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Stereochemical and Isotopic Labeling Studies of 2-Oxo-hept-4-ene-1,7-dioate Hydratase: Evidence for an Enzyme-Catalyzed Ketonization Step in the Hydration Reaction

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Abstract: 2-Oxo-hept-4-ene-1,7-dioate hydratase from *Escherichia coli C* converts 2-oxo-hept-4-ene-1,7-dioate to 2-oxo-4-hydroxy-hepta-1,7-dioate by the addition of water using magnesium as a cofactor. The enzyme is one of a set of inducible enzymes, known collectively as the homoprotocatechuate meta-fission pathway. The entire pathway enables the organism to utilize aromatic amino acids as its sole sources of carbon and energy. Expression and purification of 2-oxo-hept-4-ene-1,7-dioate hydratase to homogeneity permitted kinetic, isotopic labeling, and stereochemical studies. Kinetic studies show that the enzyme processes either 2-oxo-hept-4-ene-1,7-dioate or 2-hydroxy-2,4-heptadiene-1,7-dioate to product with comparable facility. Isotope labeling studies show that the hydratase catalyzes the incorporation of a solvent deuteron at both C-3 and C-5 when the reaction is performed in ^2H_2O. The enzyme also accelerates the exchange of the C-3 proton of the alternate substrate 2-oxo-1,7-heptadioate with solvent deuterons. The results are consistent with a mechanism in which the enzyme catalyzes the isomerization of 2-oxo-hept-4-ene-1,7-dioate to its α,β-unsaturated ketone followed by the Michael addition of water. Whether this mechanistic sequence involves a one-base or a two-base mechanism is not yet known.

Several microorganisms are able to use an aromatic compound as a sole source of carbon and energy. Initially, the aromatic hydrocarbon is converted to catechol or a substituted catechol. Subsequently, the catecholic compound is processed to the Krebs cycle by one of the many so-called meta-fission pathways.[1] The general strategy of these meta-fission pathways is exemplified by the catechol and the homoprotocatechuate meta-fission pathways. The catechol meta-fission pathway consists of an inducible set of enzymes from *Pseudomonas putida mt-2* that converts catechol and 3-substituted catechols to acetaldehyde and pyruvate.[2] The pathway is encoded by the TOL plasmid and is part of a degradative route for toluene, *m*- and *p*-xylene, 3-ethyltoluene, and 1,2,4-trimethylbenzene. The homoprotocatechuate pathway is an inducible set of enzymes from *Escherichia coli C* that converts 3,4-dihydroxyphenylacetate to succinic semialdehyde and pyruvate.[3] The enzymes of this pathway are encoded by chromosomal DNA and may be part of a degradative route for phenylalanine and tyrosine.

Our long-standing interest in these two pathways is due to the striking parallelism between enzymatic reactions.[4] Parallel

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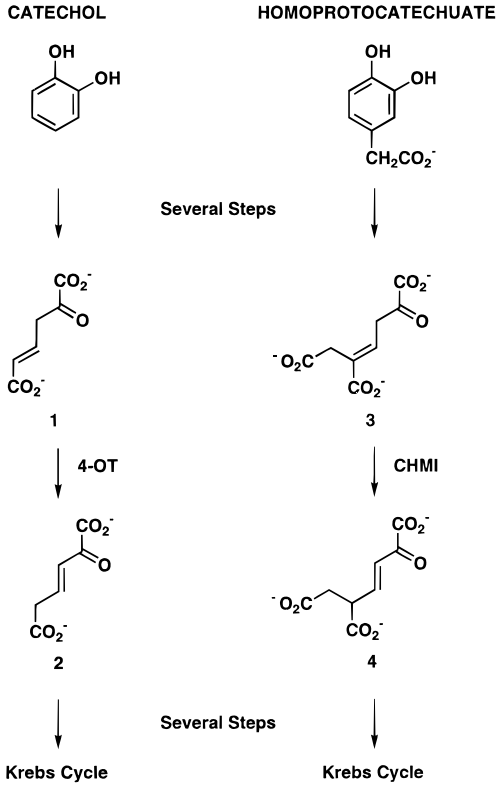
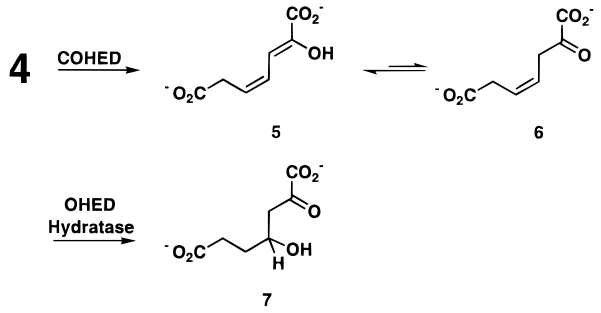
Scheme 1



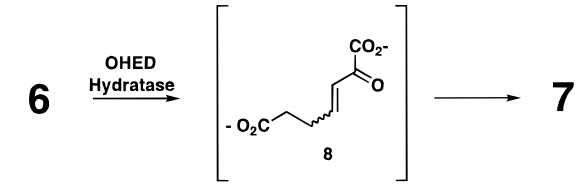
enzymes perform chemically identical reactions on substrates that differ only by the absence or the presence of a carboxymethyl group (i.e., $-CH_2CO_2^-$). The isomerization reactions catalyzed by 4-oxalocrotonate tautomerase (4-OT) and 5-(carboxymethyl)-2-hydroxymuconate isomerase (CHMI) demonstrate this parallelism (Scheme 1). While the structural resemblance between substrates (2-oxo-4*E*-hexenedioate, **1**, and 2-oxo-5-(carboxymethyl)-4*E*-hexenedioate, **3**) suggests that the enzymes might be evolutionarily related, there is no obvious sequence homology.[4−6] However, when the crystal structures were solved, it was found that the overall folds of the two proteins as well as the active site regions are nearly superimposable.[7] Moreover, the key amino acid residues involved in the mechanism are identical.[7,8] In an effort to obtain further insight into the relationship between the catechol and homoprotocatechuate meta-fission pathways, we are pursuing comprehensive mechanistic and structural studies of all of the enzymes in these two pathways including the title enzyme, 2-oxo-hept-4-ene-1,7-dioate (OHED) hydratase.

In previous work, we showed that 5-(carboxymethyl)-2-oxo-3-hexene-1,6-dioate decarboxylase (COHED) catalyzes the magnesium-dependent conversion of 5-(carboxymethyl)-2-oxo-3*E*-hexene-1,6-dioate (**4**) to 2-oxo-4*Z*-heptene-1,7-dioate (**6**) through the intermediate 2-hydroxy-2,4*Z*-heptadiene-1,7-dioate (**5**) (Scheme 2).[9,10] Although the facile nonenzymatic inter-

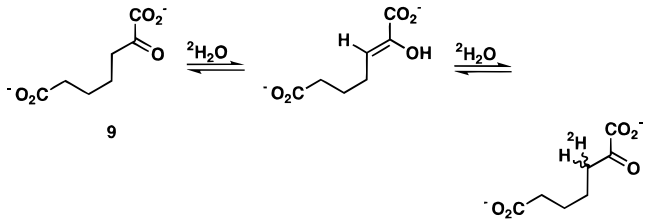
Scheme 2



Scheme 3



Scheme 4



conversion of **5** and **6** precludes a direct determination, the observation that COHED generates (3*S*)-[3-^{2}H]**6** from **5** in ^2H_2O is the basis for the conclusion that **6** is the product of the reaction and the substrate for the next enzyme in the pathway. This enzyme, OHED hydratase, converts **6** to 2-oxo-4-hydroxy-hepta-1,7-dioate (**7**) by utilizing only magnesium as a cofactor.[11] Because this transformation requires neither iron nor the B_{12} coenzyme, the most attractive mechanism for the enzyme-catalyzed addition of water to **6** involves the isomerization of **6** to 2-oxo-3-heptene-1,7-dioate (**8**) followed by the Michael addition of water to **8** (Scheme 3).[12]

To determine whether the isomerization/hydration mechanism is operative, kinetic, isotopic labeling, and stereochemical studies of the OHED hydratase-catalyzed reaction were performed. Three lines of evidence were obtained from these studies which are consistent with the proposed mechanism. First, OHED hydratase processes either **5** or an isomeric mixture of **5** and **6** to product with comparable kinetic facility. Second, the enzyme accelerates the H → D exchange of the C-3 protons of the alternate substrate, 2-oxo-1,7-heptanedioate, **9** (Scheme 4). Finally, when the reaction is performed in ^2H_2O, a deuteron is stereospecifically incorporated at both C-3 and C-5 of **7**. The stereochemistry at C-3, C-4, and C-5 of 3,5-[di-^{2}H]**7** was also assigned, but it was not possible to determine the overall stereochemical course of the reaction (**6** → **7**) because the configuration of **8** could not be established. Hence, either a one-base or two-base mechanism can be utilized in a metal-assisted isomerization/hydration sequence mediated by OHED hydratase.

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Results

Cloning, Expression, and Purification of 2-Oxo-hept-4-ene-1,7-dioate Hydratase. The cloning of the OHED hydratase gene (*hpcG*) has previously been reported.[11] It encodes one of several enzymes in the homoprotocatechuate meta-fission pathway whose gene sequences are contained on a *BamH*I fragment of *E. coli C* genomic DNA cloned into the *BamH*I site of pB*R*328.[11] The expression of recombinant enzyme from this clone (in cell-free extracts) is reportedly 10-fold higher than the expression of the wild-type enzyme from *E. coli C* grown on 4-hydroxyphenyl acetate.[11] Because the clone is not available and much greater quantities of OHED hydratase are required for mechanistic and structural studies, the *hpcG* gene was amplified by the polymerase chain reaction (PCR) using *E. coli C* genomic DNA as template and cloned into a T7 expression system. Sequencing of the gene revealed minor discrepancies in the previously reported sequence.[13] The gene sequence encodes a 267 amino acid protein with a predicted subunit $M_r = 29\,714$.

The large quantities of expressed recombinant enzyme coupled with facile precipitation at high protein concentration (>5 mg/mL) necessitated a different purification protocol than the previously published procedure. To minimize precipitation during the purification process, the cells are lysed in a large volume of buffer solution containing high salt concentration (~2 M NaCl). The new protocol results in high-purity protein as judged by SDS−PAGE. Typically, this method results in 20−30 mg of pure enzyme from 3 L of cell culture.

