Inhibition of Staphyloxanthin Virulence Factor Biosynthesis in Staphylococcus aureus: In Vitro, in Vivo, and Crystallographic Results†

Yongcheng Song,‡ Chia-I Liu,§ Fu-Yang Lin,‡ Joo Hwan No,*, Mary Hensler,‖ Yi-Liang Liu,*, Wen-Yih Jeng,§ Joo Hwan No,*, Mary Hensler,‖ Yi-Liang Liu,*, Wen-Yih Jeng,§ Joo Hwan No,*, Mary Hensler,‖ Yi-Liang Liu,*, Wen-Yih Jeng,§ Joo Hwan No,*, Mary Hensler,‖ Yi-Liang Liu,*, Wen-Yih Jeng,§ Joo Hwan No,*, Mary Hensler,‖ Yi-Liang Liu,*, Wen-Yih Jeng,§ Jennifer Low,‖ George Y. Liu,‖ Victor Nizet,‖ Andrew H.-J. Wang,§‖ and Eric Oldfield*‡

Department of Chemistry and Center for Computational Biology, University of Illinois at Urbana—Champaign, 600 S. Mathews Avenue, Urbana, Illinois 61801, Institute of Biological Chemistry, Academia Sinica, Nankang, Taipei 11529, Taiwan, National Core Facility of High-Throughput Protein Crystallography, Academia Sinica, Nankang, Taipei 11529, Taiwan, Institute of Biochemical Sciences, College of Life Science, National Taiwan University, Taipei 10098, Taiwan, Department of Pediatrics and Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA, and Division of Pediatric Infectious Diseases and Immunobiology Research Institute, Cedars-Sinai Medical Center, Los Angeles, California 90048

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The gold color of Staphylococcus aureus is derived from the carotenoid staphyloxanthin, a virulence factor for the organism. Here, we report the synthesis and activity of a broad variety of staphyloxanthin biosynthesis inhibitors that inhibit the first committed step in its biosynthesis, condensation of two farnesyl diphosphate (FPP) molecules to dehydroqu unable, catalyzed by the enzyme dehydroquVLalane synthase (CrtM). The most active compounds are phosphonoacetamides that have low nanomolar $K_i$ values for CrtM inhibition and are active in whole bacterial cells and in mice, where they inhibit S. aureus disease progression. We also report the X-ray crystallographic structure of the most active compound, N-$\alpha$-(3-phenoxyphenyl)proplyphosphonoacetamide ($IC_{50} = 8$ nM, in cells), bound to CrtM. The structure exhibits a complex network of hydrogen bonds between the polar headgroup and the protein, while the 3-phenoxyphenyl side chain is located in a hydrophobic pocket previously reported to bind farnesyl thiophosphate (FsPP), as well as biphenyl phosphonosulfonate inhibitors. Given the good enzymatic, whole cell, and in vivo pharmacologic activities, these results should help guide the further development of novel antivirulence factor-based therapies for S. aureus infections.

Introduction

Infections caused by Staphylococcus aureus are a growing cause of concern because of the widespread development of antibiotic resistance and the shortfall in the introduction of new types of anti-infective agents. An alternative strategy that is now gaining interest is targeting of bacterial virulence factors, particularly of pigmented types of anti-infective agents. An alternative strategy that is now gaining interest is targeting of bacterial virulence factors, particularly of pigmented S. aureus,6,7 one important virulence factor is a brightly colored carotenoid pigment, staphyloxanthin (STX), whose biosynthesis can be readily monitored spectrophotometrically. The carotenoid is produced by the condensation of the C15 isoprenoid farnesyl diphosphate (FPP) to form presqualene diphosphate and then dehydroquVLalane, followed by a series of oxidations and glycosylations, in a series of reactions catalyzed by the enzymes CrtM, N, O, P,...,8 and inhibition of these enzymes (e.g., CrtN glycosylations, in a series of reactions catalyzed by the enzymes

1 Crystal structure coordinates have been deposited in the Protein Data Bank and will be released upon publication (2ZY1).
2 To whom correspondence should be addressed. Phone: 217-333-3374, Fax: 217-244-0997. E-mail: eo@chad.scs.uiuc.edu.
3 Department of Chemistry, University of Illinois at Urbana—Champaign.
4 Institute of Biological Chemistry.
5 National Core Facility of High-Throughput Protein Crystallography.
6 National Taiwan University.
7 Center for Biophysics and Computational Biology, University of Illinois at Urbana—Champaign.
8 University of California, San Diego.
9 Cedars-Sinai Medical Center.
10 Abbreviations: CrtM, dehydroquVLalane synthase; FPP, farnesyl diphosphate; QSAR, quantitative structure—activity relationship; SQS, squalene synthase; STX, staphyloxanthin.

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activity. To explore some of these possibilities, we were particularly interested to see if it might be possible to reduce backbone charge/ acidity/polarity while still retaining CrtM activity, since this might improve inhibitor uptake into bacterial and host cells, as well as further reducing bone affinity. The possibility that less polar analogues might still have good activity is supported by the observation that, unlike bisphosphonate inhibitors of farnesyl diphosphate synthase (FPPS), our published CrtM results indicated that Mg\(^{2+}\) binding (which usually involves binding to two anionic groups) is not essential for potent phosphonosulfonate inhibition of CrtM.\(^{14}\) For example, while 1 binds to CrtM with two Mg\(^{2+}\) (PDB file 2ZCQ), 2 binds with only one Mg\(^{2+}\) (PDB file 2ZCR), and 3 has no Mg\(^{2+}\) at all in its X-ray crystallographic structure (PDB file 2ZCS). So, unlike the situation found with FPPS, it seemed likely that a broad range of backbone structures having fewer anionic groups might be developed, since the number of metal ions involved in binding to CrtM is quite variable.

