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Supporting Online Material for

A Cholesterol Biosynthesis Inhibitor Blocks *Staphylococcus aureus* Virulence

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Materials and Methods

Cloning of CrtM. The ATCC 27659 strain of *Staphylococcus aureus* was obtained from the Food Industry Research and Development Institute in Taiwan. The full-length CrtM was first isolated by PCR from *Staphylococcus aureus* genomic DNA using the oligonucleotides 5'-ATGACAATGATGGATATGAATTTTAAATATTG-3' (forward) and 5'-ttATATTCTATGATATTTACTATTTATTTC-3' (reverse). The complete coding sequence of CrtM was cloned into an expression vector pET-32 Xa/LIC (Novagen, Madison, WI).

CrtM Expression, purification and inhibition. CrtM with a histidine tag was overexpressed in *E. coli* BL21(DE3) cells. A 50 mL overnight culture was transferred into 1L LB medium supplemented with 100 μg/mL ampicillin. The induction was carried out with 1mM IPTG for four hours, at 37 ºC, when the cell culture reached an OD of 0.6 at 600 nm. The cell extract was loaded onto a Ni-NTA column, and CrtM eluted by using a 100 mL linear gradient of 0-0.5 M imidazole in 50 mM Tris-HCl buffer, pH 7.4. CrtM inhibition assays were carried out by using 96 well plates with 200 μL reaction mixture in each well. The condensation of farnesyl diphosphate was monitored by a continuous spectrophotometric assay for phosphate releasing enzymes (*S1*). The reaction buffer contained 50 mM Tris-HCl, 1mM $MgCl_2$, 450 μM FPP, pH 7.4. The compounds investigated were pre-incubated with 2 μg CrtM for 30 minutes at 20 °C. The IC_{50} values were obtained by fitting the inhibition data to a normal dose-response curve in Origin 6.1 (OriginLab Corporation, Northampton, MA). K_i is calculated based on the IC_{50} value, and the reported kinetic constant of CrtM (*S2*).

X-Ray Crystallography. Native CrtM was eluted from Ni-NTA beads by incubation with Factor Xa (Novagen) to cleave it from the polyhistidine-containing N-terminal thioredoxin fusion tag. The cleaved product was equilibrated with buffer containing 150 mM NaCl, 5 mM

DTT, 1 mM β-mercaptoethanol, 5 % glycerol and 20 mM Tris pH 7.5, then concentrated to 15 mg/ml. Native CrtM crystals (space group $P3_221$) were grown using the hanging-drop method by mixing equal amounts of reservoir with 0.12-0.58 M potassium sodium tartrate, at room temperature. Seleno-methionine (SeMet) substituted CrtM was purified, concentrated and crystallized as for native CrtM. X-ray diffraction data collection results are summarized in Tables S1 and S2. The data were indexed, integrated and scaled by using the HKL2000 package (*S3*). The structure of SeMet substituted CrtM was solved by the multi-wavelength anomalous diffraction (MAD) method. The programs SOLVE (*S4*) and RESOLVE (*S5*) were used to calculate the phases, and to perform automatic model building. Further model building and refinement employed XtalView (*S6*) and CNS (*S7*). Inhibitor-bound crystals were obtained by soaking with 0.05 mM BPH-652, BPH-698, BPH-700 or 0.5 mM FsPP. With the exception of the FsPP-containing system, all crystals belonged to the $P3_221$ space group and had similar lattice parameters. The CrtM/FsPP complex belonged to a different space group ($P3_121$) and best results were obtained by co-crystallization in the presence of 0.5 mM FsPP with 11-21% PEG4K, 0.3-0.4 M $MgCl_2$ and 0.1 M Tris pH 8.5. The structure of the CrtM/FsPP complex obtained by co-crystallization had improved resolution over that obtained by soaking and was determined by molecular replacement using CNS, using the refined native CrtM as a search model. Figures were obtained using pymol (*S8*).

Bacteria, mice, and chemical reagents. *S. aureus* used were the WT clinical isolate (Pig1) and the corresponding isogenic carotenoid-deficient mutant, ΔCrtM (*S9*). CD1 mice were purchased from Charles River Laboratory, Inc. (Wilmington, MA). *S. aureus* was propagated in Todd-Hewitt broth (THB) or on THB agar (Difco, Detroit, MI). For all *in vitro* pigment inhibition studies, *S. aureus* was cultured in THB (1 mL) in the presence of inhibitor compounds for 72h, in

duplicate. Prior to the specific assays, the bacteria were centrifuged and washed twice in PBS. Staphyloxanthin was extracted with MeOH and the O.D. was determined at 450 nm using a Perkin Elmer MBA 2000 (Norwalk, CT) spectrophotometer. All *in vivo* experiments made use of early stationary phase bacteria, defined as 1-2 hr post logarithmic phase of growth. All animal experiments were approved by the UCSD or Cedars-Sinai Committees on the Use and Care of Animals and performed using accepted veterinary standards.

