Synthetic and Mechanistic Studies of (2*R*,3*S*)-3-Vinylmalic Acid as a Mechanism-Based Inhibitor of 3-Isopropylmalate Dehydrogenase

Akira Chiba,[†] Tetsuya Aoyama,[†] Rieko Suzuki,[†] Tadashi Eguchi,*[,†,‡] Tairo Oshima,[§] and Katsumi Kakinuma*[,†]

Department of Chemistry, Tokyo Institute of Technology, O-okayama, Meguro-ku, Tokyo 152-8551, Japan, and Department of Molecular Biology, Tokyo University of Pharmacy and Life Science, Hachioji-shi, Tokyo 192-0392, Japan

Received November 3, 1998

(2*R*,3*S*)-3-Vinylmalic acid (VM) was designed as a mechanism-based inhibitor of *threo*-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 VM was achieved in six steps from diethyl (*R*)-malate. Besides its weak activity as a substrate, VM was shown to be a mechanism-based inhibitor ($K_I = 1.20$ mM) for IPMDH as deduced from the time-dependent and kinetic analyses. 2-Oxo-3-pentenoate was identified as the enzyme reaction product of VM by GC−MS analysis and ^{1}H NMR spectroscopy, but the supplemented 2-oxo-3-pentenoate was not, in contrast, inhibitive to the enzyme reaction. It seems likely from these results that an activated or nucleophilic amino acid of IPMDH by deprotonation during the ordinary enzyme reaction process participates in a strong interaction with the 2-oxo-3-pentenoate product, probably by the formation of a transient covalent bond, which in turn gives rise to inhibition of the enzyme reaction.

Introduction

Mechanistic enzymology is a field under intense investigation within the study of chemical biology. We have been involved in 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 is a homodimeric enzyme and catalyzes the oxidation and decarboxylation reaction of (2*R*,3*S*)-3-isopropylmalate (IPM) into 2-oxoisocaproate with the aid of NAD^+ in the penultimate step of the biosynthetic pathway of L-leucine, as shown in Figure 1.[1]

The cryptic stereochemistry of the IPMDH reaction has already been elucidated: the hydride transfer from 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 tricarboxylic acid cycle. Genetic comparison of IPMDH and ICDH also suggested an evolutionary relationship between the two enzymes.[3,4]

Recently, the separate crystal structures of the enzyme containing the NAD^+ cofactor or the IPM substrate were analyzed by X-ray crystallography, and the polypeptide

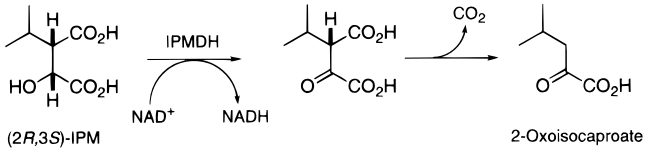


Figure 1. IPMDH enzyme reaction.

regions responsible for the binding of NAD^+ and IPM were discussed by Hurley et al. and Tanaka et al., respectively.[4−6] However, several features of the IPMDH−substrate interaction have yet to be clarified, i.e. (i) the conformation of the nicotinamide nucleotide was rather mobile in the crystals of IPMDH with NAD^+ and (ii) when the IPMDH crystals were soaked with the IPM substrate at high concentrations, the enzyme crystals were destroyed. Tanaka et al. suggested that the enzyme conformation must be altered rather significantly upon the substrate binding, and that the crystal structure deduced with the soaked specimen at low substrate concentration may not represent the features of the active IPMDH−substrate complex.[6] It is well-established that most of the enzymes utilizing a nicotinamide coenzyme bind the coenzyme first, followed by the substrate. Accordingly, in the complexation of IPMDH with the cofactor and the substrate, when an enzyme molecule captures the cofactor, significant conformational changes are induced to make the resulting enzyme−cofactor complex prone to accepting the IPM substrate. Furthermore, quite recently, a crystal of *Thiobacillus ferrooxidans* IPMDH complexed with IPM was analyzed, again

* To whom correspondence should be addressed. Fax: +81-3-5734-3713. E-mail: kakinuma@chem.titech.ac.jp.
† Tokyo Institute of Technology.
‡ Present address: Department of Chemistry and Materials Science, Tokyo Institute of Technology.
§ Tokyo University of Pharmacy and Life Science.

(1) (a) Tanaka, T.; Kawano, N.; Oshima, T. *J. Biochem. (Tokyo)* **1981**, *89*, 677. (b) Kagawa, Y.; Nojima, H.; Nukiwa, N.; Ishizuka, M.; Nakajima, T.; Yasuhara, T.; Tanaka, T.; Oshima, T. *J. Biol. Chem.* **1984**, *259*, 2956. (c) Yamada, T.; Akutsu, N.; Miyazaki, K.; Kakinuma, K.; Yoshida, M.; Oshima, T. *J. Biochem. (Tokyo)* **1990**, *108*, 449.
(2) (a) Yamada, T.; Kakinuma, K.; Oshima, T. *Chem. Lett.* **1987**, 1745. (b) Yamada, T.; Kakinuma, K.; Endo, T.; Oshima, T. *Chem. Lett.* **1987**, 1749. (c) Kakinuma, K.; Ozawa, K.; Fujimoto, Y.; Akutsu, N.; Oshima, T. *J. Chem. Soc., Chem. Commun.* **1989**, 1190.
(3) (a) Nakamoto, T.; Vennesland, B. *J. Biol. Chem.* **1960**, *235*, 202. (b) England, S.; Listowsky, I. *Biochem. Biophys. Res. Commun.* **1963**, *12*, 356. (c) Hurley, J. H.; Dean, A. M.; Sohl, J. L.; Koshland, D. E., Jr.; Stroud, R. M. *Science* **1990**, *249*, 1012.
(4) Imada, K.; Sato, M.; Tanaka, N.; Katsube, Y.; Matsuura, Y.; Oshima, T. *J. Mol. Biol.* **1991**, *222*, 725.
(5) Hurley, J. H.; Dean, A. M. *Structure* **1994**, *2*, 1007.
(6) Kadono, S.; Sakurai, M.; Moriyama, H.; Sato, M.; Hayashi, Y.; Oshima, T.; Tanaka, N. *J. Biochem. (Tokyo)* **1995**, *118*, 745.

