Anthropogenic acidification of Chinese agricultural soils will be difficult to correct as long as excessive levels of N fertilization continue. Goulding and Annis (*28*) found that each 50 kg $ha^{-1}$ of added ammonium-N generates ~4 kmol $H^+$ $ha^{-1}$ $year^{-1}$ and requires ~500 kg $CaCO_3$ $ha^{-1}$ $year^{-1}$ to neutralize in their field conditions. Similar theoretical calculations show that each kg of applied $NH_4$-N leached as $NO_3$-N demands 7.2 kg of $CaCO_3$ to neutralize the acidity generated (*29*, *30*). Adding appropriate amounts of lime in China would be arduous; intensive double-cropping systems that generate 30 to 50 kmol $H^+$ $ha^{-1}$ $year^{-1}$ would theoretically require 1.5 to 2.5 tons $CaCO_3$ $ha^{-1}$ $year^{-1}$ to counteract soil acidification—and greenhouse vegetable systems would require ten times this amount.

Overuse of N fertilizer contributes substantially to regional soil acidification in China. Since 1980, crop production has increased with rapidly increasing N fertilizer consumption (fig. S5). Decreasing N use efficiency (fig. S5) indicates that more fertilizer N is being lost to the environment (*31*), causing further negative environmental impacts. Optimal nutrient-management strategies can significantly reduce N fertilizer rates without decreasing crop yields (*14*, *32*, *33*), with multiple benefits to agriculture and the environment (*15*), including the slowing of dangerous rates of anthropogenic acidification. Fertilization based on comprehensive, knowledge-based N management practices has become one of the most urgent requirements for sustainable agriculture in China and in other rapidly developing regions worldwide.

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### Supporting Online Material

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# Peptidomimetic Antibiotics Target Outer-Membrane Biogenesis in *Pseudomonas aeruginosa*


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Antibiotics with new mechanisms of action are urgently required to combat the growing health threat posed by resistant pathogenic microorganisms. We synthesized a family of peptidomimetic antibiotics based on the antimicrobial peptide protegrin I. Several rounds of optimization gave a lead compound that was active in the nanomolar range against Gram-negative *Pseudomonas* spp., but was largely inactive against other Gram-negative and Gram-positive bacteria. Biochemical and genetic studies showed that the peptidomimetics had a non–membrane-lytic mechanism of action and identified a homolog of the β-barrel protein LptD (Imp/OstA), which functions in outer-membrane biogenesis, as a cellular target. The peptidomimetic showed potent antimicrobial activity in a mouse septicemia infection model. Drug-resistant strains of *Pseudomonas* are a serious health problem, so this family of antibiotics may have important therapeutic applications.


Naturally occurring peptides and proteins make interesting starting points for the design and synthesis of biologically active peptidomimetics. We previously synthesized libraries of β-hairpin–shaped peptidomimetics (*1*, *2*) based on the membranolytic host-defense peptide protegrin I (PG-I) (*3*). These mimetics contain loop sequences related to that in PG-I, but linked to a D-proline–L-proline template, which helps to stabilize β-hairpin conformations within the macrocycle (*4*, *5*) (Fig. 1A). One sequence variant, L8-1, had broad-spectrum antimicrobial activity like that of PG-I, but with a reduced hemolytic activity on human red blood cells (*2*). To optimize this lead, we performed iterative cycles of peptidomimetic library synthesis and screening for improved antimicrobial activity. The optimal hit from each library was used as a starting point for the synthesis and testing of variations in a subsequent library. This structure-activity trail led sequentially to mimetics L19-45, L26-19, and L27-11 (Fig. 1). L27-11


