O-Alkyl Hydroxamates as Metaphors of Enzyme-Bound Enolate Intermediates in Hydroxy Acid Dehydrogenases. Inhibitors of Isopropylmalate Dehydrogenase, Isocitrate Dehydrogenase, and Tartrate Dehydrogenase[1]

Michael C. Pirrung,*[,2] Hyunsoo Han, and Jrlung Chen

Department of Chemistry, P. M. Gross Chemical Laboratory, Duke University, Durham, North Carolina 27708-0346

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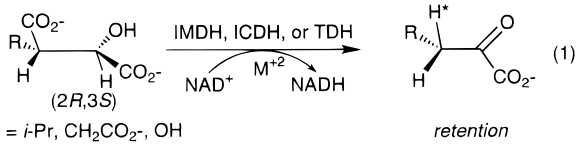
The inhibition of *Thermus thermophilus* isopropylmalate dehydrogenase by *O*-methyl oxalohydroxamate was studied for comparison to earlier results of Schloss with the *Salmonella* enzyme. It is a fairly potent (1.2 μM), slow-binding, uncompetitive inhibitor against isopropylmalate and is far superior to an oxamide (25 mM K_i competitive) that is isosteric with the ketoisocaproate product of the enzyme. This improvement in inhibition was attributed to its increased NH acidity, which presumably is due to the inductive effect of the hydroxylamine oxygen. This principle was extended to the structurally homologous enzyme isocitrate dehydrogenase from *E. coli*, for which the compound *O*-(carboxymethyl) oxalohydroxamate is a 30 nM inhibitor, uncompetitive against isocitrate. The pH dependence of its inhibition supports the idea that it is bound to the enzyme in the anionic form. Another recently discovered homologous enzyme, tartrate dehydrogenase from *Pseudomonas putida*, was studied with oxalylhydroxamate. It has a relatively low affinity for the enzyme, though it is superior to tartrate. On the basis of these leads, squaric hydroxamates with increased acidity compared to squaric amides directed toward two of these enzymes were prepared, and they also show increased inhibitory potency, though not approaching the nanomolar levels of the oxalylhydroxamates.

Introduction

Hydroxy acid dehydrogenases, a significant class of NAD-utilizing metalloenzymes, oxidatively decarboxylate substituted malic acids to produce α-keto acids (eq 1),



which can then be converted to α-amino acids. These enzymes come from a variety of bacterial,[3,4] yeast,[5,6] mammalian, and plant[7] sources, and study of their sequences has allowed their placement into homologous groups. One group includes the isopropylmalate dehydrogenase (IMDH) from *Thermus thermophilus*, whose structure has been solved by X-ray crystallography.[8] Oshima has continued studies of this enzyme and related

IMDH TGLLSLRKSQDLFANLRP
TDH GSLLKFRREFDQYVNIRP

Figure 1. Active-site sequence alignment of *T. thermophilus* isopropylmalate dehydrogenase (IMDH) and *P. putida* tartrate dehydrogenase (TDH).

mutants.[9] The isocitrate dehydrogenase (IDH) from *Escherichia coli* has been found to have a similar fold and active site structure, and sequence alignments make manifest its relation to IMDH.[10] The differences in amino acids in its active site compared to IMDH are easily rationalized on the basis of the change from a hydrophobic (isopropylmalate) to a charged (isocitrate) substrate. Recently, a novel hydroxy acid dehydrogenase, tartrate dehydrogenase (TDH), was isolated from *Pseudomonas putida*.[11] While structurally uncharacterized, its active site sequence is highly related to IMDH (Figure 1), and it is therefore expected to have a similar structure. These three enzymes are a testing ground on which we have evaluated some design concepts for inhibitors of enol-[12] and enolate-utilizing enzymes.

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(1) Dedicated to Clayton H. Heathcock on the occasion of his 60th birthday.

(2) Fellow of the John Simon Guggenheim Memorial Foundation, 1994−95.

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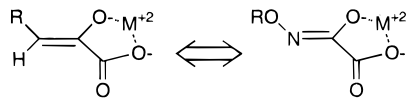
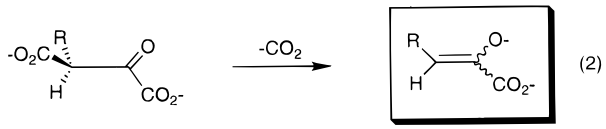


Figure 2. Suggested mode for inhibition of decarboxylating hydroxy acid dehydrogenases by *O*-alkyl hydroxamates by analogy to the mode of decarboxylation of substrate with enolate stabilization.

