Stereochemical Studies of 5-(Carboxymethyl)-2-hydroxymuconate Isomerase and 5-(Carboxymethyl)-2-oxo-3-hexene-1,6-dioate Decarboxylase from *Escherichia coli C*: Mechanistic and Evolutionary Implications

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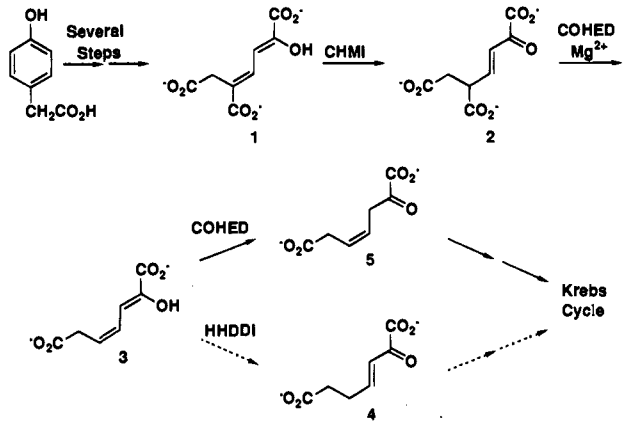
Abstract: 5-(Carboxymethyl)-2-hydroxymuconate isomerase (EC 5.3.2, CHMI) and 5-(carboxymethyl)-2-oxo-3-hexene-1,6-dioate decarboxylase (EC 4.1.1., COHED) from *Escherichia coli C* catalyze two successive reactions in the homoprotocatechuate meta-fission pathway resulting in the conversion of 5-(carboxymethyl)-2-hydroxymuconate (**1**) to 2-oxo-4-heptene-1,7-dioate (**5**). Stereochemical studies on both enzymatic reactions have been completed. It has been determined that the product of CHMI, 2-oxo-5-(carboxymethyl)-3-hexenedioate (**2**), has the *R* configuration at C-5. In addition, these studies show that the enzymatic decarboxylation of **2** by COHED generates (4*Z*)-2-hydroxy-2,4-heptadiene-1,7-dioate (**3**). Isolation and subsequent incubation of (4*Z*)-**3** with COHED, in ^2H_2O, affords predominantly (3*S*)-[3-^{2}H]**5**. On the basis of these stereochemical findings, it can be concluded that the loss of carbon dioxide and the incorporation of a deuteron occur on the same side of the dienol intermediate. These results are consistent with the working hypotheses for the mechanisms of both enzymes and indicate that the sequence of events catalyzed by these two enzymes parallel the reactions catalyzed by 4-oxalocrotonate tautomerase and 4-oxalocrotonate decarboxylase in the catechol meta-fission pathway.

5-(Carboxymethyl)-2-hydroxymuconate isomerase (EC 5.3.2, CHMI) and 5-(carboxymethyl)-2-oxo-3-hexene-1,6-dioate decarboxylase (EC 4.1.1., COHED) from *Escherichia coli C* convert 5-(carboxymethyl)-2-hydroxymuconate (**1**) to 2-oxo-4-heptene-1,7-dioate (**5**, Scheme 1).[1-6] These two enzymes are expressed as part of an inducible set of enzymes known collectively as the homoprotocatechuate (hpc) meta-fission pathway which oxidatively catabolizes 4-(hydroxyphenyl)acetic acid to intermediates in the Krebs cycle. The pathway is reportedly part of a degradative route for phenylalanine and tyrosine and enables *Escherichia coli C* to utilize these aromatic amino acids as its sole sources of carbon and energy.

It has previously been shown that 5-(carboxymethyl)-2-oxo-3-hexene-1,6-dioate (**2**) is the product of the CHMI-catalyzed ketonization of **1** and the substrate for the next enzyme in the pathway, COHED.[1-6] This observation indicates that COHED catalyzes the decarboxylation of a vinylogous analogue of a β-keto acid.[6] The generally accepted mechanism for β-decarboxylases involves the intermediate formation of a metal-stabilized enol which ketonizes to the α-keto acid product.[7] A considerable body of evidence supports the existence of an enol

Scheme 1



intermediate in this mechanism. Presumably, the same mechanism applies to the decarboxylation of vinylogous substrates (e.g., **2**) although such a mechanism would involve a dienol intermediate. The initial studies on the COHED-catalyzed reaction indicated that the decarboxylation of **2** results in a single product, 2-hydroxy-2,4-heptadiene-1,7-dioate (**3**, Scheme 1), although this conclusion was based solely on the observation and isolation of a compound with a λ_{max} at 276 nm.[2,3,8] It was

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further reported that another enzyme in the pathway, 2-hydroxyhepta-2,4-diene-1,7-dioate (HHDD) isomerase, converted **3** to an α,β-unsaturated ketone, 2-oxo-3-heptene-1,7-dioate (**4**, Scheme 1). More recently, it has been reported that a single enzyme, COHED, catalyzes both the decarboxylation of **2** and the subsequent ketonization of **3** to **4**.[5] In the absence of any further characterization of **3**, **4**, or other possible products in the reaction mixture, we reinvestigated the COHED reaction.[6]

In a preliminary account, we reported that two products, the β,γ-unsaturated ketone 2-oxo-4-heptene-1,7-dioate (**5**, Scheme 1) as well as **3**, are generated in the reaction mixture containing COHED and **2**. Moreover, a comparison of the values of k_{cat} and k_{cat}/K_M determined for **3** to those measured for **2**, demonstrated that the dienol is an excellent substrate for COHED and that it is kinetically competent to be an intermediate in the overall reaction. There was no chemical or spectroscopic evidence for the production of **4**. These results led us to propose that COHED catalyzes the decarboxylation of **2** to **5** through the dienol intermediate **3** (Scheme 1).[6]

We report herein the results of a stereochemical investigation into the reactions catalyzed by both CHMI and COHED as well as the isolation of **3** and a characterization of its properties. It has been found that CHMI ketonizes **1** to (5*R*)-**2**. Moreover, it has been determined that COHED generates (4*Z*)-**3** from (5*R*)-**2** and that the incubation of **3** with COHED in ^2H_2O results in its highly stereoselective ketonization to afford (3*S*)-[3-^{2}H]**5**. These stereochemical observations provide insight into the mechanisms of both enzymes and are analogous to the results obtained for the corresponding enzymes in the the catechol meta-fission pathway.[9-11]

Results

Isolation of 3 in the COHED-Catalyzed Decarboxylation of 2. The isolation of **3** has been described by Sparnins et al.[2] and Jenkins et al.[3] Both groups generated **3** enzymatically from 4-(hydroxyphenyl)acetic acid. The former used heat-treated cell extracts from *Pseudomonas putida U*, while the latter used a subclone containing only the necessary enzymes required for the production of **3** from 4-(hydroxyphenyl)acetic acid. Because these strains are not widely available, we generated **3** from 5-(carboxymethyl)-2-hydroxymuconate (**1**, Scheme 1) using partially purified preparations of CHMI and COHED. Ketonization of **1** by CHMI afforded a mixture of **2** and 2-oxo-5-(carboxymethyl)-4-hexenedioate (**6**, Scheme 2).[1] Decarboxylation of **2** by COHED generated **3** and **5** and resulted in the further conversion of **6** to **2**.[1,6] Subsequent acidification resulted in the isolation of **3** as determined by ^{1}H and ^{13}C NMR spectroscopy.[6] Although the mixture of **3** and **5** is slowly converted to **4**, the rate is so slow (*vide infra*) that significant quantities of **4** do not accumulate under these conditions.

