BIOTIN ANALOGUES WITH ANTIBACTERIAL ACTIVITY ARE POTENT INHIBITORS OF BIOTIN PROTEIN LIGASE

Tatiana P. Soares da Costa †, William Tieu ǂ, Min Y. Yap", Ondrej Zvarec ǂ, Jan Bell §, John D. Turnidge †§, John C. Wallace †, Grant W. Booker †, Matthew C. J. Wilce", Andrew D. Abell ǂ, Steven W. Polyak †.

† School of Molecular and Biomedical Science, University of Adelaide, South Australia 5005, Australia.

ǂ School of Chemistry and Physics, University of Adelaide, South Australia 5005, Australia.

" School of Biomedical Science, Monash University, Victoria, Australia.

§ Microbiology and Infectious Diseases Directorate, SA Pathology, Women's and Children's Hospital, South Australia 5006, Australia.

Supporting information

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Supporting Experimental Methods

General synthetic chemistry methods

All reagents were from standard commercial sources and of reagent grade or as specified. Solvents were from standard commercial sources and used without further treatment, except for anhydrous THF and anhydrous dichloromethane that were dried and distilled according to reported procedures. [1] Reactions were monitored by ascending TLC using precoated plates (silica gel 60 F_{254}, 250 μm, Merck, Darmstadt, Germany), spots were visualised under ultraviolet light at 254 nm and with basic potassium permanganate dip. Column chromatography was performed with silica gel (40-63 μm 60 Å, Davisil, Grace, Germany). RP-HPLC was performed on HP Series 1100 with Phenomenex Gemini C18 5 μM (250 x 4.60 mm). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 (300 MHz) or a Varian Inova 600 MHz. Chemical shifts are given in ppm (δ) relative to the residue signals (DMSO-d_6 was 2.50 ppm for ^{1}H and 39.55 ppm for ^{13}C and $CDCl_3$ was 7.26 ppm for ^{1}H and 77.23 ppm for ^{13}C. High-resolution mass spectra (HRMS) were recorded on a Thermo Fisher Scientific LTQ orbitrap FT MS equipment (Δ < 2 ppm) at Adelaide Proteomics Centre, the University of Adelaide. Purity of biologically assayed compounds were determined using ^{1}H NMR or analytical RP-HPLC (>90%). Compounds **2 – 7** and **12 - 15** were synthesized as previously reported. [2-4]

Specific chemical synthesis and characterisation methods

(3a*S*,6a*R*)-4-(5-Azidopentyl)-1,3,3a,4,6,6a-hexahydrothieno[3,4-*d*]imidazol-2-one (Compound 10).

To a solution of biotin tosylate **8** [5] (151 mg, 0.39 mmol) in DMF (2 mL) was added sodium azide (32 mg, 0.48 mmol) and solution was stirred overnight under nitrogen atmosphere. The reaction mixture was poured into water (20 mL) and extracted with dichloromethane (3 x 25 mL). The organic layers were pooled and washed with brine (1 x 75 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by silica gel chromatography eluting with 5% methanol in dichloromethane

to give a white solid (86 mg, 86%). **^{1}H NMR** (300 MHz, $CDCl_3$): 6.57 (1H, bs), 6.46 (1H, bs), 4.38-4.42 (1H, m), 4.20-4.24 (1H, m), 3.40 (2H, t, *J* = 6.9 Hz), 3.16-3.22 (1H, m), 2.91 (1H, dd, *J* = 5.1, 12.6 Hz), 2.66 (1H, d, *J* = 12.6 Hz), 1.37-1.78 (8H, m); **^{13}C NMR** (300 MHz, DMSO-d_6): δ 164.30, 62.31, 60.35, 55.91, 51.56, 40.62, 28.93, 28.87, 28.71, 26.85.

(3a*S*,6a*R*)-4-(5-Aminopentyl)-1,3,3a,4,6,6a-hexahydrothieno[3,4-*d*]imidazol-2-one (Compound 11)

