Chemical and Enzymatic Ketonization of 5-(Carboxymethyl)-2-hydroxymuconate

Gholamhossein Hajipour, William H. Johnson, Jr., Paul D. Dauben, Neal J. Stolowich,† and Christian P. Whitman*

Contribution from the Medicinal Chemistry Division, College of Pharmacy, The University of Texas, Austin, Texas 78712, and Center for Biological NMR, Department of Chemistry, Texas A&M University, College Station, Texas 77843

Received October 8, 1992

Abstract: The conjugated enol, 5-(carboxymethyl)-2-hydroxymuconate (**1**),[1] is a stable dienol generated in the course of bacterial catabolism of 4-hydroxyphenylacetate by the enzymes of the homoprotocatechuate pathway. The dienol ketonizes chemically in aqueous solution and enzymatically by the action of 5-(carboxymethyl)-2-hydroxymuconate isomerase (EC 5.3.2) to an α,β-unsaturated ketone, (*E*)-2-oxo-5-(carboxymethyl)-3-hexenedioate (**2**). Mechanistic studies for both processes remain largely unexplored. An examination of the behavior of **1** in phosphate buffer has been completed using UV and ^{1}H NMR spectroscopy. The results of these studies indicate that a rapid equilibrium forms between **1** and the β,γ-unsaturated ketone, 2-oxo-5-(carboxymethyl)-4-hexenedioate (**6**) before a much slower conversion to **2**. The spectroscopic results were confirmed by reduction of the isomeric ketones with sodium borohydride and subsequent identification of the products. The rapid interconversion of **1** and **6** in aqueous phosphate buffer raises the question of whether the enzyme has a preference for one isomer as its substrate. The values of k_{cat}/K_M determined for **1** and a mixture of **1** and **6** suggest that both **1** and **6** are excellent substrates for CHMI. In addition, these studies indicate that **1** is kinetically competent to be an intermediate in the overall reaction. A reasonable hypothesis to explain these observations is that 5-(carboxymethyl)-2-hydroxymuconate isomerase catalyzes the transformation of **6** to **2** through the intermediacy of **1**. The relevance of these findings to a related dienol, 2-hydroxymuconate (**3**), and the in vivo catabolism of 3,4-dihydroxyphenylacetate by the enzymes of the homoprotocatechuate pathway are briefly discussed.

5-(Carboxymethyl)-2-hydroxymuconate[1] (**1**; Scheme I) is an intermediate generated in the course of bacterial catabolism of 4-hydroxyphenylacetate by the enzymes of the homoprotocatechuate (hpc) meta-fission pathway.[2-5] It is subject to chemical ketonization in aqueous solution and enzymatic ketonization by an enzyme in the pathway, 5-(carboxymethyl)-2-hydroxymuconate isomerase (EC 5.3.2; CHMI) to generate the α,β-unsaturated ketone, 2-oxo-5-(carboxymethyl)-3-hexenedioate (**2**).[2,3] While studying the chemical and enzymatic ketonization of a related compound, 2-hydroxymuconate,[1] (**3**; Scheme I), we became interested in mechanism of these processes for **1**.[6]

Our previous work on **3** indicates that its behavior in aqueous solution and in the presence of 4-oxalocrotonate tautomerase (4-OT) is considerably more complex than previously thought.[6] In phosphate buffer, a rapid equilibrium forms between **3** and the β,γ-unsaturated ketone, (*E*)-2-oxo-4-hexenedioate (**5**), before a much slower conversion to the α,β-isomer, (*E*)-2-oxo-3-hexenedioate (**4**) (Scheme II).[6] This behavior is fully consistent with the properties of other so-called slow-reacting dienols.[7] The rapid interconversion of **3** and **5** raises the question of whether the enzyme has a preference for one isomer. The results of kinetic studies suggest that enzyme acts as an isomerase catalyzing the transformation of **5** to **4** through the intermediacy of **3**.[6]

The structures of **1** and **3** differ by the carboxymethyl group at C-5 of **1**. This modest difference suggests that the chemical properties of the two dienols as well as the mechanisms of the two enzymes might be similar. Hence, it is likely that **1** behaves like **3** and other slow-reacting dienols in that it ketonizes initially to afford the β,γ-unsaturated ketone, 2-oxo-5-(carboxymethyl)-4-hexenedioate (**6**; Scheme II) before a slower conversion to the α,β-isomer **2**.[7] The reactivity of **1**, however, is almost certainly different than that of **3** because it is well known that several factors including steric hindrance to protonation, can affect the rate and site of protonation on a dienol.[8] It is also likely that the mechanism of CHMI resembles the mechanism of 4-OT such that CHMI acts as an isomerase and transforms **6** to **2** through the dienolic intermediate **1** (Scheme II). The major differences between the two enzymes may be the amino acid residues responsible for isomerization and imparting the different specificities. Although these predictions are certainly reasonable, they have not yet been verified experimentally. Moreover, the existing literature does not adequately identify **1** and **2** nor provide any substantial characterization of their properties. In light of the recent interest in dienol chemistry[7-9] as well as our interest

* Address correspondence to this author.
† Texas A & M University.

(1) In order to simplify discussion of **1** and **3**, we use their trivial names, 5-(carboxymethyl)-2-hydroxymuconate and 2-hydroxymuconate, respectively. The systematic name for **1** is 5-hydroxy-2,4-pentadiene-1,2,5-tricarboxylic acid and the systematic name for **3** is 1-hydroxy-1,3-butadiene-1,6-dicarboxylic acid. In addition, the numbering system used throughout the results section and discussion is designated on **1** in Scheme I.

(2) Sparnins, V. L.; Chapman, P. J.; Dagley, S. *J. Bacteriol.* **1974**, *120*, 159–167.

(3) Garrido-Pertierra, A.; Copper, R. A. *Eur. J. Biochem.* **1981**, *117*, 581–584.

(4) Jenkins, J. R.; Cooper, R. A. *J. Bacteriol.* **1988**, *170*, 5317–5324.

(5) Roper, D. I.; Cooper, R. A. *FEBS Lett.* **1990**, *266*, 63–66.

(6) Whitman, C. P.; Aird, B. A.; Gillespie, W. R.; Stolowich, N. J. *J. Am. Chem. Soc.* **1991**, *113*, 3154–3162.

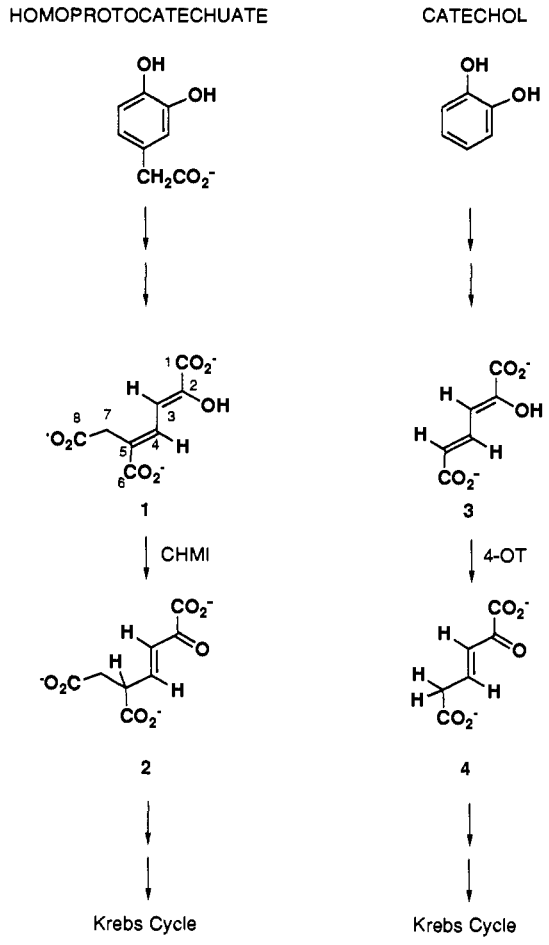
(7) Dzingeleski, G. D.; Blotny, G.; Pollack, R. M. *J. Org. Chem.* **1990**, *55*, 1019–1023.

