BNL-111806-2016-JA

***β Hydroxyacyl Acyl Carrier Protein
Dehydratase (FabZ) from Francisella
tularensis and Yersinia pestis: Structure
Determination, Enzymatic Characterization,
and Cross Inhibition Studies***

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Submitted to Biochemistry

January 2016

Biology Department

Brookhaven National Laboratory

**U.S. Department of Energy
DOE Office of Basic Energy Sciences**

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Journal:	Biochemistry
Manuscript ID	bi-2015-00832x.R2
Manuscript Type:	Article
Date Submitted by the Author:	26-Jan-2016
Complete List of Authors:	McGillick, Brian; Brookhaven National Laboratory, Biological, Environmental and Climate Sciences Department Kumaran, Desigan; Brookhaven National Laboratory, Biological, Environmental and Climate Sciences Department Vieni, Casey; Brookhaven National Laboratory, Biological, Environmental and Climate Sciences Department Swaminathan, Subramanyam; Brookhaven National Laboratory, Biological, Environmental and Climate Sciences Department

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**β -Hydroxyacyl-Acyl Carrier Protein Dehydratase (FabZ) from
Francisella tularensis and *Yersinia pestis*: Structure Determination,
Enzymatic Characterization, and Cross Inhibition Studies**

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Funding Source.

SS was partially supported from an unrestricted grant from Allergan, USA. M, B.E.

partially supported from training grant # NIH-NIGMS GM08444-23. CV was supported

in part by the U.S. Department of Energy, Office of Science, Office of Workforce

Development for Teachers and Scientists (WDTS) under the Science Undergraduate

Laboratory Internships Program (SULI).

Abbreviations Used

DMSO, dimethyl sulfoxide

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Abstract

The bacterial system for fatty acid biosynthesis (FAS) contains several enzymes whose sequence and structure are highly conserved across a vast array of pathogens. This, coupled with their low homology and difference in organization compared to the equivalent system in humans, makes the FAS pathway an excellent target for antimicrobial drug development. To this end, we have cloned, expressed, and purified the β -hydroxyacyl-Acyl Carrier Protein Dehydratase (FabZ) from both *F. tularensis* (FtFabZ) and *Y. pestis* (YpFabZ). We also solved the crystal structures and performed an enzymatic characterization of both enzymes and several mutant forms of YpFabZ. Additionally, we have discovered two novel inhibitors of FabZ, Mangostin and Stictic Acid, which show similar potencies against both YpFabZ and FtFabZ. Lastly, we selected several compounds from the literature that have been shown to be active against single homologs of FabZ and tested them against both YpFabZ and FtFabZ. These results have revealed clues as to which scaffolds are likely to lead to broad-spectrum antimicrobials targeted against FabZ, as well as modifications to existing FabZ inhibitors that may improve potency.

F. tularensis and *Y. pestis* are both pathogenic Gram-negative bacteria, classified as Tier 1 select agents by the U.S. government. This designation has been assigned due to their low infectious dose, high virulence, and relative ease by which these pathogens can be aerosolized. *F. tularensis* causes the disease Tularemia, which can have various clinical manifestations depending on the route of exposure to include ulceroglandular, oropharyngeal, respiratory, oculoglandular, and typhoidal. Of these potential manifestations, respiratory Tuleremia is the most severe, marked by chills, high fever, difficulty breathing, dry or productive cough, pharyngitis, chest pain, headache, drenching sweats, drowsiness and weakness. The possible development of pneumonia is extremely deadly with an average mortality rate of 30-60%, if left untreated.¹

Y. pestis is most commonly known as the causative agent of Bubonic Plague, but when encountered in aerosolized form it can cause a deadly pneumonic plague. This variant, in contrast to Bubonic plague, can be spread from person to person making the disease much more difficult to control.² Of further concern is that multidrug resistant forms of *Y. pestis* have been isolated in the last few decades while simultaneously the number of human cases of plague has been rising.³ This highlights the general need for novel antimicrobials to combat the growing concern not only of *Y. pestis*, but also of multidrug resistant bacteria in general.

One under exploited bacterial process for drug therapy against these pathogens is the bacterial system for fatty acid biosynthesis. In bacteria, fatty acid synthesis is carried out by series of individual enzymes, each encoded by a separate gene and collectively known as Type II FAS. This contrasts with the Type I FAS system present in humans, which is conducted by a single multifunctional polypeptide containing all of the initiation

and chain elongation enzymes of fatty acid biosynthesis. The major differences between these two FAS systems, both in structure and organization, suggests that bacterial fatty acid biosynthesis can be successfully targeted without interfering with the host process. Furthermore, many of the specific enzymes of the Type II FAS system contain significant sequence and structural homology across a wide range of bacterial pathogens making this pathway an excellent target for developing broad-spectrum antibiotics.

In bacteria, the fatty acid synthesis pathway can be understood through a description of the process in *E. coli*. Generally, the process consists of initiation followed by four sequential steps of chain elongation, each carried out by an individual enzyme, as summarized in Figure 1. In the third step of the elongation cycle, β -hydroxyacyl-ACP is dehydrated by one of two β -hydroxyacyl-ACP dehydratases (FabA or FabZ) to form an α,β -unsaturated acyl-ACP. The primary role of FabA is in unsaturated fatty acid synthesis and is only found in Gram-negative bacteria, while FabZ is the primary dehydratase in the formation of both saturated and unsaturated fatty acids. This, coupled with the ubiquitous distribution of FabZ across both Gram-positive and Gram-negative pathogenic bacteria, makes it especially suited for antimicrobial drug discovery efforts.

