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Structure, Function and Inhibition of Staphylococcus aureus Heptaprenyl Diphosphate Synthase

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Abstract

We report the first structure of heptaprenyl diphosphate synthase from *Staphylococcus aureus* (SaHepPPS), together with an investigation of its mechanism of action, and inhibition. The protein is involved in the formation of menaquinone, a key electron transporter in many bacteria, including pathogens. SaHepPPS consists of a “catalytic” subunit (SaHepPPS-2) having two “DDXXD” motifs and a “regulatory” subunit (SaHepPPS-1) that lacks these motifs. High concentrations of the substrates, isopentenyl diphosphate and farnesyl diphosphate, inhibit the enzyme, which is also potently inhibited by bisphosphonates. The most active inhibitors ($K_i \sim 200$ nM) were N-alkyl analogs of zoledronate containing $\sim C_6$ alkyl side-chains. They were modestly active against *S. aureus* cell growth, and growth inhibition was partially “rescued” by addition of menaquinone-7. Since SaHepPPS is essential for *S. aureus* cell growth, its structure is of interest in the context of the development of menaquinone biosynthesis inhibitors as potential antibiotic leads.

Introduction

With the increasing rise in resistance to antibiotics, there is a need for new drug targets, and inhibitors. Isoprenoid biosynthesis is of interest in this context because isoprenoids are involved in the production of a diverse range of compounds including sterols, membrane lipids, carotenoids, quinones, hemes, as well as in bacterial cell wall biosynthesis.^[1] Moreover, many important commercial drugs such as statins, azoles, and bisphosphonates, act in these pathways. Isoprenoid biosynthesis in general begins with the condensation of dimethylallyl diphosphate (DMAPP) with two molecules of isopentenyl diphosphate (IPP) to produce farnesyl diphosphate (FPP) in a reaction catalyzed by farnesyl diphosphate synthase (FPPS, Figure 1).^[2] FPP is then converted to e.g. the C_{55} -isoprenoid undecaprenyl diphosphate (involved in cell wall biosynthesis); to virulence factors such as staphyloxanthin or tuberculosinyl adenosine, or to quinones such as menaquinone and ubiquinone, used in

electron transport. Many of the enzymes involved in these reactions are present only in bacteria and are essential for survival or virulence, so they are drug targets.^[3] In *Staphylococcus aureus*, the major quinone is menaquinone-7 (MK-7), which contains a C₃₅ side-chain (made from 7 isoprenoid C₅ subunits). Because *S. aureus* does not contain ubiquinone (CoQ), menaquinone is the only quinone electron transporter.^[4] MK-7 is produced via the condensation of 1,4-dihydroxy-2-naphthoic acid (Figure 1) with heptaprenyl diphosphate in a reaction catalyzed by MenA, and the heptaprenyl diphosphate substrate is produced by the condensation of FPP with four molecules of IPP in a reaction catalyzed by heptaprenyl diphosphate synthase (HepPPS, Figure 1). HepPPS from *S. aureus* is a heterodimeric *trans*-prenyl transferase that consists of a regulatory protein (190 residues) and a catalytic protein (319 residues), called here SaHepPPS-1 and SaHepPPS-2, respectively. In this work, we report the X-ray structure of the heterodimeric protein (SaHepPPS), its activity, its inhibition by a series of compounds, together with the inhibition of *S. aureus* cell growth, growth rescue by menaquinone, as well as synergy assays with a range of known antibiotics.

Results and Discussion

Sequence Similarity Network for SaHepPPS

Sequence similarity networks for HepPPS-1 and HepPPS-2 were generated by using enzyme similarity tools in the Enzyme Function Initiative (EFI-EST).^[5] The sequences of close relatives were obtained by using BLAST searches^[6] and were then used to generate an output network in Cytoscape 3.1.0. Results for the putative regulatory domain (HepPPS-1) and the putative catalytic domain (HepPPS-2) are shown in Figures 2a, b, respectively. There are 3,414 relevant nodes in the HepPPS-2 network (Figure 2b), but only 212 in the HepPPS-1 network (Figure 2a).

As can be seen in Figure 2b, there are many sequences (from a broad range of organisms) with close sequence similarity to SaHepPPS and the vast majority are annotated as polyprenyl diphosphate synthases with C₂₀ side-chains, many being found in *Bacillus* species. Shorter chain synthases, such as farnesyl diphosphate synthase (FPPS), are largely absent. No human genes were identified as close relatives in the HepPPS-2 network. However, a BLAST search for SaHepPPS-2 against the *Homo sapiens* genome did reveal one homolog, decaprenyl diphosphate synthase (HsDPPS-1) having a 22% sequence identity as calculated using MultiSeq.^[7] In contrast, the BLAST analysis revealed no significant sequence similarities between SaHepPPS-1 and any human enzymes. This is of potential interest since as we show below, both SaHepPPS-1 and SaHepPPS-2 are essential for SaHepPPS activity, suggesting the possibility that selective inhibition might be possible by targeting the regulatory domain and/or its interaction with the catalytic domain.