(8) Pollack, R. M.; Bounds, P. L.; Bevins, C. L. In *The Chemistry of Enones*; Patai, S., Rappoport, Z., Eds.; John Wiley & Sons Ltd.: New York, 1989; pp 559–597.

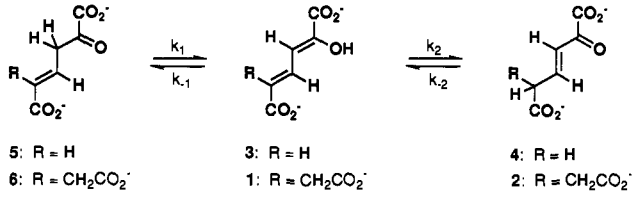
(9) (a) Whalen, D. L.; Weimaster, J. F.; Ross, A.; Radhe, R. *J. Am. Chem. Soc.* **1976**, *98*, 7319–7324. (b) Duhaime, R. M.; Weedon, A. C. *J. Am. Chem. Soc.* **1985**, *107*, 6723–6724. (c) Duhaime, R. M.; Weedon, A. C. *Can. J. Chem.* **1987**, *65*, 1867–1872. (d) Duhaime, R. M.; Weedon, A. C. *J. Am. Chem. Soc.* **1987**, *109*, 2479–2483. (e) Pollack, R. M.; Mack, J. P. G.; Blotny, G. *J. Am. Chem. Soc.* **1987**, *109*, 3138–3139. (f) Dzingeleski, G. D.; Bantia, S.; Blotny, G.; Pollack, R. M. *J. Org. Chem.* **1988**, *53*, 1540–1544. (g) Pollack, R. M.; Zeng, B.; Mack, J. P. G.; Eldin, S. *J. Am. Chem. Soc.* **1989**, *111*, 6419–6423.

0002-7863/93/1515-3533$04.00/0

Scheme I



Scheme II

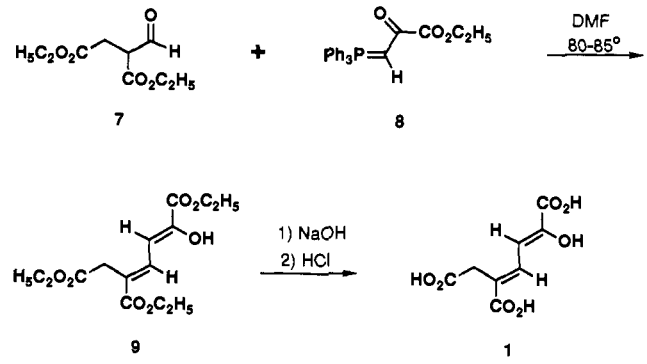


in the mechanism and evolutionary origin of CHMI and 4-OT,[6,10] we synthesized **1** and characterized its chemical and enzymatic properties.

The results of our investigation confirm that there are some similarities as well as some intriguing differences between the reactivity of the two dienols and the mechanisms of the two enzymes. In aqueous solution, we observe the formation of a rapid equilibrium between **1** and **6** before a much slower conversion to **2**. While this behavior is similar to that of **3**, we find that the partitioning of **1** to **2** and **6**, the distribution among **1**, **2**, and **6** at an estimated equilibrium, and the stability of **2** are different. With regard to the mechanism of CHMI, examination of the values of k_{cat}/K_M determined for **1** and a mixture of **1** and **6** show that both **1** and **6** are excellent substrates for CHMI. These studies suggest that CHMI is also an isomerase catalyzing the transformation of **6** to **2** through the intermediacy of **1**. More interestingly though, the proposed intermediate in the CHMI reaction is kinetically competent in the overall reaction whereas the proposed intermediate in the 4-OT reaction is not. Furthermore, CHMI does not operate near the diffusion control limit

(10) Whitman, C. P.; Hajipour, G.; Watson, R. J.; Johnson, W. H., Jr.; Bembenek, M. E.; Stolowich, N. J. *J. Am. Chem. Soc.* **1992**, *114*, 10104–10110.

Scheme III



in contrast to 4-OT. It is apparent from these data that the structural difference between **1** and **3** results in a modest difference in their corresponding chemical and enzymatic properties. Finally, these findings have implications for the proposed intermediates in the homoprotocatechuate meta-fission pathway.

Results

Synthesis of 5-(Carboxymethyl)-2-hydroxymuconate. The isolation of **1** has been described by Sparnins et al.[2] and Jenkins et al.[4] Both groups generated the intermediate enzymatically from 4-hydroxyphenylacetate. The former used heat-treated extracts of *Pseudomonas putida U* while the latter used a clone that produced only the enzymes necessary for the conversion of 4-hydroxyphenylacetate to **1**.[2,4] The isolated compound was identified as **1** based on the λ_{max} at 300 nm and the parent ion peak in the mass spectrum of a tetrasilylated derivative.[2] Because these procedures are tedious and the strains are not widely available, we devised a straightforward synthesis for **1** (Scheme III). The triester of CHM (**9**) is synthesized by a Wittig reaction between diethyl formylsuccinate (**7**) and the substituted ylide **8**. The synthesis of **7** and **8** are described elsewhere.[11,12] Alkaline hydrolysis of **9** generates the trisodium salt which is subsequently acidified to afford **1**. The isolated compound was identified as **1** by ^1H and ^{13}C NMR spectroscopy and elemental analysis.

Kinetics of Nonenzymic Ketonization of 1. The ketonization of **1** in aqueous phosphate buffer (μ = 0.2 M, NaCl, 0.5% ethanol, 30 °C) is monitored at pH values 6.50, 7.00, and 7.50 by observing the decay in UV absorbance at 300 nm in different concentrations of phosphate buffer (0.002–0.05 M). In all cases, a very rapid drop in absorbance followed by a much slower decrease is observed. A representative experiment (20 mM Na_2HPO_4/NaH_2PO_4, pH 7.49) is shown in Figure 1. The insert in Figure 1 clearly shows the initial precipitous drop in absorbance which slows after the first 36 s. The initial drop in absorbance corresponds to the rapid ketonization of **1** to form the β,γ-unsaturated ketone **6** (Scheme II). The subsequent slower decrease in absorbance results from the slower formation of **2** from **1** (Scheme II).

The initial rapid rate of decay of **1** in buffer precludes a reliable estimate of the uncatalyzed rate of ketonization (k_o) as well as the effect of pH and buffer using conventional spectrophotometry.[13] In order to provide a quantitative comparison with the CHMI reaction and the decay of **3**, the ketonization of **1** and **3** is followed in nearly identical solutions under nearly identical conditions. Accordingly, the decay of **1** and **3** are monitored in separate experiments at 300 and at 295 nm, respectively, in phosphate buffer (20 mM Na_2HPO_4/NaH_2PO_4, pH 7.49) under the conditions described above. The data in both experiments

(11) Wermuth, C. G. *J. Org. Chem.* **1979**, *44*, 2406–2408.

(12) Ernest, I.; Gostel, J.; Greengrass, C. W.; Holick, W.; Jackman, D. E.; Pfaendler, H. R.; Woodward, R. B. *J. Am. Chem. Soc.* **1978**, *101*, 8214–8222. A significantly better yield is obtained by mixing ethyl bromopyruvate and triphenylphosphine in toluene at 0 °C instead of methylene chloride at room temperature.

