

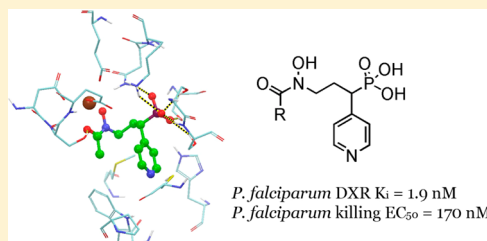
Antimalarial and Structural Studies of Pyridine-Containing Inhibitors of 1-Deoxyxylulose-5-phosphate Reductoisomerase

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Supporting Information

ABSTRACT: 1-Deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) in the nonmevalonate isoprene biosynthesis pathway is a target for developing antimalarial drugs. Fosmidomycin, a potent DXR inhibitor, showed safety as well as efficacy against *Plasmodium falciparum* malaria in clinical trials. On the basis of our previous quantitative structure–activity relationship (QSAR) and crystallographic studies, several novel pyridine-containing fosmidomycin derivatives were designed, synthesized, and found to be highly potent inhibitors of *P. falciparum* DXR (PfDXR) having K_i values of 1.9–13 nM, with the best one being $\sim 11\times$ more active than fosmidomycin. These compounds also potently block the proliferation of multidrug resistant *P. falciparum* with EC_{50} values as low as 170 nM. A 2.3 Å crystal structure of PfDXR in complex with one of the inhibitors is reported, showing that the flexible loop of the protein undergoes conformational changes upon ligand binding and a hydrogen bond and favorable hydrophobic interactions between the pyridine group and the PfDXR account for the enhanced activity.



KEYWORDS: 1-deoxy-D-xylulose-5-phosphate reductoisomerase, antimalarial activity, X-ray protein crystallography, inhibitor design

Eukaryotic parasite *Plasmodium* spp. are the causative agents of malaria, among which *Plasmodium falciparum* produces the most severe form of human malaria and is responsible for the vast majority of deaths of malaria patients (~ 1 million/year). In addition, this dreadful number could be rising because of the increased drug resistance of *P. falciparum* to inexpensive drugs such as chloroquine. Since 2001, the World Health Organization (WHO) has strongly recommended the use of artemisinin-based combination therapies to treat malaria.¹ However, *Plasmodium* parasites are known to be able to develop drug resistance. After 10 years, *P. falciparum* strains that are resistant to these new drug combinations have been observed.¹ There is therefore a pressing need to develop new antimalarial drugs.

1-Deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) is the second enzyme in the nonmevalonate isoprene biosynthesis pathway, catalyzing the reductive isomerization of 1-deoxy-D-xylulose-5-phosphate (DXP) to 2-methyl-D-erythritol-4-phosphate (MEP) using Mg^{2+} (or Mn^{2+}) and NADPH as the cofactors, as schematically illustrated in Figure 1.² This is used by most bacteria, as well as apicomplexan parasites such as *Plasmodium* spp., to make isopentenyl diphosphate and dimethylallyl diphosphate. These two diphosphate compounds are the only precursors for the biosynthesis of all isoprenoids

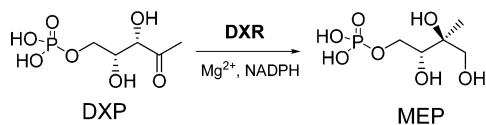
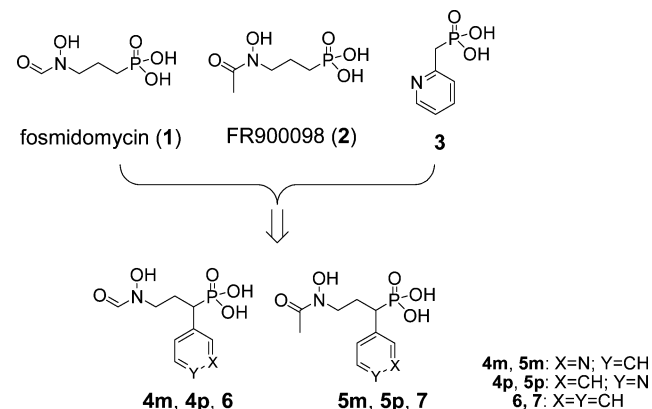