Structure of *S. aureus* Heptaprenyl Diphosphate Synthase

We next determined the structure of the heterodimeric protein, SaHepPPS, as described in the Experimental Section. Full data acquisition and refinement details are given in Table S1. The SaHepPPS (SaHepPPS-1 plus SaHepPPS-2) structure is shown in Figure 3a and contains two Asp-rich motifs: the first Asp-rich motif (FARM), DDVID, and the second

Asp-rich motif (SARM) DDVLD, involved in catalysis, shown by the red spheres. As expected for a head-to-tail trans-prenyl transferase, the catalytic domain is highly helical. Using the SSM server,^[8] we find that the closest known structure is that of hexaprenyl diphosphate synthase from *Micrococcus luteus* (PDB:3AQB)^[9] where there is a 1.27 Å C α RMSD for the catalytic domain (over 304 residues) and a 2.33 Å C α RMSD for the regulatory domain (over 128 residues).^[10] Structural superpositions of the two proteins are shown in Figure 3b. The catalytic domains are clearly more similar than are the regulatory domains, but also of interest, there is a cluster of highly conserved residues (pink spheres, Figure 3c) that lie at the interface between the two proteins. Partial sequence alignments of a series of regulatory (Figure 4a) and catalytic (Figure 4b) proteins annotated as heptaprenyl or hexaprenyl diphosphate synthases from *S. aureus*, *S. haemolyticus*, *S. epidermidis*, *Streptococcus pneumoniae*, *Bacillus subtilis*, and *Micrococcus luteus* (Figure 4) reveal that 9 out of 13 residues that are conserved in these regulatory proteins are located at the interface of HepPPS-1 and HepPPS-2. As expected, the majority of the conserved residues in the catalytic subunits are in close proximity to the substrate binding sites (FARM and SARM) but the others are at the interface, Figures 3c, 4b. So, even though there is low overall sequence homology between the different regulatory proteins, most of the residues that are conserved are located at the interface and are close to conserved (non-active site) residues in the catalytic proteins, implying that they play an important role in catalysis.

We then sought to see how the SaHepPPS monomer structures compared with the structures of a broad range of other prenyl synthases by using Q_H^[11] scores, obtained by using the MultiSeq program.^[7] The Q_H score is a structural homology score that attempts to account for errors in structural alignments when the numbers of residues in target and query proteins differ. Homologous proteins have Q_H>0.4. When Q_H has a low value (0.1–0.3), structures are considered to have low homology. As can be seen in Figure S1A, the regulatory protein SaHepPPS-1 clusters with the *M. luteus* protein, MIHexPPS-1, but the cluster is isolated and the Q_H = 0.31 value indicates low homology. On the other hand, the catalytic domain of SaHepPPS-2 is very highly homologous (Q_H = 0.80) to that found in *M. luteus* HexPPS-2 (Figure S1B), and is also clustered with other long-chain (C₄₀, C₅₀) prenyl transferases. These observations reinforce the idea that SaHepPPS-1 has an unusual structure.

Enzyme Kinetics and Substrate Inhibition

We next investigated SaHepPPS catalytic activity, and its inhibition. In SaHepPPS, we find that the two components are not active individually, but form a heterodimeric complex that is catalytically active in the presence of IPP, FPP and Mg²⁺. Proteins that were separately expressed using the pET-28a vector had the same activity as proteins co-expressed using the pRSFDuet-1 vector. As detailed in the Experimental Section, we used a coupled assay in which diphosphate released in isoprenoid biosynthesis is converted by pyrophosphatase (baker's yeast) to monophosphate, which in the presence of purine nucleotide phosphorylase (PNP) converts 7-methyl-6-thioguanosine to 7-methyl-6-thioguanine (E = 11,000 M⁻¹•cm⁻¹ at 360 nm), and the rate of increase in absorbance at 360 nm is taken to reflect the rate of HepPP synthesis.^[12]