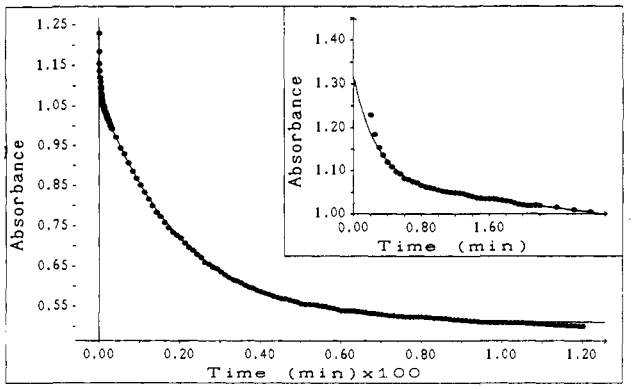


Figure 1. Plot of absorbance vs time for the reaction of 5-(carboxymethyl)-2-hydroxymuconate (**1**) in 20 mM Na_2HPO_4/NaH_2PO_4, (pH 7.49, 0.5% ethanol, μ = 0.2 M, NaCl, 30 °C).

Table I. Rate Constants for the Decay of **1** and **3** in 20 mM Na_2HPO_4 Buffer, pH 7.49[a]

$10^2 \times K_{obs}$, min^{-1}	**1**	**3**
k_1[b]	182 ± 8.6	52.5 ± 0.3
k_{-1}	354 ± 6.2	64.4 ± 0.4
k_2	3.98 ± 0.05	7.86 ± 0.02
k_{-2}	2.29 ± 0.01	0.93 ± 0.01

[a] The rate constants are derived from a double exponential curve and are defined in Scheme II. [b] Errors are standard deviations.

give a good fit to a double exponential curve. The kinetic parameters for the mechanism shown in Scheme II are estimated by nonlinear least-squares regression of the absorbance data generated in this experiment.[14] After 60 min, it is estimated that the mixture contains 32.5% **1**, 50.8% **2**, and 16.7% **6**.[15]

The rate constants are summarized in Table I along with the rate constants determined for **3** under the same conditions. Three features are apparent from a comparison of these rate constants. First, protonation of **1** occurs more rapidly at the α-carbon rather than the γ-carbon (~46-fold) under these conditions. The corresponding factor for **3** (~7) is much less. Second, the reverse rates, particularly k_{-1}, show a greater propensity for the formation of **1** than do the corresponding reverse rates for **3**. Finally, the rate of ketonization of **1** to **2** is about half the rate of ketonization of **3** to **4**.

^{1}H NMR Proof for the Formation of 6 during Chemical Ketonization of 1. The nonenzymatic decay of **1** can be monitored by ^{1}H NMR spectroscopy in order to provide confirmatory evidence for the mechanism shown in Scheme II. The ^{1}H NMR spectra (Figures 2, parts A–C) recording the fate of **1** during its nonenzymic decay in ^2H_2O are consistent with the mechanism. A ^{1}H NMR spectrum of **1** in methanol-d_4 shows the three sets of signals corresponding to the three different proton types of **1** (Figure 2, part A). The doublet furthest upfield at 6.14 ppm (J = 12.5 Hz) is assigned to the proton on C-3 while the doublet at 7.62 ppm (J = 12.5 Hz) is assigned to the proton on C-4. The methylene protons on C-7 produce a singlet at 3.22 ppm (not shown). After 2 min (Figure 2, part B), decay of **1** in buffer results in the appearance of a new singlet superimposed upon the downfield doublet (7.25 ppm). After 20 min (Figure 2, part C), only the singlet remains (Figure 2, part C). Moreover, concomitant with the appearance of the new singlet is the near disappearance of the upfield doublet assigned to the proton on C-3 of **1** (6.10 ppm).

The key to the interpretation of the spectra is the identification of the species giving rise to the singlet at 7.25 ppm.[16] The

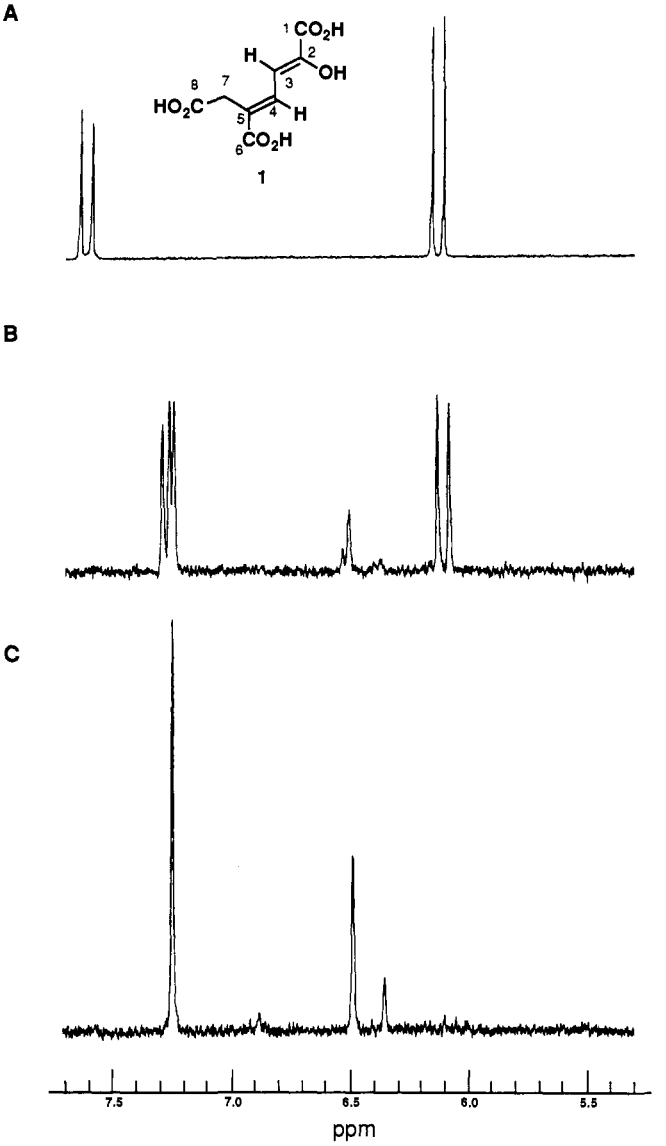


Figure 2. Partial ^{1}H NMR (250 MHz, ^2H_2O) spectra indicating the time course of the decay of 5-(carboxymethyl)-2-hydroxymuconate (**1**) in 100 mM $Na_2[^2H]PO_4$ (final pD = 7.0, 1.7% v/v dimethyl sulfoxide-d_6). (A) In methanol; (B) after 2 min in buffer; and (C) after 20 min in buffer.

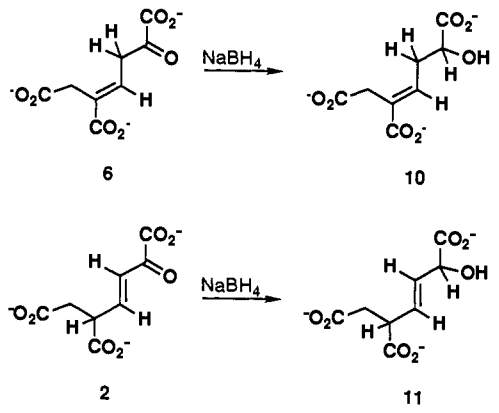
(13) Although in all cases, we observe a rapid drop in absorbance followed by a slower decrease in absorbance, there is a significant variability in the kinetic constants when the ketonization of **1** is monitored at the different pH values (~6.5, ~7.0, and ~7.5) and the various concentrations of buffer (0.002–0.05 M). At lower buffer concentration, the pH varies considerably after the addition of **1** presumably because addition of the triacid exceeds the buffering capacity. In addition, at all buffer concentrations, the estimated half-life of **1** is nearly equal to or less than the time required to initiate the experiment (~10 s). Hence, the initial part of the decay is not accurately recorded. In addition to these problems, the product, **2**, is subject to facile decarboxylation at C-5.[19] It is, however, apparent from the data that phosphate buffer has an effect on the ketonization of **1** as previously observed for the ketonization of **3**.[6] It is not possible to say whether the effect is general acid or general base catalysis.

