DXR Inhibition by Potent Mono- and Disubstituted Fosmidomycin Analogues

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Supporting Information

ABSTRACT: The antimalarial compound fosmidomycin targets DXR, the enzyme that catalyzes the first committed step in the MEP pathway, producing the essential isoprenoid precursors, isopentenyl diphosphate and dimethylallyl diphosphate. The MEP pathway is used by a number of pathogens, including *Mycobacterium tuberculosis* and apicomplexan parasites, and differs from the classical mevalonate pathway that is essential in humans. Using a structure-based approach, we designed a number of analogues of fosmidomycin, including a series that are substituted in both the $C\alpha$ and the hydroxamate positions. The latter proved to be a stable framework for the



design of inhibitors that extend from the polar and cramped (and so not easily druggable) substrate-binding site and can, for the first time, bridge the substrate and cofactor binding sites. A number of these compounds are more potent than formidomycin in terms of killing *Plasmodium falciparum* in an in vitro assay; the best has an IC_{50} of 40 nM.

INTRODUCTION

Fosmidomycin (1) and its acetyl derivative, FR-900098 (2), are natural product antibiotics with activity against a number of important pathogens.^{1,2} They work by blocking the pathway for biosynthesis of isopentenyl diphosphate and dimethylallyl diphosphate that proceeds via the intermediate 2-C-methyl-Derythritol 4-phosphate (MEP). This MEP pathway is used in Gram-negative and some Gram-positive bacteria as well as in plant chloroplasts, algae, and apicomplexan protozoa.³ The enzymes involved are essential to the organisms possessing them yet completely absent in humans, and they have therefore received considerable attention as potential targets for antimicrobial drug discovery. Fosmidomycin and 2 act by inhibiting the second (and first committed) step in the pathway,^{4,5} in which 1-deoxy-D-xylulose 5-phosphate (DXP) is converted to MEP by the enzyme 1-deoxy-D-xylulose-5phosphate reductoisomerase (DXR, also called IspC). A number of major pathogens are dependent upon the MEP pathway, although not all are sensitive to fosmidomycin and its analogues. For example, while both 1 and 2 inhibit the DXRs of the protozoan Plasmodium falciparum (PfDXR)⁶ and Mycobacterium tuberculosis (MtDXR),⁷⁻¹⁰ the Plasmodium species are sensitive to these antibiotics in vitro and in vivo,⁶ but the mycobacteria are not.¹¹ The use of fosmidomycin as a singledrug treatment for P. falciparum malaria has been hampered by low bioavailability, rapid clearance from the parasite, and recrudescent infection,¹² although the compound has been used more successfully in combination with clindamycin.¹³ Failure to obtain biological activity against mycobacteria appears to result from poor uptake.¹¹

Considerable efforts have been made to improve the efficacy of fosmidomycin, for example, modifications to the phosphonate group,^{14,15} extensions to the hydroxamic acid group,¹⁶ and changes in the linker connecting them.^{15,17} Extensive crystallographic studies on DXRs from different species have provided the structural framework needed to understand the binding of these inhibitors^{10,18-21} as well as their α -aryl-substituted analogues.^{7,22,23} Despite the lack of biological activity of fosmidomycin itself against M. tuberculosis, the well-conserved nature of the active site of DXR,²¹ combined with the antiparasite activity of some DXR inhibitors, led us to believe that our medicinal chemistry effort based on MtDXR would produce interesting compounds for use against other pathogens where the MEP pathway is essential. It also seemed possible that some fosmidomycin analogues would in fact be active against M. tuberculosis. As part of this same-target-otherpathogen (STOP) strategy, we determined the structure of

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*Mt*DXR as an apoenzyme,⁹ and together with fosmidomycin¹⁰ and 2^{21} in ternary complexes with NADPH, as well as structures with two 3,4-dichlorophenyl-substituted fosmidomycin analogues (3 and 4, Figure 1).⁷ As expected, these C α -



Figure 1. Structures of fosmidomycin (1), 2, and the 3,4-dichlorophenyl-substituted fosmidomycin analogues (3 and 4).

substituted dichlorophenyl compounds bound to the substrate site, coordinating the metal ion in the same way as fosmidomycin, but they otherwise showed an unexpected mode of binding that forces disorder in an active-site flap. To prove that this mode of binding was not a crystallographic artifact, we determined the structure of MtDXR with one of these analogues in a new crystal form; it showed an essentially identical mode of binding.⁷ Our expression and crystallization conditions were used elsewhere to determine the structure with one of these analogues, confirming our conclusions.²² This class of compounds had previously been reported to have high activity against P. falciparum growth in a blood assay,² although the most promising was later found to be inactive in an in vivo mouse model.²⁵ We conclude that novel compounds need to be explored and that existing compounds need to be tested against other organisms.

