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Enediol mimics as inhibitors of the D-arabinose 5-phosphate isomerase (KdsD) from *Francisella tularensis*

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ABSTRACT

We explored the D-arabinose 5-phosphate isomerase (KdsD, E.C. 5.3.1.13) from *Francisella tularensis*, a highly infectious Gram-negative pathogen that has raised concern as a potential bioweapon, as a target for the development of novel chemotherapeutics. *F. tularensis* KdsD was expressed in *Escherichia coli* from a synthetic gene, purified, and characterized. A group of hydroxamates designed to be mimics of the putative enediol intermediate in the enzyme's catalytic mechanism were prepared and tested as inhibitors of *F. tularensis* KdsD. The best inhibitor, which has an IC_{50} of 7 μM, is the most potent KdsD inhibitor reported to date.

Francisella tularensis, a Gram-negative bacterium from the gamma subdivision of the proteobacteria, is a highly infectious pathogen that has raised concern as a potential bio-weapon.[1] While standard antibiotics like streptomycin, tetracyclines and fluoroquinolones are effective against natural strains of *F. tularensis*, the potential for resistance, natural or artificial, creates a need for an effective antibiotic that works through a novel target.

One source of novel antibiotic targets is the biosynthetic pathway of the outer membrane component lipopolysaccharide (LPS). LPS biosynthesis is an attractive target because it is both unique to Gram-negative bacteria and essential to their viability.[2] Defects in LPS biosynthesis also increase the susceptibility of Gram-negative bacteria to standard antibacterials because they increase the permeability of the outer membrane to these drugs. A key component of LPS is the unique eight-carbon sugar 3-deoxy-D-*manno*-octulosonic acid 8-phosphate (KDO). KDO serves as the bridge between the membrane anchor of LPS, known as lipid A, and the inner oligosaccharide core of LPS. Biosynthesis of KDO requires four steps starting from the pentose pathway intermediate D-ribulose 5-phosphate (Ru5P). The first, committed step in the biosynthesis of KDO is the isomerization of Ru5P to D-arabinose 5-phosphate (A5P), which is catalyzed by the enzyme D-arabinose 5-phosphate isomerase (KdsD, E.C. 5.3.1.13). Cells of the model Gram-negative bacterium *Escherichia coli* lacking this enzyme require exogenous A5P for LPS synthesis and cellular growth.[3] The lack of A5P in mammalian serum makes KdsD essential for viability in the host, and a good target for the discovery of novel antibiotics targeting *F. tularensis*.

We took a classic approach to finding inhibitors of KdsD from *F. tularensis* by testing compounds that mimic the putative high-energy 1,2-enediol intermediate in the enzymatic inter-conversion of Ru5P and A5P (Fig. 1). This approach is not without precedent. Bingham et al.[4] used aldonic acids and alditols as mimics of the enediol intermediate when searching for inhibitors of *E. coli* KdsD. Their most potent inhibitor was a four-carbon aldonic acid (**1**, Fig. 2), formally derived from D-erythrose 4-phosphate, which had an IC_{50} of

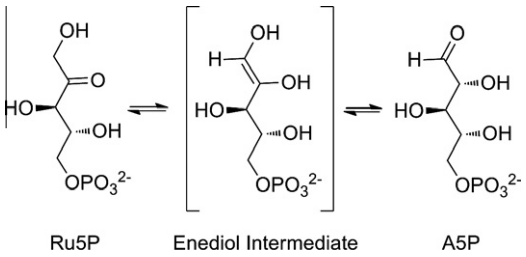

Figure 1. Isomerization catalyzed by KdsD and the enzyme-bound enediol intermediate.

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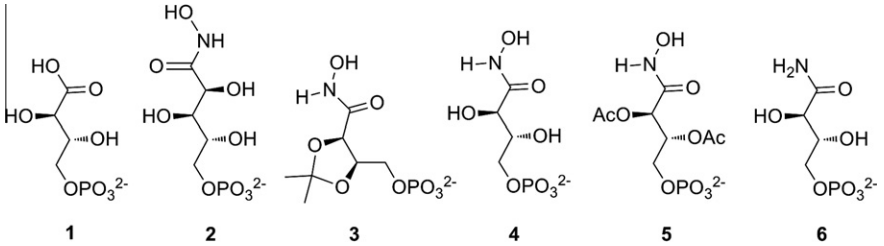

Figure 2. Chemical structures referred to in this study.