(14) (a) Veng-Pederson, P. *J. Pharmacokin. Biopharm.* **1977**, *5*, 513–531. (b) Moore, J. W.; Pearson, R. G. *Kinetics and Mechanism: A Study of Homogeneous Chemical Reactions*, 3rd ed.; John Wiley & Sons: New York, 1981. (c) Capellos, C.; Bielski, B. H. J. *Kinetic Systems: Mathematical Description of Chemical Kinetics in Solution*; Robert E. Krieger Publishing Co.: Huntington, NY, 1980.

(15) An equilibrium constant for this reaction was estimated based on the assumption that a sufficient quantity of enzyme produces an equilibrium mixture of **1**, **2**, and **6** before decarboxylation of **2** at C-5 significantly perturbs the equilibrium. The quantity of **1** remaining after the CHMI-ketonization of **1** was measured directly by UV spectrophotometry. The quantities of **2** and **6** were estimated from their peak areas in ^{1}H NMR spectral data. The composition of the equilibrium mixture reported here after 60 min estimated is in reasonably good agreement with the composition estimated using peak areas in the ^{1}H NMR spectra.

(16) The large upfield shift observed between Figure 2, parts A and B (from 7.62 to 7.25 ppm) does not result from deuterium incorporation at C-3 of **1**. It merely reflects a change in solvents (methanol to phosphate buffer).

Scheme IV



6 10

2 11

concomitant loss of the upfield doublet at 6.10 ppm indicates that this singlet can only result by deuteriation at C-3 of **1** such that [3-$^2H_1$]**1** is the major species present. Rapid exchange of the proton on C-3 of **1** with a solvent deuteron through the intermediate [3-$^2H_1$]**6** affords [3-$^2H_1$]**1**. The formation of [3-$^2H_1$]-**1** from [3-$^2H_1$]**6** is favored as a result of an isotope effect and the greater stability of the dienol **1**. This result is entirely consistent with previous results following the decay of **3** in phosphate buffer.[6]

Rapid exchange of the proton on C-3 of **1** is accompanied by the appearance of a set of ^1H NMR signals that can reasonably be assigned to the protons on C-4 of the 4*E* and 4*Z* isomers of **6** (Figure 2, part C). The singlet at 6.50 ppm corresponds to the *E* isomer while the singlet at 6.36 corresponds to the *Z* isomer.[17] The methylene protons at C-7 of these two isomers generate singlets at 3.10 ppm and 3.07 ppm, respectively (not shown). The intensities of the signals indicate that the *E* isomer predominates in solution. Presumably, these isomers result from free rotation about the 4,5 bond of **2**. Finally, the protons on C-3 of **6** are observed as a broadened singlet centered at 3.53 ppm (not shown) and are present only in early spectra (2 min) following the decay of **1**. The broadening and eventual loss of the signal corresponding to the protons on C-3 along with the simplification of the signal assigned to the proton on C-4 of **6** are consistent with the exchange of these methylene protons with solvent deuterons by the interconversion of **1** and **6**.[18]

Chemical Trapping of 6 in the Chemical Ketonization of 1. The presence of **6** during the chemical ketonization of **1** can be confirmed chemically by trapping the unstable β,γ-unsaturated isomer through its immediate conversion to 2-hydroxy-5-(carboxymethyl)-4-hexenedioate (**10**) with $NaBH_4$ (Scheme IV). Our strategy for the trapping of **6** is based on its generation from the chemical decay of **1** in phosphate buffer. The nearly exclusive formation of **10** can be achieved by the addition of $NaBH_4$ at short intervals (3–5 min) because of the nearly 46-fold differences in the rate of formation of **6** and **2** from **1**. Conversion of **6** to its respective product with $NaBH_4$ effectively removes it from the equilibrium mixture and allows complete ketonization of **1**.

After repeated additions of $NaBH_4$ to a solution containing buffer and **1**, the reduced alcohol, 2-hydroxy-5-(carboxymethyl)-4-hexenedioate (**10**), is the only product recovered in quantitative yield by ion-exchange chromatography as determined by ^1H and ^{13}C NMR analysis. The upfield region (2.3–4.4 ppm) of its ^1H NMR spectrum (Figure 3) shows the characteristic coupling pattern for **10**. The methylene protons on C-3 of compound **10**, adjacent to both C-4 and the chiral center at C-2, give rise to overlapping multiplets, centered at 2.49 ppm, because they are diastereotopic. Accordingly, the signal assigned to the proton on C-2 of **10** is a doublet of doublets (4.22 ppm; J = 5 and 7 Hz). The remaining signal in this region (3.19 ppm) corresponds to the methylene protons on C-7 and appears as a singlet—consistent with the structure of **10**. The triplet corresponding to the olefinic proton at C-4 is located at 6.81 ppm (J = 7 Hz; not shown). The ^{13}C NMR chemical shifts can be found in the Experimental Section and are fully consistent with the structure of **10**.

Identification of the Product of the CHMI-Catalyzed and Chemical Decay of 1. The enzyme-catalyzed and chemical ketonization of 5-(carboxymethyl)-2-hydroxymuconate (**1**) result in the formation of the same product. At pH 7.20 (20 mM Na_2HPO_4/NaH_2PO_4) UV analysis reveals (Figure 4, part A) facile chemical decay of **1** (λ_{max} = 300 nm) accompanied by the formation of a product (λ_{max} = 236 nm) with two isosbestic points at 222 and 266 nm. Addition of CHMI (0.4 unit) from *Escherichia coli C* to an identical mixture affords the same spectral changes (Figure 4, part B) although at a faster rate. After 21 min, the peak at 236 nm slowly diminishes in intensity.[19]

The product with a λ_{max} at 236 nm was identified as (*E*)-2-oxo-5-(carboxymethyl)-3-hexenedioate (**2**) by its immediate conversion to (*E*)-2-hydroxy-5-carboxymethyl-3-hexenedioate (**11**) with $NaBH_4$ (Scheme IV). ^1H NMR analysis reveals the presence of two products, **10** and **11**, which are separated by anion-exchange chromatography. The reduced compounds are readily distinguishable from one another by characteristic coupling patterns for the methylene protons (C-3 of **10** and C-7 of **11**) and the C-2 protons. Analysis of the upfield region (1.8–4.3 ppm) of a ^1H NMR spectrum (Figure 5) of the separated isomer **11** is consistent with its predicted coupling pattern. The methylene protons on C-7 of **11** are adjacent to the chiral center at C-5, giving rise to separate signals (2.00 and 2.35 ppm) because they are diastereotopic. The signal corresponding to the proton on C-5 of **11** appears to be a doublet of triplets (2.95 ppm) although not well defined. The signal assigned to the proton on C-2 is observed as a doublet (4.10 ppm) because it is coupled to the single proton on C-3. Finally, the downfield region of the spectrum (not shown) is characterized by the trans-coupled (J = 15.9 Hz) olefinic protons at 5.27 and 5.42 ppm located on C-3 and C-4, respectively, indicative of a 1,2-reduction.[6] Complete ^1H–^1H coupling constants and ^{13}C NMR chemical shifts can be found in the Experimental Section and are fully consistent with the structure of **11**. Therefore, the final product of enzymic ketonization is **2**, the α,β-unsaturated ketone. Because nonenzymic and enzymic ketonization of **1** results in an identical final UV spectrum, it can be inferred that the product of chemical ketonization of **1** is also **2**. Hence, the final product in both processes is **2**.

While the behavior of **1** is generally reminiscent of **3**, there are three distinct differences. First, **2** is subject to facile decarboxylation at C-5, while **4** is not. Although both **2** and **4** are vinylogous β-keto acids, the additional carboxylate group in **2** presumably causes unfavorable steric and electronic interactions which are relieved by decarboxylation. Second, all the spectra coincide at the isosbestic point (266 nm) creating the false impression that the decay of **1** to **2** is first order. The equilibrium between **1** and **6** has already been established during the interval (3 min) between the recording of the first and second spectrum. Hence, the

(17) Jackman, L. M.; Sternhell, S. In *Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry*; Barton, D. H. R.; Doering, W., Eds.; Peragon Press: Oxford, 1969; pp 184–192, 222–225.

