Supplemental Information

Structure-guided design and biosynthesis of a novel FR-900098 analogue as a potent *Plasmodium falciparum* 1-deoxy-D-xylulose-5-phosphate reductoisomerase (Dxr) inhibitor

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Cloning, Expression, and Purification

The complete *dxr* gene from *Plasmodium falciparum* was codon-optimized for expression in *E. coli* by DNA 2.0 (Menlo Park, CA). The core enzyme sequence (without the nucleotides 1-216 corresponding to the 72 amino acid leader peptide) was PCR amplified with *Nde*I and *Hind*III restriction sites and cloned into the respective sites of vector pET28a, introducing an N-terminal His_6 tag to the protein. *E. coli* BL21(DE3) was transformed with the resulting construct by electroporation. Cells were grown in LB media supplemented with kanamycin (50 μg/mL) at 37 °C to an OD_{600} of ~0.8, after which induction was carried out by addition of 0.5 mM IPTG at 25 °C. After 18 hr, the cells were harvested by centrifugation at 15,000 rpm for 15 min and resuspended in 20 mM Tris-HCl (pH 7.65), 0.5 M NaCl, and 15% glycerol supplemented with 1 mg/mL lysozyme. After a freeze-thaw cycle at -80 °C, the cell suspension was sonicated to ensure sufficient lysis. The lysate was clarified by centrifugation, after which the His_6-tagged protein was purified by affinity chromatography on TALON Superflow Co^{2+} resin (Clontech, Mountain View, CA) coupled to fast-performance liquid chromatography (Bio-Rad, Hercules, CA). The eluted protein was washed three times in 50 mM HEPES (pH 7.25), concentrated, and stored in 15% glycerol at -80 °C. FR-900098 pathway enzymes were expressed as previously described.[1]

Synthesis of FR-900098P

The *N*-propionyl analog FR-900098P was synthesized *in vitro* using purified FrbF, FrbG, FrbH, and FrbI enzymes from the downstream FR-900098 biosynthetic pathway. First, cytidine monophosphate-5'-3-aminopropylphosphonate (CMP-5'-3APn) was synthesized in 50 mM HEPES buffer, pH 7.25, containing 1.5 mM cytidine triphosphate (CTP), 3 mM 2-amino-4-phosphonobutyrate (2APn), 10 mM $MgCl_2$, and ~10 μM FrbH with incubation at 30°C for 4 hours, after which nearly complete conversion was observed. Following removal of FrbH with a 10 kDa cutoff filter, CMP-5'-FR-900098P was synthesized by addition of 1.5 mM NADPH, 1.5 mM propionyl-CoA, ~10 μM FrbF, and ~15 μM FrbG with incubation at 30 °C for 4 hours. At this point, side products such as CMP-5'-2-amino-4-phosphonobutyrate (CMP-5'-2APn), CMP-5'-N-propionyl-3-aminopropylphosphonate (CMP-5'-P3APn), CMP, CDP, and $NADP^+$ were removed via fractionation on an Agilent 1100 Series HPLC using an Alltech Prevail C18 column with isocratic 15 mM ammonium formate elution. The resulting CMP-5'-FR-900098P was lyophilized and resuspended in double-distilled H_2O. Finally, CMP cleavage was carried out in 10 mM Tris-HCl buffer, pH 7.5, by addition of ~10 μM FrbI with incubation at 30 °C for 4 hours. The final product titer was estimated from the concentration of CMP-5'-FR-900098P (measured by UV absorbance at 254 nm) and confirmed by dry weight of the final lyophilized product. Correct identity and purity of the final product was determined using an Agilent XCT ion-trap MSD mass spectrometer, where the anticipated m/z = 212^+ parent ion and m/z = 138^+ daughter ion were observed (Figure S2). Other FR-900098 analogues were synthesized in an analogous manner.

