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Synthesis of Cyclopropane Substrate Analog for 3-Isopropylmalate Dehydrogenase and Its Mechanism-Based Inhibition

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Abstract

A cyclopropane substrate analog **1** was designed as a mechanism-based inhibitor for 3-isopropylmalate dehydrogenase (IPMDH), the rate-determining enzyme responsible for the penultimate step in the biosynthetic pathway of the essential amino acid L-leucine. The synthesis of **1** was pursued in 5 steps from diethyl (*R*)-malate. As was deduced from the time-dependent and kinetic analyses, **1** appeared to be a competitive and mechanism-based inhibitor for IPMDH (*K*i = 1.9 mM). Thus, **1** was recognized as a substrate and oxidized by IPMDH. Inhibitory mechanism is suggested to be slowering the decarboxylation step.

Keywords; Cyclopropanes; Enzymes and enzyme reactions; Enzyme inhibitors; Hydroxy acids and derivatives

Introduction

Mechanistic enzymology is among the most interesting area in chemical biology. We have been involved in the mechanistic and molecular recognition studies on *threo*-D-3-isopropylmalate dehydrogenase (IPMDH, EC 1.1.1.85) derived from the extremely thermophilic bacteria *Thermus thermophilus* HB8. IPMDH catalyzes an oxidation and decarboxylation reaction of (2*R*,3*S*)-3-isopropylmalate (IPM) into 2-oxoisocaproate with an aid of NAD^+ in the penultimate step of the biosynthetic pathway of L-leucine (Scheme 1).[1]

The cryptic stereochemistry of the IPMDH reaction was already elucidated; *i.e.*, the hydride transfer from

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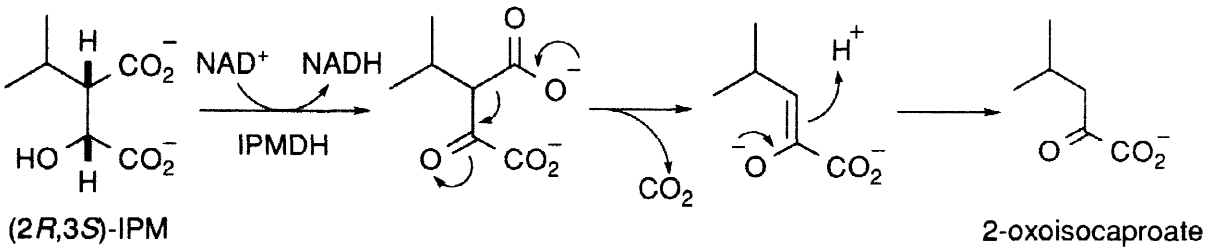


Scheme 1. IPMDH enzyme reaction.

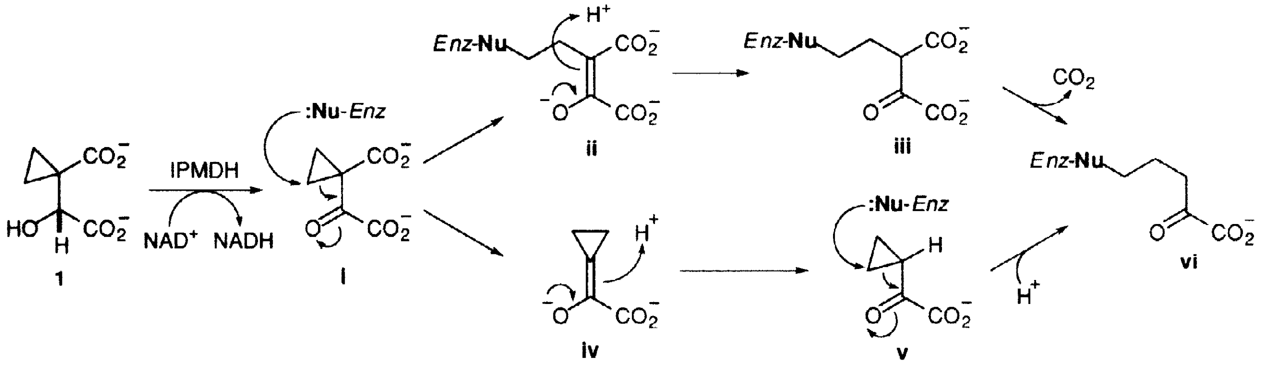
the C-2 position of IPM to NAD$^+$ is *pro-R* (A-site) specific, and the decarboxylation proceeds with retention of configuration at C-3 of the substrate.[2] These stereochemical features of IPMDH demonstrate significant similarity to those of *threo*-D-isocitrate dehydrogenase (ICDH, EC 1.1.1.42) functioning in the tricarboxylate cycle. Genetic comparison of IPMDH and ICDH also suggested an evolutionary relationship between the two enzymes.[3,4]

