Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 4427–4433
Bioorganic & Medicinal Chemistry

Brucella suis histidinol dehydrogenase: Synthesis and inhibition studies of a series of substituted benzylic ketones derived from histidine

Marie-Rose Abdo,[a,†] Pascale Joseph,[b,†] Rose-Anne Boigegrain,[b] Jean-Pierre Liautard,[b] Jean-Louis Montero,[a] Stephan Köhler[b,*] and Jean-Yves Winum[a,*]

[a]*Institut des Biomolécules Max Mousseron (IBMM), UMR 5247 CNRS-UM1-UM2, Bâtiment de Recherche Max Mousseron, Ecole Nationale Supérieure de Chimie de Montpellier, 8 rue de l'Ecole Normale, 34296 Montpellier Cedex, France*
[b]*Centre d'Etudes d'Agents Pathogènes et Biotechnologies pour la Santé (CPBS), UMR 5236 CNRS-UM1-UM2, Université Montpellier II, cc100, Place E. Bataillon, 34095 Montpellier, France*

Received 10 January 2007; accepted 17 April 2007
Available online 24 April 2007

Abstract—*Brucella* spp. is the causative agent of brucellosis (Malta fever), which is the most widespread zoonosis worldwide. The pathogen is capable of establishing persistent infections in humans which are extremely difficult to eradicate even with antibiotic therapy. Moreover, *Brucella* is considered as a potential bioterrorism agent. Histidinol dehydrogenase (HDH, EC 1.1.1.23) has been shown to be essential for the intramacrophagic replication of this pathogen. It therefore constitutes an original and novel target for the development of anti-*Brucella* agents. In this work, we cloned and overexpressed the HDH-encoding gene from *Brucella suis*, purified the protein and evidenced its biological activity. We then investigated the inhibitory effects of a series of substituted benzylic ketones derived from histidine. Most of the compounds reported here inhibited *B. suis* HDH in the lower nanomolar range and constitute attractive candidates for the development of novel anti-*Brucella* agents.

1. Introduction

Brucella species are Gram-negative cocco-bacillary organisms, of which three are pathogenic in humans (*Brucella melitensis*, *B. abortus* and *B. suis*).[1] They are facultative intracellular pathogens that can survive and multiply within the phagocytic cells of the mammalian hosts where they are at least partially protected from the immune system.[2,3] These bacteria are the causative agent of brucellosis (Malta fever), the most widespread zoonosis worldwide. They are capable of establishing persistent infections in humans which are extremely difficult to eradicate even with antibiotic therapy.

Brucella is considered as a potential bioterrorism agent and was the first pathogenic organism to be weaponized by the U.S. Military as it is highly transmissible by aerosol. In this context, the CDC (Center for Disease Control) classified *Brucella* as a class-B bioterrorism agent (http://www.bt.cdc.gov/agent/brucellosis/). Its known virulence, the strongly invalidating character of the disease and its potential ability to develop resistance to antibiotics used in treatments make this pathogen particularly dangerous. Moreover, a vaccine for humans is not available, and the isolation of antibiotic-resistant strains is easily conceivable. In the case of an accident or a bioterrorism attack with such modified strains a classical therapy would be without effect.

The large-scale analysis of the virulome of intracellular bacteria such as *Brucella* is an original approach for the identification of pathogen-restricted targets essential for the adaptation to and multiplication within the replicative niche. Indeed, the virulence of these bacteria depends on their capacity of survival and replication in macrophages.[4,5] The major goal of our project is to use the analysis of the virulome of *B. suis* to develop a non-antibiotic, target-directed therapeutic approach.[6] Amino acid biosynthesis enzymes are targets of choice, as these pathways are absent from mammalian cells.

Keywords: *Brucella*; Histidinol dehydrogenase; Enzyme; Inhibitor.
* Corresponding authors. Tel.: +33 4 6714 7234; fax: +33 4 6714 4344; e-mail addresses: kohler@univ-montp2.fr; winumj@univ-montp2.fr
† These authors contributed equally to this work.

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.04.027

The drugs will therefore inhibit the biosynthesis of certain amino acids by *Brucella*, which are essential for the pathogen within the human macrophage host cell. As a consequence, the development of the pathogen will be blocked specifically inside the host cell niche, without, however, affecting the host itself and the commensal flora. The targeted action of these anti-infectious agents most likely reduces the selective pressure on the microorganism, resulting in reduced rates of resistance. Finally, the specificity of such a therapeutic agent for a defined species of bacteria may be useful to protect a population against an epidemic as it may occur as a consequence of a bioterrorism attack.[6]

Recent work in our group has shown that the enzyme histidinol dehydrogenase (HDH, EC 1.1.1.23), encoded by gene *hisD* (BR0252) in *B. suis*, is essential for intramacrophagic replication.[4] L-histidinol dehydrogenase is a NAD-linked four-electron dehydrogenase that catalyzes the last two steps in the L-histidine biosynthesis, and it is found in microorganisms such as bacteria, fungi, and in plants. This enzyme is a homodimeric zinc metalloenzyme which contains one Zn^{2+} ion in each identical subunit. Although histidine biosynthesis has been elucidated in various organisms, histidinol dehydrogenases have been cloned and characterized to date from only two microorganisms, *Salmonella typhimurium* and *Escherichia coli*.[7–9]

Ten years ago, Dancer et al. reported that HDH is a suitable target for the development of potential herbicides.[10] The approach developed by this group was to prepare HDH inhibitors which target the lipophilic binding pocket adjoining the active site of the enzyme. Some of their compounds exhibited nanomolar inhibition activity against HDH cabbage enzyme.[10] To date, no other work has been published on the inhibition of this enzyme except a computational modelling study in 2001.[11]

As this enzyme could also constitute an attractive target for antibacterials, further development of specific HDH-inhibitors would be highly beneficial for obtaining novel types of anti-brucellosis agents, devoid of side effects.

