Inhibitors of *Thermus thermophilus* Isopropylmalate Dehydrogenase

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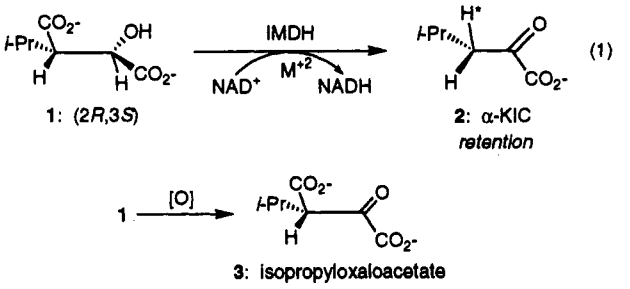
In an attempt to use mechanism-based design for the discovery of inhibitors of the isopropylmalate dehydrogenase from *T. thermophilus*, we have prepared and studied a number of potential mimics for an intermediate in the oxidative decarboxylation of isopropyl malate, the enol or enolate of α-ketoisocaproate. Because hydroxamate and dicarboxylate enolate mimics are strong, uncompetitive inhibitors of the enzyme and vinyl fluoride enol mimics are weak, competitive inhibitors, it is suggested that the reaction involves the enolate. The uncompetitive inhibition by a number of anionic compounds suggests, in combination with previous studies in other laboratories, that they mimic the enolate product of the decarboxylation. An explanation for the potency of the inhibition of IMDH by these compounds is proposed based on the electrostatic interaction of product and cofactor.

Introduction[1]

We have recently reported on the kinetic mechanism and reaction pathway of *Thermus thermophilus* β-isopropylmalate dehydrogenase (IMDH, E C 1.1.1.85),[2] the penultimate enzyme of the leucine biosynthetic pathway in bacteria, yeast, and higher plants. It conducts a simple transformation in which (2*R*,3*S*)-isopropylmalate (**1**)[3] is oxidatively decarboxylated (eq 1) to produce α-ketoisocaproate (**2**), the leucine keto acid, which is subsequently transaminated. The enzyme follows an ordered sequential bi–tri mechanism, with cofactor NAD binding before substrate β-isopropylmalate. The release of products occurs in the order CO_2, α-ketoisocaproate, and NADH. The chemistry of IMDH occurs in two discrete steps: dehydrogenation to isopropyloxaloacetate (**3**) followed by decarboxylation to give an enol or enolate intermediate which is stereoselectively protonated[4] to give product. We established the intermediacy of **3** and demonstrated its capacity to be reduced by IMDH/NADH (the reverse reaction) with a 22 μM K_m. Stereoelectronic considerations require that the decarboxylation occurs with the C–CO_2 bond parallel to the carbonyl p-orbitals, an orientation that can be achieved via two conformations that differ by a 180° rotation of the α-keto acid unit (Figure 1). Of course,



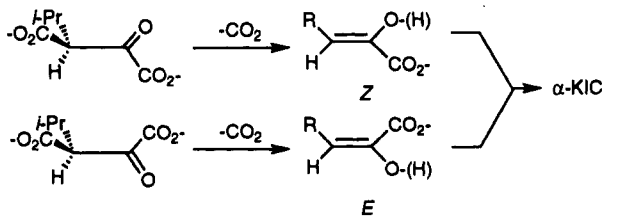


Figure 1. Stepwise reaction pathway for IMDH via the (*E*) or (*Z*) enols or enolates.

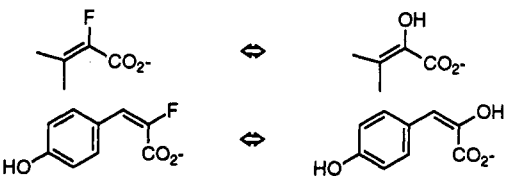


Figure 2. Vinyl fluoride metaphors for enols.

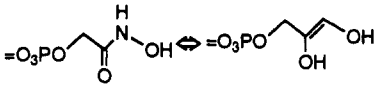


Figure 3. Amide metaphors for enols.

it is likely that **3** is unable to rotate freely within the active site and that therefore only one pathway (*via* the (*E*) or (*Z*) enol/enolate) is followed.

We have aimed to use our mechanistic findings in the design of specific inhibitors for IMDH and, in a complementary way, to use the properties of putative inhibitors to gain insight into the mechanism. We earlier investigated vinyl fluoride metaphors for unstable enzyme-bound intermediates such as enols (Figure 2),[5] with the aim of determining individual stereochemical events during substrate processing which might further help to locate active site residues. Amides are classical metaphors for enols (Figure 3), particularly of α-keto acids,[6] but have the disadvantage that they are configurationally mobile.

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(1) Abbreviations: IMDH: isopropylmalate dehydrogenase; ICDH: isocitrate dehydrogenase; α-KIC: α-ketoisocaproate; α-IM: α-isopropylmalate; β-IM: β-isopropylmalate; α-KG: α-ketoglutarate; NAD: nicotine adenine dinucleotide; Ser: serinc; Leu: leucine; Asn: asparagine; Thr: threonine.

(2) Pirrung. M. C.; Han, H.; Nunn, D. S. *J. Org. Chem.*, preceding paper in this issue.

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(4) Kakinuma, K.; Ozawa, K.; Fujimoto, Y.; Akutsu, N.; Oshima, T. *J. Chem. Soc., Chem. Commun.* **1989**, 1190.

(5) Pirrung, M. C.; Ha, H.-J.; Holmes, C. P. *J. Org. Chem.* **1989**, *54*, 1543. Pirrung, M. C.; Holmes, C. P.; Horowitz, D. M.; Nunn, D. S. *J. Am. Chem. Soc.* **1991**, *113*, 1020. Pirrung, M. C.; Chen, J.; Rowley, E. G.; McPhail, A. T. *J. Am. Chem. Soc.* **1993**, *115*, 7103.

