POTENT INHIBITORS OF A SHIKIMATE PATHWAY ENZYME FROM MYCOBACTERIUM TUBERCULOSIS: COMBINING MECHANISM- AND MODELING-BASED DESIGN

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Running title: Potent active site inhibitors for M. tuberculosis DAH7PS

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Tuberculosis (TB) remains a serious global health threat, with the emergence of multi-drug resistant strains highlighting the urgent need for novel antituberculosis drugs. The enzyme 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAH7PS) catalyzes the first step of the shikimate pathway for the biosynthesis of aromatic compounds. This pathway has been shown to be essential in Mycobacterium tuberculosis, the pathogen responsible for TB. This enzyme catalyzes a condensation reaction between phosphoenol pyruvate (PEP) and erythrose 4-phosphate to give 3-deoxy-D-arabino-heptulosonate 7-phosphate. The enzyme reaction mechanism is proposed to include a tetrahedral intermediate, which is formed by attack of an active site water on the central carbon of PEP during the course of the reaction. Molecular modeling of this intermediate into the active site reported in this study shows a configurational preference consistent with water attack from the re face of PEP. Based on this model, we designed and synthesized an inhibitor of DAH7PS that mimics this reaction intermediate. Both enantiomers of this intermediate mimic were potent inhibitors of M. tuberculosis DAH7PS, with inhibitory constants in the nanomolar range. The crystal structure of the DAH7PS-inhibitor complex was solved to 2.35 Å. Both the position of the inhibitor and the conformational changes of active site residues observed in this structure correspond closely to the predictions from the intermediate modeling. This structure also identifies a water molecule which is located in appropriate position to attack the re face of PEP during the course of the reaction, allowing the catalytic mechanism for this enzyme to be clearly defined.

INTRODUCTION

Tuberculosis (TB) remains a serious global health threat with over a million deaths per year. The recent emergence of multi-drug resistant strains of Mycobacterium tuberculosis, the pathogen that causes the lung disease, highlights the need for rapid development of new antibacterial drugs to combat TB (1-4).
pathway is absent in humans, and inhibitors of amino acid biosynthesis have been shown to be effective antimicrobial and herbicidal agents (8, 9). Gene disruption studies have demonstrated that *M. tuberculosis* is not viable if the shikimate pathway is not operational (10). These findings make DAH7PS an attractive target for drug development.

DAH7PS catalyzes the aldol-like condensation of phosphoenol pyruvate (PEP) and D-erythrose-4-phosphate (E4P) to yield 3-deoxy-D-arabino-heptulosonate 7-phosphate (Figure 1 (a)). The reaction mechanism has been subject to extensive study, and many of the key details of the mechanism have been elucidated (11-16). The reaction occurs stereospecifically with respect to both substrates, with the *si* face of PEP attacking the *re* face of E4P. A divalent metal ion present in the active site is essential for activity. The reaction takes place with cleavage of the C-O bond of PEP rather than the O-P bond, requiring water to attack C2 of PEP at some stage during the reaction.

**Figure 1**

A mechanism consistent with the data published to date starts with nucleophilic attack of PEP at the E4P aldehyde moiety resulting in the formation of oxocarbenium species 1 (Figure 1 (b)). This oxocarbenium ion 1 can be attacked by an active site water to form phosphohemiketal 2. It is noteworthy that water can potentially attack from either face of 1, giving rise to two possible diastereoisomers of tetrahedral intermediate 2, differing in their absolute configuration at C2. Although this stereogenic centre is transient, and the stereochemical information is lost by elimination of phosphate in the final step to generate the product DAH7P (3), the geometry of the enzyme active site is likely to favor stereoselective attack of water to form one diastereoisomer of 2 preferentially. In this way DAH7P is formed in its acyclic form, and cyclizes into its cyclic pyranose form following release from the enzyme.

3-Deoxy-D-manno-octulosonate 8-phosphate synthase (KDO8PS), an enzyme involved in the synthesis of the lipopolysaccharide of cell wall of Gram-negative bacteria, is structurally and evolutionarily related to DAH7PS. KDO8PS catalyzes an analogous reaction between PEP and the five-carbon sugar arabinose 5-phosphate (A5P) (17). Studies of the reaction mechanism of KDO8PS suggest that water is activated by the active site metal prior to attack at the reaction intermediate (18). Computational and structural studies of KDO8PS indicate that after activation, water or hydroxide attacks from the *si* face of PEP resulting in overall *syn* addition of A5P and a hydroxyl group to the double bond of PEP (19). Due to lack of comparable data for the reaction catalyzed by DAH7PS it is unclear whether these findings also apply to the reaction catalyzed by DAH7PS. Despite the similarities in their reaction chemistry, a number of key structural and mechanistic differences of DAH7PS and KDO8PS such as divalent metal ion requirement and substrate specificity have also been identified (20).