In the present study, we cloned and overexpressed the HDH-encoding gene from *B. suis*, and we purified the protein. We then investigated the inhibitory effects of a series of substituted benzylic ketones derived from histidine on the enzyme activity. The data show that some are effective inhibitors targeting this bacterial HDH.

2. Results and discussion

2.1. Chemistry

The compounds chosen in this study belong to the same class as the previously described cabbage HDH inhibitors.[10] The chemistry employed for the preparation of the compounds reported here is shown in Scheme 1.

Starting from the commercially available L-histidine, we synthesized on a 10-grams-scale the fully protected histidine compound **4** in four steps. Reaction of L-histidine with thionyl chloride in methanol gave the methyl ester **1**, which was then protected by *tert*-butoxycarbonyl group on the NH position by action of di-*tert*-butyl dicarbonate to give compound **2**. Selective deprotection of imidazole NH with potassium carbonate afforded compound **3** in good yield. This latter was then protected on the same position by a methoxytrityl group to lead to compound **4** in good yield. This key compound was then condensed with the dianion of various substituted phenylacetic acids obtained by action of lithium bis(trimethylsilyl-amide). The resulting compounds were purified by silica-gel chromatography and then deprotected with dioxane HCl solution to afford compounds **5** as dihydrochloride salts (Fig. 1).

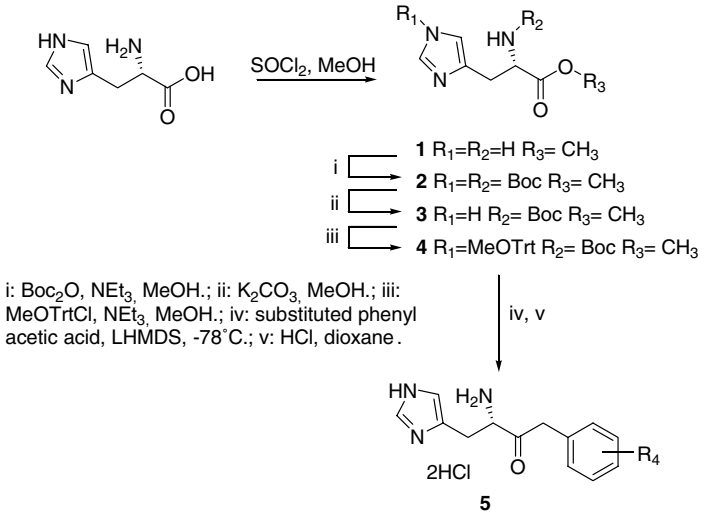
2.2. Preparation of recombinant *B. suis* HDH protein

The HDH-encoding gene was cloned into *E. coli* expression vector pET15b (Novagen) downstream the T7 promoter, enabling inducible expression of the recombinant protein. The resulting plasmid, pET15bHDH, was used to transform *E. coli* BL21(DE3). HDH protein was overproduced in *E. coli* BL21(DE3) as N-terminal hexahistidine ($6\times$(His))-tagged fusion protein after induction with IPTG. After cell lysis by sonication and centrifugation, His-tagged HDH protein was detected in the soluble fraction (data not shown). The recombinant HDH was then purified to homogeneity by Cobalt-affinity chromatography as described in Section 4. The protein fractions of the different purification steps were analyzed by SDS–PAGE (Fig. 2). The purified protein shows a single Coomassie-stained band at ≈49 kDa on SDS–PAGE (Fig. 2A, lane 6). This molecular mass determined by SDS–PAGE is in good agreement with the calculated value (≈48.2 kDa) deduced from the recombinant $6\times$(His)-tagged HDH. In addition, immunoblotting of the elution fraction with antibodies raised against the His-tag shows a single band, indicating that the recombinant HDH protein is produced as stable protein in *E. coli* (Fig. 2). The yield of *B. suis* HDH protein purification was 43 mg/L of bacterial culture. The specific activity of *B. suis* HDH is about 15 U mg^{-1} of protein, equivalent to a k_{cat} of about 12 s^{-1}. The enzyme affinity for the substrate, $K_{m\ app}$, histidinol is approximately 12 μM.

2.3. Inhibition study

Inhibition data against *Brucella suis* HDH with compounds **5a**–**5o** are presented in Table 1.

The compounds **5a** to **5o** showed IC_{50} in the range of 3–200 nM. The most effective compounds were the bromo-**5d** and the benzyloxy-**5i** derivatives with IC_{50} of 6 and 3 nM, respectively. The most ineffective inhibitors were compounds **5g** and **5m** which exhibited an IC_{50} of 150 and 200 nM, respectively. The other compounds have an inhibitory effect for concentrations ranging from 10 to 40 nM.



Scheme 1. Synthesis of the inhibitors.

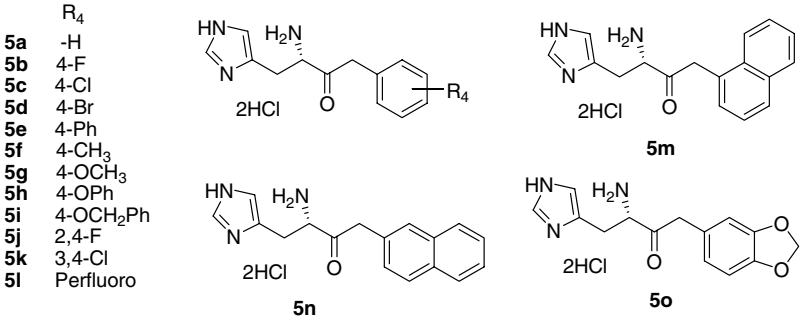


Figure 1. Structures of the inhibitors.