(6) Novoa, W. B.; Schwert, G. W. *J. Biol. Chem.* **1961**, *236*, 2150. Novoa, W. B.; Winer, A. D.; Glaid, A. J.; Schwert, G. W. *J. Biol. Chem.* **1959**, *234* 1143–1148. Northrop, D. B.; Cleland, W. W. *J. Biol. Chem.* **1974**, *249*, 2928–2931.

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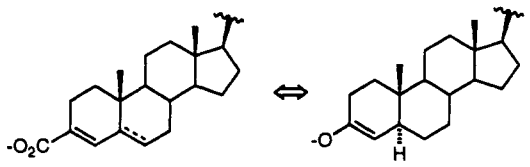
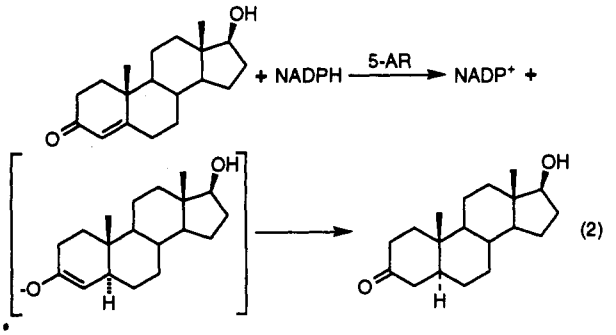


Figure 4. A carboxylate metaphor for an enolate.

Metaphors for enolates have been discovered by Holt & Metcalf[7] in the course of a search for inhibitors of steroid 5α-reductase. This enzyme follows an ordered sequential mechanism with NADPH binding first and $NADP^+$ being released last. Because steroid 5α-reductase conducts a conjugate reduction of testosterone (eq 2) it produces either



(2)

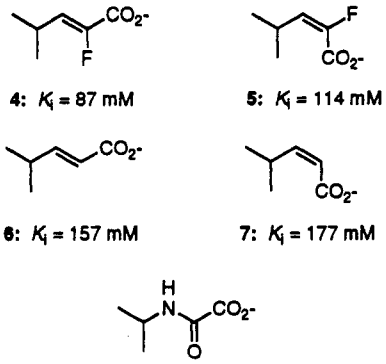
an intermediate enol or enolate. Because the enol/enolate is produced in the enzyme form bearing *oxidized* cofactor, if it could be studied it would be *uncompetitive* against substrate, which is bound to the form of the enzyme bearing *reduced* cofactor. Neutral inhibitors of steroid 5α-reductase, such as unsaturated nitro compounds and a lactam,[8] 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. It is reasonable to suggest that the carboxylates are uncompetitive inhibitors because they mimic the product enolate (Figure 4).

Our approach to addressing the intermediacy of the enol or enolate in IMDH has centered on the inhibition properties of metaphors for each. This manuscript reports the preparation of inhibitors for IMDH from each of the three classes mentioned above. Additionally, a new metaphor based on squaric acid derivatives has been investigated. An explanation for the potencies of several classes of compounds with enolate-producing oxidoreductases is proposed based on the electrostatic interactions of the product and cofactor.

Results

The synthesis of the vinyl fluoride inhibitors **4** and **5** is summarized in another publication.[9] To evaluate the contribution of the fluorine atom to their binding, the corresponding acrylic acids **6** and **7** were desired. Described in the Experimental Section are literature procedures used to obtain them, as is the straightforward preparation of amide **8**. The alkylidenemalonic acids **9-11** were produced by a Knoevenagel condensation of the corresponding aldehydes with malonic ester followed by hydrolysis. The most challenging of the targets were the squaric acids **12** and **13**, which can be accessed using the standard route developed by Moore: organometallic additions to the commercially-available squarate esters (eq 3).[10] The overall yields are reasonable, and the

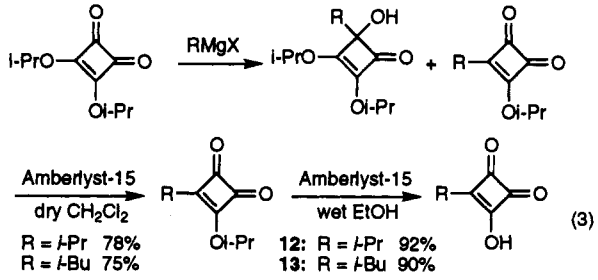
Chart 1. Competitive Inhibition Data for Enol Analogues for IMDH



4: K_i = 87 mM **5:** K_i = 114 mM

6: K_i = 157 mM **7:** K_i = 177 mM

8: K_i = 25 mM



reactions are made significantly more convenient to conduct by the use of an ion-exchange resin for the acidic steps. Hydrolysis of the tertiary alcohols resulting from the initial Grignard addition is accomplished with Amberlyst-15 acidic resin in anhydrous dichloromethane. The water solubility of the semisquaric acids presents a significant difficulty to their isolation following hydrolysis of the esters resulting from the first sequence. This problem was circumvented by the use of the same Amberlyst resin in moist ethanol. The semisquarates can be readily obtained following filtration and removal of the solvent.

The data for these compounds are grouped in Charts 1 and 2 based on whether the inhibition of IMDH is competitive or uncompetitive against isopropylmalate. The chart legends also identify them as enol or enolate mimics; for the former, this assignment is based on the substructures that have earlier served as such; for the latter, it is based on their binding to the reduced enzyme by analogy to steroid 5α-reductase. It should be acknowledged that their inhibition could be explained by action as product analogues. Their potency can be evaluated against the K_m (IM) of ~80 μM at [NAD^+] = 700 μM. Compounds **4-7** are very weak, competitive inhibitors of IMDH, while