10.1021/jo982206c CCC: $18.00 © 1999 American Chemical Society
Published on Web 08/05/1999

by X-ray crystallography.[7] The structure was in a fully closed conformation in contrast to the open conformation of the *Thermus* IPMDH−IPM complex. The authors suggest that the isopropyl group is recognized by a unique hydrophobic pocket, which includes Glu88, Leu91, and Leu92 of the subunit 1 and Val193′ of the subunit 2 (corresponding to Glu87, Leu90, Leu91, and Val188′ in *Thermus* IPMDH, respectively). In particular, the side chain, C_β and C_γ moiety, of Glu88 interacts with the isopropyl group of IPM and plays a central role in the recognition of the substrate. However, a description of the structure of the ternary complex of IPM is desirable since the enzyme−cofactor−substrate interaction has not been fully rationalized.

One approach to this objective is to prepare an appropriate ternary complex with a highly potent inhibitor, so that one may obtain appropriate crystals of enzyme−cofactor−inhibitor complex and thus elucidate the precise nature of the substrate and cofactor binding in the enzyme active site. Wittenbach et al.[8] and Pirrung et al.[9] have reported several inhibitors for IPM. In particular, *O*-isobutenyl and *O*-methyl oxalylhydroxamate were shown to be potent inhibitors against *Salmonella* IPMDH (K_i = 31 and 15 nM, respectively).[8] They suggested that the hydroxamates could be deprotonated (because of the acidic amide NH group) and that the deprotonated form, which mimics the intermediary enolate of the IPMDH reaction, was bound to the enzyme active site. In other words, these inhibitors bound to the enzyme in such a way that the protonation of the enolate intermediate was inhibited in the later productive step. Therefore, while these inhibitors are highly potent, it seems rather difficult to analyze the initial binding of IPMDH with the substrate and cofactor by use of these hydroxamates.

We have been involved for quite some time in designing substrate analogues and inhibitors for IPM.[10−13] In this paper we describe the first successful example of the design and synthesis of a mechanism-based inhibitor for IPMDH, (2*R*,3*S*)-3-vinylmalate (VM, **1**). In our previous studies (Figure 2), 3-(1-fluoro-1-methylethyl)malic acid (F−IPM) was found to be converted to **iii** via oxidation and decarboxylation by IPMDH, but was not inhibitory to the enzyme.[12] The observation that F−IPM had no inhibitory activity may be explained in two ways. One possibility is that the expected Michael addition to an electron-deficient conjugated olefin functionality cannot take place due to the steric hindrance of **iii**. The other possibility is that HF-elimination of **ii** to yield **iii** did not take place within the enzyme active site. To investigate the latter possibility, we designed VM as a new mechanism-based inhibitor, anticipating that it can be converted in situ to a Michael acceptor in the enzyme active site.

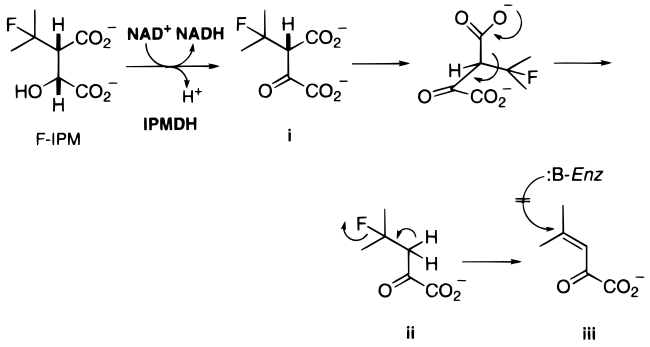


Figure 2. Enzyme reaction of F−IPM.

VM was expected to be recognized by the enzyme and be oxidized accordingly as a substrate since 3-ethylmalate was a good substrate for IPM[13] (Figure 3). The second step of the enzyme reaction may proceed via two possible pathways. In one pathway (path A), the conjugated enolate (**v**), formed after the direct decarboxylation as in the normal reaction, may abstract a proton of a neighboring amino acid to give a product (**vi**). Subsequently, the deprotonated residue of the enzyme may attack the electron-deficient olefin functionality of **vi** to give an enzyme−inhibitor complex (**ix**). In the other pathway (path B), a double bond migration may preferentially give rise to the ethylidenoxaloacetate (**vii**). The electron-deficient conjugated olefin functionality of **vii** is susceptible to attack by a nearby nucleophilic group of the IPMDH active center to give **viii**, which then spontaneously decarboxylates to 3-substituted 2-oxopentanoate (**ix**). In either case, the enzyme reaction can conceivably result in the formation of a covalent bond between the reaction product and the enzyme as **ix**.

Results and Discussion

VM was synthesized from readily available diethyl (*R*)-malate **2**, as shown in Scheme 1. First, the well-established diastereoselective α-alkylation reaction of *β*-hydroxycarboxylic ester was applied to the starting material **2**.[14] Thus, **2** was treated with 2 equiv of LDA at −78 °C to form an intermediary lithium alkoxide enolate, which was then alkylated with benzyl 2-iodoethyl ether at −20 °C in THF−HMPA to give stereoselectively 3-alkylated malate in a 15:1 ratio. The desired major diastereomer **3,** possessing the (2*R*,3*S*)-configuration, was obtained after chromatographic separation in 55% yield.[15] After acetylation of the hydroxyl group of **3** (71% yield), the benzyl group of **4** was removed by catalytic hydrogenation to afford alcohol **5** in 98% yield. The primary hydroxyl group of **5** was converted to a phenylselenenyl group by the method of Grieco et al.,[16] in which **5** was treated with *N*-phenylselenophthalimide and tributylphosphine in THF at 0 °C to give phenylselenenide **6** in 68%

(7) Imada, K.; Inagaki, K.; Matsunami, H.; Kawaguchi, H.; Tanaka, H.; Tanaka, N.; Namba, K. *Structure* **1998**, *6*, 971.

(8) (a) Wittenbach, V. A.; Rayner, D. R.; Schloss, J. V. *Curr. Top. Plant Physiol.* **1992**, *7*, 69. (b) Wittenbach, V. A.; Teaney, P. W.; Hanna, W. S.; Rayner, D. R.; Schloss, J. V. *Plant Physiol.* **1994**, *106*, 321.

(9) (a) Pirrung, M. C.; Han, H.; Nunn, D. S. *J. Org. Chem.* **1994**, *59*, 2423. (b) Pirrung, M. C.; Han, H.; Ludwig, R. T. *J. Org. Chem.* **1994**, *59*, 2430. (c) Pirrung, M. C.; Han, H.; Chen, J. *J. Org. Chem.* **1996**, *61*, 4527.

(10) Terasawa, H.; Miyazaki, K.; Oshima, T.; Eguchi, T.; Kakinuma, K. *Biosci. Biotechnol. Biochem.* **1994**, *58*, 870.

