Investigación Científica y Técnica) for financial support. C.S. thanks CONICET for a fellowship.

Note added in proof: After the submission of this manuscript for publication, a similar approach to compound **6** appeared in the literature (Li, T.-T.; Wu, Y.-L. *Tetrahedron Lett.* **1988**, *29*, 4039).

Registry No. (±)-**1**, 112420-42-5; (±)-**6**, 118798-10-0; (±)-**7a**, 118798-07-5; (±)-**7b**, 118798-08-6; (±)-**7c**, 118798-09-7; (±)-**8d**, 118798-06-4; (±)-**8b**, 60078-94-6; (±)-**8c**, 60078-92-4; **9**, 72324-39-1; **10**, 118798-11-1; **11**, 118798-12-2; (±)-**12**, 118798-13-3; (±)-**19**, 118798-14-4; (±)-**14**, 118798-15-5; (±)-**15**, 118798-16-6; (±)-**16**, 118798-17-7; (±)-**17a**, 118798-18-8; (±)-**17b**, 118916-42-0; (±)-**17c**, 114375-37-0; (±)-**17d**, 118798-19-9; (±)-**18**, 118798-21-3; (±)-**19a**, 118798-22-4; (±)-**19b**, 118798-23-5; (±)-**19c**, 118798-20-2; (±)-α-cyclocitral, 59462-59-8; α,2,6,6-tetramethyl-2-cyclohexene-1-methanol, 118798-24-6; (±)-1-(2,6,6-trimethyl-2-cyclohexen-1-yl)ethanone, 72717-26-1; 1-(1,3,3-trimethyl-7-oxabicyclo[4.1.0]-hept-2-yl)ethanone, 118798-25-7.

Purification and Inhibition of Spinach α,β-Dihydroxyacid Dehydratase†

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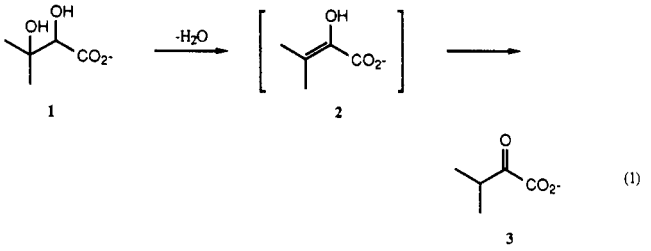
The α,β-dihydroxyacid dehydratase (E.C. 4.2.1.9) responsible for the production of α-oxoisovaleric acid in the valine biosynthetic pathway has been purified from spinach leaves. Its properties are similar to those given in a previous report using a less pure preparation. Its monomer mass is estimated to be 55 kDa. Evidence for an enol intermediate in the reaction mechanism has been obtained by a deuterium labeling study. Several inhibitors have been screened against the enzyme. Four of particular effectiveness are 4-fluoro-2,3-dihydroxyisovaleric acid, 1-hydroxy-1-isobutanesulfonic acid, *N,N*-dimethylglycine *N*-oxide, and 2-fluoro-3,3-dimethylacrylic acid. As an enol analogue, the latter compound gives further evidence for an enol intermediate.

The biosynthetic pathway for the branched-chain amino acids valine, leucine, and isoleucine in higher plants has recently been identified as a site of herbicide action. Three classes of compounds, the sulfonylureas,[1-4] imidazolinones,[5,6] and triazolopyrimidines,[7] have been show to inhibit the first and rate-limiting enzyme in the pathway, E.C. 4.1.3.18, acetolactate synthase (ALS) or acetohydroxyacid synthase (AHAS). These compounds have found commercial success as soybean and small grain herbicides, but still lack selectivity for grasses, and some resist soil metabolism. Consequently, two other enzymes in this pathway draw attention as potential targets for developing new meristematic inhibitors. The acetohydroxyacid reductioisomerase[8] has thus far not been subjected to serious scrutiny. This work has focused on the subsequent enzyme in the pathway, α,β-dihydroxyacid dehydratase (DHAD). This enzyme catalyzes the transformation of 2,3-dihydroxyisovaleric acid (**1**) into 2-oxoisovaleric acid (**3**) with loss of water (eq 1).



1 2 3 (1)

Much of the detailed information about the valine biosynthetic pathway has come from studies on bacterial enzymes. DHAD has been partially purified from *Escherichia coli*,[9] *Neurospora crassa*,[10] and *Salmonella typhimurium*.[11] The stereochemical course of the *E. coli*[12] and *Salmonella*[13] enzymes has been well-studied. It has been shown that a 2*R* configuration is uniformly required for both the natural substrates and analogues. Evidence from tritium labeling studies implicates an enol intermediate in the reaction catalyzed by the *Salmonella* dehydratase.[14] DHAD has also been identified as the site of hyperbaric oxygen poisoning in *E. coli*.[15,16] It has been postulated that this is due to excessive superoxide levels, and superoxide generated from Paraquat has been shown to decrease DHAD activity in vivo.[17]

For the purpose of herbicide design, the plant enzyme is required. DHAD activity has been studied in 29 plant species[18] and has been found to strongly correlate with seedling growth. A previous publication reported the purification (120-fold, 1% activity yield) of the spinach enzyme.[8] It was shown to require Mg^{2+} for activity, as further evidenced by inhibitors such as fluoride and

†Supported by the National Science Foundation (CHE 84-51324) and American Cyanamid Co.