Our structure-based approach to improving DXR inhibitors focused initially on modifying the 3,4-dichlorophenyl-substituted analogues in an attempt to utilize a conserved water-filled pocket we had earlier identified. However, this did not result in improved IC_{50} values.⁷ Here we describe a new strategy in which we extend from the hydroxamic acid group of fosmidomycin and related analogues. While we were still unsuccessful in terms of obtaining compounds active against *M. tuberculosis*, some were highly active against *P. falciparum* growth in an in vitro blood cell assay. Thus, the STOP approach has allowed us to design inhibitors that, for the first time, bridge the substrate and cofactor binding sites and provide useful new leads in the search for antimalarial drugs.

RESULTS AND DISCUSSION

General Considerations. The active site of DXR is made up of two main components, the substrate (DXP/MEP) and NADPH-binding sites. The substrate-binding site is quite small and polar/charged and so has limitations when trying to develop improved drug-like molecules; however, this has been the target area of essentially all inhibitor design to date. Structural work has demonstrated the dynamic nature of an active-site flap involved in the binding of fosmidomycin and analogues substituted in the $C\alpha$ position. Attempts have been made to extend beyond the hydroxamate group; these changes often have adverse effects on activity, although a few analogues have been obtained with submicromolar IC₅₀-values for *EcDXR* and *PfDXR*.²⁶ In our search for DXR inhibitors with in vivo activity, we decided to evaluate some of these acyl-substituted analogues on *MtDXR* and *M. tuberculosis* and to probe this binding pocket further with new analogues. Crystallographic studies on a select group of enzyme—inhibitor complexes were used to guide our design efforts.

Design, Synthesis, and Biochemical Evaluation of Hydroxamate-Substituted Analogues of Fosmidomycin. The synthetic route to the compounds shown in Table 1 was

HO HO H HO N R HO N R				
	R	MtDXR IC ₅₀ (µM)	EcDXR IC ₅₀ (µM)	PfDXR IC ₅₀ (µM)
Fosmidomycin (1)	Н	0.08 ± 0.02^{a}	0.050°	0.032°
2	methyl	$0.16 \pm 0.03^{\text{b}}$	0.051°	0.018 ^c
8a	ethyl	27.2 ± 7.7		
8b	2-furyl	48.7 ± 6.2		
8c	cyclopropyl	156 ± 68	10-4.44 ^d	
8d	phenyl	2.0 ± 0.6	0.13 ^c	0.061°
8e	3-pyridyl	1.6 ± 0.1		
8f	N NH2	3.6 ± 0.5		
8g	2-naphthyl	2.0 ± 0.3		
8h	benzyl	67.7 ± 23.3	13	n.d
8i	тът NH	>200	7.1°	3.3°
8j	°, °, °, NH O. Ph	>200	1.0°	0.4°

 Table 1. Inhibition Constants for Fosmidomycin Analogues
 (Literature Values Are Included for Comparative Purposes)

^aReference 10. ^bReference 43. ^cReference 26. ^dReference 44.

based on the work of Puyau et al. and Kurz et al.^{27,28} Synthesis started from the O-benzylhydroxylamine compound 5 (Scheme 1, which was prepared in three steps according to published procedures).^{29*} Next, acylation was performed by adding an excess of acyl chloride to a solution of 5 in dichloromethane (DCM) or via a HATU-mediated amide coupling (6i-6i).²⁹ Hydrogenolysis of the benzyl group was conducted in the presence of 10% Pd/C with methanol as solvent under hydrogen atmosphere at room temperature. Finally, phosphonate ester hydrolysis was performed using trimethylsilyl bromide (TMSBr) in dry DCM to obtain the target compounds 8a-8j in moderate to good yields. The inhibitory capacity of the analogues was evaluated in a spectrophotometric assay, in which the MtDXR-catalyzed NADPH-dependent rearrangement and reduction of DXP to form MEP was monitored at 340 nm.

Table 1 shows that none of these compounds inhibited MtDXR more effectively than fosmidomycin. The acetyl derivative, **2**, showed a small drop in activity,^{7,10} which we attribute to minor changes in the interaction with the indole ring of Trp203 in the active site of MtDXR. In the first crystal structure of a DXR ternary complex in a conformational state suitable for catalysis,¹⁰ we observed a well-defined active-site flap that covered the fosmidomycin bound in the substrate site. The relative orientation of the NADPH-binding domain suggested that the conformation would allow hydride transfer from the nicotinamide ring to the substrate. In this state, the

Scheme 1. Synthesis of Fosmidomycin Analogues 8a-8j^a



"Reagents and conditions: (i) **6a–6h**, acid chloride, DCM, rt, or **6i–6j**, carboxylic acid, HATU/DIPEA, DMF, rt; (ii) H₂, 10% Pd/C, MeOH, rt; (iii) TMSBr, DCM, rt.

indole ring of Trp203 from the flap forms a close set of interactions with the fosmidomycin backbone. The *Mt*DXR–Mn–2–NADPH ternary complex showed a similar active-site structure and enzyme–inhibitor interactions, with the newly introduced methyl group of the analogue forming good van der Waals interactions with the indole ring.²¹ Equivalent studies on ternary complexes of *Pf*DXR¹⁸ confirm the generality of these interactions, although there are subtle differences, particularly in the interaction of the phosphonate group with the enzyme,²¹ that probably underlie small differences in relative affinity of the two compounds with *Ec*DXR and *Pf*DXR (Table 1).