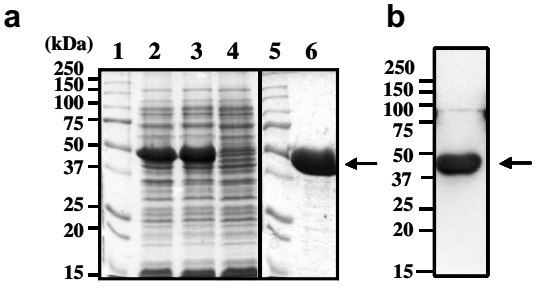


Figure 2. (a) SDS–PAGE of 6×(His)-tagged HDH from *B. suis* expressed from pET15b-HDH in *E. coli* BL21(DE3). Recombinant HDH was purified by Cobalt-affinity chromatography as described in Section 4. Lanes 1 and 5, molecular weight marker; lane 2, soluble fraction of whole cell lysate; lane 3, proteins from soluble fraction after dialysis; lane 4, proteins from soluble fraction after binding to the cobalt resin; lane 6, proteins from soluble fraction after elution from cobalt resin. (b) Western blot analysis of pure, His-tagged HDH protein with antibodies raised against His-tag. Pure HDH is indicated by an arrow.

Table 1. Inhibition of *B. suis* histidinol dehydrogenase with compounds **5a**–**5o**

Compound	HDH (*Brucella suis*) IC_{50}[a] (nM)	HDH (*Cabbage*) IC_{50}[b]
5a	40	100
5b	15	—
5c	12	300
5d	6	40
5e	12.5	40
5f	15	—
5g	150	1000
5h	16	—
5i	3	—
5j	10	—
5k	20	—
5l	70	—
5m	200	—
5n	14.5	—
5o	25	—

[a] The values are means of three independent assays. Variations were in the range of 5–10% of the shown data.
[b] Ref. 10.

The following should be noted regarding data of Table 1: (i) increase in the volume of R_4 substituent led in general to more potent inhibitors. This can be observed for example in the case of the compounds **5a** and **5f**; **5b** and **5d** or **5g** and **5h**. The presence of the phenoxy moiety in **5h** led to a 10-fold increase of drug inhibitory properties as compared to the corresponding methoxy-substituted compound **5g**. (ii) An interesting observation can be made with the two naphthyl derivatives **5m** and **5n**. Depending on the substitution position on the naphthyl group, a difference in behaviour could be observed. Substitution on the position 2 of the naphthyl moiety gave a very potent inhibitor with an IC_{50} of 14.5 nM. Substitu-

tion on position 1 led to an important decrease of activity with an IC_{50} of 200 nM. This could be explained by the alternative orientation of the bulky naphthyl moiety. In the case of compound **5n**, the naphthyl moiety could probably better accommodate in the lipophilic pocket near the active site than in the case of compound **5m**. In the same way, the effect of compound **5i**, which was a stronger inhibitor than **5h** and which was characterized by a longer R-chain, may give an indication of the distance between the active site and the lipophilic pocket. (iii) Five of the inhibitors investigated here such as **5a**, **5c**, **5d**, **5e** and **5g** also showed selectivity against *B. suis* HDH compared to cabbage HDH. Thus, the potent HDH inhibitors **5a**, **5d**, **5e** and **5g** were between 2.5 and 6.6 times, and the inhibitor **5c** (chloro-derivative) 25 times more efficient on *B. suis* HDH than on cabbage HDH.

3. Conclusion

For the first time, histidinol dehydrogenase protein from *Brucella suis* has been isolated, purified, and its biological activity characterized. Inhibition studies with this enzyme are presented here. A library of new histidine derivatives is reported and has been assayed for the inhibition of HDH. Effective inhibitors were detected with inhibitory activity below 10 nM. Our data suggest that HDH from *Brucella suis* constitutes a suitable target for such compounds, which represent valuable candidates for the potential development of novel, non-classical anti-brucellosis therapy. Moreover, the study of the interactions of the drugs with the active site of the HDH enzyme will give a better understanding of the drug effect, allowing the design of new potential drugs with improved inhibitory activity.

4. Experimental

4.1. Chemistry

4.1.1. General. All chemicals, reagents and solvents for the synthesis of the compounds were of analytical grade, purchased from commercial sources and used without further purification, unless otherwise specified. TLC analyses were performed on silica gel 60 F_{254} plates (Merck Art.1.05554). Spots were visualized at 254 nm under UV illumination, or by ninhydrin solution spraying. Melting points were determined on a Büchi Melting Point 510 apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on Brüker DRX-400 spectrometer using DMSO-d_6 as solvent and tetramethylsilane as internal standard. For ^1H NMR spectra, chemical shifts are expressed in δ (ppm) downfield from tetramethylsilane, and coupling constants (*J*) are expressed in Hertz. Electron Ionization mass spectra (10 eV) were recorded in positive or negative mode on a Water MicroMass ZQ.

4.1.2. Preparation of the protected histidine methyl ester (4). This compound was prepared in a four-step synthesis.

4.1.2.1. Preparation of L-histidine methyl ester dihydrochloride (1). Thionyl chloride (1.2 equiv) was added dropwise to a suspension of L-histidine (1 equiv) in anhydrous methanol (50 mL) at 0 °C, and the resulting mixture was refluxed for 16 h. The reaction mixture was allowed to cool down to room temperature and was concentrated. Additional methanol (50 mL) was added and the solution was evaporated again to afford a solid which was then triturated with ethylic ether to give a white powder. After filtration the resulting product was used in the next step without further purification: ^1H NMR (DMSO-d_6, 400 MHz) 9.05 (d, 1H, J = 1.3 Hz), 7.51 (d, 1H, J = 1.22 Hz), 4.47 (t, 1H, J = 7.0 Hz), 3.72 (s, 3H), 3.31 (dd, 2H, J = 1.2 Hz, J = 7.0 Hz); MS ESI^+ *m/z* 170.20 $(M+H)^+$.