220 μM. This is the most potent inhibitor of KdsD described in the literature. To improve upon this, we chose to explore hydroxamic acids, which more closely resemble the putative enediol intermediate.

Hydroxamates have been used as enediol mimics in many similar systems. In three classic cases, hydroxamates were used to help elucidate the structure of the active site and the enzyme mechanism. Collins[5] first demonstrated that phosphoglycolohydroxamate, a two-carbon hydroxamate, inhibits triose phosphate isomerase (TIM) with a K_i of 4 μM. Details of the active site and the mechanism of catalysis were elucidated with the help of X-ray crystal structures (e.g., 2VXN[6]) of TIM–phosphoglycolohydroxamate complexes. The inter-conversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate phosphate catalyzed by TIM is thought to proceed via a proton transfer mechanism, through a cis-enediol intermediate. Allen et al.[7] found D-threonohydroxamic acid, a four-carbon hydroxamate, to be a slow-binding inhibitor of xylose isomerase, with a K_i of 100 nM. As with TIM, the details of the xylose isomerase active site were elucidated with the help of an X-ray crystal structure (2GYI)[7] of the hydroxamate-xylose isomerase complex. Xylose isomerase catalyzes the inter-conversion of five-carbon sugars xylose and xylulose, but is also used industrially to inter-convert the six-carbon sugars glucose and fructose. This metallo-enzyme follows a slightly different mechanistic path than TIM, involving the transfer of hydride. Arsineiva et al.[8] used 5-phospho-D-arabinohydroxamic acid (**2**, Fig. 2) to study the mechanism of phophoglucose isomerase (PGI), a cytosolic enzyme that catalyzes the reversible isomerization of glucose 6-phosphate to fructose 6-phophate. The K_i for 5-phospho-D-arabinohydroxamic acid versus rabbit PGI is 100 nM.[8] An X-ray crystal structure of the PGI–hydroxamate complex (1KOJ)[8] suggests a proton transfer mechanism with a cis-enediol intermediate similar to that of TIM.

In addition to these classic cases, hydroxamates have been used as chemical probes with a number of other sugar isomerases, in some cases proving to be potent inhibitors of enzymes derived from pathogens. Pastiet al.[9] and Dardonville et al.[10] synthesized and tested hydroxamates as inhibitors of *Trypanosoma brucei* 6-phosphogluconate dehydrogenase (6PGDH), which catalyzes the oxidation and subsequent decarboxylation of 6-phosphogluconate to form Ru5P. Pasti et al.[9] found that 5-carbon hydroxamate **2** (Fig. 2) inhibited *T. brucei* 6PGDH with an IC_{50} of 5.8 μM. Dardonville et al.[10] found that 4-carbon hydroxamates **3–5** (Fig. 2) provided K_i values below 100 nM, and amide **6** (Fig. 2) a K_i of 1.52 μM versus *T. brucei* 6PGDH. Hydroxamate inhibitors have also been found to inhibit RpiB from *Mycobacterium tuberculosis*.[11,12] RpiB catalyzes the inter-conversion of ribose 5-phosphate (R5P) and Ru5P, but its putative physiological role is to inter-convert the six-carbon sugars allose 5-phosphate and allulose 5-phosphate. The four-carbon hydroxamate **4** (Fig. 2) had a K_i of 57 μM against the *M. tuberculosis* RpiB, while the aldonic acid **1** had a K_i of 1.7 mM.[11,12] A five-carbon hydroxamate mimic of the putative enediol intermediate in the inter-conversion of allose to allulose was a poorer inhibitor (K_i of 400 μM) than **4**, but still better than aldonic acid **1**. These precedents reveal that hydroxamates can be potent inhibitors of carbohydrate isomerases, and that the best starting point is to prepare hydroxamates one carbon shorter than the shortest sugar substrate. In order to find a starting point for *F. tularensis* KdsD inhibitor design, we decided to prepare and test the four most potent inhibitors of *T. brucei* 6PGDH, the four-carbon analogs **3–6** (Fig. 2).

Compounds **3–6** were synthesized according to procedures outlined by Dardonvilleet al.[10] The reaction scheme is presented in Figure 3; the full experimental details relating to the synthesis of these compounds are delineated in the Supplementary data.

