Structural Basis for the Recognition of Peptide RJPXD33 by Acyltransferases in Lipid A Biosynthesis*

Received for publication, March 13, 2014, and in revised form, April 15, 2014 Published, JBC Papers in Press, April 16, 2014, DOI 10.1074/jbc.M114.564278

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Background: Peptide RJPXD33 binds to and inhibits both LpxA and LpxD acyltransferases.

Results: The crystal structure of the antibacterial peptide RJPXD33 complexed to E. coli LpxA was determined.

Conclusion: RJPXD33 binds to E. coli LpxA in a unique modality that mimics the (R)-β-hydroxyacyl pantetheine moiety of substrate acyl-ACP.

Significance: Bioactive, dual binding LpxA/LpxD peptides raise the possibility of designing less resistance-prone peptidomimetics and/or small molecule antibacterials.

UDP-N-acetylg glucosamine acyltransferase (LpxA) and UDP-3-O-(acyl)-glucosamine acyltransferase (LpxD) constitute the essential, early acyltransferases of lipid A biosynthesis. Recently, an antimicrobial peptide inhibitor, RJPXD33, was identified with dual affinity for LpxA and LpxD. To gain a fundamental understanding of the molecular basis of inhibitor binding, we determined the crystal structure of LpxA from Escherichia coli in complex with RJPXD33 at 1.9 Å resolutions. Our results suggest that the peptide binds in a unique modality that mimics the (R)-β-hydroxyacyl pantetheine binding to LpxA and displays how the peptide binds exclusive of the native substrate, acyl-acyl carrier protein. Acyltransferase binding studies with photo-labile RJPXD33 probes and truncations of RJPXD33 validated the structure and provided fundamental insights for future design of small molecule inhibitors. Overlay of the LpxA-RJPXD33 structure with E. coli LpxD identified a complementary peptide binding pocket within LpxD and serves as a model for further biochemical characterization of RJPXD33 binding to LpxD.

Gram-negative microbes are encapsulated by an asymmetric lipid bilayer composed mainly of lipopolysaccharide (LPS) on the environmental face and phospholipids on the periplasmic face (1, 2). This protective sheath imparts a physiochemical barrier that helps defend these pathogens from hydrophobic antibacterial compounds (3, 4). The glycolipid, lipid A, which is the minimal motif required for the survival of most Gram-negative organisms (5) and is responsible for the endotoxic effects associated with Gram-negative bacterial sepsis, anchors LPS to the outer cell membrane (2, 5). Furthermore, although lipid A has proven to be a viable target for antimicrobial drug discovery (6, 7), there are currently no clinically approved antimicrobial agents targeting lipid A.

The first and third enzymes of the Raetz lipid A pathway (UDP-N-acetylg glucosamine acyltransferase, LpxA; UDP-3-O-(R-3-hydroxyacyl)-glucosamine acyltransferase, LpxD) are type II acyl-carrier protein (ACP)3-dependent acyltransferases. Both enzymes utilize R-3-hydroxyacyrl-ACP and UDP-glucosamine core substrates (Fig. 1) in Escherichia coli (8, 9) and display a unique left-handed β-helix motif that was first identified in LpxA (10–12). Such structural and functional similarities provide the basis for the possibility that a single inhibitor that targets both enzymes could be identified.

Recently, through a phage display screen against LpxD, an inhibitor (RJPXD33; TNLYMLPKWDIP-NH2) was identified that demonstrated affinity and inhibitory activity for both LpxA and LpxD (13). Although peptides display susceptibility to proteases and poor bioavailability, they have become a coveted tool in chemists’ and biologists’ repertoires because of the ease of synthesis and functional utility. Thus, much attention has been given to designing “peptide mimics,” or peptidomimetics, which display greater bioavailability, enhanced three-dimensional structural characteristics, and proteolytic stability compared with their natural counterparts (14). However, a structural and biochemical understanding of how the peptide interacts with its host complex is crucial to the development of peptidomimetics.

The molecular mechanism by which RJPXD33 interacts with either LpxA or LpxD is currently unknown. Herein, we have utilized x-ray crystallography and peptide photoaffinity probes to provide insights into the binding interactions between the LpxA acyltransferase and RJPXD33 (13). Furthermore, acyltransferase binding studies using truncations of RJPXD33 were undertaken to provide biochemical verification of the structural data obtained.