Several FabZ structures from a variety of different organisms have been solved in recent years and include *Helicobacter pylori* (HpFabZ),⁴ *Pseudomonas aeruginosa* (PaFabZ),⁵ *Campylobacter jejuni* (CjFabZ),⁶ *Plasmodium falciparum* (PfFabZ),⁷ *Burkholderia thailandensis* (BtFabZ),⁸ and *Neisseria meningitidis* (NmFabZ) (PDB ID: 4I83). Additionally, several co-crystal structures of inhibitors bound to either HpFabZ or PfFabZ have also been reported.^{4, 7, 9-13} Most inhibitors reported thus far for HpFabZ adopt either of two general binding poses referred to as “gate-blocking” or “channel

blocking". Channel blocking inhibitors have been found to bind at or near the FabZ enzymatic active site, making specific interactions near the strictly conserved catalytic histidine and glutamic acid residues found at this location. Alternatively, gate-blocking inhibitors bind far from the active site making interactions with conserved residues surrounding the entrance to the tunnel leading to the FabZ active site.⁴ Inhibitors for PfFabZ generally adopt only a single pose close to the active site and make contacts with the catalytic residues.¹²

In this work, we report the crystal structures of FabZ from *F. tularensis* (FtFabZ) and *Y. pestis* (YpFabZ) as well as their enzymatic properties. In addition, we have identified two novel inhibitors of FabZ: the natural compounds Stictic Acid and Mangostin. Lastly, we tested our newly discovered inhibitors, as well as several inhibitors identified for HpFabZ, against both FtFabZ and YpFabZ. As will be described, these two FabZ homologs together represent much of the natural variation that occurs between all FabZ molecules, particularly in key areas within the active site tunnel that have been shown to interact with inhibitors. Therefore, an understanding of the relative strength of known inhibitors against these two homologs should be informative for the future design of broad-spectrum inhibitors of FabZ.

Experimental Section

Cloning, Expression and Purification. Plasmids for both FtFabZ and YpFabZ were obtained from the DNASU Plasmid repository (DNASU Plasmid ID's: FtCD00023067 and YpCD00016324). The FabZ gene for each of these constructs was sub-cloned into a pET28b vector, yielding the plasmids pET28b-FtFabZ and pET28b-YpFabZ, both of which contain an N-terminal 6-His tag. Several YpFabZ point mutations were also

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constructed using pET28b-YpFabZ as a template. These mutations were inserted within 5' phosphorylated primers that were used to prime extensions around the entire plasmid. After amplification the plasmid was reconstituted using T4 ligase. The primers used were as follows: H19A (Forward: 5'-GCACGTTTTCCGTTCTTGCTG-3' Reverse: 5'-CGGGAGTAGATCCAAAATCTC-3'), F79A (Forward: 5'-GCAAAGAGCCGCGGTAAACTG -3' Reverse: 5'-CGCCAAAATCCCTGTAGCCTG-3'), and Y91A (Forward: 5'- GCATACTTTGCTGGTATTGAT -3' Reverse: 5'-AAGCTCGCCCGGTTCCAGTTT -3'). Proper insertion of mutations was confirmed by sequencing.

All FabZ constructs were transformed into *E. coli* BL21(DE3) and inoculated into ZYP-5052 medium containing 100 µg/mL of Kanamycin and grown at 37°C until A₆₀₀ reached approximately 0.6. These cells were then allowed to autoinduce overnight at 20°C.¹⁴ The cells were harvested at 5000g for 20 minutes and resuspended in buffer A (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 5 mM imidazole), supplemented with 1X Bugbuster (Novagen) reagent. After 20 min of stirring at room temperature, benzonase was added and the solution was stirred for an additional 10 minutes. The mixture was centrifuged at 17,000g for 20 min at 4°C, yielding a clear supernatant, which was applied to a column with Ni-NTA resin (Qiagen) pre-equilibrated in buffer A. The column was washed several times with buffer B (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 20 mM imidazole) and eluted with buffer C (20 mM, Tris-HCl, pH 8.0, 500 mM NaCl, and 200 mM imidazole). Fractions containing FabZ were then concentrated and loaded onto a size exclusion (S-200) column previously equilibrated with buffer D (2mM dithiothreitol, 200mM NaCl, and 20mM HEPES buffer at pH 7.4).

Crystallization and Structure Determination. FtFabZ crystals were grown at room temperature using the sitting drop vapor diffusion method in a 1:1 solution of protein (6 mg/ml in buffer D) and reservoir solution consisting of 0.2M ammonium sulfate, 0.1M BIS-TRIS pH5.5, 25% PEG 3350. Rectangular shaped crystals appeared after approximately 48 hours and were flash cooled at liquid nitrogen temperature by dipping crystals in the mother liquor containing 20% ethylene glycol or 20% glycerol (final volume) as a cryoprotectant. Hexagonal shaped YpFabZ crystals were grown in a similar fashion using a reservoir solution of 8% tacsimate pH 8.0, 400mM sodium thiocyanate and 20% PEG 3350. X-ray diffraction data were collected at the x29 beamline at the National Synchrotron Light Source with an ADSC Quantum 315 detector. FtFabZ and YpFabZ crystallized in space groups C2 and P6₃, respectively. FtFabZ diffracted to at least 2.5Å, while for YpFabZ this value was 1.9 Å for the glycerol bound structure and 1.8Å for the ethylene glycol bound structure. All data were processed using the HKL2000 suite.¹⁵ Both structures were determined by molecular replacement via the CCP4 suite module, MOLREP.¹⁶ One monomer from the structure of PfFabZ (1ZHG) was used as a search model and the appropriate sequence changes were made after the molecular replacement solution was obtained. Following the initial structure determination, several iterative rounds of refinement via the CCP4 module,¹⁷ REFMAC,¹⁸ and model building with COOT,¹⁹ were performed. During the last stages of refinement, water molecules were selected based on peaks above 1σ in the 2F_{obs}-F_{calc} electron density maps. Further significant residual density, not corresponding to water molecules, was determined to be glycerol and ethylene glycol, which had been used as cryoprotectants for various crystals. Density corresponding to the planar structure of the