Kinetics of the Nonenzymic Ketonization of 3. The ketonization of **3** in aqueous phosphate buffer (μ = 0.2 M, NaCl, 0.4% ethanol, 23 °C) was monitored at pH values 5.80, 6.30, and 6.80 by observing the decay in UV absorbance at 276 nm in different concentrations of phosphate buffer (0.002−0.05 M). In all cases, a rapid drop in absorbance followed by a much slower decrease is observed. The slower decrease in absorbance is noticeable only after a lengthy period of time.[6] The rapid drop in absorbance (~5 min) corresponds to the ketonization of **3** to form the β,γ-unsaturated ketone **5** (Scheme 1). The slower decrease in absorbance corresponds to the formation of **4** from **3**. The identities of **5** and **4** were established by ^{1}H and ^{13}C NMR spectrosopy and by their separate conversions to 2-hydroxy-4-heptene-1,7-dioate and 2-hydroxy-3-heptene-1,7-dioate, respectively, with $NaBH_4$ and subsequent identification by ^{1}H and ^{13}C NMR spectrosopy.[6] At equilibrium, **4** is the predominant product (>95%).

Table 1. Rate Constants for the Phosphate-Catalyzed Ketonization of 2-Hydroxy-2,4-heptadiene-1,7-dioate (**3**)[a]

k_{obsd}	k_o ($\times 10^{-4}$ s^{-1})[b]	k_{H^+} (M^{-1} s^{-1})	$k_{H_2PO_4^-}$ ($\times 10^{-2}$ M^{-1} s^{-1})	$k_{HPO_4^{2-}}$ ($\times 10^{-2}$ M^{-1} s^{-1})
k_1	45.8 ± 0.8	313 ± 82	2.4 ± 0.4	3.7 ± 2.1
k_2	15.9 ± 0.3	64 ± 28	1.1 ± 0.1	1.5 ± 0.7

[a] The rate constants are derived from eq 1. [b] 23.0 °C, ionic strength = 0.2 (NaCl), 0.4% ethanol. Errors are standard deviations.

The absorbance data collected during the first 12 min in each individual experiment were fitted to a single exponential by nonlinear least-squares regression analysis in order to estimate the rate constants for the interconversion of **3** and **5**.[12] From this analysis, it is estimated that, after 12 min, the solution contains 28.4% **3** and 71.6% **5**. The conversion of **3** to **4** was not taken into account in this analysis because the reaction is very slow. Although at equilibrium **4** is the predominant product, it constitutes only about 10% of the mixture after 8 h.[6]

$$k_{obsd} = k_o + k_{H^+}[H^+] + k_{H_2PO_4^-}[H_2PO_4^-] + k_{HPO_4^{2-}}[HPO_4^{2-}] \quad (1)$$

The kinetic constants obtained for all experiments were fitted to eq 1 by multiple linear regression to estimate the kinetic parameters listed in Table 1. The rate constant k_1 is the rate of ketonization for **3** to **5** and the rate constant k_2 is the rate of enolization for **5** to **3**. The results indicate that $H_2PO_4^-$, HPO_4^{2-}, and hydronium ion have a catalytic effect on the ketonization of **3** to **5**. The value of k_o determined for k_1 in Table 1 provides an upper limit on the uncatalyzed rate constant for the ketonization of **3** to **5**. This value can only be considered an upper limit because the rate due to the concentration of hydroxide ion, hydronium ion, and other buffer species cannot be determined within the narrow pH range studied. Nonetheless, the measured k_o value is substantially smaller than the corresponding rate reported for 1,3-cyclohexadienol (~33-fold) and larger than the rates reported for 2-hydroxymuconate (~3-fold), (*E*)-1,3-butadienol (~2-fold), and (*Z*)-1,3-butadienol (~1.4 fold).[10,13]

Stereochemistry of 5-(Carboxymethyl)-2-hydroxymuconate Isomerase. The stereochemical analysis of CHMI was based on the enzymatic and chemical conversion of 5-(carboxymethyl)-2-hydroxymuconate (**1**) to 3-carboxyadipic acid (**11**, Scheme 2). In the presence of CHMI, ketonization of **1** afforded the desired product **2** and 2-oxo-5-(carboxymethyl)-4-hexenedioate (**6**).[1] Reduction of the two ketones by $NaBH_4$ generated 2-hydroxy-5-(carboxymethyl)-3-hexenedioate (**7**) and

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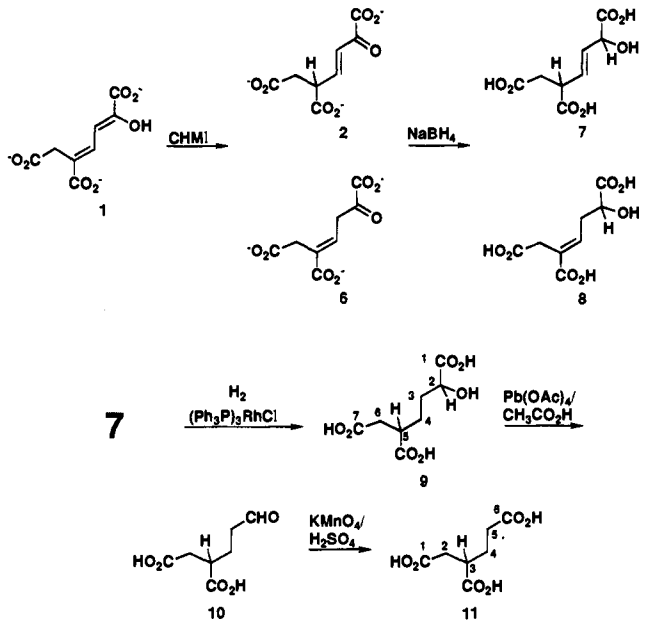
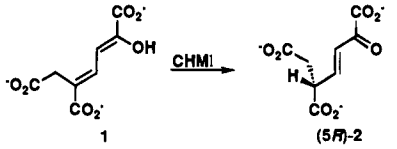
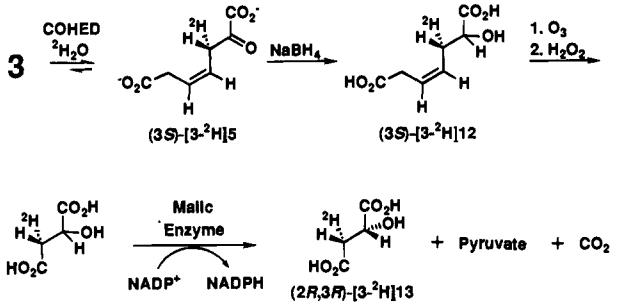
Scheme 2



Scheme 3



Scheme 4



2-hydroxy-5-(carboxymethyl)-4-hexenedioate (**8**), respectively, and should make C-5 of the resulting **7** nonepimerizable. The two isomeric alcohols were the major products isolated by anion exchange chromatography as determined by ^{1}H NMR spectroscopy.[1] The strategy for the stereochemical analysis of **7** was based on its further chemical conversion to **11**. However, subjecting **8** to the same series of reactions would result in a loss of the stereochemical purity of **11**, making a separation of the two isomers necessary. Accordingly, the undesired isomer, **8**, was crystallized from a mixture of ethyl acetate and hexanes while the desired isomer, **7**, was recovered from the mother liquor. Subsequently, the fully saturated compound, **9**, was obtained by hydrogenation of **7** in the presence of Wilkinson's catalyst.[14] Homogeneous catalytic hydrogenation prevents the scrambling of the allylic hydrogens which would also lead to a loss of the chirality.[14] Oxidative decarboxylation of **9** by lead tetraacetate resulted in the formation of **10**, which was not isolated.[15,16] Oxidation of **10** by potassium permanganate afforded **11**.[15,17]

The optical rotation of the purified 3-carboxyadipic acid (**11**) derived from the above procedure was measured and determined to be $[\alpha]_D = -9.35°$ (acetone). It was previously reported that the *R* isomer of **11** exhibits a positive optical rotation of 16.4°.[18] It can be concluded, therefore, that the 3-carboxyadipic acid derived from our chemical and enzymatic procedures is the *S* isomer. Thus, the product of the CHMI reaction was (5*R*)-2-oxo-5-(carboxymethyl)-3-(*E*)-hexenedioate because the priority numbering changes with the presence of the double bond at C-3 (Scheme 3). The lower value of the optical rotation is most likely due to the racemization of **11** that may occur during the oxidation of **10** to **11** and to the residual presence of **8** (~8%) and its subsequent processing to **11**.[18] The optical rotation is, however, unquestionably negative.