The observation that the two individual HepPPS components were inactive individually, but were active when mixed, is basically as observed with MIHexPPS^[9] and other heterodimeric prenyl synthases.^[13] However, the activity of the SaHepPPS heterodimeric complex at high substrate concentrations deviated very considerably from the hyperbolic dependence of velocity on substrate concentration seen with most enzymes, decreasing with increases in either IPP or FPP substrate concentration. One possible explanation for this behavior is formation of a ternary substrate-enzyme-substrate (SES) complex in which a second molecule of substrate binds to the ES complex, forming an inactive, ternary SES complex.^[14] The rates of reaction observed with the active SaHepPPS heterodimer as a function of IPP and FPP substrate concentrations are shown in Figure 5. If this mechanism of substrate inhibition (i.e. negative feedback) is correct, then the effect of second substrate binding (and SES complex formation) on activity will be given by^[14] the following equation, where K_d represents the dissociation constant for the inhibitory SES ternary complex:

$$V = \frac{V_{max}}{1 + \frac{K_m}{[S]} + \frac{[S]}{K_d}}$$

As shown in Figure 5, we estimated the K_m and K_d values by splitting the velocity function domain at the maximum, because at low substrate concentrations HepPPS follows Michaelis-Menten kinetics (Figures 5a, b). The results suggest strong substrate inhibition, in particular with FPP. As can be seen in Figures 5c, d, the experimental results for both IPP as well as FPP inhibition (at high concentrations) can be fit by using the above equation using a K_d of $300 \pm 40 \mu\text{M}$ for IPP, and $35 \pm 5 \mu\text{M}$ for FPP (Figure 5c–e). We interpret these results to indicate that—at high concentrations—both FPP as well as IPP may bind to the regulatory protein, SaHepPPS-1, and indeed, Suzuki et al. have found, using atomic force microscopy, that FPP can bind to the regulatory domain in a *Bacillus subtilis* HepPPS.^[15] It is thus possible that at high levels, FPP disrupts the HepPPS-1/HepPPS-2 protein-protein interaction required for catalysis. There might also, in principle, be feedback inhibition by HepPP (or other, not fully-elongated) products. However, this latter possibility seems unlikely since the longer (C₂₀) chain species geranylgeranyl diphosphate (GGPP) did not exhibit the substrate-inhibition effect at high concentrations, having a lower V_{max} and a much larger K_m ($V_{max} = 2.2 \mu\text{M}/\text{min}$ and $K_m = 652 \mu\text{M}$) than did FPP (Figure S2). As controls, we also investigated whether high FPP and IPP might inhibit the coupling enzymes (phosphatase and purine nucleoside phosphorylase), but as can be seen in Figure S3, this is not the case. It thus appears that FPP may play a role in regulating menaquinone biosynthesis, in *S. aureus*.

SaHepPPS Inhibition by Other Compounds

We next investigated a series of compounds that in earlier work were found to inhibit a diverse range of prenyl synthases since one of the objectives of our work is to find new drug leads having new targets. The structures of the 55 compounds (**1–55**) tested are given in Table S2, with their dose-response curves shown in Figure S4 and their syntheses and characterization were previously reported.^[3a, 16] Of the several families of compounds tested

—benzoic acids, sulfonic acids, diketo acids, bisphosphonates, and dicationic species—only the lipophilic bisphosphonates were active. Representative dose-response curves for imidazolium and pyridinium bisphosphonates are shown in Figures 6 a, b, and Table S2 shows more extensive data for zoledronate (**15**; Figure 1) and 28 analogs (containing 1-H and 1-OH groups, and varying N-alkyl substituents). The effects of chain length on activity for both 1-H and 1-OH analogs are illustrated in Figures 6 c, d.

What is clear from the results shown in Figure 6 (and Table S2) is that the most potent inhibitors have ~C₆–C₈ side-chains, which results in an overall length of ~22 Å, about that of the FPP substrate (~24 Å). Surprisingly, zoledronate itself (and its short chain N-alkyl chain-containing analogs) had very little activity with IC₅₀ values of ~100 μM.

Zoledronate (**15**) has potent activity against human FPPS^[17] as well as a (bifunctional) *Plasmodium* geranylgeranyl diphosphate synthase (*P. vivax* GGPPS; PvGGPPS)^[18] and both of these proteins have similar folds. However, zoledronate has much less activity against human GGPPS due, it has been suggested, to the lack of the third Asp in the second aspartate rich motif (DDYAN) that coordinates to Mg²⁺ in the catalytic site.^[19] So, the pattern of inhibition activity we observe is far more similar to that found in human GGPPS than in human FPPS or PvGGPPS. We also find that the lipophilic bisphosphonates are competitive inhibitors of HepPPS, as shown in Figure 6e for **5**, an N-alkyl analog of zoledronate with a C₆ side-chain addition (and no 1-OH group). As shown in Figure 6f, the reaction rate is significantly decreased in the presence of 1 μM **5** and does not increase at high FPP concentrations, due to the FPP substrate-induced inhibition discussed above.