Kinetic Properties of OHED Hydratase. The previous enzyme in the pathway, COHED, generates (3*S*)-[3-^{2}H]**6** from **5** in ^2H_2O.[10] This observation is the basis for the conclusion that **6** is the product of the COHED-catalyzed reaction and the substrate for OHED hydratase. Because **6** cannot be synthesized nor isolated (it exists only in rapid equilibrium with **5**), kinetic studies of OHED hydratase were carried out using **5** and an isomeric mixture of **5** and **6**.

In previous studies, it was established that the highest concentrations of **6** are typically obtained after a 9−12 min equilibration period in aqueous phosphate buffer.[10] After 12 min, the solution consists of ~28% **5** and ~72% **6**. Although **8** is the predominant isomer (>95%) at thermodynamic equilibrium, there is no significant amount of **8** present after 12 min. Hence, the rate of product formation (**7**) by OHED hydratase was examined using **5** and an isomeric mixture of **5** and **6** generated by the equilibration of **5** in buffer for 9 min.[14] The production of **7** was linked to the decrease in absorbance of nicotinamide adenine dinucleotide (NADH) at 340 nm ($\epsilon = 6220\ M^{-1}\ cm^{-1}$) by two coupled enzymatic reactions involving 2-oxo-4-hydroxyhepta-1,7-dioate (OHHD) aldolase and lactate dehydrogenase.[15] The action of OHHD aldolase on **7** results in the formation of pyruvate and succinate semialdehyde. The pyruvate is reduced to lactate by the inclusion of lactate dehydrogenase with the concomitant oxidation of NADH.

Two observations result from the kinetic studies that shed light on the mechanism. First, the initial rate of product formation using either the single isomer or the isomeric mixture (in which the total concentration of **5** and **6** is equal to the concentration of **5**) is comparable at all concentrations measured. Second, the results show that the K_m for the hydratase using the isomeric mixture is about half that measured using the single isomer (**5** and **6**, 8 ± 1 μM; **5**, 16 ± 2 μM) whereas the values of k_{cat} are comparable (**5** and **6**, 42 ± 1 s^{-1}; **5**, 47 ± 1 s^{-1}). As a result, the value of k_{cat}/K_m is 1.8-fold greater for the mixture than that obtained for the single isomer (**5** and **6**, $5.3 \times 10^6\ M^{-1}\ s^{-1}$; **5**, $2.9 \times 10^6\ M^{-1}\ s^{-1}$). A comparison of the specificity constants (i.e., k_{cat}/K_m) suggests that the enzyme has a slight preference for the isomeric mixture (consisting primarily of **6**). The combination of these two observations though suggests that product formation is not significantly influenced by a change in the isomeric composition of the substrate solution and that OHED hydratase readily processes either isomer.

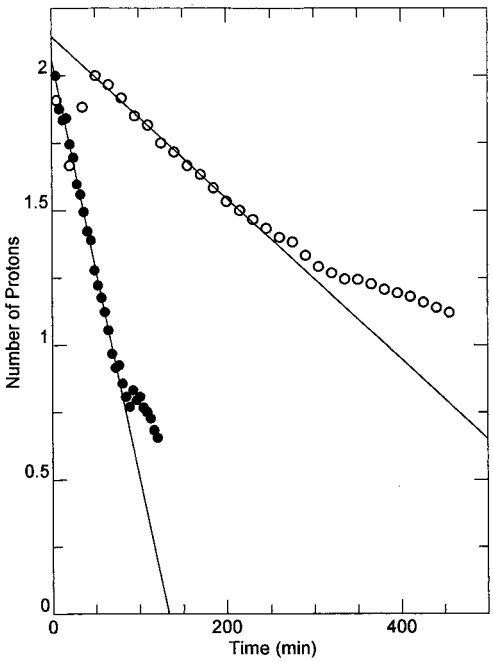

Figure 1. Rate of the buffer- and OHED hydratase-catalyzed C-3 proton exchange with solvent deuterons of 2-oxo-1,7-heptadioate (**9**). Each time point of the buffer-catalyzed exchange (open circles) and OHED-catalyzed exchange (filled circles) represents the fraction of protons remaining at C-3 of **9**. For the buffer-catalyzed exchange, spectra were acquired at 15 min intervals for 8 h. The initial spectrum was obtained after 5 min after mixing **9** with buffer. For the OHED hydratase-catalyzed exchange, spectra were acquired at 4 min intervals for 2 h. The initial spectrum was obtained after 6 min after the addition of OHED hydratase to a solution containing **9** in buffer. In both experiments, the initial intensity of the signal at 2.66 ppm (corresponding to the C-3 proton) is set equal to two protons. The subsequent measurements were divided by this initial intensity to give the fraction of the protons remaining. The data were fitted as described in the text.

OHED Hydratase-Catalyzed Exchange of the C-3 Protons of 2-Oxo-1,7-heptadioate (9) with ^2H_2O. The exchange of the C-3 protons for deuterons of the alternate substrate, **9**, was followed by ^{1}H NMR spectroscopy in the presence and in the absence of enzyme. Monitoring of the decrease in signal intensity at 2.66 ppm as a function of time allowed a first-order rate constant for each process to be obtained (Figure 1). For the nonenzymatic process, the rate was 0.003 min^{-1} (in 100 mM $Na_2[^2H]PO_4$ buffer, p[^{2}H] = 7.1). For the enzyme-catalyzed process, the rate was 0.016 min^{-1} (using 10 μM of OHED hydratase). Hence, the enzyme-catalyzed rate is 5.2-fold faster than the nonenzymatic rate. Interestingly, the nonenzymatic reaction appears to level off after the exchange of only one proton, whereas the enzymatic reaction does not. Presumably, the nonenzymatic and stereorandom incorporation

(13) GenBank accession number AF036583.

(14) An isomeric mixture of **5** and **6** having a similar composition can be generated by the addition of COHED to a solution containing **5**. If the enzymatically generated mixture is used in kinetic studies of OHED hydratase, comparable results are obtained.

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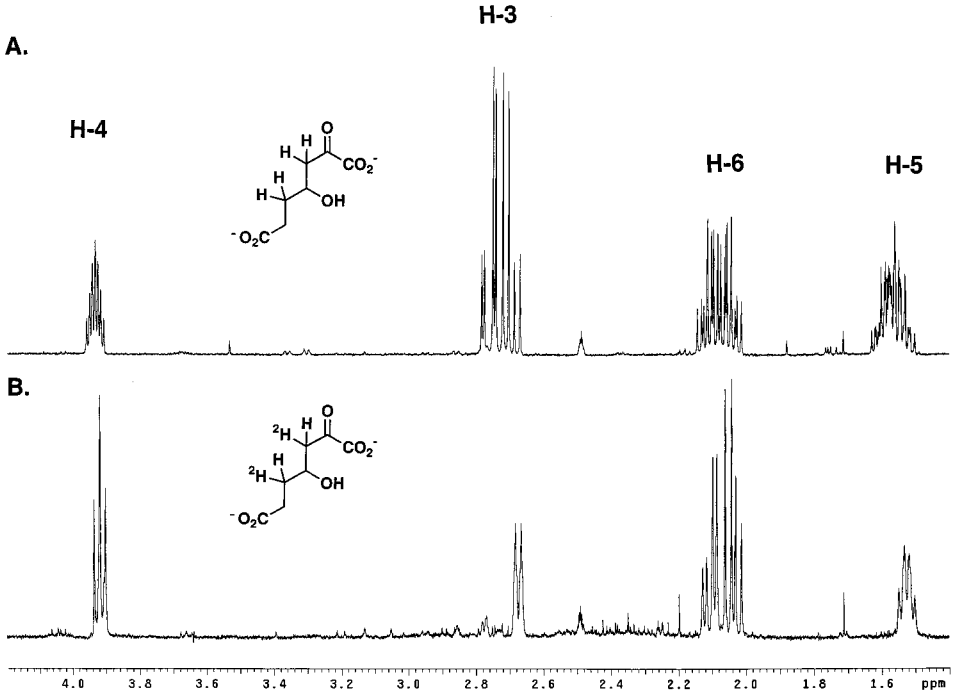


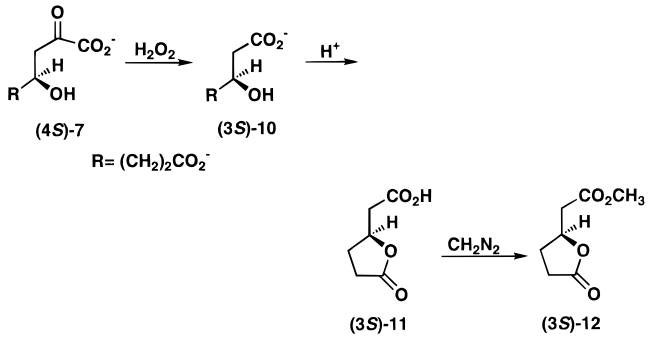
Figure 2. Partial 500 MHz ^{1}H NMR spectra of (A) the fully protio **7** generated from **5** by OHED hydratase in H_2O and (B) the dideuterated **7** generated from **5** by OHED hydratase in ^2H_2O. The 3-*pro*-*R* and 5-*pro*-*S* protons of **7** are replaced with deuterons.

of deuterium results in a substantial isotope effect on the reaction and greatly slows the rate of exchange. Thus, the observation that the nonenzymatic reaction levels off after the exchange of one proton may be a reflection of the reduced rate. With regard to the enzyme-catalyzed process, there may be some loss in the stereospecificity of the reaction when the enzyme uses **9**. Nonetheless, the enzyme-catalyzed reaction is clearly accelerated and this observation is consistent with an enzyme-catalyzed enolization and subsequent ketonization at C-3 (Scheme 4).

