to the 24-h-old biofilm. The next day, the plates were washed three times with PBS, fixed with 4% (vol/vol) paraformaldehyde, and stained with 0.1% crystal violet (CV) for 10 min at room temperature (RT). Excess stain was removed with water and the plates were dried overnight at RT. CV stain extracted with 95% (vol/vol) ethanol and the absorbance of a 1:5 dilution in 95% (vol/vol) ethanol was measured at 570 nm.

Growth Phase-Dependent Activity. Bacterial death was evaluated with the *Bac*Light kit (Invitrogen) (4). First, a 6-mL aliquot was removed from an overnight culture of *P. aeruginosa* PAO1 and stored on ice. The rest of the overnight culture was diluted to $OD_{600} = 0.07$ in LB and incubated at 37 °C with shaking. Aliquots were removed at $OD_{600} = 0.01$ (lag phase) and $OD_{600} = 1$ (exponential phase). Bacteria were washed, resuspended in PBS to $OD_{600} = 0.1$, and treated with 150 μg/mL $_D(KLAKLAK)_2$, 30 μg/mL cecropin A (positive control), 150 μg/mL control peptidomimetic, or vehicle (PBS). After 1 h, bacteria were harvested, washed, and resuspended to $OD_{600} = 0.03$ in PBS. An equivolume mixture of SYTO9 and propidium iodide (3 μL each) was added to each 1-mL sample and incubated for 15 min at RT in the dark. Fluorescent micrographs of stained bacteria mounted on glass slides were acquired on an Olympus IX71 inverted microscope attached to a Lumen 200 Fluorescence Illumination system with fluorescein and Texas Red band pass filter sets (Prior Scientific) at 400× magnification.

Transmission Electron Microscopy. *P. aeruginosa* was grown to $OD_{600} = 0.15$. Bacterial cells were recovered by centrifugation, resuspended in saline, and treated with freshly prepared $_D(KLAKLAK)_2$ at increasing concentrations for 25 min. Bacteria were collected, washed, and fixed with a solution of 3% (vol/vol) gluteraldehyde and 2% (vol/vol) paraformaldehyde in 0.1 M cacodylate buffer at pH 7.3. After fixation, samples were washed in 0.1 M cacodylate buffer, postfixed in 4% (wt/vol) Millipore-filtered potassium permanganate for 1 h, washed in distilled water, and stained en bloc with 1% Millipore-filtered aqueous uranyl acetate for 1 h. Stained samples were dehydrated in increasing concentrations of ethanol, three changes in propylene oxide, infiltrated and embedded in LX-112 epoxy resin medium, and polymerized in a 70 °C oven for 3 d. Ultrathin sections were subsequently cut in a Leica Ultracut microtome (Leica), stained with uranyl acetate and lead citrate in a Leica EM Stainer, and subsequently examined in a JEM 1010 TEM (JEOL) at an accelerating voltage of 80 kV. Digital images were obtained with the AMT Imaging System (Advantage Microscopy Techniques) (5).

Liposome Formation. *E. coli* extract devoid of LPS, 1,2-distearoyl-sn-glycero-3-phospho-L-serine (PC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (PE), 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (PG), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (PS), cholesterol, *N*-(7-nitro-2,1,3-benzoxaidasol-4-yl)-PE (NBD-PE), *N*-(rhodamine B sulfonyl)-PE (Rh-PE) and cholesterol were procured from Avanti Polar Lipids. Fluorescent lipids were dissolved together with the unlabeled lipid. After solvent evaporation under a steady stream of argon, lipids were resuspended to a concentration of 1 mg/mL PBS and sonicated for 10 min. Liposomes were used immediately or stored at 4 °C for a maximum of 2 wk (6).

Fluorescence Resonance Energy Transfer. To determine the ability of $_D(KLAKLAK)_2$ to disrupt liposomal membranes, increasing peptide concentrations were added to an equal mixture of Rh-labeled and NBD-labeled liposomes. After 24 h, FRET was measured with a Spectramax M5 spectrophotometer. The excitation and emission wavelengths were 460 nm and 534 nm for NBD-PE and 550 nm and 590 nm for Rh-PE, respectively. The emission spectrum was measured between 500 and 620 nm with 9-nm excitation and 15-nm emission band pass widths. For the FRET measurements, liposomes were prepared with 5% fluorescent lipid (NBD-PE or Rh-PE) and 95% unlabeled lipid, by weight (7). For specific lipid interactions, increasing weight percentages of PC, PS, PE, PG (each, 25%, 45%, or 65% wt/vol) or cholesterol (10%, 20%, 30%, or 40% wt/vol) were added to *E. coli* extracts (7).