Hydrogen peroxide susceptibility assay. *S. aureus* was cultured in THB with or without inhibitor (100 μM). After 2 days, $5x10^6$ bacteria were washed twice in PBS, diluted to a concentration of $5x10^6$ colony forming units (cfu) per 100 μL reaction mixture in a 96 well plate. Hydrogen peroxide (H_2O_2) in PBS was added to a 1.5% final concentration and the plate was incubated for 1 hr at 37°C with shaking. The reaction was stopped by addition of 1000U/ml exogenous catalase (Sigma-Aldrich, St. Louis, MO), and bacterial viability assessed by dilutions on THA plates.

Whole blood killing assay. The assay was performed as previously described (9). Essentially, *S. aureus* were washed twice in PBS, diluted to an inoculum of 10^4 cfu in 25 μL PBS, and mixed with 75 μL of freshly drawn human blood in heparinized tubes. The tubes were incubated at 37°C for 4 h, with agitation, at which time dilutions were plated on THA for enumeration of surviving cfu.

Murine model of nasal colonization. A 1:1 mixture of 3×10^8 each of WT and ΔCrtM mutant *S. aureus* was inoculated into noses of 10 wk old CD1 mice in 10 μL PBS. After 5 days, the mice were sacrificed and the noses were harvested and homogenized. The numbers of surviving WT and ΔCrtM *S. aureus* were enumerated on THA plates. WT and ΔCrtM were differentiated based on pigmentation.

Murine model of kidney infection. 10 to 12 wk old CD-1 male mice were injected intraperitoneally (i.p) with 10^8 early stationary phase *S. aureus*. After 3 d, animals were euthanized, kidneys homogenized in PBS, and plated on THA for quantitative bacterial culture. For the treatment study, mice were randomized into two groups at the start of the experiment and administered either 0.5 mg of BPH-652 or PBS control, i.p., twice a day, starting on d-1 to d2 (a total of 8 doses). Intraperitional challenge by 10^8 early stationary phase *S. aureus* was performed on d0. The mice were sacrificed on d3 for enumeration of cfu in kidney homogenates. 14 mice were used in the first experiment, 13 in the second. All results were pooled, for statistical analysis.

Statistics. The significance of experimental differences in hydrogen peroxide sensitivity, blood killing, and mouse *in vivo* challenge studies were evaluated by use of the Student's *t* test.

Fig. S1. Sequence alignment of CrtM and human SQS showing conserved DXXXD repeats (red boxes) and 30% identity, 36% similarity.

Fig. S2. Electron densities for FsPP bound to *S. aureus* CrtM. Chain A, contoured at 1σ/3σ. Chain B, contoured at 1σ/3σ. FsPP-1 is in green, FsPP-2 is in yellow. The Mg^{2+} is in magenta; S, O, and P atoms are in gray, red and orange, respectively.

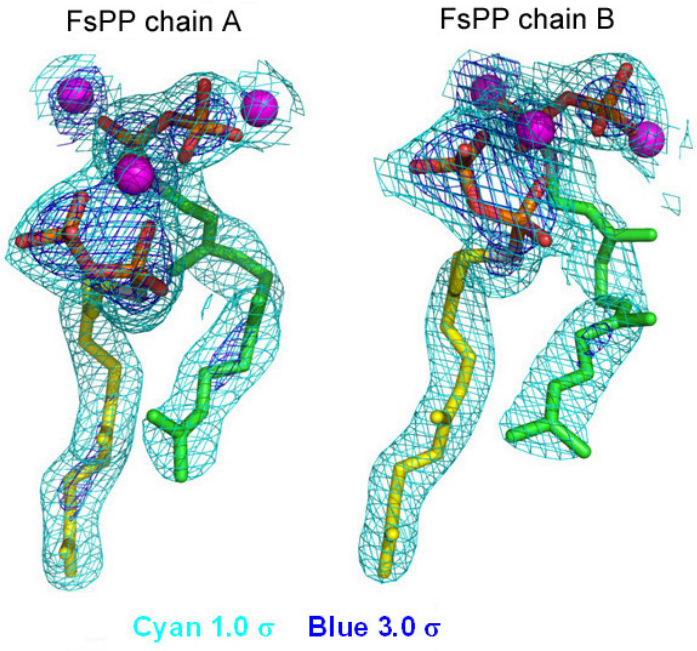


Fig. S3. Ligplot interaction diagram for the two FsPP ligands in CrtM (Chain A). The interactions in Chain B are essentially identical.