Recently, the crystals of the enzyme containing the NAD$^+$ cofactor and the ones containing the IPM substrate were analyzed separately by X-ray crystallography, and the polypeptide regions responsible for the binding of NAD$^+$ and IPM were discussed by Hurley *et al.* and Tanaka *et al.*, respectively.[4-6] However, even at this stage, several features of the IPMDH-substrate interaction have yet to be clarified, *i.e.* i) the conformation of the nicotinamide moiety of NAD$^+$, affecting the enzyme reaction, was rather mobile in the crystalline complex of IPMDH with NAD$^+$, and ii) upon soaking with the IPM substrate, enzyme crystals were destroyed at high substrate concentrations. Tanaka *et al.* argued with these observations that the enzyme conformation must be altered rather significantly upon substrate binding, and the crystal structure deduced with the soaked specimen at low substrate concentration may not represent the features of the native IPMDH-substrate complex.[6] It is well-established that most of the enzymes utilizing a nicotinamide coenzyme bind the coenzyme first and the substrate binding is followed. Accordingly, when an enzyme molecule captures the cofactor in the binding process to the cofactor and the substrate, significant conformational change must be induced to make the resulting enzyme-cofactor complex prone to accept the IPM substrate. Furthermore, quite recently, a crystalline complex of IPMDH from *Thiobacillus ferrooxidans* containing IPM was analyzed crystallographically.[7] The *Thiobacillus* IPMDH appeared to be related to the *Thermus* IPMDH with 50 % amino acid sequence identity. The crystal structure of the *Thiobacillus* IPMDH-IPM complex was in a fully closed conformation, whereas the *Thermus* IPMDH-IPM complex was in a open conformation. In order to get more insight into the precise enzyme-cofactor-substrate interaction, it is highly desirable to develop suitable substrate analogs which can be appropriately retained in the ternary complex.

Among other approaches to this end is to prepare potent inhibitors. Wittenbach *et al.*[8] and Pirrung *et al.*[9] reported several inhibitors for IPM. Especially, *O*-isobutenyl and *O*-methyl oxalylhydroxamate were shown to be highly inhibitory against *Salmonella* IPMDH (*K*i = 31 nM and 15 nM, respectively).[8] They suggested that the hydroxamates could be good mimics to the intermediary enolate in IPMDH reaction. The inhibition mechanism by these hydroxamates appeared to be competitive at the later enzyme reaction step. Therefore, it seemed difficult to elucidate the initial binding of the substrate and cofactor to IPMDH from the studies with these hydroxamates.

We have been involved for quite some time in designing the substrate analogs and inhibitors for IPM.[10-14] We synthesized conformationally restricted substrate analogs which possess an epoxide or an oxetane ring,[11] and showed that IPMDH recognizes an *anti*-conformation of the dicarboxylates of IPM. In designing mechanism-based inhibitors of IPMDH, we further synthesized 3-(1-fluoro-1-methylethyl)malic acid (F-IPM)[12] and 3-vinylmalic acid (VM).[14] Whereas F-IPM showed no inhibitory activity, VM was proved to have a highly potent inhibitory activity. The inhibition mechanism of VM has been elucidated that the enzyme reaction product of VM forms a transient covalent bond with IPMDH.

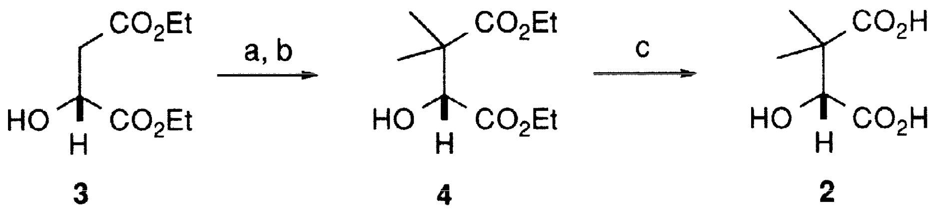
Cyclopropanoid compounds have been reported as mechanism-based inhibitors.[15] The cyclopropylamines,[15d-f] *e.g. trans*-phenylcyclopropylamine, which are known as classical antidepressant inhibitors of monoamine oxidase, are oxidized to the cyclopropylimine equivalents, and the resulting imines are thought to inactivate the monoamine oxidase by forming an adduct with the enzyme or a flavin cofactor. Thus, it is intriguing to introduce a cyclopropane unit into the IPM substrate for a design of mechanism-based inhibitor. We envisioned a scenario depicted in Scheme 2. If a cyclopropane substrate analog **1** is recognized as a substrate and oxidized accordingly by IPMDH, compound **i** may be produced in the enzyme active site. The cyclopropane ring in the resulting compound **i** can accept an attack of a nearby nucleophilic group of IPMDH active center, thereby forming a covalent bond to the enzyme. Alternatively, a covalent bond may form between the end product **v** with the enzyme at the later stage of the enzyme reaction. This paper describes the synthesis of the cyclopropane analog and its inhibition of IPM.