* This work was supported, in whole or in part, by National Institutes of Health Grant ST32GM008597-14 (University of Michigan Chemistry-Biology Interface training program; to R. J. J.). This work was also supported in part by a VanLeesth Research Award administered by the College of Pharmacy, University of Michigan and by a Pre-doctoral Research Grant administered by the University of Michigan Rackham Graduate School.

The atomic coordinates and structure factors (code 8J09) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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3 The abbreviations used are: ACP, acyl-carrier protein; Fmoc, N-(9-fluorenyl)methoxycarbonyl; PPan, phosphopantetheine; r.m.s.d., root mean square deviation.
Structure of LpxA Complexed with RJPXD33

FIGURE 1. Lipopolysaccharide biosynthesis in E. coli. Acyltransferases LpxA and LpxD catalyze functionally similar acylations of their respective UDP-glucosamine-based substrates using R-3-hydroxymyristoyl-ACP as the acyl donor.

EXPERIMENTAL PROCEDURES

Materials—Bio-Rad Protein assay and BioGel P2 size exclusion gel were purchased from Bio-Rad. LB (Lennox) broth and agar were purchased from Difco. Fluorescein isothiocyanate (FITC) was purchased from Acros Organics. Natural amino acids, Rink amide resin, and Fmoc-OSu were purchased from AnaSpec. Photo-leucine (PhotoLeu) was purchased from Thermo Fisher/Pierce. Benzonase was purchased from Novagen. All buffers and antibiotics were purchased in the highest grade from Sigma or Fisher.

Protein Crystallography—Protein purification was carried out as previously described (15). Before crystallization, purified LpxA (10 mg/ml) was incubated for 1 h at 4 °C in the presence of 600 μM RJPXD33 (final concentration of DMSO was 2% v/v). The mixture was centrifuged at 10,000 × g for 10 min at 4 °C to pellet any insoluble material. Crystals were grown by vapor diffusion in a sitting drop tray. Droplets contained 2 μl of the LpxA-RJPXD33 solution and 2 μl of a well solution containing 0.8–1.8 M phosphate buffer (pH 6.3–6.8) and 31–34% DMSO. Crystals formed within 24 h. Crystals were cryoprotected in well solution containing 20% glycerol and flash-frozen in liquid nitrogen. X-ray data were collected at LS-CAT 21-ID-F and -G (24) using Equation 3,

\[ K_d = \frac{[I]_{50}}{K_i} \times \frac{[P]_0 + K_i}{K_d + 1} \] (Eq. 3)

where \([I]_{50}\) is the unlabelled peptide concentration (inhibitor) at 50% inhibition, \([L]_{50}\) is the free ligand (fluorescent tracer) concentration at 50% inhibition, \([P]_0\) is the free protein concentration at 0% inhibition, \(K_i\) is the dissociation constant of fluorescent peptide (tracer), and \(K_d\) is the calculated dissociation constant for unlabelled peptide.

Fmoc-Photoleucine—Fmoc-PhotoLeu was synthesized as previously described (25). In an aluminum foil-covered round-bottom flask, PhotoLeu (L-2-amino-4,4’-azi-pentanoic acid; 1.4 mmol, 200 mg) and sodium bicarbonate (2.8 mmol, 236 mg) were dissolved in 20 ml of H₂O and chilled to 0 °C on ice. Fmoc-OSu (2.1 mmol, 708 mg) was added dropwise over 15 min. The resulting slurry was stirred on ice for 10 min and then
allowed to warm to room temperature for 8 h. The flask was placed on ice, and the reaction was stopped by the addition of 25 ml of H₂O and 50 ml of ethyl acetate (EtOAc) followed by acidification to pH 2 with concentrated HCl. The product was extracted (3×) with 50 ml EtOAc and concentrated by rotary evaporation. The resulting white solid was purified by flash chromatography with 1% methanol (v/v) and 1% acetic acid (v/v) in dichloromethane. The product was obtained as a white solid (464 mg, 91%). 1H NMR (400 MHz, DMSO) δ 12.70 (s, 1H), 7.87 (d, J = 7.5, 2H), 7.30 (t, J = 7.5, 2H), 4.38–4.16 (m, 3H), 3.89–3.76 (m, 2H), 3.19 (m, 3H), 2.53–2.41 (m, 2H), 2.12–2.02 (m, 2H), 1.93–1.81 (m, 1H), 1.63 (dd, J = 7.4, 2H), 7.71 (dd, J = 7.4, 2H), 7.30 (t, J = 7.4, 2H), 4.38–4.16 (m, 3H), 3.89–3.76 (m, 2H), 3.19 (m, 3H), 2.53–2.41 (m, 2H), 2.12–2.02 (m, 2H), 1.93–1.81 (m, 1H), 1.63 (dd, J = 14.8, 10.8, 1H), 0.99 (s, 3H).