Effects of Bisphosphonates on *S. aureus* Cell Growth

We next tested zoledronate and 28 analogs against *S. aureus* cell growth. 14 out of 28 inhibitors showed modest activity with a similar chain-length dependence to that seen with SaHepPPS enzyme inhibition, the most active inhibitors (IC₅₀ ~ 16–23 μM; ~ 7–10 μg/mL) being the C₈–C₁₃ N-alkyl analogs (Table S3). Dose-response curves for the 14 bisphosphonates inhibiting *S. aureus* cell growth are shown in Figure S5 and their structures are shown in Table S3. As can be seen in Figure 7a, there is a correlation between the pIC₅₀ (= -log₁₀IC₅₀ [μM]) values for SaHepPPS and *S. aureus* cell growth inhibition (a Pearson r-value correlation-coefficient r = 0.91). This correlation suggests that HepPPS might be a target, but there could be other targets, for example enzymes such as farnesyl diphosphate synthase (which is known to be inhibited by bisphosphonates), undecaprenyl diphosphate synthase (involved in cell wall biosynthesis and inhibited by some bisphosphonates) as well as MenA, another enzyme involved in quinone biosynthesis.

To test whether FPPS or UPPS inhibition might be contributing to *S. aureus* cell growth inhibition, we investigated synergistic interactions with a series of known drugs that target, or do not target, bacterial cell wall biosynthesis since synergistic activity would be expected with FPPS or UPPS inhibitors.^[20] We carried out 12 fractional inhibitory concentration index (FICI) determinations using one of the most potent bisphosphonates **10** (zoledronate containing an N-C₁₃ alkyl substituent and a 1-H group; IC₅₀ = 7 μg/mL; Figure 7b) together with either a known antibacterial isoprenoid/cell wall biosynthesis inhibitor (fosmidomycin, carbenicillin, vancomycin, ampicillin, bacitracin, cefotaxime, fosfomycin) or an antibacterial

compound that does not target these pathways (kanamycin, tetracycline, chloramphenicol, spectinomycin, sulfamethoxazole, trimethoprim). We obtained FICI values for each combination using the FICI formula:[21]

$$FICI = FIC_A + FIC_B = \frac{MIC(AB)}{MIC(A)} + \frac{MIC(BA)}{MIC(B)}$$

where FIC_A and FIC_B are fractional inhibitory concentrations of drugs A and B, $MIC(A)$ and $MIC(B)$ are the MIC values of drugs A and B acting alone, and $MIC(AB)$ and $MIC(BA)$ are the MIC values of the most effective combination of drug A or B in the presence of drug B and A. FICI values of <0.5 are taken here to represent synergy, >0.5 and <1.0 represent additivity, >1 and <2 represent an indifferent effect, and >2 represents antagonism.[22] In addition, we evaluated isobolograms.[23] FICI values are shown in Table S4 and isobolograms are in Figures S6 and S7. We actually obtained data on two organisms: *B. subtilis*, chosen because it utilizes the non-mevalonate (MEP) pathway and is inhibited by fosmidomycin (which targets 1-deoxy-*D*-xylulose-5-phosphate reductoisomerase, DXR) as well as of course *S. aureus*, which uses the mevalonate pathway. The results shown in Table S4 show that in both organisms, there is—in almost all cases—an indifferent interaction with drugs in combination with **10** ($FICI_{avg} = 1.43 \pm 0.37$, $n = 24$). The only exception is with fosmidomycin where there is a synergistic interaction, in *B. subtilis* ($FICI = 0.29 \pm 0.094$) (Figure 7c–f). The observation of a synergistic interaction between fosmidomycin and **10** but a lack of any synergistic interaction with the other cell wall biosynthesis inhibitors suggests that FPPS and UPPS are not major targets for **10** since these cell wall biosynthesis inhibitors would be expected to act in synergy with an FPPS or UPPS inhibitor. However, HepPPS and MenA remain as possible candidates because fosmidomycin targets 1-deoxy-*D*-xylulose 5-phosphate reductoisomerase, reducing FPP levels and thence, HepPP biosynthesis.

We next tested whether *S. aureus* cell growth inhibition was “rescued” by addition of menaquinone-7 (MK-7). As can be seen in Figure 7b, we investigated the effects of 200 μ M MK-7 on *S. aureus* growth inhibition by the two most potent *S. aureus* cell growth inhibitors (**10** and **23**) finding that there was, on average, an ~ 8 x increase in the IC_{50} for cell growth inhibition, consistent with an HepPPS target. However, this is not indicative of HepPPS being the sole target since we also find that **23** inhibits MenA (from *E. coli*) with an IC_{50} of ~ 10 μ M (Figure S8), and MK-7 should show a similar rescue effect if SaMenA were being targeted. It thus appears that, based on the totality of the evidence: SaHepPPS inhibition, MenA inhibition, fosmidomycin synergy and MK-7 rescue experiments, that both HepPPS as well as MenA are being targeted by the lipophilic bisphosphonates, in cells.

