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Synthesis and Biochemical Evaluation of Selective Inhibitors of Class II Fructose Bisphosphate Aldolases: Towards New Synthetic Antibiotics

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Abstract: We report the synthesis and biochemical evaluation of selective inhibitors of class II (zinc-dependent) fructose bisphosphate aldolases. The most active compound is a simplified analogue of fructose bisphosphate, bearing a well-positioned metal chelating group. It is a powerful and highly selective competitive inhibitor of isolated class II aldolases. We report crystallographic studies of this inhibitor bound in the active site of the *Helicobacter pylori* enzyme. The compound also shows activity against *Mycobacterium tuberculosis* isolates.

Keywords: aldolases · antibiotics · bioorganic chemistry · enzyme inhibitors

Introduction

Bacterial infections, including nosocomial diseases contracted in hospital from antibiotic-resistant strains of pathogenic bacteria, are a public health problem of major concern. Tuberculosis (TB) is one of the most common infectious diseases known to man. The increasing occurrence of multiple-drug resistant TB, even in developed countries, is also alarming because there are only a few effective drugs available, and infection with drug-resistant *Mycobacterium tuberculosis* (*M. tb*) could give rise to a potentially untreatable form of disease. It must be noted that there have been no new major anti-TB drugs introduced into widespread use since rifampicin in the 1960s. *Helicobacter pylori* is another very widespread bacterium, responsible for most gastric ulcers and the sole known microorganism directly involved in the development of cancer.[1]

There is clearly an urgent need for new drugs with bactericidal mechanisms different from those of currently available agents.[2] This implies identification of new metabolic targets, specific to microbes, as likely sites of action for rationally designed drugs. The search for new therapeutic targets resulted in our interest in ATP synthesis from glucose in microbial pathogens, and more specifically in fructose-1,6-bisphosphate aldolase (Fba; E.C. 4.1.2.13).

Fba is an enzyme involved in glycolysis, in which it catalyses the retro-ketolic cleavage of fructose-1,6-bisphosphate (FBP) to yield dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P), and it also plays a role in gluconeogenesis and the Calvin cycle, in which it catalyses the condensation of DHAP with G3P.

Selective inhibition of microbial Fba would be expected to disrupt glycolysis, thereby affecting a major metabolic pathway and hindering survival and persistence of pathogens within their human host.[3] Although ubiquitous in living organisms, Fbas can be divided into two classes that differ in their structures and catalytic mechanisms. Class I Fbas are found in mammals and higher plants and form Schiff-base intermediates between the carbonyl substrates (FBP or DHAP) and lysine residues in their active sites. Class II Fbas, in contrast, require divalent metal ions (usually zinc or cobalt) to polarize the carbonyl groups of the substrates (FBP or DHAP) and to stabilize the enediolate intermediates during catalysis (Figure 1). They are found mainly in micro-organisms such as bacteria, yeasts, micro-

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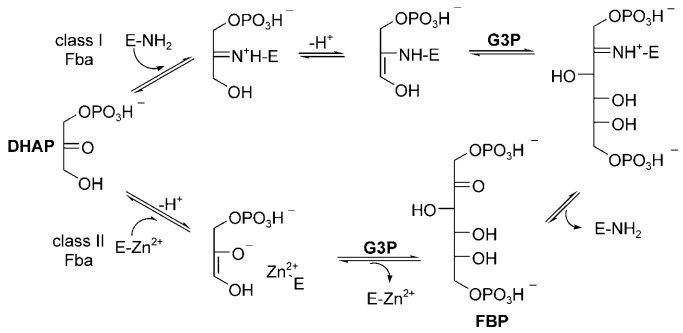


Figure 1. Mechanisms of class I (e.g., human) and class II (e.g., bacterial) FBP aldolases.

algae and protozoa (occasionally in combination with class I enzymes).

Because of their occurrence in many pathogenic bacteria (*M. tb*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *H. pylori*, *Yersinia pestis*, *Clostridium difficile*), yeasts (*Candida albicans*) and parasites (*Giardia lamblia*) and their absence in animals, it has been suggested that class II Fbas may serve as drug targets.[4] This assumption was further supported by the inability to knock out the class II FBP aldolase genes of *E. coli* and *Streptomyces*.[5,6] Also, the conditional loss of class II aldolase activity in temperature-sensitive mutants of *E. coli* harbouring point mutations in the class II FBP aldolase gene (*fda*) was found to result in loss of viability attributable to inhibition of rRNA synthesis.[7,8] *M. tb*, like *E. coli*, is distinctive in that it possesses both a class I and a class II FBP aldolase.[9,10] A recombinant form of the class II *M. tb* Fba protein was recently produced in *E. coli*.[11] However, hypoxic conditions, generally believed to be determinant in establishment of a latent infection by *M. tb*, have been shown to induce both increased levels and increased activity of the class II Fba.[9,12] The apparent essentiality of class II Fbas in various micro-organisms and the likely importance of these enzymes during latent infection thus make Fbas an attractive and specific target for the development of novel anti-TB drugs.

Surprisingly, while dozens of class I Fba inhibitors have been reported,[13] only one potent inhibitor of class II enzymes was known when we started this work. Phosphoglycolohydroxamate (PGH; Figure 2) was first prepared independently by two groups in 1973.[4,14] It is a very powerful inhibitor of the *E. coli* class II Fba ($K_i \approx 10$–50 nM). However, PGH is poorly stable in water, releasing (toxic) hydroxylamine through hydrolysis within a few hours. It has limited selectivity for class II Fbas over class I Fbas, and is a powerful inhibitor of several other enzymes. PGH has been shown to act both as a stable analogue of the high-energy intermediate of the reaction (Figure 2) and as a strong chelator of Zn^{2+}. The report of structural data on PGH-fuculose phosphate aldolase complexes[15] was a major breakthrough for understanding of the role of PGH as an inhibitor of metalloenzymes.

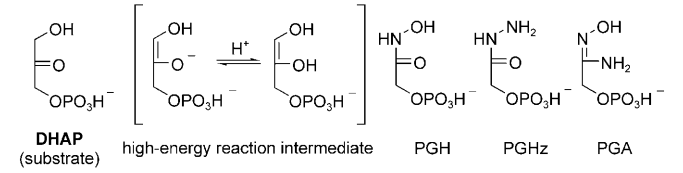


Figure 2. Substrate, reaction intermediate and inhibitors of class II Fbas.

We first reasoned that through changing the hydroxamate chelating group for other functions we might be able to retain strong inhibition while at the same time increasing selectivity and resistance to hydrolysis. This goal was only partly achieved with PGHz (phosphoglycolohydrazide) and PGA (phosphoglycoloamidoxime; Figure 2). The selectivities of both compounds for class II aldolases were increased and they were much more stable in water, but their K_i values were much higher than that of PGH (Table 1).[16]

Table 1. Activities of DHAP analogues as selective inhibitors of class II Fbas.

Inhibitor	K_i [μM] class I Fba	K_i [μM] class II Fba	Selectivity[b]
PGH[a]	2	0.05	273
PGHz	370	0.34	8900
PGA	>1000	2.3	3560

[a] Data from ref. [4]. [b] $(K_M/K_{iclass\ II})/(K_M/K_{iclass\ I})$. K_M class I (rabbit muscle) = 55 μM; K_M class II (*S. cerevisiae*) = 450 μM.

PGHz and PGA probably behaved as analogues of DHAP rather than as analogues of a high-energy intermediate. PGH was unambiguously shown to be present in its *s-Z* conformation within the active sites of several enzymes (Fbas from *E. coli* and *H. pylori*,[17–19] fuculose bisphosphate aldolase from *E. coli*,[15] methylglyoxal synthase[20] and triose phosphate isomerase[21]). Similarly, PGHz in complexation with *H. pylori* Fba chelates the Zn^{2+} ion in the *s-Z* conformation (M. Coinçon, J. Sygusch, unpublished results). Although we have no structural data on the structure of PGA in the active site of Fba yet, the *Z* configuration is very likely.