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Table I. Purification of α,β-Dihydroxyacid Dehydratase from Spinach

fraction	vol, mL	protein, mg	mg/mL	activity	specific activity	purification	yield
crude	3110	5474	1.76	3836	0.70	1	100
Polymin P	3060	3580	1.17	3264	0.91	1.3	85
$(NH_4)_2SO_4$	110.5	775	7.08	2376	3.07	4.4	59
DEAE-cellulose	103.0	53.0	0.52	1833	34.6	49.4	45
Sephacryl S-200	19.5	20.2	1.04	1693	83.8	119.7	42
AH-agarose	12.5	5.88	0.47	965	164.3	234.7	24

EDTA. *p*-Chloromercuribenzoate was also reported as a strong (0.5 mM) inhibitor. A K_m was measured at 6 mM for dihydroxyisovalerate (valine precursor) and 2 mM for dihydroxy β-methylvalerate (isoleucine precursor). The invariance of these values at various purification stages and pH's suggested that a single enzyme was present. This report also showed that the enzyme has no aconitase or fumarase activity, nor activity on α,β-dihydroxy acids bearing β-hexyl or -phenyl groups. During the course of the work described herein, an independent report appeared concerning the spinach enzyme.[19]

While important in terms of extending the accepted bacterial biosynthetic pathway to plants, this previous work did not meet our needs for substantial quantities of enzyme for inhibitor screening. Progress toward this goal as well as some success in DHAD-targeted herbicide design is reported herein.

Experimental Section

General. Poly(ethylenimine) (Polymin P) was obtained from Sigma as a 50% solution, which was diluted according to Jendrisak and Burgess.[20] DEAE-cellulose (DE52) was obtained from Sigma, Sephacryl S-200 from Pharmacia, and aminohexylagarose from Sigma. Xanthine, xanthine oxidase, and superoxide dismutase were from Sigma. 2-Keto-3-methylbutyric acid, (*R*)-pantolactone, *N,N*-dimethylglycine, and *dl*-2-hydroxy-3-methylbutyric acid were from Aldrich. 2,3-Dihydroxy-3-methylbutyric acid was obtained by the method of d'Angelo.[21] 3-Hydroxy-3-methylbutyric acid was prepared by the method of Krapcho and Jahngen.[22] *N,N*-Dimethyloxamide was obtained by the method of Vermuelen.[23] 2-Fluoro-3-methyl-2-butenoic acid was obtained by the procedure of Gillet.[24] 1-Hydroxy-2-methylpropanesulfonic acid was obtained by the procedure of Green and Hine.[25] 2,3-Epoxy-3-methylbutanoic acid was obtained by the method of Speziale and Frazier.[26] 1-Hydroxy-2-methylpropanephosphonic acid[27] was obtained from the diethyl ester[28] by cleavage with TMSBr. *N,N*-Dimethylglycine *N*-oxide[29] was obtained from ethyl *N,N*-dimethylglycinate by oxidation with hydrogen peroxide and saponification. Tetrahydrofuran (THF) was distilled from sodium/benzophenone under nitrogen immediately prior to use. ^1H NMR spectra were recorded on a Nicolet NMC-300 spectrometer. UV spectra were obtained on a Hewlett-Packard 8450A spectrophotometer. High-pressure liquid chromatography was conducted on a Hewlett-Packard 1090 liquid chromatograph using a 4 mm × 15 cm oligonucleotide column operated at 40 °C and eluted with 15% methanol in 60 mM pH 3 phosphate buffer. Protein concentration was determined by the Bio-Rad modification of the Bradford[30] dye-binding assay.[31] BSA was the standard. Buffer T consists of 50 mM Tris·HCl, pH 8.00 (25 °C), 2 mM EDTA, 5 mM mercaptoethanol, and 5 mM $MgCl_2$.

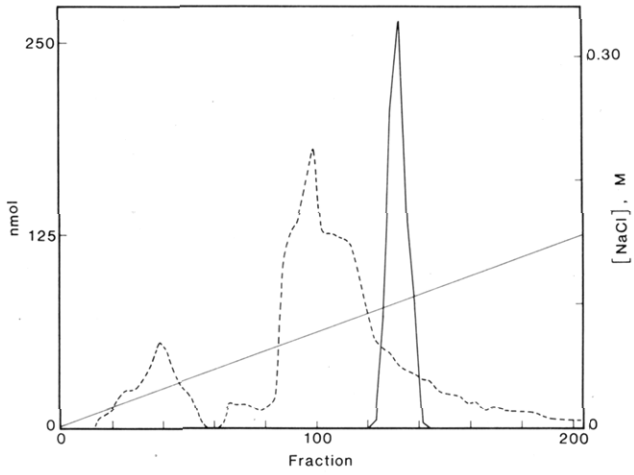

Figure 1. DE52 chromatography of DHAD: A_{280}, dashed lines; solid lines, gradient and enzyme activity. Aliquots of 100 μL were assayed as described in the Experimental Section.