Conclusions

The results we have described above are of interest for several reasons. We obtained the first structure of *Staphylococcus aureus* heptaprenyl diphosphate synthase, HepPPS, an anti-infective drug target. The protein crystallizes as a heterodimer containing a catalytic (HepPPS-2) as well as a regulatory (HepPPS-1) chain. In addition to the highly conserved

(DDXXD) residues in the catalytic protein we found 22 highly conserved residues that exist at the interface between the two subunits. HepPPS was only catalytically active when both subunits were present and we found substrate inhibition with FPP with a $K_d \sim 35 \mu\text{M}$. We also discovered several μM inhibitors, N-alkyl zoledronate analogs, whose activity was dependent on alkyl side-chain length with K_i as low as $\sim 200 \text{ nM}$. These compounds had modest activity against *S. aureus* cell growth (best $\text{IC}_{50} = 7 \mu\text{g/mL}$), which was partially rescued by menaquinone-7. These inhibitors acted synergistically with fosmidomycin but not with other, known cell-wall-targeting (or other) antibiotics, indicating that FPPS and UPPS are not being targeted. This is consistent with a HepPPS target although since we also find activity against a MenA (from *E. coli*), multi-site targeting is likely. Since SaHepPPS has high structural homology to only *M. luteus* hexaprenyl diphosphate synthase, not the more common trans-prenyl transferases such as human FPPS and GGPPS, our results open up the possibility of future in silico screening targeting both the catalytic and regulatory domains, to find selective SaHepPPS inhibitors as new drug leads.

Experimental Section

Clones and Plasmids

Staphylococcus aureus genomic DNA (ATCC, #700699) was used to amplify the gene sequences of the two components of HepPPS. PCR amplification of HepPPS-1 and HepPPS-2 was accomplished using gene-specific (IDT, Coralville, IA) primers designed from sequences identified in the *S. aureus* genome. HepPPS-1 forward 5'-GCCGCTAGCATGGAAACAACCTGTTAGCAAATTGGAAAGACAAATAGAAGAAAGA and HepPPS-1 reverse 5'-GCCCTCGAGTAATTACCTCTACTTTTTTAAATAACTTTTTTGGATATCGTGTAAGTAATGCTTTACTTCACT were used to amplify the HepPPS-1 sequence. HepPPS-2 forward 5'-GCCGCTAGCATGGCAAAGTTAAACATGAACAATGAAATTAAGAAAGTGGAACAAC and HepPPS-2 reverse 5'-GCCCTCGAGCTATGTGTTTCTTGACCCCATTTTTTCGTCAAACCTTAAAAGTAGT were used to amplify the HepPPS-2 sequence. Both HepPPS-1 and HepPPS-2 amplified sequences were incorporated into a pET28-a vector (Novagen, #69864-3). For co-expression of both components, HepPPS-1 forward 5'-GCCGGATCCCTGGTGCCGCGCGGCAGCATGGAAACAACCTGTTAGCAAATTGGAAAGACAAATAGAAGAAAGA, HepPPS-1 reverse 5'-GCCGCGGCCGCTAATTACCTCTACTTTTTTAAATAACTTTTTTGGATATCGTGTAAGTAATGCTTTACTTCACT, HepPPS-2 forward 5'-GCCAGATCTATGGCAAAGTTAAACATGAACAATGAAATTAAGAAAGTGGAACAAC, and HepPPS-2 reverse 5'-GGCCTCGAGCTGGGCGCGCGGCAGCTGTGTTTCTTGACCCCATTTTTTCGTCAAACCTTAAAAGTAGT primers were used. HepPPS-1 and HepPPS-2 genes were incorporated into multi-cloning sites 1 and 2 of a pRSFDuet-1 vector (Novagen, #71341-3), respectively, with thrombin cleavage site-containing linker sequences (DPLVPRGS) inserted between the N-terminal His6-tag and HepPPS-1, and between the C-terminal S-tag and HepPPS-2. The cloned products were transformed into *E. coli* BL21-CodonPlus (DE3)-RIPL cells, plated on LB containing 50 $\mu\text{g/mL}$ kanamycin and 34 $\mu\text{g/mL}$ chloramphenicol,

and cultured overnight at 37°C to select kanamycin/chloramphenicol resistant clones, which were then confirmed by DNA sequencing (ACGT Inc., Wheeling, IL).