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possessed an interesting spectrum of antimicrobial activity, including minimal inhibitory concentrations (MICs) in the nanomolar range against many *Pseudomonas aeruginosa* (*PA*) strains and other *Pseudomonas* spp.; the mimetic is only weakly active or inactive against other Gram-positive and Gram-negative bacteria [Table 1 (*6*)]. In contrast, PG-I displays broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria in the low micromolar range (*7*). The enantiomeric (mirror image) form of L27-11 is essentially inactive (MIC ≥ 32 μg/ml) against *PA*, unlike the two enantiomers of PG-I (*8*), suggesting that the antibacterial target of the mimetic is chiral and not the achiral lipid chains of the cell membrane targeted by PG-I (*9*–*12*). Further efforts focused on optimizing plasma stability and drug-like properties. The compounds POL7001 and POL7080 displayed much improved plasma half-lives while retaining a potent and selective action against *PA*; the MICs covering 90% of more than 100 *PA* clinical isolates tested ($MIC_{90}$) were 0.13 and 0.25 μg/ml, respectively, for these two mimetics. These isolates were of European and North American origin and most were resistant to one or more classes of clinically used antibiotics (*6*).

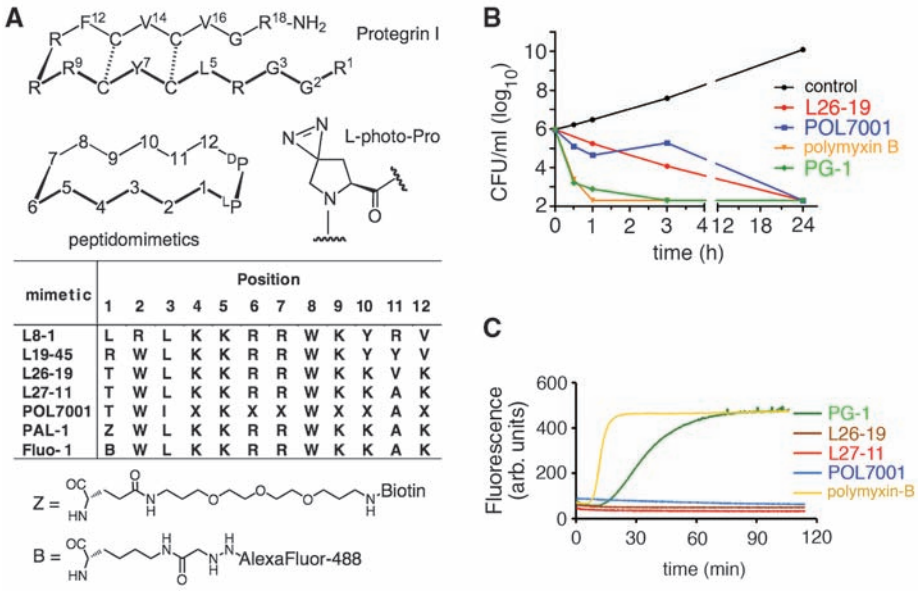


**Fig. 1.** (**A**) Structures of PG-1 and peptidomimetics. Dashed lines indicate disulfide bridges in PG-1. The single-letter code is used for amino acids (*30*), except as shown, and X is L-2,4-diaminobutyric acid, $^{D}$P= D-proline, and $^{L}$P is L-proline. PAL-1 contains L-photo-Pro in place of $^{L}$P. (**B**) The kinetics of bacterial cell death at 37°C is shown for *PA* PAO1 in MH broth after treatment with the indicated antibiotics at 4× MIC, or for the control (no drug). The remaining colony-forming units (CFU) are shown as a function of time after exposure to the antibiotic. (**C**) *PA* membrane integrity was measured by fluorescence spectroscopy. *PA* PAO1 cells in MH broth were treated with SYTOX and each of the antibiotics (added at time $t$ = 0) at a concentration of 5 μg/ml. The fluorescence change is shown versus time.