To a solution of biotin azide **10** [6] (80 mg, 0.31 mmol) in THF (2 mL) was added triphenylphosphine (106 mg, 0.41 mmol) and the solution was stirred at ambient temperature for 1 h, followed by addition of water (2 mL) and stirred for a further 3 h at 60 ^oC. The reaction mixture was filtered and the filtrand was diluted with water (20 mL) and extracted with dichloromethane (3 x 25 mL). The organic layer were pooled, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by silica gel chromatography eluting with 10% methanol in dichloromethane to give a white solid (13 mg, 18%). **^{1}H NMR** (300 MHz, $CDCl_3$): 6.51 (0.5H, bs, C(O)NH), 6.41 (0.5H, bs), 4.33-4.38 (1H, m), 4.15-4.20 (1H, m), 3.59 (2H, bs), 3.14-3.16 (1H, m), 2.87 (1H, dd, *J* = 7.5, 12.6 Hz), 2.56-2.64 (3H, m), 1.25-1.74 (8H, m); **^{13}C NMR** (300 MHz, DMSO-d_6): 163.14, 79.61, 61.47, 59.64, 55.94, 41.35, 32.35, 28.87, 28.70, 26.74. **HRMS** calcd. for (M^+ + H) $C_{11}H_{20}N_2OSNa$: requires 230.1322, found 230.1319.

(3a*S*,6a*R*)-4-Hexyl-1,3,3a,4,6,6a-hexahydrothieno[3,4-*d*]imidazol-2-one (Compound 16)

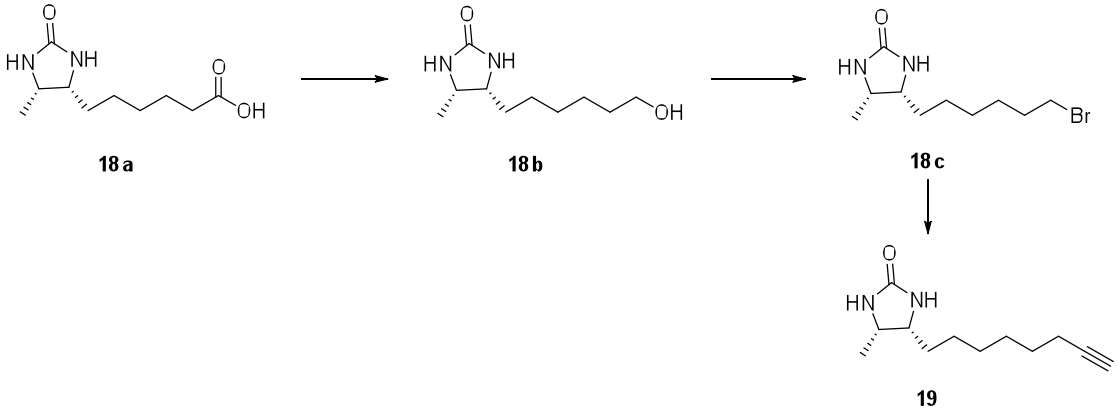
To a solution of biotin tosylate **8** [5] (52 mg, 0.14 mmol) in anhydrous THF (2 mL) was added lithium aluminium hydride (15 mg, 0.41 mmol) and the solution was stirred under reflux for 3 h. The reaction mixture was cooled to ambient temperature and were added methanol (1 mL) and saturated aqueous sodium sulphate (2 mL), followed by concentration *in vacuo* and dissolving with 1:1 dichloromethane and methanol (15 mL) and stirring for 30 min. The solution was filtered and the filtrate was concentrated *in vacuo* and purified by silica gel chromatography eluting with 3%

methanol in dichloromethane to give an off white solid (16 mg, 56%). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 5.27 (1H, bs), 5.16 (1H, bs), 4.49-4.53 (1H, m); 4.29-4.33 (1H, m), 3.14-3.20 (1H, m), 2.94 (1H, dd, *J* = 4.8, 12.6 Hz), 2.73(1H, d, *J* = 12.6 Hz), 1.62-1.70 (2H, m), 1.28-1.45 (4H, m), 0.89 (3H, t, *J* = 6.6 Hz); **^{13}C NMR** (300 MHz, DMSO-d_6): δ 163.16, 62.23, 60.41, 55.81, 40.92, 31.93, 29.52, 29.34, 28.98, 22.90, 14.39. **HRMS** calcd. for (M + H) $C_{10}H_{19}N_2OS$: requires 215.1218, found 215.1217.