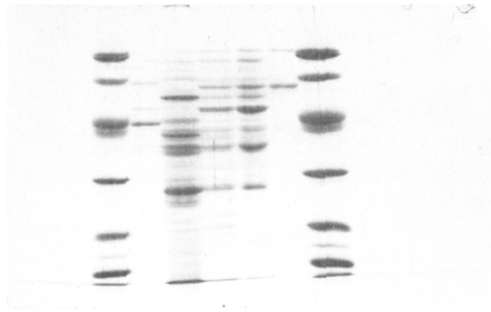

Figure 2. Polyacrylamide gel electrophoresis of DHAD. Molecular weight markers (lanes 1 and 7 left to right) are at 97.4, 66.2, 42.7, 31.0, 21.5, and 14.4 kDa. Lane 3 is crude, lane 4 is after DE52, lane 5 is after gel filtration, and lane 6 is after hydrophobic chromatography.

Purification of Spinach α,β-Dihydroxyacid Dehydratase (DHAD). All steps were carried out at 4 °C. Fresh spinach leaves (1.2 kg) obtained from a local market were chopped into small pieces and homogenized in a 1 gallon Waring blender with 2 L of buffer T. Filtration through cheesecloth and centrifugation at 12000*g* for 30 min gave a supernatant to which NaCl was added to a concentration of 200 mM. Polymin P[20] (0.005 volumes, 15.55 mL of a 12.5% solution adjusted to pH 8.0 with HCl) was added slowly with stirring. After being stirred for 30 min, the solution was centrifuged at 12000*g* for 15 min. The supernatant was brought to 30% saturation with ammonium sulfate, stirred for 30 min, and centrifuged and the pellet was discarded. The remaining solution was brought to 55% saturation, stirred for 30 min, and centrifuged, and the supernatant was discarded. The pellet was suspended in 80 mL of buffer T and dialyzed (Spectra/Por 4) exhaustively against the same buffer. Insoluble material was removed by centrifugation.

The clear yellow dialysate (110.5 mL) was applied to a 225-mL DEAE-cellulose column equilibrated with buffer T. The column was eluted with a linear 2-L gradient of NaCl (0.0 → 0.5 M) in

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buffer T. Fractions of 7.9 mL were collected. The elution profile is shown in Figure 1. Fractions showing DHAD activity were combined and concentrated to 4–5 mL by ultrafiltration using a PM-30 membrane.

This concentrate was applied to a 1.5 × 105 cm column of Sephacryl S-200 equilibrated with buffer T. The column was eluted at a flow rate of 30 mL/h, and fractions of 4 mL were collected. Fractions containing DHAD activity were combined and used without concentration.

The enzyme solution after gel filtration was loaded onto a 12.5-mL column of aminohexylagarose equilibrated with buffer T. It was eluted with a 200-mL linear gradient (0.0 → 0.5 M) of NaCl in buffer T. Four fractions of 3 mL each were pooled, brought to 10% glycerol, and quick-frozen. A representative purification is summarized in Table I.

Gel filtration on a Sepharose 6B column standardized for molecular weight determination [blue dextran (2000 kDa), β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serium albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa)] gave the M_r of DHAD as 55 500. Denaturing polyacrylamide gel electrophoresis of DHAD (specific activity 513 U/mg) against standards indicated a M_r of 62 000 (Figure 2).

Enzyme Assay. A modification of the literature method was used.[10] A standard OD curve for α-keto acid (dinitrophenyl)-hydrazone was obtained by substituting α-ketoisovaleric acid for enzyme and substrate in the procedure below. Solutions of substrate and inhibitors were prepared in buffer T and adjusted to pH 8.00 prior to use. Enzyme solution was added to a 13 × 1 cm test tube and the volume was brought to 300 μL with buffer T. A solution of α,β-dihydroxyisovaleric acid (100 μL, 80 mM) at pH 7.5 was added and the mixture was incubated for 30 min at 35 °C. Blanks were prepared by omitting the substrate or by boiling the enzyme solution. The reaction was quenched by the addition of 100 μL of 10% trichloroacetic acid. A saturated solution of 2,4-dintrophenylhydrazine (200 μL) in 2 N HCl was added and the mixture was allowed to stand at room temperature for 20 min. NaOH solution (2.5 N, 800 μL) was added, the mixture was again allowed to stand (30 min), and the coagulated protein was removed by filtration through glass wool. The A_{538} measured against the reference curve gave the amount of α-ketoisovaleric acid produced. Time course studies established that the rate of product formation was linear over the incubation time. K_m was determined to be 8 mM. V_{max} was measured at 4.56 nmol min^{-1} (mg protein)$^{-1}$ × 10^3. Inhibition studies (on sodium salts) were conducted as above with substrate at K_m (Dixon analysis) or at various substrate and inhibitor concentrations (Hanes–Woolf analysis and least squares by KINFIT, a program kindly provided by Prof. V. Anderson). For those compounds that are chiral, all values refer to the racemate.

For HPLC analysis, the reaction mixture consisted of 120 μL of buffer T, 30 μL of 80 mM dihydroxy acid, and 450 μL of enzyme solution incubated at 35 °C for 2 h. Aliquots of 100 μL were quenched into 20 μL of cold methanol and injections of 100 μL were made. Substrate loss under these conditions was 0.438 nmol min^{-1}, while product formation was 0.492 nmol min^{-1}.