*M. tuberculosis* DAH7PS (*MtudDAH7PS*) is the only member of the DAH7PS type II family that has been structurally characterized (21-23). Type II DAH7PS enzymes show very little sequence homology with their type I DAH7PS counterparts, which are relatively well characterized and are found in organisms such as *Escherichia coli* (14, 15) and *Saccharomyces cerevisiae* (16, 24). Both type I and type II DAH7PS enzymes share the common triosephosphate isomerase (TIM barrel) fold, and mechanistic studies have suggested that the key details of the reaction chemistry are similar for enzymes of both DAH7PS types (25). Despite the low sequence similarity the active site architecture of *MtudDAH7PS* shows remarkable correspondence to that of type I enzymes (Figure 2). PEP is held in place by a tightly knit network of interactions. The PEP phosphate forms salt bridges to Lys306 (*MtudDAH7PS* numbering) and Arg337 and forms a hydrogen bond to the backbone N-H of Glu283, while the PEP carboxylate forms a salt bridge to Arg126. The metal ion is coordinated by His369, Glu411, Cys87 and Asp441 in a trigonal pyramidal fashion, leaving one coordination site potentially free for the carbonyl moiety of the E4P aldehyde moiety, thereby activating this functionality to nucleophilic attack. The proposed E4P binding site is constituted mostly by a 133KPRS136-motif that is highly conserved in the type II subfamily, whereas members of the type I DAH7P synthase subfamily display a very similar, also highly conserved KPRT-motif at the equivalent position. The best indication of how E4P
is bound to the enzyme can be found in the crystal structure of *S. cerevisiae* type I DAH7PS (*Sce*DAH7PS) in complex with the E4P analogue, glycerol 3-phosphate (G3P) (16). Arginine and threonine from the KPRT motif interact with the G3P phosphate moiety, while the primary hydroxyl group of G3P forms a hydrogen bond to the backbone carbonyl of the proline residue of the KPRT motif, and the secondary hydroxyl interacts with the metal-coordinating aspartate.

Despite the many similarities in active site architecture and reaction chemistry between DAH7P synthases from different organisms and families, there are some significant distinctions of type II DAH7P synthases. For example, Trp280 forms part of the PEP-binding site in *Mtu*DAH7PS, whereas in the *E. coli* and *S. cerevisiae* enzymes this residue is replaced by Ala. However, in the type I enzymes the space occupied by Trp280 is occupied by a Tyr residue from another part of the structure. Another significant distinction between *Mtu*DAH7PS and all other DAH7P synthases characterized to date is its complex mechanism of allosteric regulation which we have recently investigated in detail (22). Furthermore, the recent discovery that *M. tuberculosis* chorismate mutase is activated in complex with DAH7PS suggests a key role of DAH7PS in the regulatory network of aromatic metabolism of *M. tuberculosis* (26).

### EXPERIMENTAL PROCEDURES

#### Analytical Methods.

Optical rotations 
\[ [\alpha]_D^{20} \]
were measured at room temperature on a Perkin Elmer Model 341 polarimeter, analyte concentrations are given in g/100 mL. Specific rotations are reported. NMR-Spectroscopy was carried out on a Varian UNITY 300 MHz or Varian INOVA 500 MHz NMR spectrometer. 
\[^1^H\] and \[^1^3^C\] NMR spectra are referenced to external tetramethylsilane; \[^3^P\] NMR spectra are referenced to external 85% phosphoric acid. Mass spectrometry was performed on Bruker maXis 3G or Micromass LCT by electrospray ionisation in positive or negative ionisation modes.

UV-Visible spectrophotometry was carried out on a Varian Cary\textsuperscript{\textregistered} One UV-Visible spectrophotometer, in stoppered quartz cells. The temperature was continuously controlled at 303 K (30°C) by the use of a jacketed multicell holder, connected to an external Varian Peltier temperature controller filled with either water or ethylene glycol.

#### Expression, purification and crystallization of *Mtu*DAH7PS.

*M. tuberculosis* DAH7PS was overexpressed and purified as previously described (21-23). A reservoir solution containing 400 µM of inhibitor was prepared by dissolving an appropriate amount of solid rac-4 in crystallization buffer (0.1 M Tris-HCl buffer at pH 8, 1.5 M ammonium sulfate, and 12% v/v glycerol). 500 µL of enzyme stock solution was concentrated to approximately 50 µL using centrifugation with a 10 kDa cutoff membrane, and the resulting solution was diluted to approximately 500 µL using the inhibitor solution. This process was repeated twice in order to buffer exchange the solution as completely as possible. 1 µL of the buffer exchanged enzyme solution...
(concentration 3-5 mg/mL) was mixed with 1 µL of crystallization buffer. Crystals were grown by sitting drop vapour diffusion over 300 µL of reservoir solution and appeared within 24 h at 25°C. Crystals were flash frozen in liquid nitrogen in a cryoprotectant solution consisting of crystallization buffer and 20% v/v glycerol.

**Structure determination.** X-ray diffraction data were collected at the MX2 beamline at the Australian Synchrotron (Table 1). The dataset was integrated using iMosflm (30). The space group and cell parameters were identical to those of previously reported structures of *Mtu*DAH7PS allowing phases calculated from the original structure (PDB-code 2B7O) (21) to be used to solve the structure. The structure was refined using the CCP4 software package (30) by methods analogous to the ones described for the native structure (21). Both enantiomers of inhibitor 4 were built using the Dundee PRODRG2 server (31). The two enantiomers of 4 were initially positioned manually into the electron density observed in the active site using COOT and then refined at half occupancy. The model was optimized using repetitive cycles of model building with COOT and refinement with REFMAC5. Water molecules were added automatically in COOT and verified using |2Fo-Fc| and |Fo-Fc| maps and potential to hydrogen bond to at least one protein atom or other water molecule. A bias removed |Fo-Fc| omit map (Figure 5 (a)) was generated by deleting the ligand and active site waters from the model and subjecting the model to simulated annealing and refinement in PHENIX. The |2Fo-Fc| map obtained by this procedure (Figure 5 (a)) shows clear continuous electron density for ligand 4. The structure was validated using SFCHECK, with 97.5% of residues in the most favored region of the Ramachandran plot (Table 1). The final model included two protein chains each containing all 462 natural residues and two residues from His tag cleavage, 554 water molecules, two manganese ions, one sulfate ion, one chloride ion and R- and S-4.