We recently described mechanistic, stereochemical, and kinetic investigations[13] of the first member of this group, isopropylmalate dehydrogenase, which uses a compulsory ordered kinetic mechanism. A key conclusion of our studies was that an enzyme-bound enolate (eq 2) is likely



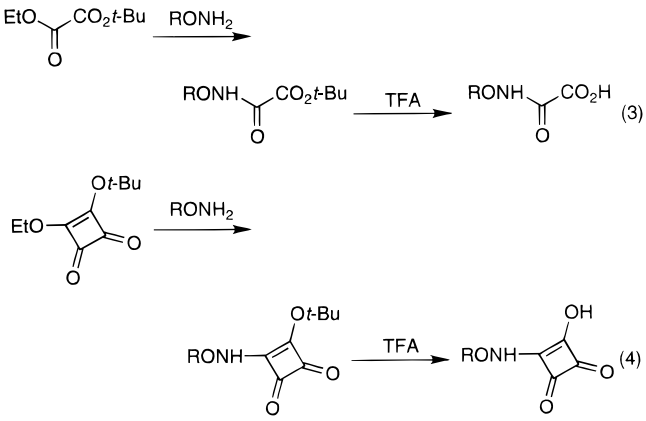
(2)

generated as an intermediate[14] and that compounds that can mimic this enolate are uncompetitive inhibitors against the substrate isopropylmalate. Functional group equivalents, usually based on an atom replacement, designed for incorporation into ligands for enzymes to produce inhibitors constitute *molecular metaphors* of the natural ligand.[12] Metaphors for enolates have been discovered by Holt and Metcalf[15] in the course of a search for inhibitors of steroid 5α-reductase. This enzyme likewise follows a compulsory ordered mechanism, with NADPH binding first and $NADP^+$ being released last. Steroid 5α-reductase conducts a conjugate reduction of testosterone, producing an intermediate enolate that is bound to the enzyme form bearing *oxidized* cofactor. If it could be studied, the enolate would be *uncompetitive* against substrate, which is bound to the form of the enzyme bearing *reduced* cofactor. Neutral inhibitors of steroid 5α-reductase, such as unsaturated nitro compounds and a lactam,[16] compete with substrate and are thought to mimic it. Negatively charged analogues, such as $\Delta^{3,4}$-steroid-3-carboxylates, phosphonates, phosphinates, and sulfonates, are *uncompetitive* against substrate.

In earlier work,[13] our data on inhibition of the *T. thermophilus* enzyme by alkyl squaric acid derivatives[17] were compared to Schloss's studies[18] of the inhibition of the *Salmonella* enzyme (which has subtle active site sequence differences and an unknown structure) by *O*-alkyl hydroxamates, and we suggested that the increased potency of the latter compared to simple amides was based on their lower NH pK_a. In this work, we have further explored these concepts with a designed inhibitor of *E. coli* isocitrate dehydrogenase and have reconciled the inhibition data with inhibitor NH acidity. A hydroxamate inhibitor of TDH has been prepared and evaluated, and the hydroxamate and squarate functionalities have been united to form novel inhibitors of these enzymes.

Results

The oxalylhydroxamates and squaric hydroxamates used in this study were prepared by a straightforward route using differentiated α-dicarbonyl synthons that permit the selective monosubstitution of an ethoxy group by hydroxylamine or a substituted hydroxylamine (eqs 3 and 4).



(3)

(4)

Inhibition of the *T. thermophilus* IMDH by *O*-methyl oxalohydroxamate (**1**) was studied for comparison with Schloss's results. It is a quite potent inhibitor with time-dependent action.[19] Initial rapid NAD^+ consumption in the presence of **1** is followed by a slower rate in the steady state. Data in the latter region were used for the K_i determination, which also showed, by the pattern of inhibition, that **1** is uncompetitive against isopropylmalate. The pK_a of **1** was determined by potentiometric titration. N-Isopropyloxamide, which is isosteric with the ketoisocaproate enolate, has a 25 mM K_i[17] and is sufficiently less acidic that its pK_a could not be determined in water.