Identification and Stereochemical Assignment of 7. The identity of the product of the OHED hydratase-catalyzed reaction, **7**, was clearly established by ^{1}H and ^{13}C NMR spectroscopy. The ^{1}H NMR spectrum (Figure 2A) of **7** shows signals at δ 1.56 (H-5), 2.08 (H-6), 2.72 (H-3), and 3.92 (H-4). The ^{13}C NMR spectrum confirmed the presence of the two carboxylate groups (C-1 and C-7) and the carbonyl carbon at C-2.[16] Under these conditions, there was no spectroscopic evidence for the presence of the enol form of **7** as has been reported.[3b,11a] This previous conclusion was based on the observation of a compound having a λ_{max} at 260 nm. The compound, which was not isolated, was generated enzymatically from 3,4-dihydroxyphenyl acetate using crude cell extracts containing the necessary enzymes in the homoprotocatechuate meta-fission pathway to generate **7**.[3b] The enol form of **7** was identified by an unpublished NMR analysis.[3b] In the absence of this analysis and further experimental detail about the composition of the mixture used to generate the compound, it is not possible to reconcile our observations with the earlier observations.

To assign the stereochemistry at C-4 of **7**, it was chemically degraded to γ-(carboxymethyl)butanolide (**11**) and esterified to form the methyl ester, γ-((methoxycarbonyl)methyl)butanolide (**12**, Scheme 5). Subsequently, the optical rotations of both compounds (**11** and **12**) were compared to the optical rotations of the configurationally known compounds. Addition of OHED

Scheme 5



hydratase to a solution containing **5** resulted in the quantitative formation of **7**. Treatment of the mixture with hydrogen peroxide resulted in the formation of 3-hydroxyadipate (**10**), which was isolated by anion exchange chromatography as the lactone, γ-(carboxymethyl)butanolide (**11**), as indicated by ^{1}H NMR spectroscopy.[17] Esterification of **11** using diazomethane afforded **12** which was purified by flash chromatography.[18]

The absolute configuration of **11** has been established by the oxidative decomposition of *S*-(−)-β-tetralol and by the synthesis and resolution of a racemic mixture.[19,20] The optical rotations of the *R* isomers of **11** and **12** were found to be −33.6 and −36.4° (c = 1, C_2H_5OH), respectively.[19] The optical rotations of the samples of **11** and **12** obtained as described above were determined to be + 33.2 and + 34°, respectively. It is therefore concluded that the *S*-isomers of **11** and **12** have been obtained by the chemical degradation of **7** and that the product of the reaction catalyzed by OHED hydratase is (4*S*)-**7** (Scheme 5).

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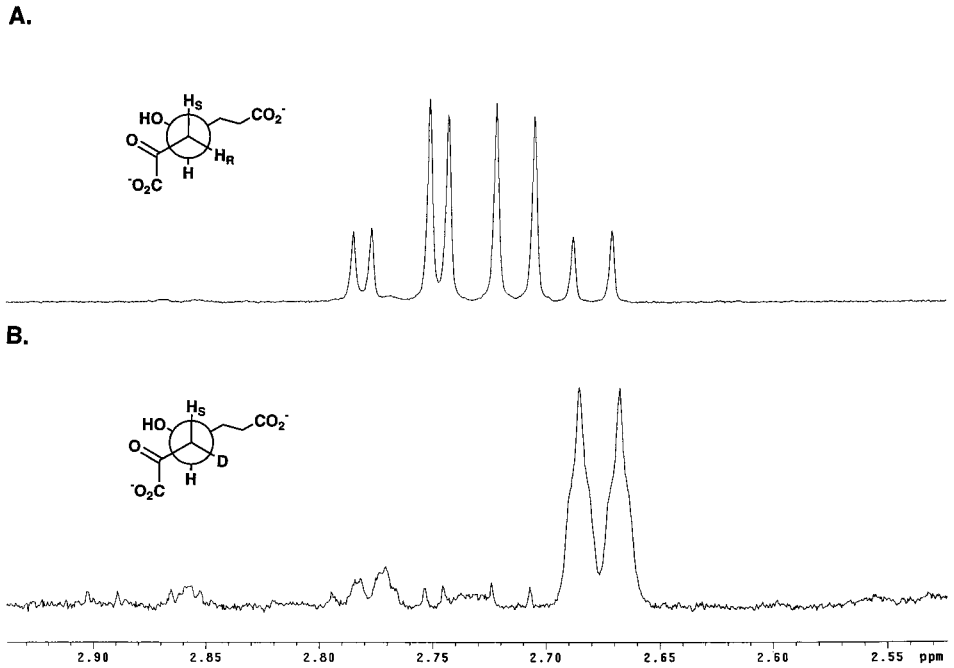

Figure 3. Partial 500 MHz ^{1}H NMR spectra showing the signals corresponding to the C-3 protons of (A) the fully protio **7** and (B) the dideuterated **7**. In (A) the downfield signal is assigned to the 3-*pro-R* proton and the upfield signal is assigned to the 3-*pro-S* proton as noted in the text. The vicinal coupling constant (8.3 Hz) for the remaining doublet in (B) indicates that OHED hydratase generates the 3*R* isomer of 3,5-di[^{2}H]**7** when the reaction is carried out in ^2H_2O.

OHED Hydratase-Catalyzed Incorporation of Deuterons at C-3 and C-5 of 7. The OHED hydratase-catalyzed generation of **7** from a solution containing **5** was carried out in ^2H_2O and followed by ^{1}H NMR spectroscopy in order to determine the position(s) of deuterium incorporation into product. A ^{1}H NMR spectrum corresponding to the fully protio **7** is shown in Figure 2A. The enzymatic reaction introduces a chiral center at C-4 of **7**. Thus, each diastereotopic proton on C-3 of unlabeled **7** results in a doublet of doublets, (one centered at 2.70 ppm and the other centered at 2.76 ppm), due to geminal coupling of the C-3 protons and vicinal coupling with the C-4 proton. The two diastereotopic protons on C-5 produce a complex signal centered at 1.56 ppm due to coupling with the adjacent protons on C-4 and C-6 and geminal coupling of the C-5 protons. The complexity of the signals centered at 2.08 ppm (corresponding to the protons on C-6) and 3.92 ppm (corresponding to the proton on C-4) is fully consistent with this analysis.

The introduction of a deuterium at C-3 and C-5 of **7** results in a considerably less complex ^{1}H NMR spectrum shown in Figure 2B. The stereospecific deuteration at C-3 of **7** by the hydratase results in the loss of the downfield quartet (2.76 ppm) and of coupling to the upfield resonance so that a broadened doublet (due to ^{1}H$-^2$H geminal coupling) at 2.68 ppm remains. The stereospecific deuteration at C-5 of **7** by the hydratase results in the loss of the downfield signal and of geminal coupling at C-5 so that a broadened doublet of doublets (due to ^{1}H$-^2$H geminal coupling) at 1.52 ppm remains. The signal assigned to the proton at C-6 now appears as a doublet of two doublets (2.08 ppm) due to ^{1}H$-^1$H geminal coupling and vicinal coupling to the single proton at C-5. Finally, the incorporation of a deuteron at both C-3 and C-5 simplifies the signal assigned to the C-4 proton (3.92 ppm) so that a doublet of doublets remains. Because only one of the prochiral hydrogens at C-3 and only one of the prochiral hydrogens at C-5 are lost (in both cases, this is reflected by the loss of the downfield signals) when the reaction is performed in ^2H_2O, the reaction is enzyme-catalyzed and both C-3 and C-5 are involved.

Assignment of the Stereochemistry at C-3 of 3,5-di[^{2}H]7. The stereochemistry at C-3 of 3,5-di[^{2}H]**7** is readily assigned by an analysis of the coupling constants in the ^{1}H NMR spectrum. Each diastereotopic proton on C-3 of unlabeled **7** results in a doublet of doublets, one centered at 2.70 ppm and the other centered at 2.76 ppm (Figure 3A). The vicinal coupling constant for the downfield doublet of doublets is 4.2 Hz whereas the vicinal coupling constant for the upfield doublet of doublets is 8.3 Hz. If the preferred conformation of **7** is dictated by hydrogen bonding between the hydroxy group at C-4 and the carbonyl group at C-2 as has been observed for similar molecules,[21] then the downfield signal can be assigned to the 3-*pro-R* proton whereas the upfield signal can be assigned to the 3-*pro-S* proton. Stereospecific incorporation of a deuteron at C-3 results in the loss of one signal and the collapse of the remaining one into a broadened doublet (Figure 3B). The vicinal coupling constant for the remaining proton at C-3 of 3, 5-di[^{2}H]**7** is 8.3 Hz. Thus, a deuteron has been incorporated at the 3-*pro-R* position indicating that OHED hydratase generates the 3*R* isomer of 3,5-di[^{2}H]**7** when the reaction is performed in ^2H_2O.

Assignment of the Stereochemistry at C-5 of 3,5-di[^{2}H]7. The stereochemistry at C-5 of 3,5-di[^{2}H]**7** was assigned by its enzymatic and chemical degradation to a monodeuterated succinate (Scheme 6). Accordingly, 3,5-di[^{2}H]**7** was generated by the addition of OHED hydratase to a solution containing **5**, in ^2H_2O. The presence of 2-oxo-4-hydroxyhepta-1,7-dioate (OHHD) aldolase in the reaction mixture resulted in the retro-aldol cleavage of 3,5-di[^{2}H]**7** to 2-[^{2}H]succinate semialdehyde (**13**) and pyruvate. Succinic semialdehyde, which was not isolated, was oxidized by potassium permanganate under acidic

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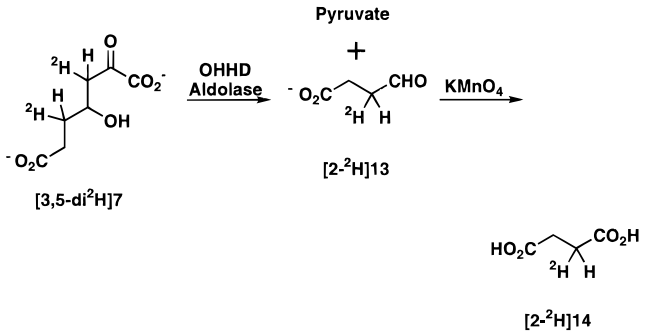
Scheme 6



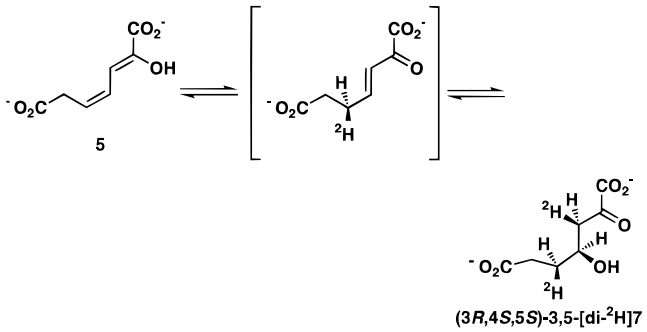
conditions to generate monodeuterated succinate (**14**).[4,22] The 2-[^{2}H]succinate was isolated by anion exchange chromatography and subjected to mass spectral analysis which indicated approximately 70% of the monodeuterated species.

