Supporting Information

Structural and Kinetic Characterization of Diazabicyclooctanes as Dual Inhibitors of Both Serine-β-Lactamases and Penicillin-Binding Proteins

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Experimental Procedures

Reagents. All chemicals of analytical grade were purchased from Sigma-Aldrich, unless otherwise stated. Nitrocefin was synthesized as described previously.[1]

β-Lactamase Protein Expression and Purification. The *Escherichia coli* CTX-M-15 and *Klebsiella pneumonia* OXA-48 constructs include the mature sequences (Q26-L288, and W25-P265) with the signal peptide removed and were prepared as previously described.[2]. Purified CTX-M-15 (with His-tag cleaved) and OXA-48 (with un-cleaved His-tag) were dialyzed into fresh crystallization buffer (CTX-M-15 buffer: 10mM Tris pH 7.5, 100mM NaCl, 250uM 2-mercaptoethanol, and OXA-48 Buffer: 20mM Tris pH 7.5, 100mM NaCl) and concentrated to ~30mg/mL. For kinetics studies CTX-M-15 and OXA-48 were purified as previously described.[3]

PBP Plasmid Construction, Protein Expression, and Purification. The *E. coli* PBP1a, PBP1b, PBP2, and PBP3 DNA corresponding to amino acid residues 1-855, 58-804, 60-633, and 57-577 were amplified from *E. coli* K12 genomic DNA. Restriction free cloning was used to produce pET-41b expression vectors containing each of the PBPs with a thrombin cleavable C-terminal 8XHis tag [4].

Vectors containing the *E. coli* PBP1b, and PBP1a membrane proteins were transformed into BL21(DE3), and C43(DE3) host cells. The transformed cells were grown at 37°C until an OD_{600} of 0.6 was reached, and the samples were cooled to room temperature for 30 min. Protein expression was induced by addition of 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the cultures were incubated at 25 °C overnight. Cell pellets were resuspended in lysis buffer (PBP1a: 25mM Tris pH 7.5, 10mM $MgCl_2$, 300mM NaCl, 1 EDTA free protease inhibitor tablet from Roche, PBP1b: 20mM Tris pH 8.0, 300 mM NaCl, 1 EDTA free protease inhibitor tablet from Roche) and lysed by 2 passes on a French press at a pressure of 1500 p.s.i. The cell lysate was then centrifuged twice at 14, 600 g for 15 minutes to remove unbroken cells and inclusion bodies. The supernatant was then centrifuged at 200, 000 g for 1 hour in order to pellet the membranes. Membranes were homogenized and incubated for 4 hours in the presence of extraction buffer (lysis buffer + 20mM n-Dodecyl-β-D-maltopyranoside, DDM; Anatrace). The solubilized protein was then purified using nickel chelation chromatography. The column was pre-incubated in the presence of equilibration buffer (PBP1a: 25mM Tris pH 7.5, 300mM NaCl, 1mM DDM, PBP1b: 20mM Tris pH 8.0, 300mM NaCl, 1mM DDM) and eluted using a linear gradient of imidazole from 0-500mM. Fractions containing purified protein were exchanged using a 100 kDa cut-off concentrator into assay buffer [PBP1a: equilibration buffer, PBP1b: 20mM Tris pH 8.0, 300mM NaCl, 4.5 mM n-Decyl-β-D-maltopyranoside (DM), Anatrace].

BL21(DE3) host cells transformed with the *E. coli* PBP2 and PBP3 expression vectors were grown at 37°C until an OD_{600} of 0.7 was attained. Protein expression was induced by addition of 1mM IPTG and the cultures (typically 9L) were incubated at 30°C for 16 hrs. Cell pellets were resuspended in lysis buffer [PBP2: 50mM Tris pH 8.0, 300mM NaCl, 1 EDTA free protease inhibitor table from Roche, PBP3: 20mM Tris pH 8.0, 10% glycerol, 300 mM NaCl, 10 mM $MgCl_2$, 40mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1 EDTA free protease inhibitor tablet from Roche] and lysed by 2 passes on a French press at a pressure of 1500 p.s.i. The cell lysate was then centrifuged at 20,000 g for 1 hour. The supernatant was purified using nickel chelation chromatography. The column was pre-incubated in the presence of equilibration buffer (50mM Tris pH 8.0, 300mM NaCl, 5% glycerol) and eluted using a linear gradient of imidazole from 0-600mM. Peak fractions containing purified protein were pooled and exchanged into assay buffer (equilibration buffer), using a 50 KDa cutoff Amicon centrifugal concentrator.

Crystallization, Data Collection and Structure Determination. FPI-1465, FPI-1523, and FPI-1602 carbamyl-enzyme complex CTX-M-15 crystals were grown using the sitting drop vapor diffusion method at 24°C. The drops consisted of 1 μL (30mg/mL protein + 2mM FPI-1465, FPI-1523 or FPI-1602), combined with an equal volume of precipitant [0.2M ammonium sulfate, 0.1M 2-(N-morpholino)ethanesulfonic acid (MES) pH6.5, 30% PEG5K monomethyl ether]. Crystals were soaked in cryoprotectant solution for 30s (mother liquor + 25% glycerol), and flash vitrified in liquid nitrogen. The FPI-1465, FPI-1523 and FPI-1602 crystals diffracted to 3.0, 1.67 and 2.70 Å at beamline CMCF-08B1-1 of the Canadian Light Source in Saskatoon Saskatchewan (CLS).

FPI-1465 and FPI-1523 carbamyl-enzyme complex OXA-48 crystals were grown using the sitting drop vapor diffusion method at 24°C. The drops consisted of 1 μL (30 mg/mL protein + 3.6 mM FPI-1465 or 3.9 mM FPI-1523), combined with an equal volume of precipitant (0.005 M $CoCl_2$, 0.005 M $CdCl_2$, 0.005 M $MgCl_2$, 0.005 M $NiCl_2$, 0.1 M HEPES pH 7.5, 12% w/v PEG 3350). Co-crystallization attempts with FPI-1602 and OXA-48 failed to yield suitable crystals so apo crystals were grown as above but without ligand and soaked in 9.0 mM FPI-1602 for 2 days. Crystals were soaked in cryoprotectant solution for 30s (mother liquor + 29 to 33% (v/v) glycerol, and 3mM inhibitor), and flash vitrified in liquid nitrogen. The FPI-1465, FPI-1523, and FPI-1602 bound OXA-48 crystals diffracted to 1.96, 1.74 and 2.09Å at beamline CMCF 08B1-1 of the CLS.