4.1.2.2. Preparation of *N*-α-(*tert*-butoxycarbonyl)-1-*tert*-butoxycarbonyl-L-histidine methyl ester (2). To a suspension (1 equiv) of (**1**) in methanol (50 mL) were added (2 equiv) of triethylamine and (2.3 equiv) of $(Boc)_2O$. The reaction mixture was stirred for two hours at room temperature and monitored with TLC until no starting material was detected. The reaction mixture was then extracted with ethyl acetate and washed with water. The resulting organic layer was dried over anhydrous magnesium sulfate and concentrated under vacuum. The crude product was purified on silica gel (ether/hexane: 5:5) to give a white solid: ^1H NMR (DMSO-d_6, 400 MHz) 8.11 (d, 1H, J = 1.2 Hz), 7.25 (d, 1H, J = 0.8 Hz), 7.18 (d, 1H, J = 8.1 Hz), 4.25 (dt, 1H, J = 5.0 Hz, J = 9.0 Hz), 3.62 (s, 3H), 2.83 (dq, 2H, J = 6.9 Hz, J = 14.8 Hz), 1.56 (s, 9H), 1.35 (s, 9H); MS ESI^+ *m/z* 370.28 $(M+H)^+$ 392.13 $(M+Na)^+$.

4.1.2.3. Preparation of *N*-α-(*tert*-butoxycarbonyl)-L-histidine methyl ester (3). To a solution of *N*-α-(*tert*-butoxycarbonyl)-1-*tert*-butoxycarbonyl-L-histidine methyl ester (**2**) (1 equiv) in 30 mL of methanol, K_2CO_3 (0.1 equiv) was added and the mixture was refluxed for 3 h. When all of the starting was consumed, the mixture was extracted with ethyl acetate and washed with water. The organic layer was then dried over magnesium sulfate and the solvent evaporated under reduced pressure to afford the resulting product as a white solid. This product was used in the next reaction without further purification:^1H NMR (DMSO-d_6, 400 MHz) 7.54 (d, 1H, J = 1.2 Hz), 7.15 (d, 1H, J = 7.8 Hz), 6.79 (s, 1H), 4.23 (dt, 1H, J = 5.7 Hz, J = 7.8 Hz), 3.58 (s, 3H), 2.86 (m, 2H), 1.36 (s, 9H); MS ESI^+ *m/z* 270.20 $(M+H)^+$ 292.16 $(M+Na)^+$.

4.1.2.4. Preparation of *N*-α-(*tert*-butoxycarbonyl)-1-methoxytrityl-L-histidine methyl ester (4). To a stirred solution of *N*-α-(*tert*-butoxycarbonyl)-L-histidine methyl ester (**3**) (1 equiv) in dimethylformamide, triethylamine (1.5 equiv) and methoxytritylchloride (1.1 equiv) were added at room temperature. The reaction was monitored by TLC until the starting material was consumed. Then the mixture was extracted with ethyl acetate and washed with brine. The combined organic layers were dried over anhydrous magnesium sulfate and the solvent was evaporated under vacuum. The resulting crude was purified by flash chromatography on silica gel (hexane:

100% to hexane/ether: 50/50) to give the pure product as a white powder: ^{1}H NMR (DMSO-d_6, 400 MHz) 7.38 (m, 5H), 7.26 (d, 1H, J = 1.4 Hz), 7.07 (m, 5H), 6.95 (m, 4H), 6.65 (s, 1H), 4.21 (dd, 1H, J = 7.6 Hz, J = 13.7 Hz), 3.76 (s, 3H), 3.55 (s, 3H), 2.80 (dd, 2H, J = 3.4 Hz, J = 6.6 Hz), 1.34 (s, 9H); MS ESI$^+$ m/z 542.25 $(M+H)^+$ 564.24 $(M+Na)^+$.

4.1.3. General procedure for the coupling of various substituted phenylacetic acids with the protected histidine (4) and obtaining of the resulting dihydrochloride salts (5). A solution of the corresponding substituted phenylacetic acid (3 equiv) in dry tetrahydrofuran (10 mL) was cooled to 0 °C, and a solution of lithium bis(trimethylsilyl)amide (6 eq, 1 M in tetrahydrofuran) was added dropwise. After 10 min, the solution was cooled to −78 °C and the protected histidine methyl ester **4** (1 equiv) in tetrahydrofuran was introduced through a canula. The reaction mixture was stirred for 16 h under argon allowing the mixture to warm to room temperature. The reaction mixture was extracted with ethyl acetate (100 mL) and washed with water (4× 25 mL). The organic layer was dried over anhydrous magnesium sulfate and the solvent evaporated under reduced pressure to give yellowish viscous oil.

The residue was purified by column chromatography on silica gel (CH_2Cl_2/hexane/acetone: 4:5:1) to give the fully protected final product, a white powder. This latter was deprotected under acidic conditions in a solution of HCl (4 M in dioxane), and the reaction was monitored by TLC until all the starting material was consumed. The final salt was filtered and washed with diethyl ether.

4.1.3.1. (*S*)-3-Amino-4-(1*H*-imidazol-4-yl)-1-phenylbutan-2-one dihydrochloride (5a). Mp: 149–151 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) 14.80 (s br, 1H), 9.11 (d, 1H, J = 1.2 Hz), 8.62 (s br, 2H), 7.50 (d, 1H, J = 0.5 Hz), 7.29 (m, 5H), 4.70 (dd, 1H, J = 4.6 Hz, J = 8.8 Hz), 4.15 (s, 2H), 3.58 (dd, 1H, J = 4.4 Hz, J = 15.5 Hz), 3.22 (dd, 1H, J = 8.9 Hz, J = 15.5 Hz); ^{13}C NMR (DMSO-d_6, 101 MHz) 202.79, 134.33, 133.30, 129.82, 128.19, 126.78, 126.57, 118.21, 56.91, 45.24, 24.17; MS ESI$^+$ m/z 230.19 $(M+H)^+$ 242.28 $(M+Na)^+$. Anal. ($C_{13}H_{17}Cl_2N_3O$) 51.63C, 5.72H, 13.85N.

