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Intermediate analogue inhibitors of mandelate racemase: *N*-Hydroxyformanilide and cupferron

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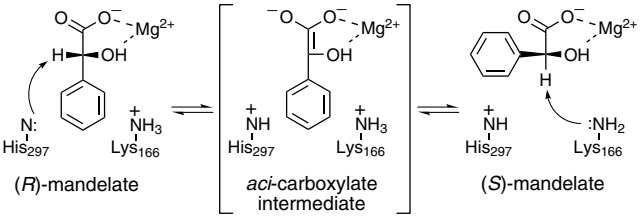
Abstract—Mandelate racemase (MR) catalyzes the 1,1-proton transfer that interconverts the enantiomers of mandelate. The transition state/intermediate analogues *N*-hydroxyformanilide (K_i = 2.79 ± 0.19 μM) and cupferron (K_i = 2.67 ± 0.09 μM) are identified as potent competitive inhibitors of MR. The pH–pK_i profile indicates that MR can bind either the protonated or deprotonated forms of *N*-hydroxyformanilide, with a 10-fold greater affinity for the latter form.

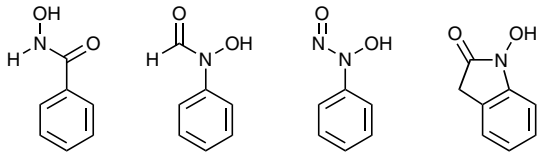
Mandelate racemase (EC 5.1.2.2; MR) from *Pseudomonas putida* catalyzes the Mg^{2+}-dependent 1,1-proton transfer that interconverts the enantiomers of mandelate via a two-base mechanism with His 297 and Lys 166 abstracting the α-proton from (*R*)-mandelate and (*S*)-mandelate, respectively, as shown in Scheme 1.[1] MR is very proficient at discriminating between the substrate in the ground state and the altered substrate in the transition state (TS), binding the latter species with an association constant equal to 5×10^{18} M^{-1} and stabilizing the TS of the reaction by 26 kcal/mol.[2,3] Consequently, MR has been studied as a paradigm for enzymes which catalyze rapid carbon-hydrogen bond cleavage of carbon acids with relatively high pK_a values.[1] Enzymes such as MR, that are extremely proficient at stabilizing the TSs and intermediates formed during catalysis, are often strongly inhibited by analogues of either the altered substrate in the TS or unstable intermediates that resemble the TS.[4,5] Our interest in understanding how protein-ligand interactions within the active site of MR stabilize the TS for α-proton abstraction led us to survey a series of reactive intermediate analogues as potential TS or intermediate analogue inhibitors.[6,7] Previously, we identified α-hydroxybenzylphosphonate (K_i = 4.7 μM) and benzohydroxamate (BzH; K_i = 9.3 μM) as potent reversible competitive inhibitors of MR.[6] Herein, we report that the *aci*-carboxylate



Scheme 1.

intermediate analogues *N*-hydroxyformanilide (HFA) and cupferron (Scheme 2) are potent competitive inhibitors of MR, binding to the enzyme with an affinity similar to that exhibited by the most effective intermediate analogue inhibitors identified to date for this enzyme.

Reverse or retro-hydroxamates (i.e., compounds bearing the *N*-formyl-*N*-hydroxyamino group) are potent inhibitors of a variety of enzymes including adenylosuccinate synthetase,[8,9] 1-deoxy-D-xylulose-5-phosphate (DXP)



Scheme 2.

Keywords: Mandelate racemase; Inhibition; Transition state analogue; pH-profile; *N*-Hydroxyformanilide; Cupferron; 1-Hydroxyoxindole.
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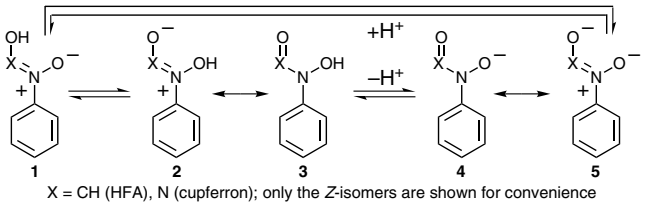
synthase and DXP reductoisomerase,[10–12] 5-lipoxygenase,[13] and numerous metalloproteinases.[14–23] Therefore, we examined HFA as an analogue of the *aci*-carboxylate intermediate. Indeed, HFA is a potent competitive inhibitor of MR, binding with an affinity exceeding that of the substrate by 300-fold (Table 1).