Results and Discussion

Chemistry: We therefore decided to design and synthesize analogues of the substrate of the forward reaction: FBP. Previous studies on class I Fbas had indicated that very simple structures can be potent inhibitors, provided that two phosphate groups, separated by an appropriate distance, are present.[13] The selectivity for class II Fbas would be induced by the presence, at the appropriate position, of a function capable of chelating the divalent Zn^{2+} ion in the active site. Taking into consideration the good inhibitory properties of PGH and PGHz,[16] we chose an N-substituted hydroxamate and an N-substituted hydrazide as basic structures. We further decided to omit the two secondary hydroxyl groups present in FBP, leading as a first step to the simplified analogues **1** and **2** (Figure 3).

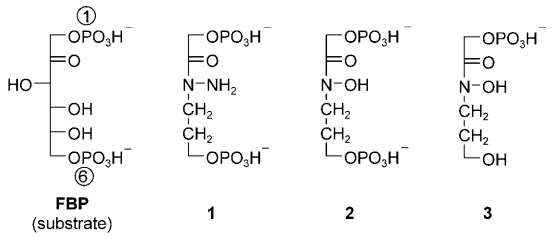


Figure 3. Substrate analogues designed as selective inhibitors of class II Fbas (with labelling of phosphates 1 and 6 on FBP).

Even if active in vitro, these bisphosphorylated compounds would be very unlikely readily to undergo passive diffusion across cellular membranes. The removal of one phosphoryl group could, however, favour permeation. The phosphoryl group at the 1-position of FBP has been reported to be essential for recognition by Fbas, while the one at the 6-position is not. Compound **3** can thus be considered a simplified analogue of fructose-1-phosphate, which is a rather weak inhibitor of class I Fbas, with $K_i > 2$ mM.[13] Syntheses of these three products are shown in Scheme 1.

Biochemical studies: All three compounds were tested as inhibitors of class I (from rabbit muscle) and class II (from *Saccharomyces cerevisiae*, *H. pylori* and *Mycobacterium bovis* BCG) aldolases.

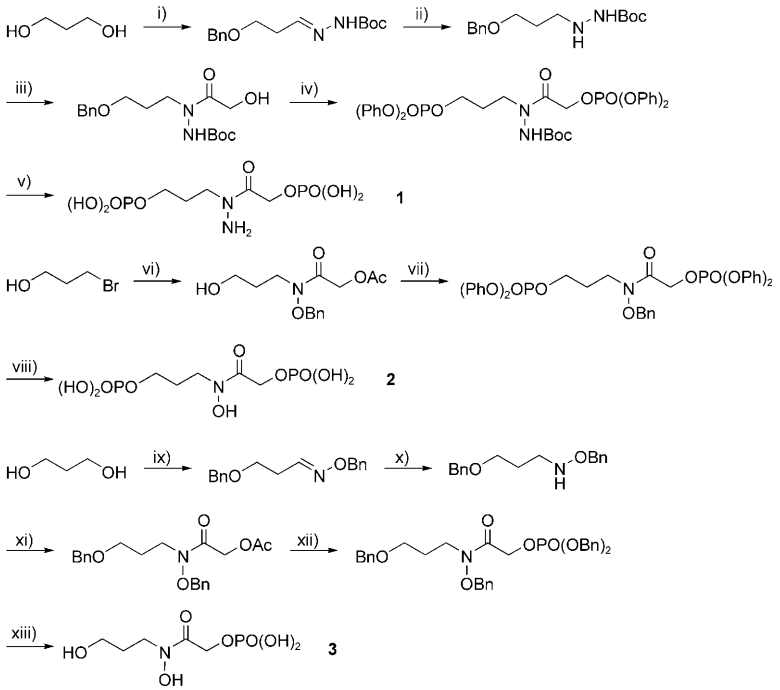
Fba from *H. pylori* was a recombinant form produced in *E. coli*. The activities of the *M. bovis* BCG (Pasteur strain; an attenuated strain of *M. bovis* used for vaccination against tuberculosis) and yeast enzymes were monitored in crude or partially purified cell-free extracts obtained by breaking fresh cells in a French press. The primary sequence of the *M. bovis* BCG enzyme is 100% identical to that of the pathogenic species: *M. tuberculosis* H37Rv. The compounds behaved as purely competitive inhibitors of all tested enzymes. Results are shown in Table 2.

Table 2. In vitro inhibition of various class I and class II Fbas by FBP analogues.

Inhibitor	Class I Fba	Class II Fba		
	Rabbit muscle	*S. cerevisiae*	*H. pylori*	*M. bovis*
	K_i [μM] (selectivity)[a]			
1	524	49 (90)	21 (23)	3.1[b] (180)
2	264	0.025[b] (86500)	0.013 (18500)	0.013 (22200)
3	2500[b]	1.6 (12800)	4.7[b] (480)	0.17 (16000)

[a] $(K_M/K_{iclass\ II})/(K_M/K_{iclass\ I})$. [b] Estimated from IC_{50} value. $K_{M\ rabbit\ muscle} = 55$ μM; $K_{M\ S.\ cerevisiae} = 450$ μM; $K_{M\ H.\ pylori} = 50$ μM; $K_{M\ M.\ bovis} = 60$ μM.

The hydrazide **1** acted as a modest inhibitor, both in terms of K_i and in terms of selectivity. In contrast, the hydroxamate **2** was very active, with a K_i comparable to that of PGH but a greatly increased selectivity for class II Fbas. The important difference between compounds **1** and **2** can be explained in part by the different affinities of a hydrazide and a hydroxamate for Zn^{2+}. Also, the ^1H NMR spectrum of **1** as a cyclohexylammonium salt indicates the presence of three cyclohexylamines (see Experimental Section), which means that one acidity of a phosphate is neutralized by the NH_2 of the neighbouring hydrazide (pK_a values of normal hydrazides are usually close to 7). Should this amphoteric form exist within the active site of the enzyme, it could lower the affinity of the hydrazide function for the zinc ion. As would be expected for N-alkylated hydroxamic acids, **2** and **3** were also much more stable in water than PGH (as verified by NMR of the product dissolved in D_2O). The higher K_i value of **3** confirmed that a phosphoryl group at the 6-position is an important, although non-essential, element of class II enzyme inhibitors.



Scheme 1. Synthesis of hydrazide **1** and of hydroxamates **2** and **3**. Reagents and conditions: i) a) NaH, BnBr (0.2 equiv), 75%; b) PCC/CH_2Cl_2, 47%; c) $BocNHNH_2$/THF, 68%. ii) DIBAH/toluene, 62%. iii) a) $ClCOCH_2OAc$/THF/pyridine, 76%; b) NEt_3/MeOH/H_2O 2:8:1, 100%. iv) a) $ClPO(OPh)_2$/pyridine, 82%; b) H_2/Pd-C, 96%; c) $ClPO(OPh)_2$/pyridine, 81%. v) a) H_2/PtO_2, 83%; b) H_2O/TFA 2:1, 100%. vi) a) $BnONH_2$EtOH reflux, 63%; b) $ClCOCH_2OAc$/MeOH/NEt_3, 70%. vii) a) $ClPO(OPh)_2$/pyridine, 81%; b) NEt_3/MeOH/H_2O 2:8:1, 100%; c) $ClPO(OPh)_2$/pyridine, 52%. viii) a) H_2/Pd-C, 98%; b) H_2/PtO_2, 98%. ix) a) NaH, BnBr (0.2 equiv), 67%; b) PCC/CH_2Cl_2, 88%. c) $BnONH_2$, EtOH/pyridine 80°C, 24 h. x) $NaBH_3CN$, EtOH/AcOH 1:1, RT, 4 h, 61% from ix. xi) $ClCOCH_2OAc$/THF/pyridine, 90%. xii) a) NEt_3/MeOH/H_2O 2:8:1, 68%; b) $P(OBn)_2NiPr$/imidazole/triazole/AcCN, 24 h RT; c) *t*BuOOH, 77% from xi. xiii) H_2/Pd-C, 100%.

Against the *M. bovis* BCG enzyme, **3** was "only" ten times less active than **2**, a particularly encouraging result for an analogue of fructose-1-phosphate. We believe that compound **3** might represent an interesting basis lead compound for the further synthesis of improved monophosphorylated compounds and lipophilic prodrugs.

