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Deciphering the Late Biosynthetic Steps of Antimalarial Compound FR-900098

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SUMMARY

FR-900098 is a potent chemotherapeutic agent for the treatment of malaria. Here we report the heterologous production of this compound in *Escherichia coli* by reconstructing the entire biosynthetic pathway using a three-plasmid system. Based on this system, whole-cell feeding assays in combination with in vitro enzymatic activity assays reveal an unusual functional role of nucleotide conjugation and lead to the complete elucidation of the previously unassigned late biosynthetic steps. These studies also suggest a biosynthetic route to a second phosphonate antibiotic, FR-33289. A thorough understanding of the FR-900098 biosynthetic pathway now opens possibilities for metabolic engineering in *E. coli* to increase production of the antimalarial antibiotic and combinatorial biosynthesis to generate novel derivatives of FR-900098.

INTRODUCTION

Malaria remains one of the most common life-threatening infections in developing countries, with up to 500 million infections and over 1 million deaths per year (Snow et al., 2005). Current antimalarial treatments include chloroquine and sulfadoxin pyrimethamine, but the rapid emergence of antibiotic-resistant strains is rendering these current therapies futile, creating an urgent need for new effective drugs (Ridley, 2002; Wiesner et al., 2003). FR-900098 and fosmidomycin represent a new class of antimalarial compounds. These compounds were first isolated from *Streptomyces rubellomurinus* and *Streptomyces lavendulae*, respectively (Okuhara et al., 1980a). Studies have shown that these two compounds bind in the active site and effectively inhibit 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), the first step in the nonmevalonate pathway for isoprenoid biosynthesis (MacSweeney et al., 2005). Genomic analyses indicate that the genes encoding the nonmevalonate pathway are present in a wide variety of Gram-negative and

Gram-positive bacteria, plants, and the parasite causing the most virulent form of malaria, *Plasmodium falciparum* (Boucher and Doolittle, 2000; Lange et al., 2000). Mammals produce isoprenoids via the mevalonate pathway; therefore, FR-900098 and fosmidomycin should not be detrimental to humans (Beytia and Porter, 1976). Both compounds are currently undergoing clinical trials and have shown great promise as malaria treatments (Borrmann et al., 2004, 2006). Earlier work revealed that FR-900098 is twice more effective than fosmidomycin against various strains of *P. falciparum* in vitro and the closely related *P. vinckei* in mice (Jomaa et al., 1999). Progress has been made in the development of chemical synthetic routes for fosmidomycin (Hemmi et al., 1982) and FR-900098 (Fokin et al., 2007; Hemmi et al., 1982), but we chose to pursue a biological route for a more cost-effective way to produce these antimalarial compounds.

Heterologous production of FR-900098 in *Streptomyces lividans* and a proposed biosynthetic pathway were reported by Eliot et al. (2008). The first step involved in FR-900098 biosynthesis, a phosphoenolpyruvate (PEP) mutase reaction catalyzed by FrbD, is common in almost all other known phosphonate biosyntheses. Currently, only three other complete phosphonate biosynthetic pathways have been identified (2-aminoethylphosphonate [Barry et al., 1988], fosfomycin [Hidaka et al., 1995; Woodyer et al., 2006], and bialaphos [Blodgett et al., 2005; Schwartz et al., 2004]), all of which have a common second step. In all of these cases, an irreversible thiamine pyrophosphate-dependent decarboxylation of phosphonopyruvate (PnPy) to form phosphonoacetaldehyde is required to provide a sufficient thermodynamic driving force to overcome the unfavorable equilibrium created by PEP mutase. However, the FR-900098 biosynthetic cluster does not contain a PnPy decarboxylase. FrbC, a homocitrate synthase homolog, catalyzes the formation of 2-phosphonomethylmalate, thus creating a novel route for phosphonate biosynthesis (Eliot et al., 2008).

The subsequent steps parallel the tricarboxylic cycle, resulting in the formation of 2-oxo-4-phosphonobutyrate, an analog of α-ketoglutarate. The final steps were proposed based only on sequence homology of the remaining genes, without any definitive assignment of order or function. A complete understanding of the FR-900098 pathway could provide significant insight into

the biosynthesis of all phosphonates. More importantly, heterologous expression inside a more genetically tractable host would act as a platform toward creating novel and potentially more potent derivatives of FR-900098 and increasing production of the antimalarial compound to industrially desirable levels. Here we report the heterologous production of FR-900098 in *E. coli* and the deciphering of the late steps in FR-900098 biosynthesis, verified through whole-cell feeding experiments and in vitro assays with purified enzymes.

RESULTS AND DISCUSSION

Heterologous Production of FR-900098 in *E. coli*

To achieve heterologous production of FR-900098 in *E. coli*, each of the genes in the biosynthetic gene cluster were first checked for soluble expression inside the new host. Each of the 11 genes was cloned, expressed, and then analyzed by SDS-PAGE and western blot. All of the proteins had high expression and showed clear overexpression bands in *E. coli*, except for FrbE and DxrB, which demonstrated low levels of soluble expression detected only by western blot analysis (data not shown). To reconstitute the FR-900098 biosynthetic pathway in *E. coli*, each of the 11 genes in the biosynthetic cluster was cloned into a set of three compatible Duet expression vectors (pETDuet, pRSFDuet, and pACYCDuet). Each individual gene is under the transcriptional control of a strong T7 promoter and expression is inducible by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG). The constructed plasmids were transformed into the heterologous host *E. coli* to create the production strain. The heterologous production of FR-900098 was confirmed using liquid chromatography/mass spectrometry (LC-MS) analysis as described in Eliot et al. (2008).