Giant Unilamellar Vesicles Formation. A thin film (~300 μL) of 1% agarose in deionized water was spread onto a glass slide and dried at 40 °C for 3 h. A mixture of *E. coli* extract and Rh-PE (30 μL) in chloroform was painted across the agarose film and allowed to dry under vacuum pressure for 2 h. Slides were subsequently placed into 150 mM KCl buffer, and the agarose was allowed to rehydrate for a minimum of 1 h. Images were collected at 30 s after the addition of $_D(KLAKLAK)_2$ or a control peptidomimetic (7).

Gram-Negative Membrane Potential Measurements. Bacteria were collected from a midexponential culture, washed in Hepes buffer (5 mM Hepes at pH 7.2 and 5 mM glucose), and resuspended in the same buffer containing 0.4 μM $diSC_3(5)$ (Invitrogen) and 0.2 mM EDTA (to $OD_{600} = 0.05$). The mixture was incubated in the dark for 1 h to allow maximal uptake of the $diSC_3(5)$ dye. The osmotic gradient was equilibrated to a final concentration of 100 mM KCl. Subsequently, the mixture was subjected to increasing amounts of $_D(KLAKLAK)_2$ in Hepes buffer. The $diSC_3(5)$-derived fluorescence was monitored with a Spectramax M5 spectrophotometer at excitation of 622 nm and emission of 670

nm. Relative fluorescence intensities were normalized to PBS (set to a value of 0%) and 99% (vol/vol) isopropanol (set to a value of 100%) standards (8, 9).

Lysozyme Permeability Assay. A 5-mL overnightculture of bacteria served to inoculate 50 mL of LB broth. Midlog phase bacteria were harvested, washed once, and resuspended in Hepes buffer (5 mM Hepes at pH 7.2 and 5 mM sodium azide) to a final optical absorbance of OD_{600} = 0.5. Bacterial suspension was added to a plastic cuvette, and the OD_{600} was measured for 10 s. Lysozyme was then added to a final concentration of 50 μg/mL, and the corresponding OD_{600} was measured for an additional 10 s. Subsequently, increasing concentrations of $_D$(KLAKLAK)$_2$ in small increments (10 μL of a 100 mg/mL stock at a time) were added to the sample. The turbidity of the sample was measured after the lysis process reached equilibrium (as seen by a stabilization in the OD_{600}) and every 10 s after stabilization for 30 s. Relative values were normalized to the effect of PBS (set to a value of 0%) and 99% isopropanol (set to a value of 100%) (8, 9).

Erythrocyte Hemolysis Assay. Erythrocytes were separated from human whole blood by centrifugation at 2,000 × *g* for 15 min. The cells were washed three times with 150 mM NaCl and resuspended in PBS at a concentration of 10^8 erythrocytes per mL. The suspension was incubated with increasing concentrations of $_D$(KLAKLAK)$_2$ at 37 °C for 2, 6, 24, or 48 h. After centrifugation, the hemoglobin released in the supernatant was measured at 415 nm (9).

Stability Assays. The stability of $_D$(KLAKLAK)$_2$ compared with $_L$(KLAKLAK)$_2$, in the presence of human liver microsomes, was determined by mass spectrometry. Briefly, 5 μL of human liver microsomes pooled from multiple donors (BD Biosciences) were diluted in 75 μL of 50 mM potassium phosphate buffer at pH 7.4. $_D$(KLAKLAK)$_2$ and $_L$(KLAKLAK)$_2$ were added to the mixture (10 μL of a 10 mg/mL solution in water) and incubated for 5 min at 37 °C. The reaction was started with the addition of 10 μL of 20 mM NADPH. Aliquots (10 μL each) were removed pre- and postaddition of NADPH at 1, 10, 30, and 60 min and 24 h. The reactions were stopped with 5 μL of 100% trifluoroacetic acid (TFA). All samples were frozen at −80 °C until processed by matrix assisted laser desorption/ionization with double time-of-flight (MALDI-TOF^2) detection as described below (10, 11).