The decreasing potency of the ethyl (8a), 2-furyl (8b), and cyclopropyl (8c) analogues can be attributed to clashes with the indole ring of Trp203. However, the introduction of larger rings with only one extra dihedral degree of freedom (8d, 8e, 8f, and **8g**) resulted in IC_{50} s in the low micromolar range. Compounds 8i and 8j are reasonably potent on EcDXR and PfDXR,²⁶ and they were therefore resynthesized and evaluated on MtDXR. Unfortunately, they have $IC_{50}s$ greater than 200 μM on that enzyme. The active compounds in this series are unlikely to be accommodated in a ternary complex containing an ordered flap. Instead, we expect the flap to be disordered, as we observed in complexes of 3 and 4 with MtDXR;7 unfortunately, we could not obtain an experimental structure to confirm this hypothesis. It is striking that the benzyl derivative (8h) in this series shows much decreased activity as judged by IC_{50} , which could indicate an inability to form suitable interactions in the opened active site that we describe in more detail below. An alternative hypothesis (also discussed below) is that these compounds extend into the cofactor-binding site at the position of the nicotinamide ring. In such a case, the measured IC₅₀ would be higher than the binding constant due to competition with NADPH. Because the concentrations of NADPH used in the assay are high (200 μ M, compared to an estimated $K_{\rm m}$ value of 7.2 μ M),⁹ this effect would only be seen when an inhibitor binds effectively in the NADPH site. We note also that our IC_{50} values for MtDXR were systematically higher than those published for the EcDXR and PfDXR enzymes, see Table 1. This results from our choice of more stringent assay conditions, which should allow better discrimination among tight-binding compounds.

Unfortunately, none of these compounds had significant in vitro activity against *M. tuberculosis*; they were not tested against *P. falciparum*.

The Disubstituted Fosmidomycin Analogues. The approximate coplanarity of the hydroxamate and dichlorophenyl groups in the experimentally determined enzyme-inhibitor structures of 3 and 4 with MtDXR,⁷ with both groups aimed out toward solvent, suggested that we could combine the C α substituent of 3 and the phenyl substituent of the hydroxamate in 8d to produce the disubstituted compound, 14a (Table 2). Compound 14a served as the lead for our second series in which various substituents were added to the phenyl ring with the aim of reaching into the NADPH-binding site (see below). Groups were selected among commercially available acyl chlorides and carboxylic acids with a variety of size and other properties, guided by molecular modeling using the experimental MtDXR-Mn-14a structures (described below). This limited set of related analogues primarily investigated substitutions at the ortho position (see Table 2). All compounds were synthesized as racemic mixtures, as outlined in Scheme 2.

The key intermediate 11 was obtained in a previously reported, three-step synthetic route,^{7,24} starting with an oxidative Heck reaction between acrolein and 3,4-dichlorophenyl boronic acid, 30,31 to furnish (E)-3-(3,4dichlorophenyl)acrylaldehyde (9). This was followed by 1,4addition of triethyl phosphate, and finally reductive amination with O-benzylhydroxylamine in the presence of sodium cyanoborohydride as reducing reagent, to produce 11. Depending on availability of starting materials, the next step was performed with acyl chlorides in DCM and triethylamine, furnishing compounds 12a-12d in 46-92% yield, or with suitable carboxylic acids using propane phosphonic acid anhydride (T3P) in ethyl acetate as a coupling reagent (compounds 12f-12k, 43-75% yields). The 2-(2thienylmethyl)benzoic acid used to obtain compound 12e was prepared from 2-(2-thienylcarbonyl)benzoic acid by refluxing for 48 h in ammonia with zinc powder. The benzyl protecting group in compounds 12a-12d and 12f-12k was removed via palladium-catalyzed (10% Pd/C) hydrogenation under atmospheric hydrogen pressure. Probably due to catalyst poisoning, the reaction did not give a product with the thiophene derivative (12e), even in harsher, acidic conditions. Debenzylation of 12e was easily achieved with trifluoroacetic acid (TFA), TMSBr, and thioanisole. Final compounds were obtained by hydrolysis of the diethylphosphonates 13a-13k by

 Table 2. Inhibition of MtDXR Activity and Plasmodium

 Growth by Disubstituted Fosmidomycin Analogues



TMSBr in DCM and purified on reversed phase HPLC using gradient elution with acetonitrile in 0.1% aqueous TFA.