Although the neutral formyl function of HFA might be anticipated to be a poor mimic of the *aci*-carboxylate group because it lacks the additional oxygen atom, HFA could exist as the hydrate in solution and thereby mimic the *aci*-carboxylate. The tetrahedral geometry of the hydrate, versus the planar geometry of the *aci*-carboxylate, does not preclude inhibition since α-hydroxybenzylphosphonate, which bears a tetrahedral phosphonate group, is a potent inhibitor of MR.[6] The ^{1}H NMR spectrum of HFA (dried in vacuo over P_2O_5) in dry DMSO-d_6 contained a broad peak at 9.68 ppm corresponding to the NOH proton and a singlet at 8.49 ppm corresponding to the proton of the formyl group (an HSQC experiment revealed one-bond correlation between the ^{1}H signal at 8.49 ppm and the signal for the carbonyl carbon at 155.81 ppm in the ^{13}C NMR spectrum). The observed ^{1}H and ^{13}C chemical shifts of the formyl group of HFA are similar to those observed for *N*-hydroxy-*N*-methylformamide[27] indicating that the formyl group has substantial amide character. Addition of D_2O to the HFA sample did not alter the chemical shift of the signal corresponding to the formyl proton from that observed in dry DMSO-d_6 solvent. Not surprisingly, the carbonyl group of HFA is not hydrated in water. It is likely that the inhibition arises because MR stabilizes an electronic configuration of HFA or its conjugate base that more closely resembles the structure of the *aci*-carboxylate intermediate (Scheme 3).

Crystal structures of metalloenzymes with bound reverse hydroxamate inhibitors reveal that the reverse hydroxamate group often coordinates the metal ion in a bidentate manner, with short distances (~2.0 to 2.5 Å) between an active site metal ion and the *N*-hydroxyl oxygen.[17,21–23] Such short distances suggest that the *N*-hydroxyl binds metal ions in a deprotonated form.[21] To explore this possibility for HFA, we investigated the pH-dependence of inhibition. The pK_a of HFA (pK_a^i) was determined to be 7.92 ± 0.09 (Table 1) by spectrophotometric titration.[20] The pK_a value of the free enzyme (pK_a^e) was determined to be 8.76 ± 0.12 by fitting the pK_m^{app} versus pH data as shown in Figure 1. This pK_a^e value is in good agreement with the value of 8.52 ± 0.04 obtained in a previous study.[6] Using these values, a good fit of the pK_i^{app} data could not be obtained when only the conjugate base of HFA was assumed to bind to MR. Previously, we demonstrated that MR binds BzH as the monoanion and that there is a marked decrease in inhibition by BzH when the pH is decreased below pH 8 (see Fig. 1).[6] On the other hand, inhibition by HFA does not exhibit as marked a decrease in inhibition when the pH is lowered below pH 8, suggesting that MR binds both the protonated and deprotonated forms of HFA. A good fit of the pK_i^{app} data was obtained using the kinetic mechanism



X = CH (HFA), N (cupferron); only the *Z*-isomers are shown for convenience

Scheme 3.

Table 1. Competitive inhibition of MR by *aci*-carboxylate analogues

Ligands	pK_a of ligand	K_i (μM)[a]
Benzohydroxamate (BzH)	8.8[b]	12 ± 1
N-Hydroxyformanilide (HFA)	7.92 ± 0.09	2.79 ± 0.19
Cupferron	4.16[c], 4.18[d]	2.67 ± 0.09[e]
1-Hydroxyoxindole (HOI)	—	510 ± 20
(*R*)-Mandelate	—	810 ± 120[f]

[a] Values are means of three experiments conducted at pH 7.5 and errors are the standard deviation. Inhibition assays[6] (see Supplementary data for details) containing HFA (1–5 μM), cupferron (1–5 μM), or HOI (0.25–0.75 mM) were conducted at 25 °C in Na^+–Hepes buffer (0.1 M, pH 7.5) and contained $MgCl_2$ (3.3 mM), BSA (0.005%), MR (150 ng/mL), and (*R*)-mandelate (0.2–10.0 mM).
[b] pK_a from Ref. 24 and K_i from Ref. 6.
[c] pK_a from Ref. 25.
[d] pK_a from Ref. 26.
[e] Conversion of the NH_4^+-salt of cupferron to the Na^+-salt using cation exchange resin did not alter K_i.
[f] K_m from Ref. 6; $K_m = K_S$.[3]