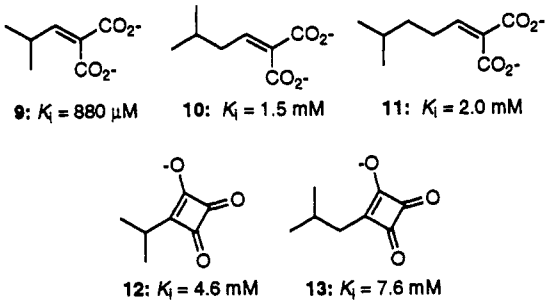
(7) Holt, D. A.; Levy, M. A.; Yen, H.-K.; Oh, H.-J.; Metcalf, B. W.; Wier, P. J. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 27–32. Metcalf, B. W.; Holt, D. A.; Levy, M. A.; Erb, J. M.; Heaslip, J. I.; Brandt, M.; Oh, H.-J. *Bioorg. Chem.* **1989**, *17*, 372–376. Holt, D. A.; Levy, M. A.; Oh, H.-J.; Erb, J. M.; Heaslip, J. I.; Brandt, M.; Lan-Harqest, H.-Y.; Metcalf, B. W. *J. Med. Chem.* **1990**, *33*, 943–950. Holt, D. A.; Levy, M. A.; Ladd, P. L.; Oh, H.-J.; Erb, J. M.; Heaslip, J. I.; Brandt, M.; Metcalf, B. W. *J. Med. Chem.* **1990**, *33*, 937–942. Levy, M. A.; Brandt, M.; Heys, X. X.; Holt, D. A.; Metcalf, B. W. *Biochemistry* **1990**, *29*, 2815–2824.

(8) Rasmusson, G. H.; Liang, T.; Brooks, J. R. *Gene Regulation Steroid Hormones 2*; Roy, A.; Clar, J. H., Ed., Springer: New York, 1983; pp 311–334. Bertics, P. J.; Edman, C. F.; Karavolas, H. J. *Endocrinology (Baltimore)* **1984**, *114*, 63.

(9) See ref 22.

(10) Moore, H. W.; Foland, L. D.; Pollart, D. T.; Perri, S. T.; Reed, M. W. *J. Org. Chem.* **1988**, *53*, 2477.

Chart 2. Uncompetitive Inhibition Data for Enolate Analogues for IMDH



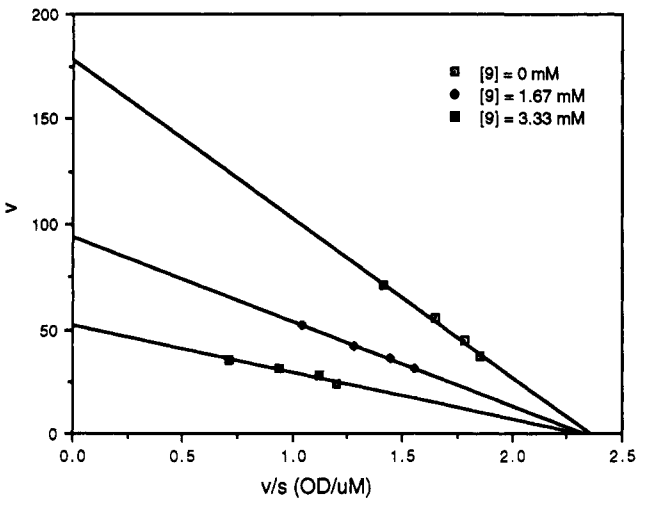


Figure 5. Uncompetitive inhibition of IMDH (forward reaction) by **9**.

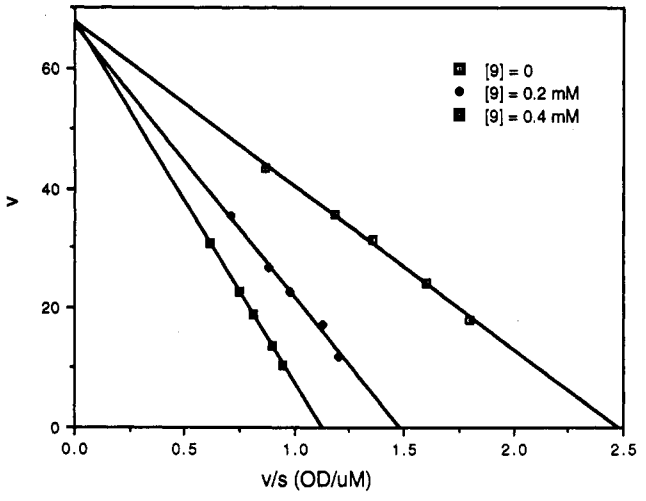


Figure 6. Competitive inhibition of IMDH (reverse reaction) by **9**.

8 is somewhat more potent but still anemic. Compound **9** proved to be the most potent new IMDH inhibitor prepared in this study and is uncompetitive against isopropylmalate (Figure 5). Further investigations established unequivocally that it binds to the reduced form of IMDH, since it inhibits the reverse reaction, reduction of isopropyloxaloacetate (**3**), in a competitive manner (Figure 6). In order to address the possibility of a mismatch between the ionic radius of magnesium and the larger span of the dicarboxylate as compared to the α-hydroxy or α-keto acid, the metal-ion dependence of the strength of inhibition of IMDH by **9** was investigated. Use of Mn^{+2} instead of Mg^{+2} resulted in little change in K_i. Compounds **10-13** are likewise uncompetitive inhibitors but were not studied in as great depth.

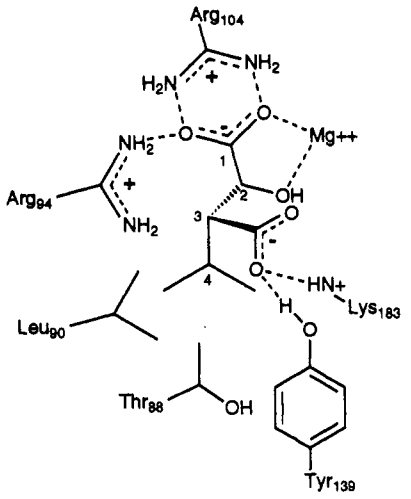


Figure 7. The active site of *Thermus* isopropylmalate dehydrogenase (IMDH).