Stereospecific Ketonization of 3 to [3-^{2}H]5 in ^2H_2O and Conversion of [3-^{2}H]5 to [3-^{2}H]Malate. Previous kinetic and spectroscopic studies suggested that the ketonization of **3** to **5** is catalyzed by COHED.[6] An additional hallmark of an enzyme-catalyzed reaction is the demonstration of stereospecificity.[19] The stereochemical question was addressed by the chemical and enzymatic conversion of [3-^{2}H]**5**, generated by the COHED-catalyzed ketonization of **3**, in ^2H_2O, to a monodeuteriated malate (Scheme 4). Ketonization of **3** by COHED, in ^2H_2O, generated [3-^{2}H]**5**.[6] Reduction of [3-^{2}H]**5** by $NaBH_4$ should make C-3 of the resulting [3-^{2}H]**12** nonepimerizable. The reduced product was the major product isolated by anion exchange chromatography, as determined by ^{1}H NMR spectroscopy.[6] The ketonization of **3** to **5** required a sufficient quantity of enzyme in order to minimize the facile nonenzymatic decay of **3** to **5** and the resulting stereorandom incorporation of deuterium at C-3. Subsequently, the purified [3-^{2}H]**12** was subjected to ozonolysis.[20,21] Treatment of the resulting ozonide with H_2O_2 generated the 2*R* and 2*S* isomers of malate because the initial $NaBH_4$ reduction of **5** was stereorandom. Incubation of the mixture with malic enzyme and purification by anion exchange chromatography afforded the 2*R* isomer of the monodeuteriated malate **13**.[21,22]

^{1}H NMR Analysis of (2*R*)-[3-^{2}H]Malate. Each diastereotopic proton at C-3 of fully protio malate appears as a doublet of doublets at 2.33 and 2.63 ppm.[23] Stereospecific incorporation of a deuteron at C-3 results in the loss of one signal and the collapse of the remaining signal into a broadened doublet.[24] The resonances for (2*R*)-[3-^{2}H]malate have been assigned by the reaction of maleic acid with maleate hydratase.[25] When the hydration is performed in ^2H_2O, (2*R*,3*R*)-[3-^{2}H]malate is obtained.[25] The resulting ^{1}H NMR spectrum shows the loss of an upfield signal and the presence of a downfield singlet.[24]

The ^{1}H NMR spectrum of the purified (2*R*)-[3-^{2}H]malate derived from the COHED reaction is shown in Figure 1. The

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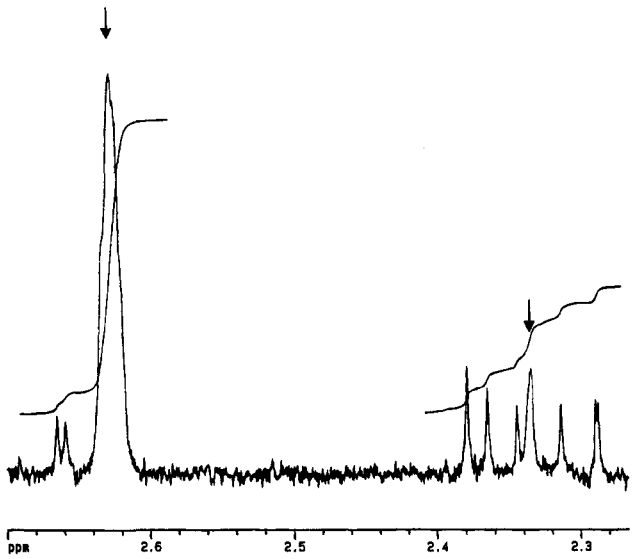


Figure 1. ^{1}H NMR (500 MHz, 20 mM $Na_2{}^2HPO_4$ in ^2H_2O) spectrum of (2*R*)-[3-^{2}H]malate obtained from the chemical and enzymatic conversion of 2-oxo-[3-^{2}H]-4-heptene-1,7-dioate (**5**) generated by the COHED-catalyzed ketonization of 2-hydroxy-2,4-heptadiene-1,7-dioate (**3**) in ^2H_2O.

two signals of interest are the larger broadened singlet centered at ~2.63 ppm and the smaller broadened singlet centered at ~2.33 ppm.[26] The larger signal corresponds to (2*R*,3*R*)-[3-^{2}H]-malate while the smaller signal corresponds to (2*R*,3*S*)-[3-^{2}H]-malate.[25,26] The height of the integral for the signal assigned to the remaining C-3 hydrogen of the (2*R*,3*R*) isomer is about 10.7 times greater than the corresponding intergral for the (2*R*,3*S*) isomer. The analysis clearly shows that (2*R*,3*R*)-[3-^{2}H]malate is the major isomer recovered. The stereochemistry at C-3 of malate indicates that the stereochemistry at C-3 of [3-^{2}H]**12** is *S* because the priority numbering changes upon unsaturation at C-4. This, in turn, indicates that, in ^2H_2O, COHED ketonizes **3** to (3*S*)-[3-^{2}H]**5** (Scheme 5).

Determination of the Configuration of 3 Generated from (5*R*)-2 by COHED. In order to assign the overall stereochemical course of the decarboxylation reaction, it is necessary to establish whether COHED generates the 4*E* or the 4*Z* isomer of **3** from (5*R*)-**2**. This question was addressed by comparing the ^{1}H NMR spectrum of **3** generated by COHED (presumably one isomer) to the ^{1}H NMR spectrum of **3** generated by the thermal decarboxylation of **1** (presumably both isomers) (Figure 2A–C). The substrate for COHED, (5*R*)-**2**, is generated by the action of CHMI on **1**.[1,6] Addition of COHED produces a complex spectrum (Figure 2A) with signals corresponding to **3** and **5** as well as signals that result from presence of residual **1** and **2**. The proton on C-4 of **3** is observed as an apparent triplet centered at 6.21 ppm while the proton on C-5 produces a doublet of triplets at 5.54 ppm.[27] The proton on C-3 of **3** appears as a doublet at 6.09 ppm. Acidification of a similar mixture results in the isolation of a compound which is identified as **3**.[6] In aqueous buffer, a ^{1}H NMR spectrum (Figure 2B) reveals that the isolated compound is identical to the one generated by COHED, indicating that the conditions of isolation had not resulted in isomerization.[28] The observed coupling constant for the proton at C-5 (J = 10.7 Hz) of **3** suggests a *cis* relationship

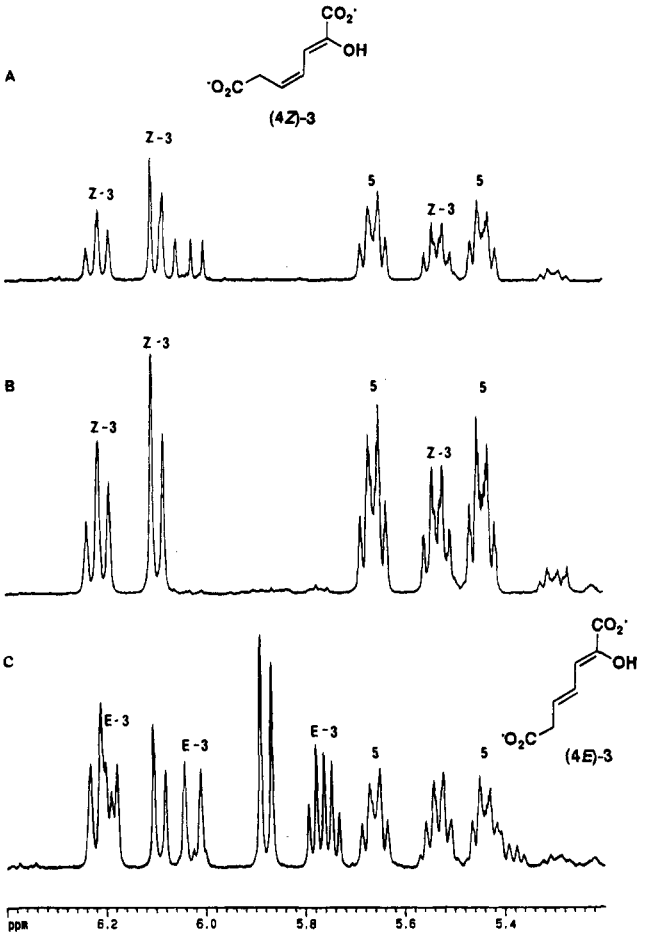


Figure 2. Partial ^{1}H NMR spectra (100 mM Na_2HPO_4, 500 MHz) indicating (A) (4*Z*)-2-hydroxy-2,4-heptadiene-1,7-dioate (**3**) generated by the action of COHED on (5*R*)-**2**; (B) (4*Z*)-**3** isolated from a mixture containing CHMI, COHED, and **1**; and (C) a mixture of (4*Z*)- and (4*E*)-**3** generated by the thermal decarboxylation of **1**. The doublet at 5.88 ppm corresponds to the proton on C-3 of **4**.