Deletion analysis was utilized to determine which genes were required for heterologous production of FR-900098. Different strains of *E. coli* were created which lacked a single gene from the pathway and each was tested for its ability to produce the compound. The strain lacking *dxrB* grew very slowly upon protein induction and, although the strain eventually grew to saturation, it produced significantly lower amounts of FR-900098. As mentioned earlier, the *dxrB* gene encodes a homolog of 1-deoxy-D-xylulose-5-phosphate reductoisomerase, the known target of the antibiotic. This provided evidence for the necessary inclusion of the *dxrB* gene in the *E. coli* FR-900098-producing strains as a means of resistance from increasing concentrations of the compound. The genes *frbI* and *frbJ* were not essential for FR-900098 production in *E. coli*; the overexpression of these two proteins more than likely results in the unnecessary consumption of cellular resources, which results in lower FR-900098 production. The final production strain (pETDuet-*frbABCD*, pRSFDuet-*frbHFG*, pACYCDuet-*frbE-dxrB*) did not include *frbI* or *frbJ*, resulting in the highest production of FR-900098, 6.3 mg/L (Figure 1). To our knowledge, this is the first known heterologous production of a phosphonate antibiotic in *E. coli*.

Whole-Cell Feeding Studies

To identify possible intermediates in the late steps of FR-900098 biosynthesis, a series of whole-cell feeding experiments was conducted. Resting cells of *E. coli* were fed with 2-amino-4-
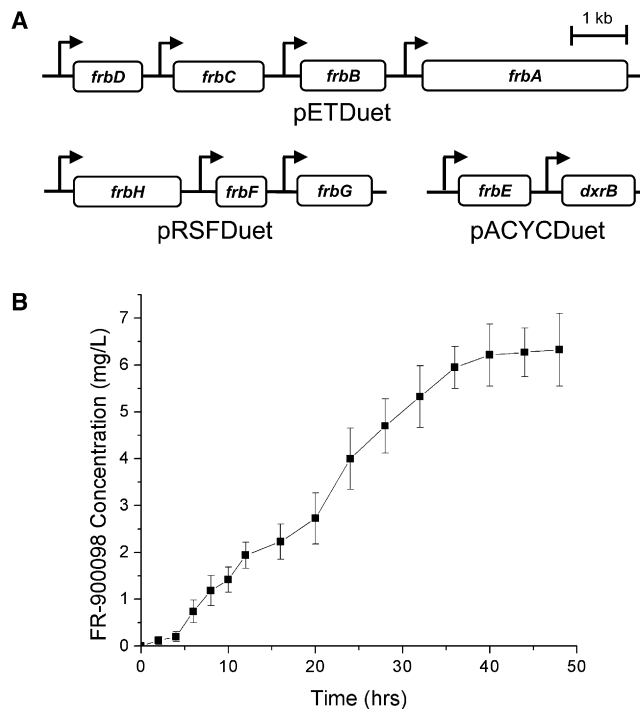

Figure 1. Heterologous Production of FR-900098 in *E. coli*
(A) Scheme for the three-plasmid system in an FR-900098-producing strain. Each gene is under transcriptional control by strong T7 promoters indicated by arrows.
(B) Time course production of FR-900098 in *E. coli*. Error bars represent standard deviations.

phosphonobutyrate (2APn), a glutamate analog which is efficiently taken up by *E. coli* and also shown to crossfeed into *S. lividans* deletion mutants (data not shown). Culture supernatants were analyzed for possible phosphonate intermediates using a coupled LC-MS approach. Cells expressing FrbH were found to accumulate CMP-5′-3-aminopropylphosphonate (CMP-5′-3APn), and cells expressing FrbH, FrbF, and FrbG had detectable levels of CMP-5′-*N*-acetyl-3-aminopropyl-phosphonate (CMP-5′-Ac3APn), CMP-5′-FR-900098, and FR-900098. Thus, these initial whole-cell feeding studies suggested that cytidine-5′-monophosphate (CMP) -conjugated phosphonate intermediates are present during the late steps of FR-900098 biosynthesis. These studies also showed that FrbG is an essential component in the late steps of FR-900098 biosynthesis, as definitive assignment of the function of the enzyme was never made (Eliot et al., 2008). It is also interesting to note that 2-oxo-4-phosphonobutyrate was detected in samples containing a negative control (empty vector), providing evidence that a ubiquitous cellular transaminase is able to carry out the transamination of 2OPn to form 2APn.