We have made some efforts to explore the spatial limits of the IMDH binding site and have collected in the supplementary material a listing of compounds that have been synthesized and tested with *Thermus* IMDH, with meager success.

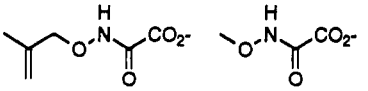
Discussion

The 345 amino acid *T. thermophilus* isopropylmalate dehydrogenase (IMDH) provides an excellent model for the design and discovery of new enzyme inhibitors with biological activity because the protein is easy to purify and study; an X-ray structure is available at 2.2-Å resolution,[11] and over a dozen cloned DNA sequences from both prokaryotes and eukaryotes are available. The only deficiency in the structural data is that no inhibitor, product, or substrate complexes have been formed with the enzyme in the crystalline state, so the interactions between micromolecule and macromolecule have not been directly observed. The IMDH structure is unlike other known dehydrogenases, but its secondary, tertiary, and quaternary structure and its cofactor and substrate binding sites closely resemble *Escherichia coli* isocitrate dehydrogenase (ICDH)[12] which has recently been solved with its substrate bound. The structure for the IMDH active site is shown in Figure 7 accompanied by the position and conformation for substrate proposed by Oshima based on the corresponding data for isocitrate bound to ICDH. Leu90 and Thr88 seem to be important elements in the recognition of IM; they together present a hydrophobic surface to the substrate's isopropyl group. The need for recognition of a substrate with an additional charge, isocitrate, by ICDH can be understood as the basis for its exchange of the Leu for Asn, but the remainder of the active site residues are conserved. The metal ion coor-

(11) Imada, K.; Sato, M.; Tanaka, N.; Katsube, Y.; Matsuura, Y.; Oshima, T. *J. Mol. Biol.* **1991**, *222*, 725–738.

(12) Hurley, J. H.; Dean, A. M.; Sohl, J. L.; Koshland, D. E., Jr.; Stroud, R. M. *Science* **1990**, *249*, 1012–1016. Hurley, J. H.; Dean, A. M.; Koshland, D. E., Jr.; Stroud, R. M. *Biochemistry* **1991**, *30*, 8671–8677. Hurley, J. H.; Thorsness, P. E.; Ramalingam, V.; Helmers, N. H.; Koshland, D. E., Jr.; Stroud, R. M. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 8635–8639. Dean, A. M.; Koshland, D. E., Jr. *Science* **1990**, *249*, 1044–1046.

Chart 3. Uncompetitive Inhibition Data for Enolate Analogues for *Salmonella* IMDH[a]



14: K_i = 31 nM **15:** K_i = 15 nM

[a] Data from Schloss *et al*

Salmonella	GARR**L**LPL**R**KHFKLFSNL**R**P
Thermus	**T**G..**L**LSL**R**KS.DLFANL**R**P

Figure 8. Comparison of the active site residues of isopropylmalate dehydrogenase (IMDH) from *Salmonella* and *Thermus*.

dinates to the C1 carboxylate and the C2 hydroxyl, positioning it to play a key role in electrophilic catalysis. The C1,C2,C3,C4 dihedral angle in bound isocitrate is −165°, almost an antiperiplanar conformation, which would lead to the (*Z*) stereochemistry of the enol/enolate on decarboxylation.

We have previously argued that vinyl fluorides are effective mimics for enols in enzymatic transformations and have found them particularly potent as inhibitors for α,β-dihydroxy acid dehydrase, an enzyme that clearly produces an enol. The inhibitory properties of **4** and **5** are so poor that it is difficult to draw any conclusions about the stereochemistry of an enol/enolate produced in IMDH from the data, and the situation is even worse for **6** and **7**. Earlier uses of amides as equivalents of enols/enolates in enzymes such as pyruvate kinase (oxamate is a competitive inhibitor) and steroid 5α-reductase (lactams are competitive inhibitors) prompted the preparation of **8**. It is a weak inhibitor, competitive against isopropylmalate. These results may not reflect a deficiency in the design of these compounds but rather may hint at mechanistic characteristics of the enzyme; that is, they are poor inhibitors not because they fail to accurately mimic an enzymatically-produced enol but because the enzyme does *not* produce an enol. Since both isomers were tested and the amide is conformationally-mobile, the problem is not the stereochemistry.

The observations with **8** are sharply contrasted with a report made by Schloss and Wittenbach while our investigation was underway.[13] They showed that hydroxamate ethers, such as the developmental herbicides **14** and **15**,[14] inhibit *Salmonella* IMDH with K_is of 15–31 nM (Chart 3). We wished to compare these data with those from our study, but it was reasonable to first question the relation of inhibition results obtained with enzymes from two different sources. An analysis of the sequence homology of the cloned *Thermus* and *Salmonella* enzymes[15] was therefore conducted using the GCG program PILEUP.[16] As shown in Figure 8, the residues that are believed to be important to the interaction of *Thermus*

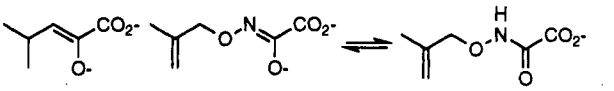


Figure 9. Comparison of the α-ketoisocaproate enolate with the hydroxamate ether anion.

IMDH with substrate (marked in outline) are, with the exception of the Thr, also present in the *Salmonella* sequence. There is significant amino acid identity at other positions around the active site. Even the lysine at position 185 in the *Thermus* IMDH is conserved in the *Salmonella* enzyme. These correlations suggest that the active site structures of the two enzymes are similar. Indeed, it might be expected that variations in buffers, assay conditions, etc., intrinsic to experiments conducted in different laboratories would be as significant a perturbation on the inhibition data as the different sources of enzyme.