to the C-4 proton.[29] The configuration was confirmed by ^{1}H NMR analysis of a mixture containing both the 4*E* and 4*Z* isomers of **3** obtained by the thermal decarboxylation of **1**. The resulting ^{1}H NMR spectrum (Figure 2C) shows a set of signals corresponding to (4*Z*)-**3** and a new set of signals that can be assigned to (4*E*)-**3**. The proton on C-5 of (4*E*)-**3** produces a doublet of triplets at 5.76 ppm with a characteristic *trans* coupling constant (J = 15.5 Hz). While the signals for the proton on C-4 of (4*E*)-**3** and the proton on C-4 of (4*Z*)-**3** appear nearly coincident (~6.21 ppm), they are separable by resolution enhancement of the spectrum. Resolution enhancement reveals that the proton on C-4 of (4*E*)-**3** generates a signal at 6.19 ppm with a characteristic *trans* coupling constant (J = 15.6 Hz) and the proton on C-4 of (4*Z*)-**3** produces a signal at 6.21 ppm with expected *cis* coupling constant (J = 10.7 Hz). The proton at C-3 of (4*E*)-**3** presents a doublet at 6.02 ppm. (The prominent doublet at 5.88 ppm corresponds to the C-3 proton of **4**.) It can be concluded, therefore, that (4*Z*)-**3** was generated from (5*R*)-**2** by the action of COHED. The combination of the stereochemical results indicates that decarboxylation and the incorporation of a deuteron occur on the same side of the dienol intermediate (Scheme 5).

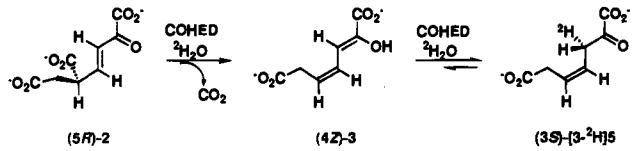
(26) The small amount of (2*R*, 3*S*)-[3-^{2}H]malate present presumably results from the nonenzymatic conversion of **3** to **5** in ^2H_2O. The doublets centered at 2.35 and 2.66 ppm correspond to residual fully protio malate. The doublet at 2.29 ppm and the singlets at 2.31 and 2.38 ppm correspond to impurities.

(27) The apparent triplet at 6.21 ppm is a doublet of doublets.

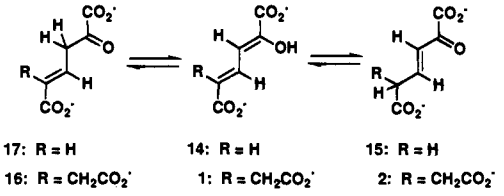
(28) The presence of signals corresponding to the protons on C-4 and C-5 of **5** (5.44 and 5.66 ppm, respectively) results from the facile nonenzymatic decay of **3** in aqueous buffer.[6]

(29) Silverstein, R. M.; Bassler, G. C.; Morrill, T. C. *Spectrophotometric Identification of Organic Compounds*, 4th ed.; John Wiley & Sons: New York, 1981; p 209.

Scheme 5



Scheme 6



Discussion

The stereochemical results provide further insight into the mechanism of the reactions catalyzed by CHMI and COHED. It has been clearly shown that CHMI processes **1** to (5*R*)-**2**—a finding which is consistent with the working hypothesis for the mechanism of CHMI.[1] It has also been found that, in ^2H_2O, COHED converts **3** into (3*S*)-[3-^{2}H]**5**, which supports the working hypothesis for the mechanism of COHED.[6] The combination of results indicates that the decarboxylation of **2** and the incorporation of a deuteron at C-3 of **5** occur in a *syn* fashion. Furthermore, the properties of the intermediate (**3**) in the reaction are characteristic of those reported for slow-reacting dienols and show that the conversion of **3** to **5** is slow in the absence of buffer or enzyme.[13] Finally, these findings confirm that **5** is the product of COHED and indicate that this reaction is analogous to the one catalyzed by 4-oxalocrotonate decarboxylase (4-OD) in the catechol meta-fission pathway.[9-11]

The stereoselective ketonization of **1** to **2** reflects the fact that the reaction is enzyme-catalyzed which was inferred from an earlier result indicating that CHMI converts 2-hydroxymuconate (**14**) into (5*S*)-2-oxo-3-(*E*)-[5-$^2H_1$]hexenedioate (**15**, Scheme 6) in ^2H_2O.[15,19] Both observations support the hypothesis that CHMI acts as an isomerase to convert 2-oxo-5-(carboxymethyl)-4-hexenedioate (**16**) to **2** through the dienol intermediate **1** (Scheme 6).[11] The reaction is entirely analogous to the allylic rearrangement of 2-oxo-4-hexenedioate (**17**) to 2-oxo-3-hexenedioate (**15**) through **14** (Scheme 6) catalyzed by 4-oxalocrotonate tautomerase (4-OT) and found in the catechol meta-fission pathway.[21] The isomerization of **17** to **15** has been shown to be predominantly a suprafacial process implicating a one-base mechanism.[21] Although the overall stereochemical course of the CHMI reaction has not yet been determined experimentally, it can be reasonably suggested on the basis of literature precedent and the foregoing example that the CHMI-catalyzed reaction also proceeds superfacially and utilizes a one-base mechanism.[30] This suggestion is further strengthened by comparing the recently solved X-ray crystal structures for both 4-OT and CHMI.[31] The overall folds of the two proteins as well as the active site regions are nearly superimposable even though there is no sequence homology between the two enzymes.[15,31]

The highly stereoselective ketonization of **3** to [3-^{2}H]**5** in ^2H_2O by COHED is compelling evidence that a reaction is enzyme-catalyzed and supports the hypothesis that **3** is an intermediate in the COHED reaction.[6,19] Kinetic studies had previously indicated that **3** is kinetically competent to be an intermediate in the overall reaction and suggested that COHED catalyzes the decarboxylation of **2** to **5** through the dienol intermediate (Scheme 1).[6] Because the substrate for COHED is a vinylogous β-keto acid and the reaction requires metal ion, the catalytic mechanism may be analogous to the mechanism described for metal-dependent β-decarboxylases although utilizing a metal-stabilized dienol intermediate.[7e,g]

The nonenzymatic properties of **3** are consistent with those observed for other slow-reacting dienols and have implications for the enzymatic mechanism. In aqueous buffer, a rapid kinetic equilibrium is formed between **3** and **5** before a much slower conversion of the mixture to **4**. Protonation is favored at C-3 and, although we were not able to measure the rate of protonation at C-5, there is clearly a large difference between the rates of protonation at C-3 and at C-5 as reflected by the slow formation of **4**.[6] Such behavior is characteristic of a slow-reacting dienol.[13] Much of the rationale for this investigation was to explain why the enzyme-catalyzed ketonization of **3** to **5** was only about 3-fold greater than the rate observed in phosphate buffer.[6] Studies on related dienols (e.g., **1** and **14**) suggested that the ketonization of **3** to **5** was catalyzed by the phosphate buffer and that the actual rate enhancement was much higher.[1,10] These results confirm that the ketonization of **3** to **5** is sensitive to the concentration of phosphate buffer and that, in the absence of either buffer or enzyme, the rate of ketonization is significantly slowed. A comparison of the turnover number for the enzyme ($\sim$160 s^{-1}) to the estimated uncatalyzed rate constant of ketonization ($\sim 4.6 \times 10^{-3}$ s^{-1}) shows an approximate 35 000-fold rate enhancement.