Bifunctional Activity of FrbH

The role of *frbH* was investigated after overexpression and purification of a histidine-tagged FrbH fusion from *E. coli*. Based on sequence homology to other known PLP-dependent enzymes, the protein was originally believed to catalyze the decarboxylation of 2APn to yield 3APn (Eliot et al., 2008). However, repeated
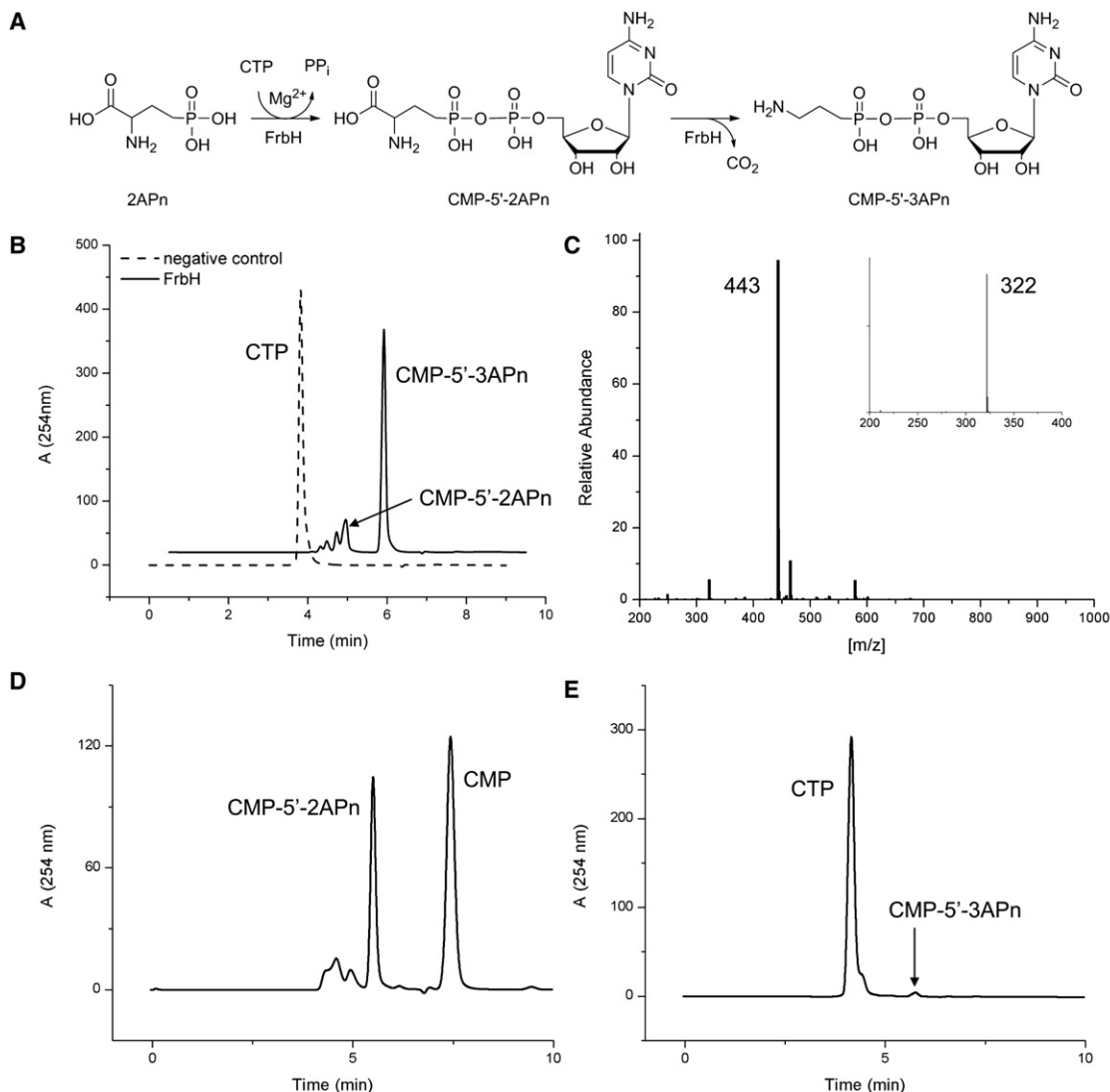
Figure 2. FrbH Reaction

(A) Proposed order of reaction where CMP attachment occurs before decarboxylation.

(B) HPLC analysis of the FrbH reaction product.

(C) MS/MS data of CMP-5′-3APn.

(D) HPLC analysis of FrbH-K466A mutant, which should remove decarboxylase activity.

(E) HPLC analysis of FrbH-K38A mutant, which should remove nucleotide transferase activity.

attempts to demonstrate this in vitro reaction failed. A BLAST search of full-length FrbH revealed two distinct domains: a nucleotide transferase domain, followed by a PLP-dependent amino-transferase/decarboxylase domain. Only the second domain was originally believed to be active, but based on the whole-cell feeding studies with 2APn, we began to investigate whether FrbH is bifunctional (Figure 2A). An in vitro reaction mixture containing both 2APn and cytidine-5′-triphosphate (CTP) showed nearly complete conversion of CTP to several compounds, including CMP, cytidine-5′-diphosphate (CDP), CMP-5′-2APn, and predominantly CMP-5′-3APn (Figure 2B). LC-MS of the products CMP-5′-2APn and CMP-5′-3APn revealed the masses m/z 487 and 443, respectively (Figure 2C). Furthermore, fragmentation of these two products in negative mode showed the

characteristic fragment ion of m/z 322, indicating the CMP moiety on both of these compounds. These results suggest FrbH condenses 2APn and CTP to form CMP-5′-2APn and then decarboxylates CMP-5′-2APn to yield CMP-5′-3APn.