4.1.3.2. (*S*)-3-Amino-1-(4-fluorophenyl)-4-(1*H*-imidazol-4-yl)butan-2-one dihydrochloride (5b). Mp: 146–148 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) 14.72 (s br, 1H), 9.10 (d, 1H, J = 1.2 Hz), 8.62 (s br, 2H), 7.50 (d, 1H, J = 0.5 Hz), 7.28 (dd, 2H, J = 5.6 Hz, J = 8.7 Hz), 7.16 (t, 2H, J = 8.9 Hz), 4.70 (dd, 1H, J = 4.6 Hz, J = 8.7 Hz), 4.16 (s, 2H), 3.57 (dd, 1H, J = 4.4 Hz, J = 15.5 Hz), 3.21 (dd, 1H, J = 8.9 Hz, J = 15.5 Hz). ^{13}C NMR (DMSO-d_6, 101 MHz) 202.76, 161.17 (d, J_{C-F} = 245.3 Hz), 134.14, 131.73 (d, J_{C-F} = 8.1 Hz), 129.50 (d, J_{C-F} = 3.3 Hz), 126.56, 118.22, 114.93 (d, J_{C-F} = 21.2 Hz), 56.85, 44.33, 24.17; MS ESI$^+$ m/z 248.29 $(M+H)^+$. Anal. ($C_{13}H_{16}Cl_2FN_3O$) 48.72C, 5.14H, 13.06N.

4.1.3.3. (*S*)-3-Amino-1-(4-chlorophenyl)-4-(1*H*-imidazol-4-yl)butan-2-one dihydrochloride (5c). Mp: 186–188 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) 14.73 (s br, 1H), 9.10 (d, 1H, J = 1.3 Hz), 8.62 (s br, 2H), 7.50 (d, 1H, J = 0.9 Hz), 7.39 (d, 2H, J = 8.5 Hz), 7.27 (d, 2H, J = 8.5 Hz), 4.70 (dd, 1H, J = 4.6 Hz, J = 8.7 Hz), 4.19 (d, 2H, J = 2.8 Hz), 3.58 (dd, 1H, J = 4.3 Hz, J = 15.5 Hz), 3.22 (dd, 1H, J = 8.7 Hz, J = 15.5 Hz) ^{13}C NMR (DMSO-d_6, 101 MHz) 202.51, 133.99, 132.42, 131.75, 131.50, 128.08, 126.53, 118.26, 56.90, 44.58, 24.09; MS ESI$^+$ m/z 264.27 $(M+H)^+$. Anal. ($C_{13}H_{16}Cl_3N_3O$) 46.33C, 4.85H, 12.58N.

4.1.3.4. (*S*)-3-Amino-1-(4-bromophenyl)-4-(1*H*-imidazol-4-yl)butan-2-one dihydrochloride (5d). Mp: 190–192 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) 14.84 (s br, 1H), 9.11 (d, 1H, J = 1.4 Hz), 8.64 (s br, 2H), 7.52 (m, 3H), 7.21 (m, 2H), 4.70 (dd, 1H, J = 4.6 Hz, J = 8.8 Hz), 4.19 (d, 2H, J = 4.2 Hz), 3.58 (dd, 1H, J = 4.3 Hz, J = 15.6 Hz), 3.23 (dd, 1H, J = 8.8 Hz, J = 15.5 Hz); ^{13}C NMR (DMSO-d_6, 101 MHz) 202.45, 134.00, 132.84, 132.14, 131.01, 126.55, 120.04, 118.25, 56.91, 44.66, 24.10; MS ESI$^+$ m/z 308.20 $(M+H)^+$. Anal. ($C_{13}H_{16}BrCl_2N_3O$) 40.92C, 4.35H, 11.20N.

4.1.3.5. (*S*)-3-Amino-4-(1*H*-imidazol-4-yl)-1-(4-phenyl)-phenylbutan-2-one dihydrochloride (5e). Mp: 152–154 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) 14.72 (s br, 1H), 9.11 (d, 1H, J = 1.1 Hz), 8.63 (s br, 2H), 7.51 (d, 1H, J = 0.7 Hz), 7.70–7.30 (m, 9H), 4.73 (dd, 1H, J = 4.6 Hz, J = 8.8 Hz), 4.20 (s, 2H), 3.60 (dd, 1H, J = 4.4 Hz, J = 15.4 Hz), 3.24 (dd, 1H, J = 8.9 Hz, J = 15.5 Hz); ^{13}C NMR (DMSO-d_6, 101 MHz) 202.77, 139.77, 138.62, 133.98, 132.60, 130.42, 128.83, 127.28, 126.53, 126.49, 126.44, 118.26, 56.93, 44.93, 24.12; MS ESI$^+$ m/z 306.34 $(M+H)^+$. Anal. ($C_{19}H_{21}Cl_2N_3O$) 60.35C, 5.68H, 11.09N.

4.1.3.6. (*S*)-3-Amino-4-(1*H*-imidazol-4-yl)-1-p-tolylbutan-2-one dihydrochloride (5f). Mp: 148–150 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) 14.77 (s br, 1H), 9.11 (d, 1H, J = 1.1 Hz), 8.62 (s br, 2H), 7.49 (s, 1H), 7.12 (s, 4H), 4.68 (dd, 1H, J = 4.5 Hz, J = 8.8 Hz), 4.09 (s, 2H), 3.56 (dd, 1H, J = 4.4 Hz, J = 15.5 Hz), 3.20 (dd, 1H, J = 8.9 Hz, J = 15.5 Hz), 2.28 (s, 3H); ^{13}C NMR (DMSO-d_6, 101 MHz) 202.92, 135.84, 134.09, 130.17, 129.66, 128.78, 126.55, 118.20, 56.82, 44.88, 24.17, 20.61; MS ESI$^+$ m/z 244.32 $(M+H)^+$. Anal. ($C_{14}H_{19}Cl_2N_3O$) 53.25C, 6.16H, 13.21N.