We next focused on identifying the mechanism of action of this family of peptidomimetics. Their mode of action against *PA* at concentrations close to the MIC was clearly different from the rapid membranolytic actions of both PG-I (*7*) and polymyxin B, a cationic macrocyclic peptide antibiotic of bacterial origin (*13*). Whereas PG-I or polymyxin at 4× MIC caused rapid cell lysis, the killing caused by the peptidomimetics was much slower (Fig. 1B). The ability of the peptidomimetics to permeabilize the *PA* cell membrane was also tested with the fluorescent nucleic acid stain SYTOX (*14*). No fluorescence increase was apparent over 120 min when *PA* cells were exposed to SYTOX and L26-19, L27-11, or POL7001, compared with a rapid fluorescence increase upon exposure to PG-I or polymyxin B [Fig. 1C (*6*)]. A direct interaction between the peptidomimetic antibiotics and lipopolysaccharide (LPS) was examined with a dansyl-polymyxin displacement binding assay (*15*). This revealed an interaction between LPS and L27-11 in the low micromolar range [median inhibitory concentration ($IC_{50}$) 0.8 μM], which was not enantioselective ($IC_{50}$ 1.1 μM for the enantiomer of L27-11), suggesting that LPS is not the primary site of antimicrobial action. Also, L26-19, L27-11, and POL7001 caused no notable lysis of human red blood cells at concentrations up to 100 μg/ml.

We tested whether the peptidomimetics have any effect on protein or nucleic acid biosynthesis in *PA* by examining the kinetics of incorporation of radiolabeled precursors into macromolecules. No notable influence was detected (*6*), suggesting that inhibition of protein, DNA, or RNA biosynthesis are not the primary modes of action. Similar attempts to monitor effects on cell wall biosynthesis by radiolabeled *N*-acetylglucosamine were frustrated by low levels of incorporation of this precursor into cell wall biopolymers.

As an alternative approach to investigate the mechanism of action, a forward genetic screen was established to characterize the genetic basis for resistance to the antibiotics. Spontaneous resistant mutants of *PA* PAO1 could be selected on the antibiotic POL7080 at 5× MIC, at an estimated frequency of ≤ $1 \times 10^{10}$. Three mutants ($PAO1^{RES1-3}$) showed MICs toward POL7080 and L26-19 of >32 μg/ml, but were more sensitive toward POL7001 (MIC 8 μg/ml, versus 0.06 μg/ml for wild-type *PA* PAO1). No other changes in growth rate were observed. Compared to wild-type *PA* PAO1, the mutants showed only minor changes in sensitivity toward several other antibiotics (Table 2). To identify the causative mutation(s), we constructed three plasmid libraries from restriction-digested fragments of *PA* $PAO1^{RES1}$ genomic DNA and transformed wild-type *PA* PAO1 with selection for growth on agar

**Table 1.** Antimicrobial activities of the peptidomimetics. The MIC values were determined by the microdilution method in MH broth in the presence of 0.02% bovine serum albumin (BSA). The MIC values are typically four to eight times as high when the assays are performed in cation-adjusted MH (MH-II) broth in the presence of BSA. ND, not determined. Strains: *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*, *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*. ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen.

| | | MIC (μg/ml) | | | | | |
|---|---|---|---|---|---|---|---|
| Strain | | L8-1 | L19-45 | L26-19 | L27-11 | POL7001 | POL7080 |
| *P. aeruginosa* | ATCC 27853 | 8 | 1 | 0.02 | 0.01 | 0.008 | 0.008 |
| *P. aeruginosa* | PAO1 | 8 | 2 | 0.03 | 0.004 | 0.008 | 0.004 |
| *A. baumannii* | DSM3008 | ND | ND | >64 | >64 | >64 | >64 |
| *K. pneumoniae* | ATCC 13883 | ND | ND | >64 | >64 | >64 | >64 |
| *S. maltophilia* | ATCC 13637 | ND | ND | >64 | >64 | >64 | >64 |
| *E. coli* | ATCC 25922 | 8 | 8 | 32 | 64 | >64 | >64 |
| *E. faecalis* | DSM 12956 | ND | ND | >64 | >64 | >64 | >64 |
| *S. aureus* | ATCC 29213 | 8 | 64 | 64 | >64 | >64 | >64 |