The counterpart to COHED in the catechol meta-fission pathway is 4-OD which is responsible for the decarboxylation of **15**.[11,32] In both the 4-OD- and the COHED-catalyzed reactions, a dienol intermediate is ketonized to a β,γ-unsaturated ketone and a solvent deuteron is incorporated at C-3 with a high degree of stereoselectivity. In addition, the stereochemical course of each reaction is a *syn* process.[11] The structural resemblance between **2** and **15** (Scheme 6), the similarities in mechanism and reaction type, and the common stereochemical course suggest that 4-OD and COHED might share additional mechanistic features. The major differences between the two enzymes may be due only to the interactions associated with the differential binding of their respective substrates. The recently uncovered structural and mechanistic similarities between 4-OT and CHMI, the enzymes preceding 4-OD and COHED in their respective pathways, foster speculation about the evolutionary relationship between 4-OD and COHED as well as the other enzymes that comprise the catechol and homoprotocatechuate pathways.[31] Further structural and mechanistic studies on these enzymes will define more precisely the relationship between them and should result in a better understanding of how these two pathways arose.

Finally, these findings raise questions about the "bifunctional nature" of COHED as well as the currently accepted version of

(30) Schwab and co-workers noted a correlation between the structural features of a substrate for allylic rearrangements and the observed stereochemical outcome of the reaction: Schwab, J. M.; Klassen, J. B. *J. Am. Chem. Soc.* **1984**, *106*, 72117-7227. A possible chemical explanation for this correlation has also been provided: Gerlt, J. A.; Gassman, P. G. *J. Am. Chem. Soc.* **1992**, *114*, 5928-5934. The substrates for those reactions which proceed suprafacially undergo facile deprotonation due to the presence of an adjacent ketone or thiol ester carbonyl group. Deprotonation of the α-proton affords a readily stabilized intermediate. Hence, the structure of **16** suggests that the CHMI-catalyzed isomerization of **16** to **2** is a suprafacial process.

(31) (a) Roper, D. I.; Subramanya, H. S.; Shingler, V.; Wigley, D. B. *J. Mol. Biol.* **1994**, *243*, 799-801. (b) Subramanya, H. S.; Roper, D. I.; Wigley, D. B. *Biochemistry*, submitted for publication. (c) Roper, D. I.; Stringfellow, J. M.; Cooper, R. A. *Gene* **1995**, *156*, 47-51.

(32) Harayama, S.; Rekik, M.; Ngai, K.-L.; Ornston, L. N. *J. Bacteriol.* **1989**, *171*, 6251-6258.

the homoprotocatechuate pathway.[5,31c] It has been suggested that COHED is a bifunctional protein which catalyzes two reactions in the pathway: the decarboxylation of **2** to **3** and the subsequent ketonization of **3** to 2-oxo-3-heptene-1,7-dioate (**4**, Scheme 1).[5] The basis for the latter conclusion is not clear. Although the enzyme catalyzes the ketonization of **2** as evidenced by the rapid loss of absorbance at 276 nm, there is not a concomitant increase in absorbance at 232 nm which would result from the formation of **4**.[6] In contrast, the present stereochemical evidence and previous spectroscopic data indicate that COHED catalyzes the decarboxylation of **2** to **3** and the subsequent ketonization of **3** to **5**.[6,33] It is also unlikely that the two reactions catalyzed by COHED occur at separate sites on the enzyme as might be construed by the suggestion that the enzyme is bifunctional. In fact, the two reactions catalyzed by COHED are reminiscent of the many enzyme-catalyzed reactions involving the generation of an enolpyruvate intermediate and its ketonization to pyruvate.[34] Although these reactions involve two separate steps, all reports to date indicate that a single enzyme catalyzes both steps at one active site. Taking these and previous results into account, the reactions of the homoprotocatechaute meta-fission pathway are analogous to those reported for the catechol meta-fission pathway.[1,6,8-11]

Experimental Section

Materials. All chemicals and solvents were purchased from Aldrich Chemical Co. with the following exceptions. Biochemicals, buffers, malic enzyme, and β-nicotinamide adenine dinucleotide phosphate (β-NADP) were obtained from Sigma Chemical Co. 2-Hydroxymuconate and 5-(carboxymethyl)-2-hydroxymuconate were prepared by procedures described elsewhere.[1,10] Centricon (10 000 MW cutoff) centrifugal microconcentrators and ultrafiltration membranes were purchased from Amicon and were used according to manufacturer's instructions. The cellulosic 0.22 μm filters were obtained from Micron Separations Inc., Westboro, MA. *E. coli* strain C was obtained from the *Escherichia coli* Genetic Stock Center, Yale University, New Haven, CT. CHMI was isolated by a procedure reported elsewhere.[1] The specific activity ranged from 1600 to 2500 units/mg.

Methods. Protein concentrations were determined using the commercially available bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL). HPLC was performed on a Waters system using a Waters Protein Pak DEAE 5PW anion exchange column (10-μm particle size), a Bio-Gel Phenyl 5-PW hydrophobic column, or a Pharmacia Superose 12 (HR 10/30) gel filtration column. Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) under denaturing conditions was performed on 15% gels and stained with Coomassie Blue as described elsewhere.[35] Kinetic data were obtained on either a Perkin-Elmer fast scan UV/vis spectrophotometer Model 553 or a Hewlett Packard 8452A diode array spectrophotometer. Ozone was generated by the passage of oxygen through a Welsbach ozonator. NMR spectra were obtained on either a Bruker AM-250 spectrometer or a Bruker AM-500 spectrometer as indicated. Chemical shifts were standardized to the $^1H^2HO$ resonance at 4.70 ppm.

Purification of 5-(Carboxymethyl)-2-oxo-3-hexene-1,6-dioate Decarboxylase (COHED). COHED from *E. coli B* and a recombinant COHED were purified previously.[5,8] It was indicated that the enzyme requires magnesium for activity although metal ions were not used in the course of either purification procedure.[5,8] In our hands, the enzyme has little or no activity after elution from either a gel filtration or an anion exchange column in the absence of magnesium. The activity does, however, significantly improve by incubating the enzyme with magnesium for about 24 h. Our procedure (*vide infra*) typically yields 1.0 mg of essentially homogeneous protein from 89 g of *E. coli C* cells and results in an overall 3400-fold purification when assayed by the disappearance of **1** at 300 nm and in an overall 17-fold purification when assayed by the disappearance of **3** at 276 nm. It is essential to include magnesium in the extraction and column buffers in order to maintain activity.

In a typical procedure, cultures of *E. coli C* were grown at 30 °C in a minimal medium described elsewhere supplemented with 10 mM 4-hydroxyphenylacetic acid.[15] After growth to late log phase (24 h), cells were centrifuged at 5000*g* for 12 min, collected, and stored at −78 °C. In a typical procedure, frozen cells (89 g) were suspended in 20 mM Na_2HPO_4 buffer (250 mL, pH 7.4), containing 1 mM 6-aminocaproic acid, 0.5 mM phenylmethylsulfonyl flouride, 50 μM leupeptin, and 0.067 μM aprotonin in order to limit proteolysis. The cells were disrupted at 4 °C with 10 pulses (1 min each) from a Heat Systems W-385 sonicator equipped with a 0.5 in. tapped horn delivering approximately 330 W/pulse. The solution was centrifuged at 27 000*g* for 30 min to remove cell debris. Nucleic acids were removed by making the supernatant 1 mg/mL in protamine sulfate (250 mg/3 mL of buffer adjusted to pH 7.4) over 10 min at 4 °C. After centrifugation (27 000*g* for 30 min), the supernatant was filtered through a cellulosic (0.22 μm) filter and injected in 12-mL portions onto a Waters Protein Pak (DEAE-5PW) anion exchange column (15 × 2.15 cm) that had been equilibrated with 10 mM Na_2HPO_4 (pH 7.3) buffer at a flow rate of 5 mL/min. Elution was carried out with a NaCl gradient (0−0.25 M over 50 min followed by 0.25−0.50 M over 10 min). The high salt buffer contained 10 mM $MgCl_2$. Activity eluting at 0.17−0.18 M NaCl was collected and concentrated by ultrafiltration (Amicon PM-10 membrane) to a final volume of 10 mL.