With that concern allayed, we seek to provide an explanation for the potent inhibition of IMDH by these hydroxamate ethers in comparison to the relatively weak inhibition by isopropyloxamate (**8**). In Figure 9 is depicted the (*Z*)-enolate of α-KIC, for which there is as yet no direct evidence. It is juxtaposed with the deprotonated form of **14**, to which it bears some resemblance. We suggest that the superior activity of the hydroxamates is related to the inductive power of the oxygen, rendering the amide NH more acidic so the inhibitor can be bound to the active site in the anionic form. The reported NH pK_a for hydroxamic acid ethers of **6**–**9** supports this idea,[17] though it is likely that the nearby carboxylate would raise this pK_a somewhat. Like the anionic inhibitors in Chart 2 and those reported by Metcalf and Holt as steroid 5α-reductase inhibitors, inhibition by **14** and **15** is uncompetitive against IM. It is interesting that **14**, which is not isosteric with the enolate (Figure 9), is a more potent inhibitor than **8** by 6 orders of magnitude. The change of Thr88 in the *Thermus* enzyme to a glycine in the *Salmonella* enzyme may afford a greater volume for **14** to bind. These data suggest that charge may be more important than sterics and that hydroxamate ethers might be used as a new metaphor for enolates.[18]

Our study also included compounds intended to mimic a possible enolate intermediate. Earlier uses of carboxylates as equivalents of enolates in enzymes such as steroid 5α-reductase (A-ring acrylates are uncompetitive inhibitors) prompted the preparation of **9**. Its uncompetitive inhibition against substrate and competitive inhibition against **3** show that it binds to the reduced form of the enzyme and suggest that it serves as an enolate mimic. Therefore, we propose that vinyl carboxylates serve primarily as enolate analogues and that decarboxylation of **3** produces the enolate. Compounds **10** and **11** were prepared even though they are not isosteric with the enolate since this had not seemed a disadvantage for **14** and **15** though, as discussed above, there may be greater freedom to accommodate larger groups in the *Salmonella* enzyme. The significance of their properties lies primarily in the fact that they are also uncompetitive inhibitors against IM.

It would be desirable to have access to enolate mimics of both configurations since in general enzymes may

(13) Wittenbach, V. A.; Rayner, D. R.; Schloss, J. V. *Proceedings of the Penn State Symposium on Plant Biochemistry*, 1992, American Society of Plant Physiologists: Rockville, MD. Wittenbach, V. A.; Rayner, D. R.; Schloss, J. V. *Cur. Topics Plant Physiol.* **1992**, *7*, 69–88. Wittenbach, V. A.; Teaney, P. W.; Rayner, D. R.; Schloss, J. V. *Plant Physiol.* **1993**, *102*, 50.

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(15) *Thermus*: Kagawa, Y.; Nojima, H.; Nukiwa, N.; Ishizuka, M.; Nakajima, T.; Yasuhara, T.; Tanaka, T.; Oshima, T. *J. Biol. Chem.* **1984**, *259*, 2956–2960. *Salmonella*: Andreadis, A.; Rosenthal, E. R. *Biochim. Biophys. Acta* **1992**, *1129*, 228–230.

(16) Devereux, J.; Haeberli, P.; Smithies, O. *Nucleic. Acids Res.* **1984**, *12*, 387–395.

(17) Miller, M. J. *Acc. Chem. Res.* **1986**, *19*, 49. Bordwell, F. G.; Fried, H. E.; Hughes, D. L.; Lynch, T.-V.; Satish, A. V.; Whang, Y. E. *J. Org. Chem.* **1990**, *55*, 3330.

(18) Forthcoming results from our laboratory explore this point with IMDH and ICDH.

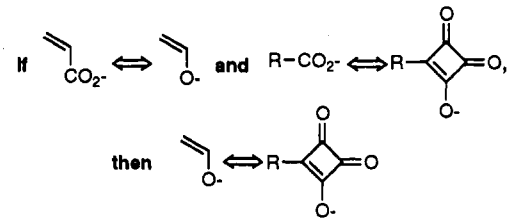

Figure 10. Syllogism describing the relations of enolate metaphors.

generate enolates of either configuration, and the stereochemistry produced by IMDH is unknown (Figure 1). These could permit the most potent inhibitor to be discovered or, in reverse logic, permit the stereochemistry of the enolate to be assigned based on the relative potency of inhibition by enolate metaphors. However, it is impossible to use either hydroxamate ether or our dicarboxylate inhibitors to address stereochemical questions because the former is configurationally mobile and the latter possesses no stereochemistry. We currently have no means to directly address the issue of the enolate stereochemistry, but the strong structural homology of IMDH and ICDH can be used to propose that both produce the (*Z*) enolate. Its protonation from the front face to yield overall retention of stereochemistry as observed could be accomplished by several residues in the active site; Tyr139 (Figure 7), a residue conserved in the IMDH sequences from a dozen plants and microorganisms,[19] is situated particularly close to this face of the α-carbon. That **14** and **15** are uncompetitive inhibitors against IM shows they bind to the form of the enzyme bearing reduced cofactor, that is, the form that binds product. Their potent inhibitory properties and the idea that they could be bound in anionic form support the idea that the immediate product of decarboxylation is the enolate, not the enol.

The use of the squarates **12** and **13** as enolate mimics followed two lines of reasoning. The results reported here and particularly by Metcalf's group have suggested that vinyl carboxylates can serve as equivalents for enolates (Figure 10), and two other pharmaceutical groups have recently reported that squaric acid derivatives can be cognate to carboxylates.[20] Combining the ideas that carboxylates are equivalent to enolates, and squarates are equivalent to carboxylates, squarates may be able to act as stable mimics for enolates. The second argument in favor of the squarate-for-enolate substitution is that, like many 1,3-dicarbonyl compounds, squarates contain a stable enol within their structures. They also show unique acidity ($pK_a \sim 1$) that makes squarates appreciably more anionic than other enols such as phenols ($pK_a \sim 10$) or cyclopentane-1,3-diones ($pK_a \sim 6$) at physiological pH. Because of the constraint of its cyclic structure, squarate can mimic only the (*Z*) enolate (Figure 11). The inhibitory potencies of these materials were not superb, but again the fact that inhibition was uncompetitive against substrate suggests that their anionic nature was the key to promoting binding to the reduced form of the enzyme. The squarates show the minimum K_i (Chart 2 and supplementary material) with an isopropyl substituent, which *is* cognate to the enolate. Their properties, particularly their uncompetitive inhibition, support the idea that they are specifically recognized as enolate metaphors.