The absolute configurations of both enantiomers of monodeuterated succinate have been unequivocally determined by their synthesis from monodeuterated malate and neutron diffraction analysis.[23] The CD spectrum of (2*S*)-[2-^{2}H]succinic acid shows a positive n to π^* Cotton effect at 210 nm with a molar ellipticity $[\theta]_{210} = +228°$ at 25 °C.[24] The purified [2-^{2}H]-**14** derived from the above reactions also exhibits a positive n to π^* Cotton effect at 210 nm with a molar ellipticity $[\theta]_{210} = +119°$ at 25 °C. It is therefore concluded that the *S* isomer of [2-^{2}H]**14** has been obtained and that OHED hydratase generates the 5*S* isomer of 3,5-di[^{2}H]**7** when the reaction is performed in ^2H_2O. The lower value observed for the molar ellipticity of our sample of [2-^{2}H]**14** is presumably due to the presence of the protio succinate (~20%) and the dideuterated succinate (~10%). Some loss of stereochemistry may also have occurred under the harsh conditions used in the oxidation of 2-[^{2}H]**13**.

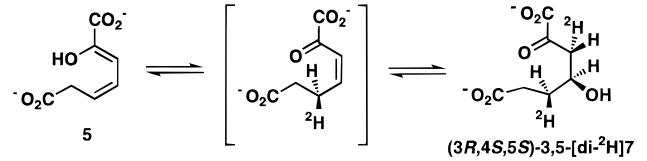
Assignment of the Absolute Stereochemical Course of the OHED Hydratase. To assign the overall stereochemical course of the isomerization/hydration reaction (i.e., **5** → **7**), it is necessary to determine whether the *E* or *Z* isomer of **8** is generated in the course of the reaction. Two experiments were carried out in an attempt to address this question. First, the conversion of **5** to **7** was monitored by a series of successive ^{1}H NMR spectra. There was no spectral evidence for the production of **8** in the course of the reaction suggesting that if OHED hydratase generates **8**, it remains enzyme-bound or it does not accumulate in solution in detectable quantities. In a second experiment, the 3*E* isomer of **8** was generated by the action of 4-OT on **5**. Subsequent incubation of 3*E*-**8** with OHED hydratase did not result in any detectable formation of **7**. Thus far, all attempts to generate or to synthesize the *Z* isomer of **8** have not been successful.

Because the configuration of **8** cannot be assigned, our results indicate that if the isomerization/hydration mechanism is operative, then the enzyme-catalyzed addition of water can occur in either a syn or an anti fashion (Schemes 7 and 8). If OHED hydratase generates the 3*E*-isomer, then the addition of a deuteron at C-5 of **5** and the subsequent hydration of the C-3 bond of **8** occur on the same face which is consistent with a

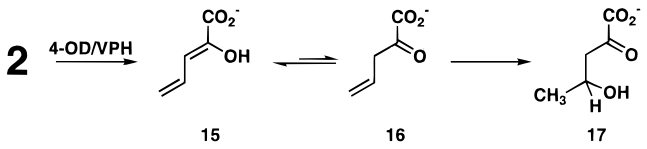
Scheme 7



Scheme 8



Scheme 9



one-base mechanism (Scheme 7).[12] If OHED hydratase generates the 3*Z*-isomer, then the enzyme-catalyzed hydration of **8** occurs in an anti fashion and the deuterons at C-3 and C-5 are incorporated on opposite faces (Scheme 8).[12] This would be consistent with a two-base mechanism.

Discussion

One step in both the catechol and homoprotocatechuate metafission pathways reportedly involves the enzyme-catalyzed addition of water to an isolated double bond using only magnesium or manganese as a cofactor.[2,11] In the former pathway, the reaction is catalyzed by vinylpyruvate hydratase while, in the latter pathway, OHED hydratase is responsible for this transformation. The proximity of a carbonyl group in both substrates suggests that these enzymes might utilize this carbonyl group in the mechanism in order to avoid the addition of water to an unactivated double bond.[12] A particularly attractive mechanism for this transformation involves a 1,3-allylic isomerization of the β,γ-unsaturated ketone to its α,β-isomer followed by the Michael addition of water.[25,26] To determine whether such a mechanism is operative for these two enzymes and to delineate further the evolutionary relationship between the two pathways, mechanistic and structural studies of both enzymes are being pursued.

Previous studies on the mechanism of a complex of 4-oxalocrotonate decarboxylase (4-OD) and vinylpyruvate hydratase (VPH) identified (4*S*)-2-oxo-4-hydroxypentanoate (**17**, Scheme 9) as the product of the complex and suggested that neither enzyme proceeded through a Schiff base intermediate.[17] The

(22) Chickos, J. S.; Bausch, M.; Alul, R. *J. Org. Chem.* **1981**, *46*, 3559−3562.

(23) (a) Cornforth, J. W.; Ryback, G.; Popjak, G.; Donninger, C.; Schroepfer, G. *Biochem. Biophys. Res. Commun.* **1962**, *9*, 371−375. (b) Englard, S.; Britten, J. S.; Listowsky, I. *J. Biol. Chem.* **1967**, *242*, 2255−2259. (c) Yuan, H. S. H.; Stevens, R. C.; Fujita, S.; Watkins, M. I.; Koetzle, T. F.; Bau, R. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 2889−2893.

(24) Ringdahl, B.; Craig, J. C.; Keck, R.; Retey, J. *Tetrahedron Lett.* **1980**, *21*, 3965−3968.

(25) (a) Schwab, J. M.; Klassen, J. B. *J. Chem. Soc., Chem. Commun.* **1984**, 296−297. (b) Schwab, J. M.; Klassen, J. B. *J. Chem. Soc., Chem. Commun.* **1984**, 298−299.

(26) Arabonate dehydrase and β-hydroxydecanoyl thiol ester dehydrase[21a,25] catalyze analogous reactions although these enzyme-catalyzed reactions involve a dehydration reaction followed by a 1,3-allylic rearrangement: Portsmouth, D.; Stoolmiller, A. C.; Abeles, R. H. *J. Biol. Chem.* **1967**, *242*, 2751−2759.

substrate for the VPH-catalyzed reaction in this complex is proposed to be **16** on the basis of a stereochemical study showing that the decarboxylase generates 2-oxo-3-[^{2}H]-4-pentenoate (**16**) stereospecifically from 2-hydroxy-2,4-pentadienoate (**15**) in ^2H_2O. This result combined with other results supported the premise that the conversion of **16** to **17** involved the allylic isomerization of **16** to its α,β-isomer followed by hydration of the α,β-isomer. Further support for this mechanism can be obtained from a stereochemical analysis of the VPH-catalyzed reaction, but such an analysis is complicated by two observations: VPH is complexed with 4-OD which introduces a deuteron at C-3 of **17** (in ^2H_2O), and a stereochemical analysis at C-5 of **17** requires the introduction of a chiral methyl group. Hence, we turned our attention to OHED hydratase because the reaction is more amenable to a stereochemical analysis.

It was previously found that the decarboxylation of **4** by COHED generates a mixture of **5** and **6**. Because **5** and **6** rapidly interconvert in aqueous buffer, it was not clear whether COHED generated a single isomer as its product.[9,10] Moreover, if COHED did produce one isomer, the identity of that isomer was unknown. To address this question (because the answer identifies the substrate for OHED hydratase), **5** was isolated and incubated with COHED in ^2H_2O. The ketonization of **5** to [3-^{2}H]**6** was highly stereoselective supporting the hypothesis that the COHED-catalyzed decarboxylation of **4** yields a single isomer (i.e., **6**) as its product and proceeds through the intermediate **5**.[10]

It can be inferred from this stereochemical finding that OHED hydratase will show a preference for one isomer. This prompted us to examine the kinetic parameters of the enzyme using **5** and a mixture of **5** and **6** (as **6** cannot be synthesized or isolated). After 12 min, the decay of **5** in buffer led to a solution consisting primarily of **6** (~72%).[10] A comparison of the kinetic parameters measured for OHED hydratase showed that there is not an appreciable difference in the rate of product formation (**7**) using either **5** or a mixture of **5** and **6** even though the concentration of **6** predominates in the latter solution. At first glance, these kinetic results appear to be at odds with the stereochemical results on COHED. There are two explanations for this apparent contradiction. If the isomerization/hydration mechanism is operative, then **5** will be an intermediate in the allylic isomerization of **6** to **8** (Scheme 3). To be a kinetically competent intermediate, **5** should be processed at a rate comparable to that of **6**. Alternately, it may be that **5** and **6** are readily interconverted in solution or at the enzyme's active site at a sufficiently fast rate so that the rate of this transformation does not limit the rate of product formation.

An isomerization/hydration mechanism for OHED hydratase predicts that the enzyme will incorporate a deuteron at both C-3 and C-5 of **7** in addition to the hydroxyl group at C-4 when the reaction is performed in ^2H_2O. In accord with this prediction, the isotopic labeling studies and subsequent stereochemical analyses clearly demonstrate that a deuteron is stereospecifically incorporated at each position. Stereospecificity is the hallmark of an enzymatic reaction so that these observations are consistent with an enzyme-catalyzed process.[4] Further evidence implicating the involvement of C-3 in the mechanism comes from the observation that OHED hydratase accelerates the exchange of the C-3 protons of **9**, a nonhydrated substrate analogue for the enzyme.