Labeling Experiments. [3-^{2}H]-α-Ketoisovaleric acid was prepared by treating the acid in D_2O with catalytic sodium carbonate for 48 h. The reaction mixture was neutralized and concentrated, and the residue was triturated with ethanol. After concentration of the ethanol, the α-keto acid obtained showed >99% deuterium incorporation by ^{1}H NMR.

[2-^{2}H]-α,β-Dihydroxyisovalerate was prepared by adding α-oxo-β-hydroxyisovaleric acid (7.03 mmol) to sodium borodeuteride (4.6 mmol) in 6 mL of H_2O. After acidification and extractive isolation, the acid was esterified with diazomethane and chromatographed on silica gel (1:1 EtOAc/hexanes). Saponification gave the sodium salt. This compound showed >90% D incorporation by integration of the residual methine proton signal versus the methyls.

Incubation of a 21.6 mM (final concentration) solution of deuterated substrate with 27 μg DHAD (specific activity 3.5 × 10^3 nmol (mg protein min)$^{-1}$) was conducted at 35 °C for 2 h in 800 μL of buffer T. After being quenched with 100 μL of 3 M HCl, the reaction mixture was extracted with ether. After drying (Na_2SO_4), the ether extract was treated with excess diazomethane, concentrated to 100 μL, and analyzed by GC/MS (Hewlett Packard 5890, 25 m × 0.2 mm cross-linked Me-Silicone; 50 °C, 2 min; 10°/min to 200 °C; t_R = 5.8 min). Analysis of the ions at m/e 70, 71, and 72 (isobutyroyl ion fragment) permits the determination of the percent deuterium. When deuterated substrate was used, percent deuterium was <9%. When the deuterated product was incubated under the same conditions, either in the absence or presence of enzyme, and reisolated, analysis showed greater than 90% retention of deuterium.

General Procedure for the Aldol Condensation of *tert*-Butyl (Benzyloxy)acetate with Ketones. To a solution of LDA (prepared from 4.40 mL of diisopropylamine and 18.8 mL of a 1.44 M solution of *n*-BuLi in hexane (27.1 mmol) in 15 mL of THF at −78 °C was added a solution of 18.0 mmol of *tert*-butyl(benzyloxy)acetate in 5 mL of THF. After 2 h, the ketone (21 mmol) was added neat. After 20 min of being stirred at low temperature, the reaction was quenched with NH_4Cl solution. The reaction mixture was extracted with ether and the organic phase dried over $MgSO_4$. Evaporation and chromatography (10% EtOAc in hexane) afforded the pure product in 90–95% yield.

1,1-Dimethylethyl 4-fluoro-3-hydroxy-3-methyl-2-(phenylmethoxy)butanoate: IR (thin film) 3480, 2985, 1736, 1372, 1161, 1144, 1032 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.23 (s, 3 H), 1.52 (s, 9 H), 3.17 + 3.26 (s, 1 H), 3.85 + 3.93 (s, 1 H), 4.19–4.35 (m, 1 H), 4.37–4.50 (m, 2 H), 4.72–4.78 (m, 1 H), 7.35 (s, 5 H). Anal. Calcd for $C_{16}H_{23}FO_4$: C, 64.41; H, 7.77; F, 6.37. Found: C, 64.37; H, 7.89; F, 6.28.

1,1-Dimethylethyl 2-(phenylmethoxy)-3-hydroxy-3-methyl-4,4,4-trifluorobutanoate: IR (thin film) 3460, 2990, 1740, 1375, 1165 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.46 + 1.57 (s, 3 H), 1.48 + 1.50 (s, 9 H), 3.79–4.00 (m, 2 H), 4.45–4.77 (m, 2 H), 7.31–7.42 (m, 5 H); exact mass calcd for $C_{12}H_{12}O_4F_3$ (M − C_4H_9) 277.0688, found 277.0678.

1,1-Dimethylethyl α-(phenylmethoxy)-1-hydroxycyclobutaneacetate: IR (thin film) 3470, 2990, 1729, 1378, 1196 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.51 (s, 9 H), 1.78–1.92 (m, 2 H), 1.99–2.13 (m, 2 H), 2.20–2.41 (m, 2 H), 2.95 (bs, 1 H), 3.85 (bs, 1 H), 4.46 (d, J = 12, 1 H), 4.80 (d, J = 12, 1 H), 7.28–7.43 (m, 5 H); exact mass calcd for $C_{13}H_{15}O_4$ (M − C_4H_9) 235.0970, found 235.0974.

General Procedure for Hydrogenolysis of *tert*-Butyl α-(Benzyloxy)-β-hydroxy Carboxylates. Aldol adduct from above (14 mmol) and 200 mg of 10% Pd/C were alternately placed under vacuum and 1 atm H_2. EtOH (100 mL) and 100 μL of concentrated HCl were added, and the vessel was repeatedly evacuated and filled with hydrogen. After being stirred vigorously for 16 h, the reaction mixture was filtered and evaporated. Chromatography using 25% EtOAc in hexanes afforded pure diol.

1,1-Dimethylethyl 2,3-dihydroxy-4-fluoro-3-methylbutanoate: IR (thin film) 3460, 2985, 1722, 1460, 1373, 1160, 1100, 1028 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.25 (s, 3 H), 1.54 (s, 9 H), 2.95 + 3.22 (s, 1 H), 3.19 + 3.26 (d, J = 5, 1 H), 3.98–4.03 (d, J = 6, 1 H), 4.20–4.56 (m, 2 H); exact mass calcd for $C_9H_{15}O_4F$ (M − H_2) 206.0955, found 206.0948.