Biological studies—growth inhibition of cultivated bacteria: Minimal inhibitory concentration values of compound **2** against *M. tb* H37Rv were determined in 7H9-ADC broth at 37 °C by the colorimetric resazurin microtiter assay described by Martin and collaborators.[22] The bacteria were grown aerobically under conditions compatible with class II Fba expression[9] and similar to those used for the preparation of the *M. bovis* BCG extracts (see above). Compound **2** inhibited the growth of *M. tb* H37Rv at a concentration of 1 mM. Although modest, this result was unexpected in view of the high polarity of compound **2** at physiological pH. The presence of the class I aldolase (not verified) might in part have offset the effect of **2**. We have no evidence yet that growth inhibition of cultivated *M. tuberculosis* by **2** correlates with inhibitory properties observed in vitro. If it is assumed, however, that this product kills *M. tb* through the inhibition of Fba, this effect could also be accounted for by an extra-cytosolic location of the enzyme.[12] We are now investigating the question of the permeation of compound **2** into *M. tb* cells (active transport or passive diffusion) and whether the inhibition of mycobacterial growth results directly from the effect of this compound on the *M. tb* class II Fba. Experiments to measure the MIC of **2** against *M. tb* grown under anaerobic conditions—under which substrate-level phosphorylation-dependent (and thus Fba-dependent) synthesis of ATP would be expected to prevail—are also underway.

Crystallographic studies: To examine the high affinity and selectivity of **2** for class II aldolases, crystallographic structures of Fba from *H. pylori* alone and bound with compound **2** were solved to 1.8 and 2.3 Å resolutions, respectively. Crystals of *H. pylori* Fba used for the studies were grown by vapour diffusion from a protein solution (10 mg mL^{-1}) diluted with an equal volume of precipitant buffer [PEG 1000 (12 %), PEG 8000 (12 %), calcium acetate (0.2 M) and Tris/HOAc (pH 8, 50 mM)] equilibrated against a reservoir of the same buffer. The *H. pylori* Fba structure was solved by molecular replacement with use of *Thermus aquaticus* Fba as search model. The refined native structure was then used as search model to determine the structure of *H. pylori* Fba bound with **2** by molecular replacement.

Superposition of native Fba with bound Fba (RMSD 0.48 Å based on all Cα atoms) revealed several significant localized conformational changes (Figure 4).

A loop (residues 177–191) in native Fba undergoes a 16 Å displacement (calculated from Cα atoms) upon binding of **2**, allowing Gly181 and Lys184 to interact with the P_1 phosphoryl group. The same loop also repositions His180, which binds the catalytic zinc ion. A second, adjacent loop (residues 210–214) adjusts by 1.7 Å, enabling Ser213 also to bind the P_1 phosphoryl group. Concomitantly with this movement, the catalytic zinc ion is displaced by 3.7 Å from its buried position in the native structure towards the active site surface, chelating the C=O and N−O oxygens of **2** while maintaining its interaction with the chelating His residues of the active site. Interactions between **2** and neighbouring residues in the *H. pylori* Fba active site are summarized in Figure 5.

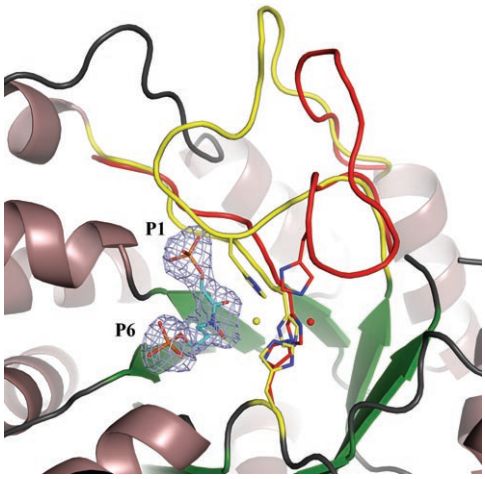


Figure 4. Difference electron density (F_0–F_C) annealed omit map showing fit of compound **2** bound in the active site of *H. pylori* Fba. Conformational changes induced upon binding of **2** are also highlighted. Loops and catalytic zinc ion that undergo positional changes are shown in red for the native Fba structure and in yellow for the bound Fba structure. P1 and P6 phosphoryls are identified. Figure and superposition were prepared with the program PyMOL.[23]

The structural data obtained with **2** in *H. pylori* Fba indicate, consistently with data previously reported for PGH bound in *E. coli* Fba,[17,18] that the interaction with the hydroxamate-based inhibitor results in a loosely coordinated trigonal bipyramid geometry around the catalytic Zn^{2+} ion (Figure 6). The conformational change stabilized by attachment of the P_1 phosphoryl results in tighter oxyanion binding (seven interactions; Figure 5) in relation to the interactions stabilizing attachment with the P_6 phosphoryl (three interactions; Figure 5) and corroborates P_1 phosphoryl as essential for active site binding. The hydroxamate moiety atoms C2, O2, N3 and O3 in compound **2** are coplanar (dihedral angle $<1°$) and in the s-*Z* conformation (Figure 6 A), mimicking an enediolate transition-state analogue, which would favour a potent K_i value. Because the catalytic Zn^{2+} ion is significantly out of the plane containing the putative enediolate, however, optimal chelation is unlikely. The same hydroxamate atoms in active site complexes formed by PGH bound with *E. coli*[17,18] and *H. pylori* Fba,[19] although not coplanar (dihedral angle $\approx 45°$), do chelate the catalytic Zn^{2+} ion in an approximately s-*Z* conformation (Figure 6B). Further synthetic exploration of simplified substrate analogues using the PGH scaffold that optimizes Zn^{2+} ion che-

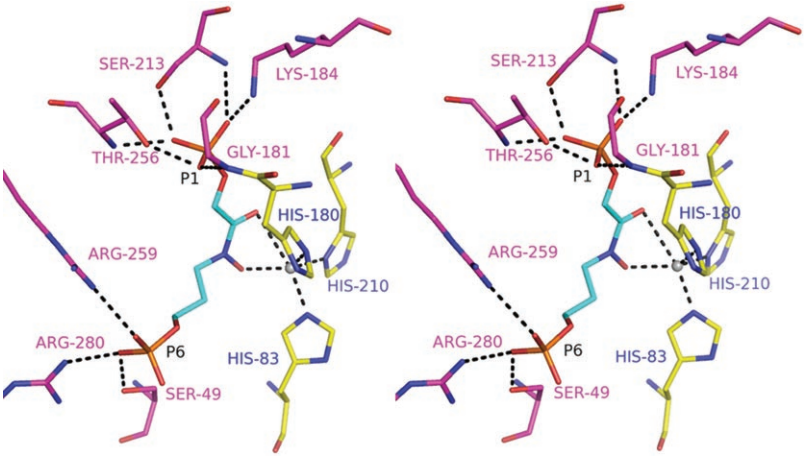


Figure 5. Interactions of inhibitor **2** with proximal residues in the *H. pylori* Fba active site (P_1 and P_6 phosphates of **2** are explicitly labelled).

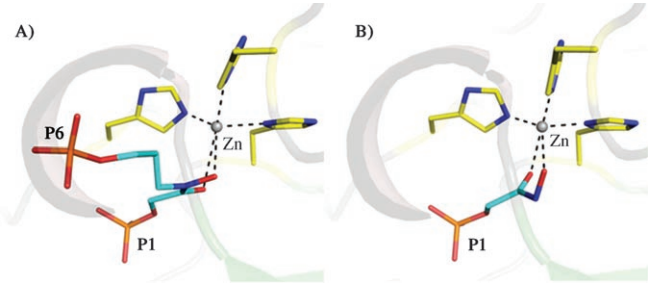


Figure 6. Coordination of catalytic Zn^{2+} ion in *H. pylori* Fba structure in binding of A) compound **2**, and B) PGH. View was obtained by first overlaying bound structures.

lation while ensuring hydroxamate coplanarity should afford compounds with more potent K_i values.