The observation that 3*E*-**8** is not chemically competent in the reaction and that neither isomer of **8** can be detected spectroscopically has three possible explanations.[27] First, the 3*Z*-isomer may be the actual intermediate in the reaction. One difficulty with this explanation is that the 3*Z* geometry puts the two carboxylate groups in what appears to be a less favorable conformation because of their closer proximity. Moreover, the fact that this isomer cannot be detected in a base-catalyzed conversion of **5** to **8** suggests that it is less stable. On the enzyme, however, the two charged carboxylate groups may be neutralized by active site residues thereby stabilizing the intermediate. A second possibility is that the protonation state of the ground-state enzyme enables it to catalyze an isomerization reaction but not a hydration reaction. Thus, the enzyme is in the wrong protonation state to hydrate **8**. Finally, the concentration of **8** may not reach a detectable level (under steady-state conditions) in the reaction because it is readily hydrated to generate product.[28] If such is the case, then it may be possible to observe the intermediate in a one-turnover experiment. Such an experiment is in progress.

The proposed mechanism involves an enzyme-catalyzed isomerization reaction followed by an enzyme-catalyzed hydration reaction. The stereochemical courses of such reactions have been used to establish whether the corresponding enzymes utilize a one-base or a two-base mechanism. Hanson and Rose first noted that several isomerases catalyzing a suprafacial 1,3-allylic rearrangement (with intramolecular proton transfer) utilize a single catalytic residue.[12a] Likewise, syn eliminations (and, conversely, syn additions) are observed for enzymes in which the double bond is adjacent to either a carbonyl or carboxylate group and are catalyzed by a single catalytic residue.[12a] Schwab and colleagues further developed a correlation between the structural features of the substrate for an allylic isomerization and its stereochemical course.[12b] Gerlt and Gassman presented an elegant argument indicating that these trends could be explained by the pK_a value of the proton adjacent to a carboxylate anion, an aldehyde, a carbonyl group, or a thioester.[29] The higher pK_a value of the α-proton to a carboxylate anion necessitates an anti addition (or elimination) while the lower pK_a values of the protons adjacent to either an aldehyde, a carbonyl group, or a thioester allow for a syn addition (or elimination). Thus, the observed stereochemical course reflects a mechanistic necessity.

On this basis, the stereochemical course of the OHED-catalyzed reaction can be predicted. Inspection of the substrate for OHED hydratase (assuming that the substrate is **6**) suggests that it will undergo facile deprotonation at C-3 because of the adjacent carbonyl group.[12b,29] Hence, the putative allylic isomerization catalyzed by OHED hydratase will proceed suprafacially which is consistent with a one-base mechanism. Likewise, inspection of the product, **7**, shows that it will undergo facile deprotonation at C-3 due to the adjacent carbonyl group indicating that the elimination of water from **7** (and, by microscopic reversibility, the addition of water to **8**) will be syn also implicating a one-base mechanism in this transformation.[12b,29]

Because the configuration of **8** has not been established, the overall stereochemical course of these reactions cannot be assigned and this prediction cannot yet be verified. As shown in Scheme 7, if 3*E*-**8** is the intermediate, then both the allylic

(27) A fourth explanation is that **8** is not the intermediate in the reaction and the isomerization/hydration mechanism is not operative.

(28) There are examples of other enzymatic reactions in which a chemically reasonable intermediate is not processed by the enzyme: (a) Cleland, W. W. *Biochemistry* **1990**, *29*, 3194−3197. (b) Anderson, K. S.; Johnson, K. A. *Chem. Rev.* **1990**, *90*, 1131−1149.

(29) Gerlt, J. A.; Gassman, P. G. *J. Am. Chem. Soc.* **1992**, *114*, 5928−5934.

isomerization and hydration reactions occur on the same face of the substrate. This suggests that the same single base may be involved in both reactions and the OHED hydratase-catalyzed transformation of **6** to **7** and the COHED-catalyzed decarboxylation of **4** to **6** take place on opposite faces. If, on the other hand, 3*Z*-**8** is the intermediate, then the allylic isomerization is antarafacial and two residues may be involved: one residue (B_1) abstracts a proton at C-3 of **6** while a second residue (B_2) places a proton at C-5 of **7**. The same two residues are then used by the enzyme to catalyze the addition of water to **8**. In one mechanistic scenario, one residue (B_2) removes a proton from water activating it for attack at C-4 of **8** while a second residue (B_1) supplies the proton at C-3 of **7**.

It is tempting to invoke the body of literature precedence and conclude that OHED hydratase uses a single residue to catalyze a suprafacial allylic rearrangement followed by a syn hydration. Recently, however, Mohrig[30] and Gerlt[31] provided evidence questioning the validity of the relationship between the substrate structure and reaction stereochemistry and the mechanism of addition−elimination reactions. Mohrig and colleagues found that the nonenzymatic hydration of (*S*)-crotonyl *N*-acetylcysteamine (**18**) favored the anti addition of water whereas the enzyme, crotonase, catalyzed a syn addition of water to **18**.[30] This discovery indicates that there is not a mechanistic imperative for the syn addition of water to **18**. Gerlt and co-workers found that glucarate dehydratase (GlucD) catalyzed a syn β-elimination of water using D-glucarate as the substrate and an anti β-elimination of water using L-idarate as the substrate. These two substrates differ only by the configuration at C-5. Because both reactions involve the abstraction of a proton adjacent to a carboxylate anion, the predicted stereochemical course of the dehydration reaction is anti. Nonetheless, the experimental finding is that GlucD shows no preference for one route of elimination. The stereochemical courses of these reactions may result not from a mechanistic imperative but from the conservation of the active-site scaffolding (and the stereochemical course) of the progenitor enzyme.[30,31] In view of these results, further experimentation will be required to determine whether the reaction catalyzed by OHED hydratase involves a one-base or a two-base mechanism.

Experimental Section

Materials. All reagents, buffers, and solvents were obtained from either Aldrich Chemical Co. or Sigma Chemical Co. unless noted otherwise. 5-(Carboxymethyl)-2-hydroxymuconate, 2-oxo-3-pentynoic acid, and 4-oxalocrotonate tautomerase were obtained by procedures described elsewhere.[6,32,33] Tryptone and yeast extract were obtained from Difco (Detroit, MI). Centricon (10 000 MW cutoff) centrifugal microconcentrators were obtained from Amicon. Isopropyl-β-D-thiogalactoside (IPTG) and thin-walled PCR tubes were obtained from Ambion, Inc. (Austin, TX). The expression vector pET24a(+) was obtained from Novagen, Inc. (Madison, WI). Restriction enzymes, T4 DNA ligase, agarose, and PCR reagents were obtained from either Promega Corp. (Madison, WI), GibcoBRL (Gaithersburg, MD), or Boehringer Mannheim Corp. (Indianapolis, IN). Oligonucleotides for the PCR, colony screening, and DNA sequencing were synthesized and provided deprotected and desalted by Oligos Etc. Inc. (Wilsonville, OR).

Strains. *Escherichia coli* strain C was obtained from the *Escherichia coli* Genetic Stock Center, Yale University, New Haven, CT. *E. coli* strain DH5α from GibcoBRL (Gaithersburg, MD) was used for the transformation and propagation of recombinant plasmids. *E. coli* lysogen B strain BL21(DE3) was obtained from Novagen and used for expression of the recombinant proteins. Cells for general cloning and expression were grown in Luria-Bertani media (LB) supplemented with kanamycin (Kn, 100 μg/mL). The composition of LB media is described elsewhere.[34]

General Methods. Techniques for restriction enzyme digestions, ligation, transformation, and other standard molecular biology manipulations were based on methods described elsewhere.[34] Plasmid DNA was introduced into cells by electroporation using a Cell-Porator Electroporation System (GibcoBRL, Gaithersburg, MD). The colony screening technique is based on a method described in the pET System Manual (Novagen, Inc., sixth ed., Aug 1995). DNA sequencing was done at the University of Texas (Austin) Sequencing Facility on a Perkin-Elmer/ABI Prism 377 DNA Sequencer according to the instructions provided with the ABI Prism Dye-Terminator kit. The base sequence is determined by analyzing fluorescent dye-labeled nucleotide fragments. Kinetic data were obtained on either a Hewlett-Packard 8452A diode array spectrophotometer or a Perkin-Elmer Lambda Bio 10 UV/vis spectrometer. HPLC was performed on a Waters system using a Waters Protein Pak DEAE 5PW anion exchange column (10-μm particle size). Protein was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) under denaturing conditions on 15% gels.[35] Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard.[36] NMR spectra were obtained using either a Bruker AM-250 spectrometer or a Varian Unity INOVA-500 spectrometer as noted. The lock signal is dimethyl-d_6 sulfoxide unless noted otherwise. Chemical shifts are standardized to the dimethyl-d_6 sulfoxide signal at 2.49 ppm unless noted otherwise.

Isolation of Genomic *E. coli* Strain C DNA. The genomic DNA was prepared by a published procedure with the following modifications.[37] The cells were suspended in a buffer consisting of 20 mM Tris-HCl, 200 mM NaCl, and 10 mM EDTA, pH 8.0, and made 1 mg/mL in lysozyme. After incubation at 37 °C for 30 min, the solution was made 0.5% in SDS and incubated at 50 °C for 1 h. The solution was chilled on ice and extracted with an equal volume of phenol equilibrated to pH 7.8 with Tris buffer (US Biochemical, Cleveland, OH). The aqueous layer was removed and extracted (2×) with an equal volume of $CHCl_3$. The DNA was precipitated from the aqueous layer using sodium acetate and ethanol as described elsewhere.[34] The DNA was suspended in sterile water and treated with RNase (∼14 000 units) at 37 °C for 30 min. Subsequently, the solution was extracted with a mixture of phenol/$CHCl_3$/isoamyl alcohol (US Biochemical, Cleveland, OH) and centrifuged for 10 min (14000*g*). The DNA was precipitated from the aqueous layer as described above, suspended in sterile water (1 mL), and stored at ∼20 °C.