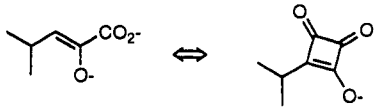

Figure 11.

The less potent inhibition by the α-KIC enolate mimic **9** as compared to the steroid 5α-reductase inhibitors, despite the fact that both are uncompetitive, is intriguing. It can be argued that their high affinity does not necessarily imply that the 5α-reductase produces the enolate. The electrostatic stabilization provided by binding of an anionic compound close to the positively-charged, oxidized cofactor might explain a great deal of their affinity. An obvious distinction between the two enzymes is that the latter is a reductase and the former is a dehydrogenase, with the consequence that the form of a reductase that binds product enolate bears oxidized cofactor NAD(P)$^+$, whereas the form of a dehydrogenase that binds product enolate bears reduced cofactor NADH. The binding of anionic inhibitors to reductases may exploit the positive charge on the cofactor. The concept can be advanced, then, that anionic compounds will bind uncompetitively against IM in mimicking the enolate, but the strength of their binding will be dependent on electrostatic and steric interactions. For example, the squarates, other charged IMDH inhibitors that are relatively weak in binding to the enzyme, are also uncompetitive against isopropylmalate. However, charge is not the only consideration, since the hydroxamate ethers are potent inhibitors of these dehydrogenases and are suggested to bind in the anionic form. Schloss has reported that they are slow binding inhibitors, adding another complication. The study of these and other classes of anionic compounds as enolate metaphors with both types of enolate-producing enzymes to determine the molecular properties germane to the strength and mode of enzyme inhibition is warranted. This line of reasoning strengthens the argument that carboxylates and squarates act as enolate equivalents and predicts that they can be both potent and uncompetitive inhibitors when used with reductases. Experiments to verify this idea are underway.

A highly speculative inference could be drawn from the foregoing arguments that *enzymes strongly inhibited by carboxylates use enolate intermediates.* With many more examples of differential inhibition among unique substructural types, diagnosis of enzyme mechanism may someday be possible on the basis of their inhibition. Efforts to build such a data base and set down the related rules of enzyme inhibition are therefore worthy of pursuit.

One lesson that can be drawn from this study is that structures with the same bond connectivity/shape as the naturally-occurring intermediates are not necessarily the most potent inhibitors. A term commonly used to identify structures that can be used to replace others in bioactive molecules is *bioisosterism.*[21] It directly translates as *same shape as one present in a biological molecule.* The

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strongest inhibitors of IMDH do not have the same shape as the intermediates they putatively mimic, however, and they are stronger than those that do (*viz.*, 8 and 14). The basis for similar function of two different molecules is similar distribution of charge in space, the key to biological recognition, not shape. We have referred to functionalities that can serve as replacements for others as *molecular metaphors*, since those molecules that function similarly may have very different structures. Bioisosterism connotes that two molecules are alike and is a term that would best fall into disuse.

Experimental Section

General. Isopropylmalate dehydrogenase was a gift from Professor T. Oshima. (*E*)- and (*Z*)-2-fluoro-4-methyl-2-pentenoic acids (4 and 5) and (*Z*)-4-carboxyl-2-fluoro-2-pentenoic acid (15) were prepared as described elsewhere.[22] Unless otherwise noted, other materials were obtained from commercial suppliers and were used without further purification. Dichloromethane, ether, pentane, 2-bromopropane, 2-chloropropane, and triethylamine were distilled from calcium hydride. Ethanol was dried over molecular sieves and distilled. THF was distilled from sodium/benzophenone ketyl. Reactions were conducted in oven-dried glassware under an atmosphere of dry nitrogen. Brine refers to a saturated aqueous solution of sodium chloride. Flash chromatography refers to the method of Still[23] and was performed using EM Reagents 0.042–0.063-mm grade silica gel (Kieselgel 60). Infrared spectra were recorded on a Bomem MB-100 instrument. Only the largest or most diagnostic lines are reported. NMR spectra were recorded on Varian XL300 or GE QE300 instruments.

(*E*)-4-Methyl-2-pentenoic Acid (6).[24] Isobutanal (5.48 g, 76.1 mmol), malonic acid (5.00 g, 48.0 mmol), pyridine (15 mL), and morpholine (75 μL) were mixed and stirred at room temperature for 24 h. The temperature was raised to 115 °C and stirring was continued for 17 h. The reaction mixture was poured into 80 mL of 1 N H_2SO_4, extracted with ether, and evaporated. The residue was redissolved in 1 N NaOH solution and extracted with ether. The aqueous phase was acidified with concd HCl and extracted again with ether. The ether extract was dried over $MgSO_4$ and evaporated to give the desired product (4.05 g, 74%): ^{1}H NMR ($CDCl_3$): δ 1.07 (d, *J* = 6.9 Hz, 6H), 2.50 (m, 1H), 5.78 (d, *J* = 15.7 Hz, 1H), 7.07 (dd, *J* = 15.7, 6.6 Hz, 1H).

1,3-Dibromo-4-methyl-2-pentanone.[25] 3-Methyl-2-pentanone (10.00 g, 100.0 mmol) was added to a precooled 48% HBr solution (10 mL) at 4 °C. Bromine (10 mL) was added dropwise over 6 h at this temperature. After the addition of bromine was complete, water was added and the heavier organic layer was separated and immediately fractionated *in vacuo* through a Widmer column. The fraction boiling between 74–77 °C at 1.0 torr was collected (14.19 g, 55%): ^{1}H NMR ($CDCl_3$) δ 1.03 (d, *J* = 6.7 Hz, 3H), 1.13 (d, *J* = 6.6 Hz, 3H), 2.30 (m, 1H), 4.04 (d, *J* = 12.4 Hz, 1H), 4.32 (d, *J* = 12.4 Hz, 1H), 4.43 (d, *J* = 8.8 Hz, 1H).