1,1-Dimethylethyl 2,3-dihydroxy-3-methyl-4,4,4-trifluorobutanoate: IR (thin film) 3530, 2995, 1729, 1376, 1295, 1175 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.51 + 1.59 (s, 3 H), 1.52 + 1.53 (s, 9 H), 3.22 + 3.23 (d, J = 7, 1H) 3.70 + 3.73 (s, 1 H), 4.01–4.07 (m, 1 H); exact mass calcd for $C_9H_{16}O_4F_3$ (M + H) 245.1001, found 245.0979.

1,1-Dimethylethyl α,1-dihydroxycyclobutaneacetate: IR (thin film) 3450, 2990, 2950, 1725, 1371, 1253, 1159, 1107, 853 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.51 (s, 9 H), 1.83–1.98 (m, 2 H), 1.99–2.15 (m, 2 H), 2.30–2.49 (m, 2 H), 2.50 (s, 1 H), 3.17 (d, J = 5, 1 H), 4.08 (d, J = 5, 1 H). Anal. Calcd for $C_{10}H_{18}O_4$: C, 59.39; H, 8.97. Found: C, 59.20; H, 9.08.

General Procedure for Hydrolysis of *tert*-Butyl α,β-Dihydroxy Carboxylates. To a solution of the above diol (12 mmol) in 20 mL of methylene chloride was added trifluoroacetic acid (4 mL). After stirring overnight, the volatiles were removed and the residue was chromatographed by using 10% MeOH in CH_2Cl_2.

2,3-Dihydroxy-4-fluoro-3-methylbutanoic acid: IR (thin film) 3520, 2995, 1726, 1378, 1251, 1050 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.23 + 1.30 (d, J = 2, 3 H), 4.07 (s, 1 H), 4.20 (s, 1 H), 4.22–4.42 (m, 3 H). Anal. Calcd for $C_5H_9FO_4$: C, 39.48; H, 5.96; F, 12.49. Found: C, 39.90; H, 6.24; F, 12.29.

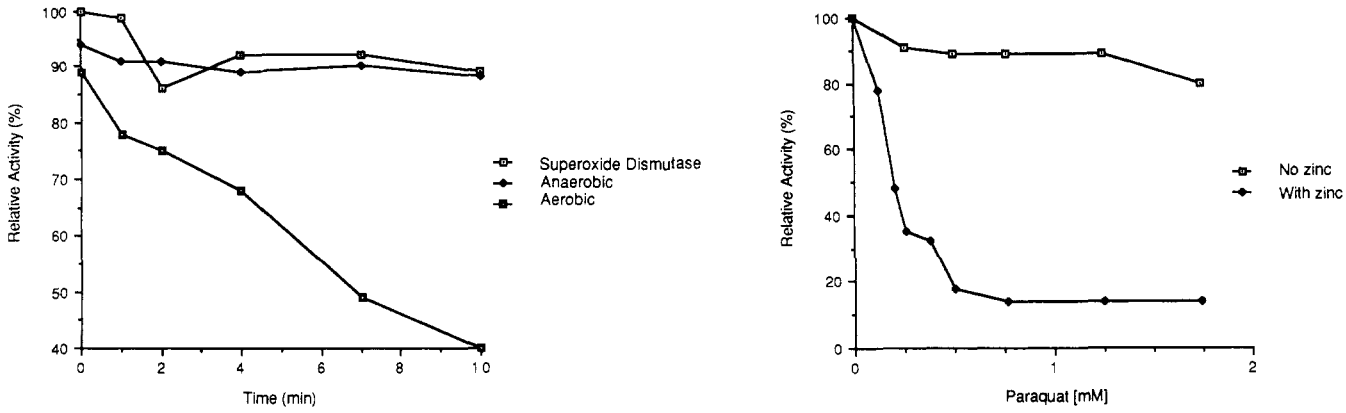


Figure 3. Time and concentration dependence of the inactivation of DHAD in the presence of reduced Paraquat (right) and oxygen (left, [Paraquat] = 10 mM).

2,3-Dihydroxy-4,4,4-trifluorobutanoic acid: IR (thin film) 3520, 1730, 1385, 1192, 1178 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.53 + 1.56 (d, J = 1, 3 H), 3.64–3.92 (m, 3 H), 4.31 + 4.33 (s, 1 H). Anal. Calcd for $C_5H_7F_3O_4$: C, 31.93; H, 3.75; F, 30.30. Found: C, 32.12; H, 3.93; F, 30.31.