High resolution MS (ESI⁺): calculated for [M + Na]⁺ was 388.1278; observed was 388.1276.

**Results and Discussion**

Recognition of RJPXD33 by LpxA—RJPXD33 is an antimicrobial peptide that inhibits both E. coli LpxA (Kᵣ = 22 μM) and LpxD (Kᵣ = 6 μM) acyltransferases in early lipid A biosynthesis. The peptide binds to acyltransferases in a mutually exclusive manner with the acyl-ACP substrate. To design potent peptide mimetics and/or small molecule antimicrobials with the same unique dual binding properties of the parent peptide, a fundamental understanding of the molecular interactions between peptide and the target protein is vital. In this present study the EclpxA-RJPXD33 crystal structure has assisted in elucidating the molecular mechanism of this peptide-protein interaction.

The co-crystal structure of EclpxA-RJPXD33 (TNLYMLPKWDIP-NH₂) diffracted to a resolution of 1.9 Å. The structure was solved by molecular replacement using a previously solved structure of LpxA (PDB code 1LXA), containing no bound ligands, as the model structure (12). The structure was refined to a R_factor and R_free of 0.179 and 0.188, respectively, while maintaining a good MolProbity score and low clash score (21).

**Table 1**

| Data collection and refinement statistics | EclpxA + RJPXD33 |
|------------------------------------------|-----------------|
| Space group                              | P₂₁₂₁₂ |
| Unit cell a, b, c (Å)                     | 96.40, 96.40, 96.40 |
| Resolution (Å)                           | 1.9 (1.93-1.90) |
| R_sym (%)                                | 5.7 (29.7) |
| I/σ(I)                                   | >20 (5) |
| Completeness (%)                         | 99.9 |
| Redundancy                               | 10.6 (10.1) |

The complete crystallography and refinement statistics can be found in Table 1. One molecule of LpxA and RJPXD33 occupied the asymmetric unit. However, three asymmetric units form around a 3-fold crystallographic axis, which is in agreement with previous structures and data showing that LpxA is a homotrimer (9, 26, 27). All of the 262 amino acids of LpxA, with the exception of the N-terminal methionine, were visible in the electron density map. The N-terminal half of RJPXD33 binds within the region between two monomeric subunits, constituting the LpxA active site, with a portion of the Met-5 side chain missing (28). RJPXD33 binds to LpxA in an unforeseen binding mode (Fig. 2).

The six N-terminal amino acids of RJPXD33, TNLYML, run vertically up the bifurcated active site with Thr-1 positioned at the cleft. Upon binding of RJPXD33, the His-160 side chain rotates 5% of the wild-type enzyme (28). A noticeable difference between our current structure and that of unbound EcLpxA is the conformation of the His-160 side chain (Fig. 3). In unbound EcLpxA this side chain faces inward toward the hydrophobic cleft. Upon binding of RJPXD33, the His-160 side chain rotates outward toward the solvent front. This conformational change is what is commonly observed among all LpxA structures containing a bound acylated ligand (26).

The co-crystal structure of EcLpxA-RJPXD33 (TNLYMLPKWDIP-NH₂) diffracted to a resolution of 1.9 Å. The structure was solved by molecular replacement using a previously solved structure of LpxA (PDB code 1LXA), containing no bound ligands, as the model structure (12). The structure was refined to an R_factor and R_free of 0.179 and 0.188, respectively, while maintaining a good MolProbity score and low clash score (21). The complete crystallography and refinement statistics can be found in Table 1. One molecule of LpxA and RJPXD33 occupied the asymmetric unit. However, three asymmetric units form around a 3-fold crystallographic axis, which is in agreement with previous structures and data showing that LpxA is a homotrimer (9, 26, 27). All of the 262 amino acids of LpxA, with the exception of the N-terminal methionine, were visible in the electron density map. The N-terminal half of RJPXD33 binds within the region between two monomeric subunits, constituting the LpxA active site, with a portion of the Met-5 side chain missing (28). RJPXD33 binds to LpxA in an unforeseen binding mode (Fig. 2).