(*Z*)-4-Methyl-2-pentenoic acid (7).[25] 1,3-Dibromo-4-methyl-2-pentanone (4.85 g, 18.8 mmol) was slowly added to a 0.8 M solution of $KHCO_3$ (188 mL). The resulting mixture was stirred for 24 h at room temperature and extracted with ether. The aqueous phase was acidified to pH 1–2 using cold 1 N HCl at 4 °C. The resulting solution was extracted with ether and dried over $MgSO_4$. Ether was evaporated at 5–10 °C using a dry ice trap to give an analytically pure product (1.39 g, 65%): ^{1}H NMR ($CDCl_3$) δ 1.03 (d, *J* = 6.6 Hz, 6H), 3.61 (m, 1H), 6.57 (d, *J* = 11.3 Hz, 1H), 6.14 (dd, *J* = 11.3, 10.3, 1H).

Isopropyloxamic Acid (8). Ethyl oxalyl chloride (3.46 g, 25.4 mmol) and pyridine (2.01 g, 25.4 mmol) were added to a solution of isopropylamine (1.50 g, 25.4 mmol) in dichloromethane (20 mL) at 4 °C. After being stirred for 2 h, the reaction mixture was allowed to warm to room temperature and was washed three times with 5 mL of 1 N HCl. The organic phase was shaken with 25 mL of 1 N NaOH for 15 min. The aqueous phase was separated and acidified with 3 N HCl. Ether extraction and evaporation gave a crude product which was recrystallized from hexane–ether (2.53 g, 76%): mp 110–113 °C; ^{1}H NMR ($CDCl_3$) δ 1.25 (d, *J* = 6.5 Hz, 6H), 4.07 (m, 1H), 7.25 (broad s, 1H), 9.4 (broad s, 1H); IR (KBr) 3294, 2980, 1770 1677 1558 1360 cm^{-1}; HRMS calcd for ($C_5H_9NO_3$ + H) 132.0660, found 132.0656.

Diethyl Isobutylidenemalonate.[26] Isobutanal (4.00 g, 55.6 mmol), diethyl malonate (8.00 g, 50.0 mmol), morpholine (0.174 g), acetic acid (0.6 g), and benzene (5 mL) were mixed in a round-bottom flask attached to a water separator and refluxed for 3 h. The reaction mixture was cooled and washed twice with water. The combined aqueous layer was extracted with ether. The ether extract was dried over $MgSO_4$ and evaporated. The remaining residue was distilled *in vacuo* through a Widmer column. The fraction boiling between 94–95 °C (4.5 torr) was collected (8.67 g, 81%): ^{1}H NMR ($CDCl_3$) δ 1.07 (d, *J* = 6.6 Hz, 6H), 1.29 (t, *J* = 7.1 Hz, 3H), 1.32 (t, *J* = 7.2 Hz, 3H), 2.67 (m, 1H), 4.22 (q, *J* = 7.2 Hz, 2H), 4.29 (q, *J* = 7.1 Hz, 2H), 6.78 (d, *J* = 10.6 Hz).

Isobutylidenemalonic acid (9). Diethyl isobutylidenemalonate (1.00 g, 4.67 mmol) was placed in 1 N NaOH solution (10 mL) and refluxed for 1 h. The reaction mixture was acidified with concd HCl and extracted with ether. The ether was dried over $MgSO_4$ and evaporated. The residue, which was a mixture of a desired product and malonic acid, was fractionally recrystallized from $CHCl_3$ to give 0.457 g (62%): mp 102–104 °C; ^{1}H NMR (acetone-d_6) δ 1.07 (d, *J* = 6.5 Hz, 6H), 3.02 (m, 1H), 6.96 (d, *J* = 10.5 Hz, 1H), 11 (broad s, 2H); IR (KBr) 2843 (broad), 1718, 1603, 1453, 1243 cm^{-1}; HRMS calcd for $C_7H_{10}O_4$ 158.1579, found 158.0581.

Ethyl 5-Methyl-2-(ethoxycarbonyl)hex-2-enoate[27] (6). The same procedure used for the preparation of diethyl isobutylidenemalonate was applied to 3-methylbutanal on a 20-mmol scale, and the title compound was obtained in 75% yield: ^{1}H NMR ($CDCl_3$) δ 0.92 (d, *J* = 7 Hz, 6H), 1.27 (t, *J* = 7 Hz, 3H), 1.31 (t, *J* = 7 Hz, 3H), 1.79 (m, 1H), 2.17 (t, *J* = 8 Hz, 2H), 4.21 (q, *J* = 7 Hz), 4.28 (q, *J* = 7 Hz, 2H), 7.00 (t, *J* = 7 Hz)

5-Methyl-2-carboxyhex-2-enoic acid (10). The same procedure used for the preparation of diethyl isobutylidenemalonate was applied to ethyl 5-methyl-2-(ethoxycarbonyl)hex-2-enoate, and the title compound was obtained in 32% yield: ^{1}H NMR ($CDCl_3$) δ 0.98 (d, *J* = 7 Hz, 6H), 1.90 (m, *J* = 7 Hz, 1H), 2.71 (t, *J* = 8 Hz, 2H), 7.85 (t, *J* = 8 Hz, 1H), 10.75 (broad s, 2H); IR (neat) 1720, 1610, 1447, 1230; HRMS calcd for $C_8H_{12}O_4$ 172.0735, found 172.0740.