containing carbenicillin and POL7080. Resistant clones were isolated from all three libraries. Plasmid DNA isolated from 12 clones contained a common 5.4-kb overlapping DNA fragment [nucleotides (nt) 652,070 to 657,452 in the genome sequence (*16*) of *PA* PAO1] containing two contiguous open reading frames identified as homologs of *surA* and *ostA* [also called *imp* and more recently *lptD* (used below)] (*17*). The 5.4-kb DNA fragment conferred the resistant phenotype on *PA* PAO1, whereas a smaller fragment containing only *surA* had no effect on resistance. The *surA* gene possessed the expected wild-type nucleotide sequence, whereas the *lptD* homolog from *PA* PAO1$^{RES1}$ (here called *lptD1*) contained a mutation, consisting of a single 18–base pair tandem duplication of nt 628 to 645, corresponding to a tandem duplication of residues 210 to 215 with the sequence LRDKGM (*6*), with no other changes in the coding or upstream promoter sequences. The *lptD1* gene appears to act as a dominant resistance marker in the wild-type *PA* PAO1 background. This does not appear to be a gene dosage effect, because introduction of the wild-type allele on plasmid pVLT31 (*18*) does not influence antibiotic sensitivity for *PA* PAO1 or PAO1$^{RES1}$. Moreover, neither wild-type *PA* PAO1, containing plasmid-borne copies of *lptD1*, nor PAO1$^{RES1}$, containing copies of *lptD*, has altered sensitivity to most of the other antibiotics tested (Table 2). This argues against a general change in the permeability of the outer membrane caused by this mutation.

LptD is an outer-membrane protein widely distributed in Gram-negative bacteria that functions in the assembly of LPS in the outer leaflet of the outer membrane (*17*, *19*–*21*). LptD is an essential low-abundance outer-membrane protein in *Escherichia coli* (*19*), depletion of which causes stress in outer-membrane biogenesis (*21*, *22*). LptD in *PA* PAO1 is predicted to contain a C-terminal β-barrel domain (approximately residues 300 to 924) embedded in the outer membrane and an N-terminal domain (approximately residues 34 to 300) (*19*), which may reside in the periplasm and/or partly plug the β barrel (Fig. 2A). The β-barrel sequence is highly conserved in LptD homologs in Gram-negative bacteria (*17*), although the N-terminal domain is more variable in length, comprising about 300 residues in *PA* but only about 180 residues in *E. coli* K12 (*6*). The size difference in the periplasmic domains of LptD in *E. coli* and *PA*, the location within this domain of the *lptD1* mutation, and the key function of LptD in outer-membrane biogenesis suggest that LptD may be a primary target of the peptidomimetic antibiotics.

Photoaffinity labeling experiments (*23*) were performed to determine whether the antibiotics bind to LptD in intact cells. For this task, an analog (PAL-1) was produced that contains a L-4,4-diazarinylproline (L-photo-proline) in place of L-proline, as well as a biotin tag at position 1 (MIC of PAL-1 against *PA* PAO1 = 0.05 μg/ml) (Fig. 1A) (*6*). Photoaffinity labeling with PAL-1 consistently revealed a major photolabeled protein with an apparent mass by SDS–polyacrylamide gel electrophoresis of ≈100 kD, close to that expected for *PA* LptD (calculated molecular weight = 100,751) (Fig. 2B). When the photoaffinity labeling was repeated in the presence of a 100× excess of L27-11, the labeled band disappeared from the blot, demonstrating a competition between L27-11 and PAL-1 for binding to LptD. The identity of the photolabeled protein was proven to be LptD (PA0595) by two-dimensional (2D) gels and in-gel protease digestion/LC-ESI-MS-MS (liquid chromatography–electrospray ionization–tandem mass spectrometry) analysis and by immunoblotting with polyclonal antibodies raised against a synthetic C-terminal peptide fragment of LptD (*6*). When the photolabeling experiment was repeated with the resistant PAO1$^{RES1}$ mutant, photolabeling of LptD was not detected, indicating that the protein was no longer able to bind the antibiotic with comparable affinity.