The six N-terminal amino acids of RJPXD33, TNLYML, run vertically up the bifurcated active site with Thr-1 positioned at the cleft. Upon binding of RJPXD33, the His-160 side chain rotates 5% of the wild-type enzyme (28). A noticeable difference between our current structure and that of unbound EcLpxA is the conformation of the His-160 side chain (Fig. 3). In unbound EcLpxA this side chain faces inward toward the hydrophobic cleft. Upon binding of RJPXD33, the His-160 side chain rotates outward toward the solvent front. This conformational change is what is commonly observed among all LpxA structures containing a bound acylated ligand (26).

**Table 1**

| Data set | EclpxA + RJPXD33 |
|----------|-----------------|
| Resolution | 1.90 |
| Unique reflections | 23,751 |
| R_factor (%) | 0.179 |
| R_free (%) | 0.188 |
| Protein atoms | 2004 |
| Solvent molecules | 213 |
| Root mean square deviations | 0.010 |
| Bond angles (°) | 1.06 |
| MolProbity | 1.21 |
| Protein Data Bank code | 4092 |
the unbound form of LpxA (12, 27, 31). These results demonstrated that His-160 of LpxA must kink outward to allow the fatty acyl chain to access the hydrophobic cleft and that RJPXD33 is able to mimic this binding motif. Although the full electron density of the RJPXD33 Met-5 side chain cannot be accounted for, these results would suggest that Met-5 would occupy the binding region formed when His-160 is kinked outward (Fig. 3). This conformational change may allow His-160 to form polar contacts with acidic residues of ACP and thus would be important in substrate binding and release.

Comparison of RJPXD33 Binding with Other E. coli LpxA Ligands—The hydroxyl moiety of the Thr-1 side chain of RJPXD33 forms polar contacts with His-122, Gln-73, and an active site water of LpxA (Fig. 3). These moieties have been implicated as binding contacts to the R-3-hydroxymyrystoyl moiety of UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc, the LpxA product (26). When the structures of RJPXD33-bound EcLpxA and the product-bound EcLpxA (PDB code 2QIA) were superimposed, the Thr-1 β-hydroxyl group was shown to occupy the same space as the R-3-hydroxy functional group of the myristate chain (Fig. 4). In addition to the Thr-1 residue, the Leu-3 and Met-5 side chains of RJPXD33 also occupy the fatty acyl binding region.

RJPXD33 binds to LpxA in a unique fashion when compared with the LpxA-specific peptide P920 (WMLDPIAGKWSR; Fig. 5) despite a similar primary sequence motif (YMLP versus WMLDP) between the two (32). P920 forms a β-hairpin fold oriented perpendicular to the LpxA symmetry axis, where the head of the loop, isoleucine, occupies a small portion of the fatty acid binding cleft of LpxA similar to Leu-3 of RJPXD33 (27). The N and C termini of P920 face out of the pocket toward the solvent face, overlapping the nucleotide binding region identified from several UDP ligand-bound structures (26, 31). In contrast, RJPXD33 is oriented parallel to the LpxA symmetry axis, occupying most of the hydrophobic fatty acid binding cleft whereas demonstrating only a minimal overlap between its Asn-2/Tyr-4 side chains and the UDP binding site. Unlike RJPXD33, P920 does not extend into the lower or upper R-β-hydroxy acyl binding pocket occupied by Thr-1 and Met-5 of RJPXD33, respectively. This is highlighted by the lack in conformational change of His-160 in the P920-bound structure compared with unbound LpxA.
Comparison of RJPXD33 Binding with (R)-β-Hydroxylauroyl-methylphosphopantetheine—Although derivatives of phosphopantetheine (PPan), the post-translational modification necessary for acyl chain assembly on ACP, are not substrates for EcLpxA, R-3-hydroxylauryl methyl-phosphopantetheine has been shown to be a substrate for *L. interrogans* LpxA (30, 33).

The co-crystal structure of EcLpxA-RJPXD33 was superimposed onto the C-chain of the LiLpxA-(R)-3-hydroxylauroyl-methyl-PPan co-crystal structure (Fig. 4; PDB code 3I3A) (30). As with the EcLpxA product-bound structure, the Thr-1, Leu-3, and Met-5 side chains of RJPXD33 overlay with the laurate chain of the LiLpxA-bound (R)-3-hydroxylauroyl-methyl-phosphopantetheine (PDB code 3I3A; gray) and EcLpxA (PDB code 1LXA; green) with no bound ligands. PyMOL alignment r.m.s.d. = 0.35 over 256 residues.