**Inhibition assays.** Enzyme activity was monitored by following the loss of absorbance at 232 nm (ε=2800 L mol⁻¹ cm⁻¹) due to the consumption of PEP (32). Assays were carried out in 50 mM BTP buffer at pH 7.5 containing 1 mM tris(2-carboxyethyl)phosphine (TCEP) at 30°C. The buffer solution was prepared using ultrapure water which was stirred over Chelex resin (Bio-Rad) and filtered before use. Manganese(II) sulfate stock solution (10 mM) was made up in ultrapure water pretreated with Chelex resin. PEP and E4P substrate solutions were made up to approximately 10 mM by dissolving the PEP monopotassium salt and E4P monosodium salt (Aldrich) in buffer solution. Accurate substrate concentrations were determined by enzymatic reaction: The absorbance change of solutions containing one substrate in excess were determined before and after conversion of the limiting substrate by added DAH7PS had occurred and the extinction coefficient of PEP was used to calculate the concentration of the limiting substrate. The absorbance change was corrected by the absorbance change caused when DAH7PS was added to a control solution which did not contain E4P. For inhibition studies, assay solutions containing manganese (II), varying concentrations of PEP and inhibitors and buffer were equilibrated at 30°C. 2 µL (1.3 mg/mL) *M. tuberculosis* DAH7PS was added and the mixture equilibrated for 2 min before the reaction was initiated by addition of E4P (11 µL). Final assay conditions were 10 µM Mn²⁺, 5.2 nM DAH7PS, 100 µM E4P, 57.6-115.2 µM PEP, 0-940 nM inhibitor with an appropriate amount of buffer to make up a final volume of 1 mL.

**Lanzetta phosphate assay.** Lanzetta reagent (33) was prepared fresh as required from the following components: 3 parts 0.045% w/v malachite green in water, 1 part 4.2% w/v ammonium molybdate in 4 M HCl, 0.1 parts 1.5% v/v Triton X-100 in water. The components were mixed in the dark and stirred for 1 h before the solution was filtered through a 0.45 µM syringe filter. For the qualitative detection of phosphate-containing fractions after anion exchange chromatography, a 20 µL sample of each fraction was mixed with 250 µL of Lanzetta reagent and the color change judged by optical inspection. For the quantitative determination of inhibitor concentration, 300 µL of the inhibitor solutions were incubated with 10 µL calf alkaline phosphatase solution (5 units/mL in 4 mM MgCl₂) for at least 2 h. To 100 µL of the digested sample was added 700 µL Lanzetta reagent and the absorbance at 630 nm determined after 20 minutes. A calibration curve for the determination of phos-
phate concentration was obtained from analogous analysis of solutions of appropriate concentrations (6-150 µM) of KH₂PO₄ which had been dried in high vacuum for at least 3 h before use. As a control, a glucose-6-phosphate solution of known concentration was also digested with calf alkaline phosphatase and analyzed.

**Modeling procedure for linear intermediate and the designed inhibitors.** Modeling studies were carried out using software packages from Schrödinger Suite 2006 software package (34-37), more detailed computational methods can be obtained from the Supplemental Data. The previously reported crystal structure of *Mtu*DAH7PS (PDB-code 2B7O) (21) was used as the receptor. As a starting point for docking studies, an ensemble of low-energy conformations of the ligands (R)- and (S)-4 and both epimers of the tetrahedral intermediate 2 was identified by conformational searches. The modeling of the tetrahedral intermediate epimers into the active site of *Mtu*DAH7PS was carried out with the Schrödinger Suite 2006 Induced Fit Docking protocol (36). The induced fit docking procedure lead to an intermediate adapted protein receptor in which several residues in the active site adopted conformations different to the ones observed in the native crystal structure (PDB-code 2B7O). This intermediate adapted structure of *Mtu*DAH7PS was then used as a rigid receptor for docking studies of compounds (R)- and (S)-4 which were conducted using Glide (37).

**Synthesis.** All reactions were carried out under an inert atmosphere of nitrogen in flame-dried glassware unless otherwise stated. Organic solvents were dried before use by standard methods (38). Dess-Martin Periodinane (39, 40) was prepared according to literature procedures. m-Chloroperbenzoic acid was recrystallized from dichloromethane before use, all other reagents were purchased from Sigma-Aldrich and used without further purification.

Preparative procedures and characterization of previously unreported compounds can be found in the Supplemental Data. A representative procedure for the final step of the synthesis and characterization of inhibitor 4 is outlined in detail below.