![Chemical structures](image)

In this work, we describe the synthesis of, and inhibition by, 18 compounds encompassing the 11 basic structural motifs (a–k) shown in Figure 2 in which Ar is an aromatic fragment. Since some compounds were prepared as salts while others were free acids, these motifs are shown for simplicity in their protonated or free acid forms, a point we discuss later in the text. These motifs were designed on the basis of the following ideas: In a, we reduced the (potentially) −3 formal side chain charge found in the phosphonosulfonates (−PO\(_3\)H/−PO\(_3\)\(^{2−}\), −SO\(_3\)H/−SO\(_3\)\(^{2−}\)) to −2 but added a H-bond donor/acceptor amide site, a feature suggested at least in part because of synthetic accessibility. In all cases, the number of methylene groups \(n\) in the “spacer” (Figure 2) was in the range \(n = 2−4\), typically \(n = 3\), and the Ar groups were diphenyl ethers or biphenyls. In b and c we reduced the side chain formal charge to −1, but of course the sulfonic and carboxylic acids are expected to have very different pKa values. In d and e we investigated whether methyl substitutions might affect activity, while in f we investigated the effect of modifying H-bond donor ability. In g–i we further investigated the role of H-bonding, while in j, we attempted to design a novel motif that might facilitate metal binding. Finally, compound k (a phosphino- methylphosphonate) was included as a nonhydrolyzable diphosphate analogue (replacing two −O− linkages with two −CH\(_2\)−). We also report the X-ray crystallographic structure of one of the most potent CrtM/STX biosynthesis inhibitors (containing motif a) bound to CrtM, which gives interesting new insights into how this compound binds to its CrtM target.

### Results and Discussion

We synthesized a total of 18 compounds (4−21) based on the 11 motifs shown in Figure 2, and the structures of these compounds are shown in Table 1, rank-ordered in terms of decreasing activity in CrtM inhibition. The \(K_i\) values were determined by using a coupled diphosphate/phosphate release assay,\(^{17}\) as described in the Experimental Section, and are also reported in Table 1. As can be seen in Table 1, the most potent inhibitors (4−6) were all diphenyl ether phosphonocacetamides (motif a, Figure 2) containing a (CH\(_2\))\(_3\) spacer between the aromatic and amide moiety and had \(K_i\) values in the range 30−70 nM, slightly greater than the 20 nM found for the lead phosphonsulfonate 1 in the same assay. The activities of these three compounds in the inhibition of STX biosynthesis in S. aureus are also shown in Table 1, from which we see that 5 is the most active compound in this bacterial cell-based assay, with an IC\(_{50}\) of 8 nM, much less than the IC\(_{50}\) of ~100 nM reported for 1, due presumably to improved cellular uptake. Substitution of one and two Cl atoms on the diphenyl ether side chain had relatively little effect on CrtM inhibition but decreased bacterial STX production by ∼3−5× (with respect to 5) (Table 1). Shortening the (CH\(_2\))\(_3\) spacer by one CH\(_2\) group (14) decreased activity in both assays (CrtM ∼40×; STX ∼400×), while lengthening the spacer by one CH\(_3\) group (16) had an even larger effect (CrtM IC\(_{50}\) ∼130×, STX ∼400×, again versus 5). We also found that replacing the diphenyl ether side chain by a biphenyl group (10) reduced both CrtM inhibition (by ∼20×) and STX biosynthesis (∼600×) (Table 1). So the diphenyl ethers containing a (CH\(_3\))\(_3\) spacer had the most activity, both in the enzyme and in the whole bacterial cell assays.

We next investigated the effects of changing the PO\(_2\)H\(_2\) group to a SO\(_3\)H group (Figure 2, motif b) or a −CO\(_2\)H group (Figure 2, motif c). The sulfonocacetamide (11) had weak activity in both assays: a \(K_i = 0.81\) \(\mu\)M in CrtM inhibition (∼20× higher than 5) and an IC\(_{50} = 15\) \(\mu\)M in STX biosynthesis inhibition (∼600× higher than 5) (Table 1). The results obtained with the carboxylic acid analogue (21) in which the −PO\(_2\)H\(_2\) group was replaced with a −CO\(_2\)H group were even worse, with \(K_i > 7\) \(\mu\)M and an IC\(_{50}\) (STX) of 200 \(\mu\)M, ∼200× and ∼2500× worse than with the phosphonocacetamide 5 (Table 1). So the ordering of activity is −PO\(_2\)H\(_2)/−PO\(_2\)\(^{2−}\) > −SO\(_3\)H/−SO\(_3\)\(^{2−}\) > −CO\(_2\)H/−CO\(_2\)\(^{−}\) in both CrtM and STX biosynthesis inhibition, suggesting the likely importance of multiple electrostatic interactions (and/or H-bonding) between CrtM and the inhibitor’s anionic group. Also of interest is the observation that, at least for the compounds where \(K_i\) values are accurately known (i.e., they are not limit values, Table 1), the cell-based activity values cover a larger range than do the CrtM enzyme inhibition results, suggesting the importance of variations in bacterial cell uptake between the different inhibitors.

On the basis of these observations, we next investigated the effects of methyl substitutions on activity. In the case of the d motif, dimethyl substitution on the acetamide CH\(_2\) (13) would increase hydrophobicity, but this compound had worse CrtM activity (≥20×) and much worse (≥6250× versus 5) activity in STX biosynthesis, where it was essentially inactive (IC\(_{50}\) ∼0.5 mM, Table 1). In the case of the N-Me substituent (Figure 2, motif e), the single methyl group on nitrogen (12) reduced activity by a factor of ∼20× versus 5 (to \(K_i = 0.91\) \(\mu\)M), essentially the same as that seen with the dimethyl analogue 13, but in contrast to 13, there was still measurable STX inhibition activity (IC\(_{50} = 8.6\) \(\mu\)M, ∼1000× worse than with 5). That is, the N-Me (12) and C(Me)\(_2\) (13) analogues have essentially the same activity in CrtM inhibition (0.91 and 0.96 \(\mu\)M, respectively), while STX biosynthesis inhibition is very different (8.6 and >500 \(\mu\)M for 12 and 13, respectively), supporting again the idea that the −CO\(_2\)H\(_2\)PO\(_2\)H\(_2\) group is important for cell-based activity. These results also suggested to us that the amide NH group might be important in hydrogen...
bonding to the protein, since the NH to NMe substitution reduced CrtM activity by \( \sim 20 \times \) (Table 1).