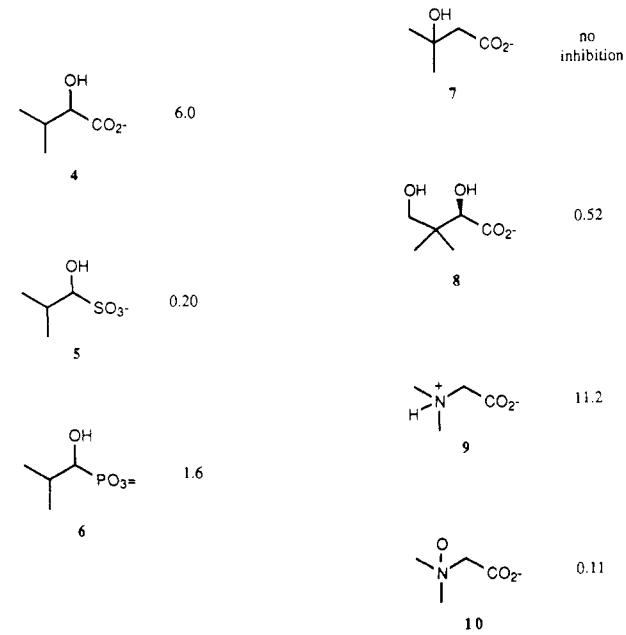
α,1-Dihydroxycyclobutaneacetic acid: IR (thin film) 3200, 2920, 1720, 1250, 1100 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.56–1.71 (m, 1 H), 1.78–1.92 (m, 1 H), 1.96–2.17 (m, 2 H), 2.30–2.42 (m, 1 H), 2.42–2.58 (m, 1 H), 4.15 (s, 1 H), 4.20–4.55 (m, 3 H). Anal. Calcd for $C_6H_{10}O_4$: C, 49.31; H, 6.90. Found: C, 49.44; H, 6.81.

Results

Purification and Properties of α,β-Dihydroxyacid Dehydratase. A previous report on the plant enzyme[8] involved the initial preparation of an acetone powder. In our hands, homogenization in aqueous buffer followed by standard precipitation methods proved much more convenient. This procedure provides 7-fold greater specific activity in the crude extract than generation of the acetone powder according to another procedure,[19] and thus less material is needed. Chromatographic techniques including hydrophobic chromatography[32] give an overall 24–34% yield (see Table I). The enzyme demonstrates remarkable stability, maintaining >75% of its activity in the crude homogenate for 3 days at 0–4 °C. For long-term storage freezing is essential, and no loss of activity is seen on a cycle of freezing and thawing in 10% glycerol. The K_m (8 mM) that is measured for this enzyme preparation with α,β-dihydroxyisovaleric acid as substrate is comparable to Wixom's (6 mM). V_{max} is 4.56 nmol min^{-1} (mg protein)$^{-1}$ $\times$ 10^3. In one instance a homogeneous preparation of enzyme (513 U/mg) was obtained from the Polymin P supernatant, as judged by SDS-PAGE (Figure 2). The specific activity of this preparation was quite similar to that obtained by Flint and co-workers.[19] It did not prove necessary to achieve this level of purity for all of the kinetic studies, since preliminary data suggest that only a single enzyme activity is present even when homogeneity has not been achieved. Monitoring the enzymatic reaction by HPLC permits the simultaneous measurement of both substrate and product. A stoichiometric relationship between substrate lost and product formed is observed. The M_r determined for DHAD from the SDS-gel is 62 000, comparable to the 63 500 found by Flint and co-workers.[19] Interestingly, the 55 500 Da value found from gel filtration under reducing (DTT)conditions suggests that the dimer seen in native gels[19] is disulfide linked.

Preliminary results on the susceptibility of the enzyme to superoxide were obtained for comparison to previous reports on the *E. coli* enzyme. Exposure of enzyme solutions to oxygen, xanthine, and xanthine oxidase leads to loss of activity (20% in 30 min). The addition of superoxide dismutase or exclusion of oxygen protects the enzyme from this inactivation (data not shown). More effective inactivation was observed with superoxide generated from zinc-reduced Paraquat. As summarized in Figure 3, inhibition is time-, oxygen-, and concentration-dependent.

Chart I. Inhibitors of DHAD with K_i/K_m Values. Substrate Analogues



Structural Requirements. In order to assess where flexibility in structure could be exploited in inhibitor design, some simple substrate analogues were studied as inhibitors of DHAD. The seven structures (with K_i/K_m values) collected in Chart I summarize the results. Because α-hydroxyisovaleric acid acts as a competitive inhibitor but its β-hydroxy isomer does not, it is evident that the α-hydroxy acid functionality is most important for binding. To further explore this point, the corresponding α-hydroxy phosphonic and sulfonic acids were prepared. Previous work has shown the latter are effective at inhibiting α-hydroxy acid oxidases.[33] In this case, the sulfonic acid shows strong, mixed inhibition (Figure 4).

Evidence for Enol. In order to differentiate between two potential mechanisms for DHAD, namely, the β-elim-

(32) Shaltiel, S. *Methods Enzymol.* **1984**, *104*, 69–96.

(33) Zelitch, I. *J. Biol. Chem.* **1957**, *224*, 251–260.