(3a*S*,6a*R*)-4-Heptyl-1,3,3a,4,6,6a-hexahydrothieno[3,4-*d*]imidazol-2-one (Compound 17)

To a solution of homobiotin tosylate **9** [3] (5 mg, 0.013mmol) in anhydrous THF (1 mL) was added lithium aluminium hydride (3 mg, 0.079 mmol) and the solution was stirred under reflux for 3 h. The reaction mixture was cooled to ambient temperature and were added methanol (1 mL) and saturated aqueous sodium sulphate (2 mL), followed by concentration *in vacuo* and dissolving with 1:1 dichloromethane and methanol (15 mL) and stirring for 30 min. The solution was filtered and the filtrate was concentrated *in vacuo* and purified by silica gel chromatography eluting with 3% methanol in dichloromethane to give an off white solid (2 mg, 69%).**^{1}H NMR** (300 MHz, $CDCl_3$): δ 4.93 (1H, bs), 4.86 (1H, bs,), 4.50-4.54 (1H, m); 4.32 (1H, ddd, *J* = 1.5, 4.5, 7.5 Hz), 3.19 (1H, dt, *J* = 4.5, 7.5 Hz), 2.94 (1H, dd, *J* = 5.1, 12.9 Hz), 2.73 (1H, d, *J* = 12.9 Hz), 1.63-1.68 (2H, m), 1.26-1.45 (6H, m), 0.89 (3H, t, *J* = 6.9 Hz); **^{13}C NMR** (75 MHz, DMSO-d_6): δ 163.16, 62.23, 60.41, 55.81, 40.92, 31.93, 29.52, 29.34, 28.98, 22.90, 14.39. **HRMS** calcd. for (M + H) $C_{11}H_{21}N_2OS$: requires 229.1374 found 229.1369.

(4*S*,5*R*)-4-methyl-5-(oct-7-ynyl)imidazolidin-2-one (Compound 19)



Compound **18b** was prepared from desthiobiotin **18a** (200 mg, 0.93 mmol) and according to the procedure outlined for compound **7.** [3] Yield (153 mg, 82%); **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.33 (3H, d, *J* = 6.6 Hz), 1.25-1.77 (11H, m), 3.62-3.71 (3H, m), 3.84 (1H, ddd, *J* = 13.5, 6.6, 6.6 Hz), 4.69 (1H, s), 5.11 (1H, s); **^{13}C NMR** (75 MHz, DMSO-d_6): δ 163.47, 62.65, 56.00, 51.38, 32.45, 29.50, 29.09, 26.38, 25.52, 15.74; **HRMS** calcd. for (M + Na) $C_{10}H_{20}O_2N_2Na$: requires 223.1422, found 223.1432.

To a stirred solution of triphenylphosphine (152 mg, 0.58 mmol) and 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) (130 mg, 0.58 mmol) in dry dichloromethane (10 mL) under a stream of nitrogen at ambient temperature was added tetrabutylammonium bromide (186 mg, 0.58 mmol) followed by addition of the compound **18b** (97 mg, 0.48 mmol) and stirred for 1 h. Volatiles where removed *in vacuo* and the crude residue purified by silica gel chromatography to give compound **18c** as an orange powder (113 mg, 89%).**^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.46 (3H, d, *J* = 6.3 Hz), 1.29-1.47 (8H, m), 1.86 (2H, ddd, *J* = 14.25, 6.9, 6.9 Hz), 3.41 (1H, t, *J* = 6.9 Hz), 3.67-3.74 (1H, m), 3.86 (1H, ddd, *J* = 13.65, 6.6, 6.6 Hz), 4.44 (1H, s), 4.64 (1H, s); **^{13}C NMR** (300 MHz, DMSO-d_6): δ 163.06, 56.27, 51.37, 33.78, 32.58, 29.57, 28.71, 27.95, 26.34, 15.77; **HRMS** calcd. for (M + Na) $C_{10}H_{19}O_2N_2Na$: requires 285.0578, found 285.0584.

Compound **18c** (86 mg, 0.32 mmol) was reacted according to the procedure outlined for compound **14.** [3] The residue was purified by silica gel chromatography eluting with 5% methanol in dichloromethane to give the title compound (**19**) as an orange

powder (34 mg, 50%) **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.14 (3H, d, *J* = 6.3 Hz), 1.25-1.59 (10H, m), 1.95 (1H, dt, *J* = 2.55, 0.9 Hz), 2.19 (1H, ddd, *J* = 6.9, 6.9, 2.7 Hz), 3.67-3.73 (1H, m), 3.85 (1H, ddd, *J* = 13.8, 6.6, 6.6 Hz), 4.36 (1H, s), 4.54 (1H, s); **^{13}C NMR** (300 MHz, DMSO-d_6): δ 163.75, 84.45, 68.16, 55.96, 51.23, 40.77, 29.52, 28.93, 28.36, 28.17, 26.20, 18.19, 15.57; **HRMS** calcd. for (M + Na) $C_{12}H_{20}ON_2Na$: requires 231.1473, found 231.1470.