Figure 1. Reaction catalyzed by DXR.

and terpenoids, including important substances such as C15–C55 isoprenyl (e.g., farnesyl and undecaprenyl) diphosphates for anchoring proteins to the cell membrane. DXR is essential for the growth of all of these species including *P. falciparum*. In addition, humans and animals use the mevalonate pathway to synthesize IPP and DMAPP, making DXR an attractive target for discovering novel antimalarial drugs.^{3,4}

Fosmidomycin (1, Chart 1) and its close analogue FR900098 (2) were found to be potent DXR inhibitors⁵ and possess antibacterial and -malarial activities.⁶ Particularly, several recent

Chart 1. Structures of DXR Inhibitors



Received: November 25, 2012

Accepted: December 11, 2012

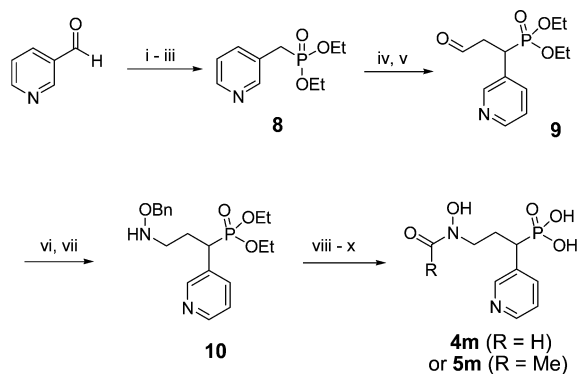
Published: December 11, 2012

clinical trials showed that in combination with clindamycin, **1** is safe for human use and effective against *P. falciparum* malaria.^{7,8} However, because of a short half-life in plasma (~1 h) as well as poor oral availability of **1**, considerable interest has therefore been generated to develop potent, lipophilic DXR inhibitors with the hope that these compounds could have broad anti-infective activities as well as improved pharmacokinetic properties.^{9–20}

In our early work,^{19,20} a series of lipophilic, pyridine- or quinoline-containing phosphonate compounds, such as compound **3** in Chart 1, were found to be a new class of DXR inhibitors. Our structure–activity relationship (SAR) and quantitative SAR (QSAR) studies all showed the importance of the presence of an electron-deficient aromatic ring, such as a pyridine, at the α -position of the phosphonate group, as shown in Figure S1a in the Supporting Information. In addition, superposition of the crystal structures of *Escherichia coli* DXR (*Ec*DXR) in complex with **1** and **3** (Figure S1b in the Supporting Information) suggests fosmidomycin derivatives with an α -pyridine substituent, such as compounds **4m**, **5m**, **4p**, and **5p** (**m** stands for *meta*-substituted pyridinyl compound and **p** stands for *para*-substituted pyridinyl) shown in Chart 1, could be particularly active, since these compounds likely have all favorable interactions with the protein as found for both **1** and **3**. Compounds **6** and **7** (Chart 1) with a more electron-rich phenyl ring were included in the study as a comparison.

The general method for synthesizing compounds **4–7** is shown in Scheme 1. Pyridine-3-carboxaldehyde was reduced

Scheme 1. General Synthesis for Compounds **4–7**^a



^aReagents and conditions: (i) NaBH₄, MeOH. (ii) SOCl₂. (iii) HP(O)(OEt)₂, NaH, THF, 43% for three steps. (iv) *n*-BuLi, then allyl bromide, –78 – 0 °C. (v) O₃, –78 °C, then Me₂S. (vi) NH₂OBn. (vii) NaBH₃CN, pH 3, MeOH, 40% from **8**. (viii) For R = H, HCOOH/Ac₂O; for R = Me, Ac₂O. (ix) TMSBr. (x) H₂, Pd/C, 76% from **10**.