(11) Chiba, A.; Eguchi, T.; Oshima, T.; Kakinuma, K. *Tetrahedron* **1997**, *53*, 3537.

(12) Aoyama, T.; Eguchi, T.; Oshima, T.; Kakinuma, K. *J. Chem. Soc., Perkin Trans 1* **1995**, 1905.

(13) (a) Kakinuma, K.; Terasawa, H.; Li, H.-Y.; Miyazaki, K.; Oshima, T. *Biosci. Biotechnol. Biochem.*, **1993**, *57*, 1916. (b) Miyazaki, K.; Kakinuma, K.; Terasawa, H.; Oshima, T. *FEBS Lett*, **1993**, *332*, 35.

(14) Seebach, D.; Aebi, J.; Wasmuth, D. *Organic Syntheses;* Wiley: New York, 1990; Collect. Vol. VII, p 153 and references therein.

(15) The diastereoselectivity was estimated from the ^{1}H NMR spectra of a crude reaction mixture, and the stereochemistry of **3** was deduced from the ^{1}H NMR coupling constant ($^3J_{H2,H3}$ = 3 Hz); see refs 10 and 13.

(16) Grieco, P. A.; Gilman, S.; Nishizawa, M. *J. Org. Chem.* **1976**, *41*, 1485.

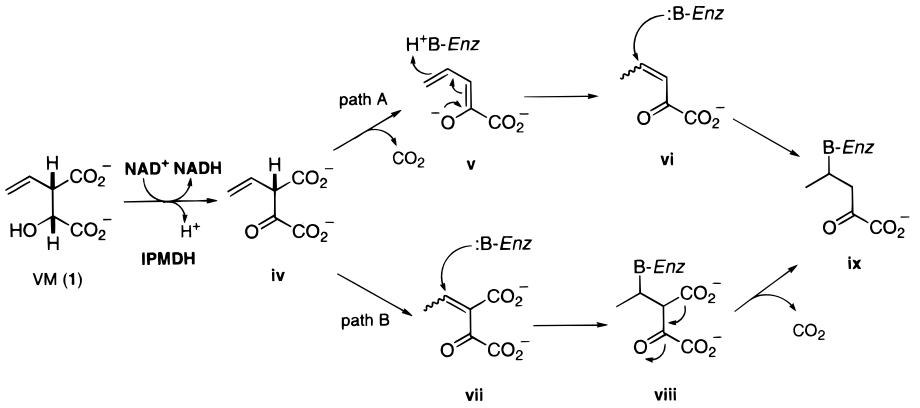
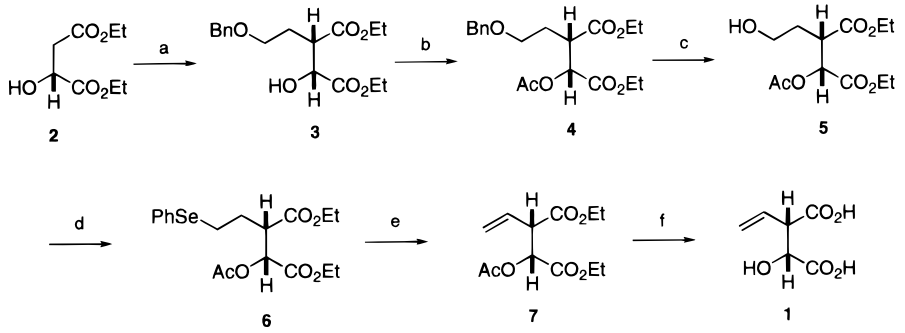


Figure 3. Possible pathways of the enzyme reaction of VM.

Scheme 1[a]



[a] Reagents and conditions: (a) 2 equiv of LDA, $BnOCH_2CH_2I$, THF−HMPA, −20 °C; (b) Ac_2O−DMAP/Py; (c) H_2/Pd-C, EtOH; (d) PhSeNPht, $^nBu_3P$, THF; (e) 30% H_2O_2−THF; (f) 6 M HCl−THF.

yield.[17] Oxidative elimination of the phenylselenenyl group of **6** was accomplished by treatment with hydrogen peroxide in THF to give a 3-vinylmalate derivative **7** in 82% yield. Finally, acid hydrolysis of **7** with 6 M HCl in THF afforded, after chromatographic purification and recrystallization, the desired VM (**1**) in 32% yield.

The synthesized VM was incubated with thermophilic IPMDH derived from *T. thermophilus* HB8 as described previously.[10−13] VM showed a very weak substrate activity (K_m = 1.85 mM, $k_{cat} = 5.10 \times 10^{-2}$ s^{-1}) and a potent inhibition activity against IPMDH. The inhibition appeared to be mechanism-based since incubation of IPMDH with VM resulted in a time-dependent inactivation as shown in Figure 4.[19] To obtain K_I and k_{inact} values for VM, the half-time ($t_{1/2}$) for inactivation at each inhibitor concentration was plotted against 1/[VM], referred to as a Kitz and Wilson plot[20] (Figure 5) and VM was found to be remarkably inhibitory (K_I = 1.20 mM, $k_{inact} = 8.16 \times 10^{-2}$ s^{-1}). The partition ratio for VM (k_{cat}/k_{inact} = 0.625) suggested an extremely strong interaction between the inactivator and the enzyme, probably by the formation of a covalent bond. Interestingly, however, we found that the enzyme activity recovered after dialysis of a VM incubation mixture. These results may be rationalized in two possible ways. Covalent bond formation may take place as anticipated, but the bond formation between the enzyme and the active species is reversible. Alternatively, the chemically feasible double bond migration from the isolated to the conjugated double bond after the initial oxidation might be faster than the decarboxylation. This would lead to formation of **vii**, which afterward resides in the active site of IPMDH for inhibition.

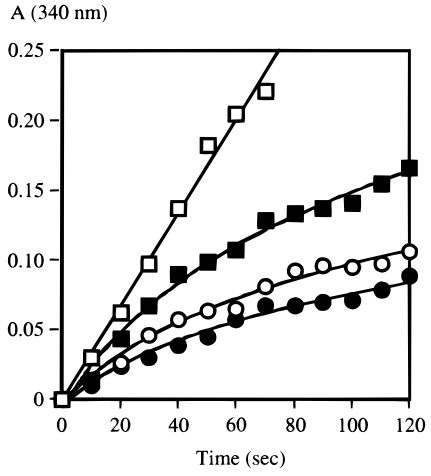


Figure 4. Progress curves for the inactivation of IPMDH by VM. The reaction was initiated by adding enzyme to an assay mixture containing IPM (0.1 mM), NAD^+ (5 mM), $MgCl_2$ (5 mM), KCl (100 mM), and VM (0−2 mM) in 50 mM HEPES−NaOH (pH 7.8) at 60 °C. VM concentrations were 0 (open square), 0.5 (closed square), 1.0 (open circle), and 2.0 mM (closed circle).