Scheme 2. The basic concept of cyclopropane substrate analog **1**.

Results and Discussion

First, (2*R*)-3,3-dimethylmalic acid (**2**) was synthesized to see whether or not a 3,3-disubstituted malate may act as a substrate. For the synthesis of **2**, α-alkylation of a β-hydroxycarboxylic ester was repeated.[16] Diethyl (*R*)-malate **3** was treated with two equivalents of LDA at -78°C to form an intermediary lithium alkoxide enolate, which was then alkylated with iodomethane to give 3-methylmalate. After the second

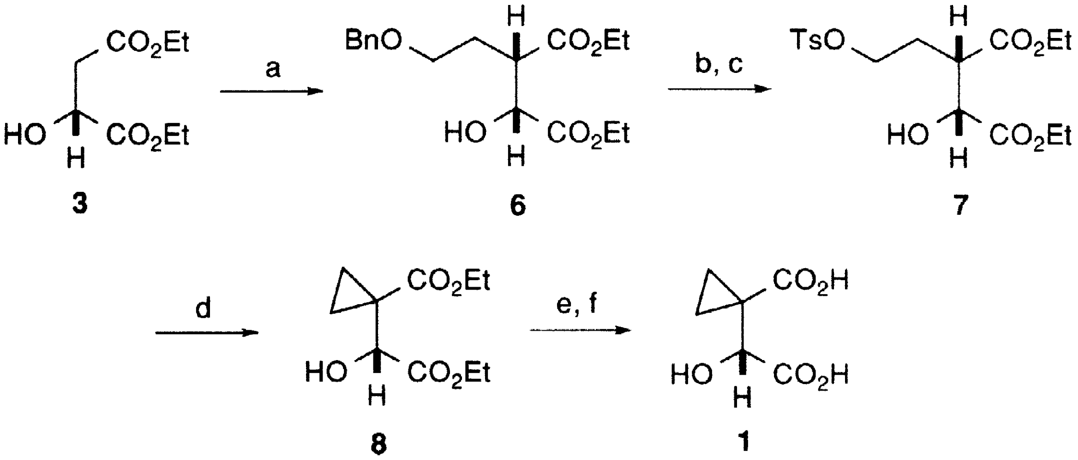


Scheme 3. *Reagents and conditions*; a, 2eq. LDA, MeI, THF-HMPA, -20°C; b, 2eq. LDA, MeI, THF; c, LiOH, THF-H_2O.

alkylation of 3-methylmalate in a similar way (41 % yield, 2 steps), diester **4** was hydrolyzed under basic conditions to afford **2** in 53 % yield.

Dimethylmalate **2** was subjected to the incubation with the thermophilic IPMDH derived from *Thermus thermophilus* HB8 as described previously.[10-14] A moderate substrate activity was obtained (Km = 81.5 μM, k_{cat} = 14.7 s^{-1}). While k_{cat} value of **2** was the same order as IPM, **2** showed a lowered binding affinity than IPM (Km = 1.84 μM, k_{cat} = 19.6 s^{-1}), probably because of steric hindrance between the enzyme and an additional CH_3-group of **2**. Since **2** was recognized as a substrate by IPMDH in spite of the effect of the steric hindrance, the cyclopropane substrate analog **1** was anticipated to be similarly recognized.

The synthesis of **1** started again from readily available diethyl (*R*)-malate **3**. Malate **3** was treated with two equivalents of LDA at -78°C to form an alkoxide enolate, which was then alkylated with benzyl 2-iodoethyl ether (**5**) at -20°C in THF-HMPA to give stereoselectively 3-alkylated malate in a 15:1 ratio (54 % yield).[16] The major diastereomer **6** possessed the (2*R*,3*S*)-configuration.[17] After removal of the benzyl group of **6** by catalytic hydrogenation, the primary hydroxyl group was immediately tosylated with an equivalent of *p*-TsCl in pyridine at 0°C. Since the tosylation of the intermediary diol was not selective as expected and the diol was rather unstable on silica gel, **7** was obtained in a low yield (19 % yield, 2 steps). Subsequently, a ring closure to cyclopropane was accomplished by the treatment of tosylate **7** with two equivalents of LDA to give **8** in 22 % yield. Finally, basic hydrolysis of **8** with lithium hydroxide in THF-water afforded, after chromatographic purification, the desired **1** in 68 % yield.