MALDI-TOF^2 Mass Spectroscopy. Samples were centrifuged at 8,000 × *g* for 30 min at 4 °C to remove liver microsomal debris and then 2 μL of each supernatant was added to 198 μL of 0.1% TFA solution. Samples were then further diluted 1:10 in α-cyano-4-hydroxyl cinnamic acid (10 mg/mL in 50% acetonitrile 0.5% TFA). Each sample was loaded onto three spots on a MALDI-TOF plate, and MS data was acquired for each spot in duplicates. ^{13}C-$_D$(KLAKLAK)$_2$ served as the internal standard for quantification. Quantification was performed by integrating the peak area of individual spectra, which was graphically displayed relative to the internal control (10, 11).

Protoplast Susceptibility Assay. Overnight cultures of *S. aureus* and *E. faecalis* were diluted to OD_{600} = 0.05 in fresh medium and grown to OD_{600} = 0.8. Cells (5×10^8) were collected by centrifugation, washed once in PBS, and resuspended in 200 μL of cell wall degradation buffer (20 mM Tris at pH 8.0, 10 mM $MgCl_2$, and 30% raffinose) containing 200 μg/mL lysostaphin or mutanolysin, respectively. After 15-min incubation at 37 °C, protoplasts were collected by centrifugation at 1,500 × *g* and resuspended in 20 mM Tris at pH 8.0 and 30% (wt/vol) raffinose (12). An equal number of intact cells and protoplast was exposed to 300 μg/mL peptidomimetics or vehicle. To determine the number of viable bacteria, aliquots were diluted and plated in duplicate onto brain heart infusion agar. Percent cell survival was calculated as the number of live cells after exposure to peptidomimetics divided by the number of live cells before treatment.

LPS Binding Assays. Microtiter plates were coated overnight with 50 μL of a 10 mg/mL solution of peptidomimetics or cathelicidin. To determine the number of viable bacteria, aliquots were diluted and plated in duplicate onto brain heart infusion agar. Blocking of exposed nonspecific binding sites was performed with PBS containing 2% (vol/vol) BSA. Purified LPS was added to the wells at 50 EU per mL. After 1 h of incubation, specific binding was detected by Limulus Amebocyte Lysate assay (Charles River) according to the manufacturer's instruction.

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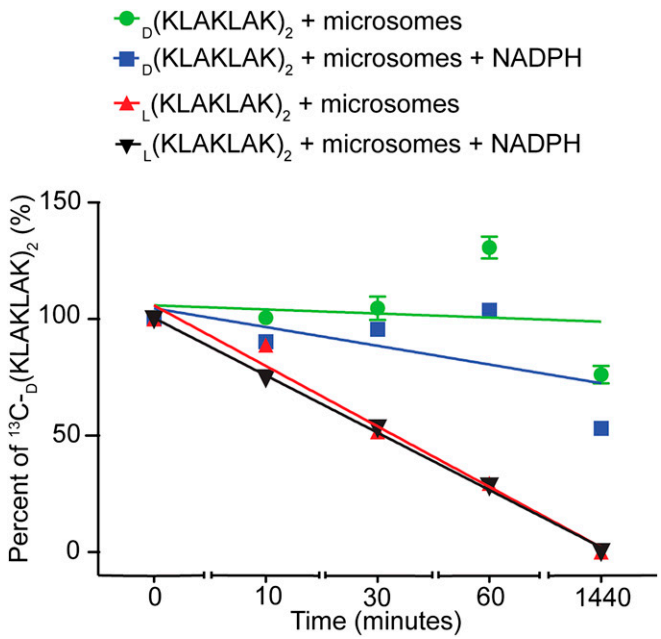

Fig. S1. $_{D}$(KLAKLAK)$_2$ is resistant to degradation by human liver-derived microsomes. Intact $_{D}$(KLAKLAK)$_2$ was detected even 24 h after exposure to microsomal enzymes in the presence (blue line) or absence (green line) of NADPH. $_{L}$(KLAKLAK)$_2$ is rapidly degraded over time by microsomal enzymes both in the presence (black line) or absence (red line) of NADPH.