The retentate was diluted with 10 mM ethylenediamine buffer (10 mL, pH 7.4) containing 2 M $(NH_4)_2SO_4$ and injected in 10-mL portions onto a Bio-Rad Phenyl 5-PW column (75 × 7.5 mm) equilibrated with the same buffer at a flow rate of 5 mL/min. After washing the column with equilibrating buffer (10 min), activity was eluted using a decreasing $(NH_4)_2SO_4$ gradient (2.0−1.0 M in 5 min, 1.0−0 M in 50 min). The activity eluting at 0.38 M $(NH_4)_2SO_4$ was collected and made 1 mM in $MgCl_2$ in order to restore activity. The pooled fractions (20 mL) were exchanged into 20 mM Na_2HPO_4 (pH 7.3) buffer containing 10 mM $MgCl_2$ and concentrated by ultrafiltration (Amicon PM-10 membrane) to a final volume of 12 mL.

The retentate was injected in 2-mL portions onto a Bio-Rad MA7Q anion exchange column (100 × 19 mm) equilibrated with the same buffer at 0.8 mL/min. After washing the column with equilibrating buffer (5 min), activity was eluted using a NaCl gradient (0−0.3 M in 55 min). The activity eluting at 0.18−0.19 M NaCl was collected, concentrated to 6 mL, and reinjected onto the Bio-Rad MA7Q column using the same buffer at a flow rate of 2 mL/min. After a 5-min wash, the activity was eluted using a NaCl gradient (0−0.3 M NaCl in 75 min). Activity eluting at 0.1 M NaCl was collected and concentrated to by ultrafiltration (Amicon PM-10 membrane) to a final volume of 2 mL.

The retentate was injected in 0.5-mL portions onto two Pharmacia Superose columns connected in series and equilibrated with 10 mM NaH_2PO_4 buffer (pH 7.4) containing 0.15 M NaCl and 5 mM $MgCl_2$ at a flow rate of 0.4 mL/min. The activity eluting at 64 min was collected and concentrated to a final volume of 3.6 mL.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions on 15% gels revealed that the fractions containing COHED were homogeneous. The enzyme migrated as a single band on SDS-PAGE with an apparent subunit size of 57 000 Da using phosphorylase b (97 400 Da), bovine serum albumin (66 200 Da), ovalbumin (42 699 Da), bovine carbonic anhydrase (31 000 Da), and lysozyme (14 400) as standards. The final specific activity of the protein was 160 units/mg utilizing **3** as the substrate and 70 units/mg utilizing **2** as the substrate. These assays and the definition of a unit of enzyme activity are explained below.

Determination of the Molecular Mass of 5-(Carboxymethyl)-2-oxo-3-hexene-1,6-dioate Decarboxylase. The native molecular mass for the purified COHED was estimated by chromatography on a Superose 12 column using blue dextran (2 × 10^6 Da), bovine serum albumin (66 200 Da), ovalbumin (42 699 Da), β-lactoglobulin (35 000 Da), soybean trypsin inhibitor (21 500 Da), and cytochrome *c* (12 400

(33) We have recently isolated the next enzyme in the pathway, 2-oxo-4-heptene-1,7-dioate hydratase, and determined that it processes **5** to 2-oxo-4-hydroxy-1,7-heptadioate (**18**). When **4** is generated from **3** by the action of 4-OT,[6] it is not processed to **18** by the hydratase.

(34) Some examples are oxaloacetate decarboxylase,[7e] malic enzyme,[7e] and pyruvate kinase: Kuo, D. J.; Rose, I. A. *J. Am. Chem. Soc.* **1978**, *100*, 6288−6289. More examples can be found elsewhere.[7d]

(35) Laemmli, U. K. *Nature* **1970**, *227*, 680−685.

Da) as standards. A plot of log molecular mass for the standards *vs* $(V_e - V_o)/(V_t - V_o)$ (where V_e = elution volume, V_o = void volume, and V_t = total volume) gives a linear curve from which the molecular mass is determined. Purified COHED was injected along with the various protein standards onto a Superose column (Pharmacia) in 100-μL portions equilibrated with 10 mM NaH_2PO_4 buffer (pH 7.4) containing 0.15 M NaCl and 5 mM $MgCl_2$ at a flow rate of 0.4 mL/min. Protein was monitored by measuring absorbance at 280 nm. Under these conditions, the enzyme elutes at 25.5 min. When the logarithm of the molecular masses was plotted against $(V_e - V_o)/(V_t - V_o)$ for the standard proteins, a molecular mass of 44 000 Da was estimated for the native COHED. The native molecular mass of COHED estimated by gel filtration chromatography (44 000 Da) coupled with the subunit molecular mass (57 000 Da) determined from SDS-PAGE suggest that COHED is a monomer. The difference between the two masses may be due to the fact that the denaturing conditions of SDS-PAGE remove the magnesium from the enzyme and result in a randomly coiled polypeptide chain whereas the conditions used to elute the protein from the gel filtration column do not remove the magnesium so that the enzyme elutes in a native conformation. These observations are in accord with those previously reported.[5]

Enzyme Assays. Enzyme activity was monitored by three different assays. The two methods used during the purification process monitor either the rate of disappearance of **3** at 276 nm or the rate of disappearance of **1** at 300 nm. During the early stages of purification, it is necessary to use a previously described assay that monitors the decay of **1** at 300 nm in the mixture of **1** and **2** generated by 5-(carboxymethyl)-2-hydroxymuconate isomerase (CHMI).[5,8] The second assay follows the decay of **3** at 276 nm.[5,8] It is not possible to use this assay during the early stages of purification due to the large absorbance of nucleic acids and other proteins present at 276 nm. For all kinetic studies, with homogeneous enzyme, enzyme activity was monitored by following the rate of disappearance of **3** at 276 nm or the rate of disappearance of **2** at 236 nm. A unit of enzyme results in the loss of 1 μmol of **3**/min, 1 μmol of **2**/min, or 1 μmol of **1**/min at pH 7.3.

Isolation of 3 in the COHED-Catalyzed Decarboxylation of 2. A mixture of partially pure CHMI (~200 units) and partially pure COHED (~280 units) was added to a solution of **1** (1.0 g, 4.6 mmol) dissolved in 10% $NaHCO_3$ (1 mL) and diluted with 10 mM NaH_2PO_4 buffer (100 mL). After stirring at 4 °C overnight, the solution was adjusted to pH 1 with HCl. A white precipitate was filtered off, and the filtrate was extracted with ethyl acetate. Evaporation of the ethyl acetate left a yellow residue which was dissolved in a minimal amount of acetone and precipitated with CH_2Cl_2. After allowing the cloudy solution to stand at 4 °C for 24 h, it was rapidly filtered to afford 0.2 g (25%) of a hygroscopic solid (**3**) that was stable for several weeks in a desiccator. The ^1H and ^{13}C NMR spectra were consistent with the assigned structure: ^1H NMR (250 MHz, CD_3OD) δ 3.15 (2H, d, J = 8.3 Hz, H-6), 5.61 (1H, dt, J = 8.3 Hz, H-5), 6.23 (1H, d, J = 12.5 Hz, H-3), 6.43 (1H, dd, J = 10.4 Hz, H-4); ^{13}C NMR (500 MHz, CD_3OD) δ 33.3 (C-6), 106.1 (C-3), 123.8 (C-4), 124.9 (C-5), 142.4 (C-2), 165.7 (C-1), 172.2 (C-7).

Kinetics of the Nonenzymic Ketonization of 3. Buffers were made up by the addition of calculated amounts of 100 mM stock solutions of $NaHPO_4$ and Na_2HPO_4, plus the calculated amount of a 0.5 M NaCl solution necessary to maintain constant ionic strength, followed by dilution with H_2O in volumetric flasks. The ionic strength of all buffers was calculated to be 0.2 M. If necessary, small amounts of HCl or NaOH were added to adjust the pH of the buffer solution before use.