Biochemical/Biological Evaluation of the 14a Series. Table 2 summarizes results for the compounds in the second series. The fact that these compounds were tested as racemic mixtures should be remembered in the interpretation of the data. While the crystallographic work (see below) indicates that the S-enantiomer is the predominant form bound to the enzyme, we have no proof at this time that activity of the *R*-enantiomer is entirely lacking. Therefore, the reported IC₅₀s should be viewed as upper limits, with the true value probably closer to one-half of this. The lead compound **14a** had submicromolar *Mt*DXR activity (measured IC₅₀ = 0.32 μ M) and remarkably good in vitro growth inhibition in the *P. falciparum* parasite assay (IC₅₀ = 0.04 μ M). Enzyme inhibition by this compound was less efficient than the C α -substituted formyl derivative **3** but better than the acetyl derivative **4**

(Table 2). Introduction of progressively larger substituents at the ortho position of the phenyl ring resulted in a decline of MtDXR inhibitory activity. Surprisingly, all of the orthosubstituted compounds were inactive in the P. falciparum parasite growth assay. When the triazole substituent was moved from the ortho (14i) to the meta (14i) and para positions (14k), the MtDXR IC₅₀ values were improved by 2 and 1 orders of magnitude, respectively. The activity in the P. falciparum parasite assay was also recovered in these two compounds. In fact, 14j had an MtDXR IC50 value that is comparable to 2. The best in vitro growth inhibition of P. falciparum, however, is shown by the lead compound, 14a (0.04 μ M); it has five times the activity of 14k (0.19 μ M), and 10 times the activity of 14j (0.39 μ M). 14a also has eight times the published activity of 2 $(0.32 \ \mu M)^{24}$ and at least an 8-fold improvement over fosmidomycin in our in vitro growth inhibition assays (fosmidomycin was used as a control in our assays, and had IC₅₀ values in the range 0.2–0.4 μ M, compared to values of 0.3–1.1 μ M found in the literature).^{6,24}

General Comments on Crystallographic Results. Details of the crystallographic work are included in the Supporting Information. All of the complexes represent monoclinic and tetragonal crystal forms that we have described previously, although there is a large variation in the unit cell constants for crystals of the monoclinic form. In the monoclinic crystals described here, as for most of our MtDXR structures, the asymmetric unit contains a complete dimer where the NADPH-binding site is more closed in one subunit (chain A; conformational state being described using a yardstick¹⁰ to describe relative orientations of the N- and C-terminal domains) and more open in the other (chain B). Somewhat counterintuitively, the active-site flap in the closed (inhibitorbound) site is disordered in each complex, while the flap of the more open (empty) active site is generally well ordered and directed away form the active site because of interactions with the closed-chain active site of a crystallographically related dimer. The A chain in this crystal form has a higher overall temperature factor than the B chain, particularly in the edge helix of the NADPH-binding domain. Our concern that conclusions about ligand binding could be influenced by crystal contacts had led us to carry out extensive screening of crystallization conditions that ultimately identified a second crystal type. This tetragonal form requires NADPH, and the asymmetric unit also contains a dimer.²¹ In this case, the two subunits adopt the same active-site conformation and are related by an almost perfect noncrystallographic 2-fold axis. In the MtDXR-Mn-2-NADPH ternary complex studied earlier in the tetragonal form (Protein Data Bank (PDB) entry 4A03), the active-site flap is in a well-defined closed substrate-binding conformation that makes extensive interactions with the inhibitor.²¹ However, the flaps were not well ordered in the MtDXR-Mn-14a-NADPH complex studied here. Overall, therefore, the behavior of the active-site flap in structures of the complexes described in the present study was more similar to that seen in complexes with the dichlorophenyl C α -substituted fosmidomycin analogues 3 and 4 than to the structures in complex with fosmidomycin or 2.

Mode of Binding to 14a. We were able to crystallize **14a** in complex with *Mt*DXR in both of the known space groups, with and without the NADPH cofactor, and so can be certain that our conclusions are unbiased by any crystallographic artifacts. Figure 2a shows a superposition of the three independent views we obtained of **14a** binding to *Mt*DXR.

Scheme 2. Synthesis of Disubstituted Analogues^a



^{*a*}Reagents and conditions: (i) Pd(OAc)₂, acrolein, dmphen, *p*-benzoquinone, 100 °C, MW; (ii) (a) TEP, phenol, 100 °C, (b) 2 M HCl, acetone, 100 °C; (iii) (a) *O*-benzylhydroxylamine hydrochloride, pyridine, EtOH, rt, (b) NaCNBH₃, MeOH, HCl, rt; (iv) acyl chloride, TEA, DCM, rt or carboxylic acid, T3P, TEA, EtOAc, 0 °C then rt; (v) H_2 , 10% Pd/C or TMSBr, TFA, thioanisole; (vi) TMSBr, DCM, rt.



Figure 2. (a) Superposition of MtDXR-14a structures from the monoclinic (silver carbons) and tetragonal (carbons in darker shades of gray) crystal forms. Side chains from residues that coordinate the active-site metal ion are included, as well as the nicotinamide ring of the NADPH cofactor from each active site in the MtDXR-Mn-14a-NADPH ternary complex structure. Interactions with the metal are shown with small bubbles. (b) Interactions at the active site of the 14a complex in the monoclinic form. Green bubbles indicate interactions of the phosphonate group with the enzyme, gold are interactions at the active site metal, and blue are intramolecular ring-ring interactions in the inhibitor. (c) Superposition of 14a (monoclinic form, gray carbons), 3 (light-red carbons, PDB entry 2Y1D), and 4 (yellow carbons, PDB entry 2Y1G). Superpositions were performed using the enzyme coordinates as described in the Experimental Section.