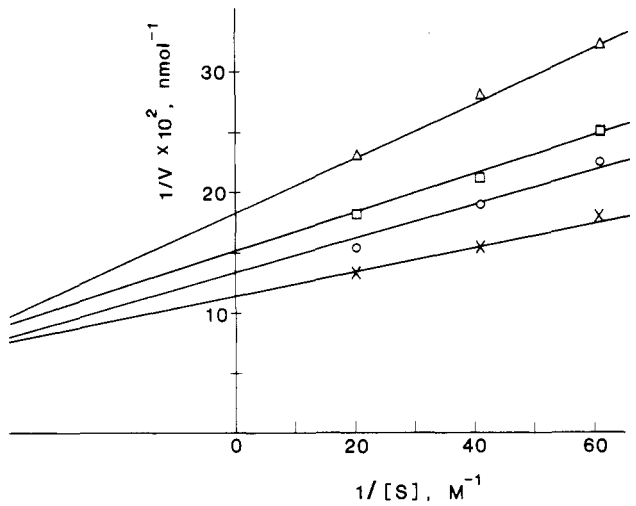
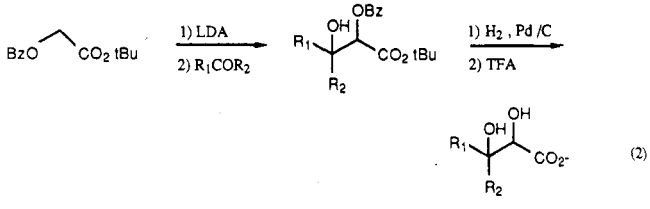


Figure 4. Lineweaver–Burk plot of the inhibition of DHAD by **5**. Best-fit lines are shown for each concentration of inhibitor: (○) 0.68 mM; (□) 1.36 mM; (△) 2.73 mM.

ination of water to form an enol or a 1,2-hydride shift, a deuterium labeling study was conducted. The deuterated substrate was prepared by $NaBD_4$ reduction[34] of α-keto, β-hydroxyisobutyric acid.[35] On incubation with the enzyme, α-oxoisovaleric acid is produced, which is subjected to extractive isolation and esterification with diazomethane. GC/MS analysis (of the isobutyroyl ion fragment) demonstrates that the product is devoid of deuterium. Control experiments demonstrate that deuterated product is stable to exchange under the incubation and isolation conditions. Thus, an enol is implicated. Furthermore, it is likely that the enzymic acid which protonates the enol is not the conjugate acid of the base which removes the α proton (if it is, exchange with solvent must be kinetically competent).

Inhibitor Design. It was thought that the *enol* was a useful target for the design process. Two major strategies were developed. The first was simple structural mimicry. The second aim was to exploit the nucleophilic nature of the enol in promoting a β-elimination reaction. The product of such a reaction, an enone, could be reactive toward an enzymic nucleophile, leading to mechanism-based inactivation. Finally, it was thought that cyclobutane ring strain could be used to prevent elimination to form the enol. The putative inhibitors that were prepared to meet these criteria are collected in Charts II and III.

Synthesis. A general procedure was used to prepare α,β-dihydroxyacid analogues from ketones (eq 2). This



(2)

method has been applied to acetone[21] and was used to prepare the substrate. When applied to fluoroacetone and trifluoroacetone, the products are obtained as (1:1) diastereomeric mixtures that were not rectified. Naturally, they are racemic. The other inhibitors are either known

Chart II. Inhibitors of DHAD with K_i/K_m Values

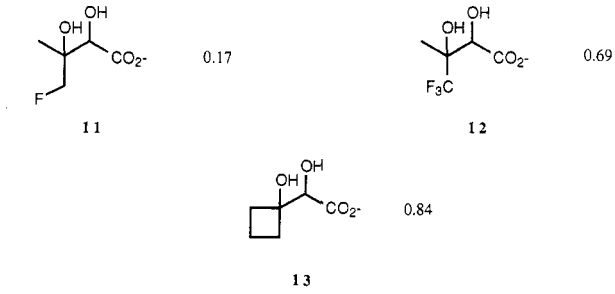
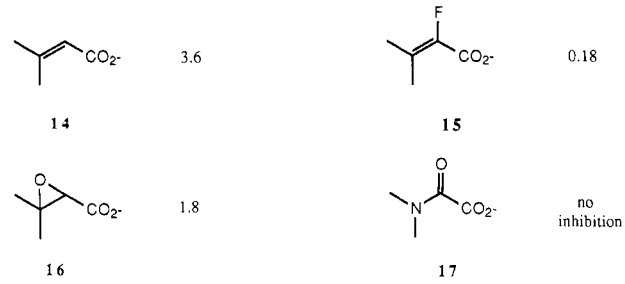


Chart III. Inhibitors of DHAD with K_i/K_m Values. Enol Mimics



compounds or were prepared by literature procedures (see Experimental Section).

Inhibition Studies. None of the dihydroxy acids in Chart II are substrates for DHAD. Each of these compounds shows competitive inhibition by graphic analysis. No evidence for mechanism-based inactivation (i.e., noncompetitive kinetics, time-dependent loss of activity) was observed for either **11** or **12**. The acids in Chart III are also competitive inhibitors.

Discussion

The three α-hydroxy acid analogues **4**–**6** provide an unusually simple system to test carboxylate surrogates. While the nature of the inhibition by **5** is difficult to interpret, it is clear that sulfonic acids are worthy of further investigation in this regard. Based on the properties of the fluorinated analogues discussed below, it was postulated that the enzymatic reaction mechanism has E1 character, with positive charge accumulation at the β-carbon. Studies of steroid and terpene biosynthesis[36-41] suggest that an ammonium ion would effectively mimic the transition state for such an elimination. The properties of *N*,*N*-dimethylglycine (**9**) clearly do not meet this expectation. The lack of an α-hydroxyl group may be the reason for this result. On the other hand, the *N*-oxide **10** is the most effective inhibitor we have studied.