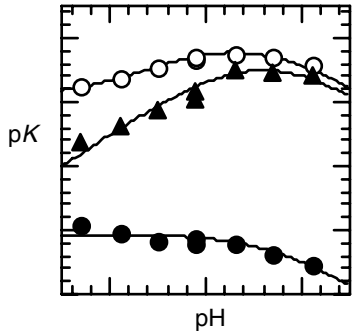


Figure 1. pH-dependence of the competitive inhibition of MR by HFA and BzH with respect to (*R*)-mandelate. Assays were conducted at 25 °C in Na^+–Hepes buffer (0.1 M, pH 6.7, 7.1, 7.5, and 7.9) or Na^+–Taps buffer (0.1 M, pH 7.9, 8.3, 8.7, and 9.1). The pK_m^{app} data (●) were fit to Eq. 1, where K'_m is the pH-independent Michaelis constant, and K_a^e represents the ionization constant for the free enzyme. The values of pK'_m and pK_a^e are 2.94 ± 0.04 and 8.76 ± 0.12, respectively. The pK_i^{app} data for HFA (○) were fit to Eq. 2, where K_a^i represents the ionization constant for the free inhibitor, K_i^- is the competitive inhibition constant for unprotonated HFA, and K_i^H is the competitive inhibition constant for protonated HFA. The values of pK_i^- and pK_i^H are 6.04 ± 0.02 and 5.07 ± 0.06, respectively, with pK_a^e = 8.76 (from pK_m data) and pK_a^i = 7.92 (from titration). The pK_i^{app} data for BzH (▲) are from Ref. 6 and were fit to Eq. 3, where K_a^e represents the ionization constant for the free enzyme, K'_i is the pH-independent inhibition constant, and K_a^i represents the ionization constant for the free BzH. The values of pK'_i and pK_a^i are 6.2 ± 0.2 and 8.7 ± 0.2, respectively, with pK_a^e = 8.51 (from pK_m data in Ref. 6). Data were fit using nonlinear regression analysis and the program *KaleidaGraph* v. 3.5 from Synergy Software (Reading, PA).

shown in Scheme 4. The curve fit of the pK_i^{app} data gave values for the pH-independent, competitive inhibition constants for HFA and its conjugate base equal to 9 ± 1 μM ($pK_i^H = 5.07 \pm 0.06$) and 0.91 ± 0.04 μM ($pK_i^- = 6.04 \pm 0.02$), respectively. This latter value is similar to the value of the pH-independent, competitive inhibition constant for the BzH monoanion ($K_i' = 0.63 \pm 0.29$ μM). Our conclusion that MR can bind the protonated or deprotonated forms of HFA seems reasonable since either the hydroxyl function (substrate mimic) or the corresponding conjugate base would be expected to chelate the active site Mg^{2+}. That MR binds the conjugate base of HFA with ~10-fold greater affinity than it binds the conjugate acid is in agreement with our expectation that metal chelation by hydroxamic acids commonly involves deprotonation of the ligand.[28–31]

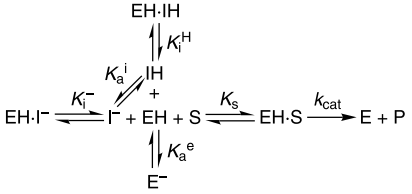
Reverse hydroxamates may exist in solution as a mixture of *Z*-(*syn*) and *E*-(*anti*) conformational rotomers with respect to the RCO–N(OH)R′ bond.[20] While it is the *Z*-rotomer that chelates the metal ion, this rotomer is disfavored in aqueous media.[20,32,33] If the *Z*-conformer of HFA inhibits MR by chelating the Mg^{2+}, we hypothesized that some of the entropic 'cost' of binding this rare form of HFA in aqueous solution could be overcome using the *Z*-restricted 1-hydroxyoxindole (HOI; Scheme 2). However, HOI was only a weak inhibitor of MR (Table 1). Weak inhibition may arise from unfavorable interactions with the methylene group, preferred binding of the *E*-conformation of HFA, or failure of the phenyl ring to bind in the correct orientation due to restricted rotation about the C_α–C_{phenyl} bond.