**Heptanoic acid 2,7-bisphosphate 4.** Protected bisphosphate 11 (177 mg, 0.24 mmol) was dissolved in ethyl acetate (15 mL) and palladium on charcoal (10% Pd/C, 57 mg, 0.053 mmol Pd) was added. The reaction flask was purged with hydrogen gas with three freeze-pump-thaw cycles. The reaction mixture was stirred for 16 h after which TLC indicated complete consumption of starting material 11. The suspension was filtered through a celite pad, the celite washed with ethyl acetate and methanol and the solvent evaporated under reduced pressure. The resulting residue was dissolved in 1 M aqueous potassium hydroxide solution (7 mL) and stirred vigorously for 2.5 h. The reaction mixture was passed down a column consisting of freshly regenerated DOWEX 50 W x4-200 (H+) resin (approximately 5x1 cm) and the column eluted with 50 mL water. The eluate was neutralized with 1 M potassium hydroxide solution and lyophilized. The crude product was purified by anion exchange chromatography on SOURCE-Q resin eluting with a gradient of water/aqueous ammonium bicarbonate solution. Fractions containing the desired product were identified using the qualitative Lanzetta phosphate assay (see below), pooled and lyophilized to give 42.5 mg of a white powder consisting of the product and residual ammonium bicarbonate. Lanzetta phosphate assay (see above) established the purity of the white powder yielded was 80% by weight, the corrected yield for this step is thus 34%.

**RESULTS**

**Modeling of the reaction intermediate into MtuDAH7PS active site.** In order to inform
inhibitor design, the predicted tetrahedral reaction intermediate (2, Figure 3) was modeled into the active site of MtuDAH7PS. Since it is unknown from which side the water attacks the PEP substrate during the reaction, both possible epimers of the linear intermediate were modeled.

The modeling study produced a total of 30 possible poses for the two possible C2 diastereoisomers of the tetrahedral intermediate in the active site, out of which 17 retained the expected PEP binding interactions. All 17 poses with correct PEP orientation are from the S-isomer of the intermediate, consistent with water attacking the C3 carbon of PEP from its re face, which is the side opposite the active site Mn$^{2+}$ ion.

**Figure 3**

The best pose from the modeling study (Figure 3 (a)) was selected based on two criteria. The first is that the PEP part of the tetrahedral intermediate retains the same interactions as observed in the original crystal structure (PDB-code 2B7O). The second is that the positioning of the E4P phosphate group should be as close as possible to the phosphate group of G3P in the crystal structure of SceDAH7PS (PDB-code 1OF8), which is considered to provide the best prediction for the E4P phosphate position. In the best pose (Figure 3 (a)), the PEP part of the molecule interacts with Arg126, Lys306, Arg337, Arg284 and Glu283. Arg284 bridges the phosphate groups of PEP and E4P through salt bridges and hydrogen bonds. The E4P phosphate group interacts with Arg284, Arg135, and Ser136, which suggests that part of the KPRS motif (Arg135 and Ser136) plays a key role in positioning of the E4P phosphate, as expected from the G3P-bound SceDAH7PS structure. The E4P aldehyde group, now converted to a hydroxyl group in the linear intermediate, is at a distance of 2.2 Å from the metal ion. This indicates that the aldehyde of E4P may be held in place by coordination to the metal ion before reaction occurs. The hydroxyl group that corresponds to the incoming water molecule forms hydrogen bonds with Trp280, Glu248, and Lys133. Two of these residues, Glu248 and Lys133, are conserved across the entire DAH7PS family, whereas Trp280 is only conserved in the type II enzymes.

**Inhibitor Design.** Combining results from the intermediate modeling and previous work on mechanism-based inhibitors (27, 28), we designed bisphosphate 4 (Figure 3 (b)) as a simplified analogue of the tetrahedral intermediate. The 2-phosphorylcarboxylic acid moiety targets the PEP-binding site and resembles phospholactate, which has been reported as an efficient inhibitor of E. coli DAH7PS (27). Bisphosphate 4 represents a simplified analogue of tetrahedral intermediate 2: the phosphoryl and carboxyl-moieties of compound 4 are proposed to act as charged anchors arranged in a similar geometry to those of intermediate 2, and held together by a non-functionalized hydrocarbon linker. Given its low degree of functionalization, bisphosphate 4 may be used as a lead structure for specific introduction of functionality in order to enhance enzyme-ligand affinity. In light of the results for molecular modeling of the tetrahedral intermediate 2 which suggest a preference for the S configuration at C2, we devised two syntheses leading to target compound 4 in order to assess possible enantio-discrimination: One racemic synthesis which had already been proven useful in our laboratory, as well as a chiral pool synthesis which allowed control of the absolute configuration at C2.

**Synthesis of racemic 4.** The synthetic strategy towards rac-4 relies on the establishment of the key $\alpha$-hydroxyester moiety by a Darzen’s-type chain elongation of an aldehyde, a methodology that has been used for the synthesis of related compounds (Figure 4) (29, 41). The final deprotection was achieved by hydrogenolysis of the benzyl groups followed hydrolysis of the isopropyl ester. The final product, rac-4 was purified by anion exchange chromatography eluting with an ammonium bicarbonate gradient.