Recombinant protein expression and purification

A single colony was used to inoculate 100 mL of LB broth containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol at 37°C, overnight. The overnight culture was transferred to six 1 L flasks of LB broth containing the same antibiotic concentrations. Flasks were shaken at 200 RPM at 37°C until the OD₆₀₀ reached ~0.5. Recombinant protein expression was induced with 1 mM IPTG and cells were incubated at room temperature, overnight. Cells were then harvested by centrifugation at 10,800 ×g for 10 minutes. Protein was obtained by lysing the cells by three freeze-thaw cycles, followed by sonication for 6 minutes. Cell lysates were centrifuged at 57,200×g for 30 minutes. Recombinant proteins were purified from the supernatant by Ni-NTA affinity chromatography using 10 mM HEPES, pH 7.5 buffer containing 500 mM NaCl, 35 mM imidazole, then eluted with increasing concentrations of imidazole in a gradient (35 mM to 500 mM) in the same buffer. The purified protein fractions were run on a 4–20% SDS polyacrylamide gel to ascertain size and purity. SaHepPPS-1 had an apparent MW of 22 kDa and SaHepPPS-2 had an apparent MW 36 kDa. For co-expression of the two components using the pRSFDuet-1 vector, basically the same protocol was used except that the kanamycin concentration was reduced to 30 µg/mL, and protein expression was induced with 0.3 mM IPTG. For purification of co-expressed proteins, 20 mM HEPES, pH 7.0 buffer containing 150 mM NaCl, 2 mM CHAPS, 10 mM β-mercaptoethanol, 35 mM imidazole, 6% glycerol was used, and protein was eluted with increasing concentrations of imidazole in a gradient (35 mM to 500 mM) in the same buffer. SaHepPPS-1 was cloned with a His6-tag and SaHepPPS-2 with an S-tag, however, Ni-NTA affinity chromatography alone was sufficient to purify both enzymes in the heterodimeric structure because of strong intermolecular binding affinity. For the crystallization experiments, the HepPPS-1 gene was inserted into an ampicillin resistant pET46 EK/LIC (Novagen) by using an EK/LIC cloning kit, creating a pET46-HepPPS-1 construct which expressed HepPPS-1 with an N-terminal His6-tag. The HepPPS-2 gene was inserted into a kanamycin resistant pET28a (Novagen) and the pET28a-HepPPS-2 construct expressed HepPPS-2 without any tag. Both plasmids were transformed into *E. coli* BL21(DE3) cells and target proteins were expressed by inducing with IPTG. Cells overexpressing HepPPS-1 and HepPPS-2 were mixed and harvested by centrifugation at 5,000×g for 20 minutes. The mixed cells were re-suspended in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 mM imidazole and 10% glycerol. Cells were disrupted by a French press and the debris removed by centrifugation at 17,000×g for 1 hour. The supernatant was then loaded onto a Ni-NTA column. HepPPS-1 and HepPPS-2 formed a complex and the target proteins eluted at ~150 mM imidazole. The purified protein fractions were run on a 4–12% SDS-PAGE to ascertain size and purity. The complex showed two bands: at 22 kDa for SaHepPPS-1 and at 36 kDa for SaHepPPS-2. Proteins were concentrated to 1 mL and applied to a size exclusion column (Sephacryl S-200 26/60, 320 mL, GE Healthcare) equilibrated with buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl) at a flow rate of 1 mL/min. Unknown proteins, HepPPS complex, and HepPPS-1 showed three peaks on the elution profile. The second peak contained the HepPPS complex. The purified

proteins were then concentrated to 5 mg/mL in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl buffer.

Crystallization, data collection, structure determination and refinement

SaHepPPS crystals were obtained using the No. 40 solution of the PEG/Ion 2 screen kit (Hampton Research) (0.03 M citric acid, 0.07 M bis-tris propane / pH 7.6, 20% w/v polyethylene glycol 3350) using the sitting-drop vapor method. In general, 1 μ L 5 mg/mL SaHepPPS was mixed with 1 μ L of reservoir solution in 48-well Cryschem plates, then equilibrated against 100 μ L of the reservoir at 25°C. Within 3 days, the crystals reached dimensions suitable for X-ray diffraction. X-ray diffraction datasets were collected at beam lines BL13B1, BL13C1, and BL15A1 of the National Synchrotron Radiation Research Center (NSRRC, Hsinchu, Taiwan) and were processed by using the HKL2000 program.^[24] Prior to structure refinement, 5 % randomly selected reflections were set aside for calculating R_{free} as a monitor of model quality.^[25] The SaHepPPS structure was solved by using molecular replacement (MR) using the crystal structure of hexaprenyl diphosphate synthase from *Micrococcus luteus* B-P 26, which shares 44% identity with SaHepPPS-2 (PDB:3AQB), as template. Model building was carried out by using Coot.^[26] Refinement employed PHENIX^[27] or REFMAC.^[28] The final MR model gave R and R_{free} values of 0.24 and 0.29 respectively at 2.68 Å resolution after refinement against the native SaHepPPS dataset using the CNS program.^[29] All graphics for the protein structures were prepared by using the PyMOL program (<http://pymol.sourceforge.net/>).