(18) The signals for **2** do not appear during this 20-min time interval. The slow nonenzymatic formation of **2** from **1** is consistent with the data presented in this paper and elsewhere.[6]

(19) Two experiments indicate that the observed decrease at 236 nm results from the decarboxylation of **2** at C-5. First, the ketonization of **1** in buffer is monitored for 20 h. This results in a decrease in the absorbance at 236 and 300 nm concomitant with an increase at 276 nm. The product with a λ_{max} at 276 nm is 2-hydroxy-2,4-hepatadiene-1,7-dioate,[2,4] which has been identified as an intermediate in the decarboxylation of **2**: Johnson, W. H., Jr.; Hajipour, G.; Whitman, C. P. *J. Am. Chem. Soc.* **1992**, *114*, 11001–11003. Second, the addition of $NaBH_4$ to this mixture affords 2-hydroxy-4-heptene-1,7-dioate and 2-hydroxy-3-heptene-1,7-dioate, which result from the reduction of 2-oxo-4-heptene-1,7-dioate and 2-oxo-3-heptene-1,7-dioate, respectively. An alternate possibility involves an allylic isomerization of **6** by the transfer of a proton at C-7 to C-4 to form a fumarate derivative. However, there is no evidence indicating the presence of such a derivative.

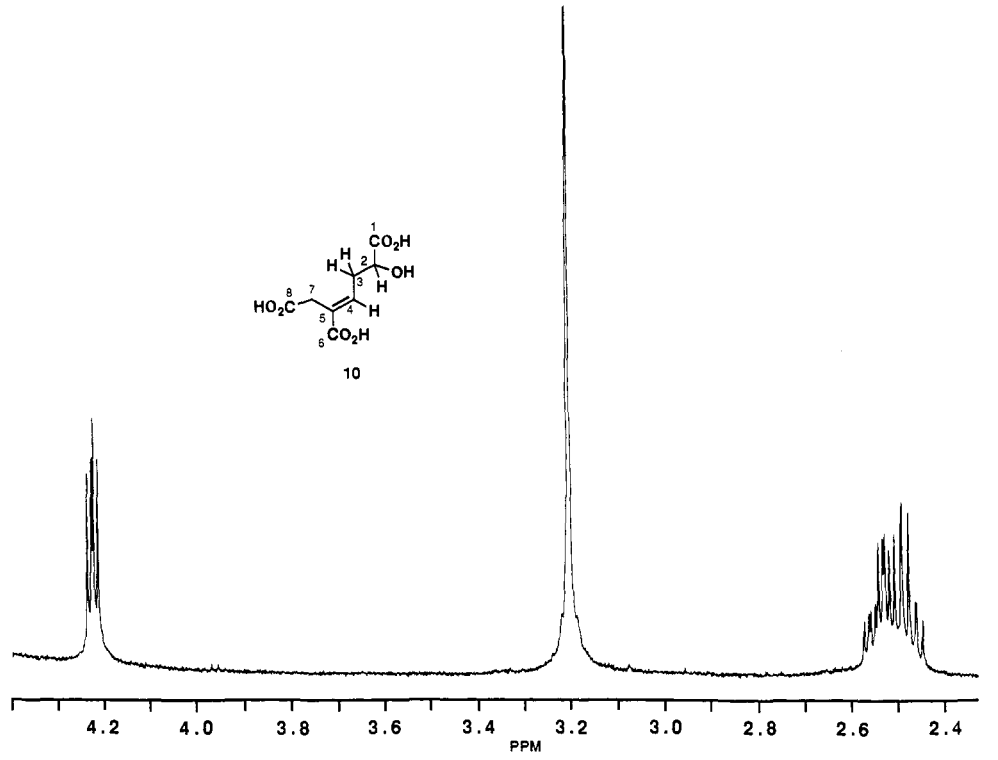


Figure 3. Partial ^{1}H NMR (500 MHz) spectrum of 2-hydroxy-5-(carboxymethyl)-4-hexenedioate (**10**) generated by the $NaBH_4$ reduction of 2-oxo-5-(carboxymethyl)-4-hexenedioate (**6**), which is the product of the nonenzymatic decay of 5-(carboxymethyl)-2-hydroxymuconate (**1**).

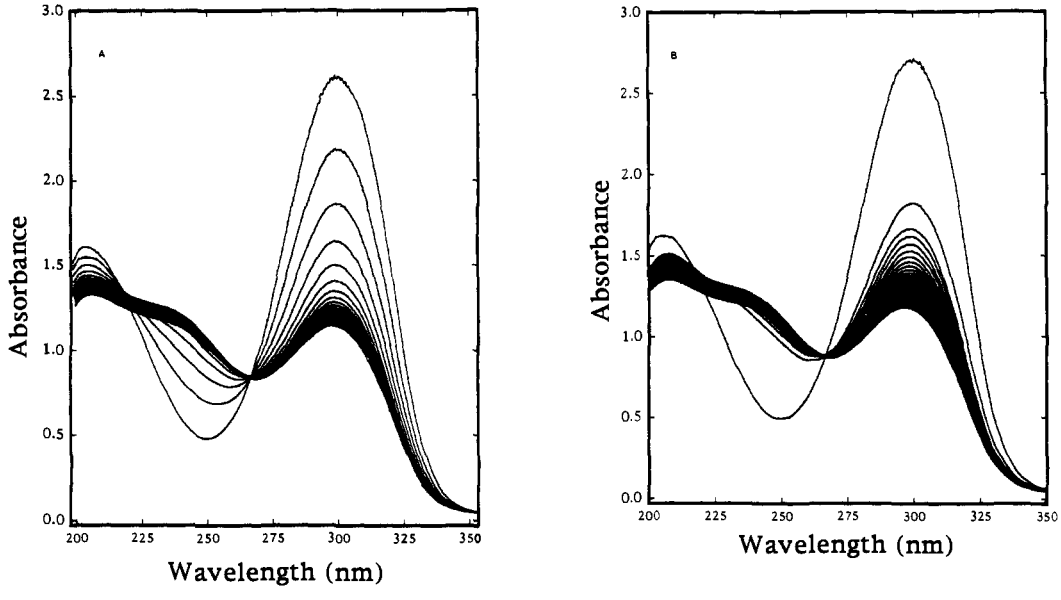


Figure 4. UV spectra (1-cm pathlength) of the chemical and enzymatic ketonization of 5-(carboxymethyl)-2-hydroxymuconate (**1**; 0.2 mM; λ_{max} = 300 nm) in 20 mM Na_2HPO_4 (pH 7.23). (A) Nonenzymatic decay (6-min intervals) over a 3-h period; (B) reaction with CHMI (0.4 unit; 3-min intervals) over a 3-h period.

isosbestic point results from the apparent first-order decay of a mixture of **1** and **6**. Finally, after 1 h, there is a considerable amount of **1** left (32.5%) in the mixture whereas much less **3** (11%) is present in the corresponding mixture of **3**, **4**, and **5**.