**Figure 4**

**Crystal structure.** The racemic inhibitor 4 was co-crystallized with DAH7PS from M. tuberculosis. Whereas generally both purification of the enzyme and crystal growth are difficult in the absence of PEP in the buffer solution, x-ray diffraction quality crystals were obtained by buffer exchange of the purified enzyme solution with a solution containing inhibitor 4 (Table 1). The crystal structure was solved by molecular replacement using the
previously determined *Mtu*DAH7PS structure (PDB-code 2B7O) (21) as search model and was refined using x-ray diffraction to 2.35 Å (Table 1). The asymmetric unit contains two enzyme molecules as in other *Mtu*DAH7PS crystal structures, tertiary and quaternary structures are essentially unaffected by binding of inhibitor 4. Superimposition on the wild-type protein (PDB-code 3NV8) (22) showed that 5029 atoms could be matched with an rms positional difference of 0.22 Å.

**Table 1**

Clear continuous electron density for inhibitor 4 was found in both active sites. The |Fo-Fc| omit map generated by removing the inhibitor and active site waters from the model and subjecting the resulting model to simulated annealing and refinement shows clear continuous density for inhibitor 4, and for a water molecule in the active site (Figure 5 (a)). Based on the electron density maps obtained, it could not be concluded whether there is preferential binding of one of the enantiomers from the racemic mixture. In order to represent this ambiguity, our final model includes both (R)-4 and (S)-4 at half occupancy.

**Figure 5**

Inhibitor 4 binds as predicted with its 2-phosphoryl and carboxylate moieties in the PEP binding site, and in an extended conformation so that the phosphate moiety on C7 is located near the proposed E4P phosphate binding site (Figure 5 (b)). Active site residues interacting with the PEP-mimicking portion of ligand 4 show little movement, consistent with this portion of the inhibitor being a good mimic of PEP. As expected, the conformations of the metal-binding residues were unaffected by binding of compound 4. The 2-phosphoryl moiety of inhibitor 4 forms salt bridges with Arg284, Arg337 and Lys306 and a hydrogen bond to the backbone N-H of Glu283. The carboxyl group forms salt bridges with Lys133 and Arg126 and coordinates to the manganese ion present in the active site. The 7-phosphoryl moiety extends into the tentative E4P phosphate binding site, forming salt bridges with Lys380 and Arg135 and hydrogen bonds with the Ser136 hydroxyl-group and backbone N-H. Conformational changes relative to the PEP-liganded structure were observed for the KPRS motif associated with inhibitor binding (Figure 5 (c)). Arg135 and Ser136 interact with the 7-phosphate moiety of inhibitor 4, moving closer into the active site pocket. The side chain of Lys133 shows the biggest conformational change of all active site residues, establishing a salt bridge to the carboxylate group of ligand 4. This big movement may also be partly due to increased electrostatic repulsion with the guanidinium group of Arg135, which has moved closer into the active site. Interestingly, the side chain movement of Lys133 correlates well with the movement that the induced fit molecular modeling results predict for this residue in order to accommodate the proposed reaction intermediate (Figure 5 (d)). The superposition of structures of the DAH7PS-4 complex and the induced fit-modeling results (Figure 5 (d)) show clearly that the experimental binding mode of biphosphate 4 closely matches the *in silico* modeling of the proposed reaction intermediate into the active site. The carboxylate and both phosphate groups of inhibitor 4 occupy positions that were predicted for the corresponding moieties of the intermediate model, with minor deviations found in the carbon chain. A crystallographically located water molecule occupies a position very close to the predicted position of the intermediate 2-hydroxyl group. This water is held in place by hydrogen bonds to Trp280 and Glu248. The distance of this water to C2 of the ligand is 3.4 Å when (R)-4 is modeled into the active site, and 3.2 Å for the model containing (S)-4. Preliminary testing of *rac*-4 indicated that its *K*_i against *Mtu*DAH7PS was in the sub-micromolar range.

Encouraged by these results, and in order to assess whether one enantiomer of 4 is preferentially bound by the enzyme, we synthesized compounds (R)-4 and (S)-4 for inhibition assays.

*Synthesis of (R)-4 and (S)-4.* Since the previously described synthesis of *rac*-4 offers little potential with respect to stereoselective introduction of the α-hydroxyester moiety, another synthetic route was developed in which the chirality is derived from a cheap and readily available chiral pool starting material (Figure 6). The chiral epoxyster 14 of appropriate configuration was prepared from D- or L-serine respectively following a previously
reported method (42). Ethyl glycidate 14 could be ring opened regioselectively α-hydroxyester 15. Hydrogenolysis of ester 15 liberated the 7-hydroxyl group to generate dihydroxyester 16, which could then be converted to \((R)-4\) or \((S)-4\) using the same procedures as described above for the racemic synthesis.