The foregoing suggested that the corresponding *O*-(carboxymethyl)oxalohydroxamate (**2**) would have high affinity for isocitrate dehydrogenases (IDH). Some time ago, an amide inhibitor of pig heart IDH, oxalylglycine (**3**), was reported (K_i = 22 μM, competitive).[20] This porcine enzyme has little sequence similarity to the *Thermus* enzyme, so **2** and **3** were examined with the *E. coli* IDH.[21] The data show that insertion of the "hydroxylamine" oxygen results in over a 1000-fold increase

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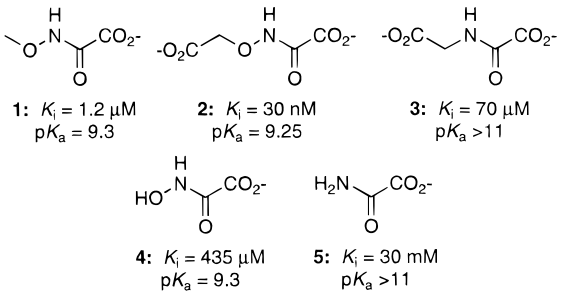
(20) Northrop, D. B.; Cleland, W. W. *J. Biol. Chem.* **1974**, *249*, 2928−2931. It is reasonable that **3** acts as a substrate analog. The K_i of **2** against the porcine enzyme is 1 μM.



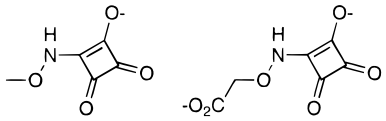
1: K_i = 1.2 μM, pK_a = 9.3 **2:** K_i = 30 nM, pK_a = 9.25 **3:** K_i = 70 μM, pK_a >11

4: K_i = 435 μM, pK_a = 9.3 **5:** K_i = 30 mM, pK_a >11

in affinity, with **2** showing nanomolar inhibitory potency. Furthermore, it is an uncompetitive inhibitor against isocitrate, in support of the idea that it mimics the enolate.[22] That compound **2** binds to the form of the enzyme bearing reduced cofactor (NADH) was further shown by studying its inhibition of the reverse reaction with ketoglutarate as substrate (competitive, K_i = 90 nM). The pK_a data suggest that the increased acidity of the NH in **2** as compared to **3** leads to better mimicking of the enolate. Further evidence on this point was obtained by a limited study of the pH dependence of the K_i for **2**.[23] At pH 8.0, it is essentially unchanged compared to pH 7.3, but at pH 6.8, where the k_{cat} is only 10-fold lower and the K_m is unchanged, the K_i for **2** is >40 μM, representing a loss in affinity of over 3 log units.

Tartrate dehydrogenase provides another test of the concept that more NH acidic hydroxamates can mimic enolates more effectively than amides. While oxalohydroxamate (**4**) is a relatively weak inhibitor, by comparison to the K_m for tartrate (1 mM), it has respectable affinity for the enzyme (K_m/K_i = 2.3). Again, it is nearly 2 log units more potent than the simple amide analogue **5** that has a much higher pK_a.

Influenced by our earlier studies of the weak, uncompetitive inhibition of IMDH by alkylsemisquaric acids, these results prompted our preparation (using conventional protocols[24]) of squaric hydroxamate inhibitors for IMDH and IDH. *O*-Alkyl squarohydroxamates **6** and **7** have NH pK_as comparable to those of the oxalylhydroxamates and significant acidity enhancements compared to squaric amides (data not shown). Compound **6** shows uncompetitive inhibition of IMDH with a 100-fold improvement in potency over the simple squarate. Compound **7** is also a respectable inhibitor of IDH.[25]



6: K_i = 47 μM, pK_a = 8.65 **7:** K_i = 15 μM

Discussion

Hydroxamates have earlier seen good use as enzyme inhibitors, and their properties have been studied extensively using both kinetic and structural methods. For example, phosphoglycohydroxamate is believed to mimic the enediol intermediate in triosephosphate isomerase.[26] The high acidity of hydroxamates may also permit them to mimic carboxylates. However, many properties of hydroxamate enzyme inhibitors can be ascribed to their high affinity for metal ions;[27] for example, as shown by crystallography, phosphonoacetohydroxamate binds to active site divalent metals in enolase.[28] This property is dependent on the *chelating* ability of the carbonyl and hydroxyl groups of the hydroxamate. *O*-Alkylhydroxamates, lacking one of the key chelating groups, are not expected to act in this manner.

