A Herbicidal Inhibitor of Isopropylmalate Isomerase

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Nitronate compounds resembling the *aci*-carboxylate reaction intermediate in the reaction catalyzed by isopropylmalate isomerase were investigated as enzyme inhibitors and potential herbicides. The nitronic acids of 1-hydroxy-2-nitrocyclopentane-1-carboxylic acid, nitroisopropylmalate and of 1-hydroxy-2-nitrocyclohexane-1-carboxylic acid were all potent inhibitors of isopropylmalate isomerase. The nitroalkane/nitronate pKa values of these were 7.3, 9.5 and 11.1, respectively. The perturbed pKa values of the first and last compound were understandable on the basis of the opposing effects of 5- and 6-membered ring strain on the ionization of the nitro group. As the only compound significantly in the nitronate form at physiological pH, it was not surprising that 1-hydroxy-2-nitrocyclopentane-1-carboxylic acid was the best herbicide. Its specific mode of action was confirmed by the fact that 1-hydroxy-2-nitrocyclopentane-1-carboxylic acid inhibited the growth of carrot cells in liquid culture and was specifically reversed by leucine.

Introduction

Isopropylmalate isomerase (IMI) is the second committed step in the biosynthesis of leucine. In the forward direction, it catalyzes the conversion of α- to β-isopropylmalate. Closely analogous to aconitase [1–3], it catalyzes a dehydration/rehydration reaction *via* the pairing of an active site base (proton acceptor/donor) and a Fe_4S_4 cluster (hydroxyl acceptor/donor). "Isomerization" occurs because the dehydrated alkene intermediate (dimethylcitraconate) flips through 180° within the active site to be rehydrated across either face.

1-NH_2-2-nitrocyclopentane-1-carboxylic acid (**I**) has been reported [4] to be a leucine antimetabolite with plant growth regulating properties. We investigated the properties of the 1-hydroxy analogues, **II** [9, 10] and **IV**, as potential herbicides and as inhibitors of isopropylmalate isomerase.

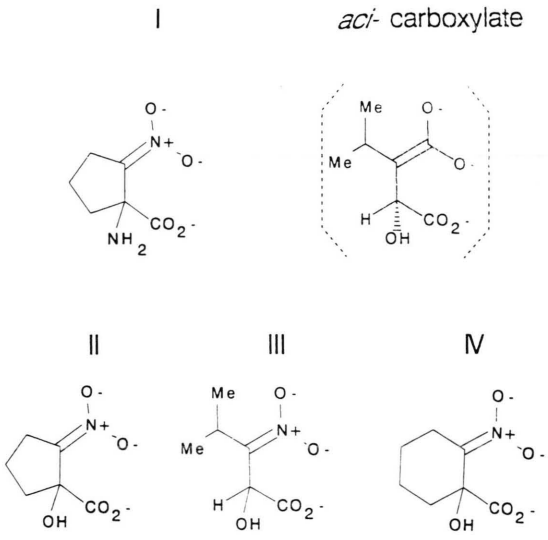

Fig. 1. Nitronate structures of **I, II, III, IV** and the *aci*-carboxylate of β-isopropylmalate (an intermediate in the reaction catalyzed by isopropylmalate isomerase).

Materials and Methods

Isolation of isopropylmalate isomerase from baker's yeast

The following procedure was developed on the basis of the stability data provided in ref. [1] and [7]. Fresh baker's yeast was mixed with an equal volume of ice-cold 0.1 M HEPES buffer (pH 7.0) containing 1 mM benzamidine, 1 mM dithioerythritol (DTE) and 0.1 M $(NH_4)_2SO_4$ and broken at 9000 psi in a French pressure cell. After low speed centrifugation the supernatant was precipitated with $(NH_4)_2SO_4$ and the 48–65% fraction resuspended in the above buffer containing 20% glycerol. This was further purified by gel filtration down a column of Sephacryl S 200 equilibrated in

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0.1 M sodium acetate buffer at pH 5.0 containing 1 mM benzamidine, 1 mM DTE, 0.1 M $(NH_4)_2SO_4$ and 20% v/v glycerol. The activity eluted in a fraction consistent with a molecular weight of *ca.* 90 kDa. Active fractions were combined (1.4 mg ml^{-1}) and stored as frozen beads over liquid nitrogen.

By the disappearance of DMC (assay 1, below), the activity of our enzyme preparation was *ca.* 0.85 μmol min^{-1} mg^{-1}. It would have been no more than 5–10% pure and, even then, mostly damaged by oxidation in air to the inactive "Fe_3S_4" form (see ref. [1]). Nevertheless the material was stable for hours as prepared at pH 5.0 and suitable for inhibition assays.