and converted to 3-picolinyl chloride, which was reacted with sodium salt of diethylphosphite to give **8**. It was alkylated with allyl bromide, followed by ozonization to give aldehyde **9**. Upon reductive amination with *O*-benzyl-hydroxylamine and NaBH₃CN, the resulting hydroxylamine **10** was formylated or acetylated, followed by hydrolysis using bromotrimethylsilane (TMSBr) and hydrogenation, to give **4m** or **5m**. Compounds **4p** and **5p** were similarly prepared, starting from pyridine-4-carboxaldehyde. However, analogous compounds with an *ortho*-substituted pyridinyl cannot be synthesized from pyridine-2-carboxaldehyde, due to instability of the corresponding aldehyde **9** (or its *O*-benzyl-hydroxylamine oxime). Phenyl-

substituted **6** and **7** were prepared similarly, starting from benzaldehyde.

The enzyme inhibitory activities of the newly synthesized compounds **4–7** were first tested against recombinant *Ec*DXR. As shown in Table 1, compounds **4p** and **5m** were found to

Table 1. Activity of DXR Inhibitors **1–7**

	enzyme K_i (μ M)		IC ₅₀ against <i>P. falciparum</i> proliferation (μ M)	
	<i>Ec</i> DXR	<i>Pf</i> DXR	3D7 strain	Dd2 strain
1	0.034	0.021	1.17	0.44
4m	0.087	0.013	0.34	0.18
4p	0.035	0.0089	0.18	0.17
5m	0.042	0.0019	0.44	0.31
5p	0.082	0.013	0.63	0.46
6	1.5	0.48	NT ^a	NT ^a
7	0.93	0.085	3.5	1.7
CQ	NT	NT	0.021	0.12

^aNT, not tested.

exhibit potent activity with K_i values of 35 and 42 nM, respectively, being as active as **1** (K_i = 34 nM). Compounds **4m** and **5p** (K_i = 87 and 82 nM) have only slightly reduced inhibition against *Ec*DXR. However, compounds **6** and **7** containing a phenyl substituent were found to be markedly less active with K_i values of 1.5 and 0.93 μ M, respectively, showing an average of ~20-fold activity reduction as compared to pyridine-containing compounds **4** and **5**.

E. coli DXR has been used in the vast majority of previous studies, although *Ec*DXR inhibition may not be relevant and predictive in the context of developing antimalarial drugs. To this end, we next tested the inhibitory activities of these compounds against recombinant *Plasmodium falciparum* DXR (*Pf*DXR) enzyme. In our previous work,²¹ **1** was determined also to be a potent inhibitor of *Pf*DXR with a K_i value of 21 nM. We were pleased to find that pyridine-containing compounds **4m,p** and **5m,p** possess considerably higher inhibitory activity against *Pf*DXR, as can be seen in Table 1 and representatively shown in Figure S2 in the Supporting Information. The K_i values of these compounds range from 1.9 to 13 nM, with the best compound **5m** (K_i = 1.9 nM) having an acetyl and a pyridin-3-yl group being ~11 \times more active than **1**. The phenyl-containing compounds **6** and **7** again exhibited significantly lowered inhibitory potencies with K_i values of 480 and 85 nM, showing that the pyridine groups in **4m,p** and **5m,p** are able to provide an average of ~30-fold enhanced activity against *Pf*DXR.

With these highly potent inhibitors of *P. falciparum* DXR in hand, we next investigated the antimalarial activities of these compounds against the proliferation of erythrocyte-stage *P. falciparum*. The 3D7 strain of *P. falciparum* is sensitive to current antimalarial drugs, while the Dd2 strain is multidrug resistant, including chloroquine (CQ), pyrimethamine, and mefloquine. Chloroquine and **1** were included in our studies as positive controls. As also shown in Table 1, **1** was found to have good activity against the growth of *P. falciparum* strains 3D7 and Dd2 with EC₅₀ values of 1.2 and 0.44 μ M, respectively, while chloroquine exhibits more activity against the 3D7 strain (EC₅₀ = 21 nM) than it does against the drug-resistant Dd2 strain (EC₅₀ = 120 nM). The antimalarial activities of **1** and chloroquine are similar to those reported previously.⁶ As compared to **1**, the novel pyridine-containing DXR inhibitors