The inhibition of IMDH by **1** as compared to *N*-isopropyloxamide strengthens the inferred relationship between potent binding and the formation of an *O*-alkylhydroxamate anion that can mimic an enolate. The pK_a of **1** supports the idea that its potent activity is related to the increased acidity of its NH. In the case of IDH, greater than a 1000-fold increase in affinity is gained by the addition of the oxygen despite the fact that **3** is isosteric with the ketoglutarate enolate and **2** is not.[29] The pK_a difference between them also supports the idea that binding of **2** as the anion is important. It is surprising that a compound as small as **2** has sufficient interactions with the enzyme to engender such a potent inhibition. While the break in activity of **2** does not occur at the titrimetrically-determined pK_a, it is reasonable that the pK_a of **2** in the active site is reduced by binding to the magnesium ion, as is postulated for the stabilization of the ketoglutarate enolate.[10] We suggest that the mode of binding of *O*-alkyl hydroxamates to these enzymes involves chelation between the carboxyl group and the hydroxamate carbonyl oxygen, analogous to the binding mode suggested by Hurley and Koshland for the product enolate in ICDH.

While such potent inhibition was not found in the case of TDH, the oxalohydroxamate has fair affinity compared to the substrate Michaelis constant. Tipton has suggested that this enzyme is a modified IMDH recruited to the job of metabolizing tartrate, which it in fact does less efficiently than it accepts isopropylmalate.

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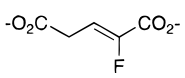
(22) A vinyl fluoride enol analogue **i** (Pirrung, M. C.; Rowley, E. G.; Holmes, C. P. *J. Org. Chem.* **1993**, *58*, 5683) has also been studied with the bacterial enzyme. It shows only moderate, competitive inhibition, further supporting the idea that an enolate, not the enol, is the intermediate in the IDH reaction.



i: K_i = 10 μM

(23) It did not prove possible to extend the range of this pH/K_i study because of the loss of divalent metal to insoluble complexes at higher pH.

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(25) Standard assays with this compound show a time-dependent increase of background absorbance that can be mostly eliminated by omission of DTT from the buffer. The K_i data were obtained from a Dixon plot.

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(29) Higher amides, including those isosteric with **2**, show no greater potency. Likewise, oxalic acid, a known metaphor for the pyruvate enolate, is a weak (K_i = 90 mM) inhibitor.

Conclusion

The principle demonstrated in this study concerning the acidification of NH groups may have use in the design of inhibitors for other enolate-utilizing enzymes. These data also provide evidence on the mode of action of enzyme inhibitors such as **1** that may have utility as herbicides.[30]

Experimental Section

***tert*-Butyl *O*-Methyl Oxalohydroxamate.** Triethylamine (0.605 g, 5.99 mmol) and methoxyamine hydrochloride (0.500 g, 5.99 mmol) were mixed in methylene chloride at 4 °C. Solutions of *tert*-butyl oxalylchloride (0.985 g, 5.99 mmol) and triethylamine (0.605 g, 5.99 mmol) were added. After being stirred for 2 h, the reaction mixture was allowed to warm to room temperature and washed three times with 5 mL of 1 N HCl. Methylene chloride extraction, drying with magnesium sulfate, evaporation of solvent, and column chromatography gave the desired product (0.954 g, 91.0%): mp 61−62 °C; IR 3226, 1749, 1696 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.54 (s, 9H), 3.82 (s, 3H), 9.55 (broad s, 1H); ^{13}C NMR ($CDCl_3$) δ 27.67, 64.45, 85.40, 154.62, 158.20. Anal. Calcd for $C_7H_{13}NO_4$: C, 47.99; H, 7.48; N, 8.00. Found: C, 48.11; H, 7.35; N, 8.08.

Di-*tert*-butyl 2-Oxo-3-azaglutarate. Following the same procedure as above with *tert*-butyl glycinate yielded the title compound (0.645 g, 88.9%): ^1H NMR ($CDCl_3$) δ 1.45 (s, 9H), 1.52 (s, 9H), 3.96 (d, J = 6 Hz, 2H), 7.49 (br t, J = 6 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 27.62, 27.92, 42.13, 82.75, 84.66, 157.47, 158.70, 167.84. Anal. Calcd for $C_{12}H_{21}NO_5$: C, 55.58; H, 8.16; N, 5.40. Found: C, 55.74; H, 8.14; N, 5.30.