Construction of the Expression Vector for the Production of OHED Hydratase. The availability of the gene sequence (*hpcG*) for OHED hydratase facilitated the design of the primers for the PCR.[11] Two oligonucleotides, 5′-GACATG*CATATG*TTCGATAAAC-3′ and 5′-CGATCG*GTCGAC*TTAAACAAAGCGGC-3′, were synthesized. The first primer contains an *Nde*I restriction site (italicized) followed by 10 bases corresponding to the coding sequence of the *hpcG* gene, whereas the second primer contains a *Sal*I site (italicized) followed by 14 bases corresponding to the complementary sequence of the *hpcG* gene. Amplification of the *hpcG* gene was carried out by the PCR in a Perkin-Elmer 480 DNA thermal cycler using the two synthetic primers, genomic DNA isolated from *E. coli C* as template, and PCR reagents supplied by GibcoBRL. Each 175 μL reaction for the PCR contained 10× buffer (200 mM Tris-HCl, 500 mM KCl, pH 8.4, 17.5

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(32) Czerwinski, R. M.; Johnson, W. H., Jr.; Whitman, C. P.; Harris, T. K.; Abeygunawardana, C.; Mildvan, A. S. *Biochemistry* **1997**, *36*, 14551−14560.
(33) Johnson, W. H., Jr.; Czerwinski, R. M.; Fitzgerald, M. C.; Whitman, C. P. *Biochemistry* **1997**, *36*, 15724−15732.
(34) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.
(35) Laemmli, U. K. *Nature* **1970**, *227*, 680−685.
(36) Bradford, M. *Anal. Biochem.* **1976**, *72*, 248−254.
(37) Chow, L. T.; Kahmann, R.; Kamp, D. *J. Mol. Biol.* **1977**, *113*, 591−609.

μL), dATP, dTTP, dGTP, and dCTP (14 μL of a stock solution containing 2.5 mM of each dNTP), primers (17.5 μL each from 10 μM stock solutions), Taq DNA polymerase (0.875 μL of a 5 units/μL solution), $MgCl_2$ (10.5 μL of a 25 mM stock solution), template DNA (3.5 μL of a 0.54 μg/μL solution), and sterile water (93.7 μL). The mixture was divided into three equal portions, placed into separate sterile 0.5 mL thin-walled reaction tubes, and layered with sterile paraffin oil. The PCR protocol consisted of 25 cycles, a 2-min incubation period at 94 °C preceding the 25 cycles, and a 5-min incubation period at 72 °C following the 25 cycles. Each cycle consisted of three steps: denaturation at 94 °C for 1 min, annealing at 55 °C for 75 s, and elongation at 72 °C for 75 s. The reaction tubes were maintained at 4 °C upon completion. The amplified products were extracted from the oil using $CHCl_3$ containing oil red O according to a published protocol.[38] The products were analyzed by electrophoresis on a 1% agarose gel using TAE buffer (4.84 g of Tris base/L, 1.1 mL of acetic acid/L, 2 mL of 0.5 M EDTA solution, pH 8.0/L) containing 0.8 μg/mL ethidium bromide, visualized by UV transillumination, excised, and extracted from the gel piece by centrifugation through gel blot paper (Schleicher and Schuell, Keene, NH) according to a published procedure.[39] The isolated DNA was precipitated from solution using 3 M sodium acetate (0.1 × vol, pH 7.0) and chilled 100% ethanol (2 × vol), incubated at −20 °C for 30 min, centrifuged, rinsed with 70% ethanol, air-dried (37 °C for 5 min), resuspended in sterile water (100 μL), and stored at −20 °C.[34] The resulting PCR product and the pET24a(+) were digested with *Nde*I and *Sal*I restriction enzymes as described elsewhere,[34] purified on a 1% agarose gel, and eluted from the gel as described above. Subsequently, the linearized vector and the PCR product were ligated using T4 DNA ligase (0.6 units) at 16 °C overnight as described elsewhere.[34] The DNA was precipitated from each ligation mixture as described above and resuspended in sterile water (10 μL). Aliquots (1 μL) were used to transform *E. coli* strain DH5α by electroporation following the manufacturer's directions. An aliquot of the transformed cells was grown on an LB/Kn (100 μg/mL) agar plate at 37 °C overnight. Single colonies were chosen at random and screened for the presence of insert by the PCR using two primers: one that is complementary to the sequence thirteen bases upstream of the first base for the T7 promoter region (5′-GATCTCGATCCCGCGAAATTAATACG-3′) and one that is complementary to the sequence of the His•Tag region of the pET-24a(+) vector (5′-CAGTGGTGGTGGTGGTGGTG-3′). The presence of the insert is indicated by the observation of a PCR product of the appropriate size (∼900 base pairs). Accordingly, the individual colonies were suspended in sterile water (50 μL) and incubated in boiling water bath for 5 min. The resulting solution was centrifuged (12 000*g*) for 1 min, and 10 μL of the supernatant was added to a 40 μL mixture containing 10× buffer (5 μL), dNTPs (1 μL), primers (1 μL each from 5 μM stock solutions), Taq DNA polymerase (0.25 μL of a 5 units/μL solution), $MgCl_2$ (3 μL), and sterile water (28.75 μL). The PCR protocol consisted of 35 cycles, a 2-min incubation period at 94 °C preceding the 35 cycles, and a 5-min incubation period at 72 °C following the 35 cycles. Each cycle consisted of three steps: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 2 min. The reaction tubes were maintained at 4 °C upon completion. The products were analyzed by agarose gel electrophoresis as described above. Positive colonies were grown in liquid LB/Kn (10 mL, 100 μg/mL) medium overnight, and the newly constructed plasmid (designated pET24a-OHEDH) was isolated by the alkaline lysis method.[34] The isolated plasmid was suspended in TE buffer (50 μL, 10 mM Tris-Cl, 1 mM EDTA, pH 8.0), treated with RNase (1 μL of a 5.5 mg/mL solution), made 2.5 M in ammonium acetate, chilled for 10 min on ice, and centrifuged. The DNA was precipitated from the aqueous layer as described above and suspended in sterile water (100 μL). Subsequently, the plasmid was introduced into *E. coli* lysogen B strain BL21(DE3) by electroporation as described above. For sequencing, a single colony was grown in liquid LB/Kn (500 mL, 100 μg/mL) medium overnight, the cells were collected by centrifugation, and washed once with STE buffer (100 mM NaCl, 10 mM Tris-Cl, 1 mM EDTA, pH 8.0). The plasmid was isolated using the Wizard Maxiprep DNA Purification System (Promega Corp., Madison, WI) following the manufacturer's directions.

Overexpression and Purification of the Recombinant OHED Hydratase. A single colony of the expression strain containing pET24a-OHEDH was used to inoculate 50 mL of LB/Kn (100 μg/mL) medium. After overnight growth at 37 °C, a sufficient portion of the culture was used to inoculate 500 mL of LB/Kn (100 μg/mL) medium in a 2 L Erlenmeyer flask so that the initial OD_{600} was 0.05. Cultures were grown to an OD_{600} of ∼1.0 at 37 °C, with vigorous shaking, and then induced with IPTG (0.5 mM final concentration). Incubation was continued for 3 h at 37 °C. Cells were harvested by centrifugation (7000*g*, 12 min) and stored at −80 °C. Typically, 3 L of culture grown under these conditions yields 8−9 g of cells.

In a typical procedure, the cells (6−10 g) are thawed and suspended in a volume of buffer A (20 mm Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 0.1 mM EDTA, 1.0 mM DTT) containing 2M NaCl that is 10× the cell weight. The cells are disrupted at 4 °C by sonication with 6−8 successive pulses (45 s) spaced approximately 10 min apart from a Heat Systems W-385 sonicator equipped with a 0.5 in. tapped horn delivering approximately 330 W/pulse. Immediately after the initial pulse, the solution is made 1 mM in two protease inhibitors (100 mM phenylmethylsulfonyl fluoride dissolved in ethanol and 100 mM 6-aminocaproic acid dissolved in buffer A containing 2 M NaCl). The sonicated solution is centrifuged (30 000*g*) at 4 °C for 30 min. The pellet is discarded, and the supernatant is centrifuged (150 000*g*) at 4 °C for 3 h. Subsequently, the supernatant is diluted with an equal volume of buffer A containing 2 M NaCl and loaded onto a 20 mL fast-flow Phenyl Sepharose 6 column (Pharmacia) at 4 °C which had been equilibrated in buffer A containing 2 M NaCl. The supernatant was loaded overnight at a flow rate of 0.2 mL/min, and fractions (6 mL) were collected. After the supernatant had been completely loaded onto the column, the fraction size was decreased (3 mL), and the column was washed with the equilibration buffer (20 mL) at a flow rate of ∼0.2 mL/min. After the wash step, the absorbance of the eluant at 280 nm decreased to a constant value. There was little significant OHED hydratase activity in these fractions. Subsequently, OHED hydratase was eluted by a two-step decrease in the NaCl concentration. In the first step, the column was washed with buffer A made 1.0 M in NaCl (∼50 mL). In the second step, the column was washed with buffer A made 0.025 M in NaCl (∼30 mL). The fractions with the highest activity in the second wash were pooled (∼40 mL) and injected in three portions (∼12 mL each) into a Waters Protein Pak DEAE 5PW anion exchange column (15 × 21.5 cm) attached to a Waters HPLC system. The column had been previously equilibrated with buffer A made 0.1 M in NaCl at a flow rate of 2 mL/min. After the column was washed for 20 min with the equilibration buffer, the protein was eluted using a linear NaCl gradient (0.1−0.4 M NaCl in 100 min) in Buffer A. The eluant was monitored at 280 nm, and 2 mL fractions were collected. The OHED hydratase activity elutes at ∼0.25 M NaCl. The appropriate fractions from each run were pooled and stored at 4 °C at concentrations below 2 mg/mL. The enzyme precipitates upon storage at higher concentrations. Typically, the yield of purified protein (>95% as assessed by SDS−PAGE) per liter of culture is 5−9 mg.