Single-point mutations were designed to eliminate the activity of each of the individual domains of FrbH to verify the proposed order of reaction. PLP-dependent enzymes are known to bind to the cofactor with a conserved lysine residue. A sequence alignment with CobD from *Salmonella typhimurium* revealed the PLP-binding motif and the lysine at position 466 (Brushaber et al., 1998). A K466A mutant was constructed, expressed, and purified which should be unable to bind the PLP and thus perform the decarboxylation. A second mutant was designed to disrupt nucleotide transferase activity of FrbH. A sequence

alignment with IspD (an enzyme in the nonmevalonate pathway for isoprenoid biosynthesis) from *E. coli* revealed a common lysine residue (K38 in FrbH) that eliminated enzymatic activity (Richard et al., 2004). Under the same in vitro reaction conditions as the wild-type FrbH, the K466A mutant produced only CMP-5′-2APn (Figure 2D). Although unable to totally abolish nucleotide transferase activity, the K38A mutant had a mostly unreacted CTP peak with a small peak corresponding to CMP-5′-3APn (Figure 2E). These results are conclusive evidence for FrbH catalyzing the CMP attachment to 2APn followed by decarboxylation, yielding CMP-5′-3APn (Figure 2A).

N-Acetyltransferase Activity of FrbF and *N*-Hydroxylase Activity of FrbG

To complete the biosynthesis of FR-900098, the amino group of CMP-5′-3APn requires both hydroxylation and acetylation. The order and identity of the enzymes catalyzing these two steps were never definitively assigned in the original pathway (Eliot et al., 2008). FrbF has strong homology to known *N*-acetyltransferases, and it was originally believed that FrbJ may be involved in the hydroxylation step. However, based on the results of the whole-cell feeding assays and deletion studies described above, FrbG, a FAD/NADPH-dependent monooxygenase, was now thought to catalyze the hydroxylation step instead of FrbJ. The whole-cell feeding studies also suggest the order of the two steps as acetylation followed by hydroxylation, because CMP-5′-Ac3APn was seen and not *N*-hydroxy-3-aminopropylphosphonate (CMP-5′-H3APn). Coupled in vitro reactions with FrbF and FrbG also supported this order of reaction, as there was always a CMP-5′-Ac3APn peak present but little to no CMP-5′-H3APn monitored (Figures 3A and 3B). To determine the order of the acetylation and hydroxylation steps, each of the potential substrates was purified and kinetic parameters for FrbF and FrbG were obtained.

Although CMP-5′-Ac3APn was believed to be an intermediate in the pathway instead of CMP-5′-H3APn, no activity was observed for FrbG toward this substrate. FrbF was able to accept either of the two potential substrates, meaning CMP-5′-Ac3APn is actually a dead-end in the FR-900098 biosynthetic pathway rather than a true intermediate (Table 1). Kinetic analysis of these two enzymes indicated that FrbF and FrbG compete for the same intermediate, CMP-5′-3APn, as they both prefer this compound compared to the alternative (CMP-H3APn for FrbF and CMP-5′-Ac3APn for FrbG). However, FrbG has 2.3-fold lower K_M for CMP-5′-3APn in comparison to FrbF, so the enzyme can effectively direct the flux away from the dead end. The reason for observing CMP-5′-Ac3APn rather than CMP-5′-H3APn throughout the feeding experiments is due to the buildup of the dead-end intermediate CMP-5′-Ac3APn, whereas the true intermediate, CMP-5′-H3APn, is prevented from accumulating because it is further processed by FrbF. These results demonstrate that the flux of the pathway must go through *N*-hydroxylation by FrbG followed by *N*-acetylation by FrbF and subsequently the more electrophilic CMP-5′-H3APn intermediate.

Nucleotide Hydrolase Activity of FrbI

The last step in the biosynthesis of FR-900098 was evident based on the presence of the intermediate CMP-5′-FR-900098.

Removal of the CMP moiety from CMP-5′-FR-900098 is necessary in order to produce the final compound FR-900098. Nucleotide hydrolases are known to catalyze the hydrolytic breakdown of a wide variety of nucleotide derivatives (McLennan, 2006). FrbI has high homology to this family of enzymes and could likely catalyze the hydrolysis of CMP-5′-FR-900098 to produce FR-900098 and CMP. In order to perform in vitro enzymatic studies, the enzyme FrbI was overexpressed in *E. coli* and purified to homogeneity. An in vitro reaction involving FrbI was tested and found to produce a significant amount of FR-900098, whereas only a very small amount of FR-900098 was present in the absence of FrbI, likely due to the instability of CMP-5′-FR-900098 (Figures 3C and 3D). Thus, it is clear that FrbI catalyzes the hydrolysis of CMP-5′-FR-900098 to form FR-900098 and CMP. Interestingly, FrbI also catalyzed the hydrolysis of all the other CMP-containing intermediates within the pathway and even showed a small amount of activity toward CTP. This is not surprising given that nucleotide hydrolases typically have broad substrate specificity (Bessman et al., 1996). This promiscuous behavior is evident from the gene deletion analysis previously mentioned. The gene *frbI* is not required for FR-900098 biosynthesis in *E. coli* as a ubiquitous nucleotide hydrolase is able to cleave the CMP, yielding the final compound FR-900098.