4.1.3.7. (*S*)-3-Amino-4-(1*H*-imidazol-4-yl)-1-(4-methoxyphenyl)butan-2-one dihydrochloride (5g). Mp: 171–173 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) 14.73 (s br, 2H), 9.12 (d, 1H, J = 1.3 Hz), 8.63 (s br, 2H), 7.49 (d, 1H, J = 1.0 Hz), 7.15 (m, 2H), 6.88 (m, 2H), 4.68 (dd, 1H, J = 4.5 Hz, J = 8.7 Hz), 4.06 (s, 2H), 3.73 (s, 3H), 3.56 (dd, 1H, J = 4.4 Hz, J = 15.4 Hz), 3.21 (dd, 1H, J = 8.9 Hz, J = 15.5 Hz); ^{13}C NMR (DMSO-d_6, 101 MHz) 203.03, 158.13, 134.14, 130.79, 126.54, 125.02, 118.17, 113.65, 56.72, 54.95, 44.37, 24.18; MS ESI$^+$ m/z 260.31 $(M+H)^+$. Anal. ($C_{14}H_{19}Cl_2N_3O_2$) 50.52C, 5.79H, 12.63N.

4.1.3.8. (*S*)-3-Amino-4-(1*H*-imidazol-4-yl)-1-(4-phenoxyphenyl)butan-2-one dihydrochloride (5h). Mp: 165–167 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) 14.80 (s br,

1H), 9.11 (d, 1H, J = 1.2 Hz), 8.66 (s br, 2H), 7.51 (m, 1H), 7.39 (m, 2H), 7.25 (m, 2H), 7.13 (m, 1H), 6.98 (m, 4H), 4.71 (dd, 1H, J = 4.6 Hz, J = 8.8 Hz), 4.15 (s, 2H), 3.58 (dd, 1H, J = 4.4 Hz, J = 15.5 Hz), 3.23 (dd, 1H, J = 8.8 Hz, J = 15.5 Hz); ^{13}C NMR (DMSO-d_6, 101 MHz) 202.85, 156.61, 155.45, 134.08, 131.44, 129.97, 128.37, 126.57, 123.33, 118.44, 118.39, 118.22, 56.85, 44.47, 24.14; MS ESI$^+$ m/z 262.24 $(M+H)^+$, 643.38 $(2M+H)^+$. Anal. ($C_{19}H_{21}Cl_2N_3O_2$) 57.83C, 5.42H, 10.54N.

4.1.3.9. (*S*)-3-Amino-1-(4-(benzyloxy)phenyl)-4-(1*H*-imidazol-4-yl)butan-2-one dihydrochloride (5i). Mp: 164–166 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) 14.75 (s br, 1H), 9.11 (d, J = 1.36 Hz, 1H), 8.6 (s br, 2H), 7.49 (d, J = 1.25 Hz, 1H), 7.47-7.28 (m, 5H), 7.16 (d, J = 8.78 Hz, 2H), 6.97 (d, J = 8.79 Hz, 2H), 5.09 (s, 2H), 4.68 (dd, J = 8.82, 4.63 Hz, 1H), 4.06 (s, 2H), 3.56 (dd, J = 15.58, 4.20 Hz, 1H), 3.20 (dd, J = 15.54, 8.86 Hz, 1H); ^{13}C NMR (DMSO-d_6, 101 MHz) 203.06, 157.22, 137.04, 134.10, 130.84, 128.34, 127.72, 127.57, 126.54, 125.35, 118.20, 114.54, 69.07, 56.75, 44.39, 24.16; MS ESI$^+$ m/z 336.27 $(M+H)^+$. Anal. ($C_{20}H_{23}Cl_2N_3O_2$) 58.80C, 5.72H, 10.21N.

4.1.3.10. (*S*)-3-Amino-1-(2,4-difluorophenyl)-4-(1*H*-imidazol-4-yl)butan-2-one dihydrochloride (5j). Mp: 195–197 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) 14.85 (s br, 1H), 9.13 (d, 1H, J = 1.3 Hz), 8.72 (s br, 2H), 7.53 (d, 1H, J = 0.7 Hz), 7.41 (dt, 1H, J = 6.8 Hz, J = 8.6 Hz), 7.23 (dt, 1H, J = 2.6 Hz, J = 9.8 Hz), 7.07(d dt, 1H, J = 0.9 Hz, J = 2.6 Hz, J = 8.5 Hz), 4.76 (dd, 1H, J = 4.6 Hz, J = 8.8 Hz), 4.24 (dd, 2H, J = 18.5 Hz, J = 54.9 Hz), 3.60 (dd, 1H, J = 4.4 Hz, J = 15.5 Hz), 3.28 (dd, 1H, J = 8.9 Hz, J = 15.5 Hz); ^{13}C NMR (DMSO-d_6, 101 MHz) 201.47, 162.23 (dd, J_{C-F} = 11.9 Hz, J_{C-F} = 98.1 Hz), 159.79 (dd, 1C, J_{C-F} = 11.8 Hz, J_{C-F} = 100.7 Hz), 134.00, 133.18 (dd, 1C, J_{C-F} = 6.2 Hz, J_{C-F} = 9.7 Hz), 126.49, 118.22, 117.02 (dd, 1C, J_{C-F} = 3.8 Hz, J_{C-F} = 16.6 Hz), 111.23 (dd, J_{C-F} = 3.6 Hz, J_{C-F} = 21.1 Hz), 103.53 (t, J_{C-F} = 25.9 Hz), 56.74, 39.43, 24.24; MS ESI$^+$ m/z 266.30 $(M+H)^+$280.33 $(M+Na)^+$. Anal. ($C_{13}H_{15}Cl_2F_2N_3O$) 46.25C, 4.58H, 12.36N.