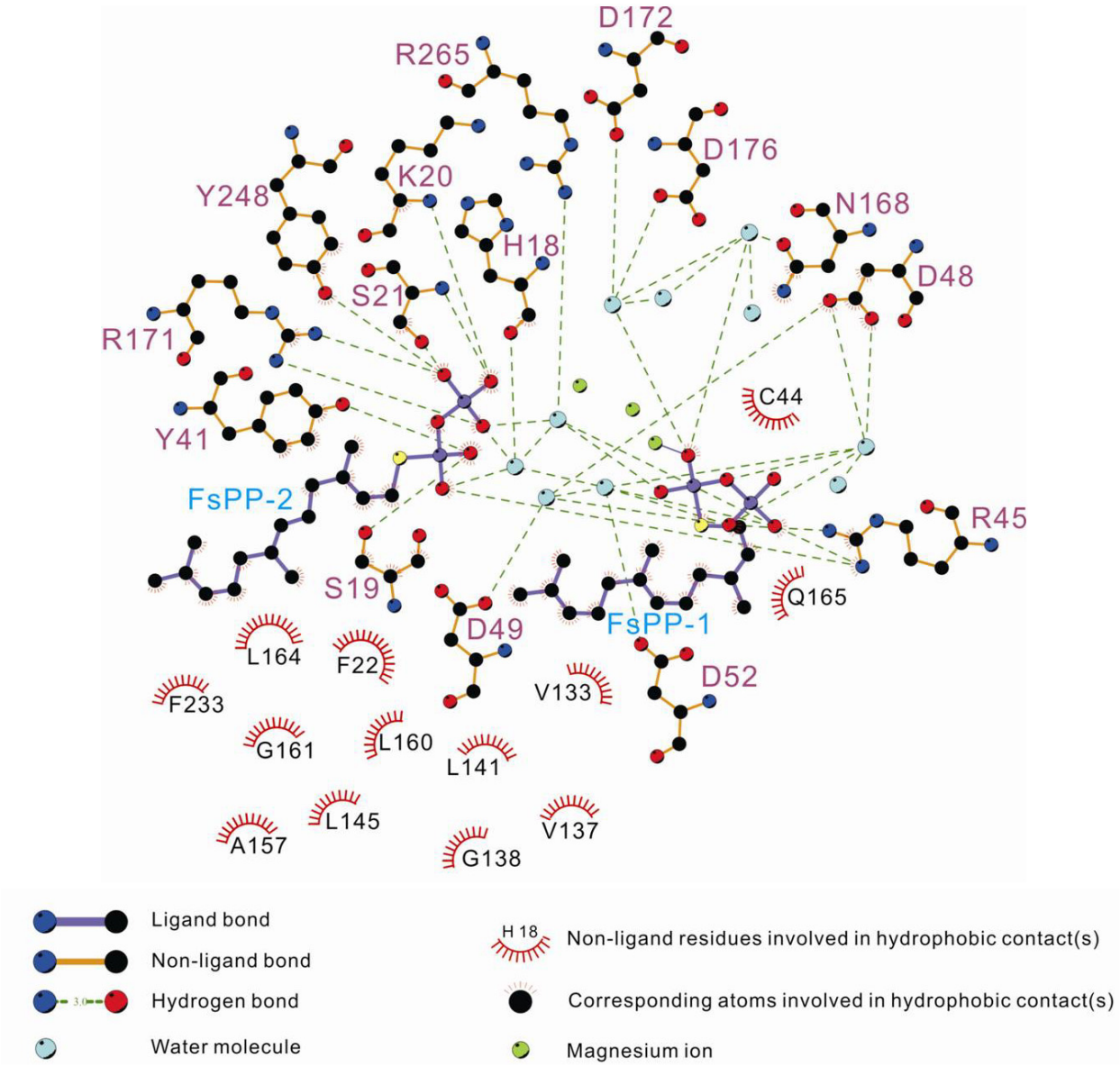
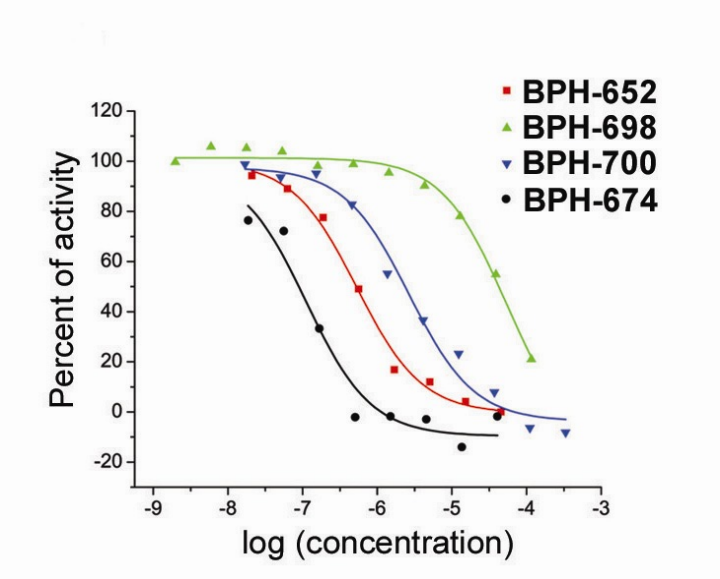