**Figure 6**

*Inhibition studies.* \((R)-4\) and \((S)-4\) were tested for inhibition of *MtuDAH7PS*. Although inhibitor 4 was designed as a bisubstrate inhibitor, it is known that DAH7PS operates by a sequential ordered reaction mechanism in which PEP has to bind to the enzyme first (25). This means that although competition with PEP is most important in order to assess the inhibitory potency, the fixed concentration of the second substrate can influence the inhibition. The kinetic assays were carried out with PEP concentrations ranging from 57 to 115 \(\mu\)M at a fixed E4P concentration of 100 \(\mu\)M, which corresponds to four times the value determined for the Michaelis constant for E4P \((K_{m}(E4P))\). The concentrations of \((R)-4\) and \((S)-4\) were varied from 0-940 nM. Even at these high levels of E4P, \((R)-4\) and \((S)-4\) gave inhibitory constants of 360 ± 50 and 620 ± 110 nM respectively (see Supplemental Data Figure S1 and S2). \((R)-4\) and \((S)-4\) represent the first potent inhibitors reported for *MtuDAH7PS* and the first inhibitors with substantially greater affinity than the substrate PEP \((K_{i}(PEP)=29\ \mu\)M for *MtuDAH7PS*) for any DAH7P synthase.

**Modeling of \((R)-4\) and \((S)-4\) into MtuDAH7PS active site.** Both \((R)-4\) and \((S)-4\) were modeled into the *MtuDAH7PS* active site and the results showed that there is little preference for binding between the two isomers of inhibitor 4. Both isomers adopt similar binding modes, which are also very similar to that of the linear intermediate in the active site, with the \((R)\)-isomer having a slightly better docking score than the \((S)\)-isomer. The interactions found in the best poses from modeling of the two isomers are the same as those observed in the crystal structure of the *MtuDAH7PS*-4-complex (Figure S3, Supplemental Data). These modeling results in conjunction with the inhibition data obtained for \((R)-4\) and \((S)-4\) and the tetrahedral intermediate modeling results suggest that a fully functionalized C2-carbon of the tetrahedral intermediate bearing carboxylate, phosphate and hydroxyl substituents is necessary in order to observe stereoselective recognition in the enzyme active site.

**DISCUSSION**

The study presented here demonstrates the successful combination of synthetic, structural and computational methods in order to inform the design of novel lead structures of inhibitors for *MtuDAH7PS*. Although both the substrate and the product of the enzymatic transformation, PEP and DAH7P, possess planar geometry at C2, mechanistic considerations suggest that the enzyme active site is also set up to accommodate preferentially a tetrahedral reaction intermediate. The high inhibitory potencies of \((R)\)- and \((S)-4\) confirm that intermediate analogues with tetrahedral C2 are a promising scaffold for high-affinity inhibitors of DAH7PS.

The fact that the experimentally determined structure and the induced-fit model are in good agreement demonstrates firstly, that inhibitor 4 is a good intermediate mimic, and secondly, verifies that our modeling approach is effective for predictions of enzyme-ligand interactions for *MtuDAH7PS*. The structural information from the enzyme-inhibitor complex can be used to inform future inhibitor design: based on the binding mode of inhibitor 4 in the active site, modifications to the lead structure allow the introduction of specific new interactions with the enzyme. For example, additional hydroxyl groups at C4 and C6 of inhibitor 4 with the appropriate absolute configuration are expected to form hydrogen bonds to Asp441, interactions which were observed in the modeling of the intermediate (Figure 3). Introduction of a C4-hydroxyl or other metal coordinating group such as an amine or thiol group could also lead to additional enzyme-inhibitor interactions through metal coordination.

Inhibitor 4 is a highly charged molecule, featuring two phosphate groups and a carboxylate moiety. This characteristic of this molecule is expected to significantly limit membrane permeability. Phosphates are abundant in naturally occurring
molecules, making them highly interesting moieties in the development of drugs. Strategies to generate phosphate prodrugs containing suitable masking groups that facilitate membrane permeability and allow the release of the active drug inside the cell have been developed, and examples of these prodrugs are in clinical trials (43-45).

Although both enantiomers of inhibitor 4 displayed a high affinity to the enzyme active site, the small difference in affinity of the two enantiomers provides valuable insight into the enzyme mechanism and its inhibition. The observation that (S)-4 proved to be a slightly less potent inhibitor may be due to the fact that the C2-hydrogen of (S)-4 points in the direction of a water molecule also present in the active site, with inter-atom distances that suggest a steric clash between these moieties. (Figure S4, Supplemental Data). For the related enzyme KD08PS, data from computational and crystallographic studies have lead to the preposition that the substrate water attacks PEP from the si side during the course of the reaction (18, 19, 46). It however so far remains unclear whether this mechanistic proposal can be transferred to MtuDAH7PS or even DAH7P synthases in general. In the crystal structure reported in this study, a water molecule was located in close proximity to C2 of the inhibitor on what would be the re side of PEP. The resemblance of this arrangement of inhibitor and water to the location of the 2-hydroxyl group of the modeled tetrahedral reaction intermediate, combined with the configurational preference observed for the docking of the intermediate, suggest that in MtuDAH7PS, water attack proceeds from the re side of PEP. This is further supported by studies of type I DAH7P synthases from S. cerevisiae (16), E. coli (14), and Thermotoga maritima (47), which located a water molecule that was suggested to attack PEP during the course of the enzymatic reaction in a very similar environment. The glutamic acid residue has been proposed as a general base catalyst to facilitate nucleophilic attack of water. The highly conserved nature of the arrangement of water in the PEP binding site suggests a common catalytic mechanism for all DAH7P synthases which involves water attacking the re face of PEP during the course of the reaction.