additive sodium thiocyanate was also resolved in the final structure. The R and R-free converged to 0.16 and 0.18 for glycerol bound YpFabZ, 0.17 and 0.20 for ethylene glycol bound YpFabZ, and 0.21 and 0.28 for FtFabZ. The final refinement statistics are given in Table 1. Representative sections of Sigmaa weighted electron density maps are given in Supplementary Material 1 and 2.

Enzymatic Characterization. The enzymatic activities of FtFabZ and YpFabZ were determined via the reported spectrophotometric method using the substrate analog crotonoyl-CoA.⁷ Initial reaction velocities were determined by monitoring the decreasing absorbance at 280 nm, resulting from the conversion of crotonoyl-CoA to β -hydroxybutyryl-CoA. The conversion of hydroxybutyryl-CoA to crotonoyl-CoA was not used in our assay. An extinction coefficient of $3600 \text{ M}^{-1} \text{ cm}^{-1}$, corresponding to the hydration of the enoyl double bond, was used to convert absorbance readings to substrate concentrations.²⁰ Reactions were performed using a Beckman-Coulter DU730 UV/Vis Spectrophotometer. Reaction volumes of 100 μL were prepared in a buffer solution consisting of 2.0 μg FtFabZ or 0.5 μg YpFabZ, 20mM TRIS pH 7.0. All reactions were performed at 25⁰C. K_m values for both FtFabZ and YpFabZ were determined by varying the substrate concentration. Inhibition reactions for IC_{50} calculations were performed under similar conditions with a fixed concentration of 100 μM crotonoyl-CoA. All tested inhibitors were dissolved in 100 % dimethyl sulfoxide (DMSO) and added to the reaction mixture to a final concentration of $\leq 1\%$ DMSO. Initial inhibitor screens were performed in identical reaction mixtures to those described above, however the assays were conducted using a Tecan Safire² Microplate Reader. Compounds for inhibitor screening were obtained from the National Cancer Institute's open repository.

Docking Studies. All docking calculations were performed with AutoDock Vina²¹ using AutoDock Tools²² for the preparation of ligand and receptor PDBQT files. Grid maps for the docking calculations were made using 60 grid points with 0.375 Å spacing in the x, y, and z direction centered at the active site.

Results and Discussion

Structure of YpFabZ. Consistent with other reported FabZ structures, YpFabZ forms a hexamer with a trimer of dimers configuration (Fig. 2A). The FabZ monomer adopts a $\beta + \alpha$ hot-dog fold, where a 6-stranded β -sheet with the topology 1-2-4-5-6-3 wraps around a long central α -helix ($\alpha 3$), with the $\beta 3$ strand containing a brief interrupted segment. A short α -helical region is formed near the N-terminus ($\alpha 1$) as well as an additional short α -helix ($\alpha 2$) between $\beta 2$ and $\alpha 3$. Short disordered regions separate individual β -strands, however the α -helices have longer loop regions in the intervening regions between themselves and adjacent secondary structure elements. These loops are defined as follows: Loop A resides between $\alpha 1$ and $\beta 1$, loop B lies between $\alpha 2$ and $\alpha 3$ and loop C is formed between $\alpha 3$ and $\beta 3$ (Fig. 2B). These loops are present on the inside of the “hot-dog” making further contacts that stabilize either the dimer or the higher order hexamer.

The dimer itself is formed by the association of each monomer along the groove of the $\beta 3$ strand creating a 12-stranded extended β sheet. The $\alpha 3$ helices of each monomer are oriented anti-parallel to one another making further contacts along the N-terminal two turns of each helix. The substrate-binding tunnel is formed in the dimeric interface and hence each dimer harbors two active sites located approximately 13 Å apart (Fig. 2C). The entrance to each active site tunnel is marked by Pro104 on one side of the entrance, opposed by Tyr92 on the opposite side (Fig. 3A). When structurally aligned, Tyr92

occupies the same position as Tyr100 in HpFabZ and Leu170 in PfFabZ, both of which are posited to act as entrance gatekeeper residues. Similar to the gatekeeper residues of HpFabZ and PfFabZ, Tyr92 is highly flexible and capable of completely blocking the tunnel entrance by adopting a “closed” conformation or alternatively can allow access to the tunnel by adopting an “open” conformation (Fig. 3B). Inside the tunnel, approximately in the region of the tunnel kink, the catalytic site formed by the conserved His54 and Glu68 residues is found. These residues are contributed by opposing monomers with His54* (*indicates the opposing monomer) coming off of loop B of one monomer and Glu68 coming off the N-terminus of $\alpha 3$ on the other monomer. Deeper into the tunnel, past the catalytic site, the tunnel narrows and largely follows the path of the $\alpha 3$ helix, passing Tyr91 and ending at Phe79. With the exception of the catalytic residues, the tunnel is almost entirely hydrophobic.