We first examined the latter possibility by comparing the reaction kinetics between VM and (2*R*)-3-ethylidenemalic acid **8**. Compound **8** was anticipated to be

(17) Nicolaou, K. C.; Claremon, D. A.; Barnette, W. E.; Seitz, S. P. *J. Am. Chem. Soc.* **1979**, *101*, 3704.
(18) Rambaud, M.; Bakasse, M.; Duguay, G.; Villieras, J. *Synthesis* **1988**, 564.
(19) Morrison, J. F.; Walsh, C. T. *Adv. Enzymol.* **1988**, *61*, 201.
(20) (a) Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245. (b) Silverman, R. B. *Mechanism Based Enzyme Inactivation: Chemistry and Enzymology*; CRC Press Inc.: Boca Raton, FL, 1988.

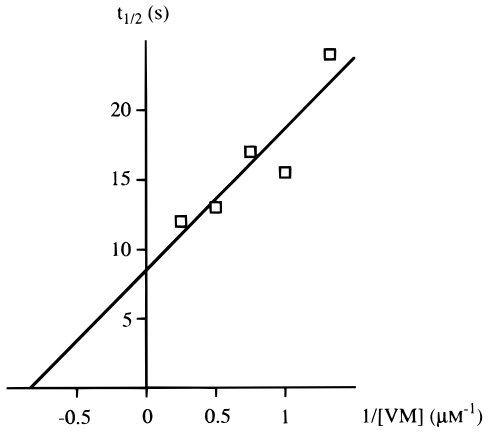
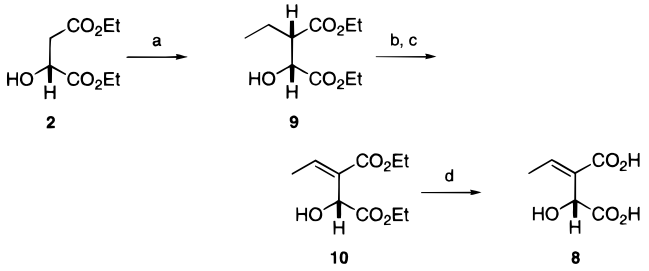


Figure 5. Kitz and Wilson plot for the inactivation of IPMDH by VM. A preincubated solution of IPMDH containing an inhibitor (0−4 mM) was diluted 100-fold into the enzyme assay mixture containing IPM (0.1 mM), NAD^+ (5 mM), $MgCl_2$ (5 mM), and KCl (100 mM) in 50 mM HEPES buffer (pH 7.8). The half-time ($t_{1/2}$) for inactivation at each inhibitor concentration was plotted against 1/[VM].

Scheme 2[a]



[a] Reagents and conditions: (a) 2 equiv of LDA, Etl, THF−HMPA, −20 °C; (b) equiv of LDA, PhSeBr; (c) 30% H_2O_2−THF; (d) LiOH, THF−H_2O.

recognized and oxidized by IPMDH to give **vii**. The synthesis of **8** also started from (*R*)-malate **2** (Scheme 2). First, (*R*)-malate **2** was treated with 2 equiv of LDA and then alkylated with ethyl iodide to give (2*R*,3*S*)-3-ethylmalate **9** in 9:1 ratio.[14] 3-Ethylmalate **9** was in turn treated with 2 equiv of LDA, and a phenylselenenyl group was introduced into the 3-position of **9**. Subsequent treatment with H_2O_2 and hydrolysis gave (2*R*)-3-ethylidenemalic acid **8** (*E*:*Z* = 25:1). Regarding the geometrical configuration of **8**, the major compound was found to have (*E*)-configuration since an NOE was observed between the CH_3 of the 3-ethylidene group and the proton at C-2.

The synthesized **8** was incubated with IPMDH as in the case of VM as described above. A very weak substrate activity (K_m = 234 mM, k_{cat} = 8.54 × 10^{-3} s^{-1}) and a modest inhibitory activity (K_I = 153 mM, k_{inact} = 8.13 × 10^{-2} s^{-1}, k_{cat}/k_{inact} = 0.105) were observed. Since the time-dependent inactivation of IPMDH was observed (Figure 6),[19] the inhibition appeared to be mechanism-based and 3-ethylidenemalate was found to be converted to **vii** by IPMDH. Further, it seems less likely that the inhibition of VM took place through the formation of **8**, because the k_{cat} and k_{cat}/k_{inact} values of **8** were distinctly different and far lower from those of VM. Thus, the enzyme reaction of the powerful inhibitor VM proceeds via path A.

We next analyzed the enzyme reaction product of VM by incubating on a large scale. The resulting reaction mixture was divided into two parts, and each was

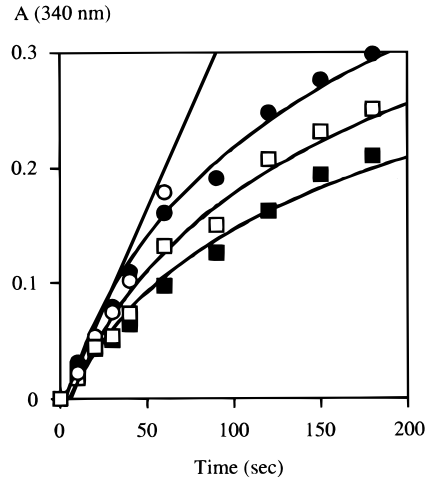


Figure 6. Progress curves for the inactivation of IPMDH by **8**. The reaction was initiated by adding enzyme to an assay mixture containing IPM (0.1 mM), NAD^+ (5 mM), $MgCl_2$ (5 mM), KCl (100 mM), and **8** (0−150 mM) in 50 mM HEPES−NaOH (pH 7.8) at 60 °C. The concentrations of **8** were 0 (open square), 50 (closed square), 100 (open circle), and 150 mM (closed circle).