The decay of **3** was followed at 276 nm in a Hewlett Packard 8452A diode array spectrophotometer equilibrated at 23 °C. Kinetic runs were initiated by the addition of 4 μL of a stock solution of **3** (19.8 mM) in ethanol to buffer (0.996 mL) which was equilibrated to 23 °C prior to use. The final concentration of **3** was 79 μM. The rate constants obtained were reproducible in multiple runs. Stock solutions of **3** were freshly made after 24 h. The reactions were monitored for 20 min.

Absorbance readings for the decomposition of **3** were measured for every curve every 2 s. The readings collected after 12 min and after 20 min were fitted to a single exponential by nonlinear regression analysis using the commercially available program known as Enzfitter (Elsevier Science Publishers, Amsterdam).[12] The model did not take into consideration the ketonization of **3** to **4**. The model fits were excellent for all runs. The individual sets of rate constants (k_1, k_2) determined from these analyses were then fitted to eq 1 over the measured pH range and buffer concentration using commercially available software known as SAS.[36] The values reported in Table 1 were those determined for the data collected after 12 min and were within experimental error of those values determined for the data collected after 20 min. The pH of the solution measured after mixing **3** and buffer was used in these analyses. Although the fit to eq 1 was excellent (correlation constant > 0.95), the narrow pH range precluded a reliable estimate of k_{H^+} and k_{OH^-}. Buffer species other than H_2PO_4 and HPO_4 were assumed to be present at insignificant concentrations. Finally, the composition of the mixture at selected time intervals was determined from the calculated rate constants using the commercially available program known as MINSQ (MicroMath Scientific Software, Salt Lake City, UT).

Ketonization of 1 to 2 by CHMI and Conversion of 2 to 2-Hydroxy-5-(carboxymethyl)-3-(*E*)-hexenedioate (7). 5-(Carboxymethyl)-2-hydroxymuconate (**1**, 450 mg, 2.08 mmol) was dissolved in dimethyl sulfoxide (0.9 mL). In 30 separate reaction mixtures, a portion (30 μL) of the solution containing **1** was added to 100 mM Na_2HPO_4 buffer (2 mL, pH = 9.26) and mixed by inversion. The addition of **1** to buffer resulted in a final buffer pH of 6.5. Subsequently, a quantity of CHMI (30 μL, ~60 units) was added to each reaction mixture. After stirring at room temperature for 5 min, $NaBH_4$ (~2 mg) was added to the separate reaction mixtures. The individual reaction mixtures were then combined (total volume of 60 mL, final pH 9.3) and allowed to stir for 30 min. The solution was subjected to chromatography on a Dowex-1 (formate) column (20 × 2.0 cm), eluting with linear gradient of aqueous formic acid (0–5 M, 100-mL total volume), followed by an additional quantity of formic acid (5 M, 50 mL). The eluent was collected in 10-mL fractions. The two isomers (**7** and **8**) eluted at the end of the gradient as two overlapping peaks. The appropriate fractions (9–13) were combined and evaporated to dryness under mechanical vacuum to yield a mixture of **7** and **8**.[1] The isomers were separated by crystallization from ethyl acetate/hexanes. Filtration of the crystallization solvent removed **8**, the undesired isomer. Evaporation of the mother liquor generated the desired isomer, **7**, as the major product (271 mg) in 60% yield. ^1H NMR analysis indicated a mixture of **7** (92%) and **8** (8%).[1]

Catalytic Reduction of 7. To a hydrogenation bottle containing a solution of **7** (271 mg, 1.24 mmol) in 1:1 ethanol/benzene (10 mL) was added a catalytic amount of $(Ph_3P)_3RhCl$ (27 mg). After the bottle had been flushed with H_2, the mixture was shaken vigorously under H_2 (45 lbs/in.^{2}) in a Parr apparatus at room temperature for 19 h. The solution was concentrated, and the concentrate was dissolved in methanol. The resulting solution was filtered, and the filtrate was evaporated to generate **9** as the major product: ^1H NMR (CD_3OD, 250 MHz) δ 1.40 (4H, brd m, H3, H4), 2.13 (1H, brd d, $J_{5,6}$ = 15.7 Hz, H6), 2.37 (1H, brd d, $J_{5,6}$ = 15.7 Hz, H6), 2.48 (1H, brd m, H5), 3.80 (1H, brd d, $J_{2,3}$ = 6.7 Hz, H2).

Conversion of 9 to 3-Carboxyadipic Acid (11). To a stirring solution of **9** (270 mg, 1.24 mmol) in 2 M acetic acid (10 mL) was added lead tetraacetate (605 mg, 1.36 mmol). After being stirred at room temperature for 2 h, the solution was diluted with H_2O and filtered, and the yellow filtrate was evaporated to dryness. The solid residue was suspended in 2 M H_2SO_4 (15 mL) and oxidized by adding solid $KMnO_4$ (300 mg, 1.9 mmol) in two portions (a 200-mg portion followed by a 100-mg portion after 30 min). After 1 h, 5% $NaHSO_3$ was added dropwise until the reaction mixture was clear. Subsequently, the pH of the solution was adjusted to 7.0. The solution was subjected to chromatography on a Dowex-1 (formate) column (0.8 × 10 cm), eluting with a formic acid gradient (0–5 M formic acid, 30-mL total volume). Fractions were collected in 3-mL portions. The appropriate fractions (8–10) were pooled and evaporated to dryness under mechanical vacuum to recover 29 mg (12 %) of **11** as the major product.

Further purification of **11** was achieved by HPLC using an analytical reverse-phase column (Alltech Associates, Inc., RPC600, 10 μm, 250 × 4.6 mm) with an acetonitrile/water (92.5:7.5) mobile phase. The

(36) SAS Institute Inc. *SAS Introductory Guide for Personal Computers, Release 6.03 Edition*; SAS Institute Inc.: Cary, NC, 1988.

eluent was monitored by a refractive index detector. At a flow rate of 0.5 mL/min, **11** eluted at 4.4 min. In this manner, it was possible to obtain **11** (13.15 mg): $[\alpha]^{23}_D = -9.35°$ ($c = 0.021$, acetone); ^{1}H NMR (CD_3OD, 500 MHz) δ 1.89 (2H, brd m, H4), 2.37 (2H, brd m, H5), 2.47 (1H, dd, H2), 2.66 (1H, dd, H2), 2.81 (1H, brd m, H3); ^{13}C NMR (CD_3OD, 500 MHz) δ 28.0 (C-4), 32.4 (C-2), 36.8 (C-5), 41.8 (C-3), 175.4 (C-3), 176.6 (C-1), 178.0 (C-6).

Stereospecific Ketonization of 3 to [3-^{2}H]5 in ^2H_2O by COHED and Conversion of [3-^{2}H]5 to [3-^{2}H]12. 2-Hydroxy-2,4-heptadiene-1,7-dioate (**3**, 46 mg, 0.27 mmol) was dissolved in dimethyl sulfoxide-d_6 (0.3 mL). In 10 separate reactions, a portion (30 μL) of the solution containing **3** was added to 100 mM $Na_2{}^2HPO_4$ buffer (0.6 mL, pD = 9.14). Addition of **3** adjusted the pD of the buffer to ~6.5. Subsequently, a quantity of COHED (100 μL, ~20 units) which had been previously exchanged into 20 mM $Na_2{}^2HPO_4$ buffer (pD ~7.0) containing $MgCl_2$ (10 mM) by repeated centrifugation of the enzyme in a Centricon-10 microconcentrator, was added to each reaction mixture. After mixing by inversion, an excess of $NaBH_4$ was added to the separate reaction mixtures. The individual reaction mixtures were then combined, and the pH of the resulting solution (6 mL) was adjusted to 9.0. The solution was subjected to chromatography on a Dowex-1 (formate) column (0.8 × 15 cm), eluting with a formic acid gradient (0−5 M formic acid, 100 mL). The eluent was collected in 10-mL fractions. The appropriate fractions (3 and 4) were combined and evaporated to dryness under mechanical vacuum to yield 40.7 mg (88%) of [3-^{2}H]**12**.[6] The ^{1}H NMR spectrum (CD_3OD, 250 MHz) corresponded to the previously published spectrum.[6]