Kinetic Analysis

The enzymatic activity of *Plasmodium falciparum* DXP reductoisomerase was determined in 100 mM Tris-HCl (pH 7.5) with 1 mM $MnCl_2$, 0.3 mM NADPH, 50 nM enzyme, and substrate ranging from 0-250 μM DXP in a final volume of 300 μL. The reaction was monitored by the oxidation of NADPH at 340 nm. Inhibition studies with either fosmidomycin FR-900098, or FR-900098P were performed at inhibitor concentrations of 0-20 nM over a similar substrate range.

Crystallization, X-ray Data Collection, and Structure Determination

Initial crystallization conditions were established by the sparse-matrix sampling methods using commercial screens. Refinement of promising conditions yielded large crystals suitable for diffraction analysis. Crystals of the *Pf*Dxr-$NADP^+$-fosmidomycin complex were grown using the hanging drop vapor diffusion method. A 2 µL drop containing the pre-incubated sample (10 mg/mL *Pf*Dxr, in 100 mM KCl, 10 mM HEPES, pH 7.5 and 10 mM of each ligand) was added to 2 µL of precipitant (1.8 M to 2 M ammonium sulfate) and equilibrated over a well containing the precipitant solution at 8 °C. Crystals grew within a week and reached a maximum size of 0.2 mm x 0.1 mm x 0.2 mm. Crystals were briefly soaked in the precipitant solution supplemented with 25% glycerol, and vitrified by plunging directly into liquid nitrogen prior to data collection.

Diffraction data were collected to a limiting resolution of 1.9 Å for the *Pf*Dxr-fosmidomycin complex and 2.2 Å for the *Pf*Dxr-FR900098 complex at an insertion device line (LS-CAT-Sector 21ID-D, Advanced Photon Source, Argonne, IL), and integrated and scaled using the HKL2000 package.[2] Crystallographic phases were determined by the molecular replacement method[3] using the refined coordinates of *M. tuberculosis* Dxr[4] (35% identity over 373 residues omitting all solvent molecules and bound ligands). The quality of the resultant phases was poor but was subsequently improved by multiple cycles of noncrystallographic symmetry averaging. After convergence, the atomic model was subject to automatic rebuilding using ARP/wARP,[5] resulting in placement of roughly 70% of the main chain and 50% side chain atoms. Clear density for metal ions and bound ligand could be observed prior to any crystallographic refinement. The remainder of the model was fitted using COOT[6, 7] and further improved by rounds of refinement with REFMAC5.[8, 9] Cross-validation used 5% of the data in the calculation of the free R factor.[10] Metal ions, ligands, and solvent molecules were built into the model only after the free R factor dropped below 0.30. The stereochemistry of the models was routinely monitored throughout the course of refinement using PROCHECK.[11]

Table S1. Data collection, phasing, and refinement statistics

	*Pf*Dxr-fosmidomycin	*Pf*Dxr-FR900098
Space group	$P2_1$	$P2_1$
Cell dimensions		
a, b, c (Å)	51.3, 88.2, 212.2	51.2, 87.8, 212.0
α, β, γ (°)	90.0, 94.8, 90.0	90.0, 94.8, 90.0
Resolution (Å)[1]	50-1.9 (1.97-1.9)	50-2.2 (2.28-2.2)
R_{sym} (%)[2]	8.0 (29.3)	9.1 (26.7)
I / σ (I)	20.2 (3.6)	15.1 (3.7)
Completeness (%)	94.1 (78.0)	94.0 (75.0)
Redundancy	3.6 (3.4)	4.1 (3.8)
Refinement		
Resolution (Å)	25.0-1.9	25.0-2.2
No. reflections	132,064	84,823
R_{work} / R_{free}[3]	18.7/22.5	18.2/22.9
Number of atoms		
Protein	13024	12990
NAD/Drug	192/48	192/52
Solvent	1259	841
B-factors		
Protein	23.5	27.4
NAD/Drug	21.3/22.8	24.5/24.9
Solvent	32.8	33.1
R.m.s deviations		
Bond lengths (Å)	0.008	0.009
Bond angles (°)	1.2	1.29

1. Highest resolution shell is shown in parenthesis.
2. $R_{sym} = \Sigma |(I_i - \langle I \rangle)| / \Sigma I_i$ where I_i = intensity of the *i*th reflection and $\langle I \rangle$ = mean intensity.
3. R-factor = $\Sigma(|F_{obs}|-k|F_{calc}|)/\Sigma |F_{obs}|$and R-free is the R value for a test set of reflections consisting of a random 5% of the diffraction data not used in refinement.