The ligand conformations are very similar, as are most of the interactions with the enzyme (Figure 2b). Metal-ion coordination is essentially unchanged with distances in the expected range; the hydroxamic acid group in all cases adopts the synperiplanar conformation. The largest difference observed is that in the MtDXR-Mn-14a complex (i.e., that lacking NADPH), a small change in the side chain of Asp151 (Figure 2a) modifies its coordination to the metal ion, with small rearrangements of the next shell of side chains, in particular the Lys128-Asp151-His154-Glu225 cluster. The phosphonate group is involved in an extensive set of hydrogen-bond and salt-link interactions that we have described earlier for complexes with 1, 2, 3, and 4. Despite the differences in substitution at the hydroxamate, the conformations of 14a, 3, and 4 are also extremely similar (Figure 2c). The orientation and interactions of each dichlorophenyl ring within the enzyme are identical within the limits of the diffraction experiments. The conformation of the phenyl ring at the hydroxamate in 14a allows an intramolecular interaction between the two aromatic groups, which could enhance binding by increasing the

population of the "correct" conformation of the ligand in solution (Figure 2b). The ring planes are inclined to one another by \sim 47°, and their centers are separated by \sim 4.3 Å. Some ring-edge atoms make van der Waals contact (3.3–3.5 Å), while others are further apart (5–5.5 Å). The other face of the phenyl ring packs against the side chain of Met267, with distances to the side-chain sulfur atom in the range 3.7–4.3 Å. In this conformation, the faces of both phenyl rings are inaccessible to solvent, while portions of the edge of each ring are exposed. The proximity of the phenyl ring and the nicotinamide ring of NADPH (Figure 2a) inspired us to design analogues that could reach into the cofactor-binding pocket.

Crystal Structures of *Mt***DXR in Complex with 14f and 14i.** Despite their relatively high IC₅₀ values (~15 μ M), good solubility of the compounds allowed us to obtain crystal structures for complexes of *Mt*DXR with **14f** and **14i**. Both structures lack electron density for a metal ion in their A-chain active sites, although MnCl₂ was included in the soak solution of the former and the cocrystallization experiments of the latter. The restraints of metal coordination via their hydroxamic acid

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Figure 3. (a) Superposition of 14a (monoclinic form, gray carbons), 14f (light-blue carbons), and 14i (magenta carbons), shown in stereo. The gold sphere represents the active-site metal ion in the 14a complex. The superposition was performed using the enzyme coordinates (as described in the Experimental Section) and therefore shows differences in ligand position within the active site. (b) Superposition of the same three structures was performed using the fosmidomycin backbone atoms to highlight differences in the inhibitor conformation.

groups have thus been removed, and the three metalcoordinating side chains of the enzyme are free to take on other conformations. Despite their close chemical similarity, 14f and 14i show striking differences in their mode of binding to the enzyme (Figure 3a). Their propyl chain conformations are very similar (Figure 3b, root-mean-squares fit for these atoms 0.13 Å), and the differences arise from a rigid body rotation (17°) around and translation (0.9 Å) along an axis close to the phosphonate groups. As a result, the hydroxamate group of 14f is pulled significantly closer to the metal-binding site (\sim 2 Å at the hydroxamate carbonyl oxygen atom). Close to the rotation axis, the interactions with the enzyme are essentially identical, with the same residues involved in interactions with the phosphonate (hydrogen bonds with main-chain nitrogen of residue 177, and side chains of Ser177, Ser213, Asn218, and Lys219, as well as two water molecules), and with the dichlorophenyl ring (Figure 2b). In neither structure is the phenyl ring completely buried. At their respective hydroxamate groups, the differences in binding mean that quasi-equivalent sets of interactions are seen. For example, the hydroxyl group of Ser152 interacts with the hydroxamate oxygen in each complex, although these inhibitor oxygens are separated by 2.8 Å. Likewise, the N-hydroxyl oxygen of each analogue interacts with the side chain of Glu153, although the detailed sets of interactions differ. In the *Mt*DXR-14i structure, a portion of the active-site flap in chain B is disordered (corresponding to residues A204-208), although it is ordered in the structure with 14f.

The ortho-substituted phenyl ring in both metal-free complexes (14f and 14i) makes intramolecular ring-ring interactions with the dichlorophenyl ring. In each case, the rings are closer to coplanarity than is observed in the MtDXR-Mn-14a complex (Figure 3b). The ortho groups of the two compounds are directed toward, and entering, the NADPH-binding site. Both have an intramolecular contact to the *para*-chlorine atom (in the range 3.5–3.7 Å), but no intramolecular hydrogen-bonding interactions are observed.

Direct Measurement of K_D **Using Fluorescence Spectroscopy.** As the structures of complexes with 14f and 14i showed that their ortho substituents impinge on the NADPH site, it seemed likely that the measured IC₅₀ would not be a good measure of ligand affinity, as discussed above for compound 8h. This led us to seek other methods for determining the affinity of *Mt*DXR for the various compounds.