^{1}H NMR Detection of 2 in the CHMI Reaction. Compound **2** is generated by the addition of **1** to a NMR tube containing CHMI in phosphate buffer made up in H_2O. After 15 min, a complex ^{1}H NMR spectrum (Figure 6) results which is indicative of the four compounds present. The signals in the downfield region corresponding to **1** (6.14 and 7.30 ppm) and the *E* and *Z* isomers of **6** (6.42 and 6.56 ppm) are readily assigned by comparison to the spectra generated by the nonenzymatic decay of **1** (Figure 2). The remaining set of signals can be reasonably assigned to the protons of **2** based on their chemical shifts, characteristic coupling constants, and the results of the UV spectra and the $NaBH_4$ trapping experiment. Thus, the proton on C-4 of **2** is observed downfield as a doublet of doublets at 6.96 ppm while the proton on C-3 appears as an upfield doublet at 6.22 ppm. The observed coupling constant (J = 17.4 Hz) between the protons on C-3 and C-4 indicates that the configuration of the newly formed bond of **2** is *E*—inconsistent with a cyclic transition state required for a 1,5 sigmatropic rearrangement. Furthermore, the methylene protons of **2** (C-7), now adjacent to the chiral center at C-5, give rise to separate signals (2.36 and 2.60 ppm)

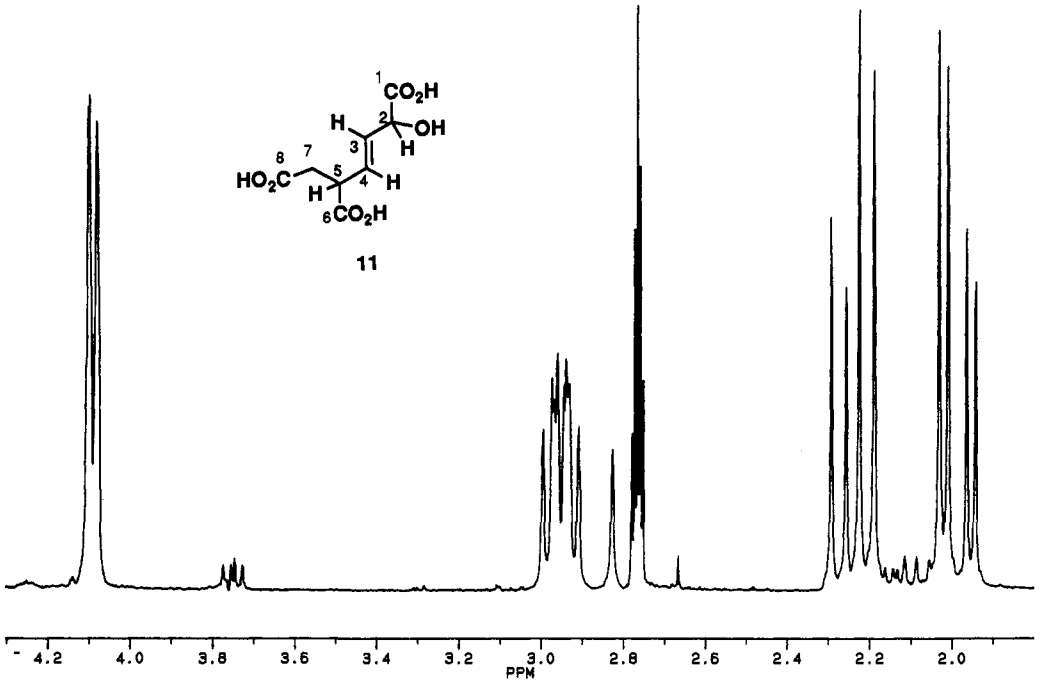


Figure 5. Partial ^1H NMR (250 MHz) spectrum of (*E*)-2-hydroxy-5-(carboxymethyl)-3-hexenedioate (**11**) generated by the $NaBH_4$ reduction of (*E*)-2-oxo-5-(carboxymethyl)-3-hexenedioate (**2**), which is the product of 5-(carboxymethyl)-2-hydroxymuconate isomerase-catalyzed ketonization of 5-(carboxymethyl)-2-hydroxymuconate (**1**).

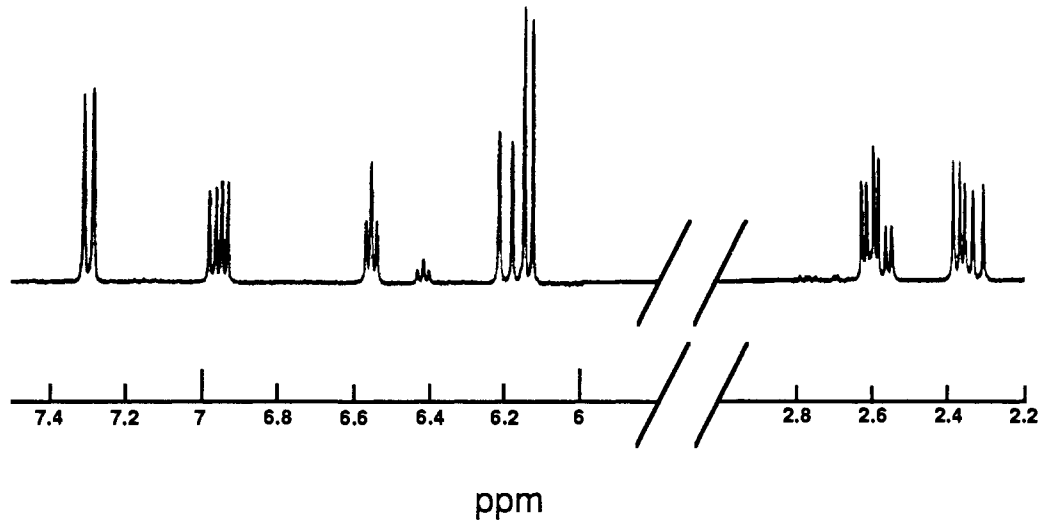


Figure 6. Partial ^1H NMR (500 MHz; H_2O) spectrum indicating the presence of (*E*)-2-oxo-5-(carboxymethyl)-3-hexenedioate (**2**) recorded 15 min after the addition of 5-(carboxymethyl)-2-hydroxymuconate isomerase (9 units) to a solution of 5-(carboxymethyl)-2-hydroxymuconate (**1**; 34.7 mM) in Na_2HPO_4 buffer (final pH 6.54; 1.7% v/v dimethyl sulfoxide-d_6).

because they are diastereotopic. Each signal appears as a doublet of doublets due to geminal and vicinal coupling. Finally, the proton on C-5 is observed as a doublet of triplets centered at 3.47 ppm (not shown).

The series of ^1H NMR spectra following the generation of **2** from **1** enabled us to estimate the quantities of **1**, **2**, and **6** near equilibrium using the relative peak areas. The spectrum chosen for this purpose was the one acquired after incubating **1** and CHMI for 55 min. In this spectrum, the signals corresponding to the protons of the decarboxylation products were not yet present and the quantity of **1** was at its minimum while the quantity of **2** was at its maximum. The areas of the signals corresponding to the methylene protons at C-7 indicated that the mixture consisted of 33.5% **1**, 47.2% **2**, and 19.3% **6**. These values are in good agreement with those determined for 60 min using the estimated kinetic constants.

Substrate Specificity of CHMI for 1 and 6. The rapid interconversion of **1** and **6** in buffered solution raises the question of whether the enzyme has a preference for one isomer as its substrate. The answer has considerable mechanistic implications. The question is most readily answered by determining the specificity constant (k_{cat}/K_M) of the enzyme for each isomer.[20] While this value is readily obtained for **1**, a straightforward determination of k_{cat}/K_M for **6** is complicated by the fact that **6** has never been isolated or synthesized. It is therefore necessary to generate a mixture of **1** and **6** in order to ascertain the value for **6**.[6]

The specificity constant for **1** is determined by adding **1** to a cuvette containing buffer (20 mM Na_2HPO_4, pH 7.49, 30 °C) and a quantity of CHMI. The rate of increase in product (**2**) is monitored at 236 nm for 10 concentrations of substrate ranging from 20 to 350 μM. The observed rate is due to enzymic ketonization of **1**. The specificity constant for **6** is determined by adding a quantity of enzyme to a cuvette containing isomer

(20) Fersht, A. R. *Enzyme Structure and Mechanism*, 2nd ed.; Freeman, W. H. and Co.: San Francisco, CA, 1985; pp 105–106.