Construction of the Expression Vector for the Production of 2-Oxo-4-hydroxyhepta-1,7-dioate (OHHD) Aldolase. Primers for the amplification of the gene for OHHD aldolase (*hpcH*) by PCR were designed using the reported gene sequence.[40] Two oligonucleotides, 5′-ATCAGTACTGAT*CATATG*GAAAACAGCTTTA-3′ and 5′-TGACAGATCAGT*CTCGAG*TCAATACACGCCGGGCTT-3′ were synthesized. The first primer contains an *Nde*I restriction site (italicized) followed by 13 bases corresponding to the coding sequence of the *hpcH* gene, whereas the second primer contains a *Xho*I site (italicized) followed by 14 bases corresponding to the complementary sequence of the *hpcH* gene. The PCR was carried out as described above using reagents supplied by Promega Corp. and Boehringer Mannheim Corp. in a 200 μL reaction. The mixture was divided into four equal portions and processed using the PCR protocol described above for the

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amplification of the *hpcG* gene with an initial incubation time of 3 min. The resulting PCR product was purified, digested with *Nde*I and *Xho*I restriction enzymes, and ligated into the similarly digested pET24a(+) using the procedures described above. The DNA was precipitated and used to transform *E. coli* strain DH5α as described above. Transformed cells were grown on an LB/Kn (100 μg/mL) agar plate at 37 °C overnight. Single colonies were chosen at random and screened for the presence of insert by the PCR as described above using two different primers. One primer (5′-TAATACGACTCACTATAGG-3′) is complementary to the sequence for the T7 promoter region of the pET-24a(+) vector, and the second primer (5′-TAGTTATTGCT-CAGCGGT-3′) is complementary to the sequence for the T7 terminator region. The presence of insert is indicated by the observation of a PCR product of the appropriate size (~1000 base pairs). A positive colony was grown in liquid media, and the newly constructed plasmid (designated pET24a-OHHDA) was isolated and used to transform the expression strain by the procedures described above.

Overexpression and Partial Purification of OHHD Aldolase. A single colony of the expression strain containing pET24a-OHHDA was grown in liquid media and induced with IPTG as described above. Cells were harvested by centrifugation (7000*g*, 12 min) and stored at −80 °C. Typically, 3 L of culture grown under these conditions yields 8−9 g of cells. The cells (~3 g) are suspended in a volume of buffer A (10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 0.1 mM EDTA, 1.0 mM DTT) containing 0.1 M NaCl that is 5× the cell weight. The cells are disrupted by sonication and centrifuged (30 000*g*) at 4 °C for 30 min. The pellet is discarded and the supernatant is centrifuged (150 000*g*) at 4 °C for 3 h. The enzymatic activity was identified in a coupled assay using lactate dehydrogenase as described below. The supernatant is stored at 4 °C and used as part of a coupled assay to monitor OHED hydratase activity.

Assay of OHED Hydratase Activity. OHED hydratase activity was monitored in 10 mM NaH_2PO_4, 5 mM $MgCl_2$ (pH 7.3) by either a noncoupled or a coupled assay. In the noncoupled assay, OHED hydratase activity was monitored by following the decrease in the absorbance at 276 nm ($\epsilon = 12\,340\ M^{-1}\ cm^{-1}$) due to the consumption of **5**. For the coupled assay, the assay mixture contained 950 μL of 10 mM NaH_2PO_4 buffer made 5 mM in $MgCl_2$ (pH 7.3), 20 μL of a 10 mg/mL solution of NADH (disodium salt) dissolved in 10 mM NaH_2-PO_4 buffer (pH 7.3), 20 μL of a 2 mg/mL solution of lactate dehydrogenase in buffer A (10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 0.1 mM EDTA, 1.0 mM DTT) made 0.1 M in NaCl and in 50% glycerol, 10 μL of OHHD aldolase, and OHED hydratase (0.1 μg in 0.3 μL). The assay was initiated by either the addition of **5** or the isomeric mixture of **5** and **6** (1−14 μL). The reported kinetic parameters were obtained using the coupled assay in a nonequilibrium and in an equilibrium experiment. An increase in the concentration of either lactate dehydrogenase or OHHD aldolase did not affect the rate of product formation. In the nonequilibrium experiment, **5**, dissolved in ethanol, was added to the cuvette without preequilibration so that the enzyme processed a mixture containing only **5**. In the equilibrium experiment, **5** had been allowed to preequilibrate for 9 min so that the enzyme was presented with a mixture of **5** and **6**. A 10 mM (based on the concentration of **5**) preequilibration solution contained **5** (4 mg) in 0.6 mL of 100 mM Na_2HPO_4 (pH ~9.1) buffer, water (1.7 mL), and 5.5 μL of a 2M $MgCl_2$ solution. The addition of **5** to the preequilibration solution adjusted the pH to ~7.3. After 9 min, the solution contained ~30% **5** and ~70% **6**. There was no appreciable amount of **8** present. Cuvettes were mixed by stirring. The kinetic data were fitted by nonlinear regression data analysis using the Grafit program (Erithacus Software Ltd., Stained, U.K.) obtained from Sigma Chemical Co. All results are reproducible in multiple runs.

2-Hydroxy-2,4-heptadiene-1,7-dioate (5). The isolation of **5** was performed using a published procedure with the following modifications.[10] The solution of **3** was made up as described, and the pH was adjusted to 7.1 using 1 M NaOH. Subsequently, it was made 5 mM in $MgCl_2$ by the addition of 2 M $MgCl_2$ (~0.25 mL). CHMI and COHED were added in four equal portions (50 μL each) at 1 h intervals (200 μL total). The flask was covered with aluminum foil, and the reaction mixture was stirred at 23 °C overnight. After the pH of the solution was adjusted to 1 (by the addition of aliquots of 1 M HCl), it was filtered through a layer of Celite on a Whatman No. 1 filter paper packed in a sintered glass funnel. The filtrate was extracted with ethyl acetate (3 × 100 mL), and the organic layers were pooled, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness to yield **5** as a yellow residue. The compound was purified further by crystallization as described. A ^1H NMR spectrum corresponded to the previously reported spectrum.[10] The compound is stored at −20 °C in a container wrapped in aluminum foil.

2-Oxo-1,7-heptadioate (9). The synthesis of **9** was accomplished by the adaptation of a literature procedure as follows.[41,42] To a well-stirred suspension of NaH (60% oil dispersion, 1.04 g, 43 mmol) in anhydrous THF (60 mL) under argon was added dropwise a solution of ethyl 5-bromovalerate (5.4 g, 26 mmol) and ethyl 1,3-dithiolane-2-carboxylate (5.0 g, 26 mmol) in a mixture of THF (10 mL) and anhydrous DMF (5 mL).[42] The reaction vessel was chilled in an acetone/ice bath. Subsequently, it was allowed to warm to room temperature and stirred overnight. The mixture was diluted into 200 mL of water, and the pH was adjusted to ~4.5 using a 10% solution of NaH_2PO_4. The resulting mixture was extracted with a mixture of hexanes/ethyl acetate (1:1, 2 × 200 mL), the organic layers pooled, washed with water, and dried over anhydrous $MgSO_4$. Evaporation in vacuo resulted in the dithiolane-protected diethyl ester of **9** (9.2 g) as an oil which was subjected to flash chromatography (12:1 hexanes, ethyl acetate). Subsequently, the compound was processed to the diethyl ester of **9** following the method of Corey and Erickson.[43] Accordingly, a solution of the crude material (1 g, 3.3 mmol) in acetonitrile was added dropwise over 2 min to a solution of *N*-bromosuccinimide (2 g, 5.6 mmol) and $AgNO_3$ (0.25 g, 1.5 mmol) in aqueous acetonitrile (20 mL, 10% H_2O, v/v). The reaction vessel was chilled in an acetone/ice bath and allowed to react for 3 min. It was then diluted with water (100 mL), extracted with ethyl acetate (3 × 200 mL), dried over anhydrous Na_2SO_4, and evaporated to dryness. The oily residue was purified further by flash chromatography (2:1 hexanes, ethyl acetate) to give the diethyl ester of **9** as a pale yellow oil (0.42 g, 56% yield). ^1H NMR (^2H_2O, 250 MHz): δ 1.20 (6H, t, CH_3 of C_2H_5), 1.63 (4H, brd quintet, H-4, H-5), 2.28 (2H, brd t, H-6), 2.61 (2H, brd t, H-3), 4.08 (4H, overlapping q, CH_2 of C_2H_5). ^{13}C NMR ($CDCl_3$, 250 MHz): δ 13.9, 14.1 (CH_3 of C_2H_5), 22.3, 24.1 (C-4, C-5), 33.9 (C-6), 38.8 (C-3), 60.3, 62.4 (CH_2 of C_2H_5), 161 (C-1), 173 (C-7), 193 (C-2). The free acid of **9** was obtained by alkaline hydrolysis using the procedure described elsewhere.[6] The product was crystallized from benzene to afford 0.25 g of **9**: ^1H NMR (^2H_2O, 500 MHz) δ 1.48 (4H, quintet, H-4,5), 2.10 (2H, t, H-6), 2.66 (2H, t, H-3).

OHED Hydratase-Catalyzed Exchange of the Proton at C-3 of 2-Oxo-1,7-heptadioate (9) with ^2H_2O. Two separate reactions (0.6 mL) measuring the buffer-catalyzed and the OHED hydratase-catalyzed rate of exchange of the proton at C-3 of 2-oxo-1,7-heptadioate (**9**) with ^2H_2O were performed at 23 °C in 100 mM $Na_2[^2H]PO_4$ buffer, p[^2H] ~9.6. Both reactions were followed by ^1H NMR spectroscopy. The addition of **9** as the free acid dissolved in dimethyl-d_6 sulfoxide (30 μL) lowered the p[^2H] of each reaction to 7.1. The final concentration of **9** in the experiment measuring the buffer-catalyzed exchange was 37 mM, while that in the experiment measuring the OHED hydratase-catalyzed exchange was 32 mM. In the experiment measuring buffer-catalyzed exchange, the first ^1H NMR spectrum was obtained 5 min after mixing. Subsequently, spectra were acquired at 15 min intervals for 8 h. In the experiment measuring the OHED-catalyzed exchange, the reaction was initiated by the addition of enzyme (0.18 mg), and the first spectrum was obtained 6 min after mixing. Subsequent spectra were acquired at 4 min intervals for 2 h. Successive ^1H NMR spectra were recorded on a Varian Unity INOVA-500 spectrometer. The enzyme solution had been previously exchanged in 20 mM Na[$^2H_2$]-PO_4 buffer (p[^2H] ~7) as described above. The intensity of the resonance observed at C-3 (δ = 2.66 ppm) was measured at timed intervals. The initial intensity of the resonance which was observed

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at 5 min (no enzyme) and 6 min (OHED hydratase) was set equal to two protons. Subsequent measurements were divided by this initial intensity to give the fraction of the protons remaining. The observed rate constants were determined from a nonlinear least-squares fit of the data obtained for the initial linear portion of the decrease in the signal intensity as a function of time to the equation for a first-order decay.