Fig. S4. CrtM inhibition by the phosphonosulfonates and a bisphosphonate.



Ki (BPH-652) = 1.5 nM

Ki (BPH-698) = 135 nM

Ki (BPH-700) = 6.0 nM

Ki (BPH-674) = 0.2 nM

Fig. S5. Electron densities for phosphonosulfonates bound to *S. aureus* CrtM. (**A**) BPH-652. (**B**) BPH-698. (**C**) BPH-700. The Mg^{2+} is in magenta; S, O, and P atoms are in gray, red and orange, respectively.

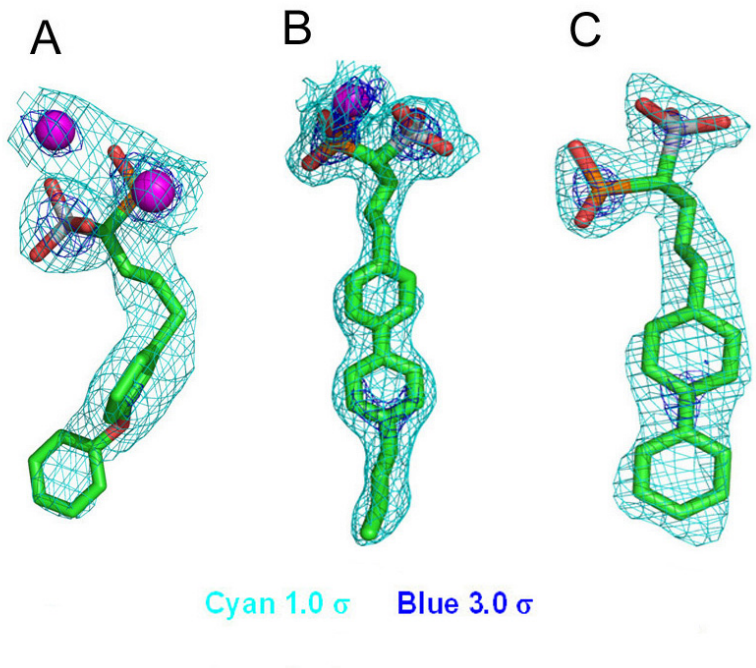


Fig. S6. Ligplot interaction diagram for BPH-652 bound to *S. aureus* CrtM.