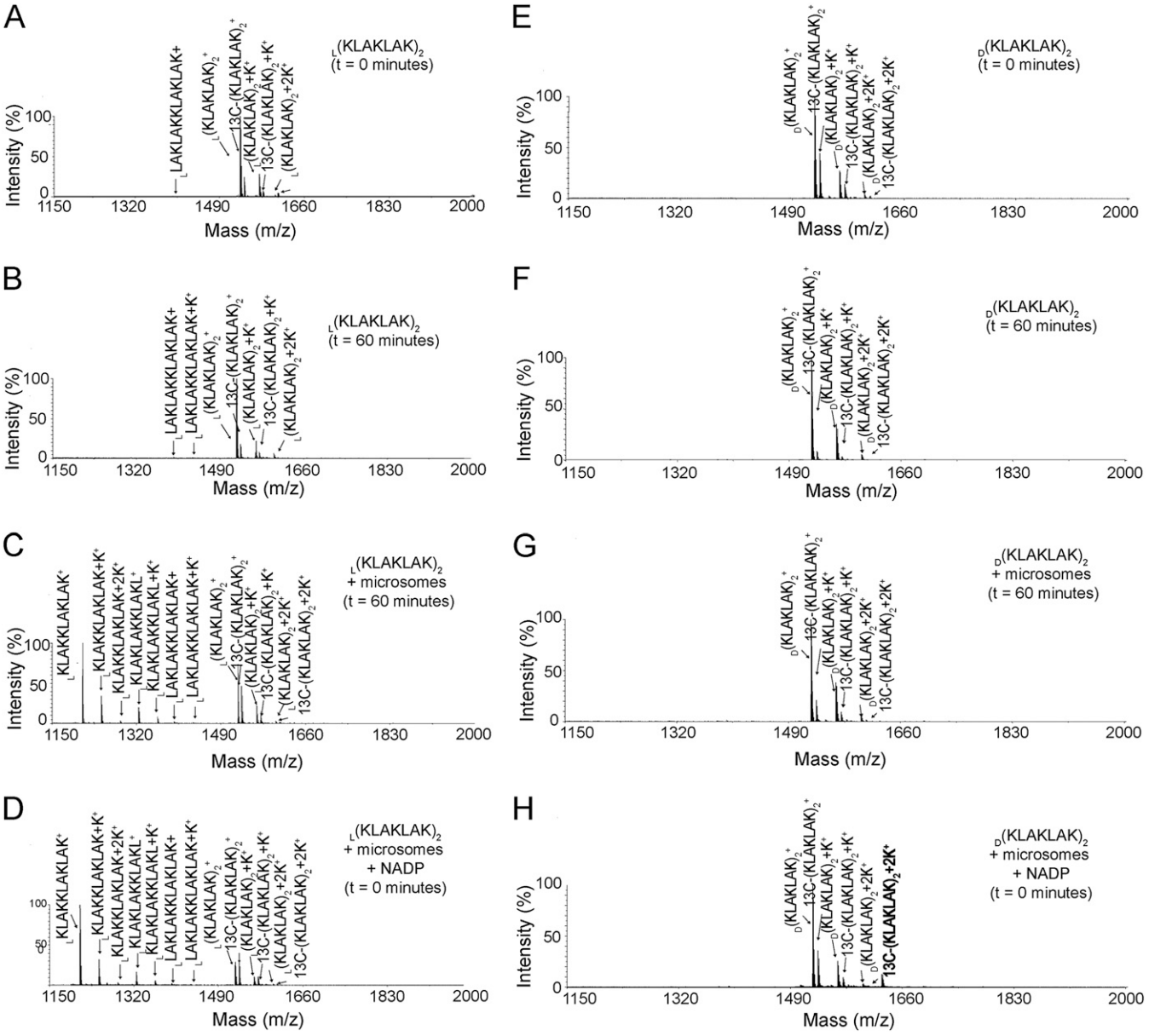

Fig. S2. Mass spectrum profiles of L- and D-enantiomers of $(KLAKLAK)_2$ after 60 min of exposure to human liver-derived microsomes. $_L(KLAKLAK)_2$ is rapidly degraded during exposure to human liver microsomes. Degradation products resulting from both N-terminal and C-terminal proteolysis of $_L(KLAKLAK)_2$ were found at 10, 30, and 60 min. Representative mass spectrum profiles are shown at preincubation (*A*), 60 min in buffer at 37 °C (*B*), 60 min after incubation with pooled human liver microsomes (*C*), and 60 min after incubation with pooled human liver microsomes in the presence of NADPH (*D*). $_D(KLAKLAK)_2$ is resistant to degradation by human liver-derived microsomes. No degradation products of $_D(KLAKLAK)_2$ were found for up to 24 h after continuous exposure to microsomal enzymes. Representative