If the function of LptD is impaired upon binding to the peptidomimetic, then some effect on outer-membrane structure and biogenesis should become apparent (*19*, *24*). When grown in Mueller-Hinton (MH) broth for several hours in the presence of growth-inhibitory amounts of L27-11, *PA* cells became more sensitive to detergents (Triton X-100 and SDS) and to various antibiotics, including tetracycline and rifampicin (*6*). *PA* PAO1 cells grown in MH broth with L27-11 or POL7001 were also examined in thin sections by transmission electron microscopy (TEM). This revealed internal accumulation of membrane-like material in many apparently intact cells (Fig. 2C), by two different fixation methods, an effect not seen in cells grown without the antibiotic. Similar accumulations have been observed in *E. coli* cells depleted of *lptD* (*21*, *22*) and in other bacteria exposed to antimicrobial peptides (*25*). Some cells (≈10%) grown in this way formed filaments comprising multiple concatenated cells (Fig. 2D), suggesting an impairment in cell division, which was not observed with untreated *PA* PAO1 cells or with L27-11–treated PAO1$^{RES1}$ cells. Cells grown in the presence of growth-inhibitory amounts of L27-11 could be uniformly stained with the membrane dye 3,5-dipropylthiacarbocyanine [diSC$_3$(5)] (Fig. 2E), whereas untreated *PA* PAO1 or L27-11–treated PAO1$^{RES1}$ cells exposed to this dye did not fluoresce. Thus, the action of L27-11 during growth impairs the outer-membrane permeability barrier.

**Table 2.** Antibacterial activities (MICs in MH-II broth) of various antibiotics toward wild-type *PA* PAO1 (PAO$^{WT}$) and resistant mutant (PAO1$^{RES1}$), as well as toward bacterial cells containing plasmid-borne copies of wild-type *lptD* or the resistance gene (*lptD1*) isolated from PAO1$^{RES1}$. The plasmid vector used for these experiments (pVLT31) has no effect on resistance to the antibiotics shown, when introduced into *PA* PAO1$^{WT}$ or PAO1$^{RES1}$.

| Antibiotic | MIC (μg/ml) | | | | |
|---|---|---|---|---|---|
| | PAO1$^{WT}$ | PAO1$^{RES1}$ | PAO1$^{WT}$ +*lptD1* | PAO1$^{RES1}$ +*lptD* | PAO1$^{RES1}$ +*lptD1* |
| POL7080 | 0.06 | 64 | 4 | 64 | 64 |
| L26-19 | 0.1 | >64 | 16 | >64 | >64 |
| Gentamicin | 0.5 | 1 | 0.5 | 1 | 1 |
| Tobramycin | 0.25 | 0.25 | 0.12 | 0.25 | 0.25 |
| Ciprofloxacin | 0.5 | 0.06 | 1 | 0.12 | 0.12 |
| Colistin | 1 | 0.5 | 0.5 | 0.5 | 0.25 |

Interference with the function of LptD may also allow entry of phospholipids into the outer leaflet of the outer membrane. In *E. coli* and *Salmonella* spp., this is known to activate the outer-membrane enzyme PagP, which modifies LPS by converting the hexa-acyl form of lipid A into the hepta-acyl form by transferring a palmitate group from outer-leaflet phospholipids to lipid A (*26*, *27*). The same effect has been observed in *lptD*-depleted *E. coli* (*21*). *PA* can also modify its lipid A by addition of a C16 fatty acid, to generate from the normal penta-acylated form (molecular weight 1447) a hexa-acylated derivative (molecular weight 1686) (*28*). This palmitate-containing hexa-acylated form has been observed in lipid A from *PA* clinical isolates from patients with cystic fibrosis (*29*), although it is normally absent from laboratory-adapted strains (such as PAO1) (*28*).