***O*-Methyl Oxalohydroxamic Acid (1).** *tert*-Butyl *O*-methyl oxalhydroxamate (0.500 g, 2.86 mmol) was dissolved in 5 mL of 1:1 trifluoroacetic acid:methylene chloride at 4 °C. The reaction mixture was stirred for 15 min at room temperature. The solvent was removed *in vacuo* to give a white solid (0.313 g, 92.0%): mp 132−135 °C; IR (KBr) 2400−3000 (br), 1677 cm^{-1}; ^1H NMR (acetone-d_6) δ 3.75 (s, 3H), 7.0 (broad s, 1H), 11.3 (broad s, 1H); ^{13}C NMR ($CDCl_3$/DMSO-d_6) δ 63.61, 154.64, 160.41. Anal. Calcd for $C_3H_5NO_4$: C, 30.25; H, 4.20; N, 11.76. Found: C, 30.20; H, 4.15; N, 11.58.

2-Oxo-3-azaglutaric acid (3).[31] Following the same procedure as above with di-*tert*-butyl 2-oxo-3-azaglutarate yielded the desired product (0.212 g, 93.6%): ^1H NMR (acetone-d_6) δ 4.06 (d, J = 6 Hz, 2H), 8.5 (br, 3H); ^{13}C NMR (D_2O) δ 41.29, 160.13, 161.71, 172.57.

Ethyl *tert*-Butyl Oxalate.[32] 2-Methyl-2-propanol (0.54 g, 7.32 mmol) and pyridine (0.58 g, 7.32 mmol) in CH_2Cl_2 (5 mL) were added dropwise to a CH_2Cl_2 solution of ethyl oxalyl chloride (15 mL) at 4 °C. The resulting mixture was refluxed for 4 h, and after cooling, 1 N HCl solution was added. Methylene chloride extraction, drying over magnesium sulfate, and evaporation of the solvent gave the desired product (1.10 g, 86%): ^1H NMR ($CDCl_3$) δ 1.35 (t, J = 7.1 Hz, 3H), 1.54 (s, 9H), 4.30 (q, J = 7.1 Hz, 2H); IR (neat) 2987, 1726, 1710, 1372 cm^{-1}; HRMS calcd for ($C_8H_{14}O_4$ + H) 175.0970, found 175.0967.

***tert*-Butyl *N*-Hydroxyoxamate.** A few drops of phenolphthalein solution (0.05 g in 100 mL 1:1 EtOH:water) were added to NaOH solution (0.237 g, 5.90 mmol) in 40 mL of EtOH. To this solution was added hydroxylamine hydrochloride (0.412 g, 5.90 mmol) in 40 mL of EtOH until the pink color disappeared, and ethyl *tert*-butyl oxalate (1.03 g, 5.90 mmol) was added. The reaction mixture was stirred for 15 h at room temperature. The EtOH was evaporated, and the residue was recrystallized from acetone (0.380 g, 40%): mp 144−147 °C; ^1H NMR (acetone-d_6) δ 1.51 (s, 9H), 2.90 (br s, 1H), 9.5 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 27.27, 83.84, 154.42, 158.08; IR (KBr) 3191 (br), 1678, 1646, 1233 cm^{-1}. Anal. Calcd for $C_6H_{11}NO_4$: C, 44.72; H, 6.88; N, 8.69. Found: C, 44.73; H, 6.80; N, 8.73.

***N*-Hydroxyoxamate (4).**[33] *tert*-Butyl hydroxyoxamate (0.1544 g, 0.96 mmol) was dissolved in 20 mL of 1:1 trifluoroacetic acid:methylene chloride at 4 °C. The reaction mixture was stirred for 15 min at room temperature. The solvent was removed *in vacuo* to give a white solid (0.096 g, 95.0%). The hydroxamic group was confirmed on the basis of the observation that it gave distinct reddish purple color when stained with $FeCl_3$ solution: ^{13}C NMR (D_2O) δ 157.06, 161.47.