Figure S1: a-b) Omit electron density maps (blue is contoured at 3σ and red is contoured at 10 σ) near the substrate-binding site, calculated with Fourier coefficients Fobs – Fcalc with the phosphonate omitted from structure factor calculations. Protein residues are shown in yellow, the phosphonate is shown in green and the nicotinamide of NADP+ is shown in pink. Dashed red lines indicate metal coordination and black lines indicate hydrogen bonds; a) shows the PfDxr-fosmidomycin complex, and b) shows the PfDxr-FR-900098 complex.

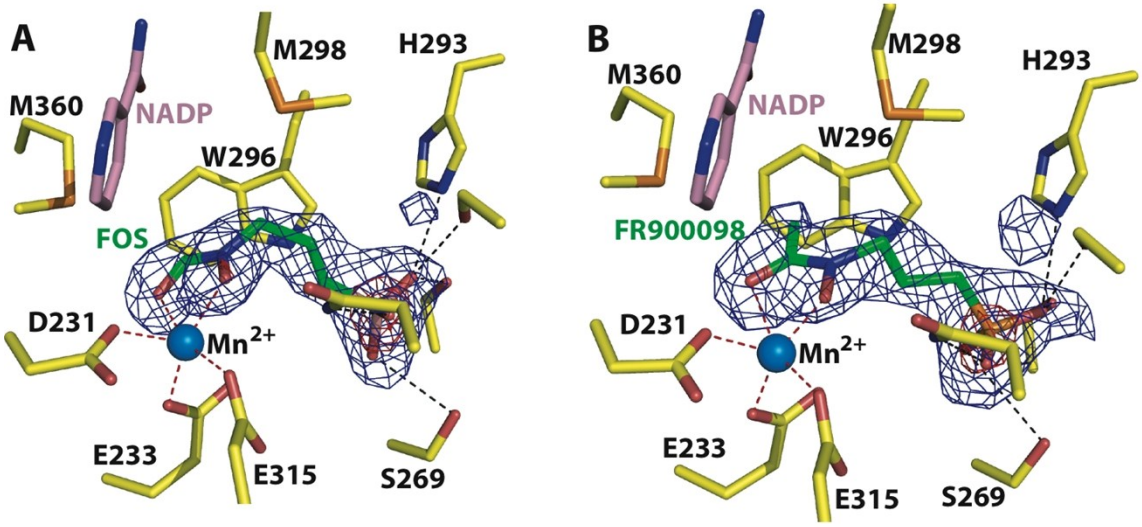


Figure S2: a) Extracted ion chromatograms showing production of CMP-conjugated FR-900098 derivatives (M, malonyl; AA, acetoacetyl; B, butyryl). All peaks exhibit the expected m/z = 322 peak upon MS/MS fragmentation, indicative of CMP. b) Tandem MS trace (selective ion monitoring of m/z = 212^{+}) showing of FR-900098P. Inset: MS/MS fragmentation of 14.2 min peak showing the expected 212→138 fragmentation.

