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Inhibition of mandelate racemase by the substrate–intermediate–product analogue 1,1-diphenyl-1-hydroxymethylphosphonate

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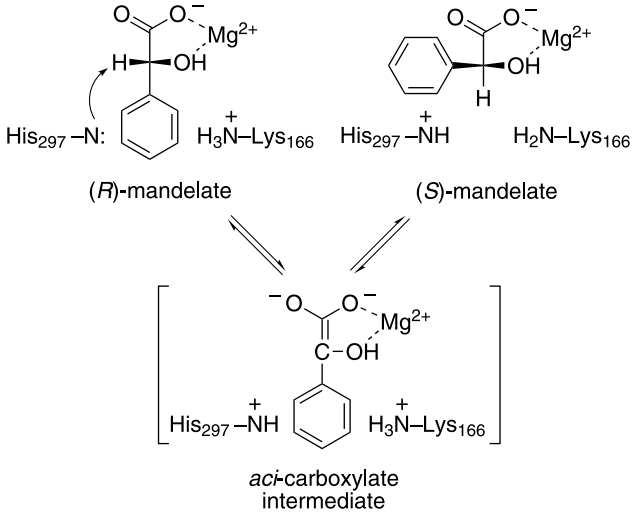
Abstract—Mandelate racemase has been studied as a paradigm for enzyme-catalyzed abstraction of a proton from carbon acids with relatively high pK_a values. 1,1-Diphenyl-1-hydroxymethylphosphonate is a substrate–intermediate–product analogue and is a modest competitive inhibitor of the enzyme (K_i = 1.41 ± 0.09 mM), suggesting that simultaneous binding of the two phenyl groups obviates mimicry of the *aci*-carboxylate function of the intermediate by the phosphonate group.

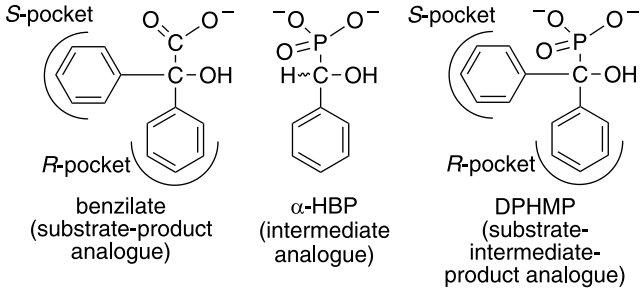
Mandelate racemase (MR; EC 5.1.2.2) from *Pseudomonas putida* catalyzes the Mg^{2+}-dependent 1,1-proton transfer that interconverts the enantiomers of mandelate via a two-base mechanism as shown in Scheme 1.[1–5] MR has been studied as a paradigm for enzymes that catalyze rapid carbon–hydrogen bond cleavage of carbon acids with relatively high pK_a values.[6]

Our interest in understanding how protein–ligand interactions within the active site of MR stabilize the transition state for α-proton abstraction led us to survey a series of intermediate analogues as potential transition state or intermediate analogue inhibitors.[7,8] We identified α-hydroxybenzylphosphonate (α-HBP) as a potent, competitive intermediate analogue inhibitor (K_i = 4.7 μM) of MR.[7] Recently, we demonstrated that MR binds benzilate as a competitive inhibitor (K_i = 0.67 mM) with an affinity similar to that exhibited for substrate.[9] Benzilate may be regarded as a substrate–product analogue because it bears two phenyl groups that mimic the phenyl groups of (*R*)- and (*S*)-mandelate as if they were bound simultaneously to MR (i.e., in an *R*-pocket and an *S*-pocket; see Scheme 2).

To further explore the topology of the active site, we examined the interaction of 1,1-diphenyl-1-hydroxymethylphosphonate (DPHMP) with MR. This sub-



Scheme 1.



Scheme 2.

Keywords: Mandelate racemase; Inhibition; Phosphonate.
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strate–intermediate–product analogue combines the structural features of α-HBP and benzilate.

All reagents were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). Dimethyl DPHMP[10] and DPHMP[11] were prepared using modified literature procedures. Chemical shifts (δ) for ^{1}H, ^{13}C, and ^{31}P NMR spectra are reported in ppm relative to the calibrated deuterium lock signal. Circular dichroism (CD) assays were conducted using a JASCO J-810 spectropolarimeter. Elemental analyses were conducted by the Canadian Microanalytical Service Ltd. (Delta, BC, Canada). MR was overexpressed, purified, and assayed as described previously.[12] Inhibition assays containing DPHMP (1.5–6.0 mM) were conducted at 25 °C in Na^{+}–Hepes buffer (0.1 M, pH 7.5) containing $MgCl_2$ (3.3 mM), (*R*)-mandelate (0.25–10.0 mM), and MR (150 ng/mL). Apparent values of V_{max} and K_m were determined from nonlinear regression analysis of Michaelis–Menten plots, and the competitive inhibition constants (K_i) were determined from plots of the apparent K_m/V_{max} values versus inhibitor concentrations.[13]