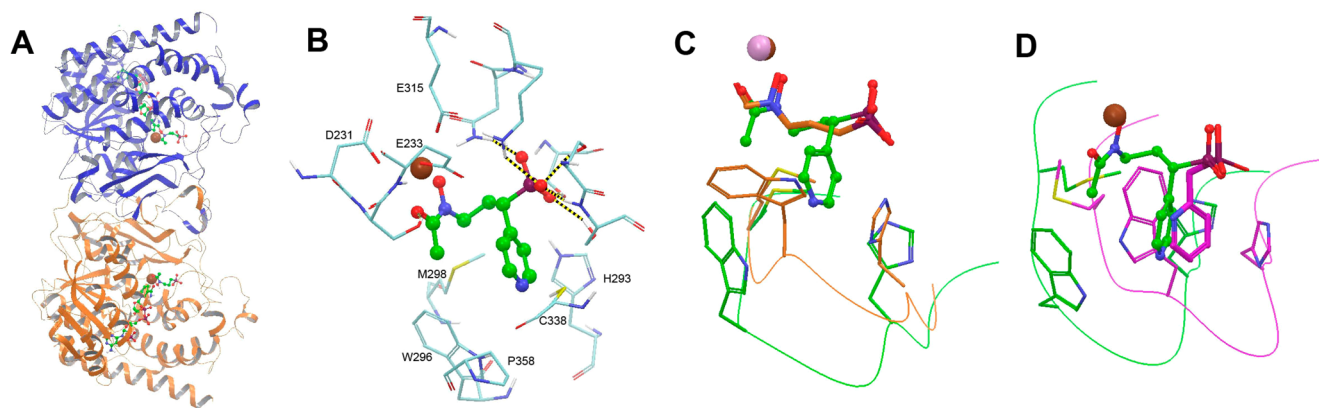


Figure 2. (A) Overall structure of *Pf*DXR in complex with Mn^{2+} (brown sphere), **5p**, and NADPH. (B) Close-up view of the active site of *Pf*DXR:**5p**. (C) Active sites of the aligned structures of *Pf*DXR:**5p** (in green) and *Pf*DXR:**1** (in orange) complexes. For clarity, only the flexible loops containing residues 290–299 of the two structures are shown as curved lines. (D) Aligned structures of *Pf*DXR:**5p** (in green) and *Ec*DXR:**3** (in purple) complexes. The colored curved lines represent the flexible loops of the proteins.

were found to have enhanced antimalarial activities. Formyl compounds **4m** and **4p** exhibit potent antimalarial activity ($\text{EC}_{50} = 340$ and 180 nM, respectively) against the 3D7 strain. Moreover, regardless of the drug resistance of the Dd2 strain, these two compounds possess even more pronounced activity against this strain of the parasite with EC_{50} values of 180 and 170 nM. The acetyl-containing compounds **5m** and **5p** have, however, relatively lowered antimalarial activity, as compared to **4m** and **4p** with a formyl group. The EC_{50} values of **5m** are 440 and 630 nM against 3D7 and Dd2, respectively, and those of **5p** are 310 and 460 nM. Compound **7** with a phenyl group was found have a relatively weak antimalarial activity against *P. falciparum* strains 3D7 and Dd2 ($\text{EC}_{50} = 3.5$ and 1.7 μM , respectively).

It is noteworthy that all of these DXR inhibitors including **1** exhibit more activity against chloroquine-resistant *P. falciparum* Dd2 strain, demonstrating that these compounds working in a different mechanism have the potential to become a new class of antimalarials without cross-drug resistance. It is also interesting that compounds **4m** and **4p** possess enhanced activities against the proliferation of *P. falciparum*, although they are relatively less active against the recombinant enzyme than the acetyl-containing inhibitor **5m**. This could be attributed to lowered cell membrane permeability of **5m**, since these inhibitors need to pass both membranes of erythrocyte and the parasite to reach their cellular target *Pf*DXR.