Assay techniques

Dimethylcitraconate (DMC) was prepared as described by Schloss *et al.* [6]. Assays in 0.1 M HEPES buffer (pH 7.0) containing 1.4 mM DMC were carried out at 25 °C in a 2 mm pathlength cell. As DMC was removed, the absorbance at 240 nm declined (mM extinction coefficient 2.9). Due to enzyme instability, assay rates only remained linear over the first few minutes.

The second assay method was based on the formation of DMC. β-Isopropylmalate was synthesized as described in ref. [6] as a mixture of the two diastereomers. All concentrations refer to the total compound of which only about a quarter would have been the substrate enantiomer, *erythro*-(*R,S*)-β-isopropylmalate. Assays following the appearance of DMC at 240 nm were as described in ref. [1] but at 25 °C HEPES buffer (pH 7.0). As expected [6], V_{max} was about 40% of the activity observed using assay method 1; in our hands, K_m was too low to measure (< = 30 μM).

Slow enzyme inhibition

This was analyzed according to the methods described in ref. [11] according to the scheme

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^*.$$

The observed rate constant (k_{obs}) for the onset of tighter inhibition was estimated graphically (Fig. 2) as described. k_4 was estimated directly (Fig. 3). k_3 was then estimated graphically (Fig. 4) from the relationship

$$\frac{1}{(k_{obs} - k_4)} = \frac{1}{I}\frac{K_i}{k_3}(1 + S/K_m) + \frac{1}{k_3}.$$

Measurement of pKa values

These were measured by conventional titration methods (taking into account the slow rates of nitronic acid protonation and deprotonation) and recording absorbance changes at 240 nm.

Chemicals tested as inhibitors

Compounds **I, II** and **III** (see Fig. 1) were synthesized as described in the literature [1, 5, 9, 10]. 1-Hydroxy-2-nitrocyclohexane carboxylic acid (**IV**) was made by hydration of 2-nitrocyclohex-1-ene carboxylic acid in a similar manner to that described for the cyclopentane [9]; the olefin was prepared by adding elements of nitryl iodide to *t*-butyl cyclohex-1-ene carboxylate followed by elimination and deesterification.

Results and Discussion

At pH 7.0, **II** (but not the amine, **I**) was a potent slow binding inhibitor (k_{off} *ca.* 0.07 min^{-1}) of IMI (Fig. 2–5). Similar to **III** which was described by

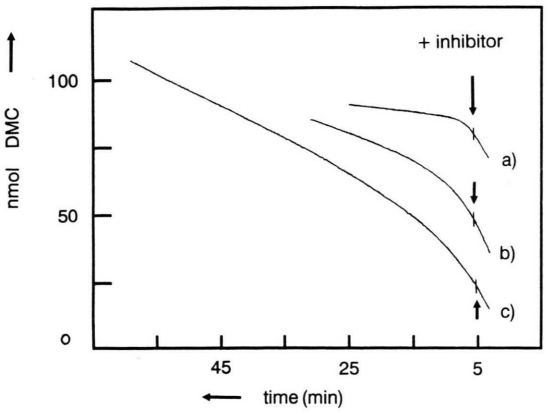

Fig. 2. Biphasic inhibition after addition of **II.** Assays containing 10 mM β-isopropylmalate were started with addition of 10 μl of enzyme. At the point marked by the arrow, inhibitor **II** was added to final concentrations of a) 5.6, b) 1.41 and c) 0.56 μM. DMC, dimethylcitraconate.

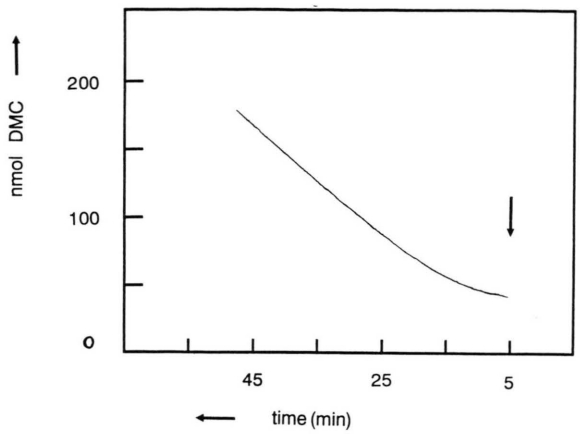

Fig. 3. Measurement of inhibitor "off" rate (k_4). 6 µl of enzyme were incubated at 25 °C with 20 µl of 0.1 M Na HEPES (pH 7.0) and 6 µl of 5 µM inhibitor **II** (equilibrated at pH 7.0) for 10 min. The assay was then started (arrow) with the addition of 3 ml of 20 mM β-isopropylmalate in 0.1 M HEPES pH 7.0 and recorded at 240 nm. The gradual increase in the observed activity corresponds to an approximate value of 0.07 min^{-1} for the rate constant k_4 (see Materials and Methods).