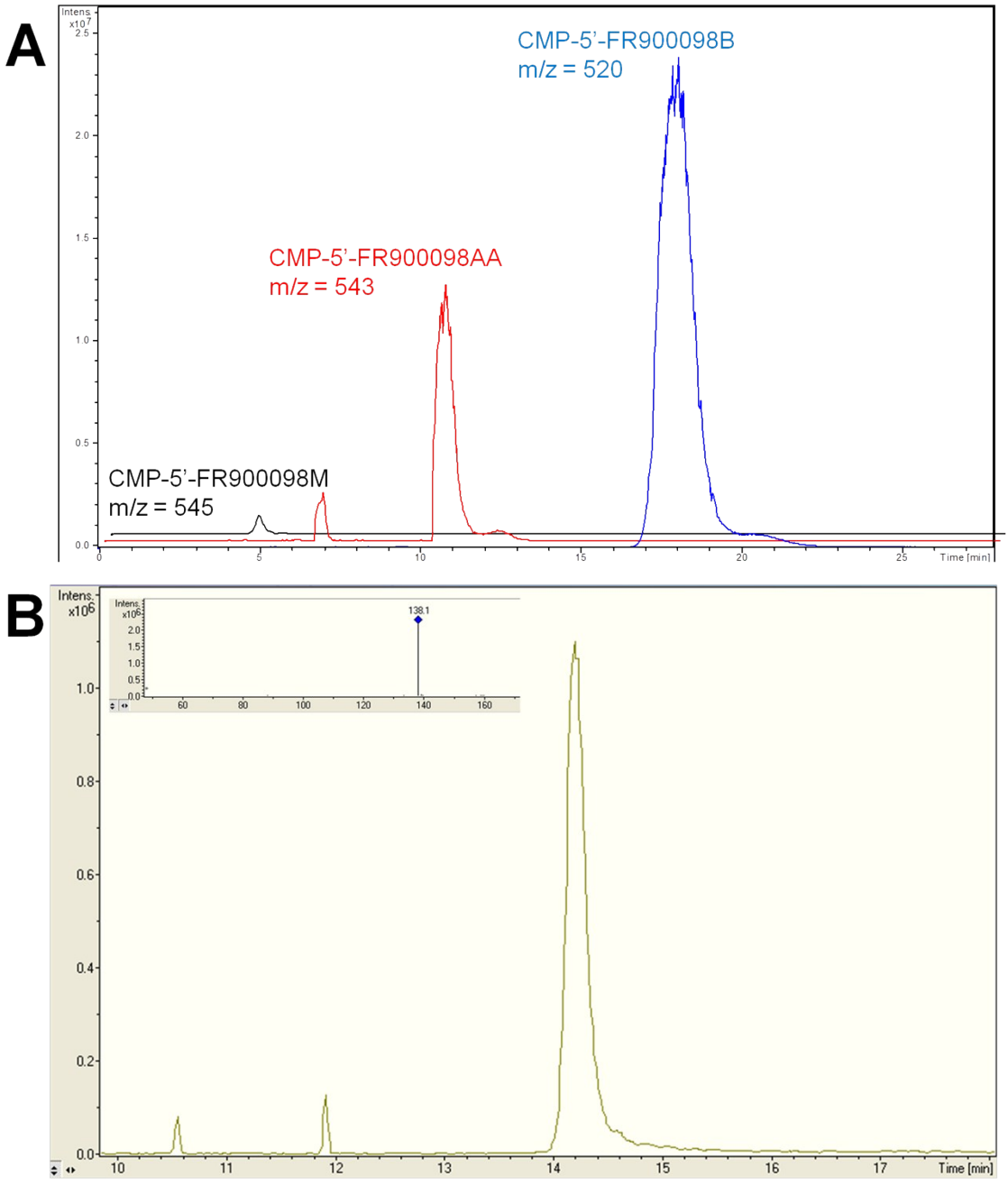


Figure S3: Separation of compounds on an Alltech Prevail C18 column following the FrbHGF *in vitro* reaction with propionyl-CoA. Compounds (confirmed by MS) are as follows: a, CDP; b, CMP; c, CMP-5'-nitroso-3APn; d, CMP-5'-3APn; e, CMP-5'-propionyl-3APn; f, $NADP^+$; and g, CMP-5'-FR-900098P.