Compound **2** differs from the aldolase substrate and class I aldolase inhibitor hexitol-bisphosphate ($K_i \approx 0.5$ μM)[17] in the absence of hydroxyl moieties at its C_4 and C_5 atoms. In class I aldolases, both C_4 and C_5 hydroxyls participate directly and indirectly in hydrogen-bonding interactions with active site residues.[25] We postulate that **2**, because it lacks hydroxyl moieties at positions C_4 and C_5, is less able to participate in hydrogen bonding interactions with active site residues of class I aldolases and that this, together with the apparent coplanarity of the hydroxamate moiety atoms in **2**—that would further hinder optimal fit into the class I aldolase active site—forms a basis for the high selectivity of **2** for class II aldolases.

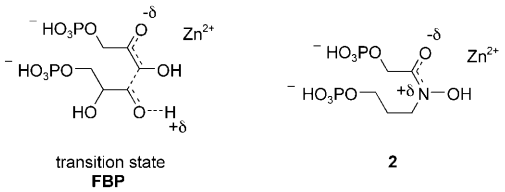


Figure 7. Presumed transition state of the retro-ketolic cleavage of FBP and mesomeric structure of compound **2**.

Although our initial goal was the synthesis of simplified analogues of the substrate FBP, the potent K_i value of compound **2** and the planarity of its hydroxamate moiety suggests that it behaves more like a transition-state analogue. One reasonable hypothesis is that the planar geometry enables internal electronic delocalization of the hydroxamic acid function in **2** that mimics in part the movement of electrons during the retro-ketolic cleavage of FBP (Figure 7).

Conclusion

Taking advantage of previous results on analogues of DHAP as inhibitors of Fba, we have prepared several analogues of FBP. We introduced a well-positioned chelating function in the hope of making these inhibitors selective for class II (zinc-dependent) Fbas. Compound **2** gave the best results, with low K_i values (10–20 nM) and good selectivity for class II over class I (more than 20000). The inhibition results, however, with $K_M/K_i > 2000$, indicate that **2** is not only a simple substrate analogue, but probably acts as a transition-state analogue. This inhibitor was co-crystallized with *H. pylori* Fba (representative of class II Fbas). The resolved structure of the complex unambiguously indicates that the inhibitor is linked to the active site around the catalytic zinc ion. Compound **2** is weakly active against cultivated *M. tuberculosis* (MIC 1 mM). The discrepancy between in vitro inhibition tests and biological assays can be partly explained by the high polarity of **2**, bearing four negative charges at physiological pH, and thus unlikely to cross biological membranes by simple diffusion. Compound **3**, in which the non-essential phosphoryl group is missing, is "only" ten times less efficient than **2** in inhibition tests. It could be a lead compound for further synthesis of lipophilic derivatives acting as prodrugs.

Experimental section

Chemical syntheses

N-(3-Hydroxypropyl)-glycolohydrazide-bisphosphate (compound 1)

Compound 1a: NaH (8 g) was added in portions to propane-1,3-diol (60 g, 0.79 mol) dissolved in anhydrous DMF (350 mL), followed by drop-by-drop addition of benzyl bromide (34.2 g, 0.2 mol). After 12 h at RT with stirring, the solvent was evaporated, the residue was dissolved in Et_2O, and the organic phase was washed with water, dried and concentrated. The product was purified by flash chromatography (Et_2O/pentane 1:1) to give **1a** (25 g, 75%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 7.36$ (m, 5H), 4.55 (s, 2H), 3.80 (dt, $J = 11$ Hz, 6 Hz, 2H), 3.68 (t, $J = 6$ Hz, 2H), 1.89 ppm (q, $J = 6$ Hz, 2H).

Compound 1b: PCC (19.4 g, 90.2 mmol) was suspended in anhydrous dichloromethane (250 mL) with vigorous stirring. Compound **1a** (10 g) was added dropwise, and the mixture was stirred overnight at RT. The black mixture was then filtered on a column of silica gel (200 mL), washed through with dichloromethane. After evaporation, the crude product was purified by flash chromatography (Et_2O/pentane 1:1) to give **1b** (4.61 g, 47%). ^{1}H NMR (250 MHz, $CDCl_3$): δ=9.78 (s, 1H), 7.36 (m, 5H), 4.55 (s, 2H), 3.82 (t, J=6 Hz, 2H), 2.68 ppm (t, J=7 Hz, 2H); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ=201.15, 137.99, 128.47, 127.79, 127.72, 73.21, 63.89, 43.87 ppm.

Compound 1c: Boc-hydrazine (1.74 g, 13.2 mmol) was added to compound **1b** (2.18 g, 13.45 mmol) in THF (25 mL). The mixture was stirred overnight at RT and was then concentrated. The residue was recrystallized from anhydrous pentane, giving **1c** (2.5 g, 68%). ^{1}H NMR (250 MHz, $CDCl_3$): δ=7.31 (m, 5H), 7.25 (t, J=5 Hz, 1H), 4.51 (s, 2H), 3.67 (t, J=6 Hz, 2H), 2.60 (q, J=6 Hz, 2H), 1.48 ppm (s, 9H); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ=152.58, 144.64, 134.07, 129.74, 128.54, 128.41, 127.69, 127.16, 81.02, 73.00, 67.59, 32.76, 28.00 ppm; HR-MS (ESI): m/z: calcd for $C_{15}H_{22}N_2O_3$ [M+Na$^+$]: 301.1523; found: 301.1524.

Compound 1d: DIBAL-H (20% in toluene, 45 mL, 55 mmol) was added dropwise under Ar at 0°C over 1 h to **1c** (5 g, 18.3 mmol) in toluene (100 mL). After the system had been kept for 5 h at RT, NaOH solution (1 M, 100 mL) was added slowly (exothermic reaction). After evaporation of the toluene, the remaining aqueous phase was extracted with dichloromethane. The organic phase was then washed successively with NaOH (1 M) and brine. Compound **1d** was purified by flash chromatography (pentane/ethyl acetate/NEt_3 30:70:0.1, 3.18 g, 62%). ^{1}H NMR (250 MHz, $CDCl_3$): δ=7.32 (m, 5H), 6.3 (brs, 1H), 4.52 (s, 2H), 3.56 (t, J=6 Hz, 2H), 2.96 (t, J=6 Hz, 2H), 1.79 (quint, J=6 Hz, 2H), 1.47 ppm (s, 9H); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ=156.67, 138.38, 128.27, 127.55, 127.44, 80.24, 72.88, 68.47, 49.41, 28.29, 28.04 ppm; HR-MS (ESI): m/z: calcd for $C_{15}H_{24}N_2O_3$ [M+Na$^+$]: 303.1679; found: 303.1681.

Compound 1e: Pyridine (1.19 mL, 15.7 mmol) was added to **1d** (3.18 g, 11.34 mmol) in anhydrous THF (50 mL), followed dropwise by acetoxyacetyl chloride (2.15 g, 15.7 mL) in THF (25 mL). After the system had been kept for 3 h at RT, water (20 mL) and dichloromethane (100 mL) were added, and the organic phase was washed with water and brine, dried over sodium sulfate and concentrated. Compound **1e** was obtained after flash chromatography (pentane/Et_2O/Et_3N 1:1:0.01, 3.38 g, 76%). ^{1}H NMR (250 MHz, $CDCl_3$): δ=7.40 (m, 5H), 4.85 (m, 2H), 4.57 (s, 2H), 4.26 (m, 1H), 3.61 (m, 2H), 3.19 (m, 1H), 2.24 (s, 3H), 1.93 (m, 2H), 1.54 ppm (s, 9H); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ=170.39, 168.87, 154.22, 137.64, 128.23, 127.23, 81.90, 72.97, 68.35, 61.10, 46.02, 27.88, 26.64, 20.39 ppm; HR-MS (ESI): m/z: calcd for $C_{19}H_{28}N_2O_6$ [M+Na$^+$]: 403.1840; found: 403.1850.