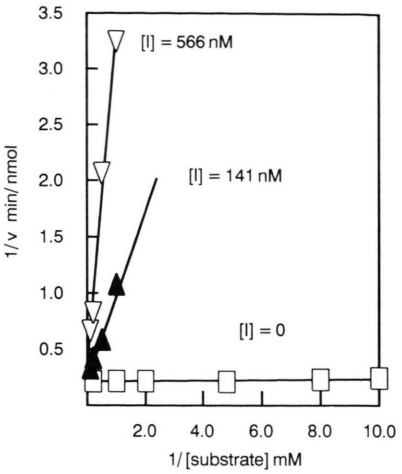

Fig. 5. Lineweaver-Burk plot of inhibition by **II.** The graph plots $1/v$ *versus* $1/S$ at different concentrations of inhibitor as indicated. Rates are *final* steady state rates (for points at high [S] and low [I] it took longer than 40 min to reach equilibrium). The graph shows that inhibition is competitive. It also shows that K_m was too low to measure (initial rates were maintained for too short a time at [S] < 0.06 mM). Assuming a K_m of 30 µM, the K_i at pH 7.0 was *ca.* 2 nM (this value was also consistent with data using DMC as substrate).

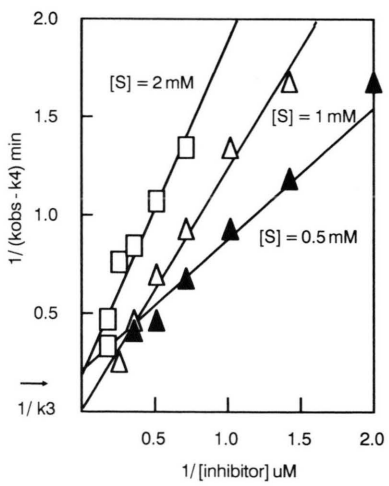

Fig. 4. Dependence of inhibitor "on" rate (k_{obs}) on inhibitor (**II**) and substrate concentration. k_4 was 0.07 min^{-1} (Fig. 3). The graph plots $1/(k_{obs} - k_4)$ *versus* 1/[inhibitor]. k_{obs}, the apparent rate of inhibitor binding was obtained graphically [11] over a range of substrate and inhibitor concentrations. $1/k_3$ is the intercept on the vertical axis. Due to the very low K_m value of β-isopropylmalate it was impossible to define this value more accurately than $k_3 >= 5\ min^{-1}$.

Emptage and Schloss [5], the nitronate forms of both **II** and **IV** resemble the *aci*-carboxylate anion intermediate in the reaction mechanism. Thus, as expected, only the nitronate forms of **II** and **IV** inhibited IMI significantly; both nitronates were of similar potency (Fig. 6).

Using DMC as the substrate (K_m *ca.* 0.2 mM), enzyme activity was too labile to allow accurate measurements of the kinetic parameters of slow binding inhibitors. Using β-isopropylmalate, steady rates were obtained for up to an hour or more (*cf.* ref. [1]). However, using synthetic material (mixed diastereomers containing only 20–25% of the correct *erythro*-(*R,S*)-β-isopropylmalate enantiomer) the natural K_m was distorted to a value too low to be measured accurately [6]. Based on an estimated apparent K_m value of 30 µM, the K_i values of the nitronate anions of **II** (Fig. 5) and **III** were *ca.* 0.6 and 0.12 nM (based on pKa values of 7.3 and 9.5). Inhibition by **II** appeared biphasic (Fig. 2) with an initial weak EI complex isomerizing to the more tightly bound (K_i 0.6 nM) complex, EI*, at a forward rate (k_3) of $>= 5\ min^{-1}$ (Fig. 4).

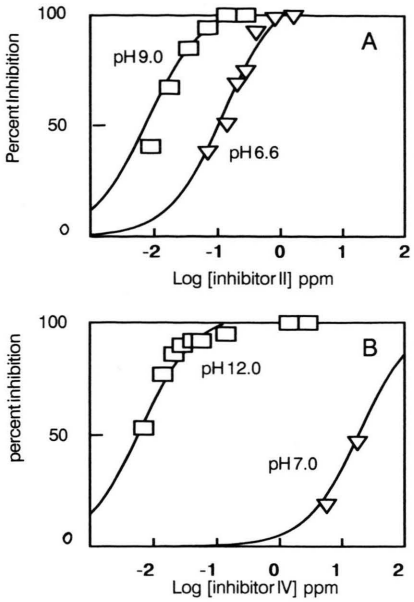

Fig. 6. pH effects on inhibition. This experiment was to distinguish between inhibition by the protonated and deprotonated (nitronate) forms of (Fig. 6A) **II** and (Fig. 6B) **IV.** The relatively slow protonation of nitronic acids (t 1/2 > 7 min) allowed the anion to be partly "trapped". Stock solutions of inhibitors **II** and **IV** were preequilibrated for an hour or more at the pH values indicated. Aliquots were then transferred to assays at pH 7.0 containing DMC as substrate. Assays were started immediately by addition of enzyme and rates recorded after 3 min. This comparative experiment (rates were not equilibrium values but the complex result of slow enzyme and protonic equilibria) indicated that both inhibitors were only significantly inhibitory as the nitronate anion and that both were about equally as potent.