When bound to LpxA and irradiated with UV light, PhotoLeu generates a reactive carbene group that can react with groups on LpxA in close proximity and covalently cross-link the peptide to the bound protein. Photopeptides 1, 3, and 4 (24 μM) were subjected to UV irradiation (10 min) in the pres-
Structure of LpxA Complexed with RJPXD33

FIGURE 5. Comparison of the binding of RJPXD33 and P920 to LpxA. A, top down view of RJPXD33 (cyan; sticks) and P920 (pink; sticks) complexed with LpxA trimer. Subunit A of each LpxA peptide complex is shown as ribbon (PDB code 4J09, gray; PDB code 2AQ9, magenta), whereas the surface of the other two trimer subunits is shown in gray, and only one molecule of RJPXD33/P920 is shown. B, frontal, close-up, stereo view of the same complexes above. PyMOL alignment r.m.s.d. = 0.18 over 237 atoms.

TABLE 2
Binding constants of RJPXD33 truncations to EcLpxA

| Peptide (sequence) | $K_d$ LpxA | $K_d$ LpxD |
|--------------------|------------|------------|
| FITC-RJPXD33       | 17 ± 1.6   | 0.6 ± 0.04 |
| FITC-Photo 1       | 32 ± 1.1   | 1.1 ± 0.1  |
| FITC-Photo 4       | 22 ± 1.7   | 0.8 ± 0.1  |
| RJPXD33Δ6          | 12 ± 1     | 22 ± 1     |
| RJPXD33Δ6-COOH     | 23 ± 2.0   | 36 ± 3.5   |

FIGURE 6. In-gel fluorescence of PhotoLeu-containing peptides. Affinity labeling was accomplished by UV irradiation ($\lambda = 365$ nm) while holding LpxA and FITC-photopeptides constant at 10 and 24 $\mu$M, respectively, in the presence or absence of unlabeled RJPXD33 (200 $\mu$M). Under these conditions photo-labeling of LpxA was linear up to 10 min, which is the time used in the above gel samples. Gels were analyzed for in-gel fluorescence using a Typhoon 9400 imaging system set to fluorescein wavelength ($\lambda_{ex} = 485$ nm and $\lambda_{em} = 525$ nm), photomultiplier tube sensitivity at 500, and pixel size at 50 $\mu$M. LpxA was stained with Coomassie Blue to demonstrate consistency of protein loading.

In competition binding assays RJPXD33 significantly decreased the fluorescent signal, indicating that the covalent cross-linking is due to specific peptide-protein interactions and that the photopeptides bind to LpxA in a similar manner as the parent peptide. As a control, reactions containing LpxA without the photopeptides were subjected to UV irradiation, and no observable fluorescence was detected.

Binding of RJPXD33 Truncation to LpxA—To corroborate our crystal structure and evaluate the potential for possible RJPXD33 truncations, FITC-RJPXD33Δ6 was synthesized. FITC-RJPXD33Δ6 lacks the six terminal amino acids of full-length FITC-RJPXD33. Fluorescence polarization binding experiments using wild-type LpxA were performed to quantify the binding affinity of the truncated RJPXD33 peptide. When the assay was performed with EcLpxA, the observed $K_d$ for FITC-RJPXD33Δ6 was 12 ± 1 $\mu$M (Table 2). This value was in close proximity to the previously published value ($K_d = 17 ± 1.6$ $\mu$M) for the full-length FITC-RJPXD33 (13). This suggested that the six terminal amino acids are not making extensive binding contacts to LpxA, in agreement with the crystal structure.

In addition to its role as the hydrocarbon ruler, His-191 may be important in binding of the phosphopantetheine moiety of ACP substrates. His-191 occupies a space in EcLpxA similar to Lys-171 of LiLpxA, which is postulated to play a similar role as the acidic C terminus to interact with the nearby basic His-191. FITC-RJPXD33Δ6-COOH binds with approximately 3 times higher affinity to EcLpxA than its amide capped homolog. This trend remains true with the removal of the fluorescein moiety.
by phage display against immobilized LpxD (13). Although no EcLpxD-RJPXD33 structure is available, this current work suggests that RJPXD33 would overlap with the acyl-PPan arm of acyl-ACP, thereby inhibiting acyl-ACP from binding to LpxD. This is in agreement with our previous results that demonstrated RJPXD33 bound exclusive of acyl-ACP to LpxD.