We first confirmed that the lipid A prepared from LPS isolated from *PA* PAO1 grown normally in MH broth was indeed the expected penta-acylated form {negative-mode matrix-assisted laser desorption/ionization (MALDI)–MS mass/charge ratio (*m/z*) 1446 [M-H]$^-$}. However, when cells were grown in MH broth with L27-11, after 5 hours the only lipid A detectable by MALDI-MS was the hexa-acylated form (observed mass *m/z* 1684 [M-H]$^-$) containing a palmitoyl residue (*6*). A further indication of a perturbed membrane structure came from fractionation of membrane extracts from cells by sucrose density ultracentrifugation. Fractions containing LPS and LptD appeared at higher density in the gradient when they came from L27-11–cultivated cells as compared to untreated cells (*6*), a result similar to that reported for *lptD*-depleted *E. coli* cells (*19*, *21*).

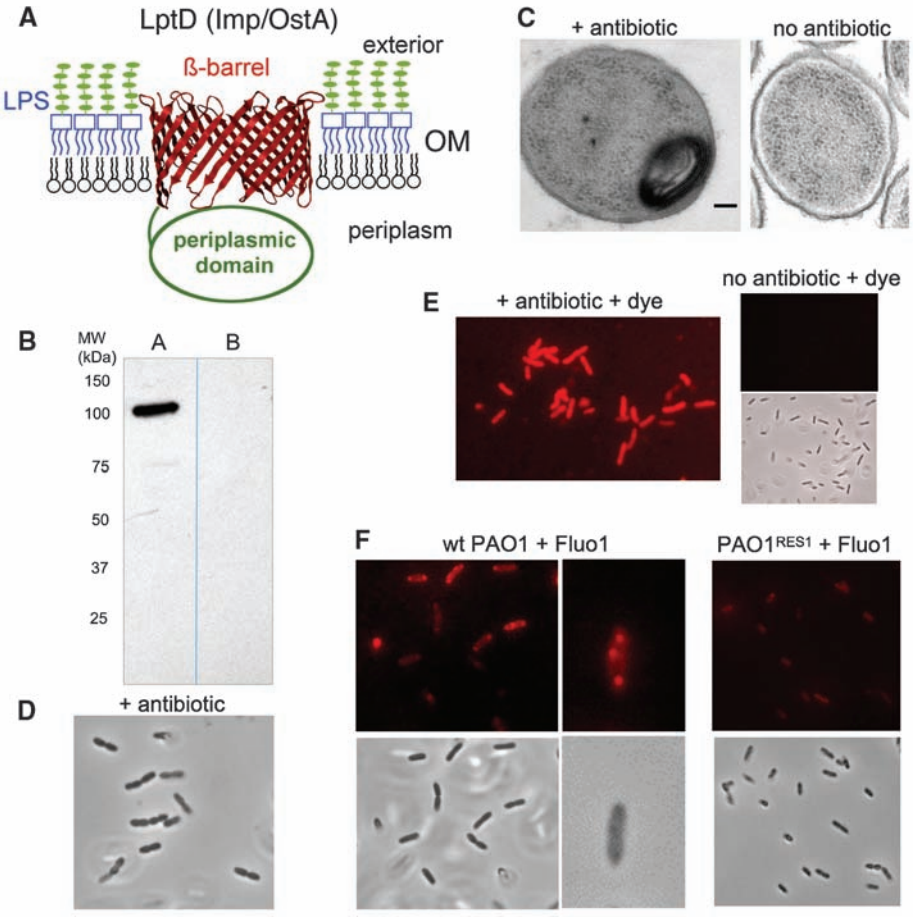


**Fig. 2.** (**A**) The predicted β-barrel domain of LptD (924 residues) is shown integrated in the outer membrane. (**B**) Photoaffinity labeling of membrane proteins in *PA* PAO1. PAL-1 (1 μg/ml) was incubated with *PA* cells at 37°C and irradiated with ultraviolet light. Membrane proteins were extracted, separated from cytoplasmic proteins by ultracentrifugation, and subjected to gel electrophoresis. Biotinylated proteins were detected by a streptavidin-based chemiluminescence detection system after blotting to a polyvinylidene difluoride membrane, with (100× excess, lane B) and without (lane A) competing L27-11 during photolysis (*6*). (**C** to **E**) Effects on *PA* cells after growth for 1 to 3 hours at 37°C in MH broth either with L27-11 (+ antibiotic) or without antibiotic (no antibiotic); (C) TEM showing accumulation of extra membrane-like material within cryo-fixed *PA* PAO1 cells (bar, 0.1 μm); (D) formation of filaments comprising multiple concatenated *PA* PAO1 cells; (E) staining *PA* PAO1 with the membrane-sensitive dye $diSC_3(5)$ and washing. (**F**) Staining and light microscope images of wild-type (wt) *PA* PAO1 (left) and resistant mutant $PAO1^{RES1}$ (right), after growth in the presence of Fluo-1 and washing.

A fluorescently labeled derivative Fluo-1 (Fig. 1A) of L27-11 showed an MIC against *PA* PAO1 of 0.1 μg/ml, suggesting that it might label potential antibiotic binding sites. *PA* PAO1 cells in MH broth were incubated with Fluo-1 (5 μg/ml) for 1 hour at 37°C, washed, and examined by fluorescence microscopy. The resulting fluorescence staining was not uniform over the cell surface, but rather appeared to be concentrated in spots (Fig. 2F), suggesting a localized binding site. A similar labeling was not observed when the *PA* $PAO1^{RES1}$ mutant was stained in this way.