Analysis of the structures of PfFabZ and HpFabZ has led to two separate residues being proposed as potential exit gate residues, with both being found to adopt either an “open” or “closed” conformation, analogous to the tunnel entrance gate residue. For PfFabZ this residue is Phe169, which corresponds to Tyr91 in YpFabZ and in HpFabZ it is Phe83, which corresponds to Phe79 in YpFabZ. However, in the structure of YpFabZ, both Phe79 and Tyr91 were found only in the “closed” conformation (Fig. 3B).

In addition to the above structural features, a glycerol molecule, added as a cryo-protectant, was found bound deep in the active site tunnel making interactions with the catalytic dyad residues, His54* and Glu68. One of the hydroxyl groups of the glycerol molecule makes a bifurcated hydrogen bond with each of the catalytic dyad residues. Another hydroxyl group forms a hydrogen bond with the conserved His19 (Fig. 3C).

Similarly, in a separate structure, ethylene glycol was found making comparable contacts (Fig. 3D). Additionally, in the ethylene glycol bound structure, a thiocyanate molecule occupies the space between the tunnel entrance and ethylene glycol.

Structure of FtFabZ. The overall architecture of FtFabZ is nearly identical to that of YpFabZ, except for an extended kinked portion of the FtFabZ $\alpha 3$ helix (Fig. 4A,C). The active sites of YpFabZ and FtFabZ are also very similar with both catalytic residues, as well as the second conserved histidine (His19 in YpFabZ and His22 in FtFabZ), having nearly identical positions in both structures (Fig. 4B). Also, Phe104 has a conserved position with respect to Tyr91 of YpFabZ. This residue corresponds to one of the two residues identified as potentially acting as an exit gate. This position is well conserved across all FabZ structures reported to date, normally occupied by a tyrosine or phenylalanine, with the exception of HpFabZ and CjFabZ where it is a valine. One difference between YpFabZ and FtFabZ is at the entrance gate residue, where FtFabZ has a non-aromatic amino acid (Met105), in contrast to the tyrosine or phenylalanine that occupies this location in most FabZ isoforms. Met105 did not demonstrate the same flexibility as seen for the YpFabZ entrance gate residue and was found to occupy only a single “closed” conformation (Fig. 4B). The region surrounding the tunnel exit of FtFabZ adopts a conformation that is unique to the FabZ structures reported to date with the $\alpha 3$ helix containing an additional turn that kinks prior to the start of loop C (Fig. 4A,C). This feature is distinct, but somewhat reminiscent of the extra α -helix formed out of loop C in the HpFabZ structure.⁴ This additional helical turn appears to derive from an approximately seven amino acid insertion in this region as compared to YpFabZ and HpFabZ. Based on sequence alignment,²³ Phe90 would be expected to occupy the exit

gate region of FtFabZ, however in the structure this residue is far removed with Glu83 taking its place (Fig. 4C). This feature is unusual in that the exit gate residue is hydrophobic in all other reported FabZ structures, with the predominant amino acid being a phenylalanine, the exceptions being PfFabZ and NmFabZ, which have a leucine and isoleucine, respectively.

Analysis of previous FabZ structures has identified three key residues that may act as gate residues. One of these residues, located at the entrance to the active site tunnel, is thought to control access to the catalytic residues. On the opposite end of the tunnel, two separate residues have been suggested as potential exit gate residues with one or both possibly functioning to control the length of the tunnel to accommodate various length carbon chains. Individually these three gate residues are most commonly either a tyrosine or phenylalanine, but often at least one will be a non-aromatic amino acid within a single FabZ isoform. The evidence to date that any of these three residue positions truly act as gatekeepers is largely based on structural information alone. A mutational study of the entrance gate of HpFabZ (Y100A) did show an approximate 50% reduction in activity over wild-type, however the mutant displayed an approximate 2.5 fold increase in K_m , which could indicate that the effects seen were not based solely on the side chain alone.⁴ It is also difficult to discern what effect, if any, the entrance gate residue would have on natural ACP bound substrates. In the case of the exit gate residues, our own mutational studies of YpFabZ (Y91A and F79A) did not show any change in either K_m or k_{cat} (data not shown), however the kinetic analysis was performed with crotonoyl-CoA as a substrate, which has a relatively short carbon chain that likely does not encounter these exit gate residues. Therefore, a proper understanding of the contribution of these residues

to enzymatic activity requires a longer chain substrate that reaches deeper into the tunnel, thus contacting these residues.