Compound 1f: Compound **1e** (3.38 g, 8.87 mmol) was stirred overnight at RT in a methanol/Et_3N/water mixture (8:2:1). After concentration, **1f** was obtained (3 g, 100%). ^{1}H NMR (250 MHz, $CDCl_3$): δ=7.25 (m, 5H), 4.45 (s, 2H), 4.11 (m, 2H), 4.06 (brs, 2H), 3.61 (t, J=7 Hz, 2H), 3.19 (m, 1H), 1.93 (quint, J=7 Hz, 2H), 1.45 ppm (s, 9H); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ=174.39, 154.16, 137.48, 128.15, 127.23, 81.75, 72.91, 68.15, 59.65, 46.02, 27.88, 26.59 ppm; HR-MS (ESI): m/z: calcd for $C_{17}H_{26}N_2O_5$ [M+Na$^+$]: 361.1734; found: 361.1738.

Compound 1g: Compound **1f** (3 g, 8.87 mmol) was dissolved in anhydrous pyridine (100 mL). Diphenylphosphoryl chloride (2.8 mL, 35.5 mmol) and DMAP (0.1 mmol) were added at once. The solution was stirred overnight at RT. Water was added, and after 10 min the reaction mixture was concentrated. Water and dichloromethane were added to the residue. The organic phase was washed with water, dried and concentrated. Compound **1f** was obtained after flash chromatography (AcOEt, 4.16 g, 82%). ^{1}H NMR (250 MHz,$CDCl_3$): δ=7.32 (m, 15H), 4.77 (m, 1H), 4.95 (s, 1H), 4.49 (s, 2H), 4.16 (m, 1H), 3.36 (t, J=6 Hz, 2H), 3.23 (m, 1H), 1.87 (dquint, J=6 Hz, 2H), 1.47 ppm (s, 9H); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ=167.00, 168.90, 154.26, 150.27, 15.,25, 137.54, 129.5, 128.35, 127.69, 125.25, 120.08, 82.18, 73.15, 68.52, 65.42, 46.15, 27.96, 26.74 ppm; ^{31}P NMR (101.3 MHz, $CDCl_3$): δ=−11.70 ppm; HR-MS (ESI): m/z: calcd for $C_{29}H_{35}N_2O_8P$ [M+Na$^+$]: 593.2023; found: 593.2037.

Compound 1h: Compound **1g** (4.16 g, 7.29 mmol) was dissolved in ethanol (50 mL). Pd/C (10%, 425 mg) was added, and the suspension was stirred overnight under dihydrogen (4 bar). The catalyst was filtered, and the solution was concentrated to give **1h** (3.38 g, 96%). ^{1}H NMR (250 MHz, $CDCl_3$): δ=8.07 (brs, 1H), 7.30 (m, 10H), 5.05 (m, H), 4.76 (m, 1H), 3.99 (m, 1H), 3.59 (m, 2H), 3.28 (m, 1H), 1.71 (m, 2H), 1.45 ppm (s, 9H); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ=168.85, 168.76, 129.65, 125.42, 120.08, 82.18, 65.58, 59.58, 45.92, 28.91, 28.00 ppm; ^{31}P NMR (101.3 MHz, $CDCl_3$): δ=−11.72 ppm; HR-MS (ESI): m/z: calcd for $C_{22}H_{29}N_2O_8P$ [M+Na$^+$]: 503.1554; found: 503.1561.

Compound 1i: Compound **1h** was phosphorylated with diphenylphosphoryl chloride as described for **1g**. Yield 2.99 g (81%); ^{1}H NMR (250 MHz, $CDCl_3$): δ=8.21 (brs, 1H), 7.24 (m, 20H), 5.28 (m, 1H), 5.03 (m, 1H), 4.31 (m, 1H), 3.88 (m, 2H), 3.88 (m, 1H), 3.40 (m, 1H), 2.01 (m, 2H), 1.48 ppm (s, 9H); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ=168.3, 154.3, 150.2, 129.70, 125.3, 119.9, 81.9, 66.7, 65.4, 45.5, 27.9, 27.2 ppm; ^{31}P NMR (101.3 MHz, $CDCl_3$): δ=−11.76, −11.66 ppm; HR-MS (ESI): m/z: calcd for $C_{34}H_{38}N_2O_{10}P_2$ [M+Na$^+$]: 735.1843; found: 735.1847.

***N*-(3-Hydroxypropyl)-glycolohydrazide bisphosphate (compound 1) (tris-cyclohexylammonium salt)**: Compound **1i** (1.87 g, 2.63 mmol) was dissolved in ethanol (20 mL). PtO_2 (200 mg) was added, and the suspension was treated under dihydrogen (40 bar) overnight with vigorous stirring. After filtration and evaporation, solid **1j** (977 mg, 83%) was recovered. The product was dissolved in a H_2O/CF_3COOH mixture (2:1, 10 mL), and the solution was kept for 12 h at RT. After evaporation, the residue was dissolved in aqueous cyclohexylamine (1 M), concentrated and recrystallized from EtOH. Yield 0.9 g (49%); ^{1}H NMR (250 MHz, D_2O): δ= 4.49 (d, $J_{H,P}$=6 Hz, 2H; -CO-CH_2-OPO_3H^-), 3.62 (dt, $J_{H,P}$=6 Hz, $J_{H,H}$= 7 Hz, 2H; $^{2-}O_3PO$-CH_2-CH_2-), 3.40 (t, J=7 Hz, 2H; -CH_2-CH_2-N), 1.74 ppm (m, 2H; -CH_2-CH_2-CH_2-) [CHA+: 2.90 (m, 3H), 1.40–1.80 (m, 15H), 0.95–1.19 (m, 15H)]; ^{13}C NMR (62.9 MHz, D_2O): δ=174.20, 174.09, 63.41, 48.36, 27.90, 27.82 ppm [CHA: 51.18, 31.34, 25.20, 24.7 ppm]; ^{31}P NMR (101.3 MHz, D_2O): δ=− 0.46 (s, 1P), −0.17 ppm (s, 1P); HR-MS (ESI, negative): m/z: calcd for $C_5H_{13}N_2O_9P_2^-$: 307.01018; found: 307.01021.

***N*-(3-Hydroxypropyl)-glycolohydroxamic acid bisphosphate (compound 2)**

Compound 2a: *O*-Benzylhydroxylamine (4.63 g, 37 mmol) was added to 3-bromopropanol (2.72 g, 18 mmol) in anhydrous ethanol (30 mL), and the mixture was heated at reflux for 45 h. After complete concentration of the mixture, ethyl acetate (200 mL) was added, and the precipitated *O*-benzylhydroxylamine hydrobromide was removed by filtration. After repeated concentration of the mixture, the oily residue was redissolved in Et_2O. This organic solution was washed with potassium hydrogencarbonate (1 M), dried and concentrated. The product was purified by flash chromatography (Et_2O/NEt_3 100:0.1). Yield 2.04 g (63%); ^{1}H NMR (250 MHz, $CDCl_3$): δ=7.33 (m, 5H), 5.62 (brs, 1H), 4.73 (s, 2H), 3.74 (t, J=7 Hz, 2H), 3.18 (t, J=7 Hz, 2H), 2.85 (brs, 1H), 1.76 ppm (quint, J= 7 Hz, 2H); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ=135.3, 128.5, 128.1, 76.3, 62.6, 51.0, 29.6, 25.20 ppm; HR-MS (ESI): m/z: calcd for $C_{10}H_{15}NO_2$ [M+Na$^+$]: 204.0995; found: 204.0996.

Compound 2b: Compound **2a** (0.839 g, 4.64 mmol) and triethylamine (0.563 g, 5.6 mmol) were dissolved in methanol (10 mL) at 0°C. Acetoxyacetyl chloride (0.76 g, 5.57 mmol) in anhydrous Et_2O was added dropwise to this solution. The reaction mixture was stirred for 15 min at RT and was then diluted with Et_2O and treated with water (30 mL). The organic phase was washed with water, dried and concentrated. The product was purified by flash chromatography (Et_2O/NEt_3 100:0.1) to yield **2b** (0.914 g, 70%). ^{1}H NMR (250 MHz, $CDCl_3$): δ=7.4 (m, 5H), 4.89 (s, 2H), 4.73 (s, 2H), 3.83 (t, J=7 Hz, 2H), 3.58 (t, J=7 Hz, 2H), 2.15 (s, 3H), 1.83 ppm (quint, J=7 Hz, 2H); ^{13}C NMR (62.9 MHz, $CDCl_3$): δ= 170.36, 168.88, 133.55, 129.01, 128.54, 76.23, 60.81, 58.39, 42.22, 29.18, 20.21 ppm; HR-MS (ESI): m/z: calcd for $C_{14}H_{19}NO_5$ [M+Na$^+$]: 304.1155; found: 304.1156.