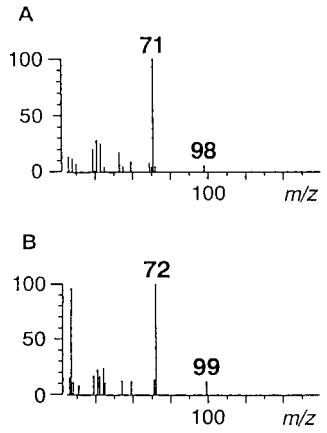
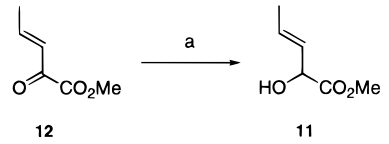


Figure 7. GC−MS analysis of the enzyme reaction product of VM. (A) Mass spectrum of the nondeuterated product at the retention time of 7.0 min. (B) Mass spectrum of the deuterated product at the same retention time.

separately reduced, with either $NaBH_4$ or $NaBD_4$, and dried by lyophilization. Both residues were treated with diazomethane, and the ethereal extracts thereof were subsequently analyzed by GC−MS. The rationale of this protocol was that, once VM was oxidized by IPMDH, the product might well contain a carbonyl group. The expected carbonyl group must then be reduced with $NaBH_4$ or $NaBD_4$ to yield a nondeuterated or deuterated alcohol derivative. The difference in the products should be only one mass unit in MS, while the GC retention time would be the same. The GC−MS ion trace showed one peak at 7 min that exhibited the desired 1 amu difference in *m*/*z* (Figure 7). Since the retention time of this peak in the GC column was shorter than that of VM dimethyl ester (t_R = 12 min), the enzyme reaction product appeared to have a lower molecular weight than VM. A possible candidate was methyl 2-hydroxy-3-pentenoate **11**. Authentic **11** was synthesized as described in Scheme 3 and compared with the derivative from the enzyme reaction by GC−MS analysis. The retention time (t_R = 7 min) and fragment ions of the authentic sample were identical to

Scheme 3[a]



12 11

[a] Reagent and conditions: (a) DIBAH, Et_2O, −78 °C.

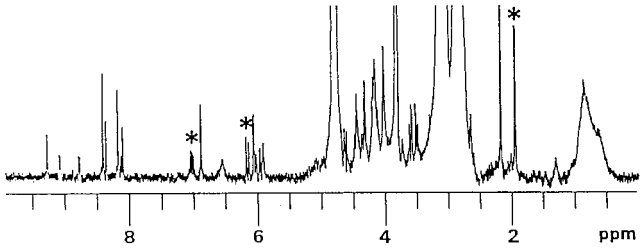


Figure 8. ^{1}H NMR spectrum of the enzyme reaction mixture of VM (0.80 mmol, final 3.98 mM) for 3 h at 25 °C. The asterisk peaks were derived from 2-oxo-3-pentenoate.

those of the enzyme reaction product. Similarly, the enzyme reaction product was identified by ^{1}H NMR spectroscopy. VM (0.80 mmol, 3.98 mM) was incubated with a large quantity of IPMDH (0.08 mmol, 0.39 mM) at 25 °C in an NMR tube. The signals of 2-oxo-3-pentenoate (δ_H 2.0, 6.2, and 7.0 ppm) clearly appeared in the ^{1}H NMR spectrum (Figure 8). It appeared, therefore, that 2-oxo-3-pentenoate was an end product from VM.

Path A is the most likely for the formation of 2-oxo-3-pentenoate from VM. This pathway involves the normal oxidation and decarboxylation processes of the IPMDH reaction. It was already reported that (2*R*,3*S*)-ethylmalate was a very good substrate for IPM.[13b] The only structural difference between the products of VM and ethylmalate is the conjugated enone moiety, which must therefore be responsible for the inhibitory reaction of VM. The most realistic scenario is as follows. In the final step of enolate quenching, the conjugated dienolate **v** may abstract a proton, from the enzyme or from the environment, and be protonated at the terminal methylene position. The amino acid residue, probably activated by the proton abstraction, may subsequently attack **vi** to form a transient covalent bond. Attempted MALDI-TOF analysis to detect an expected enzyme-inhibitor complex has so far been unsuccessful.

With regard to the other possible inhibition mechanism of VM, either the enolate **v** or the enzyme reaction product **vi** might inhibit the normal enzyme reaction without covalent bond formation. If the inhibition was due to the enolate **v** residing in the active site, it would imply that **v** is as chemically stable as the deprotonated forms of the oxalylhydroxamates, which is unlikely. The intact or free **vi** is similarly unlikely for the inhibition, since it would be expelled out to the outside of the enzyme as a regular reaction product. To figure out this possibility, the supplemented 2-oxo-3-pentenoate was incubated with IPMDH, but no inhibition was observed. Accordingly, the transient bond formation is the most likely mechanism for the inhibition by VM, although the anticipated complex **ix** has not yet been directly detected.

The most recent X-ray analysis of the *Thiobacillus* IPMDH−IPM complex suggested by analogy that C_β and C_γ of Glu87 in *Thermus* IPMDH may play an important role in the recognition of the isopropyl group of IPM.[7] Hence, the carboxylate of Glu87 may be a candidate to form the transient bond as **ix**, although there are some other possible Glu and Asp residues in the active site.

In conclusion, VM is the first successful example of a mechanism-based inhibitor that shows a very potent inhibition toward IPMDH. The involvement of ethylidenoxaloacetate seemed less likely as the results of incubation of the synthesized (2*R*)-3-ethylidenemalic acid. The enzyme reaction with VM was followed by GC−MS and NMR spectroscopic studies. These studies showed that VM was recognized by the enzyme and oxidized normally as a substrate to yield 2-oxo-3-pentenoate (**vi**). Though the enzyme−inhibitor complex **ix** was not directly detected by MALDI-TOF mass spectrometry of the enzyme reaction, transient bond formation between IPMDH and a product of VM was strongly suggested by the time-dependent and kinetic analyses. The development of a highly potent inhibitor as VM may allow us to obtain crystals of the IPMDH−inhibitor−NAD^+ complex appropriate for crystallographic analysis, and efforts are being directed toward this goal.

Experimental Section

Melting points are uncorrected. ^{1}H and ^{13}C NMR chemical shifts are reported in δ value based on internal TMS ($\delta_H = 0$) or dioxane ($\delta_C = 66.5$), or solvent signal ($CDCl_3$, $\delta_C = 77.0$; HOD, $\delta_H = 4.8$) 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. Toluene was distilled from calcium hydride. HMPA was distilled from calcium hydride under reduced pressure and stored over molecular sieves 4A under Ar.