Biochemical Properties of FtFabZ and YpFabZ. The activity of both FtFabZ and YpFabZ was assessed with a commonly used spectrophotometric assay that utilizes the substrate analog crotonoyl-CoA.⁷ The conversion of crotonoyl-CoA to β -hydroxybutyryl-CoA was measured by monitoring the decrease in absorbance at 280 nm as described in the experimental section. This reaction is analogous to the reverse reaction in vivo. The K_m for this reaction was determined to be 78.2 μ M for YpFabZ and 61.8 μ M for FtFabZ (Fig. 5 and Table 2). The k_{cat} and k_{cat}/K_m for YpFabZ is $1.45 \pm 0.060 \text{ s}^{-1}$ and $14322 \text{ M}^{-1} \text{ s}^{-1}$ and for FtFabZ these values are $0.72 \pm 0.039 \text{ s}^{-1}$ and $11650 \text{ M}^{-1} \text{ s}^{-1}$. Several FabZ homologs have been previously characterized using crotonoyl-CoA as a substrate with reported K_m values of 69.7 μ M (CjFabZ)⁶, 82.6 μ M (HpFabZ)²⁴, and 86.0 μ M (PfFabZ)⁷. The K_m values obtained for YpFabZ and FtFabZ are of similar magnitude, however the reported k_{cat} value for the other homologs fall between 0.013 s^{-1} and 0.0515 s^{-1} , which is significantly below that of FtFabZ and YpFabZ. The reason for this discrepancy is not immediately apparent. However, we found the high salt concentration of 500 mM used in previous experiments to be strongly inhibitory to both YpFabZ and FtFabZ (Fig. 6).

YpFabZ (H19A) Mutant Study. Our crystal structures of YpFabZ showed both a glycerol and ethylene glycol molecule making hydrogen bonds with both catalytic residues as well as a second histidine in the active site. The proximity of this histidine to the catalytic residues as well as its complete conservation in all FabZ isoforms suggests it may have some role in enzymatic activity. To test this, we made a FabZ H19A mutation. The H19A mutation had no effect on K_m with a value of $73.5 \pm 7.6 \mu$ M as opposed to a

K_m of $78.1 \pm 14.0 \mu\text{M}$ for wild-type YpFabZ. However, the k_{cat} of YpFabZ (H19A) was calculated to be $0.48 \pm 0.023 \text{ s}^{-1}$, which is approximately one third the value for YpFabZ. The agreement between K_m values suggests that the H19A mutation did not affect the protein fold and this coupled with a modest reduction in k_{cat} suggests only a minor catalytic role, if any for this second conserved histidine.

Inhibitor Screening and Evaluation. The NCI compound database natural product set III, containing 117 compounds, was screened using the spectrophotometric enzyme assay. This set was chosen since it contains numerous scaffolds similar to known FabZ inhibitors and most of the compounds in this set are of appropriate size for the FabZ active site tunnel. Initial screens were conducted at a concentration of $100 \mu\text{M}$ of each tested compound against YpFabZ. After screening, the two compounds Stictic Acid and Mangostin were identified as potential inhibitors of YpFabZ and were subjected to further analysis. The IC_{50} value of Mangostin against YpFabZ was determined to be $6.1 \pm 1.4 \mu\text{M}$. For Stictic Acid, the IC_{50} was $13.0 \pm 1.4 \mu\text{M}$ (Table 3). Inhibition mode characterization was conducted for each inhibitor against YpFabZ and the results indicate that both Mangostin and Stictic Acid function as competitive inhibitors with K_i values of $1.4 \pm 0.13 \mu\text{M}$ and $22.6 \pm 2.0 \mu\text{M}$, respectively (Fig. 7A,B).

Mangostin Docking Study. Docking analysis suggests that Mangostin adopts a binding pose spanning the tunnel entrance, making contacts with the catalytic residues and ending in the region just beyond the active site (Fig. 8A). In the dock pose, Mangostin's 2-methyl-2-butene groups make extensive hydrophobic interactions with YpFabZ. One of these groups is found sandwiched between Tyr92 and Pro104*, analogous to the position occupied in the "gate blocking" pose of many of the crystal structures of HpFabZ

inhibitors. The other 2-methyl-2-butene makes further interactions with the hydrophobic portion of the tunnel found beyond the catalytic residues towards the tunnel exit, making contacts with Tyr91, Ala71, and His19. The xanthone scaffold of Mangostin occupies the region between the tunnel entrance and the catalytic residues, stabilized by hydrogen bonds formed between the 1'-hydroxyl group with the backbone oxygen of Tyr91, as well as the 3'-hydroxyl group with the carboxyl group of Glu68. The 3'-hydroxyl group makes additional polar interactions with His54*. The predicted binding affinity of Mangostin is $-10.4 \text{ kcal mol}^{-1}$.

Stictic Acid Docking Study. The predicted binding location of Stictic Acid is similar to that of Mangostin, found bound in the region spanning the tunnel entrance and making contacts near the catalytic residues (Fig. 8B). The 5'-methyl group of Stictic Acid is found between Tyr92 and Pro104*, analogous to contacts seen in the Mangostin docking pose. Deeper in the tunnel, near the catalytic residues, the benzodioxepin moiety of Stictic Acid is buried in the hydrophobic pocket formed primarily by Ala71, Leu90, Phe93, Tyr91, Phe55*, His54*, Ile 60*, and Pro62*. Additional hydrophobic interactions are seen between the 8'-methyl group and Leu90. Several polar interactions between Stictic Acid constituent groups and YpFabZ are also present, as follows: (1) the 1'-hydroxyl group with the backbone oxygen of Phe61*, (2) the 4'-hydroxyl group with the backbone nitrogen of Pro104*, and (3) the 11'-aldehyde group with the backbone nitrogen of Gly63*. The predicted binding affinity of Stictic Acid is $-8.8 \text{ kcal mol}^{-1}$.