Protein expression and purification

The expression and purification of recombinant *Sa*BPL [7], *Ec*BPL [8] and *Hs*BPL [9] were performed as previously described.

Nucleic acid manipulation

To generate a construct for over-expression of *Ec*BPL in *E. coli*, the *bir*A gene was placed under control of the IPTG-inducible, strong tac promoter. The gene was amplified by PCR using oligonucleotides B130 [5′-TCATGAAGGATAACACCGTGCCAC-3′] and B131 [5′-AAGCTTAATGATGATGATGATGTCCTTTTTCTGCACTACGCAGGG-3′] upon template pCY216 containing the *bir*A. [10] The resulting PCR product was digested with *BspH*1 and *Hind*111 and ligated into *Nco*1 and *Hind*111 treated pC104 [11], a derivative of pK223-3 (Amersham). This yielded the vector pK(*Ec*BPL-H6) that was confirmed by DNA sequence analysis (DNA Sequencing Service, SA Pathology, South Australia). *E. coli* K12 was transformed with pK (*Ec*BPL-H6) to address the mechanism of antibiotic action.

X-ray crystallography

Apo-*Sa*BPL was buffer exchanged into 50 mM Tris HCl pH 7.5, 50 mM NaCl, 1 mM DTT and 5% (v/v) glycerol, and concentrated to 5 mg/mL. Each compound was then added to BPL in a 10:1 molar ratio. The complex was crystallized using the hanging drop method at 4°C in 8 – 12% Peg 8000 in 0.1 M Tris pH 7.5 or 8.0, and 10% (v/v) glycerol as the reservoir. A single crystal was picked using a Hampton cryo-loop (Hampton Research, USA) and streaked through cryo-protectant containing 25% (v/v) glycerol in the reservoir buffer prior to cryo-cooling. X-ray diffraction data was collected using the macromolecular crystallography beamline (MX1) at the Australian Synchrotron using an ADSC Quantum 210r Detector. 90 images were collected with a 1 second exposure each and an oscillation angle of 1° for each frame. Data was integrated and scaled using XDS. [12] PDB and cif files for the compounds were obtained using the PRODRG web interface. [13] The co-ordinates from the *Sa*BPL –

biotinol-5-AMP (PDBID = 4DQ2) [3], with all water and ligands removed were used as a starting model. After rigid body refinement using REFMAC, [14] the models were built using cycles of manual modelling using COOT [15] and refinement with REFMAC. [14] The electron density clearly defined the positions of the ligands. The quality of the final models was evaluated using MOLPROBITY. [16] Data collection and refinement statistics are summarized in Supplementary Table S1.

In vitro biotinylation assays

Quantitation of BPL catalysed ^{3}H-biotin incorporation into the biotin domain substrate was performed as previously described [11, 17]. Briefly, the reaction mixture contained: 50 mM Tris HCl pH 8.0, 3 mM ATP, 4.5 μM biotin, 0.5 μM ^{3}H-biotin, 5.5 mM $MgCl_2$, 100 mM KCl, 0.1 mg/mL BSA and 10 μM biotin domain of *S. aureus* pyruvate carboxylase. The reaction was initiated by the addition of BPL to a final concentration of 4 nM. After 20 minutes at 37^o C, 4 μL aliquots of the reaction were spotted onto Whatman paper pre-treated with biotin and trichloroacetic acid. The filters were washed twice with 10% v/v ice-cold trichloroacetic acid and once with ethanol before air-drying. Quantitation of protein-bound radiolabelled biotin was performed by liquid scintillation. One unit of enzyme activity was defined by the amount of BPL required to incorporate 1 nmol of biotin per minute. The IC_{50} value of each compound was determined from a dose-response curve by varying the concentration of the inhibitor under the same enzyme concentration. The data was analysed with GraphPad Prism using a non-linear fit of $\log_{10}$ (inhibitor) vs. normalized response. The K_i, the absolute inhibition constant for a compound, was determined using Eq 1: [18]

Eq 1. $$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}}$$

where [S] is the substrate concentration ([biotin] =5 μM) and K_m is the affinity of the enzyme for biotin (S. aureus BPL =1 μM [3], *E. coli* BPL = 0.3 μM [8] and human BPL = 1 μM [19]). The mode of inhibition was investigated by varying the concentrations of inhibitor alongside varying the concentrations of ^{3}H-biotin. The data was plotted as

double reciprocal plots and assessed using Lineweaver-Burk analysis. Ligand efficiency was calculated using Eq2: [20]

Eq 2. $LE = - RT \ln K_i$ / number of heavy atoms

where R is the gas constant and T is the temperature (^{o} Kelvin).