Conversion of [3-^{2}H]12 to [3-^{2}H]Malate. A solution of [3-^{2}H]**12** (20.3 mg, 0.12 mmol) in dioxane (5 mL) and methanol (0.05 mL) at 5 °C was subjected to a stream of O_3 (1 L/min) for 20 min. After the solvent was evaporated to dryness, H_2O_2 (0.5 mL, 30%) and glacial acetic acid (2.5 mL) were added to the residual oil, and the mixture was stirred at room temperature overnight.[20,21] The solution was evaporated to dryness, and 5% $NaHCO_3$ (2 mL) was added. The [3-^{2}H]-malate was subjected to chromatography on a Dowex-1 (formate) column (0.8 × 15 cm), eluting with a formic acid gradient (0−5 M formic acid, 100-mL total volume). The eluent was collected in 10-mL fractions. Malate eluted in one fraction at ~2.0−2.5 M formic acid. The appropriate fraction was evaporated to dryness under mechanical vacuum to yield 4.4 mg (28%) of [3-^{2}H]malate. The ^{1}H NMR spectrum (20 mM $Na_2{}^2HPO_4$ in ^2H_2O, 250 MHz) corresponded to the previously published spectrum.[21]

The purified [3-^{2}H]malates (4.4 mg, 0.03 mmol) were dissolved in Na_2HPO_4 buffer (20 mM, 3 mL, pH 7.5) containing $MgCl_2$ (1 mM), β-NADP (20 mg, 0.026 mmol), and malic enzyme (3.5 units) from chicken liver.[21,22] After stirring at room temperature overnight, (2*R*)-[3-^{2}H]malate was recovered by chromatography on a Dowex-1 (formate) column as described above. (2*R*,3*S*)-[3-^{2}H]**13**: ^{1}H NMR (20 mM $Na_2{}^2HPO_4$ in ^2H_2O, 500 MHz) δ 2.33 (~0.1H, brd s, H3), 4.29 (brd d, H2). (2*R*, 3*R*)-[3-^{2}H]**13**: ^{1}H NMR (20 mM $Na_2{}^2HPO_4$ in ^2H_2O, 500 MHz) δ 2.63 (~0.9H, brd s, H3), 4.28 (~0.9H, brd d, H2).

Thermal Decarboxylation of 1. In a typical reaction, a solution of **1** (200 mg, 0.93 mmol) dissolved in anhydrous dimethyl sulfoxide (0.4 mL) was placed in a test tube, sealed with a rubber septum, and purged with argon. The stirring solution was heated at 120 °C until the reaction no longer generated gas bubbles (~10 min). Subsequently, the mixture was chilled in an ice bath, diluted with ethyl acetate (100 mL), and washed with H_2O (2 × 50 mL). The organic layer was collected, dried over $MgSO_4$, and filtered, and the filtrate was evaporated to dryness to give a yellow solid (100 mg, 63%). The solid was identified by ^{1}H NMR spectroscopy as a mixture of (4*E*)- and (4*Z*)-**3**. (4*E*)-**3**: ^{1}H NMR (DMSO-d_6, 250 MHz) δ 3.10 (2H, d, $J = 7.25$ Hz, H6), 5.85 (1H, dt, $J_{4,5} = 15.7$ Hz, $J = 7.1$ Hz, H5), 6.08 (1H, d, $J = 10.7$ Hz, H3), 6.38 (1H, dd, $J_{4,5} = 15.6$ Hz, H4). (4*Z*)-**3**: ^{1}H NMR (DMSO-d_6, 250 MHz) δ 3.15 (2H, dd, $J = 7.5$ Hz, $J = 1.6$ Hz, H6), 5.61 (1H, dt, $J_{4,5} = 10.7$ Hz, $J = 7.1$ Hz, $J = 2.8$ Hz, H5), 6.23 (1H, d, $J = 12.6$ Hz, H3), 6.43 (1H, dd, $J_{4,5} = 10.7$ Hz, H4). Heating the reaction mixture for a longer period of time results in the formation of **4**: ^{1}H NMR (Na_2HPO_4 buffer, pH = 6.5, 500 MHz) δ 2.37 (2H, t, H6), 2.55 (2H, dd, H5), 6.20 (1H, d, $J_{3,4} = 17.5$ Hz, H3), 7.05 (1H, dt, $J_{3,4} = 17.5$ Hz, H4).

Determination of the Configuration of 3 Generated from (5*R*)-2 by COHED. The enzymatic generation of **3** by the action of COHED and CHMI on **1** has been described elsewhere.[6] The ^{1}H NMR spectrum presented in Figure 2A was recorded 85 min after the addition of COHED and CHMI to buffer containing **1**.[6] The addition of **3** (4.5 mg in 10 μL of DMSO-d_6), isolated according to the procedure described above, to buffer (100 mM Na_2HPO_4, 0.6 mL) results in the ^{1}H NMR spectrum presented in Figure 2B. Addition of **3** to the buffer adjusts the pH to 6.5. The thermal decarboxylation of **1** (5.6 mg, 0.03 mmol) was carried out in 30 μL of DMSO-d_6 according to the procedure described above. The resulting mixture of (4*E*)-**3**, (4*Z*)-**3**, and **4** was added to the NMR tube containing the isolated (4*Z*)-**3** in buffer (100 mM Na_2HPO_4, 0.6 mL). This procedure results in the ^{1}H NMR spectrum presented in Figure 2C. Spectra were acquired on a Bruker AM 500-MHz spectrometer in 99% H_2O solution using a selective composite pulse presaturation of the water signal with a 2-s duration. The lock signal is DMSO-d_6. Chemical shifts are standardized to the residual H_2O resonance of 4.70 ppm. (4*Z*)-**3**: ^{1}H NMR (Na_2HPO_4 buffer, pH = 6.5, 500 MHz) δ 3.15 (2H, d, $J = 9.3$ Hz, H6), 5.54 (1H, dt, $J_{4,5} = 10.7$ Hz, H5), 6.09 (1H, d, $J = 11.7$ Hz, H3), 6.21 (1H, t, $J_{4,5} = 10.7$ Hz, H4). (4*E*)-**3**: ^{1}H NMR (Na_2HPO_4 buffer, pH = 6.5, 500 MHz) δ 3.05 (2H, d, $J = 5.8$ Hz, H6), 5.76 (1H, dt, $J_{4,5} = 15.5$ Hz, H5), 6.02 (1H, d, $J = 12.6$ Hz, H3), 6.19 (1H, t, $J = 15.6$ Hz, H4). The chemical shifts and coupling constants for the protons of **5** have been reported elsewhere.[6]

^{1}H NMR Detection of (3*E*)-4 Generated by the 4-OT-Catalyzed Ketonization of 3. A solution of **3** (4.5 mg, 0.026 mmol) dissolved in dimethyl sulfoxide-d_6 (10 μL) was added to 100 mM $Na_2{}^2HPO_4$ buffer (pD = 9.14, 0.6 mL) and transferred to a NMR tube. The addition of **3** to buffer adjusts the pD to 7.22 and makes the concentration of **3** approximately 43 mM. The reaction was initiated by the addition of 4-OT (16 μg in 0.02 μL, ~150 units), which had been previously exchanged into 20 mM $Na_2{}^2HPO_4$ buffer (pD ~7.0) by repeated centrifugation of the enzyme in a Centricon-10 microconcentrator. The reaction is complete after 1.5 min. ^{1}H NMR ($Na_2{}^2HPO_4$ buffer, pD 7.22, 250 MHz): δ 2.27 (2H, d, $J = 5$ Hz, H6), 2.45 (1H, brd dd, H5), 6.10 (1H, d, $J = 15$ Hz, H3), 6.96 (1H, dd, $J_{3,4} = 15$ Hz, $J_{4,5} = 5$ Hz, H4).

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