Compound 2c: Compound **2b** (1.38 g, 4.9 mmol) was phosphorylated with diphenylphosphoryl chloride as described for **1f**. Yield 2.05 g (81%); ^{1}H NMR (250 MHz, $CDCl_3$): δ=7.15–7.4 (m, 15H), 4.87 (s, 2H), 4.69 (s, 2H), 4.31 (dt, J=12, 7 Hz, 2H), 3.73 (t, J=7 Hz, 2H), 2.15 (s,

3H), 2.06 ppm (quint, $J=7$ Hz, 2H); ^{13}C NMR (62.9 MHz, $CDCl_3$): $\delta=$ 170.4, 168.6, 150.3, 150.2, 133.6, 129.0, 129.2, 129.1, 128.7, 125.2, 119.9, 119.8, 76.5, 66.7, 66.6, 61.1, 42.4, 27.5, 27.4, 20.4 ppm; ^{31}P NMR (101.3 MHz, $CDCl_3$): $\delta=-12$ ppm; HR-MS (ESI): m/z: calcd for $C_{28}H_{28}NO_8P$ [M+Na$^+$]: 536.1445; found: 536.1452.

Compound 2d: Compound **2c** (2.2 g, 4.2 mmol) was deprotected as described for **1e**. Yield 2 g (100%); ^{1}H NMR (250 MHz, $CDCl_3$): $\delta=7.15$–7.4 (m, 15H), 4.73 (s, 2H), 4.30 (dt, $J=12$, 7 Hz, 2H), 4.21 (s, 2H), 3.75 (t, $J=7$ Hz, 2H), 2.04 ppm (quint, $J=7$ Hz, 2H); ^{13}C NMR (62.9 MHz, $CDCl_3$): $\delta=173.66$, 150.24, 150.13, 133.46, 129.61, 129.08, 128.58, 125.23, 119.81, 119.75, 76.41, 66.51, 66.43, 59.97, 42.45, 27.46, 27.36 ppm; ^{31}P NMR (101.3 MHz, $CDCl_3$): $\delta=-11.98$ ppm; HR-MS (ESI): m/z: calcd for $C_{24}H_{26}NO_7P$ [M+Na$^+$]: 494.1339; found: 494.1347.

Compound 2e: Compound **2d** (2 g, 4.24 mmol) was phosphorylated as described for **1f**. Yield 1.56 g (52%); ^{1}H NMR (250 MHz, $CDCl_3$): $\delta=$ 7.15–7.4 (m, 25H), 4.83 (d, $J=11$ Hz, 2H), 4.76 (s, 2H), 4.33 (dt, $J=7.5$, 7 Hz, 2H), 3.77 (t, $J=7$ Hz, 2H), 2.10 ppm (quint, $J=7$ Hz, 2H); ^{13}C NMR (250 MHz, $CDCl_3$): $\delta=167.91$, 150.54, 150.42, 133.58, 129.91, 129.82, 129.44, 128.95, 125.51, 120.30, 120.23, 120.13, 76.71, 66.85, 66.77, 65.41, 42.68, 27.74, 27.65 ppm; ^{31}P NMR (101.3 MHz, $CDCl_3$): $\delta=-11.98$, -11.53 ppm; HR-MS (ESI): m/z: calcd for $C_{36}H_{35}NO_{10}P_2$ [M+Na$^+$]: 726.1628; found: 726.1628.

Compound 2f: Compound **2e** (0.945 g, 1.34 mmol) was dissolved in ethanol (20 mL). Pd-C (10%, 100 mg) was added, and the suspension was vigorously stirred under dihydrogen overnight. After filtration and evaporation, **2f** (0.805 g, 98%) was recovered. ^{1}H NMR (250 MHz, $CDCl_3$): $\delta=$ 7.15–7.4 (m, 20H), 4.97 (d, $J=11$ Hz, 2H), 4.33 (dt, $J=6$ Hz, 2H), 3.67 (t, $J=6$ Hz, 2H), 2.10 ppm (quint, $J=6$ Hz, 2H); ^{13}C NMR (62.9 MHz, $CDCl_3$): $\delta=167.39$, 167.28, 150.38, 150.29, 150.20, 129.85, 129.75, 125.57, 120.19, 120.13, 12.01, 119.95, 67.04, 66.37, 65.71, 65.65, 45.09, 27.42, 27.34 ppm; ^{31}P NMR (101.3 MHz, $CDCl_3$): $\delta=-11.26$, -11.64 ppm; HR-MS (ESI): m/z: calcd for $C_{29}H_{29}N_2O_{10}P_2$ [M+Na$^+$]: 636.1159; found: 636.1171.

***N*-(3-Hydroxypropyl)-glycolohydroxamic acid bisphosphate (compound 2) tetrakis-cyclohexylammonium salt**: Compound **2g** (0.805 g, 1.31 mmol) was dephenylated as described for **1i**. The residue was dissolved in ethanol (20 mL), and an excess of cyclohexylamine was added. The solution was evaporated to afford a white powder, which was recrystallized from ethanol. Yield 0.32 g (34%); ^{1}H NMR (250 MHz, D_2O): $\delta=430$ (d, $J_{H,P}=$ 6 Hz, 2H; -CO-*CH*$_2$-OPO$_3^{2-}$), 3.54 (dt, $J_{H,P}=J_{H,H}=6$ Hz, 2H; $^{2-}$O$_3$PO-*CH*$_2$-CH$_2$-), 3.45 (t, $J=7$ Hz, 2H; -CH$_2$-*CH*$_2$-N), 1.66 ppm (quint, $J=$ 7 Hz, 2H; -CH$_2$-*CH*$_2$-CH$_2$) [CHA+: 2.85 (m, 4H), 1.25–1.80 (m, 20H), 0.80–1.25 (m, 20H)]; ^{13}C NMR (62.9 MHz, D_2O): $\delta=172.13$, 62.30, 62.24, 62.08, 50.92, 47.00, 31.04, 28.13, 28.04, 24.99, 24.49 ppm; ^{31}P NMR (101.3 MHz, D_2O): $\delta=3.52$, 3.65 ppm; HR-MS (ESI, negative): m/z: calcd for $C_5H_{12}NO_{10}P_2^-$: 307.99420; found: 307.99427

***N*-(3-Hydroxypropyl)-phosphoglycolohydroxamic acid (compound 3)**

Compound 3a: BnBr (0.1 mol, 17 g) was added dropwise over 20 minutes to a solution of propane-1,3-diol (0.3 mol, 21.67 mL) in anhydrous DMF (100 mL). After cooling of the mixture in an ice-bath, NaH (60%, 3 g, 0.125 mol) was added in portions. This mixture was stirred under argon overnight at RT. The solvent was concentrated under reduced pressure. Water (75 mL) was added, and the water phase was extracted with CH_2Cl_2 (2×75 mL). The combined organic phases were dried with Na_2SO_4 and concentrated under reduced pressure. The product was an oil (0.067 mol, 11.24 g, 67%). $R_f=0.18$ (pentane/Et_2O 8:2). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta=7.3$–7.4 (m, 5H), 4.54 (s, 2H), 3.76 (m, 2H), 3.64–3.67 (t, $J=6$ Hz, 2H), 1.9 ppm (quint, $J=5.8$ Hz, 2H); ^{13}C NMR (62.9 MHz, $CDCl_3$): $\delta=138.2$, 128.5, 127.7, 127.6, 32.31 73.2, 68.84, 61.09 ppm.