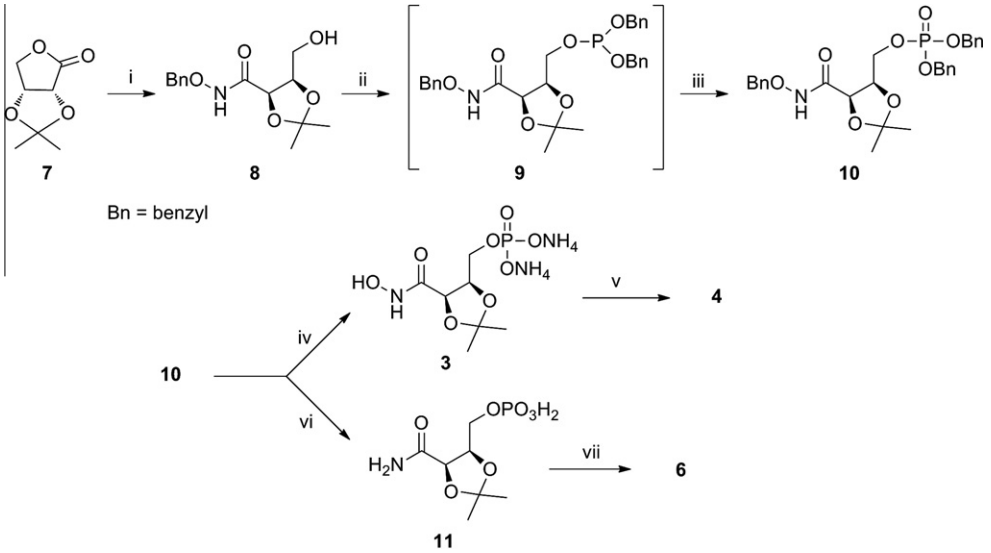

Figure 3. Synthesis of hydroxamate analogs **3–6**.

Table 1
Biological activity of compounds **3–6**

Compound	*F. tularensis* KdsD IC_{50} (μM)	*T. brucei* 6PGDH K_i[a] (μM)	*M. tuberculosis* RpiB K_i[b] (μM)	*E. coli* MIC (mM)
3	3000 ± 900	0.035		–
4	35 ± 6	0.01	57	–
5	7 ± 1	0.08		52
6	10 ± 2	1.52	>30,000	18

[a] From Dardonville et al.[10]
[b] From Roos et al.[12]

Difficulties in reproducibly generating a key protected phosphate intermediate in the synthesis of inhibitor **6** required development of a novel work-around. Rather than use the tribenzyl phosphite and iodine procedure employed by Dardonville et al.[10] to introduce the tribenzylated phosphate in compound **10**, we employed dibenzyldiisopropylphosphoramidite and tetrazole to form intermediate **9** (Fig. 3), which was oxidized without isolation using *tert*-butyl hydroperoxide. The yield of compound **10** (78% from compound **8**) was slightly superior to that reported by Dardonville et al. (73%), but had the additional advantage of being more reproducible.

The *F. tularensis* KdsD protein used in this study was derived from a commercially prepared synthetic gene based upon the published *F. tularensis* genome sequence. This synthetic gene was subcloned into the expression vector pT7LOH,[13] creating a plasmid that encodes KdsD with an N-terminal hexahistidine tag. This plasmid was used to transform *E. coli* BL21(DE3) cells that were then used to produce multi-milligram quantities of metal-affinity purified KdsD using standard growth, expression, lysis and purification techniques (see Supplementary data). The resulting protein was substantially pure, judged by SDS–PAGE.

The purified *F. tularensis* KdsD was assayed for isomerase activity in the reverse of the physiological direction, using A5P as substrate and determining the product Ru5P using the discontinuous cysteine-carbazole colorimetric method.[3] *F. tularensis* KdsD had an A5P K_m of 380 ± 80 μM and a k_{cat} of 141 ± 9 s^{-1} at 37 °C using this assay. These values are very similar to values (K_m = 610 μM and k_{cat} = 157 s^{-1}) published for *E. coli* KdsD.[3]

Compounds **3**–**6** were then assayed for inhibition of KdsD at 37 °C using the same discontinuous cysteine-carbazole colorimetric method, adapted to a 96-well microplate, used to characterize the enzyme. The full details are presented in the Supplementary data. Briefly, compound and KdsD (100 nM final concentration) were mixed in buffer at pH 8.5, and then warmed to 37 °C. An equal volume of A5P (1 mM final concentration), in the same buffer at 37 °C, was added to initiate the reaction. After a 5 min reaction time, the assay was stopped with sulfuric acid, transferred to a separate plate, mixed with the sulfuric acid–cysteine–carbazole reagent and allowed to stand at ambient temperature for 3 h for color development. Absorbance at 540 nm was then measured. IC_{50}s for each of the compounds studied are presented in Table 1.