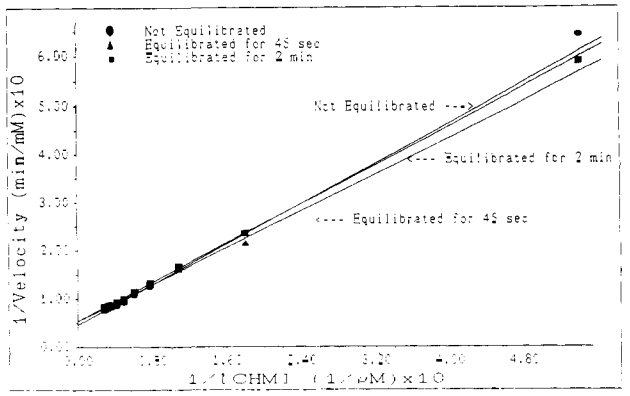


Figure 7. Comparison of the initial velocities determined in no equilibration of **1** (●), equilibration of **1** for 45 s (▲), and equilibration of **1** for 2 min (■) in 20 mM Na_2HPO_4 (pH 7.49) at 30 °C.

Table II. Kinetic Parameters for the Enzymatic Reaction of **1**, a Mixture of **1** and **6**, and **6**

substrate	K_M,[a] μM	k_{cat}, s^{-1}	k_{cat}/K_M, M^{-1} s^{-1}
1	225 ± 15	286	1.27×10^6
1 and **6** (45 s)	176 ± 9	245	1.33×10^6
1 and **6** (2 min)	186 ± 13	244	1.31×10^6
6 (45 s)	109	200	1.84×10^6
6 (2 min)	116	186	1.60×10^6

[a] Errors are standard deviations.

1, which has been previously allowed to decay in the buffer for either 45 s or 2 min. These intervals allow the system ample time to generate a significant concentration of **6**, but do not allow the system sufficient time to reach thermodynamic equilibrium where the concentration of **2** predominates. After 45 s in this buffer, a mixture of isomers **1** (68.1%), **6** (29.4%), and **2** (2.4%) results. After 2 min, the amount of **6** has not changed appreciably (28.8%) while the amount of **1** has decreased (65.3%) and that of **2** has increased (5.9%). The experiment is performed at the two different equilibrating times in order to be certain that a significant time interval elapses between these experiments and the previous experiment where there is no equilibrating time interval. The rate of increase in product (**2**) is again monitored at 236 nm for 10 concentrations of substrate ranging from 20 to 350 μM. However, the observed rate in this experiment results from the enzymic processing of **1** and **6**. The kinetic parameters for these two experiments are in excellent agreement.

The kinetic parameters, K_M and k_{cat}, for **1** and a mixture of **1** and **6** are determined from a double-reciprocal plot of the data generated in these three experiments (Figure 7). These values and the corresponding specificity constants (k_{cat}/K_M) are reported in Table II. The kinetic parameters for **6** are obtained from these experiments by using a velocity equation derived for a model in which two substrates (**1** and **6**) compete for the same active site.[21]

A comparison of the results of these three experiments provides kinetic and mechanistic insight into the CHMI reaction. First, the initial rates observed in the three experiments are about equal at lower concentrations of substrate (20–207 μM). At the higher concentrations of substrate (240–350 μM), the initial rates observed in the experiment using the single isomer, **1**, are slightly faster (3–6%) than those observed in the experiment using the two isomers (**1** and **6**). The resulting values of k_{cat} for these latter experiments are about 86% of the value of k_{cat} obtained for the former experiment. Further, the specificity constants (k_{cat}/K_M) are comparable for all three experiments. Within experimental error, CHMI processes **1** and the mixture of **1** and **6** comparably. These rates are observed even though the combined concentration of **1** and **6** is less than the concentration of **1** (~2.5% after 45 s and ~6% after 2 min) used in the experiment where the substrate is not allowed to equilibrate. Moreover, the concentration of **1** present in the mixture of **1** and **6** is 32–35% less than the concentration of **1** used in the experiment where the substrate is not allowed to equilibrate. Furthermore, there is no observable lag in the production of **2** when CHMI processes either **1** or a mixture of **1** and **6**. Examination of the separate kinetic parameters for **1** and **6** shows that their turnover numbers (k_{cat}) range from 186–286 s^{-1} with **1** being processed 43% (after 45 s) to 54% (after 2 min) faster than **6**. The values of the specificity constants (k_{cat}/K_M) determined for **6** are 26–45% faster than that determined for **1**. These observations are consistent with a mechanism in which **1** is an intermediate in the CHMI-catalyzed isomerization of **6** to **2**. The faster turnover number for **1** suggests that **1** is kinetically competent in the overall reaction.[22]

Finally, these data permit a crude estimate of the rate acceleration produced by CHMI. The rate enhancement can be determined by comparing k_2 in Scheme II to the k_{cat} determined for **1**. It is necessary to use k_2 in this analysis because the nonenzymatic isomerization of **6** to **1** is not known. A comparison of the rate constants for the conversion of **6** to **1** and **1** to **2** indicates that k_2 is the slow step in the overall process. Accordingly, the ratio of k_{cat} to k_2 for **1** to **2** is ~4×10^5.

Discussion

Our previous work on the chemical and enzymatic ketonization of 2-hydroxymuconate (**3**) compelled us to examine the behavior of 5-(carboxymethyl)-2-hydroxymuconate (**1**) in aqueous solution and in the presence of CHMI.[6] This investigation reveals similarities and differences between the properties of the two dienols and the mechanisms of 4-OT and CHMI. Our results clearly indicate that **1** is a slow-reacting dienol and has properties that are fully consistent with those reported for **3** as well as other dienols.[7-9] Kinetic studies on CHMI suggest that the enzyme is an isomerase catalyzing the transformation of **6** to **2** through the intermediacy of **1**. These studies also demonstrate that **1** is kinetically competent to be an intermediate in the overall reaction, in contrast to the behavior of **3** when processed by 4-OT. Finally, our results raise anew, questions about whether **1**, **3**, and other dienols proposed as intermediates in various meta-fission pathways are, in fact, the intermediates generated or if they result from the harsh conditions of isolation.

Kinetic, NMR, and chemical trapping experiments all indicate that the decay of **1** in aqueous phosphate buffer results in a rapid equilibrium between **1** and **6** before a much slower conversion to **2**. After 1 h, a mixture of **1**, **2**, and **6** results with **1** and **2** being the predominant species in aqueous solution. The product, **2**, a vinylogous β-keto acid, is subject to facile decarboxylation at C-5 which precludes an accurate measurement of the equilibrium constant. Presumably, this facile decarboxylation relieves steric and electronic strain. The kinetic studies also demonstrate that **1** is a so-called slow-reacting dienol which is unusually stable and has a slow rate of ketonization in comparison to **3** under the same conditions. We also observe that the reverse rates (i.e., k_1 and k_{-1}), particularly k_{-1}, show a greater propensity for the formation of **1** than do the analogous rates for **3** (Table I). The stability of **1** is presumably due to its extensive conjugation as previously noted for **3** as well as the conjugated trienol 3-hydroxy-3,5,7-estratrien-17-one.[6,9f] The slow rate of ketonization for **1**, like **3**, is likely due to its inability to undergo a 1,5 sigmatropic rearrangement. This rearrangement would require that **1** be in a s-cis conformation. This conformation is probably not a likely one because of the close proximity of the charged carboxylate groups.

These experiments indicate that protonation occurs more rapidly at the α-carbon rather than the γ-carbon (~46-fold). The partitioning factor is larger than the corresponding factor

(21) Fersht, A. R. op. cit. pp 111–112, 150.