Surface Plasmon Resonance

SPR was performed using a Biacore™ T100 (GE Healthcare). BPL was immobilised on a CM5 sensor chip following the manufacturer's instructions. Typically, 6500 resonance units of *Sa*BPL were immobilised on the sensor chip. One channel was left blank which was subtracted from sample channel to allow analysis methods to distinguish between actual and non-specific binding. All experiments were conducted at 25^{o} C at a flow rate of 30 µL/minute with running buffer containing 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.005% (v/v) surfactant. Regeneration of the surface was carried out by injecting 10 mM sodium acetate pH 5.8 at a flow rate of 30 µL/minute for 10 seconds. Zero concentration samples were used as blanks. Biotin and compound **6** showed fast on and off rates outside the range of kinetic quantification hence only K_D values could be estimated from equilibrium responses using compounds of different concentrations. In contrast, the association and dissociate rate constants for compounds **16** and **5** could be determined from the sensorgrams. The data were fitted to a 1:1 ligand binding model using the Biacore™ evaluation software. Buffers for experiments conducted with non-water soluble compounds contained 4% (v/v) DMSO.

The sensorgrams for the SPR experiments are shown in Figure 3. The concentrations of ligands employed were as follows: For biotin (Figure 1a) the concentrations used were 0 (-), 0.98 (-) , 3.9 (-), 7.8 (-), 31.3 (-), 62.5 (-) and 125 µM. For compound **6** (Figure 1b), the concentrations used were: 0 (-), 0.68 (-), 2.7 (-), 5.5 (-), 10.9 (-) and 43.8 (-) µM. For compounds **16** and **5** (Figure 1c and d), the concentrations used were: 0 (-), 0.4 (-), 0.8 (-), 1.6 (-) and 3.2 (-) µM.

Antibacterial Activity Evaluation

Minimal Inhibitory Concentrations (MICs) were determined by a microdilution broth method as recommended by the CLSI (Clinical and Laboratory Standards Institute, Document M07-A8, 2009, Wayne, Pa.) with cation-adjusted Mueller-Hinton broth (Trek Diagnostics Systems, U.K.). Compounds were dissolved using DMSO. Serial two-fold dilutions of each compound were made using DMSO as the diluent. Trays were inoculated with 5 x 10^4 CFU of each strain in a volume of 100 µL (final concentration of DMSO was 5% (v/v)), and incubated at 35° C for 16-20 hours when MICs were determined.

To address the antibiotic mechanism of action, a complementation assay was performed using *E. coli* K12 cells. Cells harbouring the *Ec*BPL over-expression vector pK(*Ec*BPL-H6) was employed. Bacteria, 5 x 10^4 CFU, were seeded in each well of a 96-well plate and treated either with compound **16** (final concentration 32 µg/mL) or DMSO control (final concentration 3.2% (v/v)) for 20 hours at 37^o C. The final optical density of the culture was measured on a Thermo Multiskan Acsent plate reader at 620 nm.

Assay of cytotoxicity

HepG2 cells were suspended in Dulbecco-modified Eagle's medium containing 10% fetal bovine serum, and then seeded in 96-well tissue culture plates at either 5 000, 10 000 or 20 000 cells per well. After 24 hours, cells were treated with varying concentrations of compound, such that the DMSO concentration was consistent at 4% (v/v) in all wells. After treatment for 24 or 48 hours, WST-1 cell proliferation reagent (Roche) was added to each well and incubated for 0.5 hours at 37° C. The WST-1 assay quantitatively monitors the metabolic activity of cells by measuring the hydrolysis of the WST-1 reagent, the products of which are detectable at absorbance 450 nm.