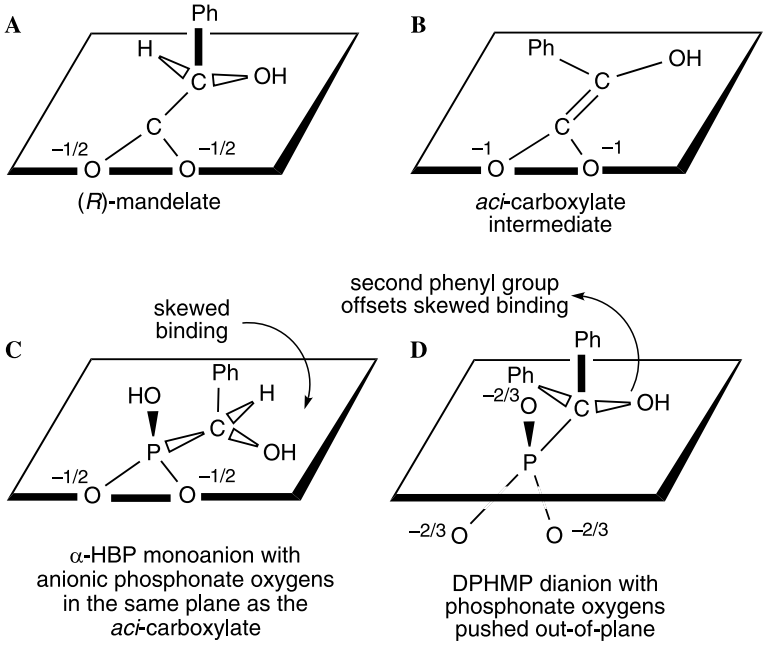
Table 1. Inhibition of MR by DPHMP

Inhibitor		K_i (mM)[a]
1,1-Diphenyl-1-hydroxymethylphosphonate (DPHMP)		1.41 (±0.09)
pH 6.3		5.91 (±0.04)
pH 8.7		6.93 (±0.04)
(*R*,*S*)-α-Hydroxybenzylphosphonate (α-HBP)		0.0047 (±0.0007)[b]
Substrate	K_m (mM)[a]	K_i/K_m
(*R*)-Mandelate	0.44 (±0.01)	3.2 (±0.2)
pH 6.3	0.95 (±0.03)	6.2 (±0.2)
pH 8.7	2.02 (±0.06)	3.4 (±0.1)

[a] Values are the means of three experiments; standard deviation is given in parentheses. All values determined at pH 7.5 unless indicated otherwise.
[b] Value from Ref. 14.

MR binds benzilate with an affinity that is similar to that which it exhibits for substrate, indicating that MR can accommodate the simultaneous binding of two phenyl groups within its active site.[9] We therefore designed DPHMP as an analogue of benzilate that incorporates the substrate and product characteristics present in benzilate (i.e., two phenyl rings), and includes the phosphonate group that, as is the case for α-HBP, mimics the *aci*-carboxylate group of the putative intermediate. Hence, DPHMP may be regarded as a substrate–intermediate–product analogue (Scheme 2). Surprisingly, MR bound DPHMP with an affinity that was ~3-fold less than that exhibited for substrate (Table 1) and 300-fold less than that exhibited for α-HBP. In previous studies, we examined the pH dependence of inhibition by α-HBP and showed that MR preferentially binds the monoanionic form of α-HBP.[7] This does not appear to be true for DPHMP. At pH values 7.5 and 8.7, the relative affinities of MR for substrate and DPHMP are not significantly different; however, the relative affinity for DPHMP is reduced 2-fold at pH 6.3, suggesting that binding of the phosphonate monoanion is not preferred relative to the dianion.

A stereoelectronic feature of the ligands that must be considered is their angular charge distribution.[7,15] The negative charges on the carboxylate of (*R*)-mandelate and on the *aci*-carboxylate of the intermediate (Scheme 3A and B) are rotationally symmetrical about the line that bisects the angle made by the carboxyl carbon and the two anionic oxygens. This line also makes an angle



Scheme 3.

of 180 ° relative to the bond between the carboxyl carbon and the α-carbon. The negative charge of the α-HBP monoanion is not rotationally symmetrical with respect to such a line. Consequently, it has been suggested that MR binds the α-HBP monoanion in a skewed orientation so that the orientation of the vector of the negative charge of the monoanionic phosphonate function aligns with that of the carboxylate group of the substrate or *aci*-carboxylate intermediate (Scheme 3C).[7] This requirement for phosphonate binding may account for why α-HBP is an inhibitor and not a substrate.[7] It may also permit the phenyl ring of α-HBP to closely approximate the binding orientation assumed by the phenyl ring of the planar intermediate. The observed weak binding of DPHMP, relative to α-HBP, suggests that binding of DPHMP in a skewed orientation similar to α-HBP and the simultaneous binding of the two phenyl groups within the active site of MR is not favored.