The combined results from intermediate modeling and the structural and kinetic study of intermediate analogue 4 allow significant refinements of the proposed catalytic mechanism of MtuDAH7PS (Figure 7). PEP and E4P binding has already been discussed in detail, we propose that the substrate water is bound on the re face of PEP through hydrogen bonding interactions with Trp280 and Glu248. Nucleophilic attack of PEP at the E4P aldehyde moiety is facilitated by metal coordination, and the resulting oxianion is protonated by Lys133. Both the intermediate modeling results and the crystal structure of the intermediate analogue show significant movement of the Lys133 sidechain upon ligand binding, positioning it in a suitable position to fulfill this role as a general acid. The substrate water is activated for attack at C2 of PEP by deprotonation with Glu248.

A similar mechanism has been previously suggested in studies of S. cerevisiae and T. maritima type I DAH7PS (16, 47). The fact that a glutamate residue is found at the equivalent position for M. tuberculosis type II DAH7PS, and the crystallographic localization of a suitable substrate water molecule in the MtuDAH7PS-4 complex in this study as well as our modeling results further support this mechanistic proposition. In the next step, the now neutral Lys133 can abstract the proton from Glu248, by either direct deprotonation or relayed via an active site water molecule. This step regenerates both the Lys133 ammonium and the Glu248 carboxylate group necessary for the next catalytic cycle. Lys306, which is positioned within hydrogen bonding distance of the PEP phosphate, could aid the elimination of phosphate from tetrahedral intermediate 2 by protonating the phosphate thus making it a better leaving group. Elimination of hydrogen phosphate yields DAH7PS protonated on the 2-keto moiety; this proton can then be removed by Lys306. After dissociation of the product DAH7P, all active site residues have returned to the protonation state that is required for the next catalytic turnover. This mechanism is the simplest that is in accordance with the data published to date, and it accounts for all protonation and deprotonation steps required on active site residues and intermediates. The only major side chain movement of an active site residue is required from
the proton-shuttling Lys133, a residue which is not well defined or possesses high B-factor values in some crystal structures of DAH7P synthases, indicating mobility of its side chain (11, 21). Further support for the important role of both Lys133 and Lys306 comes from results in our laboratory that show both residues are essential for catalytic activity.2

CONCLUSION

By combining molecular modeling-guided and mechanism-based design we have synthesized the first potent inhibitor of MtuDAH7PS. The crystal structure of the enzyme-inhibitor complex shows that the structural features of the inhibitor designed to mimic features of the natural substrates and proposed reaction intermediate indeed leads to the predicted specific enzyme inhibitor interactions. Inhibitor 4 represents a promising lead structure for anti-mycobacterial drugs and offers ample opportunity for further modification in order to establish structure-activity relationships. The crystallographic location of an active site water molecule has clarified the enzyme catalytic mechanism.

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36. Schrödinger Suite 2006 Induced Fit Protocol; Glide version 4.0; Prime version 1.5, Schrödinger, LLC, 2005.
ABBREVIATIONS USED:

AEC, anion exchange chromatography; BTP, 1,3-bis(tris(hydroxymethyl)methylamino)propane; DAH7PS, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; MtuDAH7PS, Mycobacterium tuberculosis DAH7PS; E4P, D-erythrose 4-phosphate; DCM, dichloromethane; DMP, Dess-Martin’s periodinane; G3P, glycerol 3-phosphate; Im, imidazole; KDO8PS, 3-deoxy-D-manno-octulosonate 8-phosphate synthase; m-CPBA, meta-chloroperbenzoic acid; PEP, phosphoenolpyruvate; Pr, isopropyl-; TBAF, Tetrabutylammonium fluoride; THF, tetrahydrofuran; Tris, tris(hydroxymethyl)aminomethane; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; rms, root mean square; TB, tuberculosis; TBDMS-, tert-butyldimethylsilyl-.


FIGURE CAPTIONS

Figure 1: (a) Overall reaction catalysed by DAH7PS. (b) Proposed mechanism of condensation of PEP with E4P.

Figure 2: (a) Stereo view of the active site of Mycobacterium tuberculosis DAH7PS (PDB-code 3NV8)(22) showing PEP (yellow carbons) in its binding site. The manganese iron is represented as a purple sphere. Major interactions between PEP and the enzyme and the metal coordination sphere are shown as dashed lines. (b) stereo view of the active site of Saccharomyces cerevisiae DAH7PS in complex with PEP, G3P (yellow carbons) and a Co²⁺-ion (magenta sphere)(PDB-code 1OF8) (16). A water molecule on the re side of PEP (left in this orientation) which is proposed to play the role of the substrate water is shown as a red sphere.

Figure 3: (a) The modeled best pose of the tetrahedral open chain intermediate 2 (S-isomer) from induced fit docking to the active site of MtuDAH7PS superimposed on the crystal structure of SceDAH7PS (PDB-code 1OF8). The active site carbons from the modeled structure are in green, and the active site residues of SceDAH7PS are shown with yellow carbons. The modeled linear intermediate molecule is shown with magenta carbons, the C2-hydroxyl group is pointing behind the plane. The residues are labeled according to the numbering in MtuDAH7PS. The metal ion is displayed as a purple sphere. Hydrogen bonds are represented by dashed lines. (b) left: linear intermediate 2 center: molecular model of linear intermediate 2 showing the preferred 2S-configuration. right: simplified intermediate analogue 4, synthetic target for this study.