Cupferron, bearing the diazeniumdiolate (nitroso-hydroxylamine) functional group,[34] may be regarded as a mimic of the *aci*-carboxylate intermediate (Scheme 3). Cupferron is a potent competitive inhibitor of MR and, like HFA, is bound with an affinity exceeding that of the substrate by ~300-fold (Table 1). Unlike HFA, cupferron ($pK_a = 4.16$)[25,26] exists as the monoanion over the pH range for which MR is active. Hence, MR binds the cupferron monoanion with ~3-fold less affinity than it binds the HFA monoanion (cf. K_i^- for HFA).

$$pK_m = pK'_m - \log\left(1 + 10^{(pH - pK_a^e)}\right) \quad (1)$$

$$pK_i^{app} = -log\left(1 + 10^{(pH - pK_a^e)}\right) - log\left(10^{(-pK_a^i)} + 10^{(-pH)}\right) + log\left(10^{(pK_i^- - pK_a^i)} + 10^{(pK_i^H - pH)}\right) \quad (2)$$

$$pK_i^{app} = pK_i' - \log\left(1 + 10^{(pH - pK_a^e)}\right) - log\left(1 + 10^{(pK_a^i - pH)}\right) \quad (3)$$



Scheme 4.

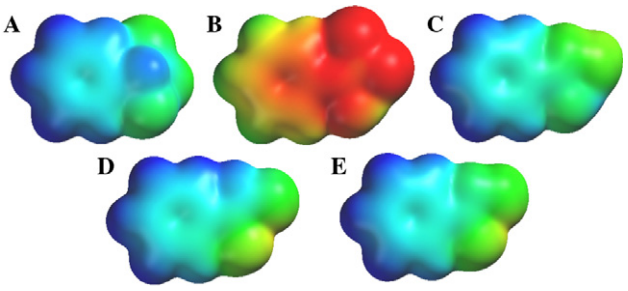


Figure 2. Molecular electrostatic potential surface (EPS) at the van der Waals radii for (*R*)-mandelate (A), the putative *aci*-carboxylate intermediate (B), the conjugate bases of *Z*-BzH (*O*-deprotonated)[24] (C), *Z*-HFA (Scheme 3: **4** or **5**, X = CH) (D), and *Z*-cupferron (Scheme 3: **4** or **5**, X = N)[39] (E). EPSs have an electron density isosurface displayed at a density of 0.002 e/a_0^3 which encompasses approximately 95% of the van der Waals radii.[40] The energy difference from the red (negative potentials) to blue (positive potentials) regions of the EPSs is 150 kcal/mol with the positive potential fixed at an upper limit of −50 kcal/mol for all molecules (as opposed to shading each molecule to represent the total variation in electrostatic potential over any single molecule).[6] Geometry optimizations and EPSs were calculated using the density functional B3LYP/6-311+G** method with *Spartan'04 Windows* v. 1.0.1 (Wavefunction, Inc.; Irvine, CA).

Enzymes effect chemistry by inducing changes in electron distribution in substrates.[35] Generation of differential charge distribution between the substrate and the altered substrate in the TS permits electrostatic interactions between the enzyme and its ligand to selectively stabilize the TS.[36] We have calculated the electrostatic potential surfaces (EPSs) of the putative *aci*-carboxylate intermediate, (*R*)-mandelate, and the conjugate bases of *Z*-BzH, *Z*-HFA, and *Z*-cupferron (assuming the *Z*-isomer is bound so that the inhibitor chelates the divalent metal ion) so that their EPSs may be compared (Fig. 2). The geometries of *Z*-BzH, *Z*-HFA, and *Z*-cupferron mimic the planarity of the *aci*-carboxylate intermediate and the calculated EPSs are similar for each of the analogues (accounting for similar binding affinities). However, there is substantial delocalization of negative charge over the dianionic *aci*-carboxylate intermediate that is not mimicked by the inhibitors. This may account for why these inhibitors capture at best only 30% of the 26 kcal/mol used to bind the altered substrate in the TS. The additional oxygen and negative charge present in the *aci*-carboxylate, but absent in the analogues, must contribute significantly to the free energy of TS binding through simultaneous interaction with the active site Mg^{2+} and the adjacent electrophilic catalyst Glu 317.[37,38]

In summary, HFA, cupferron, and BzH are the most potent TS/intermediate analogue inhibitors reported to date for MR, binding ~300-fold tighter than the substrate.

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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.2006.09.079.

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