Scheme 4. *Reagents and conditions*; a, 2eq. LDA, $BnOCH_2CH_2I$ (**5**), THF-HMPA, -10°C; b, H_2/Pd-C, EtOH; c, *p*-TsCl, py, 0°C; d, 2eq. LDA, -78°C; e, LiOH, THF-H_2O; f, Dowex 50W-X2 (H^+).

The synthesized **1** was subjected to the incubation with thermophilic IPMDH as described above. The cyclopropane substrate analog **1** showed a very weak substrate activity (Km = 255 μM, k_{cat} = 0.136 s^{-1}) and appeared to be modestly inhibitory against IPMDH (Ki = 1.90 mM). The inhibition mode of **1**, which was analyzed by a standard Lineweaver-Burk plot, was distinctly competitive for IPM, as shown in Figure 1A. Since the incubation of IPMDH with the cyclopropane substrate analog **1** resulted in time-dependent inactivation as shown in Figure 1B,[18] the inhibition appeared to be mechanism-based. However, the enzyme activity was recovered after dialysis of the incubation mixture including **1**. The reaction product was further analyzed. Thus, after the incubation mixture including **1** was dialyzed, the dialytic solution was lyophilized.

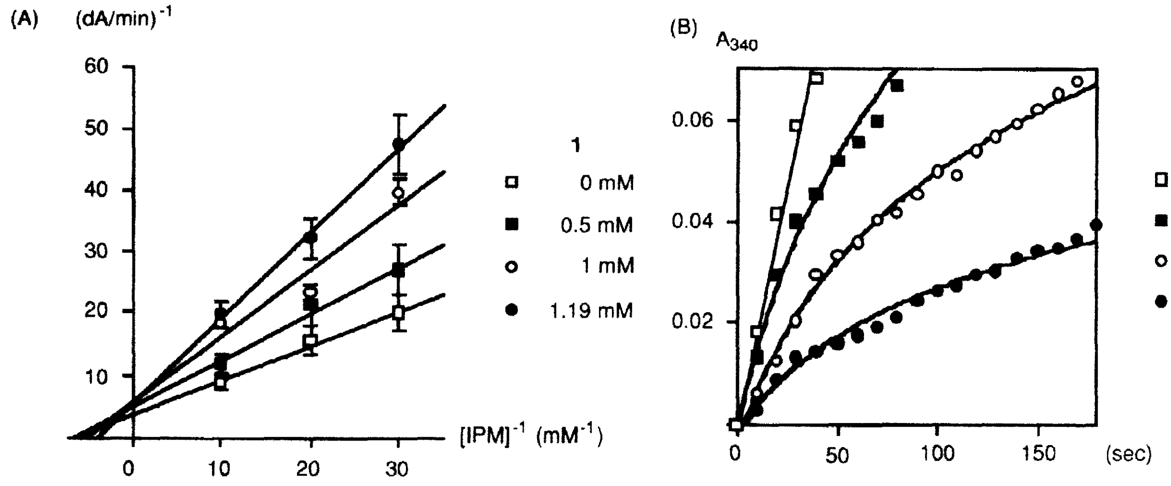


Figure 1. (A): Lineweaver-Burk plots of the effect of **1** on IPMDH reaction. (B): Progress curves for inactivation of IPMDH by **1**. The reaction was initiated by adding enzyme to an assay mixture containing IPM (0.1 mM), NAD$^+$ (5 mM), MgCl$_2$ (5 mM), KCl (100 mM), and **1** (0-3.9 mM) in HEPES (pH 7.8) at 60°C.

The residual enzyme reaction product was analyzed by ^{1}H-NMR spectroscopy. In the double-quantum filtered COSY spectrum, a cross peak which correlated with the signals (0.43 and 0.56 ppm) of the CH$_2$ group of the cyclopropane ring was observed at 1.05 ppm. This signal was ascribable to the signal of 3-H in the decarboxylated product **v**. These results suggest that the decarboxylation of compound **i** proceeds rather slowly. Therefore, mechanistically, the cyclopropane ring of **1** did not covalently react with the enzyme.

To be noted was that a catalytic efficiency and inhibitory activity of **1** were substantially different from those of **2**. These significant difference between them may be the effect of C-3 carbon on the cyclopropane ring. Naturally in the normal enzyme reaction, the sp^3-hybridized C-3 carbon must be changed to a more strained sp^2-hybridized carbon in the decarboxylation, and significant activation energy is necessary. The cyclopropane structure of **1** appeared to reduce the tendency of the transition of the hybridization state. In order to estimate the feasibility of the decarboxylation of **1**, standard enthalpies of formation for the intermediates and products in the enzyme reaction of **1** and **2** were calculated by the semiempirical molecular orbital method, MNDO-PM3 (Figure 2). The difference of the enthalpy between the substrate (**i** or **vii**) and product (**v** or **ix**) were exothermic in both cases. Taking the enthalpy of the intermediate enols (**iv** and **viii**) into account, the differential enthalpy in the decarboxylation step of **i** was more endothermic than that of **vii**. Therefore, these computation results may well support that the decarboxylation of **1** must proceed much slower than that of **2**.