Given the apparent importance of this region for activity, it was of interest to see if activity might be improved by conversion of the NHCO amide moiety to a hydroxamate (NOH\cdot CO, Figure 2, motif f), which would provide alternative possibilities for H-bond formation. We made three hydroxamates, 8, 9, and 20, the first two species containing diphenyl ether side chains, the third, a biphenyl. Interestingly, both the dichloro and unsubstituted diphenyl ether phosphonohydroxamates had good activity in both CrtM and STX biosynthesis inhibition (Table 1), although they were both less active than the three most potent phosphonoacetamides. On average, the hydroxamates were \( \sim 6 \times \) less active against CrtM and \( \sim 2 \times \) less active in the STX biosynthesis assay (Table 1). Not unexpectedly, the biphenyl hydroxamate (20) was even less active, consistent with the weaker activity of the biphenyl phosphonoacetamide, 10. So for activity, these results indicate the importance of a phosphonoacetyl group, located in either an acetamide or a hydroxamate group, suggesting the importance of both electrostatic (H-bond) interactions between the phosphonate and the protein and, most likely, hydrogen bonding between the amide (or hydroxamate) and the protein. To test these ideas further, we next investigated motifs g–i, Figure 2, using compounds 19, 17, and 18. Surprisingly, the sulfonamide 19 had no activity in either the CrtM or STX biosynthesis assay (Table 1), suggesting a critical role for the amide/hydroxamate CO group as an H-bond acceptor. Consistent with this, neither

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**Figure 1.** Schematic flowchart for staphyloxanthin and cholesterol biosynthesis from farnesyl diposphate. The first committed step in both pathways involves the head-to-head condensation of two molecules of farnesyl diposphate to form presqualene diposphate, catalyzed by the CrtM enzyme in *S. aureus*, or squalene synthase in humans. In *S. aureus*, dehydrosqualene is then formed via ring-opening and elimination of diposphate; in humans, ring-opening is accompanied by an NADPH reduction step, resulting in squalene.

**Figure 2.** Structural motifs present in the different inhibitors investigated.
Table 1. Enzyme (CrtM, $K_i$), Pigment (STX, *S. aureus*, IC$_{50}$), and Cell Growth (IC$_{50}$) Inhibition Results for 4–21

<table>
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<th>compound</th>
<th>structure</th>
<th>CrtM$^a$ $K_i$ (µM)</th>
<th>STX$^b$ (S. aureus) IC$_{50}$ (µM)</th>
<th>HSQ$^c$ $K_i$ (µM)</th>
<th>MCF-7 cells$^d$ IC$_{50}$ (µM)</th>
<th>NCI-H460 cells$^e$ IC$_{50}$ (µM)</th>
<th>SF-268 cells$^f$ IC$_{50}$ (µM)</th>
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$^a$ The value given are the $K_i$ values for CrtM inhibition, in µM. $^b$ The values given are the IC$_{50}$ values for STX (staphyloxanthin) virulence factor inhibition in *S. aureus* and are in µM. $^c$ The values given are the $K_i$ values for human squalene synthase inhibition (in vitro) and are in µM. $^d$ The values given are the IC$_{50}$ values for MCF-7 cell growth inhibition, in µM. $^e$ The values given are the IC$_{50}$ values for NCI-H460 cell growth inhibition, in µM. $^f$ The values given are the IC$_{50}$ values for SF-268 cell growth inhibition, in µM.
the ester (17) nor the ketone (18) had significant activity in either assay (Table 1).

We then investigated two additional motifs, j and k in Figure 2. The hydroxamate (j, 15) had modest activity in both assays ($K_i, IC_{50} \sim 4 \mu M$) but was $\sim 100 \times$ (CrtM) to $\sim 500 \times$ (STX) less active than was 5. The phosphinomethylphosphonate 7 (motif k) was a potent CrtM inhibitor ($K_i = 220 \mu M$), but again we believe, due to its increased polarity, it was $\sim 50 \times$ less effective in STX biosynthesis inhibition in whole cells than was the most potent phosphonoacetamide (5).

Finally, we investigated the effects of all 18 compounds on human SQS inhibition, together with their activity in growth inhibition of three human tumor cell lines (MCF-7, NCI-460, and SF-268) (Table 1). Although inhibition of human SQS may not be a particularly important toxicologic consideration, given a choice, it would seem to be preferable to have a STX biosynthesis inhibitor with poor activity against SQS, as opposed to one with potent SQS activity, and as can be seen in Table 1, several potent CrtM inhibitors do have relatively little activity in the SQS assay. More important, in essentially all cases we find no inhibition of human cell growth (in three human cell lines, $IC_{50} > 300 \mu M$), supporting the idea that these compounds will have low toxicity. In fact, only the ester 17 had significant activity ($IC_{50} \approx 70-90 \mu M$) on human cell growth, but since this compound is inactive in STX biosynthesis inhibition, it is not likely to be of interest.

**Structure and Function—Function Aspects.** The results described above are of interest, since they represent the development of several new, chemically diverse virulence inhibitors for *S. aureus*. However, the relationships between structure and activity need to be explored in more detail in order to obtain a better understanding of how, in particular, the most active compounds act on their CrtM target. What is interesting about the most active compounds, the phosphonoacetamides, is that while they contain the same 3-(3-phenoxyphenyl)propyl (PhOC$_6$H$_4$(CH$_2$)$_3$-) side chain as found in the most active phosphonosulfonates, the “backbone” of these compounds is clearly about two bond lengths greater in the new compounds. So we next examined the effects of this common feature on binding.

In the CrtM enzyme, there are two binding sites for FPP, and in previous work we found that phosphonosulfonates could bind to either one of these sites. The diphenyl ether phosphonosulfonate 1 bound to site 1 and had interactions with 2 Mg$^{2+}$, while two biphenyl phosphonosulfonates (2, 3) bound to site 2 with 1 and 0 Mg$^{2+}$, respectively. This observation complicates any QSAR analysis, since there could be 0, 1, or 2 Mg$^{2+}$ present, and two different sites might be occupied. Plus, with the phosphonoacetamides, the presence of a longer backbone complicates the situation further, since, for example, if the diphenyl ether is located in the same region as bound with 1, the headgroup will not fit. We therefore next obtained the structure of a CrtM–5 complex using X-ray crystallography to determine how 5 does in fact bind to CrtM, in order to provide a better, albeit qualitative, structure-based interpretation of the activity of one of the most potent inhibitors. Data collection and refinement statistics are given in Table 2, and full details are given in the Experimental Section.