The ability of the antibiotics to provide protection against a lethal *PA* infection in a whole animal was also tested. For this, the in vivo efficacy of POL7001 and POL7080 was evaluated in a mouse septicemia model at doses of 10, 3, 1, 0.3, and 0.1 mg per kilogram of body weight given subcutaneously at 1 and 5 hours after bacterial inoculation with either *PA* ATCC 9027 or ATCC 27853. Both antibiotics demonstrated substantial activity against both strains with calculated median effective doses ($ED_{50}$ values) in the range 0.25 to 0.55 mg/kg, as compared to gentamicin (used as a positive control), which showed an $ED_{50}$ of 3.1 and 2.9 mg/kg, respectively, against these strains.

The mechanism of action of the peptidomimetic antibiotics reported here likely includes perturbation of the critical LPS transport function of LptD. The in vivo activity reported here raises the prospect that this family of antibiotics may be useful in a clinical setting to combat nosocomial infections and lung infections in patients with cystic fibrosis, where multiple drug-resistant *PA* strains are a serious health problem.

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30. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; R, Arg; T, Thr; V, Val; W, Trp; and Y, Tyr.


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### Supporting Online Material

www.sciencemag.org/cgi/content/full/327/5968/1010/DC1
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Science

# Peptidomimetic Antibiotics Target Outer-Membrane Biogenesis in *Pseudomonas aeruginosa*

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**Killing *Pseudomonas***

Gram-negative *Pseudomonas* bacteria are opportunistic pathogens, and drug-resistant strains present a serious health problem. **Srinivas *et al.*** (p. 1010) synthesized a family of peptidomimetic antibiotics that is active only against *Pseudomonas*. These antibiotics do not lyse the cell membrane, but instead target an essential outer membrane protein, LptD, which plays a role in the assembly of lipopolysaccharide in the outer cell membrane. Activity in a mouse infection model suggests that the antibiotics might have therapeutic potential. In addition, LptD is widely distributed in gram-negative bacteria and so its validation as a target has the potential to drive development of antibiotics with a broader spectrum of activity against gram-negative pathogens.