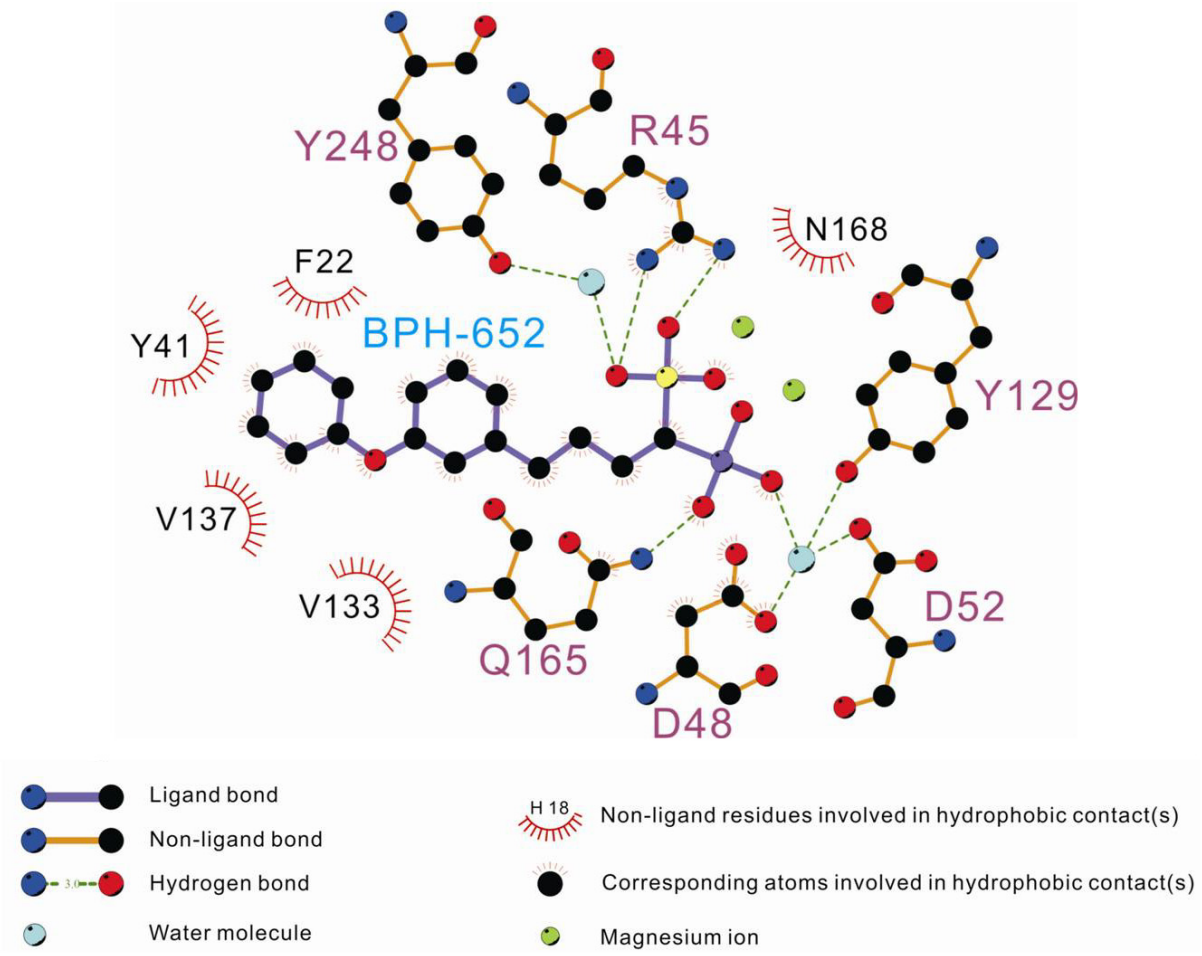


Fig. S7. Ligplot interaction diagram for BPH-698 bound to *S. aureus* CrtM.