CrtM crystallizes in the $P_3_21$ space group, and there is one molecule per asymmetric unit. The phosphonoacetamide 5 yielded well-structured 2$F_{o} - F_{c}$, densities (Figure 3A), and the refined structure of 5 (red) is shown superimposed on FsPP (bound to CrtM) in Figure 3B and on 1–3 in Figure 3C. Interestingly, 5 binds to CrtM in a completely different manner to that observed with FsPP or any of the three phosphonosulfonates reported previously. Its polar phosphonate headgroup is located in the FsPP site 1, where it makes a salt bridge with Arg45 and H-bond contacts with Gln165 and Asn168, together with a complex H-bond network with five H$_2$O molecules (Figure 4). However, unlike the situation found with 1, the diphenyl ether side chain occupies the FPP site 2. In addition, the amide CO forms H-bonds with Arg45 and two H$_2$O molecules, and the amide NH acts as an H-bond donor to Gln165 (Figure 4B). Essentially, the increased length of 5 (over that present in the phosphonosulfonates) is accommodated in the protein by 5 “bridging” both the FPP-1 and FPP-2 binding sites (Figure 3B, C).

Given this new structure (PDB 2ZY1), it is now possible to rationalize several of the SAR observations noted above. Specifically, conversion of the −PO$_3$− group in 5 to −SO$_3$− or −CO$_2$− can be expected to result in the loss of many of the H-bond/electrostatic interactions with Arg45, Gln165, and Asn168 and the five H$_2$O molecules. Second, conversion of the amide NH to NMe will prevent H-bond formation with Gln165, as will formation of an ester (17) or a ketone (18) in this region. Likewise, conversion of the amide to the hydroxamate (9) results in disruption of this H-bond interaction which may, however, be partially compensated for by other conformational changes, since 9 still retains quite good ($K_i = 320 \mu M$) CrtM activity. But how can we correlate our enzyme inhibition results with cell based inhibition of STX biosynthesis, something that is clearly desirable because we are interested in good inhibition of virulence factor formation in cells and not just good CrtM inhibitors (which might not actually get into cells)?

**In Vitro and in Vivo Results.** When considering all compounds investigated, we find that there is quite a good correlation between the CrtM enzyme $pK_i (= -\log_{10} K_i, M)$...
and cell STX biosynthesis inhibition pIC$_{50}$ (=$-\log_{10}$ IC$_{50}$, M) values, as can be seen in Figure 5A where the $R^2$ value is 0.73 (for the 14 compounds having nonlimit $K_i$/IC$_{50}$ values). However, when we add results for the 36 compounds reported

Figure 3. X-ray crystallographic results: (A) electron density for 5 in CrtM; (B) superposition of 5 (red) in the CrtM active site (2ZY1) with that of two molecules (green, yellow) of S-thiolofarnesyl diphosphate (2ZCP); (C) superposition of 5 (red) with 1 (blue), 2 (yellow), 3 (cyan) in the CrtM active site (2ZCQ, 2ZCR, 2ZCS).

Figure 4. Interactions between 5 and different residues in the CrtM active site: (A) Pymol$^{26}$ view; (B) Ligplot$^{27}$ interactions.

Figure 5. Figure showing correlations between CrtM inhibition and STX biosynthesis inhibition: (A) data for the 14 compounds reported in this work; (B) combination of 36 phosphonosulfonate inhibitor results (ref 18) with the 14 compounds reported here; (C) combinatorial descriptor search result for all 50 compounds tested (here and in ref 18) in CrtM and STX biosynthesis inhibition. The lower $R^2$ value in part B is likely due the high diversity of the large data set; the $R^2$ improves to 0.68 by using the combinatorial descriptor approach.$^{19}$
previously to the correlation, the n = 50 compound data set exhibits a much worse correlation (Figure 5B) with an $R^2 = 0.42$. This is similar to the results we reported previously where we found for 10 different enzyme/cell assays that on average the $R^2$ value for the $pK_d/pIC_{50}$ correlation was only 0.32 (ref 19), making any predictions of cell-based activity, based on enzyme activity, in some cases, impossible. The large discrepancies found were, we proposed, likely to be due to the neglect of factors that affect inhibitor uptake into cells, and we described a general method in which this aspect might be taken into consideration, by using a "combinatorial descriptor approach". That is, we described cell activity by using the following equation:

$$pIC_{50}(cell) = apK_e(enzyme) + bB + cC + d$$

where $a-d$ are linear regression coefficients and where $B$ and $C$ are mathematical descriptors (such as SlogP) chosen in a combinatorial manner from a large series of potential descriptors (such as the 230 descriptors in the program MOE). Applying this same method to the combined data set (50 compounds), we now obtain (Figure 5C) $R^2 = 0.68$, a significant improvement.

To investigate in vivo activity, we selected the most potent in vitro STX biosynthesis inhibitor (5) and carried out an intraperitoneal challenge experiment with $S. aureus$ in exactly the same manner as reported previously for 1. We treated one group of mice ($n = 9$) with 0.5 mg of 5 twice per day (days -1, 0, 1, and 2) and a second group ($n = 9$) with equivalent volume injections of PBS control. Upon sacrificing the mice at 72 h, $S. aureus$ bacterial counts in the kidneys of the mice treated with 5 were significantly lower than those of the control group ($p < 0.001$). The median number of colony forming units (cfu) in the untreated animals was 22 500 cfu/mL compared with 850 cfu/mL for the treated animals (Figure 6), about a 96% reduction in surviving bacteria in the treatment group.

**Conclusions**

The results we have described here are of interest for a number of reasons. First, we have synthesized a broad variety of inhibitors of the dehydroconjugate synthase enzyme, CrtM, exhibiting novel structural motifs. Second, we have determined their activity in CrtM inhibition and in staphyloxanthin biosynthesis in $S. aureus$. Third, we show that STX biosynthesis activity for a broad range of inhibitors can be predicted from enzyme inhibition results with quite good $R^2$ values by using a combinatorial descriptor approach. Fourth, we have demonstrated that the most potent STX biosynthesis inhibitor in vitro also has activity in vivo in preventing infection. Fifth, we show that these compounds have no activity against three human cell lines. Sixth, we have obtained the X-ray crystallographic structure of the most potent STX biosynthesis inhibitor investigated here (5) bound to CrtM. The structure is unusual in that the inhibitor actually bridges the FPP-1 and FPP-2 sites observed previously, whereas the phosphonosulfonates reported previously bind to one or other of these sites but not to both. Many of the major changes in CrtM activity between the different motifs investigated can be interpreted in terms of the crystallographic results, opening up the way to the further development of this class of compound as novel anti-infective agents targeting inhibition of the biosynthesis of the staphyloxanthin virulence factor in $S. aureus$.