Enzyme kinetics and Inhibition

All putative inhibitors were prepared in 10 mM stock solutions in either DMSO or sodium carbonate buffer, and were then serially diluted in a gradient from 1 mM to 1 nM. Inhibitors were incubated with 25 μ g of co-expressed enzymes at room temperature for 10 minutes in assay buffer (10 mM HEPES, 150 mM NaCl, 1 mM MgCl₂, pH 7.5) before adding “reaction mixture” containing 20 μ M FPP, 80 μ M IPP, 3 U/mL purine nucleoside phosphorylase, 1 U/mL phosphatase, and ~ 600 μ M MESG, again in the assay buffer. Reactions were monitored for 15 minutes. The rate of increase in absorbance at 360 nm was taken as the rate of HepPP synthesis. The 50% inhibitory concentration (IC₅₀) values were calculated by using Prism 5 (GraphPad Software, Inc., La Jolla, CA).

MenA Inhibition Assay

The MenA inhibition assay was carried out as described previously. [30]

S. aureus and *B. subtilis* Growth Inhibition Assay

IC₅₀ values for *S. aureus* cell growth inhibition were determined by using a microdilution method. An overnight starter culture of *S. aureus* (Newman strain) in tryptic soy broth was diluted 1000-fold in fresh tryptic soy media to create a “working solution”. 200 μ L of working solution was transferred into each well of a 96-well culture plate (Corning 3370). Inhibitors were then added at 1 mM and sequentially diluted 3 $\times$ to 46 nM, keeping volume and culture broth composition constant. Plates were incubated for 12 hours at 37°C, shaking at 200 RPM, then absorbance at 600 nm was measured to assess bacterial cell growth. IC₅₀

values were determined using nonlinear regression whereas minimum inhibitory concentration (MIC) values in the synergy assays were calculated by using a Gompertz function in Prism 5 (GraphPad Software, Inc., La Jolla, CA). For the *B. subtilis* cell growth inhibition assay, an overnight starter culture (in LB broth) of *B. subtilis* (subsp. *subtilis* (Ehrenberg) Cohn ATCC 6051) was diluted 1000-fold (in fresh LB media) to create a “working solution”. Working solutions were then transferred into flat-bottom 96-well plates and inhibitors were added at 1 mM and sequentially diluted 3× to 46 nM. Plates were incubated at 37°C, shaking at 200 RPM overnight. The OD₆₀₀ was then measured to determine bacterial growth inhibition.

Synergy/Antagonism Assays

In order to investigate possible synergistic interactions between compound 10 and a range of antibiotics, we carried out two-drug combination assays. Bacteria were incubated with a 3× gradient of antibiotic typically ranging from 40 µg/mL to 18 ng/mL (200 µg/mL to 90 ng/mL for bacitracin, fosfomycin, and sulfamethoxazole) in the presence of half-MIC concentrations of **10**, in addition to a 3× gradient of **10** ranging from 40 µg/mL to 18 ng/mL in the presence of half-MIC concentrations of each antibiotic. New MIC values were calculated by using a Gompertz function in Prism 5 (GraphPad Software, Inc, La Jolla, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schematic illustration of the biosynthesis of several isoprenoids in *Staphylococcus aureus*.

Condensation of one DMAPP and two IPP to produce FPP is carried out by FPPS.

Condensation of 8 IPP with FPP yields a C₅₅-isoprenoid, undecaprenyl diphosphate, which is an essential intermediate in cell wall biosynthesis. Sequential condensation of FPP with 4 IPP yields heptaprenyl diphosphate, which then alkylates dihydroxynaphthoic acid in a reaction catalyzed by MenA to generate demethylmenaquinol (DMK-7), an intermediate in menaquinone biosynthesis.

Figure 2.