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(a) (b)

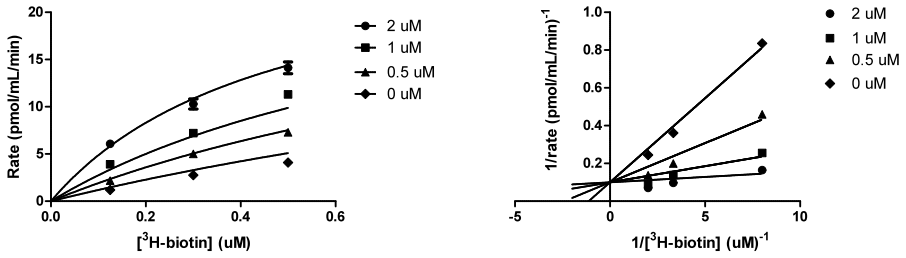

Figure S1. Mechanism of inhibition is via competition with biotin.

Concentration dependent inhibition of *S. aureus* BPL by compound **16** and associated double reciprocal Lineweaver-Burk plot show that this compound is a competitive inhibitor of biotin. (a) Rate plot with different fixed concentrations of inhibitor and varying concentrations of ^{3}H-biotin, b) Double reciprocal Lineweaver-Burke plot of initial velocity curves against varying concentrations of ^{3}H-biotin.

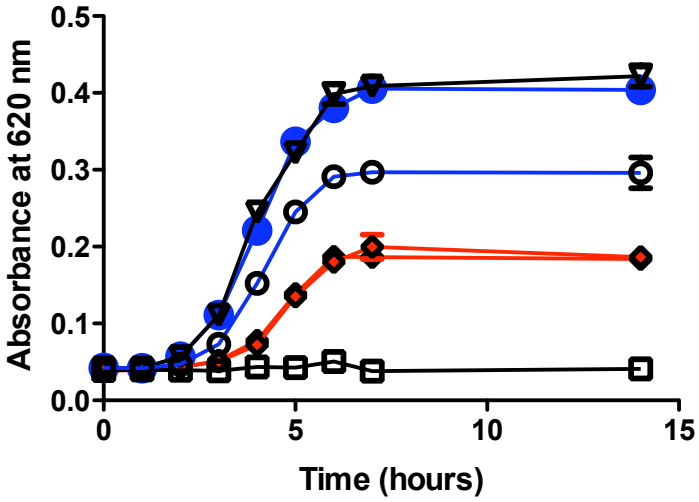

Figure S2. Mechanism of antibacterial activity is via inhibition of BPL.

E. coli K12 cells were transformed with either the parent vector pKK-223-3 (diamonds with red lines) or pK(*Ec*BPL-H6) (circles with blue lines) for the over-expression of *E. coli* BPL. Cells were the grown for 14 hours at 37°C in the presence or absence of 32 μg/ml compound **5** when the absorbance of the culture was measured at 600 nm. Protein expression was induced by the addition of IPTG into the growth media (solid symbols contain IPTG vs open symbols with no IPTG). A control with no compound was included (▽), as was a control with no bacteria (□).

Supporting Table S1. X-ray data collection and refinement. Data collection and refinement statistics for holo *Sa*BPL structures with compounds **6** and **14** bound.

	Compound 6	**Compound 14**
Data collection		
Space group	$P4_22_12$	$P4_22_12$
Unit cell (*a,b,c)*	93.6, 93.6, 130.4	93.1, 93.1, 129.9
Resolution	50.00 -2.60 (2.67- 2.60)	50.00 - 2.65 (2.72-2.65)
Number of unique reflections	18454 (1329)	17103 (1234)
Multiplicity	6.9 (7.1)	5.4 (5.6)
Completeness	99.3% (99.6%)	99.3% (97.4%)
I/σ	13.9 (4.5)	17.4 (2.5)
R_{merge}	12.5% (70.5%)	8.1% (83.1%)
R_{pim}	2.8% (26.4%)	2.2 % (27.0%)
Refinement		
R_{factor}	19.7	19.7
R_{free}	26.8	24.2
r.m.s. deviations		
Bond lengths	0.022	0.022
Bond angles	1.96	2.05
Ramachandran plot		
Allowed regions	99.4%	98.7%
Outliers	0.7%	1.3%

[a] Values in parentheses refer to the highest resolution shell.

[b] $R_{merge} = \sum |I - \langle I \rangle| / \sum \langle I \rangle$ where I is the intensity of individual reflections.

[C] $R_{pim} = \sum [1/(N-1)]^{1/2} \sum |I - \langle I \rangle| / \sum \langle I \rangle$ [21]

[d] $R_{factor} = \sum_h |F_o - F_c| / \sum_h |F_o|$, where F_o and F_c are the observed and calculated structure-factor amplitudes for each reflection "h".

[e] R_{free} was calculated with 5% of the diffraction data selected randomly and excluded from refinement.