The X-ray analysis of *Thiobacillus* IPMDH-IPM complex has been reported recently.[7] The *Thiobacillus* IPMDH is known to be highly homologous to the *Thermus* IPMDH. The crystallographic structure of the *Thiobacillus* IPMDH-IPM complex indicates that the hydrophobic pocket of *Thermus* IPMDH, which is responsible for recognition of the 3-alkyl chain of the substrate, is analogous and includes Glu87, Leu90, Leu91 and Val188' (a prime indicates the residue of the second subunit). Especially, Cβ and Cγ of Glu87 may play an important role on the recognition of the isopropyl group of IPM.[7] Among these residues, Glu87 is the only reactive residue, which might have been involved in a covalent bond formation, which did not take place in the actual incubation. This may be because the carboxylate of Glu87 does not face to the cyclopropane ring

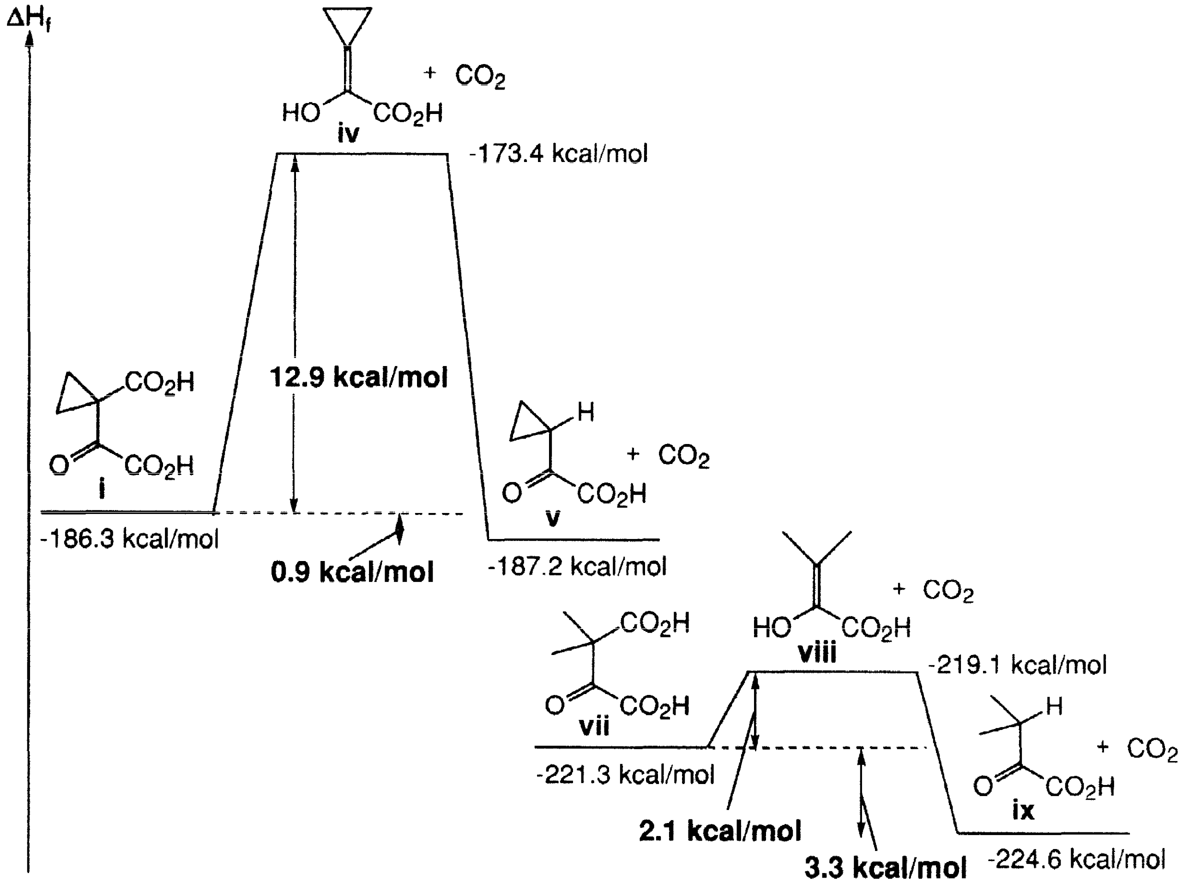


Figure 2. Diagram of the enthalpy of the intermediates and products in the decarboxylations. The standard enthalpy of formation were calculated by the semiempirical molecular orbital method, MNDO-PM3.

of **1** and there may be no other suitable functional residue neighboring the cyclopropane ring. Or, the reactivity of Glu87 may not be sufficient enough to attack the cyclopropane ring.

