3-Isopropylidenemalic Acid: A Mechanism-based Inhibitor of 3-Isopropylmalate Dehydrogenase

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3-Isopropylidenemalic acid (**1**) was designed and synthesized as an inhibitor of 3-isopropylmalate dehydrogenase (IPMDH). While being weakly active as substrate, **1** appeared to be a mechanism-based inhibitor (K_I = 60.2 μM) for IPMDH as deduced from the time-dependent and kinetic analyses.

Specific enzyme inhibitors are of use for mechanistic enzymology as well as for pharmaceuticals, fungicides and herbicides. 3-Isopropylmalate dehydrogenase (IPMDH, EC 1.1.1.85) catalyzes the oxidation and decarboxylation reaction of (*2R,3S*)-3-isopropylmalate (IPM) into 2-oxoisocaproate with an aid of NAD^+ in the penultimate step of the biosynthetic pathway of an essential amino acid L-leucine, as shown in Figure 1.[1-3] We have been involved in the mechanistic enzymology of IPMDH, derived from the extremely thermophilic bacteria *Thermus thermophilus* HB8.

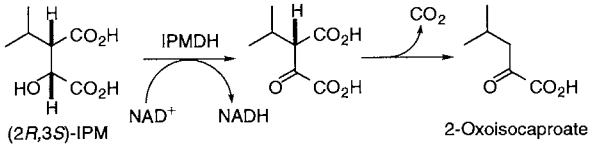


Figure 1. IPMDH enzyme reaction.

So far, several crystal structures of IPMDH complexed with either a substrate or an NAD^+ cofactor were reported,[4] and recently, the *Thiobacillus ferrooxidans* IPMDH-IPM complex has also been determined crystallographically.[5] However, the most important and intriguing ternary structure of IPMDH-IPM-NAD^+–metal complex has not been elucidated to date. To this end, it appears to be necessary to develop potential inhibitors which can be trapped in the enzyme active site. While we and others have reported several inhibitors for IPMDH,[6-8] more elaborated design seems desirable for appropriate inhibitors.

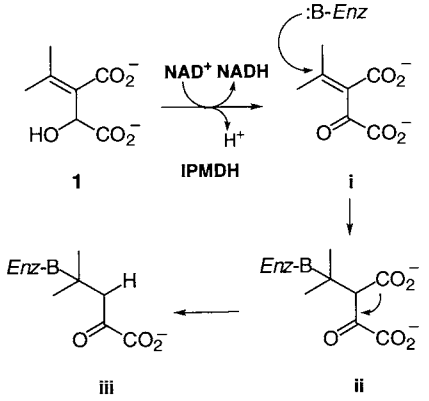
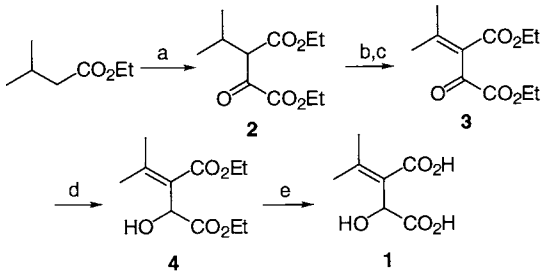


Figure 2. The possible pathway of the enzyme reaction of 3-isopropylidenemalic acid.

Based on the two-step mechanism of the IPMDH reaction, an inhibitor which cannot be susceptible for the second decarboxylation reaction may reside in the active site. This paper describes the design and synthesis of 3-isopropylidenemalate (**1**) as a mechanism-based inhibitor. Once **1** is oxidized by the first oxidation step, the resulting isopropylidenoxaloacetate (**i**) should not be decarboxylated, and hence, may stay on in the active site. Alternatively, a neighboring nucleophilic residue in the active site may attack the electron deficient double bond to form a covalent bond as shown in Figure 2.

Preparation of **1** was rather straightforward as depicted in Scheme 1. The synthetic inhibitor **1**[9] was incubated with thermophilic IPMDH derived from *Thermus thermophilus* HB8 and kinetic analysis was carried out as described previously. 3-Isopropylidenemalate showed a very weak substrate activity (K_m = 69.7 μM and k_{cat} = 1.54 x 10^{-3} s^{-1}) against IPMDH. The inhibition appeared to be mechanism-based since an incubation of IPMDH with **1** resulted in a time-dependent inactivation as shown in Figure 3A.[10]



Scheme 1. Reagents; a, NaH, $(CO_2Et)_2$, THF, 61%; b, PhSeCl, pyridine; c, 31% H_2O_2, CH_2Cl_2, 0 °C, 92% in 2 steps; d, $NaBH_4$, $CeCl_3 \cdot 7H_2O$, MeOH, -78 °C, 63%; e, $LiOH \cdot H_2O$, THF-H_2O, 50 °C, 49%.