**Experimental Section**

**General Synthesis Methods. General Method A.** Triethyl phosphonoacetate, or diethyl cyanomethylphosphonate (3.3
mmol), was added dropwise to NaH (145 mg, 60% in oil, 3.6 mmol) suspended in dry THF (7 mL) at 0 °C. To the resulting clear solution was added a benzaldehyde (3 mmol), and after being stirred at room temperature for 0.5 h, the reaction mixture was partitioned between diethyl ether (50 mL) and water (50 mL). The organic layer was dried and evaporated. The oily residue was then hydrogenated in MeOH (15 mL) in the presence of 5% Pd/C (50 mg). The catalyst was filtered and the filtrate concentrated and dried in vacuo.

**General Method B.** The nitrile (or ester) obtained using general method A was added slowly to 2 equiv of LiAlH4/AlCl3, or LiAlH4, in dry THF at 0 °C. After the mixture was stirred at room temperature for 2 h, the reaction was carefully quenched by adding a few drops of water and the reaction mixture filtered and evaporated.

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**Scheme 1.** General Synthetic Routes to Phosphonoacetamides and N-Hydroxyphosphonoacetamides

Reagents and conditions: (i) BuLi, then CO2, −78 °C, 63%; (ii) oxalyl chloride (2 equiv), 100%; (iii) NaH, diethyl cyanomethylphosphonate; (iv) H2, Pd/C (5%); (v) LiAlH4, (2 equiv), AlCl3 (2 equiv); (vi) NaH, triethyl phosphonoacetate; (vii) LiAlH4, (2 equiv); (viii) MsCl, NEt3, then NaI (5 equiv); (ix) O-benzylhydroxylamine (2 equiv), diisopropylethylamine, DMF, 80 °C, 50% overall from 24; (x) EDC, HOBt; (xi) NEt3; (xii) TMSBr (2 equiv), then MeOH.

**Scheme 2**

Reagents and conditions: (i) NEt3; (ii) TMSBr (2 equiv), then MeOH, 48% for two steps; (iii) EDC, HOBt; (iv) DOWEX ion-exchange resin, H+ form, 85% for two steps; (v) H2, Pd/C (5%); (vi) KOH, MeOH/H2O, 66% for two steps; (vii) NaCN, DMF; (viii) LiAlH4, (2 equiv), AlCl3 (2 equiv).
General Method C. To a solution of a carboxylic acid (1 mmol) and an amine (1 mmol) in CH₂Cl₂ (5 mL) were added N-ethyl-N-(3-dimethylaminopropyl)carbodiimide (EDC) (1.5 mmol) and 1-hydroxybenzotriazole (1 mmol). After the mixture was stirred for 2 h at room temperature, 50 mL of ethyl acetate was added and the reaction mixture was washed successively with 1 N HCl (5 mL), water (5 mL), and saturated NaHCO₃ (5 mL), dried, and evaporated. The amide was purified using flash chromatography (silica gel, ethyl acetate).

General Method D. A DMF solution (3 mL) containing a halide (1 mmol), O-benzylhydroxylamine (3 mmol) and diisopropylethylamine (3 mmol) was heated at 90 °C for 24 h. After the mixture was cooled, diethyl ether (50 mL) was added and the mixture was washed with H₂O (20 mL), dried, and evaporated. The alkylated hydroxylamine, such as 29, was purified by using column chromatography (silica gel; hexane/ethyl acetate, 6/1).

General Method E. To a diethyl phosphate (1 mmol) in dry CH₂CN (3 mL) was added TMSBr (2 mmol) at room temperature. The reaction mixture was then partitioned between ethyl acetate and water and washed successively with 1 N HCl and saturated NaHCO₃, dried, and evaporated to dryness. The oily residue was treated with NaI (1.35 g, 9 mmol) in acetone (7 mL) followed by neutralization with KOH to pH 8, followed by evaporation to give the residue. The residue was treated with NaHCO₃ (5 mL), dried, and evaporated. The amide was purified using column chromatography (silica gel; hexane/ethyl acetate, 6/1).

Diethylphosphonoacetyl Chloride (23a). Compound 23a was prepared in the same way as 26, but using 3-(4-chlorophenoxy)benzaldehyde (1 mmol) as starting material, as a white powder (267 mg, 58% overall yield). Anal. (C₁₇H₁₇ClK₂NO₅P) C, H, N. 1H NMR (400 MHz, D₂O): δ 7.60–7.20 (m, 2H, PhCH₂); 6.70–6.50 (m, 2H, PhCH₂); 2.98 (t, J = 7.6 Hz, 2H, PhCH₂); 2.98 (t, J = 7.6 Hz, 2H, PhCH₂); 2.98–2.80 (m, 2H, PhCH₂); 2.46 (t, J = 7.6 Hz, 2H, PhCH₂); 2.35 (m, 2H, PhCH₂); 2.35 (m, 2H, PhCH₂); 1.70 (m, 2H, CH₂); 1.50 (m, 2H, CH₂); 1.30 (m, 2H, CH₂); 1.30 (m, 2H, CH₂); 1.20 (m, 2H, CH₂); 1.20 (m, 2H, CH₂); 1.00 (s, 1P); 39.9 (s, 1P). 31P NMR (D₂O): δ 13.7.

N-[3-(3-Phenoxynaphenyl)propyl]phosphonooacetamide Dipotassium Salt (5). Amine 26 was prepared from 3-phenoxynaphthaldehyde (1 mmol) using general method A and was then coupled with dibenzylphosphonoacetamic acid according to general method C to give the dibenzyl ester of 4. The benzyl groups were removed by catalytic hydrogenation (5% Pd/C in methanol for 1 h) followed by neutralization with KOH to give compound 5 as a white powder (245 mg, 48% overall yield). Anal. (C₁₇H₁₇ClK₂NO₅P) C, H, N. 1H NMR (400 MHz, D₂O): δ 7.60–7.20 (m, 2H, PhCH₂); 6.70–6.50 (m, 2H, PhCH₂); 2.98 (t, J = 7.6 Hz, 2H, PhCH₂); 2.98 (t, J = 7.6 Hz, 2H, PhCH₂); 2.98–2.80 (m, 2H, PhCH₂); 2.46 (t, J = 7.6 Hz, 2H, PhCH₂); 2.35 (m, 2H, PhCH₂); 2.35 (m, 2H, PhCH₂); 1.70 (m, 2H, CH₂); 1.50 (m, 2H, CH₂); 1.30 (m, 2H, CH₂); 1.30 (m, 2H, CH₂); 1.20 (m, 2H, CH₂); 1.20 (m, 2H, CH₂); 1.00 (s, 1P); 39.9 (s, 1P). 31P NMR (D₂O): δ 13.7.