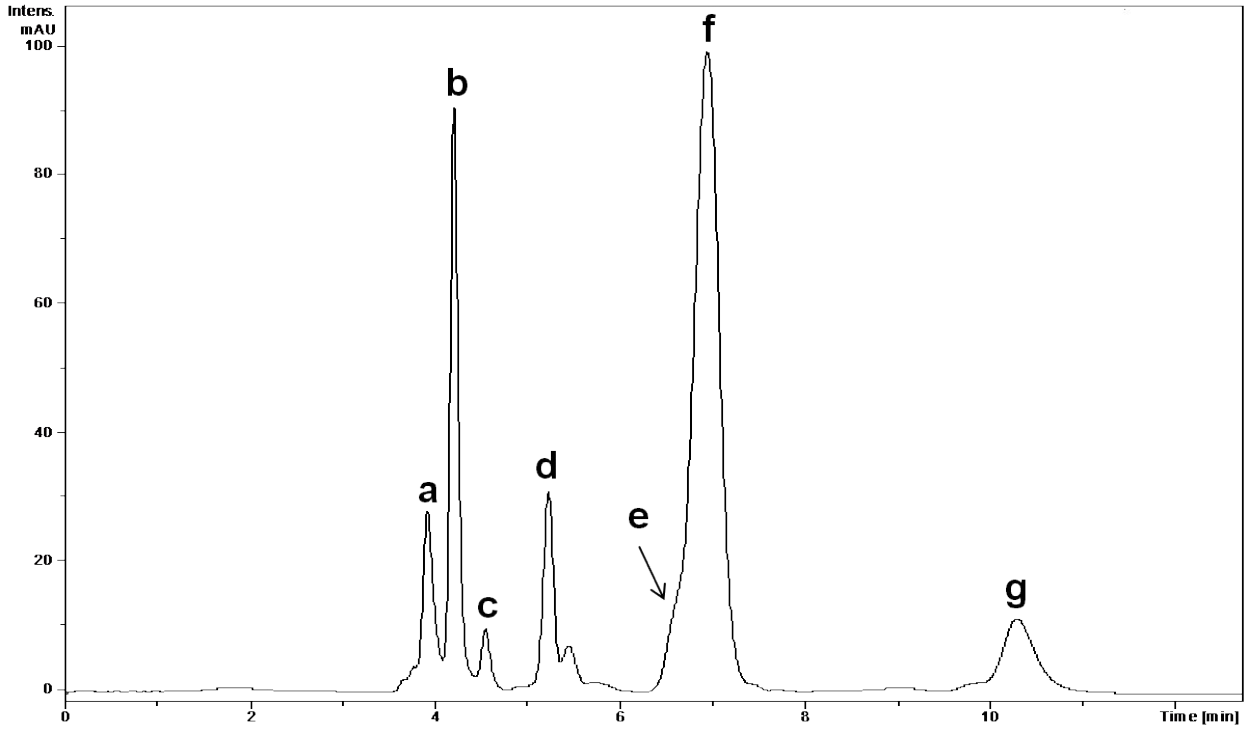
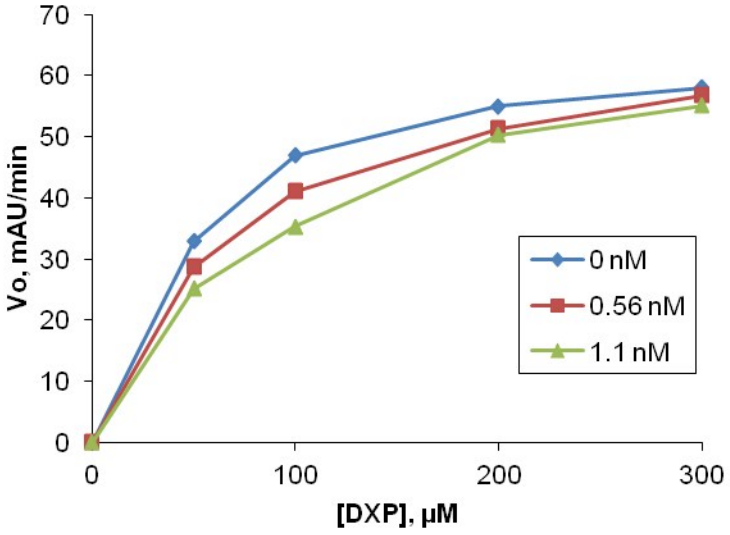


Figure S4: Representative kinetic data at three concentrations of FR-900098P. Calculated K_m values at 0 nM, 0.56 nM, and 1.1 nM inhibitor are 50 µM, 72 µM, and 102 µM, respectively.



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