Diethyl (2*R*,3*S*)-3-(2-Benzyloxyethyl)malate (3). A solution of BuLi (2.0 mL, 3.2 mmol, 1.60 M) was added dropwise to a stirred solution of diisopropylamine (0.54 mL, 3.8 mmol) and 2,2′-dipyridyl (5 mg) in THF (6.3 mL) at 0 °C. After 30 min, the mixture was cooled to −78 °C. A solution of diethyl (*R*)-malate **2** (301 mg, 1.58 mmol) in THF (0.2 mL) was added dropwise at −78 °C. After completion of addition, the mixture was warmed to room temperature and stirred for 1 h. HMPA (1.3 mL) was added to the solution. Then the solution was recooled to −20 °C, and a solution of benzyl 2-iodoethyl ether (500 mg, 1.90 mmol) in THF (0.2 mL) was added. After 15 min, the reaction was quenched by addition of water, and the mixture was extracted with ethyl acetate (50 mL). The organic phase was subsequently washed with saturated aqueous NH_4-Cl solution and brine, dried over Na_2SO_4, and evaporated. The resulting residue was chromatographed over silica gel with hexanes−ether (5:1−1:1) to give **3** (280 mg, 55%) as an oil: IR (film) 1740, 1640 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.25 (t, J = 7 Hz, 3H), 1.34 (t, J = 7 Hz, 3H), 2.00 (m, 1H), 2.23 (m, 1H), 3.19 (dt, J = 3 and 8 Hz, 1H), 3.33 (d, J = 8 Hz, 1H), 3.63 (m, 2H), 4.15 (m, 2H), 4.28 (m, 2H), 4.33 (dd, J = 3 and 8 Hz, 1H), 4.51 (s, 2H), 7.35 (m, 5H); ^{13}C NMR (125.8 MHz, $CDCl_3$) δ 14.0, 14.1, 28.1, 45.4, 60.8, 61.8, 67.5, 70.9, 72.9, 127.6, 128.3, 138.2, 172.3, 173.3. Anal. Calcd for $C_{17}H_{24}O_6$: C, 62.95; H, 7.46. Found: C, 62.86; H, 7.65.

Diethyl (2*R*,3*S*)-2-*O*-Acetyl-3-(2-benzyloxyethyl)malate (4). A mixture of **3** (2.69 g, 8.29 mmol), DMAP (5 mg), and acetic anhydride (5.4 mL) in pyridine (27 mL) was stirred for 30 min. The reaction was quenched by addition of water at 0 °C. The reaction mixture was extracted with ethyl acetate (150 mL × 2). The combined organic phase was subsequently washed with 6 M HCl, saturated aqueous $NaHCO_3$ solution, and brine, dried with Na_2SO_4, and evaporated. The resulting residue was chromatographed over silica gel with hexanes−ether (5:1−1:1) to give **4** (2.16 g, 71%) as an oil: IR (film) 1740

cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 1.24 (t, J = 7 Hz, 3H), 1.29 (t, J = 7 Hz, 3H), 1.82 (dq, J = 14 and 6 Hz, 1H), 2.03 (s, 3H), 2.05 (m, 1H), 3.27 (ddd, J = 5, 6, and 8 Hz, 1H), 3.55 (t, J = 6 Hz, 2H), 4.15 (m, 2H), 4.23 (m, 2H), 4.49 (s, 2H), 5.29 (d, J = 5 Hz, 1H), 7.32 (m, 5H);^{13}C NMR (50.3 MHz, $CDCl_3$) δ 14.1, 20.6, 27.6, 43.4, 61.0, 61.4, 67.3, 72.2, 72.9, 127.5, 128.3, 168.2, 170.0, 170.3. Anal. Calcd for $C_{19}H_{26}O_7$: C, 62.28; H, 7.15. Found: C, 62.46; H, 6.85.

Diethyl (2*R*,3*S*)-2-*O*-Acetyl-3-(2-hydroxyethyl)malate (5). A mixture of **4** (811 mg, 2.21 mmol) and 10% Pd/C (80 mg) in ethanol (10 mL) was vigorously stirred for 6 h under 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 **5** (631 mg, 98%) as an oil: IR (film) 3550, 1740 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.27 (t, J = 7 Hz, 3H), 1.29 (t, J = 7 Hz, 3H), 1.81 (m, 1H), 2.03 (m, 1H), 2.15 (s, 3H), 3.12 (dt, J = 9 and 5 Hz, 1H), 3.74 (t, J = 6 Hz, 2H), 4.20 (m, 2H), 4.22 (m, 2H), 5.33 (d, J = 5 Hz, 1H); ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 14.1, 20.6, 30.3, 43.6, 60.3, 61.2, 61.7, 72.4, 168.2, 170.0, 171.2. Anal. Calcd for $C_{12}H_{20}O_7$: C, 52.17; H, 7.30. Found: C, 52.21; H, 7.25.

Diethyl (2*R*,3*S*)-2-*O*-Acetyl-3-(2-phenylselenenylethyl)-malate (6). *N*-(Phenylseleno)phthalimide (1.0 g, 3.35 mmol) was added to a stirred solution of **5** (615 mg, 2.23 mmol) and Bu_3P (0.98 mL, 4.8 mmol) in THF (12 mL), and the mixture was stirred for 1.5 h. The reaction was quenched by addition of saturated aqueous NH_4Cl solution. The mixture was extracted with ethyl acetate (100 mL). The organic phase was subsequently washed with brine, dried with Na_2SO_4, and evaporated. The resulting residue was chromatographed over silica gel with hexanes−ethyl acetate (5:1) to give **6** (628 mg, 68%) as an oil: IR (film) 1740 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 1.23 (t, J = 7 Hz, 3H), 1.24 (t, J = 7 Hz, 3H), 1.84 (m, 1H), 2.09 (s, 3H), 2.19 (m, 1H), 2.88 (dt, J = 13 and 8 Hz, 1H), 3.01 (ddd, J = 6, 9, and 13 Hz, 1H), 3.22 (dt, J = 9 and 4 Hz, 1H), 4.15 (m, 4H), 5.25 (d, J = 5 Hz, 1H), 7.25 (m, 3H), 7.49 (m, 2H); ^{13}C NMR (67.9 MHz, $CDCl_3$) δ 14.00,14.05, 20.5, 25.0, 27.9, 46.1, 61.1, 61.6, 72.0, 127.1, 129.1, 132.9, 168.1, 170.0, 170.5. Anal. Calcd for $C_{18}H_{24}O_6Se$: C, 52.05; H, 5.82. Found: C, 52.14; H, 5.77.