Figure 4: Synthesis of racemic inhibitor 4: (i) TBDMSCl, Im, DCM, 61% (ii) DMP, DCM, 70% (crude) (iii) Isopropyl dichloroacetate, KOPr, ¹PrOH, Et₂O (iv) NaCNBH₃, ¹PrOH, 60% (2 steps) (v) TBAF, THF, 56% (vi) (1) (BnO)₂PN(¹Pr)₂, 1H-tetrazole, DCM (2) m-CPBA , 50% (vii) (1) H₂, Pd/C (2) KOH, H₂O (3) DOWEX-H⁺ (4) Source-Q AEC, 42%.

Figure 5: Stereo view of the active site of the enzyme in complex with 4 (only the (R)-enantiomer is shown for clarity): (a) |Fobs-Fcalc| simulated annealing omit map contoured at 3σ showing the electron density for inhibitor 4 (blue carbons) in the active site. (b) interactions of 4 with the active site residues (green carbons) and metal ion (purple sphere). (c) overlay of PEP (yellow carbons)-DAH7PS structure 3NV8.
(cyan carbons) with DAH7PS-4-complex (green carbons). (d) overlay of the preferred pose of the linear intermediate (magenta carbons) and 4 (blue carbons, the oxygens on the C2-phosphate were omitted for clarity). The enzyme structure experimentally obtained from the DAH7PS-4-complex (green carbons) superimposes well onto the enzyme structure obtained by induced fit modeling of the linear intermediate (peptide: yellow carbons, intermediate: magenta carbons).

Figure 6: Synthesis of enantiopure inhibitor 4: yields are given for the synthesis of (R)-4, analogous procedures using L-serine as the starting material were used for the synthesis of (S)-4 in comparable yields, please refer to the Supplemental Data for more detail. (i) HNO₂, KBr, H₂O then KOH/EtOH, 37% (2 steps) (ii) EtBr, Bn(Et)₂NCl, CH₂Cl₂, 66% (iii) BnO(CH₂)₄Br, Mg, THF, r.t. then CuBr•SMe₂, -78°C, then 14, 32% (iv) H₂, Pd/C, EtOAc, 66% (v) (BnO)₂P=N(Pr)₂, 1H-tetrazole, DCM, then m-CPBA 64% (vi) H₂, Pd/C, EtOAc, then KOH/H₂O, then DOWEX-H⁺, anion exchange chromatography, 27%.

Figure 7: Refined catalytic mechanism for MtuDAH7PS in accordance with the results obtained from this study.
**TABLES**

Table 1: Data collection and refinement statistics for *M. tuberculosis* DAH7Ps in complex with 4.

<table>
<thead>
<tr>
<th>A. Data collection</th>
<th>B. Refinement</th>
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<tr>
<td><strong>Crystal system</strong></td>
<td>Resolution (Å)</td>
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<tr>
<td>space group</td>
<td>$R_{cryst}$ (%)</td>
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<tr>
<td>Unit cell parameters</td>
<td>$R_{free}$ (%)</td>
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<tr>
<td>a (Å)</td>
<td>Amino acids (chain length 464 residues)</td>
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<tr>
<td>b (Å)</td>
<td>Water molecules</td>
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<tr>
<td>c (Å)</td>
<td>Other</td>
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<td>Resolution range (Å)</td>
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<td>Redundancy</td>
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<td></td>
<td>Disallowed (%)</td>
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$^a$ refined at reduced occupancy.
FIGURES

(a)  
\[
\begin{align*}
\text{E4P} & \quad \text{PEP} \\
\begin{array}{c}
2\text{-O}_3\text{P} \\
\text{H} \\
\text{OH} \\
\end{array} & \quad \begin{array}{c}
\text{O}_3\text{P} \quad \text{CO}_2^- \\
\text{H}_2\text{O} & \quad \text{O}_3\text{P} \quad \text{CO}_2^- \\
\end{array}
\end{align*}
\]

DAH7PS \quad \text{Shikimate pathway}

aromatic amino acids

(b)  
\[
\begin{align*}
\text{M}^{2+} & \quad \text{H-Enz} \\
\begin{array}{c}
2\text{-O}_3\text{P} \\
\text{O} \\
\text{H} \\
\end{array} & \quad \begin{array}{c}
\text{O}_3\text{P} \quad \text{CO}_2^- \\
\text{H}_2\text{O} \\
\end{array}
\end{align*}
\]

\[
\begin{align*}
\text{1} & \quad \text{2} \\
\begin{array}{c}
\text{O}_3\text{P} \quad \text{O}_3\text{P} \\
\text{OH} \\
\end{array} & \quad \begin{array}{c}
\text{O}_3\text{P} \quad \text{O}_3\text{P} \\
\text{OH} \\
\end{array}
\end{align*}
\]

\[
\begin{align*}
\text{3} & \\
\begin{array}{c}
\text{O}_3\text{P} \quad \text{O}_3\text{P} \\
\text{OH} \\
\end{array}
\end{align*}
\]

Figure 1
Figure 2
Figure 4
Figure 5
Figure 6
Figure 7
Potent inhibitors of a shikimate pathway enzyme from *Mycobacterium tuberculosis*: combining mechanism- and modeling-based design

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