mass spectrum profiles are shown at preincubation (*E*); 60 min in buffer at 37 °C (*F*); 60 min after incubation with pooled human liver-derived microsomes (*G*); and 60 min after incubation with pooled human liver-derived microsomes in the presence of NADPH (*H*).

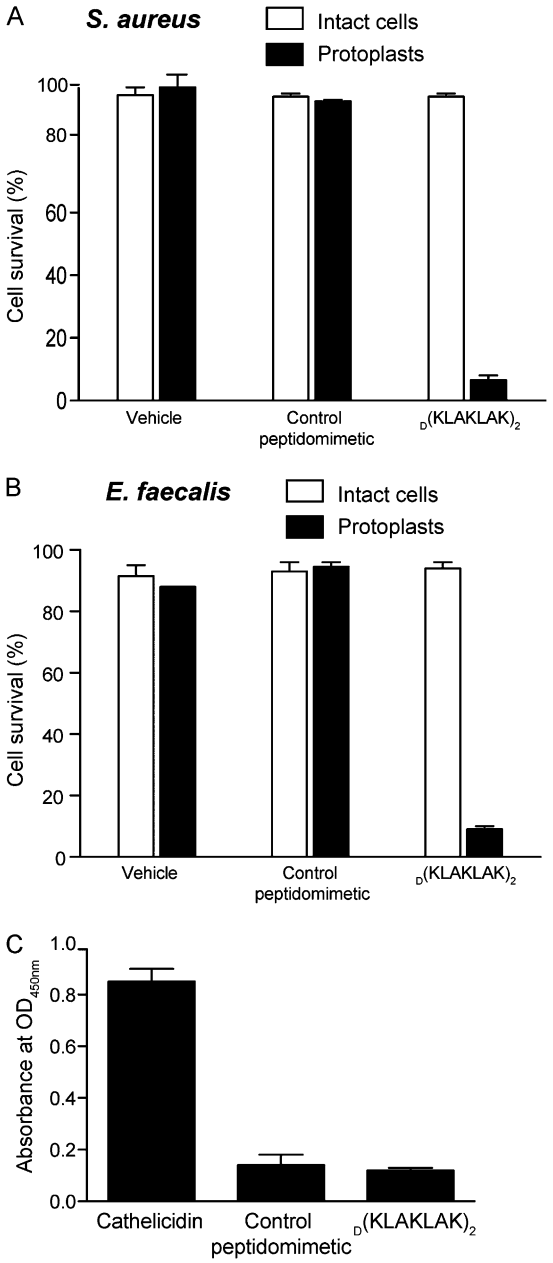

Fig. S3. Peptidoglycan and LPS effect on the susceptibility of Gram-positive bacteria to $_D$(KLAKLAK)$_2$. Intact cells and protoplasts of *S. aureus* (*A*) and *E. faecalis* (*B*) were exposed to $_D$(KLAKLAK)$_2$, control peptidomimetic, or vehicle. Cell survival is shown as percentage of remaining live cells after treatment. (*C*) LPS was incubated with either $_D$(KLAKLAK)$_2$ or control peptidomimetic immobilized on plates. Cathelicidin, an LPS-binding AMP, was used as a positive control. LPS binding was detected by LAL assay.