Monooxygenase Activity of FrbJ

FrbJ is homologous to α-ketoglutarate-dependent monooxygenases capable of functionalizing an inactivated C-H bond. Although not required for FR-900098 biosynthesis, we believed the enzyme may be involved in the synthesis of another phosphonate antibiotic, FR-33289. Production of this compound was observed in both the native *S. rubellomurinus* and heterologous *S. lividans* 4G7 host (data not shown). FR-33289 has similar antibacterial properties compared to FR-900098 and could also be another effective antimalarial compound (Okuhara et al., 1980b). An in vitro reaction mixture containing active FrbJ was able to convert FR-900098 to FR-33289 (Figures 3E and 3F). Expression of *frbJ* along with the remaining genes in the pathway could provide a means for production of FR-33289.

In summary, we have heterologously produced FR-900098 in *E. coli* and demonstrated that the late steps of FR-900098 biosynthesis follow a series of novel intermediates (Figure 4). Of special note, the nucleotide attachment to the intermediates in FR-900098 biosynthesis seems to play an unprecedented role. It is traditionally believed that attachment of the nucleotide serves only as an activator for otherwise challenging chemistry in natural product biosyntheses containing nucleotide-conjugated intermediates. For example, in the biosynthesis of phosphinothricin tripeptide, PhpF catalyzes the condensation of CTP with phosphonoformate to form CMP-5′-phosphonoformate (CMP-5′-PF). This reaction was required so that the activated intermediate would be primed for transfer of the phosphonoformate group to the appropriate acceptor molecule (Blodgett et al., 2007). As part of the nonmevalonate pathway, IspD has been shown to catalyze the formation of 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) from 2-C-methyl-D-erythritol 4-phosphate and CTP, which facilitates the subsequent formation of 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (Rohdich et al., 1999). However, in FR-900098 biosynthesis, nucleotide attachment clearly does not activate the bond for subsequent
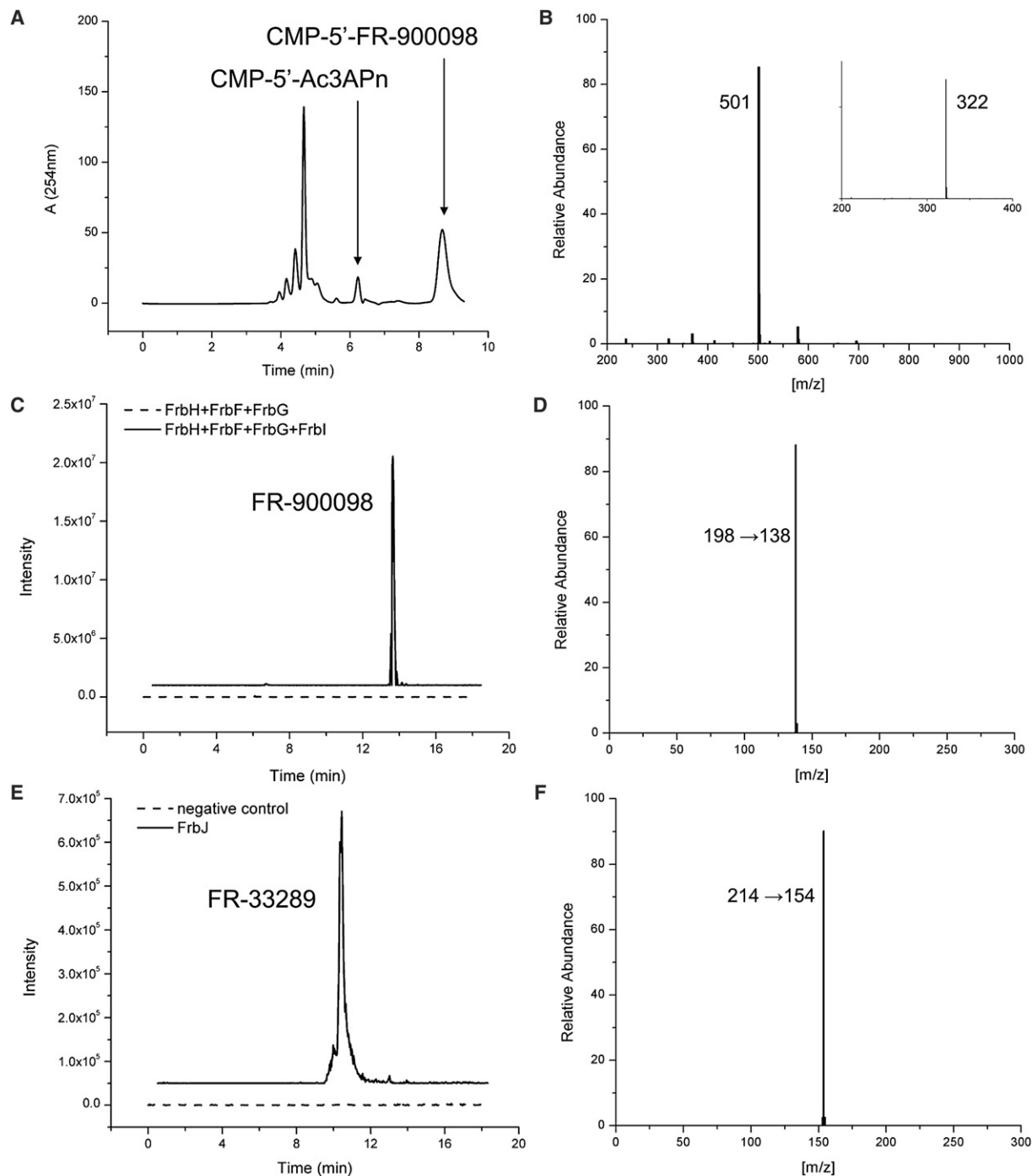
Figure 3. FrbG, FrbF, FrbI, and FrbJ Reactions
(A) HPLC analysis of the FrbG and FrbF combined reaction product.
(B) MS/MS data of CMP-5′-FR-900098.
(C) HPLC analysis of the FrbI reaction product.
(D) MS/MS data of FR-900098.
(E) HPLC analysis of the FrbJ reaction product.
(F) MS/MS data of FR-33289.