A direct fluorescence assay, based on changes in the environment of one or more of the six tryptophan residues of the protein, proved a feasible option; the results with a number of compounds are presented in Table 3. For five

Table 3. Binding Constants Determined by Fluorescence
Spectroscopy for a Selection of Compounds against MtDXR
in the Presence of 1.5 mM $MnCl_2^a$

	$K_{\rm d}$ with MnCl ₂ (μ M)	Mt DXR IC ₅₀ (μ M)		
1	0.04	0.08		
3	0.12	0.15		
4	0.36	0.70		
8h	0.32	67.7		
14a	0.21	0.32		
14d	0.09	8		
14f	0.09	19		
14i	0.04	13		
14j	0.17	0.14		
14k	0.20	1.2		
^a IC ₅₀ values for <i>Mt</i> DXR are also shown for easier comparison.				

compounds (1, 3, 4, 14a, and 14j), the measured dissociation constant (K_D) in the presence of MnCl₂ agrees well with the degree of enzyme inhibition as indicated by the IC₅₀s. The three compounds with ortho substitutions (14d, 14f, and 14i) showed tighter binding than the unsubstituted lead (14a). The IC₅₀s of these compounds, however, were much higher than their K_D values (Table 2), and so we conclude that, like 14f and 14i, other compounds with ortho substituents are likely to interfere with the binding of NADPH. The one para-substituted compound in the study, 14k, seems to have similar behavior, but more compounds will need to be synthesized and tested to determine if the effect is a general one.

Structure–Activity Relationship (SAR) of Analogues 14a–14k. None of the new compounds inhibited the growth of *M. tuberculosis* strain H37Rv in a microplate Alamar blue assay (data not shown). In contrast, the disubstituted analogues (14a–14k) did show a range of activities against the *P. falciparum* 3D7 strain in an in vitro growth assay (Table 2). Having obtained the crystal structures of three of the analogues in Table 2, we could address the SAR of this set of compounds. The *Mt*DXR inhibition of 14a is poorer than that of the formyl derivative 3 but better than the acetyl derivative 4 described

earlier (Table 2).⁷ The structures of complexes with these three compounds (3, PDB entries 2Y1D and 2Y1F; 4, 2Y1G; 14a, 3ZHX and 3ZHY) all show a disordered active-site flap and otherwise share a great deal of similarity. In particular, the fosmidomycin backbone, dichlorophenyl ring, and metal-ion coordination are highly conserved in these structures (Figure 2c). Despite the poorer enzyme inhibition relative to 1, 2, and 3, the activity of 14a on *P. falciparum* growth is superior (40 nM, Table 2) and closely comparable to the reference compound artemisinin (IC₅₀ in the range 5–25 nM in our assays, similar to the value of 10 nM reported by Baniecki et al.³²

Introducing groups at the ortho position in the phenyl ring at the hydroxamic acid resulted in compounds that have poorer MtDXR inhibition as judged by IC₅₀ values; only the analogue with a methyl substituent (14b) had an IC₅₀ that is submicromolar. In the MtDXR-Mn-14a-NADPH structure, the ortho position and the edge of the nicotinamide ring make van der Waals contact (~3.5 Å). If substituents are introduced here, clashes would become severe in this orientation of the ring. If the substituted phenyl ring is flipped by 180°, clashes with the dichlorophenyl ring or protein side chains would develop. Similar conclusions follow if we superimpose the MtDXR-Mn-14a-NADPH and MtDXR-14f structures to look for potential clashes between inhibitor and cofactor. However, the high concentration of NADPH needed for the activity assay dictates that the IC₅₀ measurements for compounds entering the cofactor site will underestimate their actual binding constants. Our modeling of meta and para substitutions suggested that these would be easier to accommodate, which is borne out by the results with a limited number of modifications (involving a triazole ring) at each of the three positions (Table 2). Although both 14j and 14k show good inhibition of MtDXR activity and P. falciparum growth, neither improved on 14a.

CONCLUSIONS

The natural product antibiotic fosmidomycin (1) and its acetyl derivative 2 have attracted a great deal of attention because they inhibit the growth of Plasmodium species in vitro and in vivo. Haemers et al.²⁴ synthesized a number of fosmidomycin analogues with an α -aryl substituent, several of which showed improved activity against P. falciparum in an in vitro growth assay. Complexes with the most active (3) and its slightly less potent acetyl derivative $(4)^{7,22}$ showed that these inhibitors destabilize the active-site flap that packs against the inhibitors in the metal-free complexes of $EcDXR^{20}$ and ternary complexes of $PfDXR^{18}$ and $MtDXR^{10,21}$ The $C\alpha$ -substituents of 3 and 4 bind in a manner that leaves one face of the phenyl ring accessible to solvent. The coplanarity of the phenyl ring and the hydroxamic acid in these structures suggested new designs that would optimize this mode of binding by replacing the methyl group of 4 with a phenyl group. We have now tested this hypothesis by preparing compound 14a, which indeed has submicromolar MtDXR activity (IC₅₀ = 0.32 μ M) and remarkably good in vitro growth inhibition in a P. falciparum parasite assay (IC₅₀ = 0.04 μ M). The crystal structures of MtDXR together with 14a confirm the success of the design