In conclusion, cyclopropane substrate analog **1** was developed as a new mechanism-based competitive inhibitor, which was recognized as a substrate by the enzyme and oxidized. However, a covalent bond formation did not take place between the enzyme and the inhibitor. That the intermediate **i** was slowly decarboxylated and resided for a prolonged time in the enzyme active site appeared to be the reason of the inhibitory activity. These results may suggest that an appropriately oriented functional group is not available around the substrate recognition site of the *Thermus* IPMDH. Attempts to cocrystallize IPMDH with **1** has been continuing.

Experimental

Melting points were measured with a Yanagimoto BY-1 micromelting point apparatus and are uncorrected. IR spectra were taken on a Horiba FT-710 fourier transform infrared spectrometer. ^{1}H and ^{13}C NMR were recorded on JEOL GSX-270, LA-300, and/or LA-400 spectrometers. ^{1}H and ^{13}C NMR chemical shifts were reported in δ-values based on internal $SiMe_4$ ($\delta_H = 0$) or dioxane ($\delta_C = 67.4$), or solvent signal ($CDCl_3$: $\delta_C = 77.0$; HOD: $\delta_H = 4.65$) as reference. Silica gel column chromatography was carried out with

Kieselgel 60 (70-230 mesh, Merck). All reactions, except for catalytic hydrogenation reaction, were carried out in an inert (Ar or N_2) atmosphere. Tetrahydrofuran was distilled immediately prior to use from sodium/benzophenone ketyl. Pyridine was distilled from potassium hydroxide. HMPA was distilled from calcium hydride under reduced pressure and stored over molecular sieves 4A under Ar.

Diethyl (2*R*)-3,3-Dimethylmalate (4)

A solution of BuLi (69.0 mL, 110 mmol, 1.60 M) was added dropwise to a stirred solution of diisopropylamine (16.2 mL, 115 mmol) and 2,2'-dipyridyl (5 mg) in THF (160 mL) at 0°C. After 1 hr, the mixture was cooled to -78°C. A solution of diethyl (*R*)-malate **3** (9.97 g, 52.4 mmol) in THF (10 mL) was added dropwise at -78°C. After completion of addition, the mixture was warmed to -20°C. To the stirred mixture, HMPA (40 mL) was added. A solution of iodomethane (6.50 mL, 104 mmol) in THF (6.5 mL) was added dropwise and the mixture was stirred for 1 hr. The reaction was quenched by addition of saturated aqueous NH_4Cl solution. The mixture was diluted with ethyl acetate and 2M-HCl. The organic phase was separated. The aqueous layer was extracted twice with ethyl acetate. The combined organic phase was subsequently washed with saturated aqueous $NaHCO_3$ solution and brine, dried over Na_2SO_4, and evaporated. The resulting residue was chromatographed over silica gel with hexane-ethyl acetate (4:1-2:1) to give diethyl 3-methylmalate (8.35 g, 78 %).

A solution of BuLi (22.7 mL, 36.2 mmol, 1.60 M) was added dropwise to a stirred solution of diisopropylamine (5.54 mL, 39.5 mmol) and 2,2'-dipyridyl (5 mg) in THF (70 mL) at 0°C. After 1 hr, the mixture was cooled to -78°C. A solution of diethyl 3-methylmalate (3.36 g, 16.5 mmol) in THF (3.5 mL) was added dropwise at -78°C. After completion of addition, the mixture was warmed to 0°C. After 1 hr, the mixture was recooled to -78°C. A solution of iodomethane (2.05 mL, 32.9 mmol) in THF (2 mL) was added dropwise to the mixture at -78°C. After 1 hr, the mixture was warmed to -20°C and stirred for 2 hr. The reaction was quenched by addition of saturated aqueous NH_4Cl solution. The mixture was diluted with ethyl acetate and 2M-HCl. The organic phase was separated. The aqueous layer was extracted twice with ethyl acetate. The combined organic phase was subsequently washed with saturated aqueous $NaHCO_3$ solution and brine, dried over Na_2SO_4, and evaporated. The resulting residue was chromatographed over silica gel with benzene-ethyl acetate (25:1) to give **4** (1.88 g, 52 %): IR (neat) 3500, 2980, 1740 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.17 (s, 3H), 1.27 (s, 3H), 1.28 (t, *J* =7 Hz, 3H), 1.29 (t, *J* =7 Hz, 3H), 3.19 (d, *J* = 7 Hz, 1H), 4.20 (m, 4H), 4.36 (d, *J* =7 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 14.1, 20.0, 22.1, 46.5, 60.8, 62.0, 75.2, 173.2, 175.4. Anal. Calcd. for $C_{10}H_{18}O_5$: C, 55.03; H, 8.31. Found C, 54.74; H, 8.54.