chemical reactions. Conjugation to CMP likely functions as a recognition mechanism to help distinguish among other metabolites within the cell and, more importantly, as a requirement for

enzymatic activity. This hypothesis was substantiated by our recent determination of the cocrystal structure of FrbG with the cofactor NADPH (data not shown). Examination of this structure

Table 1. Kinetic Parameters for FrbF and FrbG Reactions with Each of the Potential Substrates

Enzyme	Substrate	k_{cat} (min^{-1}) (Mean ± SD)	K_M (μM) (Mean ± SD)	k_{cat}/K_M (mM^{-1} min^{-1})
FrbF	CMP-5′-3APn	3.46 ± 0.16	35.2 ± 3.2	98.0
	CMP-5′-H3APn	2.04 ± 0.43	52.3 ± 3.0	39.0
FrbG	CMP-5′-3APn	5.92 ± 0.38	15.1 ± 1.5	391
	CMP-5′-Ac3APn			ND

ND, no detectable activity; SD, standard deviation.

reveals that there is no room for the binding of the substrate in the active site. Thus, we proposed a novel reaction mechanism for the *N*-hydroxylation catalyzed by FrbG. Briefly, the substrate and NADPH cofactor compete for the same binding pocket in the enzyme, and the nucleotide helps dock the substrate in the correct position. The remaining enzymes in the pathway likely evolved to accept substrates conjugated to CMP rather than those proposed in the original pathway.

SIGNIFICANCE

We have successfully produced a potent antimalarial agent, FR-900098, in a recombinant *E. coli* strain and elucidated the biosynthetic mechanisms of the late steps involved in FR-900098 biosynthesis. Notably, a nucleotide CMP is conjugated to several of the intermediates, which seems

to play an unprecedented role in natural product biosynthesis. Instead of activating a chemical bond, the nucleotide conjugation seems to serve as a substrate recognition mechanism. In addition, a biosynthetic route to a second phosphonic acid antibiotic, FR-33289, was discovered, which is embedded in the biosynthetic route to FR-900098, and its production is regulated by a single enzyme, FrbJ. This compound could also be effective in the treatment of malaria. These studies now open possibilities for metabolic engineering in *E. coli* to increase production of the antimalarial antibiotic and combinatorial biosynthesis to generate novel derivatives of FR-900098 with more potent antimalarial activity.**

EXPERIMENTAL PROCEDURES

Heterologous Production of FR-900098 in *E. coli*

An overnight culture of the production strain (pETDuet-*frbABCD*, pRSFDuet-*frbHFG*, pACYCDuet-*frbE-dxrB* in *E. coli* BL21[DE3] cells) was grown at 37°C in Luria-Bertani (LB) medium broth using the following antibiotic concentrations: 50 μg/ml ampicillin, 25 μg/ml kanamycin, and 12.5 μg/ml chloramphenicol. The cultures were diluted 1:100 in fresh medium and grown at 37°C to an OD$_{600}$ of ∼0.7. At that point, the expression of the recombinant genes was induced with 0.3 mM IPTG and the culture was incubated at 30°C with shaking (250 rpm). Samples (1 ml) were then removed from the cultures at specific time points and stored at −80°C. Frozen samples were thawed at room temperature and centrifuged at maximum speed for 2 min. Supernatants from the samples were then analyzed by LC-MS to determine the concentration of FR-900098.
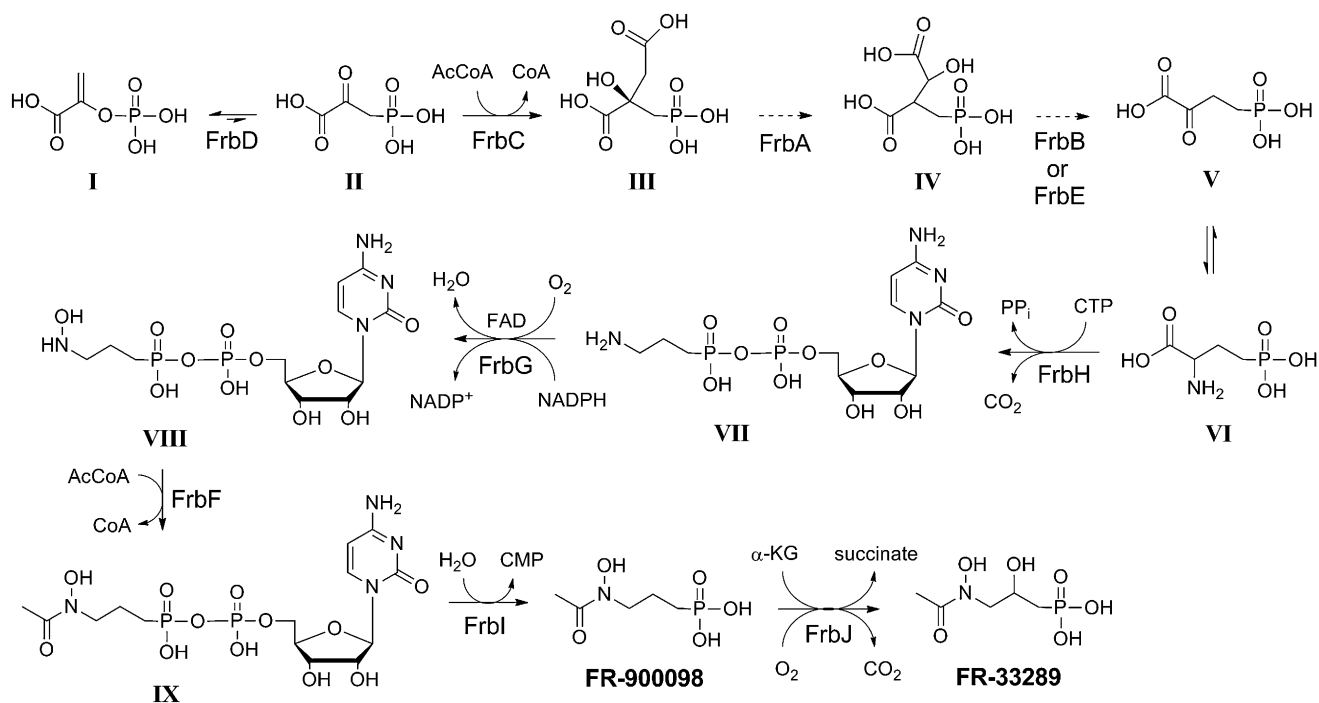