Sequence similarity networks for SaHepPPS-1 and SaHepPPS-2. a) Network based on regulatory domain (SaHepPPS-1) sequence constructed with $E=10^{-50}$. Major clusters are: 1, HepPPS from *Staphylococcus* spp.; 2, HepPPS from *Geobacillus* spp.; 3, HepPPS from *Paenibacillus* spp.; 4, HepPPS from *Bacillus* spp.; 5, ParB-like protein from *Burkholderia* and *Ralstonia* spp.; 6, HepPPS from *Anoxybacillus* spp.; 7, HepPPS from *Planomicrobium* spp.; 8, tyrosine recombinase from *Rhodopirellula* spp.; 9, peptidase from *Prevotella* spp.; 10, HexPPS from *Micrococcus* spp. b) Network based on the catalytic domain (SaHepPPS-2) sequence constructed with an $E=10^{-70}$. Major clusters are: 1, HepPPS and polyprenyl pyrophosphate synthase (PPPPS) from *Bacillus*, *Staphylococcus* and sulfate reducing bacteria; 2, HepPPS and PPPPS from *Lactococcus*, *Enterococcus*, *Streptococcus*, and *Lactobacillus* spp.; 3, PPPPS from *Selonomonas*, *Thermosinus* and *Paelosinus* spp.; 4, eukaryotic DPPS from *Drosophila* and *Polysphondylium* spp.; 5, solanesyl pyrophosphate synthase (SPPS) from eukaryotes and bacteria, plant species and Cyanobacteria; 6, octaprenyl pyrophosphate synthase (OPPS) from soil bacteria and *Pseudomonas* spp.; 7, PPPPS from flavobacteria, *Prevotella* and *Bacteriodes* spp.; 8, FPPS and GGPPS from methanogenic archaea; 9, GGPPS and PPPPS from *Streptomyces*, *Micrococcus* and *Mycobacterium* spp.; 10, GGPPS from sulfate reducing *Desulfitobacteria* spp.

Figure 3.

X-ray structures of heptaprenyl and hexaprenyl diphosphate synthases. a) Structure of the dimeric protein SaHepPPS (PDB:5H9D) where HepPPS-1 is shown in teal, and HepPPS-2 is shown in yellow. The FARM and SARM Asp-rich motifs in the active site are shown as red spheres and are in the HepPPS-2 catalytic protein. b) Superimposition of SaHepPPS (cyan) and MlHexPPS (green; PDB:3AQB) structures. c) View of the highly conserved residues (in pink) that are located at or near the interface between SaHepPPS-1 (teal) and SaHepPPS-2 (yellow); FARM/SARM motifs are shown in red. The conserved residues at or near the interface were deduced from the ClustalW alignments shown in Figure 4.

Figure 4.

ClustalW alignments of regulatory and catalytic proteins in heptaprenyl and hexaprenyl diphosphate synthase heterodimers. a) *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. pneumoniae*, *B. subtilis*, and *M. luteus* HepPPS-1 and HexPPS-1 sequences. Conserved residues in the interface of the two subunits are shown in orange. This is the regulatory protein. b) *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. pneumoniae*, *B. subtilis*, and *M. luteus* HepPPS-2 and HexPPS-2 sequences. Conserved residues at the interface of the two subunits are shown in orange. This is the protein that contains the Asp-rich domains involved in catalysis.

Figure 5. Rates of reaction of SaHepPPS as a function of substrate (IPP, FPP) concentration. a) Low [IPP]. b) Low [FPP]. c) High [IPP]. d) High [FPP]. e) Kinetic parameters for SaHepPPS. Data points are shown as mean $\pm$ SD, for duplicate experiments.

Figure 6. Dose-response curves and chain-length dependence of log(IC₅₀) for HepPPS by bisphosphonates. a) Dose-response curves for SaHepPPS inhibition by 1-H analogs of zoledronate. b) Dose-response curves for SaHepPPS inhibition by pyridinium bisphosphonates. c) Effects of chain length on SaHepPPS inhibition (log IC₅₀) for 1-H analogs of zoledronate. d) Effect of chain length on SaHepPPS inhibition (log IC₅₀) for zoledronate analogs. e) Lineweaver-Burk plot for SaHepPPS in the presence of 1 μM **5**. f) Enzyme kinetics of SaHepPPS in the presence of 1 μM **5**. Data points are shown as mean ±SD, for duplicate experiments.

Figure 7.

Identification of targets of bisphosphonates inhibiting *S. aureus* cell growth. a) Correlation between *S. aureus* cell growth inhibition and SaHepPPS inhibition based on pIC₅₀ (= $-\log_{10} \text{IC}_{50} [\mu\text{M}]$) results. b) Addition of 200 μM menaquinone-7 (MK-7) to the growth medium increases the IC₅₀ of **10** and **23** for cell growth inhibition by a factor of ~8. Data points are shown as mean±SD, for duplicate experiments. c) **10**+fosmidomycin in *B. subtilis* showing synergy (FICI=0.27) of **10** with a cell wall biosynthesis inhibitor (that targets DXR, 1-deoxy-*D*-xylulose-5-phosphate reductoisomerase, in the non-mevalonate pathway). d) **10**+sulfamethoxazole in *B. subtilis* showing an indifferent effect (FICI=1.73) of **10** with a nucleic acid biosynthesis inhibitor (that targets dihydropteroate synthase). e) **10**+ampicillin in *S. aureus* showing an indifferent effect (FICI=1.40) of **10** and a cell wall biosynthesis inhibitor (that targets transpeptidase). f) **10**+tetracycline in *S. aureus* showing an indifferent effect (FICI=1.85) of **10** with a protein synthesis inhibitor (that targets ribosome function).