Direct K_D measurements, as well as the IC₅₀s, show the binding to *Mt*DXR is ordered **1**, **3**, **14a**, and **4** with fosmidomycin the tightest binder. Compound **14a**, however, has improved inhibition of the growth of *P. falciparum*

compared to fosmidomycin and is 2.5-fold more effective than 3, the best compound described by Haemers et al.²⁴ The improved in vitro activity of 14a may reflect differences between *Mt*DXR and *Pf*DXR or properties of the compound that increase uptake or disfavor export and metabolism. A series of decorations at the ortho position on the ring did not improve inhibitory properties in either enzymatic or parasite growth assays (Table 2), although substitutions at the meta and para positions gave superior results. The micromolar or better enzymatic and in vitro activities of compounds 14j and 14k suggest that these triazole substitutions may be making specific interactions with the enzyme.

The structures of the metal-free enzyme in complex with two closely related inhibitors indicate new binding poses that may help in the design of additional inhibitors. Restraining a hydroxamate group to coordinate with the active-site metal ion while maintaining the extensive set of interactions in the phosphonate binding site may limit the ability of this series of compounds to form optimal intra- and intermolecular interactions within the boundaries of the MtDXR active site. The propyl chain conformations of 14f and 14i are very similar in the metal-free active sites despite the different detailed set of interactions and show only small changes when coordinating the metal ion (Figure 3b). This suggests that these backbone conformations are close to a local energy minimum. The metalfree complexes show more pronounced face-to-face ring interactions, which are not associated with optimal electrostatic interactions between aromatic rings in proteins. This may be a consequence of accommodating the extensions associated with these compounds as well as the optimization of proteininhibitor interactions. Further, the structures indicate that ortho substitutions to the phenyl ring at the hydroxamate enter the NADPH binding site and, therefore, make it difficult to interpret the enzymatic IC₅₀ measurements as indicators of tight binding. The fluorescence-based binding data, however, indicate that 14f and 14i have comparable dissociation constants to fosmidomycin and that these compounds bind more tightly than 14a. The relatively poor in vitro $IC_{50}s$ of 14f and 14i relative to 14a, therefore, we attribute to competition with the NADPH cofactor. The good enzymatic and in vitro activity of the meta- and para-substituted triazoles, 14j and 14k, on the other hand, we attribute to a 14a-like mode of binding where the substitutions are directed away from the nicotinamide ring binding site of the cofactor into the additional volume created by the disordered active-site flap. This space is also the likely binding site for the extensions showing good enzyme inhibition in the series shown in Table 1.

Our work provides new insights on how DXR interacts with a promising series of novel compounds, and suggests new routes for the creation of even better, biologically active molecules.

EXPERIMENTAL SECTION

Chemistry. The microwave reactions were performed in a Smith synthesizer producing controlled irradiation at 2450 MHz with a power of 0–300 W. The reaction temperature was determined using the built-in online IR sensor. Flash column chromatography was performed on Merck silica gel 60 (40–63 μ m). Analytical thin layer chromatography was done using aluminum sheets precoated with silica gel 60 F₂₅₄. Analytical RPLC-MS was performed on a Gilson HPLC system with a Finnigan AQA quadrupole mass spectrometer with detection by UV (DAD) using an Onyx Monolithic C18 column (50 mm × 4.6 mm). Acetonitrile in 0.05% aqueous HCOOH at a flow rate of 4 mL/min was used as a mobile phase. Preparative RP-HPLC was

performed on a system equipped with a Zorbax SB-C8 column (150 mm \times 21.2 mm) in H₂O/MeCN gradient with 0.1% CF₃COOH or with 0.05% HCOOH as mobile phase at a flow rate of 5 mL/min, unless stated otherwise in experimental details. Purity of the final compounds was determined by RP-HPLC on a Restec Allure biphenyl column (5 μ m, 4.6 mm \times 50 mm) and an ACE 5 C18 column (5 μ m, 4.6 mm \times 50 mm) in a H₂O/MeCN gradient with 0.1% CF₃COOH and UV detection at 220 nm. All the compounds showed purity above 95%, except compound 14h (93% pure). ¹H and ¹³C NMR spectra were recorded on Varian Mercury Plus instrument; ¹H at 399.8 MHz and ¹³C at 100.5 MHz. The chemical shifts for ¹H NMR and ¹³C NMR were referenced to TMS via residual solvent signals (¹H, CDCl₃ at 7.26 ppm, CD₃OD at 3.31 ppm; ¹³C, CDCl₃ at 77.16 ppm, CD₃OD at 49.00 ppm). ³¹P spectra were recorded on a Varian Mercury 300 Plus instrument at 121.4 MHz; the chemical shifts were referenced to 85% H₂PO₄ which was used as an external standard. Molecular masses (HR-ESI-MS) were determined on a Micromass Q-Tof2 mass spectrometer equipped with an electrospray ion source. Compounds 9, 10, and 11 were synthesized as previously reported.⁷

Materials. Most reagents were purchased from commercial suppliers and used without further purification. DCM was distilled under nitrogen immediately before use; calcium hydride was used as a drying agent.