Inhibition Studies of Known FabZ Inhibitors. Inhibitor bound crystal structures exist for both HpFabZ and PfFabZ. A common finding among a majority of the inhibitors identified against HpFabZ is that they often have a binding pose at the entrance to the

active site tunnel whereby an aromatic portion of the inhibitor is located between the phenol ring of the Tyr100 gate residue and the pyrrolidine ring of Pro112*, forming a sandwich structure.^{4, 9-11, 13} Additionally, these compounds often have a second pose that is deeper into the tunnel and bound in the vicinity of the catalytic and/or exit gate residues. Unlike HpFabZ, the entrance gate residue of PfFabZ is non-aromatic leucine and inhibitors show only a single pose residing deep in the tunnel near the catalytic residues.¹² In total, there are approximately 17 residue positions that make side chain interactions with one or more of the known inhibitors of either HpFabZ or PfFabZ. A sequence alignment of the eight structurally characterized FabZ isoforms demonstrates that these 17 positions are all highly conserved with seven positions being strictly conserved (Supplementary Material 3). Of the remaining positions that are not strictly conserved, all are substitutions of chemically similar amino acids with a few exceptions. One exception, as has been described above, is at the exit gate residue of FtFabZ where there is a charged residue (Glu83), while the other 7 isoforms have a hydrophobic exit gate residue, which is typically a phenylalanine. Another exception is at the position corresponding to Leu90 on YpFabZ. This position is a hydrophobic residue in all isoforms except FtFabZ where it is substituted to a threonine. Lastly, the conserved proline at the tunnel entrance, corresponding to Pro104 on YpFabZ, is substituted to a valine in BtFabZ, and a glutamine for NmFabZ.

With the exception of the conserved proline marking the tunnel entrance, YpFabZ and FtFabZ together represent much of the natural variation that exists between all of the FabZ isoforms. For this reason, we tested our newly identified inhibitors, as well as several commercially available inhibitors of HpFabZ, against both YpFabZ and FtFabZ

to determine whether or not these inhibitors are generally cross reactive or specific to a single isoform (Table 3).

When tested, the compounds Apigenin and Quercetin, showed no inhibition against YpFabZ or FtFabZ up to a concentration of 200 μ M (Table 3). The reasons for the inability of Quercetin and Apigenin to inhibit either YpFabZ or FtFabZ are not obvious. However, a study of the structure-activity relationship between a series of flavonoids against PfFabZ demonstrated that even slight alterations of the hydroxylation pattern of these compounds had major effects on their potency.²⁵ In fact, this study revealed that Quercetin, but not Apigenin, is an inhibitor of PfFabZ. Furthermore, examination of the co-crystal structures of HpFabZ, with both Quercetin and Apigenin reveals a pronounced shift in the β 3 and β 6 strands, as compared to unliganded enzyme, resulting in the tunnel entrance opening up by approximately 3 Å. A similar magnitude shift is seen in other HpFabZ inhibitor bound structures, but in the case of PfFabZ the shift appears less pronounced or even absent. This suggests that inhibitor binding to FabZ may be augmented by an induced fit mechanism. Therefore, the relative flexibility of these regions between isoforms could influence inhibitor binding. This is also supported by a published FabA-ACP complex structure with a crosslinked substrate (Supplementary material 4).²⁶

The remaining compounds we tested inhibited both FabZ isoforms. A direct comparison of each compound against YpFabZ identified Juglone and Mangostin as the most potent inhibitors with IC_{50} values of $5.3 \pm 1.0 \mu$ M and $6.1 \pm 1.4 \mu$ M, followed by Stictic Acid ($13.0 \pm 1.4 \mu$ M) and Emodin ($29.7 \pm 6.0 \mu$ M). When these compounds were tested against FtFabZ, they generally showed little to no difference in potency as

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3 compared to YpFabZ (Table 3). At a minimum, this suggests that these compounds are
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5 not dependent on an aromatic amino acid as the entrance gate residue since FtFabZ
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7 contains a methionine at this position rather than a tyrosine as in YpFabZ and HpFabZ.
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9 Supporting this finding is a previous mutational study of the HpFabZ entrance gate
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11 residue (Y100L), which showed no impact on the potency of the inhibitors Quercetin,
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13 Apigenin, or (S)-Sakuranetin.¹³ More importantly, the entire range of residue
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15 substitutions and structural variations between YpFabZ and FtFabZ appear to be
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17 unimportant for the activity of these compounds. Taken together, this data demonstrates
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19 that the scaffolds of Mangostin, Stictic Acid, Emodin, and Juglone are all excellent
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21 starting points for further inhibitor design against all FabZ variants, while the flavonoids
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23 may be more isoform specific.
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29 Our inhibition studies also allowed for a side-by-side comparison of Mangostin
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31 and Emodin, which share similar scaffolds. The results show Mangostin to have an
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33 approximate 5-6 fold lower IC₅₀ than Emodin. Inspection of the dock pose for Mangostin
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35 with YpFabZ suggests that this effect could be due to Mangostin's ability to exploit
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37 hydrophobic regions of YpFabZ on both the entrance and exit sides of the active site
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39 tunnel through its dual 2-methyl-2-butene groups. Additionally, Mangostin makes polar
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41 contacts with both catalytic residues. This information provides insights into potential
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43 functional groups that could be added to existing inhibitor scaffolds for the design of
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45 more potent compounds.
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53 Conclusion

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In summary, we have reported the crystal structure of two new variants of FabZ (YpFabZ and FtFabZ) as well as their enzymatic characterization, including evidence of a minor catalytic role, if any for the second histidine located in the active site. Additionally, we have discovered two new inhibitors of FabZ, Mangostin and Stictic Acid. Comparison of the dock pose of Mangostin with the crystallographic pose of Emodin has provided insight into rational modifications of existing inhibitors that may improve their potency. Lastly, we have compared the strength of multiple inhibitors against two isoforms of FabZ demonstrating which scaffolds tend to be cross-reactive. In total, we expect the results of this study to help direct the synthesis of more potent FabZ inhibitors in the future.