Diethyl (2*R*,3*S*)-2-*O*-Acetyl-3-vinylmalate (7). Aqueous 30% H_2O_2 (1.62 mL) was added to a stirred solution of **6** (599 mg, 1.44 mmol) in THF (6 mL) at 0 °C. The mixture was stirred for 1.5 h at 0 °C, and then at room temperature for 5 h. The mixture was extracted with ethyl acetate (50 mL × 2). The combined organic phase was subsequently washed with saturated aqueous Na_2SO_3 solution and brine, dried with Na_2SO_4, and evaporated. The resulting residue was chromatographed over silica gel with benzene−ethyl acetate (100:1) to give **7** (492 mg, 82%) as an oil: IR (film) 1740, 1640 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 1.26 (t, J = 7 Hz, 3H), 1.27 (t, J = 7 Hz, 3H), 2.12 (s, 3H), 3.60 (dd, J = 7 and 9 Hz, 1H), 4.20 (m, 2H), 4.21 (m, 2H), 5.28 (dt, J = 17 and 1 Hz, 1H), 5.28 (d, J = 10 Hz, 1H), 5.28 (d, J = 7 Hz, 1H), 5.85 (ddd, J = 9, 10, and 17 Hz, 1H); ^{13}C NMR (67.9 MHz, $CDCl_3$) δ 14.0, 20.4, 51.5, 61.3, 61.5, 72.7, 120.6, 130.2, 168.1, 169.7, 169.9. Anal. Calcd for $C_{12}H_{18}O_6$: C, 55.80; H, 7.03. Found: C, 55.66; H, 6.80.

(2*R*,3*S*)-3-Vinylmalic Acid (1). To a stirred solution of **7** (2.50 g, 9.67 mmol) in THF (75 mL) was added 6 M HCl (35 mL). The mixture was stirred for 26 h at 60 °C and concentrated to dryness. The residue was chromatographed over LiChroprep RP-18 with water. Crystallization from CH_3CN−$CHCl_3$ gave **1** (496 mg, 32%) as a colorless powder: mp 115−118 °C; IR (KBr) 3000, 1710, 1650 cm^{-1}; ^1H NMR (300 MHz, D_2O) δ 3.65 (dd, J = 5 and 9 Hz, 1H), 4.56 (d, J = 5 Hz, 1H), 5.35 (d, J = 16 Hz, 1H), 5.36 (d, J = 11 Hz, 1H), 5.93 (dt, J = 16 and 9 Hz, 1H); ^{13}C NMR (67.9 MHz, D_2O) δ 53.4, 71.3, 120.7, 130.8, 174.8, 175.4; $[a]^{27}_D$ −43.7° (c = 1.04, H_2O). Anal. Calcd for $C_6H_8O_5$: C, 45.01; H, 5.04. Found: C, 44.91; H, 5.06.

Diethyl (2*R*)-3-Ethylmalate (9). A solution of BuLi (27.6 mL, 44.1 mmol, 1.60 M) was added dropwise to a stirred solution of diisopropylamine (6.5 mL, 46.4 mmol) and 2,2′-dipyridyl (5 mg) in THF (80 mL) at 0 °C. After 30 min, the mixture was cooled to −78 °C, and a solution of diethyl (*R*)-malate **2** (4.01 g, 21.1 mmol) in THF (4 mL) was added dropwise at −78 °C. After completion of addition, the mixture was warmed to −20 °C, and HMPA (80 mL) was added. After 1 h, a solution of ethyl iodide (3.4 mL, 42.5 mmol) in THF (3.4 mL) was added dropwise to the mixture at −20 °C. After 10 min, the reaction was quenched by addition of saturated aqueous NH_4Cl solution. The solution was diluted with ethyl acetate and 2 M 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 hexanes−ethyl acetate (5:1) to give **9** (2.90 g, 63%) in a 9:1 ratio: IR (neat) 3500, 2980, 1740 cm^{-1}; ^1H NMR of the major product (300 MHz, $CDCl_3$) δ 1.02 (t, J = 7 Hz, 3H), 1.25 (t, J = 7 Hz, 3H), 1.31 (t, J = 7 Hz, 3H), 1.70 (m, 1H), 1.89 (m, 1H), 2.78 (dt, J = 3 and 7 Hz, 1H), 3.22 (d, J = 7 Hz, 1H), 4.15 (q, J = 7 Hz, 2H), 4.27 (m, 3H); ^{13}C NMR of the major product (75.4 MHz, $CDCl_3$) δ 11.9, 14.1, 21.4, 50.2, 60.7, 61.8, 70.8, 172.7, 173.5. Anal. Calcd for $C_{10}H_{18}O_5$: C, 55.03; H, 8.31. Found: C, 55.29; H, 8.49.

Diethyl (2*R*)-3-Ethylidenemalate (10). A solution of BuLi (50.5 mL, 80.8 mmol, 1.60 M) was added dropwise to a stirred solution of diisopropylamine (11.9 mL, 84.9 mmol) and 2,2′-dipyridyl (5 mg) in THF (180 mL) at 0 °C. After 30 min, the mixture was cooled to −78 °C, and a solution of **9** (8.39 g, 38.4 mmol) in THF (20 mL) was added dropwise at −78 °C. After completion of addition, the mixture was warmed to 0 °C and stirred for 2 h. The mixture was recooled to −78 °C, and a solution of phenylselenenyl bromide (13.6 g, 57.6 mmol) in THF (25 mL) was added dropwise at −78 °C. After 20 min, the reaction was quenched by addition of saturated aqueous NH_4Cl solution. The solution was diluted with ethyl acetate and 2 M 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 hexanes−ethyl acetate (10:1−3:1) to give the phenylselenenyl compound (2.81 g).

To a solution of the phenylselenenyl compound (2.81 g) in CH_2Cl_2 (30 mL) was added 30% aqueous H_2O_2 solution (1.50 mL, 15.2 mmol) at 0 °C. After 5 h, the mixture was diluted with ethyl acetate and water. The organic phase was separated. The aqueous layer was extracted twice with ethyl acetate. The combined organic phase was subsequently washed with saturated aqueous Na_2SO_3 solution and brine, dried over Na_2SO_4, and evaporated. The resulting residue was chromatographed over silica gel with hexanes−ethyl acetate (10:1−3:1) to give **10** (1.06 g, 13%) as the E−Z mixture (E:Z = 10:1): IR (neat) 1740, 1650 cm^{-1}; ^1H NMR of (*E*)-isomer (300 MHz, $CDCl_3$) δ 1.26 (t, J = 7 Hz, 3H), 1.28 (t, J = 7 Hz, 3H), 1.98 (d, J = 7 Hz, 3H), 3.62 (d, J = 7 Hz, 1H), 4.17 (qd, J = 11 and 7 Hz, 1H), 4.18 (qd, J = 11 and 7 Hz, 1H), 4.26 (qd, J = 11 and 7 Hz, 1H), 4.27 (qd, J = 11 and 7 Hz, 1H), 5.03 (d, J = 7 Hz, 1H), 7.12 (q, J = 7 Hz, 1H);^{13}C NMR of (*E*)-isomer (75.4 MHz, $CDCl_3$) δ 14.06, 14.07, 14.3, 60.9, 61.9, 66.2, 130.8, 142.5, 165.8, 172.9. Anal. Calcd for $C_{10}H_{16}O_5$: C, 55.55; H, 7.46. Found: C, 55.74; H, 7.76.