Figure 4. Revised FR-900098 Pathway
Each of the final steps in the FR-900098 biosynthetic pathway has been demonstrated in vitro using purified enzymes. Intermediates in the FR-900098 biosynthetic pathway are as follows: I, phosphoenolpyruvate; II, phosphonopyruvate; III, 2-phosphonomethylmalate; IV, 3-phosphonomethylmalate; V, 2-oxo-4-phosphonobutyrate; VI, 2-amino-4-phosphonobutyrate; VII, CMP-5′-3-aminopropylphosphonate; VIII, CMP-5′-*N*-hydroxy-3-aminopropylphosphonate; IX, CMP-5′-FR-900098.

Whole-Cell Feeding Studies

E. coli BL21(DE3) cells expressing empty vector (control) and FrbH and cells overexpressing FrbH, FrbF, FrbG, and DxrB were cultured overnight at 37°C in Terrific Broth medium with appropriate antibiotics. The cultures were diluted 1:100 in fresh medium and grown at 37°C to an OD_{600} of ~0.7. Protein expression was induced by the addition of 0.3 mM IPTG final concentration and cultures were grown at 25°C for 24 hr. Cultures were then centrifuged at 3200 xg for 15 min and the supernatant was discarded. The cell pellet was resuspended in 1 ml of 10 mM Tris-HCl buffer (pH 7.0). Resuspended cells were mixed with 20 mM final concentration (±)-2-amino-4-phosphonobutyrate (2APn) and incubated at 30°C for 24 hr. Samples were lysed by freeze/thaw and centrifuged at maximum speed in a microcentrifuge for 10 min. The supernatant (1 ml) was mixed with 9 ml of methanol, vortexed, and centrifuged at 3200 xg for 15 min. The pellet was discarded and the supernatant was rotary evaporated down to a volume of 1 ml. Samples were placed in a −80°C freezer until analyzed by LC-MS using a 7 Tesla LTQ-FT mass spectrometer (Thermo Finnigan, San Jose, CA, USA).

Cloning, Expression, and Purification

The cloning of genes *frbH*, *frbF*, *frbG*, *frbI*, and *frbJ* from fosmid 4G7 was similar to that in Eliot et al. (2008). Briefly, the genes were PCR amplified with NdeI and HindIII sites and cloned into corresponding sites of pET28a, designed to create an N-terminal histidine tag and transformed BL21(DE3) cells. The cells were grown in LB medium supplemented with kanamycin (50 μg/ml) at 37°C to OD_{600} ~0.7 and then induced at 25°C by the addition of IPTG to a final concentration of 0.3 mM. During the purification of FrbH, PLP was added to the media for a final concentration of 50 μg/ml. After 6–8 hr, the cells were harvested by centrifugation and resuspended in 20 mM Tris-HCl (pH 7.65), 15% glycerol, and 0.5 M NaCl at 4°C. The sample was freeze-thawed and then lysed further by sonication. After cellular debris was removed by centrifugation, the N-His$_6$-tagged proteins were purified using immobilized metal-affinity chromatography with TALON Superflow Co^{2+} resin coupled to fast-performance liquid chromatography. The purified protein was washed three times with 50 mM HEPES-Na$^+$ buffer and concentrated a final time. Concentrated protein was stored in 20% glycerol at −80°C.