^{1}H and ^{13}C NMR Spectroscopic Identification of 7. A solution of **5** (4.0 mg, 0.02 mmol) dissolved in dimethyl-d_6 sulfoxide (30 μL) was added to 100 mM Na_2HPO_4 buffer (0.6 mL, pH 9.1) and transferred to an NMR tube. The final pH of the solution was 6.45. Subsequently, an aliquot of OHED hydratase (10 μL, 0.018 mg) in 10 mM NaH_2PO_4 buffer (pH 7.3) containing 5 mM $MgCl_2$ was added to initiate the reaction. The progress of the reaction was monitored by recording successive ^{1}H NMR spectra on a Varian Unity INOVA-500 spectrometer at 23 °C. After 16 min, only a set of signals most reasonably assigned to **7** were present. Spectra are recorded in 100% H_2O using selective presaturation of the water signal with a 2-s presaturation interval. **7**: ^{1}H NMR (H_2O, 500 MHz) δ 1.56 (2H, m, H-5), 2.08 (2H, m, H-6), 2.70 (1H, dd, J = 8.3 Hz, 16.7 Hz, H-3$_S$), 2.76 (1H, dd, J = 4.2 Hz, 16.7 Hz, H-3$_R$), 3.92 (1H, septet, H-4). A ^{13}C NMR spectrum was obtained on a Bruker AM-250 spectrometer using a separate sample made up as follows. A solution of 10 mM Na_2HPO_4 buffer (0.5 mL, pH = 7.3) made 5 mM in $MgCl_2$ was added to **5** (11.4 mg, 0.07 mmol) dissolved in dimethyl-d_6 sulfoxide (100 μL). The pH of the solution was adjusted to 7.05 using 5 M NaO[^{2}H] (~30 μL), and the mixture was transferred to an NMR tube. An aliquot of OHED hydratase (10 μL, 0.018 mg) in 10 mM NaH_2PO_4 buffer (pH 7.3) containing 5 mM $MgCl_2$ was added to initiate the reaction. After 3 h, only a set of signals most reasonably assigned to **7** were present. **7**: ^{13}C NMR (^2H_2O, 250 MHz) δ 34.7 (C-5), 35.3 (C-6), 48.0 (C-3), 68.6 (C-4), 171.2 (C-7), 184.0 (C-1), 206.0 (C-2). In the course of these experiments, there was no ^{1}H NMR spectral evidence for the production of **8**.

Assignment of the Stereochemistry at C-4 of 7. A solution of **5** (100 mg, 0.58 mmol) dissolved in 100 mM Na_2HPO_4 buffer (50 mL, pH 9.1) was prepared. The pH of the solution was adjusted to 7.2, and OHED hydratase (1 mL of a 1 mg/mL solution) was added. The hydration reaction was followed spectrophotometrically by monitoring the loss in absorbance at 276 nm and was complete in 20 min. The mixture was treated with H_2O_2 (5 mL of a 30% solution) added slowly over a 10-min period. After the mixture was allowed to stir for 30 min, a solution of catalase (5 mg in 1 mL of 20 mM NaH_2PO_4 buffer, pH 7.3) was added over a 5-min period. The solution was swirled in order to prevent foaming. When the reaction mixture no longer generated gas bubbles, it was allowed to stand for a 10-min period. Subsequently, the pH of the solution was adjusted to 8 and subjected to chromatography on a Dowex-1 (formate) (2 × 16 cm) column. The column was washed with water (20 mL) and eluted with a formic acid gradient (0−4 M formic acid, 300 mL total volume). (3*S*)-3-Hydroxyadipic acid (**10**) eluted from ~0.9−1.6 M formic acid. Appropriate fractions were collected, pooled, and evaporated to dryness to give **10** (61.5 mg, 65%) predominantly as the lactone (**11**): $[\alpha]^{23}_D$ = +33.2° (c = 15.4, ethanol). **10**: ^{1}H NMR (H_2O, 500 MHz) δ 1.70 (2H, m, H-4), 2.20 (2H, m, H-5), 2.34 (2H, ddd, H-2), 3.87 (1H, septet, H-3); ^{13}C NMR (CD_3OD, 250 MHz) δ 31.1 (C-4), 33.0 (C-5), 43.1 (C-2), 68.5 (C-3), 173.4 (C-6), 179.8 (C-1).

To a solution of (3*S*)-**11** (61.5 mg, 0.38 mmol) dissolved in methanol (2 mL) was added an ethereal solution of freshly generated CH_2N_2 until the yellow color persisted. After the mixture had stirred for 10 min, the excess CH_2N_2 was titrated with a dilute solution of CH_3CO_2H until the yellow color was no longer present. The resulting mixture was concentrated to give an oil which was purified further by flash chromatography (5% methanol, 95% CH_2Cl_2) to afford **12** (62.3 mg, 86%): $[\alpha]^{23}_D$ = +34° (c = 5.7, ethanol); ^{1}H NMR (CD_3OD, 250 MHz) δ 1.92 (2H, m, H-4), 2.46 (2H, m, H-5), 2.70 (2H, ddd, $-CH_2CO_2CH_3$), 3.70 (3H, s, $-CO_2CH_3$), 4.85 (1H, quintet, H-3); ^{13}C NMR ($CDCl_3$, 250 MHz) δ 27.4 (C-4), 28.3 (C-5), 39.6 ($-CH_2CO_2CH_3$), 51.8 ($-CH_2CO_2CH_3$), 77.5 (C-3), 169.8 ($-CH_2CO_2CH_3$), 176.3 (C-1).

OHED Hydratase-Catalyzed Incorporation of Deuterons at C-3 and C-5 of 7 and Assignment of the Stereochemistry at C-3. A solution of **5** (4.0 mg, 0.02 mmol) dissolved in dimethyl-d_6 sulfoxide (30 μL) was added to 100 mM $Na_2[^2H]PO_4$ buffer (0.6 mL, p[^{2}H] 9.6) and transferred to an NMR tube. The addition of **5** as the free acid adjusted the p[^{2}H] of the solution to 7.4. The enzyme solution had been previously exchanged by repeated dilution and concentration in 20 mM $Na[^2H_2]PO_4$ buffer (p[^{2}H] ~7) in a Centricon-10 microconcentrator and stored overnight. The first spectrum was acquired 3 min after the addition of a quantity of OHED hydratase (0.05 mg). Subsequent spectra were recorded at 2 min intervals. Successive ^{1}H NMR spectra were recorded on a Varian Unity INOVA-500 spectrometer. After 11 min, the intensities of the signals corresponding to 3,5-di[^{2}H]**7** reached a maximum. 3,5-di[^{2}H]**7**: ^{1}H NMR (^2H_2O, 500 MHz) δ 1.52 (1H, brd dd, H-5), 2.08 (2H, ddd, H-6), 2.68 (~0.7H, d, J = 8.3 Hz, H-3$_S$), 3.92 (1H, brd t, H-4).

Assignment of the Stereochemistry at C-5 of 3,5-di[^{2}H]7. A solution of **5** (44 mg, 0.25 mmol) dissolved in 100 mM $Na_2[^2H]PO_4$ buffer (7.5 mL, p[^{2}H] = 9.6) was incubated with OHED hydratase (0.3 mg/mL, 4 mL). The addition of **7** to the buffer adjusted the p^{2}H of the solution to 7.2. After 60 min, the reaction was judged to be complete as indicated by the absence of any significant absorbance between 200 and 300 nm. Subsequently, an aliquot of partially pure OHHD aldolase (0.4 mL) was added to the mixture. After a 45 min incubation period, there was no further production of pyruvate as determined by the aldolase assay. The p[^{2}H] of the mixture was adjusted to ~1.9 by the addition of phosphoric acid (8.5%), and solid $KMnO_4$ (93.2 mg) was added. After the mixture was stirred for 1 h, $NaHSO_3$ (5%) was added until the solution became clear at which time the p[^{2}H] was adjusted to ~8 by the dropwise addition of a 5 M NaOH solution. The reaction mixture was filtered to remove a white precipitate, and the filtrate was subjected to chromatography on a Dowex-1 (formate) (2 × 16 cm) column. The column was eluted with a formic acid gradient (0−4 M formic acid, 200 mL total volume). The monodeuterated succinic acid eluted from ~2.8−3.3 M formic acid. Appropriate fractions (5 mL) were collected, pooled, and evaporated to dryness to give 2-[^{2}H]**14**: $[\theta]_{210}$ = + 119° at 25 °C. The ^{1}H and ^{13}C NMR spectra of 2-[^{2}H]**14** corresponded to those reported previously. HRMS (m/z): calcd for $C_4H_5{}^2HO_4$ (MH^+), 120.0405; found, 120.0407.

Incubation of (3*E*)-8 with OHED Hydratase. A solution of **5** (4 mg, 23 μmol) dissolved in dimethyl-d_6 sulfoxide (30 μL) is added to 100 mM Na_2HPO_4 buffer (0.6 mL, pH 8.8) and transferred to an NMR tube. The addition of **5** as the free acid adjusts the pH to 6.5. The reaction was initiated by the addition of 4-OT (5 μg in 50 μL of 20 mM NaH_2PO_4, pH 7.1). Spectra are recorded in 100% H_2O as described above. After 10 min, (3*E*)-**8** was the predominant product present in solution as determined by the corresponding NMR signals: ^{1}H NMR (20 mM NaH_2PO_4, pH 7.1, 500 MHz) δ 2.20 (2H, dq, H-6), 2.40 (2H, m, H-5), 6.04 (1H, d, H-3, $J_{3,4}$ = 20 Hz), 6.88 (1H, dq, H-4, $J_{3,4}$ = 20 Hz). Subsequently, 4-OT was inactivated by the addition of 2-oxo-3-pentynoate (60 μg in 50 μL of 20 mM NaH_2PO_4, pH 7.1). 2-Oxo-3-pentynoate does not lead to significant inactivation of OHED hydratase at this concentration. After 12 min, OHED hydratase (50 μg in 50 μL of storage buffer) was added to the mixture. Subsequently, successive ^{1}H NMR spectra were acquired at regular intervals for 3 h. There was no spectral evidence for the formation of **7**.

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