The four-carbon hydroxamate **4** inhibits *F. tularensis* KdsD with a potency (IC_{50} 35 μM), which is more in line with its inhibition of *M. tuberculosis* RpiB (K_i = 57 μM)[12] than its inhibition of *T. brucei* 6PGDH (K_i = 0.01 μM).[10] The acetonide **3,** in which the sugar hydroxyl groups are masked in a five-membered ring, is 35-fold less potent than **4** against *F. tularensis* KdsD, while its potency decreases only 3.5-fold toward *T. brucei* 6GPDH.[10] The potency ratio we observed toward KdsD seems more reasonable than the ratio observed toward *T. brucei* 6GPDH, and Dardonville et al.[10] apparently agree, since they attributed the small ratio they observed to hydrolysis of the acetonide. The diacetate **5** and the amide **6** were both slightly more potent than the hydroxamate **4,** a result that was unexpected. The hydroxyl groups on hydroxamate **4** are positioned analogous to those in the substrate Ru5P, and the acetyl groups in **5** would be expected to disrupt the natural interactions with the protein. The presence of the acetyl groups led to an eight-fold decline in potency against *T. brucei* 6PGDH,[10] but a fivefold increase in potency toward *F. tularensis* KdsD. The amide **6**, while it maintains the substrate-like positioning of the hydroxyl groups, lacks the enediol mimicry of the hydroxamate. This led to easy-to-rationalize losses in potency against *T. brucei* 6PGDH (150-fold),[10] and *M. tuberculosis* RpiB (>750-fold),[12] but a 3.5-fold increase in potency against *F. tularensis* KdsD.

To further investigate the properties of these inhibitors, we tested the ability of the two most potent compounds, **5** and **6**, to inhibit the growth of *E. coli* in culture.[14] We used this model Gram-negative organism for the sake of convenience, as *F. tularensis*is not generally available for these types of studies. As shown in Table 1, the minimum inhibitory concentrations (MICs) were in the tens of millimolar range, approximately 1000-fold their IC_{50}s for *F. tularensis* KdsD.

Compound **5** now represents the most potent KdsD inhibitor reported to date, which is clearly a step in the right direction. The limited selectivity and cytotoxicity data available, however, suggest that work will be required to convert compound **5** into a solid lead. Its K_i against *T. brucei* 6PGDH is 80 nM and its K_i against sheep 6PGDH is 360 nM.[10] The in vitro antiparasitic activity and the cytotoxicity against L6 cells were 229 μM or greater—at least 10^3 higher than the enzyme inhibitory level[10]—but perhaps lower than the 52 mM *E. coli* MIC (Table 1). Clearly, a round or two of optimization is necessary before one would expect to find selectivity. Information that would be helpful for optimizing the inhibitors described here is currently limited.

Unlike *T. brucei* 6PGDH[11] and *M. tuberculosis* RpiB,[12] there are no crystal structures of *F. tularensis* KdsD. The closest model available is the very recently reported X-ray crystal structure of a fragment, the sugar isomerase domain, of an inactive site-directed mutant of *E. coli* KdsD.[15] While sufficient for making predictions about which residues are involved in catalysis, this model is insufficient to explain the inhibition results presented here. A high-resolution structure of the full-length, wild-type *F. tularensis* KdsD protein containing a small molecule in the active site is needed to make rapid progress. In the absence of this structure, however, there are still steps that can be taken. Although the hydroxamates in this study did not inhibit KdsD as potently as they inhibit *T. brucei* 6PGDH, this does not rule out the use of other enediol mimics to increase potency. Other enediol mimics include the oxime used by Bearne and Blouin[16] as an inhibitor of glucosamine-6-phosphate synthase, and the reverse-hydroximate found in the antibacterial fosmidomycin,[17] an inhibitor of bacterial 1-deoxy-D-xylulose 5-phosphoate reductoisomerase. Finally, the assay described here could be used to screen individually synthesized analogs of **5**, or collections of randomly selected compounds as a means of identifying promising chemical matter for drug discovery efforts.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.12.066.

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