Acknowledgements

The authors gratefully acknowledge data collection support from beamline X29 (NSLS).

Supporting Information

Supporting information is provided in Supplementary Materials included as a separate file. The electron density maps are provided as a validation of the crystal structure. A sequence alignment is included as Supplementary Material 3. Finally, Supplementary Material 4 is given to support our hypothesis that inhibitor binding to FabZ may be augmented by an induced fit mechanism.

Associated Content

Chemical Names

Mangostin: 1,3,6-Trihydroxy-7-methoxy-2,8-bis(3-methylbut-2-en-1-yl)-9H-xanthen-9-one

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Stictic Acid: 1,3-Dihydro-1,4-dihydroxy-10-methoxy-5,8-dimethyl-3,7-dioxo-7H-isobenzofuro(4,5-b)(1,4)benzodioxepin-11-carboxaldehyde

Accession Codes

The structure information has been deposited in the PDB under the accession codes 5BUY (FtFabZ), 5BUX (YpFabZ with glycerol bound), and 5BUW (YpFabZ with ethylene glycol bound).

Author Information

The authors declare no competing financial interest.

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Table 1. Crystal data and refinement statistics of YpFabZ and FtFabZ

Name/code [*]		YP(Gly)	YP(EG)	FT
Cell dimensions	a (Å)	104.76	104.08	136.71
	b (Å)	104.76	104.08	56.03
	c (Å)	87.96	88.33	140.09
	α (°)	90.0	90.0	90.0
	β (°)	90.0	90.0	113.0
	γ (°)	120.0	120.0	90.0
Space group		P6 ₃	P6 ₃	C2
No. of molecules/ASU		2	2	6
Resolution range (Å)				
Overall		50–1.9	50–1.8	50 – 2.55
Last shell		1.96 -1.9	1.85-1.8	2.64 -2.55
Number of unique reflections		42366	49818	31876
Completeness (%)				
(Overall/Last shell)		98/85	99/89	99/91
R _{merge} ¹ overall/last shell		5.0/8.0	7.0/41	8.5/22
Refinement Statistics				
Resolution (Å)		50-1.9	50-1.8	50-2.55
R factor ² /R _{free} (%)		15.7/18.3	17.3/19.7	21.0/28.0
R.M.S deviations from ideality				
Bond lengths (Å)		0.03	0.01	0.01
Bond angles (°)		2.4	2.3	1.70
Average B-factors (Å ²)				
Main chain		18.4	22.5	48.1
Side chain		26.3	29.5	54.0
Waters		30.1	31.1	39.6
Ramachandran Plot statistics				
Most favored region		87.0	88.0	85.3
Additional allowed regions		13.0	12.0	14.2
Disallowed region		0	0	0.5

¹R_{merge} = $\sum_j (|I_h - \langle I_h \rangle|) / \sum I_h$, where $\langle I_h \rangle$ is the average intensity over symmetry equivalents

²R-factor = $\sum |F_{obs} - F_{calc}| / \sum |F_{obs}|$

^{*}YP(Gly): YpFabZ with glycerol bound; YP(EG): YpFabZ with glycerol bound; FT: FtFabZ

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Table 2: Kinetic values for YpFabZ and FtFabZ by spectrophotometric assay.

Enzyme	K_m (μM)	V_{max} ($\mu\text{M}/\text{min}/\mu\text{g}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)
FtFabZ	61.8 ± 8.5	22.1 ± 1.2	0.72 ± 0.039	11650
YpFabZ	78.2 ± 14.0	31.3 ± 1.3	1.45 ± 0.060	14322

Table 3: Inhibitory activities of Mangostin, Stictic Acid, Emodin, Juglone, Apigenin, and Quercetin against both YpFabZ and FtFabZ. N/O = None Observed.

Compound	Structure	IC ₅₀ for YpFabZ	IC ₅₀ for FtFabZ
Mangostin		6.1 ± 1.4 μM	7.7 ± 2.0 μM
Stictic Acid		13.0 ± 1.4 μM	27.8 ± 6.1 μM
Emodin		29.7 ± 6.0 μM	43.1 ± 9.2 μM
Juglone		5.3 ± 1.0 μM	5.4 ± 1.4 μM
Apigenin		N/O	N/O
Quercetin		N/O	N/O

Figure Legends:

Figure 1: Fatty Acid Synthesis Pathway: Initially, acetyl-CoA is carboxylated by acetyl-CoA carboxylase (AccABCD) forming malonyl-CoA. Next, malonyl-CoA is transferred to ACP by malonyl-CoA:ACP transacylase (FabD). The first cycle of chain elongation is initiated by the condensation of acetyl-CoA with malonyl-ACP by β -ketoacyl-ACP synthase III (FabH). The second step of the elongation cycle is carried out by β -ketoacyl reductase (FabG) forming β -hydroxyacyl-ACP. Next, the β -hydroxyacyl-ACP intermediate is dehydrated by FabZ to form trans-2-enoyl-ACP. Finally, the double bond of trans-2-enoyl is reduced by enoyl-ACP reductase (FabI). The acyl-ACP product of the FabI reaction feeds back into the cycle and FabF initiates subsequent rounds of elongation. Figure adapted from Campbell et. al.²⁷

Figure 2: (A) Ribbon diagram of the YpFabZ hexamer. Dimer A/B is colored cyan/green, Dimer C/D is colored grey/salmon, and Dimer E/F is colored yellow/magenta. (B) Ribbon diagram of the YpFabZ monomer with α -helices in red, β sheets in yellow, and loop regions in green. (C) Ribbon diagram of YpFabZ dimer with locations of the two individual active sites marked by stick representation of the Tyr92 gate keeper residue and the catalytic residues, His54 and Glu68.