FPI-1465 carbamyl-enzyme PBP1b crystals were grown using the sitting drop vapor diffusion method at 24°C. Drops contained 1μL (10mg/mL protein + 5mM FPI-1465), combined with an equal volume of precipitant (20% w/v PEG 3350, 0.2M potassium/sodium tartrate, 0.1M Bis Tris pH 8.5). Crystals were soaked in cryoprotectant solution for 4 min (mother liquor + 40% glycerol), and flash vitrified in liquid nitrogen. The FPI-1465-PBP1b crystals diffracted to

2.85Å resolution at beamline CMCF-08B1-1 of the CLS. All crystallographic data in this study was collected at a temperature of 100K and wavelength of 1.00Å.

Data were processed using Xia2.[5] During refinement, 5% of reflections were set aside for cross validation. All structures of CTX-M-15, OXA-48, and PBP1b were solved by molecular replacement using the program Phaser [6], with chain A of the native crystal structures as starting models (PDB ID's, CTX-M-15; 4HBT, OXA-48; 3HBR, PBP1b; 3VMA). Several iterations of manual rebuilding in Coot.[7] followed by refinement using Phenix [8] were carried out. All structures were refined using isotropic B-factors, with the notable exception of FPI-1523-CTX-M-15, which was refined using anisotropic B-factors. Water and the appropriate ligands were added manually by examination of the F_o-F_c and $2F_o$-F_c electron density maps. Ligand CIF dictionaries were created using PRODRG.[9] In all structures, all ligands were refined at full occupancy. Figs 1b-c, Fig. 2d, and Supplementary Fig. 1,2 and 7 were made using PyMol.[10]

Enzyme Assays. For all enzyme assays the buffer consisted of 50 mM HEPES pH 7.5 and Tween20 0.01%. OXA-48 experiments were performed with the addition of 50 mM $NaHCO_3$. Enzyme dilutions were made in BSA to 100 ng/µL. Acylation and deacylation experiments were performed as described previously.[2, 11].For all compounds described, on-rates were determined using a continuous assay with nitrocefin as reporter substrate. For CTX-M-15, 100 µL enzyme (0.2 nM [final]) was added to 100 µL nitrocefin (50 µM [final]; K_m = 10 µM) and inhibitor. The maximum concentration of inhibitor used for CTX-M-15 on-rates was: avibactam, 0.8 µM; FPI-1465, 9 µM; FPI-1523, 4 µM; FPI-1602, 9 µM. The same methods were applied for OXA-48 (0.03 nM [final]) with nitrocefin (100 µM [final]; K_m = 50 µM). For OXA-48, the maximum concentration of inhibitor used was: avibactam, 50 µM; FPI-1465, 100 µM; FPI-1523, 100 µM; FPI-1602, 100 µM.

For CTX-M-15, off-rates were determined continuously using the jump dilution method[12] where 1 µM enzyme was incubated with 10 µM inhibitor at 37°C for 30 minutes and then diluted 1/400 before adding 20 µL to 180 µL of nitrocefin (400 µM) in assay buffer. For OXA-48 7 µM enzyme was incubated with 10 µM inhibitor and incubated for 1 hour before 1/16000 dilution and addition to substrate (200 µM) as above. For OXA-48, discontinuous sampling was applied for all inhibitors other than FPI-1465.

For concentration-response experiments assay buffer was used as above. All enzymes (1 nM) were incubated with inhibitor for 30 minutes at 37°C before dilution in nitrocefin (20 µM). Metalloenzymes were supplemented with 10 µM $ZnSO_4$. The maximum concentration of avibactam used was 20 µM.

Antimicrobial Susceptibility Testing. MIC testing was done according to the Clinical Laboratory Standards Institute.[13] All experiments were performed in duplicate and strains were grown at 37°C for 18 hours. pGDP constructs were made with the noted gene under control of a *bla* promoter for high-level constitutive expression.

PBP Binding Assays. For bacterial membrane preparation, an overnight culture of *E. coli* K-12 (MG1665) in BHI broth was diluted in a fresh medium and was further incubated at 37°C under agitation to reach an OD_{600} of ~0.6-0.7. The cells were harvested by centrifugation at 3,000 g for 15 min at 4°C, washed and suspended in KPN (20 mM potassium phosphate - 140 mM NaCl, pH 7.5). Cells were first treated with lysozyme (500 µg/mL) for 1h at 37°C, before addition of a protease inhibitor cocktail (Sigma Aldrich Canada, Oakville, ON), deoxyribonuclease (6 µg/mL) and ribonuclease (6 µg/mL). After 30 minutes of treatment, cells were disrupted by a French press and the bacterial lysate was centrifuged at 12,000 g for 10 min to remove unbroken cells. The supernatant was then centrifuged at 150,000 g for 40 min at 4°C using a fixed-angle rotor to collect the membranes. The membranes were suspended in a minimal volume of KPN buffer and stored at -86°C. Protein concentration was estimated by the method of Bradford with the BCA kit (Pierce) using bovine serum albumin as a standard.

The relative binding affinity of test molecules for bacterial PBPs were assayed in a competition assay with the fluorescent penicillin BOCILLIN FL (Invitrogen, Carlsbad, CA) as the reporter molecule. Increasing concentrations of the test compounds were added to aliquots of the reaction mixture containing 30 µg of bacterial membrane preparation for 10 min at 37°C prior to the addition of BOCILLIN FL (100 µM) for an additional 20 min. Membrane-containing samples were then heated to 95°C for 3 min in electrophoretic loading buffer containing SDS before electrophoresis and separation of proteins on a SDS-polyacrylamide discontinuous gel system (5% stacking and 10% separating gels). After electrophoresis, the gels were quickly rinsed in water and incubated for 30 min in a fixing solution (50% methanol - 7% acetic acid). Gels were scanned with a Molecular Imager FX Pro instrument (Bio-Rad Laboratories Canada, Mississauga, ON) using the excitation and emission wavelengths of 488 nm and 530 nm, respectively, to collect the image of the PBP profile. The concentration of the test compound needed to block 50% of the subsequent binding of BOCILLIN FL to each PBP represented the IC_{50} value.