Di-*tert*-butyl 2-Oxo-3-aza-4-oxaadipate. Triethylamine (0.327 g, 3.23 mmol) and *tert*-butyl *N*-hydroxyoxamate (0.52 g, 3.23 mmol) were mixed in acetone (20 mL), and *tert*-butyl bromoacetate (0.63 g, 3.23 mmol) was added. The resulting mixture was stirred for 10 h at room temperature. The precipitate was removed by filtration, and the solvent was evaporated. Column chromatography of the residue (90:10 chloroform:ethyl acetate) gave the desired product (0.453 g, 51%): ^1H NMR ($CDCl_3$) δ 1.46 (s, 9H), 1.51 (s, 9H), 4.37 (s, 2H), 10.15 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 27.62, 27.98, 72.52, 83.22, 85.47, 154.30, 157.73, 168.27; IR (neat) 2966, 1714, 1467, 1371, 1243 cm^{-1}; HRMS calcd for ($C_{12}H_{21}NO_6$ + H) 276.1447, found 276.1450. Anal. Calcd for $C_{12}H_{21}NO_6$: C, 52.35; H, 7.69; N, 5.09. Found: C, 52.37; H, 7.68; N, 5.14.

2-Oxo-3-aza-4-oxaadipic Acid (2). Di-*tert*-butyl 2-oxo-3-aza-4-oxaadipate (110 mg, 0.4 mmol) was dissolved in 5 mL of 1:1 trifluoroacetic acid:methylene chloride at room temperature. The reaction mixture was stirred for 30 min. The solvent was removed *in vacuo* to give a white solid (63 mg, 97%): mp 145−148 °C; ^1H NMR (acetone-d_6) δ 4.79 (s, 2H), 10.10 (br s, 2H), 11.25 (broad s, 1H); ^{13}C NMR (D_2O) δ 72.20, 158.41, 164.95, 172.18; IR (KBr) 3173 (broad), 1756, 1666, 1500, 1412 cm^{-1}; HRMS calcd for ($C_4H_5NO_6$ + H) 164.0194, found 164.0186.

3-*tert*-Butoxy-4-ethoxy-3-cyclobutene-1,2-dione.[34] Potassium *tert*-butoxide solution (1 N, 17.6 mL, 17.6 mmol) was added at once to diethyl squarate (3.00 g, 17.6 mmol) in 50 mL of THF at 4 °C. After 5 min of stirring, the reaction mixture was quenched and acidified with 1 N HCl. Ether extraction, drying over magnesium sulfate, evaporation of solvent, and column chromatography (C_6H_{14}/EtOAc, v/v 9:1) gave the desired product (1.92 g, 55.1%): ^1H NMR ($CDCl_3$) δ 1.47 (t, J = 7 Hz, 3H), 1.60 (s, 9H), 4.75 (q, J = 7 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 15, 28, 70, 87, 184, 185, 188, 189.

3-*tert*-Butoxy-4-(*N*-methoxyamino)-3-cyclobutene-1,2-dione. To a solution of methoxyamine hydrochloride (0.127 g, 1.52 mmol) and 1 equiv of sodium methoxide (as sodium, 0.0350 g, 1.52 mmol) in dry methanol (10 mL) was added 3-*tert*-butoxy-4-ethoxy-3-cyclobutene-1,2-dione (0.300, 1.52 mmol) at room temperature with stirring. Evaporation of solvent and column chromatography (CH_2Cl_2/Et_2O, v/v 85:15) gave the desired product (0.148 g, 48.9%): IR 2700−3600 (br), 1795, 1706, 1591 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.62 (s, 9H), 3.83 (s, 3H), 9.70 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 28.75, 65.67, 86.48, 171.05, 177.51, 179.96, 186.26. Anal. Calcd for $C_9H_{13}NO_4$: C, 54.27; H, 6.53; N, 7.04. Found: C, 54.48; H, 6.62; N, 6.88.

***tert*-Butyl *O*-[(2-*tert*-Butoxy-3,4-dioxocyclobut-1-enyl)-amino]-2-hydroxyacetate.** Following the same procedure as above with aminooxyacetate hydrochloride gave the desired product (0.259 g, 64.9%): IR 2900−3600 (br), 1798, 1721, 1592 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.45 (s, 9H), 1.56 (s, 9H), 4.37 (s, 2H), 9.0 (broad s, 1H); ^{13}C NMR ($CDCl_3$) δ 28.02, 28.69, 73.34, 83.33, 86.42, 168.17, 172.08, 177.56, 181.47, 185.12; HRMS calcd for ($C_{14}H_{21}NO_6$ + H) 300.1447, found 300.1447.