That the fluorinated inhibitors **11** and **12** are not turned over suggests that E1 mechanism mentioned above. That is, it becomes unfavorable to develop positive charge at the β-carbon when a more powerful electron-withdrawing group is present. The cyclobutane analogue reveals the power of ring strain in influencing reactivity. The re-

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quirement that **13** be converted to an enol, which is also a methylenecyclobutane totally shuts down turnover. That **13** is not a better inhibitor reveals the subtlety of the recognition of this enzyme for its substrate. The isoleucine precursor α,β-dihydroxy-β-methylvaleric acid differs from **13** only by the addition of two hydrogens.

The activity of vinyl fluoride **15**, possibly acting as a stable enol surrogate, implicates an enol intermediate[42] in the reaction mechanism of spinach DHAD. Empirical force field calculations on **15** and the putative enol **2** show a very close correspondence in structure. The extremely poor activity of oxamide **17** is disappointing, since in principle this should be an excellent enol mimic. These examples serve to illustrate the need for a larger data base to aid in inhibitor design.

(42) Rose, I. A. *Methods Enzymol.* **1982**, *87*, 84-97.

Given the ultimate goal of this work, the screening of all of the inhibitors prepared in this study against whole plants was conducted. At an application rate (spray, greenhouse) of 4 kg/h, only **12** showed activity, with complete kills against quack grass, bindweed, and wild mustard and some activity against other species. That some postemergent herbicidal activity is detected is encouraging, but it should be emphasized that there is no evidence as yet which indicates that inhibition of DHAD is the mode of action. There is also little correlation between activity in vivo and in vitro.

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Total Synthesis of (±)-Fawcettimine

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(±)-Fawcettimine (**7**) has been prepared in 13 steps from cyano enone **14** along the line: **14** → **16** → **17** → **19** → **22** → **23** → **24** → **25** → **35** → **42** → **43** → **45** → **54** → **7**. The overall yield is 16.6%, and no protecting groups are required in the synthesis. The tautomeric ring–chain equilibria of keto carbinolamines **7**, **57**, and **60** and diketo amines **56** and **59** have been investigated by NMR spectroscopy and molecular mechanics calculations. In the 4*R* ("epi") series, diketo amine **56** seems to predominate over keto carbinolamine form **57**. Isomer **60** cannot be observed spectrally, and the molecular mechanics calculations suggest that it should be 5 kJ/mol less stable than **57**. These experiments and calculations are in agreement with the observation that compound **54** exists wholly in the keto amine form, with none of the carbinolamine tautomer being observable spectrally. In the 4*S* ("natural") series, the keto carbinolamine form **7** greatly predominates over the diketo amine form **59**.

Background

In 1959, from extracts of alkaloids of *Lycopodium fawcetti* collected in the Blue Mountain Range of Jamaica in the Spring, R. H. Burnell isolated a compound of intermediate basicity, which he designated as base A.[1] Subsequent investigation gave the formula $C_{16}H_{25}NO_2$ and revealed that, whereas the infrared spectrum of the free amine in CCl_4 shows one carbonyl stretch (1730 cm^{-1}) and a hydroxyl absorption (3585 cm^{-1}), the acetyl and methiodide derivatives show two carbonyl absorptions (1690, 1730 cm^{-1} and 1710, 1730 cm^{-1}, respectively) and no hydroxyl absorption.[2-4] These early investigations led to the proposal of structure **1** for fawcettimine and structure **2** for the acetyl derivative (Scheme I).[4]

The gross structural assignment was confirmed by the chemical correlation of fawcettime with serratinine (**3**), the structure of which was proven by X-ray analysis.[5] As shown in Scheme I, the reduction of *N*-acetylfawcettimine with either sodium borohydride in methanol or sodium/isobutyl alcohol in toluene furnishes dihydro-*N*-acetylfawcettimine (**4**).[3,4] The latter compound was also obtained from 13-acetyl-8-deoxyserratinine (**5**)[6] by reduction with zinc in the presence of acetic anhydride, followed by alkaline saponification of the acetate group. This correlation did not permit assignment of relative configuration at C-4 in fawcettimine.

In 1974, Ayer and co-workers reported an X-ray analysis of allopecuridine, which revealed it to be **6**, the 4*R*-hydroxy derivative or fawcettimine.[7] Reduction of **6** with calcium in ammonia gives fawcettimine, confirming the previous structural assignment of the latter alkaloid. It was noted that allopecuridine and fawcettimine behave similarly with acetic anhydride, giving *N*-acetyl derivatives, and that *N*-acetylfawcettimine and *N*-acetylallopecuridine have similar positive Cotton effects at 300 nm in their circular dichroism spectra. From this optical similarity, Ayer assigned fawcettimine and *N*-acetylfawcettimine the 4*S* stereochemistry, as shown in Scheme II (**7** and **9**).

In 1981, Inubushi and Harayama elucidated the absolute stereostructure of lycothunine (**10**) by X-ray analysis of its acetyl derivative, **11**.[8] This investigation revealed lycothunine to be 10,11-dehydrofawcettimine. Unlike

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