BOCILLIN FL Competition Assays using Purified *E. coli* PBPs. To assess the relative inhibition of *E. coli* PBPs by the avibactam derivatives, SDS-PAGE based concentration response experiments were performed in triplicate using BOCILLIN FL as a reporter molecule. All reagents were diluted in assay buffer prior to use. To start the reaction, various concentrations of unlabeled compound and 27.8 µM BOCILLIN FL were simultaneously added to 4.7µM of purified PBP in a final reaction volume of 36 µL. The reaction was incubated at 25°C for 20 min prior to addition of 10X SDS-PAGE loading dye. In contrast, for pre-incubation experiments various amounts of inhibitor compound was pre-incubated with 4.7µM *E. coli* PBP1b for 48 hours prior to an additional 20 min incubation in the presence of 27.8

μM BOCILLIN FL (Figure S6). The samples were then boiled for 2 min prior to loading 10μL on a 12% SDS-PAGE precast gel (Bio-Rad). Following electrophoresis, gels were imaged under UV light using a Syngene ChemiGenius2 bio imaging System. Densitometry analysis was performed using ImageJ as previously described.[14] The individual data points were normalized to the maximum value of the fluorescence intensity, which represents total saturation of protein by BOCILLIN FL in the absence of unlabeled compound. Benzyl penicillin, and Kanamycin were used as positive and negative controls, respectively. The IC_{50} values are defined as the compound concentration required to reduce the residual binding of BOCILLIN FL by 50% and were calculated using SigmaPlot.

Microscopy. Cells were cultured in a standard MIC curve, then fixed and imaged according to the methods of Czarny et al.[15] In brief, after culture densities were recorded using a spectrophotometer, cultures were diluted 1:10 in 2% glutaraldehyde buffered with 25 mM HEPES (pH 6.8) for one hour. Then, 15 uL of this solution was transferred to a 0.17 mm glass-bottom 384-well microplate, along with 5 uL of 1.5% filter-sterilized nigrosin stain. Plates were gently flushed with nitrogen gas, then heat-fixed at 50°C in a humidity-controlled incubator. Finally, plates were imaged under brightfield using a Nikon Eclipse Ti-E inverted microscope. Cell features were quantified with ImageJ[14], using the analysis pipeline in Czarny et al.[15] These image features were used to cluster drug treatments using Ward's least variance, as well as compute a correlation map and Pearson correlation values for treatments.

Accession Codes. Coordinates and structure factors for FPI-1465-CTX-M-15, FPI-1523-CTX-M-15, FPI-1602-CTX-M-15, FPI-1465-OXA-48, FPI-1523-OXA-48, FPI-1602-OXA-48 and FPI-1465-PBP1b were deposited in the PDB with accession codes (5FAO, 5FA7, 5FAP, 5FAQ, 5FAS, 5FAT, 5FGZ).