For example, either the added steric bulk of the second phenyl group or the simultaneous binding of the two phenyl groups in their respective ground state *R*- and *S*-pockets, may cause the phosphonate to bind with an orientation similar to the substrate carboxylate (i.e., with rotationally symmetrical negative charge as in Scheme 3D). Consequently, the ability of DPHMP to mimic the intermediate/transition state would be lost, although the additional negative charge on dianionic DPHMP may enhance binding to some degree. It is the presence of only the single phenyl group on α-HBP that permits this inhibitor to adopt the appropriate conformation upon binding such that it can better mimic the intermediate or transition state. Hence, substrate–product features of an inhibitor may enforce a binding mode that obviates the ability of another functional group to mimic transition state/intermediate characteristics.

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10. Dimethyl 1,1-diphenyl-1-hydroxymethylphosphonate (dimethyl DPHMP) was prepared following a procedure similar to that described by Maeda et al. for the corresponding diethyl phosphonate diester.[16] Dimethyl benzoylphosphonate[17] (3.21 g, 15 mmol) in dry THF (30 mL) was cooled to −78 °C for 10 min. Phenylmagnesium bromide (16.5 mL of a 1.0 M solution in THF) was added dropwise and after the slow addition was complete, the reaction was stirred for 30 min at −78 °C. The reaction mixture was then allowed to warm to room temperature and quenched by addition of satd aq NH_4Cl (25 mL) and stirred for 10 min. The entire reaction mixture was then added to satd aq NH_4Cl (400 mL) and stirred for an additional 10 min. The mixture was extracted with CH_2Cl_2 (3 × 50 mL) and the combined extracts were washed with satd NaCl (50 mL) and dried over anhydrous Na_2SO_4. The CH_2Cl_2 was removed in vacuo giving a yellow-white solid. This solid was transferred to a sintered glass funnel and washed with cold THF to give a white solid (1.05 g, 24%): mp 167–169 °C (lit.[18] 171 °C); ^{1}H NMR ($CDCl_3$, 250 MHz, δ) 7.28–7.37 (m, 6H), 7.68–7.71 (m, 4H), and 3.65 (d, J = 10.5 Hz, 6H); ^{13}C NMR (^{1}H-decoupled, $CDCl_3$, 500 MHz, δ) 54.24 (d, J = 7.5 Hz), 78.75 (d, J = 160.2 Hz), 127.09 (d, J = 5.3 Hz), 127.86, 128.19, and 141.19 (d, J = 2.6 Hz). ^{31}P NMR (^{1}H-decoupled, $CDCl_3$, 500 MHz, δ) 22.81. Anal. Calcd for C, 61.63; H, 5.87; P, 10.60. Found: C, 61.60; H, 6.14; P, 10.97.
11. Disodium 1,1-diphenyl-1-hydroxymethylphosphonate (DPHMP) was prepared by slowly adding trimethylsilylbromide (3.0 mL, 22.8 mmol) to a solution of dimethyl DPHMP (1.0 g, 3.42 mmol) in dry CH_3CN (30 mL). After refluxing the solution for 30 min under argon, the solvent was removed in vacuo. Methanol (10 mL) and water (10 mL) were added and then removed in vacuo leaving a light brown oil. CH_2Cl_2 (20 mL) was added to this oil which produced the phosphonic acid as a white solid after 10–20 min (0.85 g, 94%). The phosphonic acid (0.5 g, 1.89 mmol) was dissolved in ethanol/water (1:1, 10 mL each) and passed through an AG 50-X8 column (3 × 15 cm, Na^+-form). Fractions containing the phosphonate were combined and the water was removed using lyophilization to yield a white solid (0.50 g, 86%): mp 157 °C (dec); ^{1}H NMR (D_2O, 250 MHz, δ) 7.35–7.38 (m, 6H), 7.58–7.61 (m, 4H); ^{13}C NMR (^{1}H-decoupled, D_2O, 500 MHz, δ) 79.06 (d, J = 154 Hz), 127.43 (d, J = 5 Hz), 127.52, 128.14, and 142.88. ^{31}P NMR (^{1}H-decoupled, D_2O, 500 MHz, δ) 18.35. Anal. Calcd for C, 50.66; H, 3.60; P, 10.05. Found: C, 51.07; H, 4.54; P, 11.37.
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