(2*R*)-3,3-Dimethylmalic Acid (2)

To a stirred solution of compound **4** (986 mg, 4.52 mmol) in THF (20 mL) and water (10 mL), $LiOH \cdot H_2O$ (947 mg, 22.6 mmol) was added. After 5.5-hr stirring at room temperature, the mixture was acidified with 6M-HCl at 0°C. The solution was evaporated. The residue was chromatographed over LiChroprep RP-18 with water. Crystallization from CH_3CN-$CHCl_3$ gave **2** (389 mg, 53 %): mp 99-100°C; IR (KBr) 1710 cm^{-1}; ^1H NMR (D_2O) δ 1.00 (s, 3H), 1.34 (s, 3H), 4.34 (s, 1H); ^{13}C NMR (D_2O) δ 20.2, 22.1, 47.1, 75.7, 176.4, 181.1. Anal. Calcd. for $C_6H_{10}O_5$: C, 44.45; H, 6.22. Found C, 44.33; H, 6.35.

Diethyl (2*R*,3*S*)-3-(2-Benzyloxyethyl)malate (6)

A solution of BuLi (41.9 mL, 64.1 mmol, 1.53 M) was added dropwise to a stirred solution of diisopropylamine (9.80 mL, 69.9 mmol) and 2,2'-dipyridyl (5 mg) in THF (120 mL) at 0°C. After 1 hr, the mixture was cooled to -78°C. A solution of diethyl (*R*)-malate **3** (5.55 g, 29.2 mmol) in THF (5 mL) was added dropwise at -78°C. After completion of addition, the mixture was warmed to -10°C. To the stirred mixture, HMPA (24 mL) was added. A solution of iodide **5** (11.5 g, 43.8 mmol) in THF (20 mL) was added dropwise to the mixture. After 30 min, the reaction was quenched by addition of saturated aqueous NH_4Cl solution. The mixture was diluted with ethyl acetate and 2M-HCl. The organic phase was separated. The aqueous layer was extracted twice with ethyl acetate. The combined organic phase was subsequently washed with saturated aqueous $NaHCO_3$ solution and brine, dried over Na_2SO_4, and evaporated. The resulting residue was chromatographed over silica gel with hexane-ethyl acetate (5:1-2:1) to give **6** (5.12 g, 54 %): IR (neat) 3500, 2980, 1740 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.22 (t, *J*=7 Hz, 3H), 1.31 (t, *J*=7 Hz, 3H), 2.00 (m, 1H), 2.20 (m, 1H), 3.17 (dt, *J*=3, 7 Hz, 1H), 3.32 (d, *J*=7 Hz, 1H), 3.59 (m, 2H), 4.12 (m, 2H), 4.26 (m, 2H), 4.30 (dd, *J*=3, 7 Hz, 1H), 4.51 (s, 2H), 7.33 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 14.06, 14.13, 28.1, 45.4, 60.8, 61.8, 67.5, 70.9, 72.9, 127.6, 128.4, 138.2, 172.4, 173.4. Anal. Calcd. for $C_{17}H_{24}O_6$: C, 62.95; H, 7.46. Found: C, 62.74; H, 7.67.

Diethyl (2*R*,3*S*)-3-(2-*p*-Toluenesulfonyloxyethyl)malate (7)

A mixture of **6** (6.52 g, 20.1 mmol) and 10 % Pd/C (684 mg) in ethanol (30 mL) was vigorously stirred under a hydrogen atmosphere. After 10 hr, additional 10 % Pd/C (675 mg) in ethanol (10 mL) was added and the mixture was vigorously stirred for 12 hr under a hydrogen atmosphere. The catalyst was filtered off through a Celite pad and washed with ethanol. The filtrate and washings were combined and evaporated to give a diol (4.98 g).