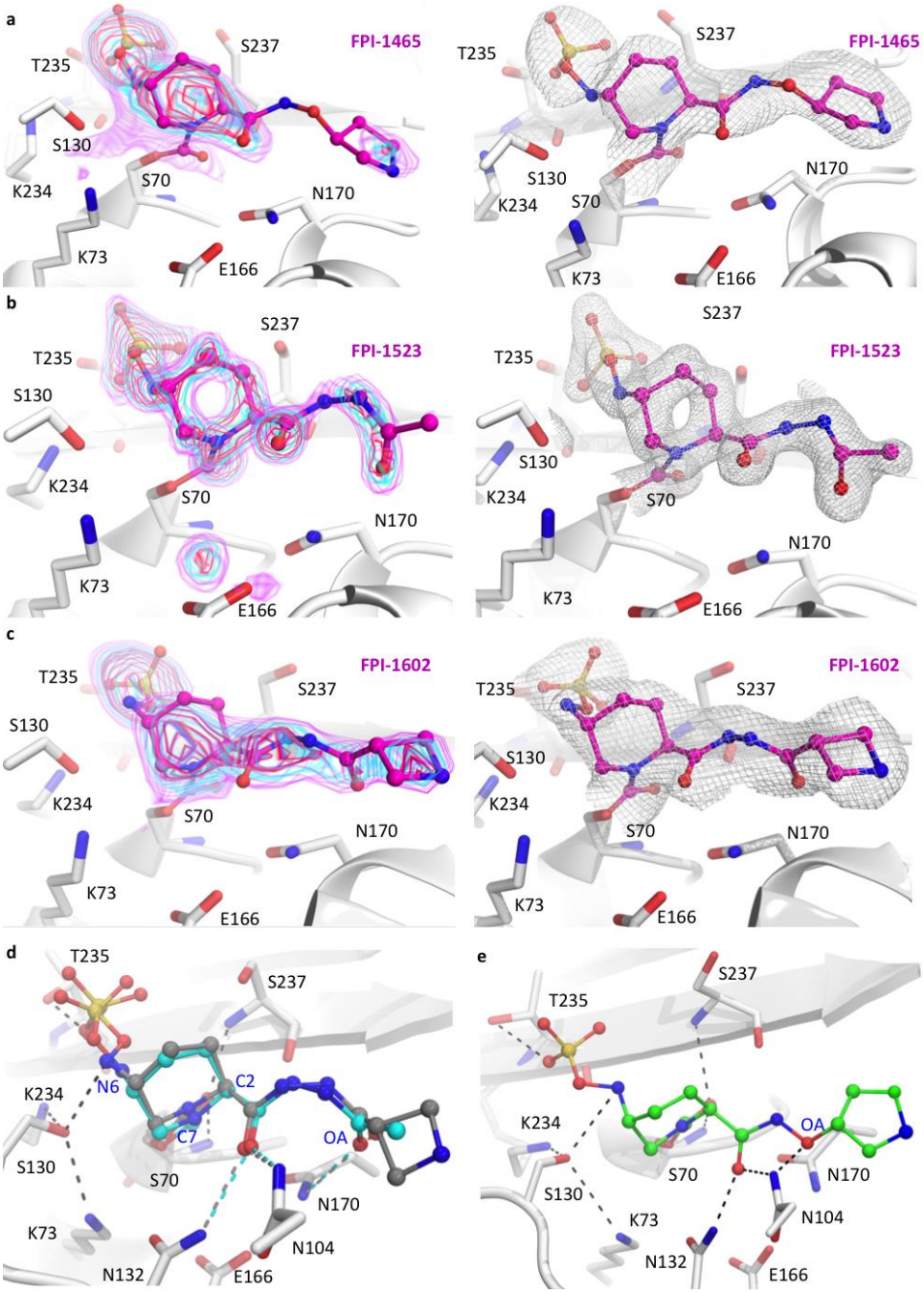


Figure S1. Avibactam derivative electron density maps and ligand protein interactions for CTX-M-15 co-crystal complexes. a, Carbamyl FPI-1465-CTX-M-15 ligand electron density maps. **b,** Carbamyl FPI-1523-CTX-M-15 ligand electron density maps. **c,** Carbamyl FPI-1602-CTX-M-15 ligand electron density maps. In **(a-c)**, the carbamyl-avibactam derivative is represented as pink sticks with atoms colored by type. The left panel for **(a-c)** shows F_o-F_c ligand omit maps contoured at 3.0, 4.0 and 5.0 σ displayed as pink, cyan and red transparent surfaces. The right panel in **(a-c)** shows final refined $2F_o$-F_c electron density maps for each ligand contoured at 1.0σ. **d,** Active site overlay of FPI-1523 and FPI-1602 bound to CTX-M-15 (rmsd= 0.2Å on all CA atoms). The carbamyl -FPI-1523 and -FPI-1602 are displayed in cyan and grey stick representation with non-carbon atoms colored by atom type. **e,** Active site close-up of FPI-1465 bound to CTX-M-15. The bound FPI-1465 is displayed as green sticks with non-carbon atoms colored by type. In all panels, the CTX-M-15 cartoon is shown in white with selected active site residues displayed in stick representation with all non-carbon atoms colored by type.

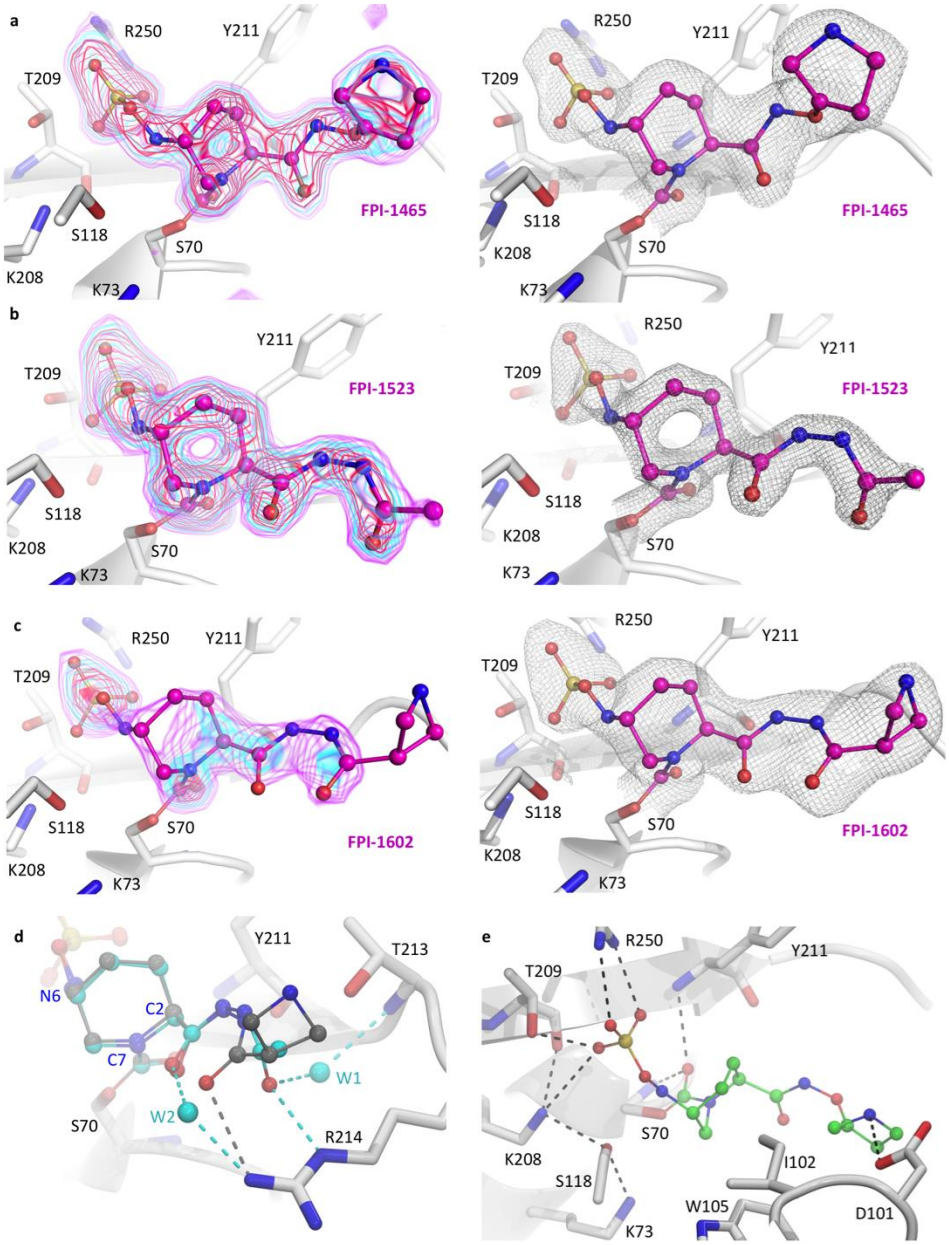


Figure S2. Avibactam derivative electron density maps and ligand protein interactions for OXA-48 co-crystal complexes. a, Carbamyl FPI-1465-OXA-48 ligand electron density maps. **b,** Carbamyl FPI-1523-OXA-48 ligand electron density maps. **c,** Carbamyl FPI-1602-OXA-48 ligand electron density maps. In **(a-c)**, the carbamyl-avibactam derivative is represented as pink sticks with atoms colored by type. The left panel for **(a-c)** shows F_o-F_c ligand omit maps contoured at 3.0, 4.0 and 5.0 σ and illustrated as pink, cyan and red transparent surfaces. The right panel in **(a-c)** shows final refined $2F_o$-F_c electron density maps for each ligand contoured at 1.0σ. **d,** Active site overlay of FPI-1523 and FPI-1602 bound to OXA-48 (rmsd= 0.2Å on all CA atoms). The carbamyl -FPI-1523 and -FPI-1602 are displayed in stick representation with non-carbon atoms colored by atom type. **e,** Active site close-up of FPI-1465 bound to the OXA-48 active site. The bound FPI-1465 is displayed as green sticks with atoms colored by type. In all panels, the OXA-48 cartoon is shown in white with selected active site residues displayed in stick representation with all non-carbon atoms colored by type.