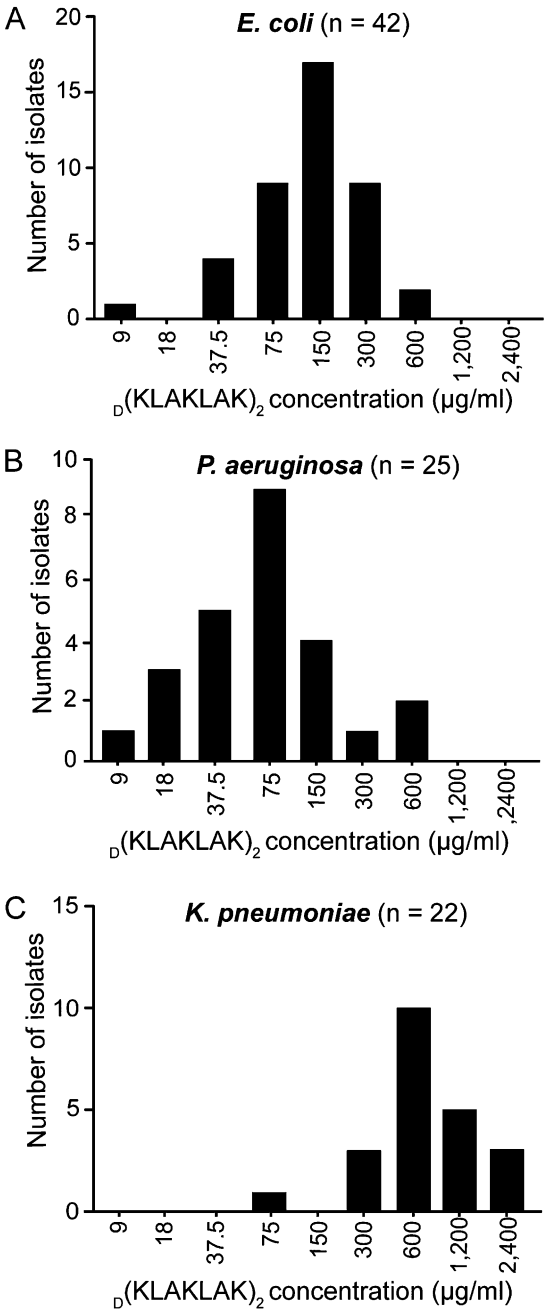

Fig. S4. Susceptibility testing of Gram-negative clinical isolates. The distribution of MIC values for *E. coli* ($n = 42$) (*A*), *P. aeruginosa* clinical isolates ($n = 25$) (*B*), and *K. pneumoniae* ($n = 22$) (*C*) represented in μg/mL. Susceptibility testing was performed by standard broth microdilution assay as described in *Materials and Methods* (2).

Table S1. $_D$(KLAKLAK)$_2$ MIC values for selected microorganisms

Organism	MIC, μg/mL	
	$_L$(KLAKLAK)$_2$	$_D$(KLAKLAK)$_2$
Gram-negative bacteria		
Acinetobacter baumannii ATCC 19606	300	300
Escherichia coli ATCC 25922	150	150
Klebsiella pneumoniae	300	300
Pseudomonas aeruginosa (PA01)	150	150
Pseudomonas aeruginosa (PA14)	150	150
Stenotrophomonas maltophilia	300	300
Gram-positive bacteria		
Enterococcus faecalis	Not active*	Not active
Staphylococcus aureus	Not active	Not active
Staphylococcus epidermidis	Not active	Not active
Streptococcus pneumoniae	Not active	Not active

MIC, minimum inhibitory concentration.
*MIC > 1,200 μg/mL.

Table S2. Comparative analysis of susceptibility to $_D$(KLAKLAK)$_2$ and routinely tested antibiotics for Gram-negative clinical isolates

$_D$(KLAKLAK)$_2$ MIC, μg/mL	No. of isolates resistant to routinely tested antibiotics*					Total no. of strains
	0	1–4	5–8	9–12	13–16	
E. coli (28)						
37.5	0	3	0	0	0	3
150	**5**	9	2	**1**	0	17
300	3	4	1	0	0	8
P. aeruginosa (14)						
37.5	2	0	0	1	0	3
150	2	4	**3**	0	0	9
1,200	1	0	**1**	0	0	2
K. pneumoniae (16)						
75	0	0	0	0	**1**	1
600	0	10	0	0	0	10
1,200	0	5	0	0	0	5

MIC, minimum inhibitory concentration.
*Bold denotes clinical isolates (discussed in the text).