N-[3-(3-Chlorophenoxy)phenyl]propylphosphonoacetamide Dipotassium Salt (6). 6 was prepared in the same way as 5, but using 3-(4-chlorophenoxy)benzaldehyde (1 mmol) as starting material, as a white powder (267 mg, 58% overall yield). Anal. (C₁₇H₁₇ClK₂NO₅P) C, H, N. 1H NMR (400 MHz, D₂O): δ 7.60–7.20 (m, 2H, CH₂); 2.98 (t, J = 7.6 Hz, 2H, PhCH₂); 2.98 (t, J = 7.6 Hz, 2H, PhCH₂); 2.98–2.80 (m, 2H, PhCH₂); 2.46 (t, J = 7.6 Hz, 2H, PhCH₂); 2.35 (m, 2H, PhCH₂); 2.35 (m, 2H, PhCH₂); 1.70 (m, 2H, CH₂); 1.50 (m, 2H, CH₂); 1.30 (m, 2H, CH₂); 1.30 (m, 2H, CH₂); 1.20 (m, 2H, CH₂); 1.20 (m, 2H, CH₂); 1.00 (s, 1P); 39.9 (s, 1P). 31P NMR (D₂O): δ 13.5.

3-(3-Phenyl(propynyl)phosphonophenyl)methylphosphonic Acid Tripotassium Salt (7). Triethyl methylphosphonylmethyphosphonic (1 mmol) was treated with BuLi (2.2 mmol) in THF at −78 °C for 1 h, followed by addition of iodide 28 (1.1 mmol). The reaction mixture was allowed to warm to room temperature over 3 h and was then quenched with saturated NH₄Cl. The product was purified with column chromatography (silica gel; ethyl acetate/methanol, 20/1) and deprotected using general method E to give 7 as a white powder (320 mg, 62% overall yield). Anal. (C₁₇H₁₇ClK₃O₇P) C, H, N. 1H NMR (400 MHz, D₂O): δ 1.30–1.60 (m, 6H, 2H, CH₃); 1.75–1.85 (m, 2H, CH₃); 2.43 (t, J = 7.6 Hz, 2H, PhCH₂); 6.70–7.30 (m, 9H, aromatic). 31P NMR (D₂O): δ 16.3 (s, 1P); 39.9 (s, 1P).

N-Hydroxy-N-[3-(3,4-dichlorophenoxy)phenyl]propylphosphonoacetamide Dipotassium Salt (8). 8 was prepared in the same way as 9, but using 3-(3,4-dichlorophenoxy)benzaldehyde (3 mmol) as starting material, as a white powder (428 mg, 28% overall yield).
N-Hydroxy-N-[3-(3-phenoxypbenyl)propyl]phosphonoacetamide Dipotassium Salt (9). General method D with iodide 28 gave substituted hydroxylamine 29 (1 mmol), which was reacted with the acid chloride in the presence of NEt 3 (2 mmol) in CH2Cl2 (5 mL) at 0 °C. After the mixture was stirred for 1 h, the coupling product was purified by using column chromatography (silica gel, ethyl acetate) and was then deprotected following general method E. Hydrogenation (5% Pd/C, MeOH) gave compound 9 as a white powder (312 mg, 56% overall yield). 1H NMR (400 MHz, D2O): δ 1.65–1.75 (m, 2H, CH2); 2.42 (d, J = 7.6 Hz, 2H, PhCH2); 2.66 (d, J = 20 Hz, 2H, CH2P); 3.39 (t, J = 7.2 Hz, 2H, CH2N); 6.70–7.20 (m, 9H, aromatic). 31P NMR (D2O): δ 13.8.

N-Hydroxy-2-phosphono-5-(3-phenoxypbenyl)pentamide Dipotassium Salt (15). Iodide 28 was added to a cold DMF solution containing ethyl dibenzylphosphonoacetate (1 equiv) and NaH (1 equiv). After the mixture was stirred for 3 h at room temperature, the product 30 was purified by using column chromatography (silica gel, hexane/ethyl acetate, 1/1). 30 was then treated with 3 N KOH in EtOH/H2O (3:1) for 24 h and the resulting solution was reduced in volume and then acidified with 3 N HCl to give the corresponding carboxylic acid. The acid so obtained was reacted with O-benzylhydroxylamine, according to general method C, to give protected 15, which was then hydrogenated in the presence of 5% Pd/C in MeOH for 1 h to afford, after neutralization with KOH, 15 as a white powder (28 mg, 21% overall yield). Anal. (C18H20K2NO4P) C, H, N. 31P NMR (D2O): δ 12.8.

N-Phenyl-3-(3-phenoxypbenyl)propyl]phosphonoacetamide (10). 3-(4-Biphenyl)propylamine was prepared from 4-phenylbenzaldehyde (1 mmol), using general method A, and was then coupled with dibenzylphosphonic acid according to general method C to give the dibenzyl ester of 10. The benzyl groups were removed by hydrogenation (catalyzed with 5% Pd/C in methanol) for 1 h, followed by neutralization with KOH, to give compound 10 as a white powder (222 mg, 65% overall yield). Anal. (C17H20K2NO4P·0.25CH2OH) C, H, N. 1H NMR (400 MHz, D2O): δ 1.65–1.75 (m, 2H, CH2); 2.31 (d, J = 20 Hz, 2H, CHP); 2.55 (t, J = 7.6 Hz, 2H, PhCH2); 3.03 (t, J = 7.2 Hz, 2H, CH2N); 7.20–7.55 (m, 9H, aromatic). 31P NMR (D2O): δ 17.5.

N-Methyl-N-[3-(3-phenoxypbenyl)propyl]phosphonoacetamide Dipotassium Salt (12). Amine 26 (1 mmol) was coupled with sulfonic acid (1 mmol) according to general method C (without addition of 1-hydroxybenzotriazole) to give 11. The product was purified by using column chromatography (Dowex ion-exchange resin, H+ form, methanol as eluent) as an off-white powder (315 mg, 85% overall yield). Anal. (C17H19NO2S) C, H, N. 1H NMR (400 MHz, D2O): δ 1.60–1.70 (m, 2H, CH2); 2.44 (m, 2H, PhCH2); 3.02 (m, 2H, CH2N); 3.59 (s, 2H, CH2S); 6.70–7.30 (m, 9H, aromatic).