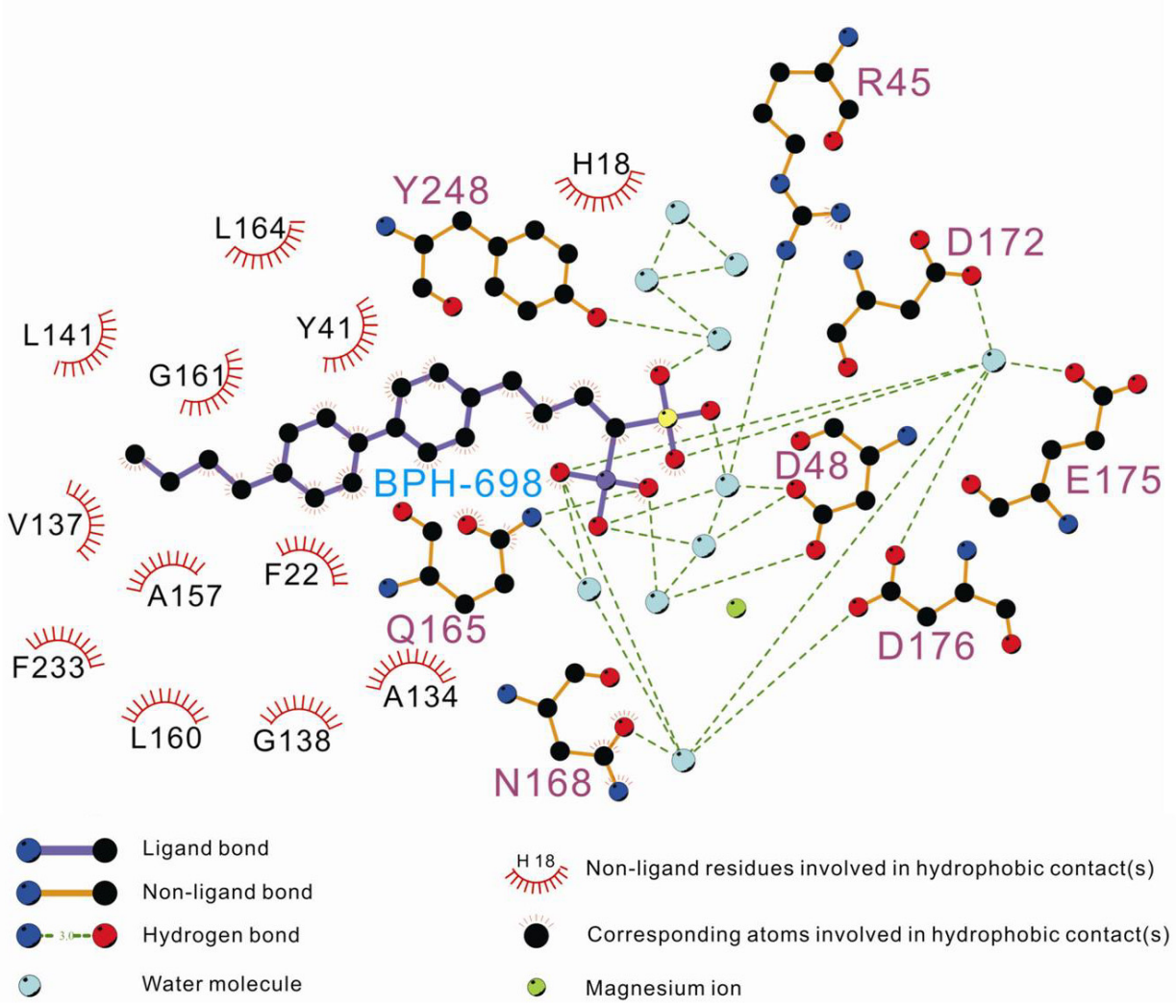


Fig. S8. Ligplot interaction diagram for BPH-700 bound to *S. aureus* CrtM.