Figure 3: Surface representation of the entrance tunnel and active site region of YpFabZ with key residues and ligands shown in stick representation. Individual YpFabZ monomers are colored green or cyan in both surface and stick representation. Hydrogen bonds between ligands and YpFabZ residues are shown as black dashed lines. (A) YpFabZ entry and exit gate residues as well as active site residues are named. (B) Overlay of entrance and exit gate residues from YpFabZ, HpFabZ, and PfFabZ

superimposed on YpFabZ surface representation. Residues from different FabZ isoforms are colored cyan (YpFabZ), magenta (PfFabZ), or orange (HpFabZ). Residues from HpFabZ and PfFabZ are shown in the “open” conformation. Tyr92 from YpFabZ is shown in both the “open” and “closed” conformation, while Phe79 and Tyr91 were found only in the “closed” conformation. (C) Interactions between glycerol and active site residues. (D) Interactions between ethylene glycol and thiocyanate with active site residues.

Figure 4: (A) Structural alignment of YpFabZ (salmon) and FtFabZ (blue) dimers. (B) Structural alignment of the gate and catalytic residues of YpFabZ and FtFabZ. FtFabZ residues are labeled and colored orange. Unlabeled YpFabZ residues are colored green and cyan. Background surface representation is of YpFabZ with individual monomers colored green and cyan. (C) Close up view of extended $\alpha 3$ helix of FtFabZ (blue) aligned to YpFabZ (salmon). Key exit gate residues are shown as sticks with colors corresponding to the ribbon representation.

Figure 5: Kinetic analysis of YpFabZ (A,B) and FtFabZ (C,D). Initial velocities were determined using increasing concentrations of crotonoyl-CoA. The data were analyzed using non-linear regression analysis and K_m values of 78.2 μM and 61.8 μM were obtained for YpFabZ and FtFabZ, respectively.

Figure 6: The effect of NaCl concentration on YpFabZ enzymatic activity. Individual NaCl concentrations were tested in triplicate.

Figure 7: Double reciprocal plots of $1/V$ vs. $1/[\text{crotonoyl-CoA}]$ at different inhibitor concentrations are shown for Mangostin (A) and Stictic Acid (B). The lines for each

inhibitor intercept at the $1/V$ axis, demonstrating that both Mangostin and Stictic Acid are competitive inhibitors with K_i values of $1.4 \pm 0.13 \mu\text{M}$ and $22.6 \pm 2.0 \mu\text{M}$, respectively.

Figure 8: Images showing the interactions of Mangostin (A) and Stictic Acid (B) with YpFabZ. The two monomers of YpFabZ are shown as $C\alpha$ chain in green and cyan. Key interacting YpFabZ residues are shown as sticks. Hydrogen bonds are shown as dashed black lines. The predicted binding energies of Mangostin and Stictic Acid are $-10.4 \text{ kcal mol}^{-1}$ and $-8.8 \text{ kcal mol}^{-1}$, respectively. These values are in relative agreement with the calculated K_i values.

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381x140mm (300 x 300 DPI)

Figure 3: Surface representation of the entrance tunnel and active site region of YpFabZ with key residues and ligands shown in stick representation. Individual YpFabZ monomers are colored green or cyan in both surface and stick representation. Hydrogen bonds between ligands and YpFabZ residues are shown as black dashed lines. (A) YpFabZ entry and exit gate residues as well as active site residues are named. (B) Overlay of entrance and exit gate residues from YpFabZ, HpFabZ, and PfFabZ superimposed on YpFabZ surface representation. Residues from different FabZ isoforms are colored cyan (YpFabZ), magenta (PfFabZ), or orange (HpFabZ). Residues from HpFabZ and PfFabZ are shown in the “open” conformation. Tyr92 from YpFabZ is shown in both the “open” and “closed” conformation, while Phe79 and Tyr91 were found only in the “closed” conformation. (C) Interactions between glycerol and active site residues. (D) Interactions between ethylene glycol and thiocyanate with active site residues.

254x193mm (72 x 72 DPI)

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Figure 6: The effect of NaCl concentration on YpFabZ enzymatic activity. Individual NaCl concentrations were tested in triplicate.
69x49mm (300 x 300 DPI)

Figure 7: Double reciprocal plots of 1/V vs. 1/[crotonoyl-CoA] at different inhibitor concentrations are shown for Mangostin (A) and Stictic Acid (B). The lines for each inhibitor intercept at the 1/V axis, demonstrating that both Mangostin and Stictic Acid are competitive inhibitors with K_i values of $1.4 \pm 0.13 \mu\text{M}$ and $22.6 \pm 2.0 \mu\text{M}$, respectively.

276x128mm (72 x 72 DPI)

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