4.1.3.11. (*S*)-3-Amino-1-(3,4-dichlorophenyl)-4-(1*H*-imidazol-4-yl)butan-2-one dihydrochloride (5k). Mp: 193–195 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) 14.91 (s br, 1H), 9.24 (d, 1H, J = 1.3 Hz), 8.80 (s br, 2H), 7.73 (d, 1H, J = 8.2 Hz), 7.69 (d, 1H, J = 2.0 Hz), 7.65 (d, 1H, J = 0.7 Hz), 7.39 (dd, 1H, J = 2.0 Hz, J = 8.3 Hz), 4.84 (dd, 1H, J = 4.6 Hz, J = 8.6 Hz), 4.38 (d, 2H, J = 5.1 Hz), 3.71 (dd, 1H, J = 4.4 Hz, J = 15.5 Hz), 3.36 (dd, 1H, J = 8.8 Hz, J = 15.6 Hz); ^{13}C NMR (DMSO-d_6, 101 MHz) 202.18, 134.63, 134.00, 131.86, 130.58, 130.42, 130.20, 129.50, 126.49, 118.32, 56.91, 44.25, 24.04; MS ESI$^+$ m/z 298.21 $(M+H)^+$. Anal. ($C_{13}H_{15}Cl_4N_3O$) 42.16C, 4.27H, 11.22N.

4.1.3.12. (*S*)-3-Amino-4-(1*H*-imidazol-4-yl)-1-(perfluorophenyl)butan-2-one dihydrochloride (5l). Mp: 193–195 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) 14.74 (s br, 1H), 9.12 (d, 1H, J = 1.3 Hz), 8.77 (s br, 2H), 7.52 (d, 1H, J = 0.9 Hz), 4.86 (dd, 1H, J = 4.7 Hz, J = 9.1 Hz), 4.42 (dd, 2H, J = 18.8 Hz, J = 40.2 Hz), 3.59 (dd, 1H, J = 4.4 Hz, J = 15.4 Hz), 3.29 (dd, 1H, J = 9.1 Hz, J = 15.5 Hz); ^{13}C NMR (DMSO-d_6, 101 MHz) 199.60, 134.21, 126.49, 118.15, 56.74, 33.68, 24.32; MS ESI$^+$ m/z 320.24 $(M+H)^+$. Anal. ($C_{13}H_{12}Cl_2F_5N_3O$) 39.95C, 3.25H, 10.58N.

4.1.3.13. (*S*)-3-Amino-4-(1*H*-imidazol-4-yl)-1-(naphthalen-1-yl)butan-2-one dihydrochloride (5m). Mp: 184–186 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) 14.78 (s br, 1H), 9.13 (d, 1H, J = 1.32 Hz) 8.70 (s br, 2H), 7.91 (m, 3H), 7.56 (d, 1H, J = 1.2 Hz), 7.49 (m, 4H), 4.91 (dd, 1H, J = 4.8 Hz, J = 8.7 Hz), 4.67 (q, 2H, J = 18.2 Hz), 3.72 (dd, 1H, J = 4.3 Hz, J = 15.5 Hz), 3.34 (dd, 1H, J = 8.6 Hz, J = 15.3 Hz); ^{13}C NMR (DMSO-d_6, 101 MHz) 202.70, 134.17, 133.23, 131.93, 130.22, 128.36, 128.29, 127.60, 126.75, 126.01, 125.66, 125.38, 124.53, 118.22, 56.89, 43.34, 24.47; MS ESI$^+$ m/z 280.32 $(M+H)^+$. Anal. ($C_{17}H_{19}Cl_2N_3O$) 57.83C, 5.58H, 11.98N.

4.1.3.14. (*S*)-3-Amino-4-(1*H*-imidazol-4-yl)-1-(naphthalen-2-yl)butan-2-one dihydrochloride (5n). Mp: 187–189 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) 14.88 (s br, 1H), 9.14 (d, J = 1.27 Hz, 1H), 8.70 (s br, 2H), 7.86 (m, 4H), 7.54 (s, 1H), 7.46 (m, 3H), 4.78 (dd, J = 8.78, 4.59 Hz, 1H), 4.35 (s, 2H), 3.64 (dd, J = 15.50, 4.35 Hz, 1H), 3.28 (dd, J = 15.45, 8.87 Hz, 1H); ^{13}C NMR (DMSO-d_6, 101 MHz) 202.94, 134.08, 132.82, 131.86, 131.10, 128.28, 128.20, 127.56, 127.45, 127.35, 126.57, 126.09, 125.73, 118.27, 56.94, 45.48, 24.20; MS ESI$^+$ m/z 280.32 $(M+H)^+$. Anal. ($C_{17}H_{19}Cl_2N_3O$) 57.72C, 5.63H, 11.82N.

4.1.3.15. (*S*)-3-Amino-1-(benzo[*d*][1,3]dioxo-6-yl)-4-(1*H*-imidazol-4- yl)butane-2-one dihydrochloride (5o). Mp: 159–161 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) 14.83 (s br, 1H), 9.11 (d, J = 1.23 Hz, 1H), 8.64 (s br, 2H), 7.50 (s, 1H), 6.85 (d, J = 7.91 Hz, 1H), 6.83 (d, J = 1.62 Hz, 1H), 6.70 (dd, J = 7.93, 1.68 Hz, 1H), 5.99 (s, 2H), 4.66 (dd, J = 8.69, 4.58 Hz, 1H), 4.06 (s, 2H), 3.55 (dd, J = 15.50, 4.38 Hz, 1H), 3.21 (dd, J = 15.45, 8.79 Hz, 1H); ^{13}C NMR (DMSO-d_6, 101 MHz) 202.92, 147.02, 146.04, 134.05, 126.82, 126.52, 122.93, 118.23, 110.16, 108.01, 100.79, 56.71, 44.88, 24.12; MS ESI$^+$ m/z 274.18 $(M+H)^+$. Anal. ($C_{14}H_{17}Cl_2N_3O_3$) 48.45C, 5.01H, 12.26N.