Fluorescence polarization binding experiments using wild-type LpxD were performed to quantitate the binding affinity of the FITC-photopeptides and truncated RJPXD33 peptides. Similar to their binding to LpxA, each of the photopeptides bound with Hill coefficients between 0.8 and 0.9. The $K_d$ values for the photopeptides (0.8–2.1 μM; Table 2) were in close proximity to the value for FITC-RJPXD33 for LpxD ($K_d = 0.6 ± 0.04$ μM) (13) and showed the same binding trend as seen in LpxA, with photopeptide 4 being least disruptive of binding. When the assay was performed with truncated peptide, the observed $K_d$ for FITC-RJPXD33Δ6 was 22 ± 1 μM, similar to its binding to LpxA (12 ± 1 μM). However, the binding of FITC-RJPXD33Δ6-COOH (36 ± 3.5 μM) did not show increased affinity to LpxD as it did to LpxA (Table 2) and reflects the fact that the role of hydrocarbon ruler in EcLpxD is fulfilled by Met-290 instead of a basic histidine residue as with LpxA (Fig. 7D) (10, 35).

Like acyl-ACP, RJPXD33 binds to LpxD without the prior binding of other ligands. To shed light on this interaction, structural overlays between our RJPXD33-EcLpxA complex, EcLpxD (PDB code 3EH0), and the recently published EcLpxD-acyl-ACP (PDB code 4IHF) were performed (Fig. 7). From these overlays, it is clear that RJPXD33 would occupy a similar fatty acyl binding groove in EcLpxD when compared with LpxA. The overlays align RJPXD33 with the R-3-hydroxymyristoyl phos- phopantetheine prosthetic group of acyl-ACP. The Thr-1, Leu-3, and Met-5 side chains of RJPXD33 overlay with the R-3-hydroxymyristoyl chain, whereas the peptide backbone of RJPXD33 aligns well with the PPan moiety. The amide bond between Thr-1 and Asn-2 of RJPXD33 superimposes with the thioester bond between the acyl chain and the PPan cysteamine moiety. The Tyr-4 and...
Leu-6 side chains of RJPXD33 occupy the O-channel, which is postulated to be the binding site for the ester-linked acyl group of substrate UDP-3-O-acyl-GlcN. The six C-terminal residues of RJPXD33 would potentially extend into a binding groove near the C-terminal α-helical domain of EcLpxD, which is not present in EcLpxA and has been identified as the ACP binding domain (35). This would explain the role of the six C-terminal residues with respect to the increased affinity of RJPXD33 for LpxD when compared with LpxA as well as the greater loss in affinity between full-length peptide and the C-terminal Δ6 peptide for LpxD over LpxA.

Our present work unravels the molecular mechanism under-lying RJPXD33 binding to acyltransferases in early lipid A biosynthesis, showing that it mimics the (R)-β-hydroxyacyl pantetheine moiety of substrate acyl-ACP both in terms of its overall chemical space occupancy and its ability to induce similar conformational changes to the acyltransferase upon binding. Considering the diverse array of protein-protein interactions between ACP and its client proteins, other acyl-PPan mimics may find use as antimicrobial inhibitors of essential ACP-protein interactions. The co-crystal structure of RJPXD33-LpxA and the fluorescence polarization experiments associated with FITC-RJPXD33Δ6 demonstrate that certain RJPXD33 truncations can bind with similar affinity to both LpxA and LpxD. Such truncations would be important in developing smaller peptidomimetics capable of crossing the cell membrane.

Acknowledgments—Use of the Advanced Photon Source, an Office of Science User Facility operated for the United States Department of Energy by Argonne National Laboratory, was supported by the United States Department of Energy Office of Science and by the Office of Biological and Environmental Research of the United States Department of Energy Office of Science. Use of the LS-CAT Sector 21 was supported by the Office of Biological and Environmental Research and the Office of Health and Environmental technologies of the United States Department of Energy Office of Science. Use of the Advanced Photon Source, an Office of Science User Facility operated for the United States Department of Energy by Argonne National Laboratory, was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, and Office of Biological and Environmental Research.

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