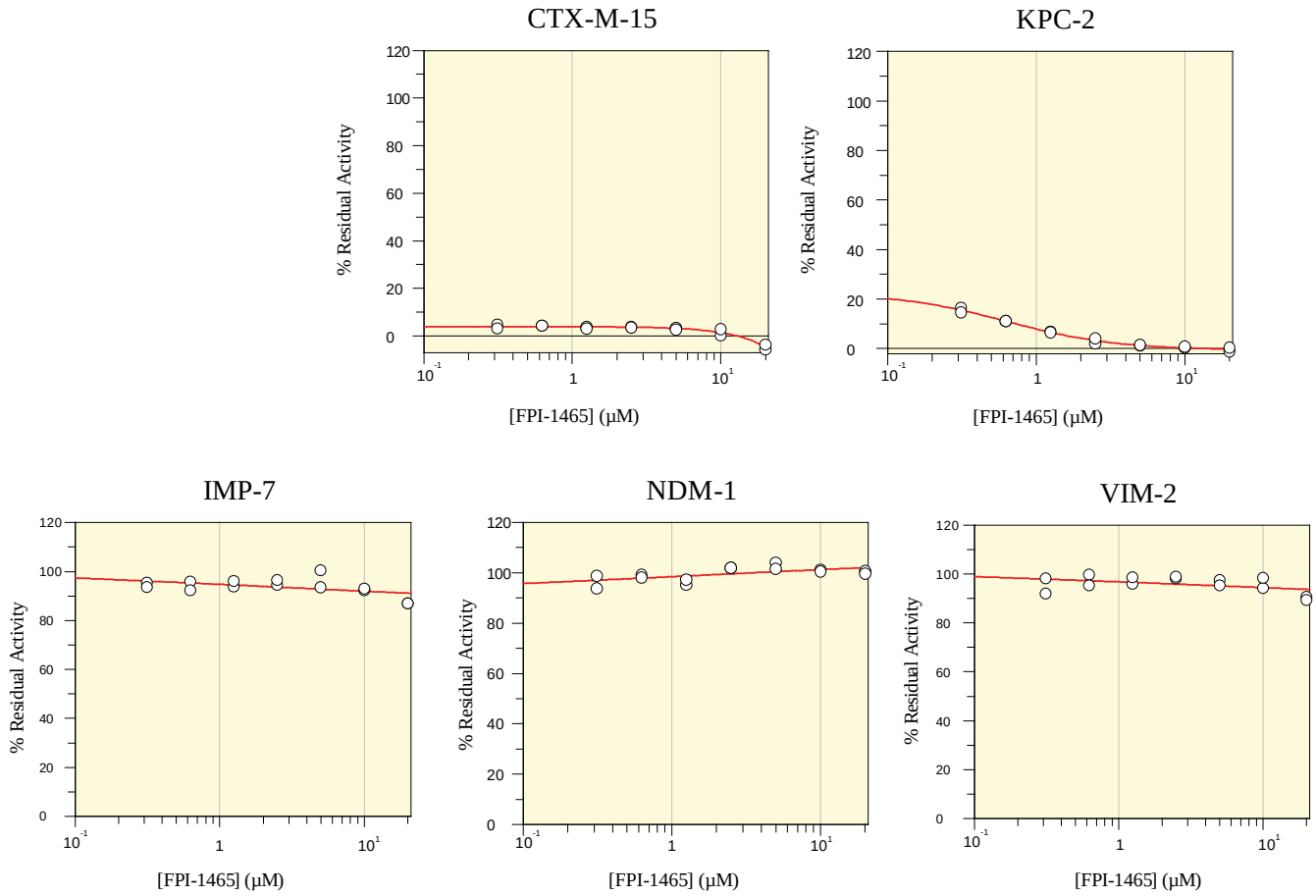
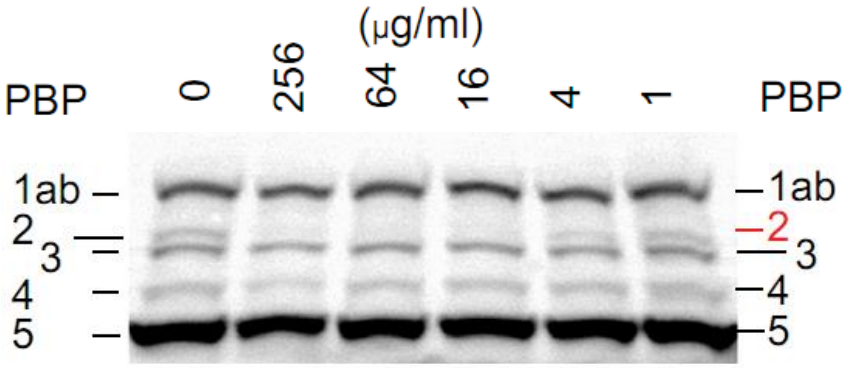
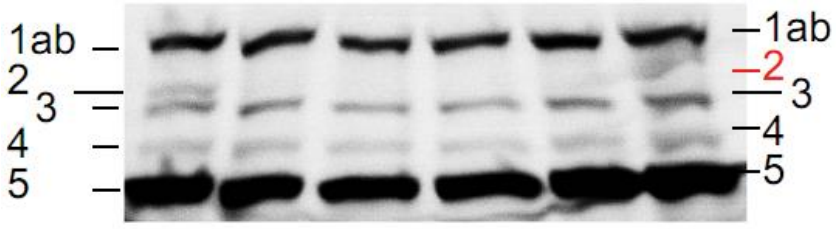


Figure S3. FPI compounds inhibit SBLs but not MBLs. Concentration-response plots show the SBLs CTX-M-15 and KPC-2 are effectively inhibited by FPI-1465 but not the MBLs IMP-7, NDM-1, or VIM-2.