FrbH Activity

Wild-type and mutant FrbH activity was assayed by HPLC and LC-MS. The assay mixture (100 μl) contained 2 mM (±)-2-amino-4-phosphonobutyric acid, 1 mM CTP, 10 mM $MgCl_2$, and His$_6$-FrbH in 50 mM HEPES-Na$^+$ buffer at pH 7.25. The reaction was initiated with the addition of 25 μg of purified wild-type or mutant FrbH and incubated at 30°C for 2 hr. Reaction mixtures were transferred into Microcon centrifugal filter devices (MWCO 10,000) and centrifuged for 15 min at maximum speed. The reactions were then analyzed by LC-MS.

FrbG and FrbF Activity

The activity of FrbG and FrbF was demonstrated in a coupled assay with FrbH. The assay mixture (100 μl) contained 2 mM (±)-2-amino-4-phosphonobutyric acid, 1 mM CTP, 10 mM $MgCl_2$, 1 mM AcCoA, 1 mM NADPH, His$_6$-FrbH, His$_6$-FrbF, and His$_6$-FrbG in 50 mM HEPES-Na$^+$ buffer (pH 7.25). The reaction was initiated with the addition of 25 μg of purified FrbF and 35 μg of purified FrbG. Reaction mixtures were transferred into Microcon centrifugal filter devices (MWCO 10,000) and centrifuged for 15 min at maximum speed. The reactions were then analyzed by LC-MS.

FrbI Activity

The activity of FrbI was demonstrated in a coupled assay with FrbH, FrbF, and FrbG. The assay mixture (100 μl) contained 2 mM (±)-2-amino-4-phosphonobutyric acid, 1 mM CTP, 10 mM $MgCl_2$, 1 mM AcCoA, 1 mM NADPH, His$_6$-FrbH, His$_6$-FrbF, His$_6$-FrbG, and His$_6$-FrbI in 50 mM HEPES-Na$^+$ buffer (pH 7.25). The reaction was initiated with the addition of 25 μg of purified FrbI and incubated at 30°C for 2 hr. Reaction mixtures were transferred into Microcon centrifugal filter devices (MWCO 10,000) and centrifuged for 15 min at maximum speed. The reactions were then analyzed by LC-MS.

FrbJ Activity

FrbJ activity was assayed by HPLC and LC-MS. The assay mixture (100 μl) contained 1 mM FR-900098, 1 mM α-ketoglutarate, 1 mM $FeSO_4$, 4 mM ascorbic acid, and His$_6$-FrbJ in 50 mM HEPES-Na$^+$ buffer (pH 7.25). The reaction was initiated with the addition of 25 μg of purified FrbJ and incubated at 25°C for 4 hr. Reaction mixtures were transferred into Microcon centrifugal filter devices (MWCO 10,000) and centrifuged for 15 min at maximum speed. The reactions were then analyzed by LC-MS.

LC-MS Analysis of CMP-Containing Compounds

Products of the enzymatic reactions were analyzed by an Agilent 1100 series LC/MSD XCT plus-ion-trap mass spectrometer (Agilent, Palo Alto, CA, USA) at the Roy J. Carver Metabolomics Center at the University of Illinois (Urbana, IL, USA). The liquid chromatography was performed using an Agilent 1100 series HPLC equipped with an online degasser, binary pump, and autosampler. Analytes were resolved on a 150 × 4.6 mm Agilent C$_{18}$ reverse-phase HPLC column. HPLC parameters were as follows: 25°C; solvent A: 15 mM ammonium formate; solvent B: 20 mM ammonium acetate; gradient: 25% B at 0 min, then to 100% B in 10 min, and finally maintain 100% B for 3 min; flow rate: 0.3 ml/min; detection by UV absorbance at 254 nm. The mass spectrometry was performed using an Agilent XCT ion-trap MSD mass spectrometer. Mass spectra were acquired in ultrascan mode using electrospray ionization (ESI) with negative polarity. The MS system was operated using a drying temperature of 350°C, a nebulizer pressure of 35 psi, a drying gas flow of 8.5 L/min, and a capillary voltage of 4500 V.

LC-MS Analysis of FR-900098 and FR-33289

LC-MS was performed using an Agilent 1100 series LC/MSD XCT plus-ion-trap mass spectrometer. The liquid chromatography was performed using an Agilent 1100 series HPLC equipped with an online degasser, binary pump, and autosampler. The mobile phase contained 0.1% formic acid in ddH$_2$O (solvent A) and 100% acetonitrile (solvent B). Analytes were resolved on a 100 × 4.6 mm Synergi 4u Fusion-RP 80A analytical column (Phenomenex, Torrance, CA, USA) at a flow rate of 200 μl/min with a gradient of 0%–40% solvent B for 10 min, a gradient of 40%–100% solvent B for 3 min, and 100% solvent B for 5 min. The mass spectrometry was performed using an Agilent XCT ion-trap MSD mass spectrometer. Mass spectra were acquired in ultrascan mode using ESI with positive polarity. The system was operated using a drying temperature of 350°C, a nebulizer pressure of 35 psi, a drying gas flow of 8.5 L/min, and a capillary voltage of 4500 V. For determination of titers, extracted ion chromatograms were generated from suitable ions of each analyte, and the integrated areas corresponding to each compound were compared to standards. Fosmidomycin was used as an internal standard to measure FR-900098 concentrations.

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