In order to obtain K_I and k_{inact} values for **1**, the half-time ($t_{1/2}$) for inactivation at each inhibitor concentration was plotted against 1/[**1**] referred to as a Kitz and Wilson plot (Figure 3B).[11] As a result, 3-isopropylidenemalic acid was found to be moderately inhibitory against IPMDH (K_I = 60.2 μM, k_{inact} = 4.74 x 10^{-2} s^{-1}). The inhibition of **1** appeared to be more potent than that of less hydrophobic 3-ethylidenemalate homolog (K_m = 234 μM, k_{cat} = 8.54 x 10^{-3} s^{-1}, K_I = 153 μM, k_{inact} = 8.13 x 10^{-2} s^{-1}).[6e] Apparently, a suitable bulkiness of the hydrophobic part of inhibitors or substrate analogs is important for the recognition by IPMDH,[6,12] and this was actually the case. The resulting partition ratio (k_{cat}/k_{inact} = 0.0325) of **1** suggested possible formation of a covalent bond between IPMDH and a putative product from **1**, however, the enzyme activity was recovered after dialysis of an incubation mixture including **1**. These

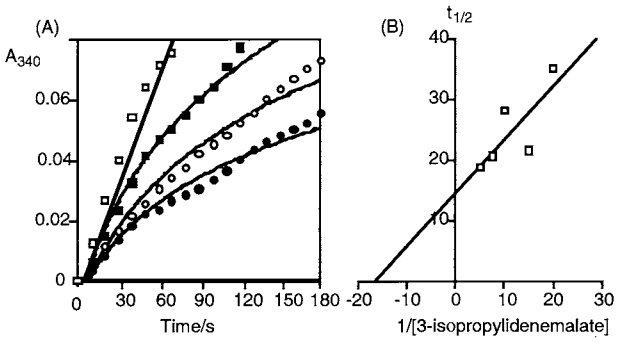


Figure 3. (A): Progress curves for the inactivation of IPMDH by **1**. The reaction was initiated by adding enzyme to an assay mixture containing IPM (0.1 mM), NAD^+ (5 mM), $MgCl_2$ (5 mM), KCl (100 mM), and **1** (0-0.3 mM) in 50 mM HEPES-NaOH (pH 7.8) at 60 °C. The concentrations of **1** were 0 (□), 0.1 (■), 0.2 (○), and 0.3 mM (●). (B): Kitz and Wilson plot for the inactivation of IPMDH by **1**. A pre-incubated solution of IPMDH containing **1** (0-20 mM) was diluted 100 fold into the enzyme assay mixture containing IPM (0.1 mM), NAD^+ (5 mM), $MgCl_2$ (5 mM), and KCl (100 mM) in 50 mM HEPES-NaOH (pH 7.8). The half-time ($t_{1/2}$) for inactivation at each inhibitor concentration was plotted against 1/[**1**].

results may be rationalized in two possible ways. Covalent bond formation may take place after an oxidation of **1**, but the bond formation between the enzyme and the active species is reversible, or the isopropylidenoxaloacetate product (**i**) simply stay on in the active site without any covalent bond formation.

More detailed analysis of the inhibition mechanism and attempts to cocrystallize the enzyme-inhibitor complex are under investigation.

References and Notes

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9 Physico-chemical data of 3-isopropylidenemalic acid (**1**): mp 106-108 °C; ν_{max} (KBr) cm^{-1} 1750 (C=O), 1660 (C=O); δ_H (D_2O) 1.72 (3H, s, CH_3), 1.81 (3H, s, CH_3), 4.99 (1H, s, CH); δ_C (D_2O) 22.7, 23.6, 68.9, 126.3, 153.9, 171.5, 177.2. Found: C, 43.59; H, 6.28 %. Calcd for $C_7H_{10}O_5$: C, 43.75; H, 6.29 %.
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