a)



b)



c)

	MIC (μg/ml) for *E. coli* K12	K12 cell shape at 1/2 or 1/4 MIC	K12 PBP2 IC_{50} (μg/ml)
Avibactam	32	Round	2.0 ± 1.2
FPI-1465	4	Round	1.0 ± 0.6
FPI-1523	4	Round	0.4 ± 0.2

Figure S4. Avibactam and derivatives specifically bind PBP2. *E. coli* PBP banding profile and PBP binding competition assay for **a,** avibactam and **b,** FPI-1523. The banding profile was generated using BOCILLIN FL and the test compound was used at increasing concentrations during the assay. Binding of BOCILLIN FL to PBP2 was specifically decreased by the presence of all DBOs tested. **c,** MICs and PBP2 IC_{50} of avibactam and derivatives for *E. coli* K12. Note that the PBP binding competition assay for FPI-1465 is shown in Fig. 2.

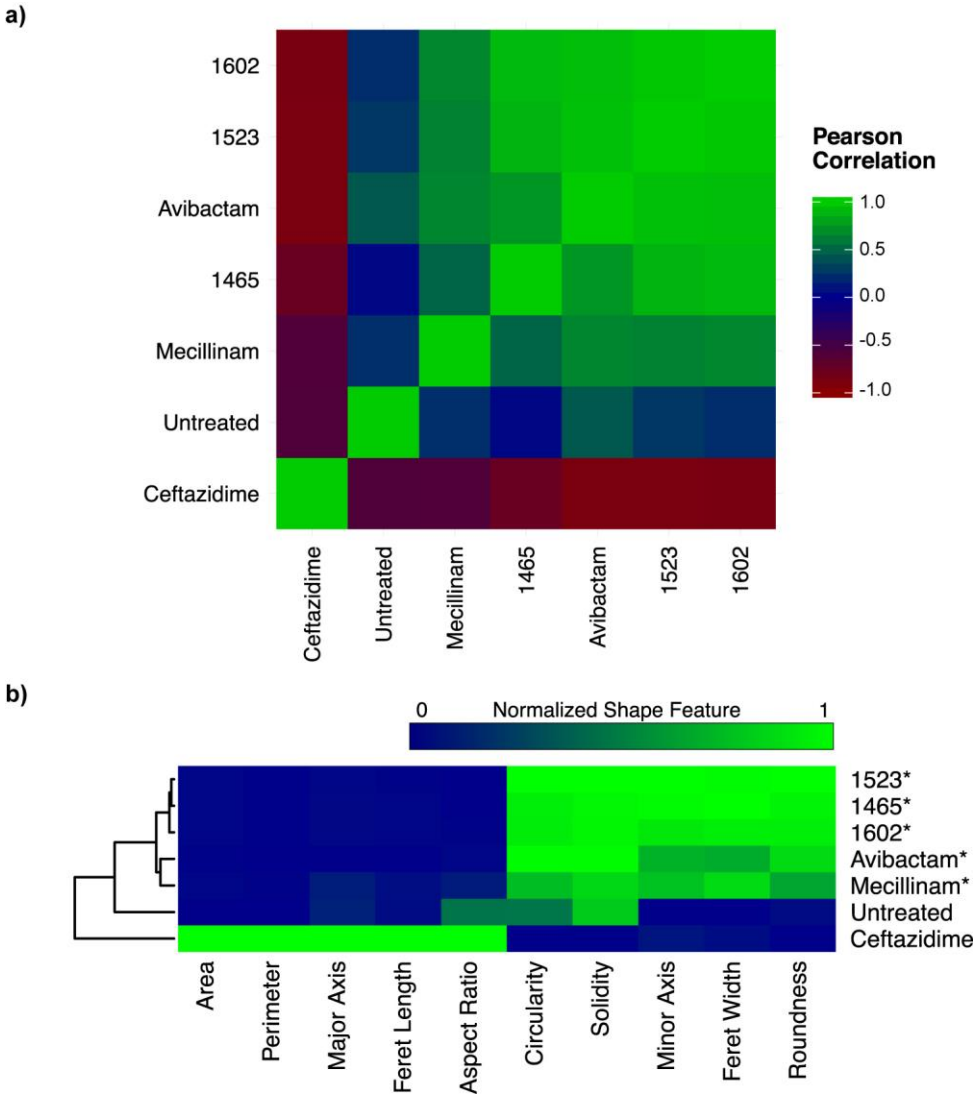


Figure S5. Pearson correlation map for morphological defects in the presence of select antibiotics. a, The correlation is based on ImageJ features shown in **b,** which were normalized for comparative purposes. It shows clear correlations between derivatives and known drugs avibactam and mecillinam, and a strong negative correlation with ceftazidime.