N-Methyl-N-[3-(3-phenoxypbenyl)propyl]phosphonodimethylacetamide Dipotassium Salt (13). Diethyl phosphonomethylacetate (3 mmol) was treated with 3 N KOH (5 mL) in ethanol (8 mL) for 24 h, followed by acidification with HCl to give the corresponding carboxylic acid. As with compound 23a, the acid was then converted to the acid chloride 23b, which was reacted with 1 equiv of amine 26 in the presence of NEt3 in CH2Cl2 (5 mL) at 0 °C. After the mixture was stirred for 1 h, the coupling product was purified by using column chromatography (silica gel, ethyl acetate) and was then deprotected following general method E to give 13 as a white powder (335 mg, 21% overall yield). Anal. (C18H22K2NO4P·0.5KBr·H2O) C, H, N. 1H NMR (400 MHz, D2O): δ 1.09 (d, J = 13.6 Hz, 6H, 2CH3); 1.60–1.70 (m, 2H, CH2); 2.46 (m, 2H, PhCH2); 2.99 (m, 2H, CH2N); 6.85–7.25 (m, 9H, aromatic). 31P NMR (D2O): δ 22.9.

N-2-(3-Phenoxyphenyl)ethyl]phosphonoacetamide Dipotassium Salt (14). 3-Phenoxybenzyl chloride (2 mmol) and NaN3 (2.2 mmol) were stirred in DMF (2 mL) overnight at 60 °C. After the mixture was cooled, diethyl ether (50 mL) was added and the mixture was washed with water and the organic layer dried and evaporated. 2-(3-Phenoxyphenyl)ethylamine was prepared from the nitrite so obtained, using general method B, and was then coupled with dibenzylphosphonic acid according to general method C to give the dibenzyl ester of 14. The benzyl groups were removed by catalytic hydrogenation (5% Pd/C in methanol for 1 h) followed by neutralization with KOH to give compound 14 as a white powder (387 mg, 45% overall yield). Anal. (C18H20K2NO5P) C, H, N. 1H NMR (400 MHz, D2O): δ 1.29 (d, J = 20 Hz, 2H, CH2P); 2.58 (t, J = 7.6 Hz, 2H, PhCH2P); 3.05 (t, J = 7.2 Hz, 2H, CH2N); 6.70–7.30 (m, 9H, aromatic). 31P NMR (D2O): δ 13.8.

N-Hydroxy-2-phosphono-5-(3-phenoxypbenyl)butyl]phosphonoacetamide Dipotassium Salt (16). Iodide 28 (2 mmol) and NaN3 (2.2 mmol) were stirred in DMF (2 mL) overnight at 60 °C. After the mixture was cooled, diethyl ether (50 mL) was added and the mixture was washed with water and the organic layer evaporated. 4-(3-Phenoxyphenyl)butylamine was prepared from the nitrite so obtained, using general method B, and was then coupled with dibenzylphosphonic acid, according to general method C, to give the dibenzyl ester of 16. The benzyl groups were removed by catalytic hydrogenation (5% Pd/C in methanol for 1 h) followed by neutralization with KOH to give compound 16 as a white powder (222 mg, 50% overall yield). Anal. (C18H20K2NO5P·0.5KBr·H2O) C, H, N. 1H NMR (400 MHz, D2O): δ 1.55–1.70 (m, 4H, CH2); 2.39 (d, J = 20 Hz, 2H, CHP); 2.48 (t, J = 7.6 Hz, 2H, PhCH2P); 3.01 (t, J = 7.2 Hz, 2H, CH2N); 6.70–7.30 (m, 9H, aromatic). 31P NMR (D2O): δ 17.5.

3-(3-Phenoxyphenyl)ethyl Phosphonoacetamide Dipotassium Salt (18). Alcohol 27 was coupled with dibenzylphosphonic acid according to general method C to give dibenzyl ester of 17. The benzyl groups were removed by catalytic hydrogenation (5% Pd/C in methanol for 1 h) followed by neutralization with KOH to give compound 17 as a white powder (180 mg, 42% overall yield). Anal. (C18H20K2O3P) C, H, N. 1H NMR (400 MHz, D2O): δ 1.60–1.70 (m, 2H, CH2); 2.38 (d, J = 20 Hz, 2H, CHP); 2.44 (t, J = 7.6 Hz, 2H, PhCH2P); 3.47 (t, J = 7.2 Hz, 2H, CH2N); 6.70–7.30 (m, 9H, aromatic). 31P NMR (D2O): δ 13.9.

2-Oxo-6-(4-phenoxypbenyl)hexylphosphonic Acid Dipotassium Salt (19). 8-BBN (0.5 M in THF, 9 mL) was added to ethyl 4-pentenoate (3 mmol) at 0 °C and the reaction mixture stirred at room temperature for 2 h. 4-Bromophenylether (3 mmol), Pd(PPh3)4 (0.15 mmol), K2PO4 (6 mmol), and H2O (2 mL) were then added, and the reaction mixture was refluxed overnight. The organic layer was evaporated and purified by using column chromatography (silica gel, hexane/ether, 0:1) to afford ester 31, which was then reacted with 2 equiv of the lithium salt of diethyl phosphonoacetate at −78 °C for 2 h. The reaction was quenched with saturated NH4Cl, diethyl ether added to extract the product, and the organic solvent removed. The oily residue was purified by using column chromatography (silica gel, ethyl acetate) and deprotected according to general method E to give compound 18.
as a white powder (312 mg, 20% overall yield). Anal. (C<sub>32</sub>H<sub>39</sub>K<sub>2</sub>O<sub>5</sub>)·0.5KBr·2H<sub>2</sub>O C, H. 1H NMR (400 MHz, D<sub>2</sub>O): δ 1.30–1.50 (m, 4H, CH<sub>2</sub>); 2.44 (t, J = 7.6 Hz, 2H, PhCH<sub>2</sub>); 2.54 (t, J = 7.6 Hz, 2H, CH<sub>2</sub>CO); 2.70 (d, J = 20 Hz, 2H, CH<sub>2</sub>P); 6.80–7.25 (m, 9H, aromatic). 31P NMR (D<sub>2</sub>O): δ 11.0.