Compound 3b: PCC (0.036 mol, 7.74 g), 3-benzyloxypropan-1-ol (**3a**, 0.018 mol, 3 g) and powdered molecular sieves were added to dry CH_2Cl_2 (72 mL). This mixture was stirred under argon for 2.5 h. Et_2O (500 mL) was then added to the reaction mixture, which was filtered through a short column of silica gel. The solvent was evaporated under reduced pressure. The product was an oil (0.0157 mol, 2.584 g, 88%). $R_f=0.77$ (pentane/ethyl acetate 8:2). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta=9.81$ (t, $J=$ 2 Hz, 1H), 7.4 (m, 5H), 4.57 (s, 2H), 3.84 (t, $J=6$ Hz, 2H), 2.72 ppm (dt, $J=6$, 2 Hz, 2H); ^{13}C NMR (62.9 MHz, $CDCl_3$): $\delta=201.3$, 137.9, 128.5, 127.7, 127.6, 73.27, 63.9, 43.9 ppm.

Compound 3c: 3-Benzyloxypropionaldehyde (**3b**, 0.0157 mol, 2.57 g) was dissolved in methanol (78.5 mL). *O*-Benzylhydroxylamine (0.024 mol, 2.89 g) and pyridine (0.145 mol, 11.72 mL) were added. The mixture was stirred at reflux at 65°C for 3.5 h and concentrated under reduced pressure. The resultant product was dissolved in CH_2Cl_2 (50 mL), washed with water (3×50 mL), dried with Na_2SO_4 and concentrated under reduced pressure. The product was an oily mixture of two *Z*/*E* stereoisomers (together with remaining benzylhydroxylamaine). Yield 4.211 g, $R_{f1}=0.94$, $R_{f2}=0.88$ (pentane/ethyl acetate 8:2). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta=7.4$ (m, 10H), 6.9 and 7.5 (t, $J=5.5$ Hz, 1H), 5.14 and 5.19 (s, 2H), 4.76 (s, 2H), 3.65 and 3.68 (t, $J=6.5$ Hz, 2H), 2.56 and 2.75 ppm (q, $J=6.5$ Hz, 2H); ^{13}C NMR (62.9 MHz, $CDCl_3$): $\delta=149.0$, 137.75, 137.5, 128.5, 128.3, 128.0, 127.9, 127.7, 127.1, 75.9, 75.7, 66.7, 30.4 ppm.

Compound 3d: Sodium cyanoborohydride (0.0188 mol, 1.18 g) was added in portions, with cooling in an ice-bath, to **3c** (0.0157 mol, 4.211 g) dissolved in a mixture of ethanol and acetic acid (1:1, 25 mL). This was stirred at RT for 7.5 h. The solvent was then evaporated under reduced pressure, and the resultant product was made alkaline (pH 8.00) with saturated $NaHCO_3$. The mixture was extracted with CH_2Cl_2 (3×150 mL), washed with water (150 mL) and sat. NaCl (150 mL), dried with Na_2SO_4 and concentrated under reduced pressure. The product was purified by column chromatography (pentane/AcOEt 9:1). Yield 2.61 g (61% from **3b**). $R_f=0.1$ (pentane/Et_2O 9:1); ^{1}H NMR (250 MHz, $CDCl_3$): $\delta=7.4$ (m, 10H), 4.76 (s, 2H), 4.55 (s, 2H), 3.6 (t, $J=6.5$ Hz, 2H), 3.12 (t, $J=6.5$ Hz, 2H), 1.9 ppm (quint, $J=6.5$ Hz, 2H); ^{13}C NMR (62.9 MHz, $CDCl_3$): $\delta=$ 138.55, 138.1, 128.55, 128.45, 128.08, 127.85, 127.7, 76.2, 73.0, 68.65, 49.65, 27.6 ppm.

Compound 3e: Compound **3d** (6.47 mmol, 1.754 g), triethylamine (9.7 mmol, 1.36 mL) and acetoxyacetyl chloride (7.764 mmol, 0.83 mL) were dissolved in dry CH_2Cl_2 (10 mL). This mixture was stirred at RT for 20 minutes. The organic phase was washed with water (3×10 mL), dried with Na_2SO_4 and concentrated under reduced pressure. The product was an oil (2.167 g, 90%). $R_f=0.49$ (pentane/ethyl acetate 6:4); ^{1}H NMR (250 MHz, $CDCl_3$): $\delta=7.4$ (m, 10H), 4.88 (s, 2H), 4.75 (s, 2H), 4.5 (s, 2H), 3.8 (brt, $J=6.5$ Hz, 2H), 3.55 (t, $J=6.5$ Hz, 2H), 2.2 (s, 3H), 2.0 ppm (quint, $J=6.5$ Hz, 2H); ^{13}C NMR (62.9 MHz, $CDCl_3$): $\delta=170.7$, 168.5, 138.4, 134.0, 129.3, 129.2, 128.9, 128.4, 127.74, 127.6, 76.5, 73.0, 67.5, 61.4, 43.45, 27.12, 20.7 ppm.

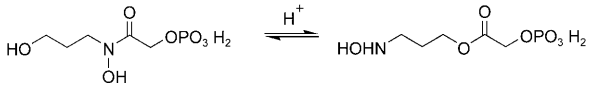
Compound 3f: Compound **3e** was dissolved in a methanol/triethylamine/water mixture (8:2:1, 44 mL). This was stirred at RT for 18 h. After complete evaporation, the product was purified by flash chromatography (pentane/AcOEt 6:4). The product was an oil (1.305 g, 68%). $R_f=0.38$ (pentane/ethyl acetate 7:3); ^{1}H NMR (250 MHz, $CDCl_3$): $\delta=7.4$ (m, 10H), 4.82 (s, 2H), 4.52 (s, 2H), 4.25 (s, 2H), 3.85 (m, 2H), 3.55 (t, $J=$ 6 Hz, 2H), 3.23 (brs, 1H), 2.0 ppm (quint, $J=6$ Hz, 2H); ^{13}C NMR (62.9 MHz, $CDCl_3$): $\delta=173.75$, 138.3, 133.95, 129.35, 128.9, 128.5, 127.8, 76.6, 73.1, 67.45, 60.25, 43.7, 27.14 ppm.

Compound 3g: A mixture of **3f** (3.96 mmol, 1.305 g), dibenzyl *N*,*N*-diisopropylphosphoramidite (7.923 mmol, 2.73 g), imidazole (11.88 mmol, 0.808 g) and 1,2,4-triazole (7.92 mmol, 0.55 g) was dissolved in dry acetonitrile (27 mL). This system was stirred at RT under argon for 27 h. *t*BuOOH (7.92 mmol, 1.08 mL, 7.3 M) and CH_2Cl_2 (30 mL) were then added. This mixture was stirred for 2 h at RT. An aqueous solution of sodium thiosulfate (1 M, 75 mL) and CH_2Cl_2 (30 mL) were added. The aqueous phase was extracted with CH_2Cl_2 (3×100 mL). The collected organic phases were washed with sat. Na_2CO_3 (200 mL), dried with Na_2SO_4, concentrated under reduced pressure and purified by flash chromatography (pentane/AcOEt). The product was an oil (1.794 g, 77%). $R_f=0.67$ (pentane/ethyl acetate 1:1); ^{1}H NMR (250 MHz, $CDCl_3$): $\delta=7.4$ (m, 20H), 5.2 (m, 4H), 4.78 (s, 2H), 4.7 (d, $J=11.25$ Hz, 2H), 4.5 (s, 2H), 3.8 (brt, $J=8.7$ Hz, 2H), 3.5 (t, $J=8.7$ Hz, 2H), 2.0 ppm (quint, $J=$ 8.75 Hz, 2H); ^{13}C NMR (62.9 MHz, $CDCl_3$): $\delta=168.5$, 138.4, 135.9, 133.9, 129.3, 128.9, 128.6, 128.5, 128.4, 128.0, 127.7, 127.6, 76.5, 73.0, 69.6, 67.5, 64.4, 43.5, 27 ppm; ^{31}P NMR (101.3 MHz, $CDCl_3$): $\delta=0.8$ ppm.