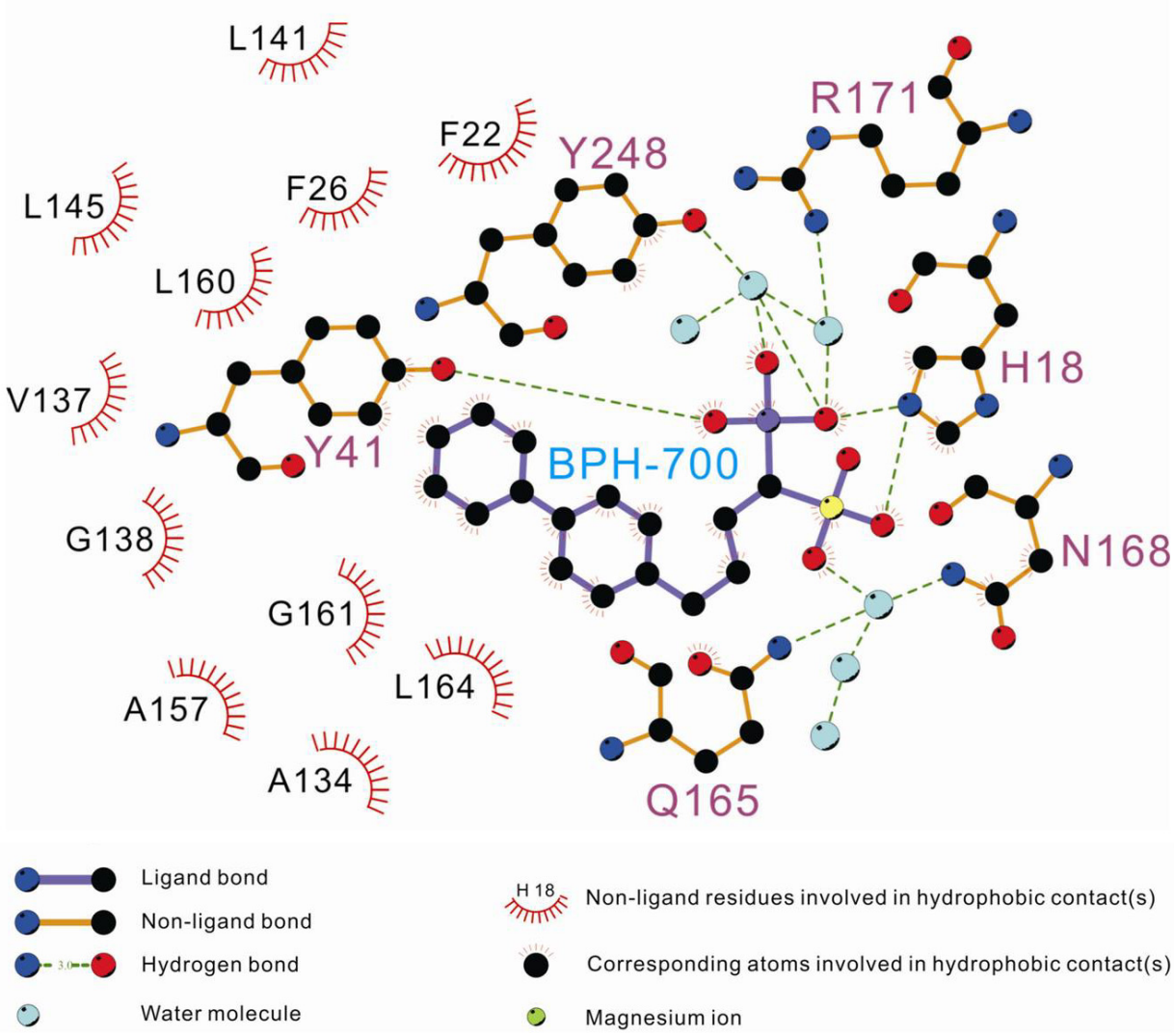


Fig. S9. Two views of the three phosphonosulfonate inhibitors bound to *S. aureus* CrtM, superimposed on the FsPP structure (FsPP ligands in green and yellow). Same inhibitor color scheme as in Fig. 2.