Potential toxicities of the newly synthesized compounds were assessed using human noncancerous fibroblast WI-38 cells. All of the pyridine-containing compounds **4** and **5** have essentially no cytotoxicity with EC_{50} values of >300 μM against the growth of WI-38 cells, showing a very high selectivity index of >1700 -fold. These compounds therefore represent promising leads for further antimalarial drug development.

The next question of interest is how these pyridine-containing inhibitors bind to *P. falciparum* DXR. Prior structural studies were mostly focused on *Ec*DXR.^{17,19,22–25} Currently, only two crystal structures of *Pf*DXR in complex with **1** and **2** are available.²⁶ However, it is known that the conformation of DXR, especially for the flexible loop at the active site, changes considerably when a ligand having a different property (e.g., hydrophobicity) from **1** and **2** binds.¹⁹ Therefore, crystallographic studies for the binding of these pyridine-containing inhibitors to *Pf*DXR are needed.

We determined the crystal structure of a quaternary complex of *Pf*DXR with compound **5p**, NADPH, and a Mn^{2+} , at 2.3 Å. Parameters for data processing and refinement are listed in Table S1 in the Supporting Information. As shown in Figure 2A, the protein crystallizes as a homodimer, with each monomer being almost identical to the other. The binding conformation of NADPH is very similar to that in previous reported structures. Each of the two Mn^{2+} ions is anchored to *Pf*DXR via coordination to three carboxylate groups of the residues Asp231, Glu233, and Glu315 (Figure 2B). The electron density and omit maps of inhibitor **5p** are shown in Figure S3 in the Supporting Information. In addition, the racemic form of **5p** was used in the study, and at the 2.3 Å resolution, it is not unambiguous to assign the absolute configuration of the α -carbon atom of compound **5p** (Figure S3 in the Supporting Information). Fifty percent occupancy for each enantiomer was therefore assigned in the final structure.

Figures 2B and S4 in the Supporting Information show the close-up view of the *Pf*DXR active site and detailed protein–ligand interactions. As always can be seen in other DXR–hydroxamate inhibitor complexes,^{22–25} the two O atoms of the hydroxamate group of **5p** chelate the central Mn^{2+} , with their distances being 2.1 and 2.4 Å. The acetyl O atom also forms a hydrogen bond with the side chain $-\text{OH}$ of Ser232. The negatively charged phosphonate group of compound **5p** is recognized by a polar environment, forming five hydrogen bonds as well as electrostatic interactions with Lys312, Asn311, Ser306, Ser270, and Ser269. The pyridine group of **5p** was found to be favorably located in a mainly hydrophobic pocket defined by residues His293, Trp296, Met298, Cys338, and Pro358, which is not present in the reported crystal structure of the *Pf*DXR:**1** complex.²⁶ In addition, a hydrogen bond between the N of the pyridine ring of **5p** and the $-\text{SH}$ of Cys338 was observed, with the distance of $\text{N}\cdots\text{H}-\text{S}$ being 3.4 Å. Because the structurally similar compound **7** cannot form a hydrogen bond with Cys338, the interaction could therefore contribute to the observed $\sim 6\times$ more inhibitory potency of **5p**. Moreover, this hydrogen bond, together with the favorable hydrophobic interactions described above, could be responsible for the enhanced activity of **5p** against *Pf*DXR as compared to **1**. It is also remarkable that the methyl group of **5p** has favorable hydrophobic interactions with Met298 and Trp296. This could explain that the acetyl-containing DXR inhibitors (e.g., **5m** and

7) in general possess increased enzyme activity than the corresponding compounds having a formyl group.