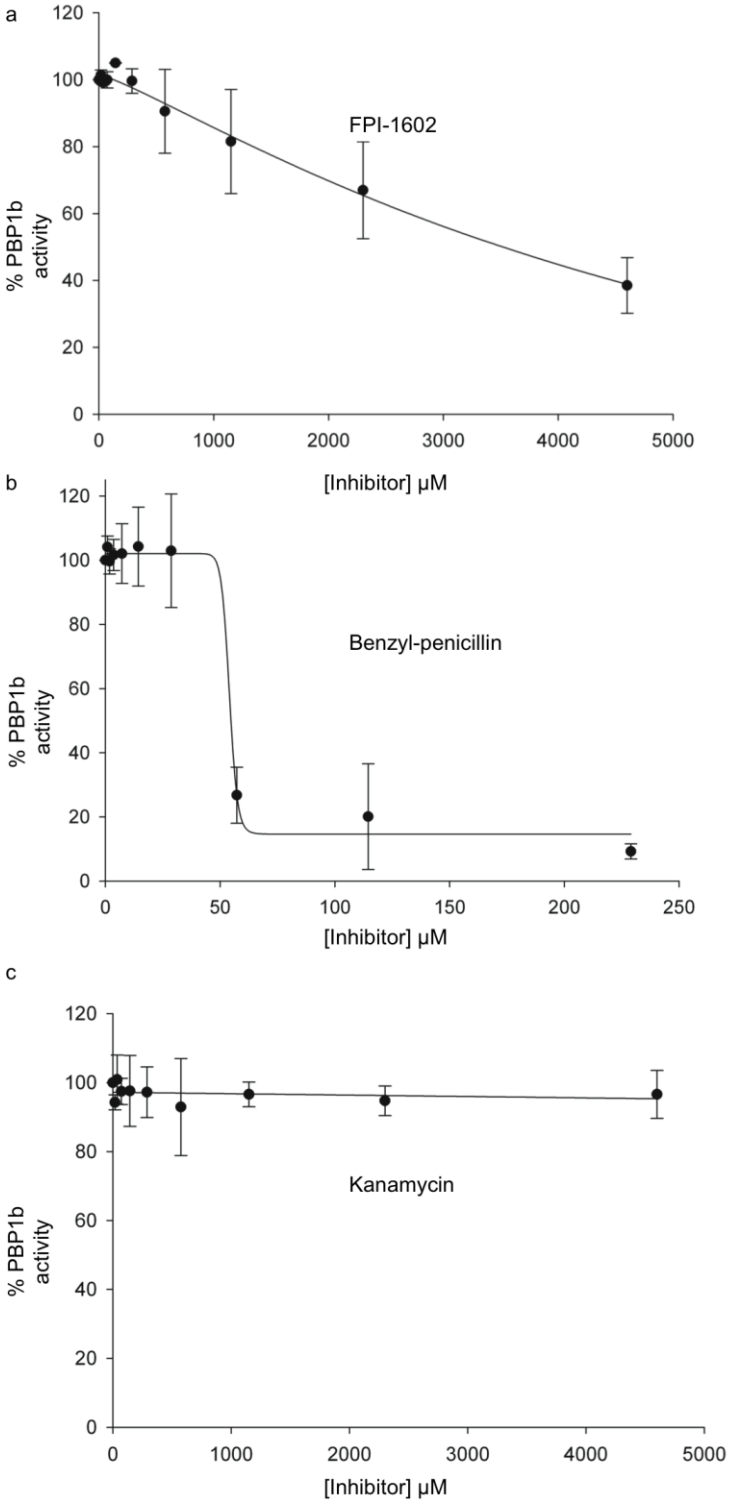


Figure S6. Gel based BOCILLIN FL competition assays to analyze the ability of unlabeled competitor compounds to bind purified *E. coli* PBP1b. In **a, b,** and **c,** various amounts of unlabeled FPI-1602, benzyl-penicillin, or kanamycin were pre-incubated with *E. coli* PBP1b for 48 hours prior to addition of BOCILLIN-FL. Error bars represent standard deviations from two separate technical replicates.

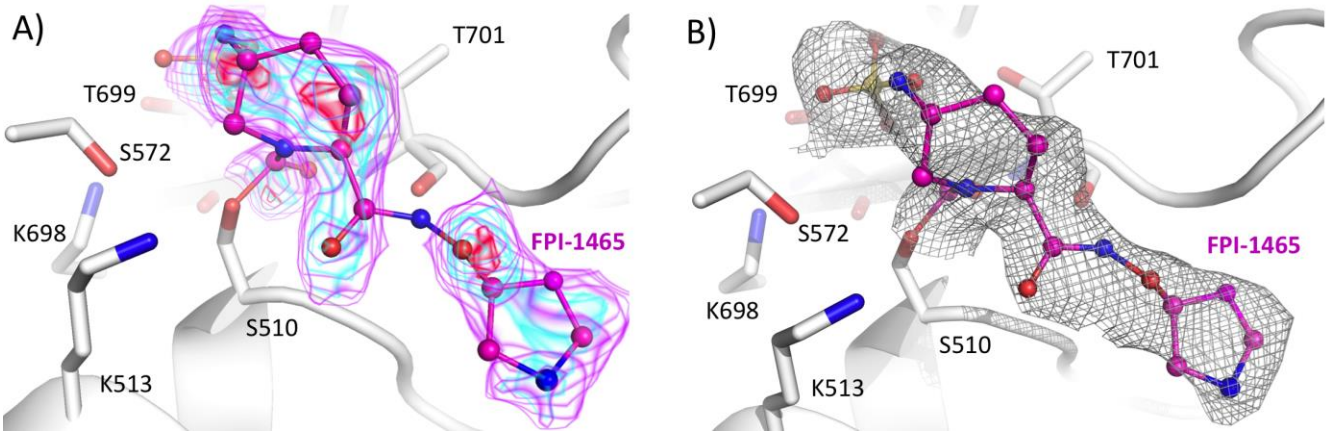


Figure S7. Avibactam derivative electron density for carbamyl-FPI-1465-*E. coli* PBP1b. a, Carbamyl-FPI-1465 PBP1b ligand omit F_o-F_c electron density. The F_o-F_c ligand omit maps are contoured at 3.0, 4.0 and 5.0 σ and are shown as pink, cyan and red transparent surfaces. **b,** Carbamyl-FPI-1523 final refined $2F_o$-F_c electron density map contoured at 1.0σ. In both panels, the PBP1b cartoon is shown in white with selected active site residues displayed as sticks with all non-carbon atoms colored by type. In all panels, the carbamyl-FPI-1465 is represented as pink sticks with atoms colored by type.

Table S1. Data collection and refinement statistics for CTX-M-15 co-crystal structures

	FPI-1465	FPI-1523	FPI-1602
Data collection			
Space group	$P2_12_12_1$	$P12_11$	$P12_11$
Cell dimensions			
a, *b*, *c* (Å)	43.9, 62.7, 175.5	62.7, 60.3, 76.0	63.1, 61.8, 73.1
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 112.8, 90.0	90.0, 104.0, 90.0
Resolution (Å)	33.28-3.00 (3.16-3.00)*	70.1-1.67 (1.71-1.67)	30.97-2.70 (2.77-2.70)
R_{sym}	0.10 (0.34)	0.053(0.42)	0.06(0.21)
$I/\sigma I$	7.8(2.4)	12.2(1.9)	11.7(3.4)
Completeness (%)	98.3(97.4)	98.4(99.7)	95.4(92.1)
Redundancy	4.4(4.3)	2.9(2.9)	2.5(2.4)
Refinement			
Resolution (Å)	33.28-3.00	70.1-1.67	30.97-2.70
No. reflections	10061(715)	59836(4407)	14478(1010)
R_{work}/ R_{free}	0.223, 0.271	0.166, 0.197	0.187, 0.227
No. atoms			
Protein	3934	3946	3926
Ligand	46	70	48
Water	34	520	85
B-factors (Å^2)			
Protein	30.4	15.3	27.8
Ligand	52.2	14.7	31.1
Water	21.2	27.6	18.1
R.m.s deviations			
Bond lengths (Å)	0.009	0.015	0.011
Bond angles (°)	1.43	1.73	1.48
Favored/allowed/disallowed (%)$^+$	94.9, 4.3, 0.8	97.2, 1.6, 1.2	96.1, 3.1, 0.8

Data corresponds to diffraction from a single crystal for each structure.
*Highest resolution shell is shown in parenthesis.
$^+$phenix.ramalyze; "allowed" is the percentage remaining after "favoured" and "outlier" residues are subtracted.