To a solution of the diol (4.98 g) in pyridine (90 mL), *p*-tosyl chloride (4.07 g, 21.3 mmol) was added at 0°C. After 4 hr, the reaction was quenched by addition of water and the mixture was extracted with diethyl ether. The organic phase was subsequently washed with 2M-HCl and brine, dried over Na_2SO_4, and evaporated. The residue was chromatographed over silica gel with hexane-ethyl acetate (5:1) to give **7** (1.47 g, 19 %): IR ($CHCl_3$) 3520, 3030, 1740 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.20 (t, *J*=7 Hz, 3H), 1.32 (t, *J*=7 Hz, 3H), 1.99 (m, 1H), 2.24 (m, 1H), 2.45 (s, 3H), 2.87 (br, 1H), 3.11 (ddd, *J*=9, 6, 3 Hz, 1H), 4.11 (m, 5H), 4.27 (q, *J*=7 Hz, 2H), 7.35 (d, *J*=8 Hz, 2H), 7.79 (d, *J*=8 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 13.9, 14.1, 21.6, 27.6, 44.2, 61.1, 62.2, 68.2, 70.7, 127.9, 129.9, 132.7, 144.9, 171.0, 173.0. Anal. Calcd. for $C_{17}H_{24}O_8S$: C, 52.57; H, 6.23; S, 8.26. Found: C, 52.57; H, 6.45; S, 8.19.

Diethyl (2*R*)-3,3-Ethylenemalate (8)

A solution of BuLi (0.53 mL, 0.85 mmol, 1.60 M) was added dropwise to a stirred solution of diisopropylamine (0.13 mL, 0.93 mmol) and 2,2'-dipyridyl (5 mg) in THF (30 mL) at 0°C. After 1 hr, the mixture was cooled to -78°C. A solution of **7** (150 mg, 0.39 mmol) in THF (0.7 mL) was added dropwise at -78°C. After 1 hr, the reaction was quenched by addition of saturated aqueous NH_4Cl solution. The mixture was diluted with ethyl acetate and 2M-HCl. The organic phase was separated. The aqueous layer was extracted twice with ethyl acetate. The combined organic phase was subsequently washed with saturated aqueous $NaHCO_3$ solution and brine, dried over Na_2SO_4, and evaporated. The resulting residue was chromatographed over silica gel with benzene-ethyl acetate (10:1) to give **8** (18.1 mg, 22 %): IR ($CHCl_3$) 3530, 2990, 1730 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.04 (m, 1H), 1.14 (m, 1H), 1.23 (t, *J*=7 Hz, 3H), 1.30 (t, *J*=7 Hz, 3H), 1.38 (m, 2H), 3.57 (s, 1H), 4.10 (dq, *J*=12, 7 Hz, 1H), 4.14 (dq, *J*=12, 7 Hz, 1H), 4.24 (dq, *J*=11, 7 Hz, 1H), 4.30 (dq, *J*=11, 7 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 13.8, 14.0, 14.1, 14.9, 27.5, 60.9, 61.8, 74.2, 172.9, 173.0. Anal. Calcd. for $C_{10}H_{16}O_5$: C, 55.55; H, 7.46. Found: C, 55.43; H, 7.66.

(2*R*)-3,3-Ethylenemalic Acid (1)

To a stirred solution of compound **8** (99.7 mg, 0.46 mmol) in THF (1 mL) and water (0.5 mL), $LiOH{\cdot}H_2O$ (98.3 mg, 2.34 mmol) was added and the mixture was stirred for 5 hr. The solution was acidified (to pH 3 by pH paper) with Dowex 50W-X2 (H^+-form) and was filtered. The filtrate was evaporated. The resulting residue was chromatographed over LiChroprep RP-18 column with water to give **1** (50.2 mg, 68 %): mp 125-127°C; IR (KBr) 1730 cm^{-1}; ^1H NMR (D_2O) δ 0.88 (m, 1H), 1.04 (m, 1H), 1.18 (m, 2H), 3.54 (s, 1H); ^{13}C NMR (D_2O) δ 14.7, 16.9, 28.1, 74.2, 177.0, 178.0. Anal. Calcd. for $C_6H_8O_5$: C, 45.01; H, 5.04. Found: C, 44.73; H, 5.29.

Enzyme assay of the analogs

The thermophilic IPMDH derived from *T. thermophilus* HB-8 was prepared and purified as described previously.[1] IPMDH reaction was monitored by measuring the NADH absorption at 340 nm on a Shimadzu UV-160A UV-Visible recording spectrophotometer. Kinetic measurements were performed at 60°C in an assay mixture (total volume 700 μL) containing 50 mM HEPES buffer (pH 7.8), 5 mM NAD^+, 5 mM $MgCl_2$ and 100 mM KCl. The reaction was started by addition of the enzyme (0.7-6.8 μg) to the reaction mixture with all required components including IPM (25-100 μM) and/or cyclopropane substrate analog **1** (0.02-4 mM). The formation of NADH was measured for 1 min. Data were graphically analyzed by Lineweaver-Burk double reciprocal plots. All standard errors are less than 20 % of the estimates.

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References and notes

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