***N*-(3-Hydroxypropyl)-phosphoglycolohydroxamic acid (compound 3) bis-cyclohexylammonium salt**: A mixture of **3g** (1.5 mmol, 0.9 g), Na_2CO_3

(1.5 mmol, 0.15 g) and Pd/C (170 mg) in water/ethanol (1:1, 40 mL) was stirred under hydrogen (3 bar) for 36 h. It was then filtered through celite and concentrated under reduced pressure. The product, dissolved in water, was then filtered through a Dowex-50 column (cyclohexylammonium form), and the eluate was concentrated under reduced pressure to give the crystalline product (0.566 g, 86%). ^{1}H NMR (250 MHz, D_2O): $\delta = 4.4$ (d, $J_{H,P} = 5.75$ Hz, 2H; CO-CH_2-OPO_3^{2-}), 3.55 (t, $J = 6.5$ Hz, 2H; -CH_2-OH), 3.5 (t, $J = 6.5$ Hz, 2H; -CH_2-N-), 1.9 ppm (quint, $J = 6.5$ Hz, 2H; -CH_2-CH_2-CH_2-) [CHA+: 3.0 (m, 2H), 1–2 (m, 22H)]; ^{13}C NMR (62.9 MHz, D_2O): $\delta = 171.7$, 63.3, 61.6, 58.8, 50.2, 45.3, 30.3, 28.4, 24.2, 23.8 ppm; ^{31}P NMR (101.3 MHz, D_2O): $\delta = 3.6$ ppm; HR-MS (ESI, negative): m/z: calcd for $C_5H_{11}NO_7P^-$ $[M-H]^+$: 228.0273; found: 228.0272.

Compound **3** is apparently subject to the following rearrangement in acidic medium:



Biochemistry

Biochemical evaluation of inhibitors 1–3

Enzymes: Rabbit muscle Fba was a commercial preparation, available from Sigma or Fluka.

H. pylori Fba was a recombinant enzyme expressed in *E. coli* strain JM109. Yeast Fba was isolated from *S. cerevisiae*. Cells were disrupted in a French press (110 psi). The enzyme was partly purified by precipitation with ammonium sulfate.[38]

M. bovis Fba was isolated from cultivated cells by disruption in a French press. Crude extracts were used.

Solutions

Glycylglycine buffer (0.1 M, pH 7.4, with 0.2 M potassium acetate for class II aldolases).

NADH 1.41 mM in buffer.

Fructose bisphosphate 2 mM in buffer.

Glycerophosphate dehydrogenase (GPDH, 274 U mL^{-1}).

Enzymatic test: Fructose bisphosphate and inhibitor at the convenient concentration, NADH (0.12 mM), GPDH (11 U) and aldolase (4 mU) were placed in a cuvette to give a final volume of 1.2 mL. The decrease in absorbance of NADH at 340 nm was monitored on a spectrophotometer over 1–2 min.

Purification and recrystallization of *H. pylori* Fba: Plasmid pKK223-3 coding for *H. pylori* Fba was transformed and over-expressed in *E. coli* strain JM109. Recombinant *H. pylori* Fba was purified by a combination of anion exchange (DEAE), hydrophobic exchange chromatography (Phenyl-Sepharose) and size exclusion chromatography.[26] Aldolase concentration was determined by use of BCA Protein Assay Reagent (Pierce) with bovine serum albumin serving as standard. Enzymatic activity was monitored by spectrophotometry with use of a coupled assay and monitoring of NADH oxidation at 340 nm. The purified protein was stored at 4°C in 85% saturated ammonium sulfate solution (25 mM Tris·HCl, pH 8).

H. pylori aldolase crystals were grown by vapour diffusion from a mixture (1:1) of protein solution (10 mg mL^{-1} initial protein concentration made up in 25 mM Tris·HCl pH 7.0) and precipitant buffer [PEG 1000 (12%), PEG 8000 (12%), calcium acetate (0.2 M) and Tris/HOAc (pH 8, 50 mM)]; 4 µ drops were equilibrated at 23°C against 1 mL reservoirs of precipitant solution. Crystals grew over two weeks.

Data collection and processing: Aldolase crystals were soaked in compound **2** buffer [mother liquor plus compound **2** (10 mM)] or in PGH buffer [mother liquor plus PGH (10 mM)]. Prior to data collection, crystals were cryoprotected by transfer through a cryobuffer solution [compound **2** or PGH buffer plus glycerol (10%)] and immediately flash frozen in a stream of gaseous N_2 cooled to 100 K. Diffraction data were collected from single crystals at beamlines X8C and X29 of the National Synchrotron Light Source (Brookhaven National Laboratory, Upton, USA). As control, a native data set was also collected with use of beamline X8C. A fluorescence energy scan, collected at beamline X8C by energy scanning about the Zn Kα edge (1.2818 Å), demonstrated that the *H. pylori* Fba crystals contained zinc, although no exogenous zinc had been added during purification or recrystallization of the protein. All data sets were processed by use of HKL2000[27], and the results are summarized in Table I in the Supporting Information.

Structure solution and refinement: A model structure of *H. pylori* aldolase was generated by feeding to the molecular replacement (MR) program AMORE[28] 20 comparative homology models obtained with the program MODELLER[29] with use of the structure of class II *T. aquaticus* aldolase[30] as template (PDB code 1RVG). The best solution was used as starting point for refinement. The native structure was solved by iterative rounds of refinement (simulated annealing and minimization) with CNS[31] and model building.[32] Initial phases used for model building of liganded structures were obtained by using the refined native structure as template for input into the program Phenix autoMR/autoBuild.[33] The native and PGH structures belong to the monoclinic space group $P2_1$ and each have one aldolase homodimer in the asymmetric unit. The structure in complexation with compound **2** belongs to space group $P1$ and also has one aldolase homodimer in the asymmetric unit. All reflections with $I/\sigma(I) > 1$ were used in refinement; however, electron density maps were calculated to the resolution shown in Table I in the Supporting Information and corresponded to completeness of greater than 70% in the highest-resolution shell. The liganded structures were subjected to iterative rounds of refinement Phenix and model building with Coot[34] and PyMol.[23] The Molprobity server[35] and the Coot validating tools were used to optimize the structures during the refinement. Water molecules were added automatically by CNS or Phenix in the initial rounds and manually near the end of refinement. Loop regions (residues 139–153) in each subunit were associated with regions of weak electron density.

Ligand modelling was based on interpretation of electron density shapes of $2F_o-F_c$ and F_o-F_c annealed omit maps and use of the phenix.elbow command for generation of topology and parameters. Binding by compound **2** and PGH were readily discernable and were associated with clearly defined electron densities in the active site. Difference electron density (F_o-F_c) annealed omit maps calculated in the final round of refinement confirmed identical binding of ligands in both subunits. Final model statistics, calculated with CNS and PROCHECK,[36] are shown in Table I in the Supporting Information. The coordinates and structure factors of *H. pylori* aldolase, as well as those of the native enzyme in complexation with PGH and with compound **2**, have been deposited with the Protein Data Bank (PDB entry codes 3C4U, 3C52 and 3C56, respectively).[37] The final structure models of native aldolase, compound **2** and PGH enzymatic complexes have R_{cryst} (R_{free}) values of 0.185 (0.207), 0.172 (0.208) and 0.204 (0.249), respectively. The corresponding Luzzati atomic coordinate errors were estimated at 0.20, 0.23 and 0.28 Å, respectively. Ramachandran analysis with PROCHECK placed at least 90% of non-glycine and non-proline residues of the four structures in the most favourable region and with the remainder found in allowed regions, attesting to good model geometry in the structures.

Comparisons: Superpositions were performed with the program PyMOL with use of C_α atom coordinates of identical regions of amino acid sequences. Root mean square (rms) deviations based on superposition of equivalent C_α atoms are reported. The dihedral angles for the hydroxamate moieties in compound **2** and PGH are the angles between the planes defined by atoms C2, N3 and O2 and by atoms C2, N3 and O3. A dihedral angle of 0° indicates coplanarity and has atoms O2 and O3 positioned *cis* to each other and coplanar with atoms C2 and N3.

Biology

Growth inhibition of cultivated bacteria: Minimal inhibitory concentration values of compound **2** against *M. tb* H37Rv were determined in 7H9-ADC broth at 37°C by the colorimetric resazurin microtiter assay described by Martin and collaborators.[22] The bacteria were grown aerobically under conditions compatible with class II Fba expression[9] and similar to those used for the preparation of the *M. bovis* BCG extracts.

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