Table S2. Data collection and refinement statistics for OXA-48 co-crystal structures

	FPI-1465	FPI-1523	FPI-1602
Data collection			
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions			
a, *b*, *c* (Å)	72.8, 75.5, 107.0	73.0, 75.8, 106.7	73.1, 75.8, 106.7
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	43.64-1.96 (2.01-1.96)	43.62-1.74 (1.79-1.74)	47.18-2.09 (2.14-2.09)
R_{merge}	0.071(0.467)	0.04(0.440)	0.072(0.540)
$I/\sigma I$	22.6(4.3)	30.6(4.1)	21.2(3.4)
Completeness (%)	99.7(99.9)	99.9(99.3)	99.7(99.9)
Redundancy	7.3(7.4)	7.4(7.4)	7.3(7.5)
Refinement			
Resolution (Å)	43.64-1.96	43.62-1.74	47.18-2.09
No. reflections	42805(3145)	61458(4464)	35720(2595)
R_{work}/ R_{free}	0.168/0.210	0.157/0.178	0.167/0.216
No. atoms			
Protein	3988	4336	4026
Ligand	46	42	48
Ion	13	13	11
Water	422	457	293
B-factors (Å^2)			
Protein	25.6	25.9	35.5
Ligand	32.7	32.7	51.3
Ion	27.1	26.2	42.6
Water	32.8	34.6	40.6
R.m.s deviations			
Bond lengths (Å)	0.017	0.013	0.010
Bond angles (°)	1.78	1.56	1.59
Favored/allowed/disallowed (%)$^+$	97.9, 2.1, 0.0	98.0, 2.0, 0.0	98.3, 1.7, 0.0

Data corresponds to diffraction from a single crystal for each structure.
*Highest resolution shell is shown in parenthesis.
$^+$phenix.ramalyze; "allowed" is the percentage remaining after "favored" and "outlier" residues are subtracted.

Table S3. Antimicrobial susceptibility patterns of *E. coli* BW25113 pGDP-2 transformants expressing β-lactamase.

Antibiotic	MIC (μg/mL)				
	CTX-M-15	***KPC-2***	***NDM-1***	***OXA-48***	***control***
Ampicillin	32	>512	>512	16	4
Avibactam	32	16	16	16	16
FPI-1465	4	4	2	2	2
FPI-1523	2	2	2	1	1
FPI-1602	<0.5	<0.5	<0.5	<0.5	<0.5

Table S4. Antimicrobial susceptibility patterns of NDM-1-positive clinical isolates to FPI-1602 alone.

Strain	MIC (μg/mL)
E. coli GN688	0.5
E. coli GN610	2
E. cloacae GN574	1
E. cloacae GN579	2
P. aeruginosa PAO1*	2
E. cloacae GN687	8
K. oxytoca GN942	32
C. freundii GN978	64
A. baumanii ATCC 17978*	>128
K. pneumoniae GN629	>128
K. pneumoniae GN529	>128
M. morganii GN575	>128
P. rettgeri GN570	>128
P. stuartii GN576	>128

*Non-NDM-1-expressing strains

Table S5. Gel based BOCILLIN FL competition assays[a].

Antibiotic	IC_{50}[b] (μM)			
	PBP2	**PBP1b**	**PBP1a**	**PBP3**
Avibactam	63 ± 5.5	NI	NI	NI
FPI-1465	14.8 ± 1.1	NI	NI	NI
FPI-1523	3.2 ± 0.4	NI	NI	NI
FPI-1602	3.6 ± 0.3	NI	NI	NI
Benzyl penicillin	318.1 ± 32.3	161.72 ± 8.6	196.9 ± 26.3	35.5 ± 3.27
Mecillinam	0.3 ± 0.1	NI	NI	NI
Kanamycin	NI	NI	NI	NI

[a] The avibactam derivatives were analyzed for the ability to inhibit binding of BOCILLIN FL to purified *E. coli* PBPs. BOCILLIN FL and competitor compound were added at the same time to start the reaction.

NI, no observable inhibition up to 2000 μM competitor compound.

[b] IC_{50} values are taken as averages from three separate experiments and represent the concentration of unlabeled compound required to reduce the residual binding of BOCILLIN FL by 50%

Table S6. Data collection and refinement statistics for FPI-1465-*E. coli* PBP1b co-crystal structure

	FPI-1465
Data collection	
Space group	$P22_12_1$
Cell dimensions	
a, *b*, *c* (Å)	62.48, 63.22, 293.9
α, β, γ (°)	90, 90, 90
Resolution (Å)	63.22-2.85(2.93-2.85)
R_{merge}	0.040(0.238)
$I/\sigma I$	23.4(4.0)
Completeness (%)	95.1(90.7)
Redundancy	3.9(2.5)
Refinement	
Resolution (Å)	63.22-2.85
No. reflections	26383(1797)
R_{work}/ R_{free}	23.7/29.0
No. atoms	
Protein	5461
Moenomycin	77
FPI-1465	23
Water	59
B-factors (Å^2)	
Protein	63.6
Moenomycin	109.5
FPI-1465	87.0
Water	36.2
R.m.s deviations	
Bond lengths (Å)	0.013
Bond angles (°)	1.65
Favored/allowed/disallowed (%)$^+$	93.9, 6.0, 0.1

Data corresponds to diffraction from a single crystal for each structure.
*Highest resolution shell is shown in parenthesis.
$^+$phenix.ramalyze; "allowed" is the percentage remaining after "favoured" and "outlier" residues are subtracted.

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