Ethyl 6-methyl-2-(ethoxycarbonyl)hept-2-enoate. The same procedure used for the preparation of diethyl isobutylidenemalonate was applied to 4-methylpentanal on a 20-mmol scale, and the title compound was obtained in 64% yield: ^{1}H NMR ($CDCl_3$) δ 0.86 (d, *J* = 7 Hz, 6H), 1.26 (t, *J* = 7H, 3H), 1.30 (t, *J* = 7H, 3H), 1.33 (q, *J* = 7 Hz, 2H), 1.55 (m, 1H), 2.26 (q, *J* = 8 Hz, 2H), 4.20 (q, *J* = 7H, 2H), 4.27 (q, *J* = 7 Hz, 2H), 6.96 (t, *J* = 8 Hz, 1H); IR (neat) 1740, 1615, 1450. 1241; HRMS calcd for $C_{13}H_{22}O_4$ 242.1518, found 242.1514.

6-Methyl-2-carboxyhept-2-enoic acid (11). The same procedure used for the preparation of diethyl isobutylidenemalonate was applied to ethyl 6-methyl-2-(ethoxycarbonyl)hept-2-enoate, and the title compound was obtained in 30% yield: ^{1}H NMR ($CDCl_3$) δ 0.91 (d, *J* = 7 Hz, 6H), 1.43 (t, *J* = 7 Hz, 2H), 1.61 (m, 1H), 2.77 (q, *J* = 8 Hz, 2H), 7.80 (t, *J* = 8 Hz, 1H), 10.85 (broad s, 2H); IR (neat) 1716, 1605, 1455, 1244 cm^{-1}; HRMS calcd for $C_9H_{14}O_4$ 186.0892, found 186.0890.

3-Isopropoxy-4-isopropyl-3-cyclobutene-1,2-dione. A solution of diisopropyl squarate (1.00 g, 5.05 mmol) under N_2 in 50 mL of dry THF was cooled to −78 °C and isopropylmagnesium chloride (1.7 mL, 3M solution in THF) was added dropwise. The reaction was kept at −78 °C for 30 min and was quenched with 40 mL of 10% aqueous NH_4Cl. The mixture was diluted with 40 mL of ether and the layers were separated. The organic layer

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was dried over Na_2SO_4 and solvents were removed by rotary evaporation. The resulting oil was dissolved in a mixture of 50 mL of CH_2Cl_2 and 2 g of Amberlyst-15 dry resin was added. The mixture was stirred for 24 h at room temperature. Solvents were removed by rotary evaporation and the residue was chromatographed on silica gel (4:1 hexane–ethyl acetate) to yield 0.68 g of the title compound (78%) as a yellow oil: ^1H NMR ($CDCl_3$) δ 5.40 (1H, septet, J=6.3 Hz), 3.02 (1H, septet, J=6.9 Hz), 1.45 (6H, d, J=6.3 Hz), 1.27 (6H, d, J=6.9 Hz); ^{13}C NMR ($CDCl_3$) δ 197.2, 194.9, 194.5, 188.8, 78.9, 26.8, 22.7, 19.0; IR (neat) 2997, 1781, 1727, 1562, 1352, 1008, 748; HRMS calcd for $C_{10}H_{14}O_3$ 182.0943, found 182.0939.

3-Isopropoxy-4-isobutyl-3-cyclobutene-1,2-dione. Following the same procedure as above yielded a title compound (75%): ^1H NMR ($CDCl_3$) δ 0.95 (d, J = 7 Hz, 6H), 1.43 (d, J = 7 Hz, 6H), 2.11 (m, 1H), 2.46 (d, J = 7 Hz, 2H), 5.40 (septet, J = 6 Hz, 1H); IR (neat) 2996, 1790, 1750, 1563, 1348, 1091 cm^{-1}; HRMS calcd for $C_{11}H_{16}O_3$ 196.1100, found 196.1095

3-Hydroxy-4-isopropyl-3-cyclobutene-1,2-dione (12). 3-Isopropoxy-4-isopropyl-3-cyclobutene-1,2-dione (0.73 g, 4.0 mmol) was dissolved in 30 mL of ethanol, and 1 g of wet Amberlyst-15 ion-exchange resin was added. The mixture was stirred while heating at 35–40 °C for 12 h. The resin was removed by vacuum filtration and washed thoroughly with ethanol. The combined filtrates were concentrated under vacuum to yield 0.52 g (92%) 12 as a white solid: mp 79 °C; ^1H NMR ($CDCl_3$) δ 3.03 (1H, septet, J=6.3 Hz), 1.33 (6H, d, J=6.3 Hz); ^{13}C NMR ($CDCl_3$) 196.5, 196.0, 187.1, 26.3, 18.4; IR (KBr) 2972, 1798, 1741, 1593, 1427 cm^{-1}; HRMS calcd for $C_7H_8O_3$ 140.0473, found 140.0475.

3-Hydroxy-4-isobutyl-3-cyclobutene-1,2-dione (13). Following the same procedure as above yielded a title compound (90%): ^1H NMR ($CDCl_3$) δ 0.99 (d, J = 7 Hz, 6H), 2.16 (m, 1H), 2.53 (d, J = 7 Hz, 2H), 10.9 (broad s, 1H); IR (neat) 2996, 1790, 1750, 1563, 1348, 1091 cm^{-1}; HRMS calcd for $C_8H_{10}O_3$ 154.0630, found 154.0630.

Enzyme Assays. All kinetic measurements were, unless mentioned, performed at 25 °C in a buffer solution containing 0.5 mM $MgCl_2$, 0.05 M KCl, 0.67 mM NAD^+, and 0.1 M KP_i, pH 7.6. 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. Data were graphically analyzed by Eadie–Hofstee plots and secondary plots of slopes and intercepts vs inhibitor concentration in the inhibition studies. All inhibitions studies were repeated at least twice and the errors associated with kinetic parameters are estimated at 10%.

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Supplementary Material Available: Listing of nine compounds prepared and tested against IMDH, including their inhibitory properties. ^1H NMR spectra of compounds characterized by high-resolution mass spectrometry (10 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.