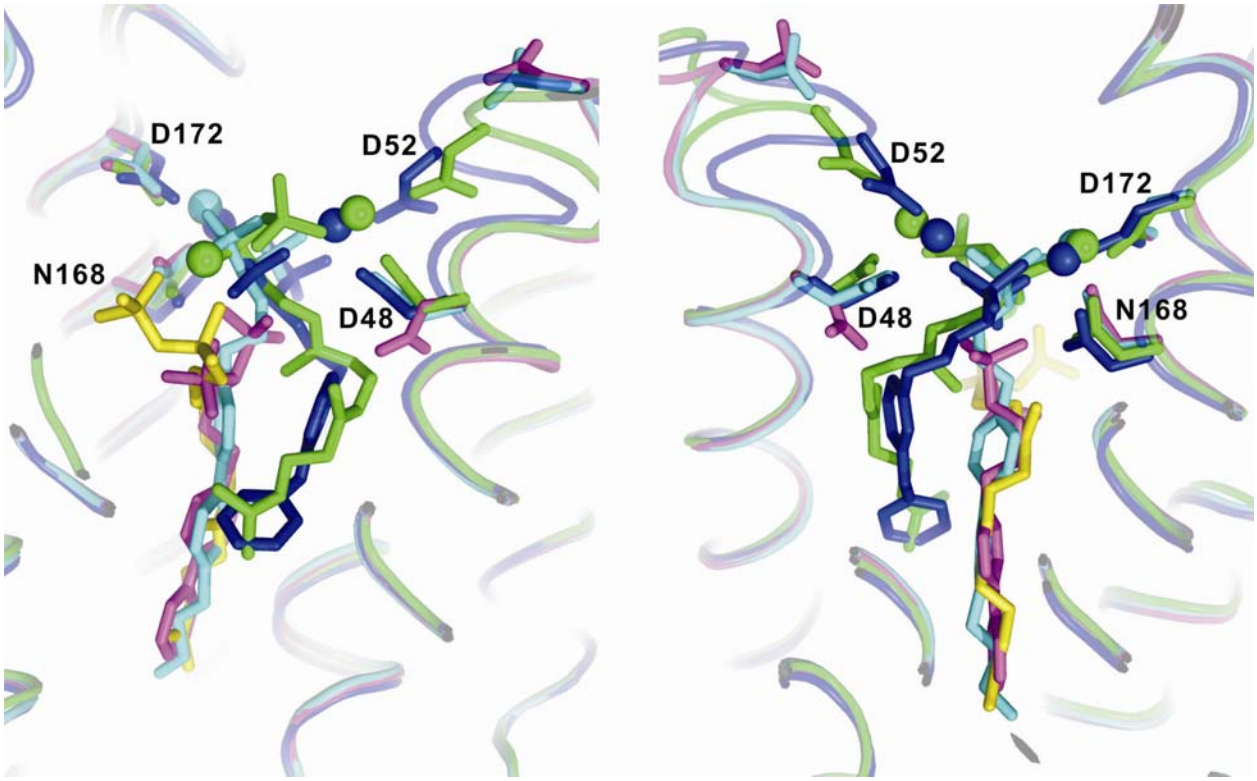


Table S1: Data Collection Statistics of SeMet-CrtM

Crystal	SeMet-CrtM		
Data Collection			
Radiation source	NSRRC BL13B1		
Wavelength (Å)	0.97918 (peak)	0.97940 (edge)	0.96394 (high remote)
Space group	$P3_221$		
Unit cell dimensions			
a (Å)	80.34		
b (Å)	80.34		
c (Å)	90.49		
Resolution (Å)[a]	30-1.84 (1.91-1.84)	30-1.85 (1.92-1.85)	30-1.83 (1.90-1.83)
No. of unique reflections	29767 (2928)	29283 (2889)	30262 (2998)
Completeness (%)	99.6 (100)	99.7 (100)	99.7 (100)
Redundancy	18.8 (19.8)	9.5 (9.7)	9.6 (9.7)
R_{merge} (%)	6.8 (34.8)	8.0 (35.9)	6.4 (35.9)
I/σ(I)	28.9 (11.0)	23.2 (7.1)	31.8 (6.9)
Phasing			
Resolution (Å)	30-2.0		
Number of Se sites	9		
Z-score (SOLVE)	80.0		
Figure of merit (SOLVE)	0.72		

[a]Values in the parentheses are for the highest resolution shells.

Table S2: Data collection and refinement statistics for CrtM with and without inhibitors

Crystals	CrtM	CrtM+FSPP	CrtM+BPH-652	CrtM+BPH-698	CrtM+BPH-700
Data Collection					
Radiation source	SPring-8 BL12B2	PF-BL5A	NSRRC BL13B1	NSRRC BL13C1	NSRRC BL13C1
Wavelength (Å)	1.00000	1.00000	1.00000	0.97315	0.97315
Space group	$P3_221$	$P3_121$	$P3_221$	$P3_221$	$P3_221$
Unit cell dimensions					
a (Å)	80.41	80.06	80.44	80.36	80.57
b (Å)	80.41	80.06	80.44	80.36	80.57
c (Å)	90.20	183.12	90.04	90.36	90.74
Resolution (Å)[a]	30-1.58	30-2.25	30-2.38	30-1.92	30-2.03
	(1.64-1.58)	(2.33-2.25)	(2.47-2.38)	(1.99-1.92)	(2.10-2.03)
No. of reflections	46486 (4600)	32134 (2665)	13919 (1386)	26220 (2578)	22419 (2187)
Completeness (%)	99.5 (100)	96.9 (82.1)	99.5 (100)	99.8 (99.9)	99.7 (100)
Redundancy	9.0 (9.0)	8.1 (5.7)	11.9 (12.4)	4.1 (4.1)	5.0(5.0)
R_{merge} (%)	4.1 (34.0)	6.8 (36.0)	5.0 (51.3)	2.7 (28.6)	3.2 (29.5)
I/σ(I)	55.3 (8.6)	31.9 (3.8)	50.1 (5.1)	41.7 (5.6)	41.7 (5.8)
Refinement					
No. of reflections	45389 (4301)	30785 (2374)	13674 (1275)	25614 (2371)	21723 (2113)
R_{work} (%)	18.7 (24.3)	20.6 (28.7)	22.3 (35.1)	18.1 (23.5)	19.8 (26.4)
R_{free} (%)	21.3 (27.8)	26.5 (30.8)	27.0 (46.9)	21.8 (30.2)	24.6 (34.6)
Geometry deviations					
Bond lengths (Å)	0.014	0.014	0.013	0.013	0.014
Bond angles (°)	1.5	1.6	1.5	1.4	1.5
Mean B-values (Å^2) / No.					
Protein atoms	24.8 / 2392	44.4 / 4784	65.5 / 2392	29.8 /2369	37.6 / 2392
Compound atoms		46.4 / 96	61.7 / 25	40.4 / 28	50.0 / 24
Mg ions		44.7 / 6	54.6 / 2	27.4 / 1	
Water molecules	48.1 / 629	56.2 / 355	74.6 / 150	46.2 / 367	53.3 / 339
Ramachandran plot (%)					
Most favored	95.6	92.8	88.9	95.5	95.2
Additionally allowed	4.1	7.2	11.1	4.1	4.4
Generously allowed	0.4	0	0	0.4	0.4

[a]Values in the parentheses are for the highest resolution shells.

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