(2*R*)-3-Ethylidenemalic Acid (8). To a stirred solution of **10** (991 mg, 4.58 mmol) in THF (25 mL) and water (25 mL) was added $LiOH·H_2O$ (968 mg, 23.1 mmol). After 3 h, the mixture was acidified by addition of 6 M 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 **8** (442 mg, 60%) as the E−Z mixture (E:Z = 25:1): mp 131−134 °C; IR (KBr) 1720, 1680, 1640 cm^{-1}; ^1H NMR of the major product (300 MHz, D_2O) δ 1.94 (d, J = 7 Hz, 3H), 5.22 (s, 1H), 7.22 (q, J = 7 Hz, 1H); ^{13}C NMR of the major product (75.4 MHz, D_2O) δ 13.9, 65.0, 130.0, 146.7, 168.9, 176.3. Anal. Calcd for $C_6H_8O_5$: C, 45.01; H, 5.04. Found: C, 45.11; H, 5.05.

Methyl (*E*)-2-Hydroxy-3-pentenoate (11). A solution of diisobutylaluminum hydride in toluene (4.0 mL, 1 M) was added dropwise to a solution of methyl (*E*)-2-oxo-3-pentenoate (**12**; 337 mg, 2.63 mmol), prepared from 1-propenylmagnesium

bromide and dimethyl oxalate according to the method of Rambaud et al,[18] in diethyl ether (20 mL) at −78 °C, and the mixture was stirred for 1.5 h at the same temperature. The reaction was quenched by addition of water. The mixture was diluted with diethyl ether and acidified with 2 M HCl. The organic phase was separated, and the aqueous layer was extracted with diethyl ether. The combined organic phase was washed successively with 2 M HCl and brine, dried over Na_2SO_4, and evaporated. The residue was chromatographed over silica gel with hexane−diethyl ether (2:1) to give **11** (34 mg, 10%): IR ($CHCl_3$) 3500, 3000, 1720 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.73 (ddd, J = 7, 2 and 2 Hz, 3H), 2.96 (d, J = 6 Hz, 1H), 3.81 (s, 3H), 4.61 (dddd, J = 6, 6, 2 and 2 Hz, 1H), 5.54 (ddq, J = 15, 6 and 2 Hz, 1H), 5.90 (dqd, J = 15, 7 and 2 Hz, 1H); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 17.6, 52.6, 71.3, 127.1, 129.7, 174.1. Anal. Calcd for $C_6H_{10}O_3$: C, 55.37; H, 7.74. Found: C, 55.29; H, 7.51.

Enzyme and Substrate Assay. The thermophilic IPMDH derived from *T. thermophiles* HB8 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−vis recording spectrophotometer or a Gilford Responce spectrometer. 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. In inhibition assay, the reaction was started by addition of the enzyme (0.68 μg) to the reaction mixture with all required components including IPM (5−100 mM) and the inhibitor (0.01−500 μM). The formation of NADH was measured for 1 min as described above. Data were graphically analyzed by Lineweaver−Burk double reciprocal plots. In substrate assay, the reaction was started by addition of the enzyme (6.8−97 μg) to the reaction mixture with all required components including VM (1−10 μM) and **8** (100−250 μM), and the enzyme reaction was monitored by the above method.

For the inactivation assay, a preincubated solution (7 μL) of IPMDH (0.7 μg) containing an inhibitor (0−20 mM) was diluted 100-fold into an assay mixture (total volume 700 μL) containing 50 mM HEPES buffer (pH 7.8), 5 mM NAD^+, 5 mM $MgCl_2$, 100 mM KCl, and 100 mM IPM at 60 °C, and the formation of NADH was measured for 1 min as described above. The half-time ($t_{1/2}$) for inactivation at each inhibitor concentration was plotted against 1/[VM], referred to as a Kitz and Wilson plot, and K_I and k_{inact} values were obtained.[20b] All standard errors are less than 20% of the estimates.

GC−MS Analysis of the Enzyme Reaction Product of VM. 3-Vinylmalic acid (128 μL, 1.25 mmol, 10 mM) was added to an assay mixture (total volume 800 μL) in 50 mM HEPES buffer (pH 7.8) containing 5 mM NAD^+, 5 mM $MgCl_2$, and 100 mM KCl. The enzyme reaction was started by addition of the enzyme (30 μL, 0.31 mmol, 10.3 mM). At 1 h after the incubation started at 25 °C, $NaBH_4$ (6.7 mg, 177 mmol) or $NaBD_4$ (7.6 mg, 182 mmol) was added, and the mixture was stirred for 4 h at room temperature. The mixture was lyophilized. After acetic acid (0.2 mL) and methanol (1 mL) were added to the residue, the mixture was treated with an excess of ethereal diazomethane and evaporated. The oily residue was dissolved in diethyl ether and filtered. The filtrate was evaporated to give a sample for GC−MS analysis. Gas chromatography−mass spectra were taken on a JEOL JMS-AX 505HA mass spectrometer using a column of HP-5. The column temperature was increased at the rate of 10 °C/min from 70 °C, and the flow rate of He gas was 50 mL/min.

A Pursuit of the Enzyme Reaction of VM by ^1H NMR Spectroscopy. VM **1** (79.6 μL, 0.796 mmol, 10.0 mM) was added to an assay mixture (total volume 200 μL) in 50 mM HEPES buffer (pH 7.8) containing 5 mM NAD^+, 5 mM $MgCl_2$, and 100 mM KCl. The assay solution was lyophilized. The residue was dissolved in D_2O (20 μL)−H_2O (150 μL). The enzyme reaction was started by addition of the enzyme (30 μL, 0.08 mmol, 2.7 mM) to the solution at 25 °C in an NMR tube. The enzyme reaction was monitored by accumulation of ^1H NMR spectral data for 3 h.

Acknowledgment. This work was supported in part by a Grant-in-Aid for Scientific Research in Priority Area from the Ministry of Education, Science, Sports and Culture, Japan.

Supporting Information Available: ^1H and ^{13}C NMR spectra of the synthesized inhibitors **1** and **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO982206C