Alignment of the structures of *Pf*DXR:5p and *Pf*DXR:1 was performed to find the conformational differences of the protein. As shown in Figures S5a in the Supporting Information, these two structures can be aligned very well with a root-mean-square (rms) deviation for the protein backbone being 0.5 Å. The only notable difference is the conformation of the flexible loop consisting of residues 290–299, which is part of the active site of *Pf*DXR. As shown in Figure 2C, the loop is located much closer to the ligand-binding site in the *Pf*DXR:1 structure, with the big, hydrophobic indole ring of Trp296 being right beneath the carbon skeleton of 1. This conformation allows the active site to be largely separated from the solvent, which is considered to be important for the enzyme activity or for the tight binding of polar inhibitor 1. For example, a previous mutagenesis study showed that replacing the corresponding Trp204 in *Synechocystis* DXR with an Ala, Val, or Leu residue renders the enzyme completely inactive.²⁷ The only weakly active mutation is Trp204Phe having a smaller, aromatic phenyl side chain. In the *Pf*DXR:5p structure, the flexible loop was observed to move up to 4.5 Å away from the center of the active site to hold the pyridine of 5p and allow the Trp296 indole and several other residues to have favorable hydrophobic interactions with the inhibitor.

We next compared the binding of inhibitor 5p to *Pf*DXR with that of another pyridine-containing inhibitor 3 to *Ec*DXR.¹⁹ Protein alignment indicated that these two structures have a rms deviation for the protein backbone of 1.9 Å (Figure S5b in the Supporting Information). The close-up view of the active site is shown in Figure 2D, in which the two phosphonate groups of 5p and 3 are very close to each other, with the distance between the two P atoms being 0.5 Å. The two pyridine rings were found also to be located in the same hydrophobic pocket. Relatively large differences can be observed with respect to the conformation of the flexible loops of these two enzymes. Not only the backbones of the loops exhibit an average distance for the C_α atoms of ~4 Å between the two structures, but the orientations of the side chains of several residues are different as well. These conformational alterations could be driven mainly by two factors. First, compound 3 does not have a hydroxamate moiety. Second, the indole ring of Trp211 of *Ec*DXR has a π–π interaction with the pyridine group of 3. The steric as well as π–π stacking interactions allow the *Ec*DXR flexible loop to shift ~4 Å on average toward the pyridine binding site. For the *Pf*DXR:5p complex, the presence of the hydroxamate moiety seems not to favor the formation of a similar π–π interaction between Trp296 and the pyridine, due likely to the steric congestion between the hydroxamate moiety and the Trp296 indole group. Rather, the hydrophobic side chains of the flexible loop, such as Met298, Trp296, and His293, together with those of residues Pro358 and Cys338 from other chains, are orientated to create a generally hydrophobic environment to have favorable interactions with the pyridine ring as well as the methyl group of compound 5p.

In summary, four pyridine-containing compounds were designed, synthesized, and found to exhibit up to 11-fold enhanced inhibitory activity (K_i, 1.9–13 nM) against *P. falciparum* DXR as compared to 1. These noncytotoxic compounds also showed considerably improved antimalarial activity against the proliferation of multidrug-resistant *P. falciparum* with EC₅₀ values as low as 170 nM, showing the

promise to overcome the widespread drug resistance of the malaria parasites. The crystal structure of the *Pf*DXR:5p complex reveals the exact binding pose of the highly potent inhibitor. The backbone of 5p binds to *Pf*DXR in a similar fashion as 1. Conformational changes were observed for the flexible loop of *Pf*DXR to form a mainly hydrophobic pocket that holds and has favorable interactions with the pyridine group of the inhibitor. In addition, the hydrogen bond between the pyridine N atom and the Cys338 should also contribute considerably to the increased potency.

■ ASSOCIATED CONTENT

📄 Supporting Information

Supplementary Figures S1–S5, Supplementary Table, and Experimental Section. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Accession Codes

The coordinates for the *Pf*DXR:5p complex have been deposited in Protein Data Bank as entry 4GAE.

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Funding

This work was supported by a grant (R21AI088123) from the National Institute of Health (NIH) to Y.S. We thank the staff of the X-ray Crystallography Facility at Baylor College of Medicine for assistance in data collection.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; *Ec*DXR, *Escherichia coli* DXR; *Pf*DXR, *Plasmodium falciparum* DXR

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