3-Hydroxy-4-(*N*-methoxyamino)-3-cyclobutene-1,2-dione (6). 3-*tert*-Butoxy-4-(*N*-methoxyamino)-3-cyclobutene-1,2-dione (0.100 g, 0.503 mmol) was dissolved in 5 mL of 1:1 trifluoroacetic acid:methylene chloride at 4 °C, and the reaction mixture was stirred for 15 min. The solvent was removed *in*

(30) The biological activities of **1** and **6** have been compared in plant cell culture (Singh, B. American Cyanamid, unpublished results). Reversal of the inhibition of growth by **1** requires all three branched-chain amino acids, suggesting that it not only inhibits IMDH *in vivo* but also the keto acid reductioisomerase (KARI), as was earlier shown.[18] Likewise, compound **6** is a growth inhibitor whose activity is reversed only in the presence of ile/val/leu.

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vacuo to give a yellowish solid (0.0683 g, 95.0%): ^{1}H NMR (D_2O) δ 3.77 (s, 3H); ^{13}C NMR (CD_3OD) δ 91.08, 177.50, 185.92, 187.43; HRMS calcd for ($C_5H_5NO_4$ + H) 144.0168, found 144.0297.

***O*-[(2-Hydroxy-3,4-dioxocyclobut-1-enyl)amino]-2-hydroxyacetic Acid (7).** Following the same procedure as above with *tert*-butyl *O*-[(2-*tert*-butoxy-3,4-dioxocyclobut-1-enyl)amino]-2-hydroxyacetic acid yielded the desired product (0.0847 g, 90.0%): ^{1}H NMR (D_2O) δ 4.54 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 86.81, 172.13, 174.16, 175.53, 185.32; HRMS calcd for ($C_6H_5NO_6$ + H) 188.0198, found 188.0222.

Enzyme Assays. Isopropylmalate Dehydrogenase. All kinetic measurements were, unless mentioned, performed at 25 °C in a buffer solution containing 0.5 mM $MgCl_2$, 0.05 M KCl, 200−670 μM NAD^+, and 0.1 M KP_i, pH 7.60. The reaction was followed by measuring the appearance or disappearance of NADH at 340 nm. The reaction was initiated by adding enzyme (0.75 μg) to the reaction mixture with all required components including the substrate (and the inhibitor) present. The initial linear portion of the reaction-progress curve (less than 5% substrate conversion) was used for data analysis, with the exception of time-dependent inhibitors. Data were graphically analyzed by Eadie−Hofstee plots with at least five points to determine the character of inhibition, and direct fitting of the data using the Enzyme Kinetics program were used to determine kinetic constants. These values were compared to those derived from secondary plots of slopes and intercepts vs inhibitor concentration. **Isocitrate Dehydrogenase**. Appropriately-diluted enzyme solution was stabilized by the addition of 1 mg/mL of bovine serum albumin at 4 °C, and an aliquot of 20 μL (69 ng) was added to assay mixtures at 21 °C to initiate the enzymatic reaction. For the forward reaction, procedures similar to isopropylmalate dehydrogenase were followed at 21 °C. The assay mixtures were 2 mM $MgCl_2$, 10 μM EDTA, 300 μM dithiothreitol (DTT), 5 μM NADP, and 33 mM imidazole, pH 6.80, or 5 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 250 μM NADP, and 30 mM MOPS, pH 7.30, with appropriate amounts of isocitrate and inhibitor. For studies of the pH dependence of the kinetic constants, the assay mixture was 5 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 250 μM NADPH, 30 mM $NaHCO_3$, 30 mM Tris, and 30 mM ethanolamine. For the reverse reaction, the assay mixture was 5 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 250 μM NADPH, 30 mM $NaHCO_3$, and 30 mM MOPS, pH 7.30, with appropriate amounts of α-ketoglutarate and inhibitor. **Tartrate Dehydrogenase**. The kinetic assays were conducted in the 100 mM HEPES, pH 8.0 containing varying concentrations of dipotassium (+)-tartrate, 1.5 mM NAD^+, 0.4 mM $Mn(OAc)_2$, and 1 mM DTT at room temperature without or with appropriate amount of inhibitor. Reactions were initiated by addition of tartrate dehydrogenase and followed by monitoring the formation of NADH at 340 nm.

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