4.2. Biology

4.2.1. Cloning and overexpression of the HDH-encoding gene from *B. suis*, and purification of the enzyme. The HDH-encoding gene, *BR0252*, was specifically amplified by PCR using *B. suis* 1330 chromosomal DNA as template and OPJ5-foward primer (5′-GCGGGCATATG GTCACAACGCTCAGACAGACCG-3′) and OPJ6-reverse primer (5′-GCGCGGGATCCTCATAGGTTCA GACGAATGGCGACG-3′) which contain BamHI and NdeI recognition sequences (underlined), respectively. The PCR products were digested with BamHI and NdeI and ligated to BamHI- and NdeI-digested pET15b (Novagen) prior to introduction into *E. coli*

strain DH5α. The integrity of the cloned gene was verified by sequencing, using primers OPJ5 and OPJ6 described above. The construct pET15bHDH was then transformed into *E. coli* strain BL21(DE3) for production of the 6 × (His)-HDH fusion protein.

Escherichia coli BL21(DE3) cells harbouring pET15bHDH were grown at 37 °C in one litre of Luria-Bertani medium supplemented with 50 μg/mL ampicillin. When the culture reached an optical density at 600 nm (OD_{600}) of approximately 0.6, expression of 6 × (His)-HDH protein was induced by the addition of isopropyl-thio-β-D-galactoside (IPTG) to a final concentration of 1 mM and growth was continued for 5 h. Cells were then harvested by centrifugation at 3500 rpm at 4 °C for 20 min and broken by sonication in buffer A (200 mM KCl, 50 mM Tris–Cl [pH 7.5], 10% glycerol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A) supplemented with 0.5 mM dithiothreitol and 0.2 mM disodium EDTA. All subsequent steps were performed at 4 °C. After centrifugation (13,000 rpm, 20 min), the soluble extract was treated with streptomycin sulfate[12] to remove ribosomes and nucleic acids. The suspension was then centrifuged at 13,000 rpm for 15 min, and the supernatants were dialysed against 2 L of sonication buffer A for 1 h. The dialysed lysates were mixed with Talon Co^{+}-affinity resin (Clontech) that had been equilibrated with buffer I (20 mM Tris–HCl [pH 8.0], 5 mM β-mercaptoethanol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A and 0.1% Nonidet P-40) supplemented with 150 mM KCl. The resin and bound His-tagged protein were collected by centrifugation at 2500 rpm for 10 min and washed with buffer I containing 500 mM KCl and 10 mM imidazole. The 6 × (His)-HDH protein was eluted with buffer I containing 125 mM KCl and 75 mM imidazole without Nonidet P-40. Elution fractions were free of detectable contaminating proteins as determined by Coomassie blue staining of sodium dodecyl sulfate (SDS)–polyacrylamide gels. The fractions containing the His-tagged proteins (estimated purity, >95%) were pooled and stored in elution buffer supplemented with 40% (v/v) glycerol at −20 °C. Protein concentration was determined with the bicinchoninic acid reagent (Pierce) using bovine serum albumin as a standard.

4.2.2. Enzyme assays. The activity and specificity of HDH were measured by monitoring the reduction of NAD^{+} to NADH directly at 340 nM (ε_M = 6200 $M^{-1}\,cm^{-1}$) as previously described.[13] The enzymatic activity was studied at 30 °C in the presence of 0.5 mM histidinol, 5 mM NAD^{+} and 0.5 mM $MnCl_2$ in 50 mM sodium glycine buffer at pH 9.2. For kinetic studies, experiments were carried out with 150 mM sodium glycine (pH 9.2) and 2 mM NAD^{+}. The K_m for the substrate was determined by varying the concentration of histidinol from 10 to 50 μM. Activity (1 U) is defined as the amount of HDH producing 1 μmol of NADH per min in the reaction. To perform IC_{50} determinations of the different inhibitors, the latter were added at various concentrations, ranging from 1 to 200 nM, and preincubated for 5 min at 30 °C with the enzyme solution prior to the initiation of the reaction. The enzyme concentration in the assay system was 4.5×10^{-11} M.

Acknowledgment

This work was supported by a grant from the German Sanitätsamt der Bundeswehr, No. M SAB1 5A002.

References and notes

1. Corbel, M. J. *Emerg. Infect. Dis.* **1997**, *3*, 213–221.
2. Dornand, J.; Gross, A.; Lafont, V.; Liautard, J.; Oliaro, J.; Liautard, J. P. *Vet. Microbiol.* **2002**, *90*, 383–394.
3. Celli, J. *Res. Microbiol.* **2006**, *157*, 93–98.
4. Köhler, S.; Foulongne, V.; Ouahrani-Bettache, S.; Bourg, G.; Teyssier, J.; Ramuz, M.; Liautard, J-P. *PNAS* **2002**, *99*, 15711–15716.
5. Delrue, R. M.; Lestrate, P.; Tibor, A.; Letesson, J. J.; De Bolle, X. *FEMS Microbiol. Lett.* **2004**, *231*, 1–12.
6. Liautard, J. P.; Jubier-Maurin, V.; Boigegrain, R. A.; Köhler, S. *Trends Microbiol.* **2006**, *14*, 109–113.
7. Grubmeyer, C. T.; Insiga, S.; Bhatia, M.; Moazami, N. *Biochemistry* **1989**, *28*, 8174–8180.
8. Teng, H.; Grubmeyer, C. T. *Biochemistry* **1999**, *38*, 7363–7371.
9. Barbosa, J. A.; Sivaraman, J.; Li, Y.; Larocque, R.; Matte, A.; Schrag, J. D.; Cygler, M. *PNAS* **2002**, *99*, 1859–1864.
10. Dancer, J. E.; Ford, M. J.; Hamilton, K.; Kilkelly, M.; Lindell, S. D.; O'Mahony, M. J.; Saville-Stones, E. A. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2131–2136.
11. Gohda, K.; Ohta, D.; Iwasaki, G.; Ertl, P.; Jacob, O. *J. Chem. Inf. Comput. Sci.* **2001**, *41*, 196–201.
12. Serror, P.; Sonenshein, A. L. *Mol. Microbiol.* **1996**, *20*, 843–852.
13. Loper, J. C.; Adams, E. *J. Biol. Chem.* **1965**, *240*, 788–795.