N-[3-(3-Phenoxypbenyl)propyl]phosphononethylsulfamidate Dipotassium Salt (19). Amine 26 prepared from 3-phenoxyzbenzyldehyde (3 mmol) using general method A was reacted with 1 equiv of methanesulfonyl chloride in CH<sub>2</sub>Cl<sub>2</sub> in the presence of 1.2 equiv of aldehyde (3 mmol) using general method A was reacted with 1 equiv of potassium salt (19).

Amine

MeOH, and the OD was determined at 450 nm using a Perkin-Elmer MBA 2000 (Norwalk, CT) spectrophotometer. The IC<sub>50</sub> values were obtained by fitting the OD data to a normal dose–response curve, using GraphPad PRISM.

Human SQS Enzyme Expression, Purification, and Inhibition Assay. E. coli expressing a human SQS construct were cultured in Luria–Berti medium supplemented with kanamycin (30 µg/mL) and chloramphenicol (34 µg/mL) at 37 °C, until the cells reached an OD of 0.4 at 600 nm, and were then induced at 37 °C for 4 h by incubation with 1 mM isopropyl-1-thio-β-galactopyranoside. Cells were harvested by centrifugation (10 min, 4000 rpm) and resuspended in 10 mM Tris-HCl, 50 mM NaCl, 0.1% Triton X-100, 0.5 mM EDTA, pH 7.4, 10 mM CHAPS, 2 mM MgCl<sub>2</sub>, 10% glycerol, 10 mM mercaptoethanol, 500 mM NaCl, 10 mM imidazole, and a protease inhibitor cocktail), disrupted by sonication, and centrifuged at 16 000 rpm for 30 min. The supernatant (40 mL) was then applied to a HitTrap nickel-chelating HP column (Amersham Biosciences).

Enzyme purification was performed according to the manufacturer’s instructions using a Pharmacia FPLC system. Unbound protein was washed off with 50 mM imidazole, and then the His<sub>6</sub>-HSQS was eluted with 1 M imidazole. Purity was confirmed by SDS–PAGE electrophoresis. Fractions containing the enzyme were pooled and dialyzed against buffer A (25 mM sodium phosphate, pH 7.4, 20 mM NaCl, 2 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 10% methanol), concentrated, then stored at −80 °C.

SQS activity was measured using the conversion of [1H]FPP to [1H]squalene. Final assay concentrations were 50 mM MOPS (pH 7.4), 20 mM MgCl<sub>2</sub>, 5 mM CHAPS, 1% Tween-80, 10 mM DTT, 0.025 mg/mL BSA, 0.25 mM NADPH, and 7.5 ng of purified recombinant human SQS. The reaction was started with the addition of substrate ([FPP, 0.1 nmol, 2.22 × 10<sup>5</sup> dpm), and the final volume of the reaction was 200 µL. After incubation at 37 °C for 5 min, an amount of 40 µL of 10 M NaOH was added to stop the reaction, followed by 10 µL of a (100:1) mixture of 98% EtOH and squalene. The resulting mixtures were mixed vigorously by vortexing. Then 10 µL aliquots were applied to 2.5 cm × 10 cm channels of a silica gel thin layer chromatogram, and newly formed squalene was separated from unreacted substrate by chromatography in toluene–EtOAc (9:1). The region of the squalene band was scraped and immersed in Hydrofluor liquid scintillation fluid and assayed for radioactivity. IC<sub>50</sub> values were calculated from the hyperbolic plot of percent of inhibition versus inhibitor concentration, using GraphPad PRISM.

Human Cell Growth Inhibition Assay. Three human cell lines MCF-7, NCI-H460, and SF-268 were obtained from the National Cancer Institute. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine at 37 °C in a 5% CO<sub>2</sub> atmosphere with 100% humidity. A broth microdilution method was used to calculate IC<sub>50</sub> values for growth inhibition by each compound. Cells were inoculated at a density of 5000 cells/well into 96-well flat-bottom culture plates containing 10 µL of the test compound, previously half-log serial diluted (from 0.316 mM to 0.1 pM) for a final volume of 100 µL. Plates were then incubated for 4 days at 37 °C in a 5% CO<sub>2</sub> atmosphere at 100% humidity, after which an MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay (ATCC, Manassas, VA) was used to quantify cell viability. The IC<sub>50</sub> values were obtained by fitting the OD data to a normal dose–response curve, using GraphPad PRISM.

Murine Model of Kidney Infection. The 10–12 week old CD1 male mice (Charles River Laboratory) were randomized into two groups at the start of the experiment and administered either 0.5 mg of BPH-652 or PBS control, ip, twice a day, starting on day 1 to day 2 (a total of eight doses). All mice were injected intraperitoneally (ip) with 10<sup>7</sup> early stationary phase S. aureus on day 0. After 3 days, animals were euthanized, kidneys homogenized in PBS, and plated on THA for quantitative bacterial culture.

Statistics. The significance of experimental differences in the mouse in vivo challenge studies were evaluated by use of the two-tailed Student’s t test.

X-ray Crystallography. Native CrTM was eluted from Ni-NTA beads by incubation with factor Xa (Novagen) to cleave it from

Staphylotherax Biosynthesis Inhibition Assay. The S. aureus strain used was the WT clinical isolate (Fig.1). S. aureus was propagated in Todd-Hewitt broth (THB) or on THB agar (TBA; Difco, Detroit, MI). For in vitro pigment inhibition studies, S. aureus was cultured in THB (1 mL) in the presence of inhibitor compounds for 72 h, in duplicate. Prior to assay, the bacteria were centrifuged and washed twice in PBS. Staphylotherax was extracted with MeOH, and the OD was determined at 450 nm using a Perkin-
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 and then concentrated to 15 mg/mL. Native CrTM crystals (space group P2₁2₁2₁) 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. BPH-830 was incorporated by soaking crystals with a solution of 5 (10 mM in DMSO) for 3 h at room temperature. X-ray diffraction data were collected at SPXF beamline BL13B1 at the National Synchrotron Radiation Research Center (NSRRC), Hsinchu, Taiwan. All diffraction images were recorded using an ADSD Q210 CCD detector, and the data were indexed, integrated, and scaled by using the HKL2000 package.⁵¹ The structure of the CrTM–5 complex was determined by molecular replacement using CNS²² using the refined native CrTM (PDB 2ZZC) as a search model. Iterative cycles of model building with Xtalview²³ and computational refinement with CNS were performed, in which 5% reflections were set aside for Rfree calculation.⁵² The stereochemical quality was assessed with the program PROCHECK.⁵⁵ Figures were obtained by using Pymol.²⁶

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