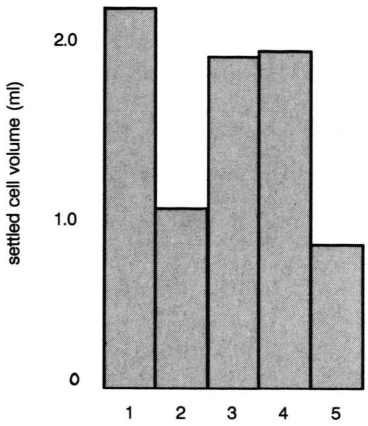

Fig. 7. Inhibition of carrot cell growth by **II** and specific reversal by leucine. Carrot cells line DC3 were subcultured to maintain log phase growth in Murashige and Skoog medium. The graph records total growth (as settled cell volume) after 7 days from subculturing in 5 ml of medium shaken at 25 °C in a 25 ml flask. Apart from the control, 1, all flasks contained 0.1 ppm of compound **II.** The settled cell volume in flask 2 represents *ca.* 60% inhibition of growth (allowing for the volume of inoculum). Reversing amino acids were added at 80 ppm each. 1) is the control, 2) has no reversing amino acid, 3) contained leu, ileu and val, 4) contained only leu and 5) only ileu. Leucine alone reversed growth inhibition by **II.**

The nitroalkane/nitronate pKa of **III** was reported to be 9.5 [1]. According to our measurements the apparent effects of ring strain were to *reduce* the pKa of the cyclopentane, **II**, to *ca.* 7.3 and to *increase* that of the cyclohexane, **IV,** to *ca.* 11.1. As the only compound significantly in the nitronate form at physiological pH, it was not surprising that **II** was the best herbicide (active at around a kilogramme per hectare against a range of dicot species). Its mode of action was confirmed by the observation that **II** inhibited the growth of carrot cells in culture and was specifically reversed by leucine (Fig. 7).

Many commercial herbicides act by inhibiting acetolactate synthase, the first common step in the biosynthesis of the three branched chain amino acids [12]. A number of experimental herbicides are now known to act at the second common step, ketol acid reductoisomerase [13, 14]. The observations in this paper indicate that herbicidal activity is also possible through inhibiting the biosynthesis of leucine alone and, more specifically, through inhibiting isopropylmalate isomerase.

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[1] M. H. Emptage, in: Biosynthesis of Branched Chain Amino Acids (Z. Barak, D. M. Chipman, and J. V. Schloss, eds.), pp. 315–328, VCH, Weinheim 1990.
[2] M. H. Emptage, in: Metal Clusters in Proteins, ACS Symposium Series 372, American Chemical Society, Washington, pp. 343–371 (1988).
[3] H. Lauble, M. C. Kennedy, H. Beinert, and C. D. Stout, Biochemistry **31,** 2735–2748 (1992).
[4] D. Broadbent and M. E. Radley, Annu. Bot. **30,** 763–777 (1966).
[5] M. H. Emptage and J. V. Schloss, Fed. Proc. **45,** 1536 (1986).
[6] J. V. Schloss, R. Magolda, and M. Emptage, Meth. Enzymol. **166,** 92–96 (1988).
[7] G. B. Kohlhaw, Meth. Enzymol. **166,** 423–429 (1988).
[8] J. Telser, M. H. Emptage, H. Merkle, M. C. Kennedy, H. Beinert, and B. M. Hoffman, J. Biol. Chem. **261,** 4840–4846 (1986).
[9] B. F. Burrows and W. B. Turner, J. Chem. Soc. (C) **1966,** 255–260.
[10] W. B. Turner, British Patent # 1040546.
[11] J. V. Schloss, in: Target Sites of Herbicide Action (P. Böger and G. Sandmann, eds.), CRC Press, Boca Raton, FL 1989.
[12] T. R. Hawkes, in: Prospects for Amino Acid Biosynthesis Inhibitors in Crop Protection and Pharmaceutical Chemistry (L. G. Copping, J. Dalziel, and A. D. Dodge, eds.), pp. 129–136, Monograph No. 42, British Crop Protection Council, Farnham, Surrey, U.K. 1989.
[13] A. Aulabaugh and J. V. Schloss, Biochemistry **29,** 2824–2830 (1990).
[14] A. Schulz, P. Sponemann, H. Kocher, and F. Wengenmayer, FEBS Lett. **238,** 375–378 (1988).