Diethyl-3-(*N*-(**Benzyloxy**)**phenylamido**)-**1**-(**3**,**4**dichlorophenyl)propylphosphonate (12a). [3-Benzyloxyamino-1-(3,4-dichlorophenyl)-propyl]-phosphonic acid diethyl ester (11) (0.10 g, 0.22 mmol), benzoyl chloride (51 μ L, 0.44 mmol), and triethylamine (94 μ L, 0.67 mmol) in DCM (3.0 mL) were stirred at room temperature for 26 h. When the reaction was completed, the mixture was diluted with water and the aqueous layer was extracted with DCM. The combined organic fractions were dried over anhydrous MgSO₄ and filtered; the solvent was removed under vacuum, and the product was purified by column chromatography using ethyl acetate/isohexane (1/4), then DCM to yield 0.056 g (46%) of the desired product.

1-(3,4-Dichlorophenyl)-3-(*N*-hydroxyphenylamido)propylphosphonic Acid (14a). A mixture of 12a (0.06 g, 0.10 mmol) and 10% Pd/C (0.01 g, 0.10 mmol) in methanol (1.5 mL) was hydrogenated at atmospheric pressure for 5 h. After reaction was finished, the mixture was filtered through a Celite pad and concentrated under vacuum to give compound 13a. To a solution of crude phosphonate diethyl ester (13a) (0.05 g, 0.10 mmol) in dry DCM (1 mL), TMSBr (68.5 μ L, 0.50 mmol) was added dropwise under N₂ at room temperature. After 10 h, the volatiles were removed under vacuum to give the corresponding phosphonic acid derivative. The product was purified on preparative RP-HPLC using gradient elution (15–60% acetonitrile in 0.1% aqueous TFA) to yield 0.02 g (53%) of 14a. ¹H NMR (400 MHz, CD₃OD) δ ppm: 2.25 (m, 1H), 2.61 (m, 1H), 3.11 (m, 1H), 3.62 (m, 2H), 7.23 (m, 1H), 7.42 (m, 7H).

Crystallography. Diffraction data were collected from single crystals at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) or at MAXlab (Lund, Sweden). The diffraction images were indexed and integrated using MOSFLM³³ and scaled with SCALA,³⁴ elements of the CCP4 package (CCP4, 1994).³⁵ The crystallized complexes belonged to space groups we have previously described $(P2_1^7 \text{ or } P4_32_12^{21})$. The *Mt*DXR-14i complex, however, had a rather large change in the unit cell parameters compared to our earlier published values, and so the structure was solved by molecular replacement using Phaser.³⁶ All complexes were refined with alternating reciprocal-space refinement with REFMAC5³⁷ and manual rebuilding with O.38 Stereochemical restraints for each ligand were generated in O with the qds tools, and the fitted model was then used to generate restraints for the refinement with a suitably edited dictionary generated by REFMAC5. The hydroxamate group was restrained to planarity with target standard deviations of 0.02 Å. Metal coordination target distances were taken from Harding³⁹ and used with standard deviations of 0.02 Å. All inhibitors were synthesized as racemic mixtures and, at the resolution of these studies, we cannot unequivocally state that one enantiomer binds exclusively. We have,

therefore, followed our earlier policy of depositing the best fitting enantiomer (in all cases, S), although both enantiomers were evaluated during the refinements. Water molecules were added using the profilebased methods implemented in O, and structural comparisons were made with the Lsq commands in O with default or close-pair C α cutoffs.40 Ring-to-ring orientations were calculated with the Ring commands available in O version 14. Figures were created in O and rendered with Molray.⁴¹ Secondary structure assignments were made with the yasspa command in O and drawn with red-blue rainbow coloring for one chain. Data collection and refinement statistics for the various new complexes are given in Supporting Information, Table S2. Briefly, two distinct 14a complexes were solved in different space groups, with and without NADPH, and refined at resolutions of 2.3 and 2.0 Å, with crystallographic R-factors of 20.1% and 18.4%, respectively. MtDXR 14f and 14i complexes were refined at resolutions of 2.25 and 1.90 Å, with crystallographic R-factors of 20.0% and 20.5%, respectively. More structural details, and figures showing the electron density around each of the ligands, are included in the Supporting Information. The electron density for each PDB entry is available at the Uppsala Electron Density Server.⁴⁴

ASSOCIATED CONTENT

Supporting Information

Additional experimental details concerning synthesis of compounds, spectroscopic data, protein expression and purification, enzyme activity/inhibition/binding studies, and crystallization. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Coordinates and structure factor data have been deposited at the PDB with entry codes: *Mt*DXR-Mn-14a (3ZHX), *Mt*DXR-Mn-14a-NADPH (3ZHY), *Mt*DXR-14f (3ZHZ), and *Mt*DXR-14i (3ZI0).

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; *EcDXR, Escherichia coli* DXR; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; *MtDXR, Mycobacterium tuberculosis* DXR; PDB, Protein Data Bank; *PfDXR, Plasmodium falciparum* DXR; SAR, structure–activity relationship; STOP, same-target-other-pathogen

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