(22) (a) Cleland, W. W. *Biochemistry* **1990**, *29*, 3194–3197. (b) Anderson, K. S.; Johnson, K. A. *Chem. Rev.* **1990**, *90*, 1131–1149.

determined for **3** under the same conditions (~7). Several factors including steric hindrance to protonation and diene conformation can influence the rate and site of protonation of a dienol.[7-9] It has been shown that small alkyl groups such as methyl groups at the γ position of dienols significantly reduce the rate of protonation relative to the α position.[8] Hence, the larger carboxymethyl group of **1** will certainly hinder protonation at the γ position and decrease its relative rate of protonation. It is also possible that in order to position the two charged carboxylate groups (C-6 and C-8) at a favorable distance, some twisting of the C-3, C-4 bond in **1** results. Slight twisting of this bond coupled with the steric hindrance to protonation can easily account for the different partitioning factors.

The similarity in the structure of **1** to **3** as well as its largely similar behavior in aqueous solution suggested that two of the mechanisms examined for 4-OT are reasonable mechanistic possibilities for CHMI.[6] In one mechanism, CHMI utilizes **1** as a substrate and catalyzes a 1,5 proton shift to yield **2**. The enzyme accelerates a kinetically unfavorable reaction (i.e, production of **2**) in order to prevent an unwanted, but a kinetically favorable reaction (i.e., the interconversion of **1** and **6**). In a second mechanism, CHMI utilizes **6** as its substrate and catalyzes a 1,3 allylic rearrangement of **6** to **2** through the dienolic intermediate **1**. In order to discriminate between these mechanistic possibilities, we determined whether the enzyme has a preference for either **1** or **6**. The results of kinetic studies into this question demonstrate that the rate of product formation (**2**) was comparable when CHMI operates on either **1** or a mixture of **1** and **6**. The similar rates are reflected in the comparable values of k_{cat}/K_M determined for **1** and the mixture of **1** and **6**. The values of k_{cat}/K_M determined for the separate isomers **1** and **6** indicate that both are excellent substrates.

These results suggest that CHMI can utilize **6** and catalyze a 1,3 allylic rearrangement of **6** to **2** through the dienolic intermediate **1**. The intermediacy of **1** in this reaction is supported by three observations. First, it is an intermediate in the chemical interconversion of **6** and **2**. Second, **1** is an excellent substrate for the enzyme and it is processed without a perceptible lag time. Finally, the turnover number for **1** is 43–54% greater than the turnover numbers determined for **2**. These rates indicate that **1** is kinetically competent to be an intermediate in the overall reaction.

Our working hypothesis for the mechanism of CHMI is chemically reasonable and based on solid literature precedent including our work on the related dienol **3**.[6] Although there are several isomerases that catalyze a 1,3 allylic rearrangement,[23] the most extensively studied isomerase is 3-oxo-Δ^5-steroid isomerase (KSI).[24] An abundance of literature implicates the existence of a dienol intermediate in this enzymic reaction. Kinetic studies of the solution chemistry of various substrates for KSI support the existence of dienol intermediates in the chemical isomerization of the β,γ-unsaturated ketones to their α,β-isomers.[9e,g] In addition, it has been clearly demonstrated by Pollack and co-workers that KSI will convert the steroidal trienol, 3-hydroxy-3,5,7-estradien-17-one and the dienol of 5-androstene-3,17-dione to their respective products at rates comparable to those observed in the overall isomerization reaction (i.e., the diffusion limit).[24a,e,f] Finally, an intermediate in the KSI reaction is consistent with the stereochemical course of the reaction and the fact that a concerted, suprafacial, 1,3 proton transfer is symmetry forbidden.[25]

Previous kinetic studies on 4-OT showed a significantly different trend between the experiments using dienol and a mixture of dienol and β,γ-unsaturated enone. It was found that the initial rates observed in an experiment using **3** and **5** were significantly faster than the initial rates observed in the experiment using **3**.[6] This resulted in turnover numbers for **3** and **5** that were on the order of 10^3 s^{-1} with **5**, the putative substrate for 4-OT, being processed about 2 times faster than **3**.[6,26] Furthermore, the rate constants indicate that **3** does not satisfy the steady-state definition of kinetic competence. Finally, the values of the specificity constants for **3** and **5** show that the 4-OT reaction is near the diffusion control limit processing either isomer.[6]

As of yet, there is no apparent reason for the difference in the behavior of the two dienols as intermediates in the two enzymatic reactions. These differences may be related to the fact that 4-OT operates near the diffusion control limit while CHMI does not. This may, in turn, be related to the fact that **1** is somewhat bulkier and carries one more charge than **3**. It is also of interest to note that 4-OT and CHMI have very different structures. 4-OT is a pentamer consisting of monomers having 62 amino acids while CHMI is a dimer consisting of monomers having 125 amino acids.[5,27] There is also no sequence homology between 4-OT and CHMI.[27] However, the specificity of each enzyme for its substrate is not absolute. CHMI and 4-OT process either **1** or **3** although each shows a clear preference for its physiological substrate.[10]

Finally, these results have implications for the widely accepted version of the homoprotocatechuate meta-fission pathway. In this version, ring cleavage of homoprotocatechuate generates 5-(carboxymethyl)-2-hydroxymuconate semialdehyde, a dienol. A NAD^+-dependent dehydrogenase oxidizes the aldehyde to the acid to generate **1**.[2-5] These steps are analogous to those proposed for the conversion of catechol to **3** by the enzymes of the catechol meta-fission pathway. If CHMI is an isomerase and utilizes **6** as its physiological substrate, then it is likely that **6**, and not **1**, is the intermediate produced in vivo. The current literature does not argue against this possibility. Although ring cleavage of homoprotocatechuate is accompanied by the observation of a characteristic yellow color (λ_{max} = 380 nm),[28] there are no data regarding the amount of the β,γ-unsaturated ketone present or whether the dienol or the ketone is the substrate for the dehydrogenase. Likewise, in the catechol meta-fission pathway, it is not possible to say whether the dienol or the ketone is the substrate for the analogous dehydrogenase.[6,29] Efforts are underway to resolve this question in both pathways.

Identification of **1** and other dienols in the homoprotocatechuate pathway as well as the analogous intermediates (e.g., **3**) in the catechol meta-fission pathway was established by their isolation from previously described mixtures after treatment with concentrated acid.[2,29] We have previously reported that the addition of HCl to a solution containing **3**–**5** results in the isolation of **3** as the major product.[6] Likewise, we find that the addition of HCl to a solution containing **1**, **2**, and **6** results in the isolation of **1** as the major product. In both pathways, it is entirely possible that the harsh conditions of isolation have resulted in the mistaken identification of the metabolites. These observations and the reactivity in aqueous solution of **1**, **3**, and presumably other dienols generated in the various meta-fission pathways emphasize that prudence must be exercised when interpreting the enzymatic chemistry in these pathways in the absence of an understanding of the nonenzymatic chemistry.

(23) Schwab, J. M.; Henderson, B. S. *Chem. Rev.* **1990**, *90*, 1203–1245.
(24) (a) Bantia, S.; Pollack, R. M.; *J. Am. Chem. Soc.* **1986**, *108*, 3145–3146. (b) Pollack, R. M.; Mack, J. P. G.; Eldin, S. *J. Am. Chem. Soc.* **1987**, *109*, 5048–5050. (c) Kuliopulos, A.; Mildvan, A. S.; Shortle, D.; Talalay, P. *Biochemistry* **1989**, *28*, 149–159. (d) Mildvan, A. *FASEB J.* **1989**, *3*, 1705–1714. (e) Eames, T. C. M.; Hawkinson, D. C.; Pollack, R. M. *J. Am. Chem. Soc.* **1990**, *112*, 1996–1998. (f) Hawkinson, D. C.; Eames, T. C. M.; Pollack, R. M. *Biochemistry* **1991**, *30*, 6956–6964.
(25) Woodward, R. B.; Hoffmann, R. *The Conservation of Orbital Symmetry*; Verlag Chemie: Weinheim, 1970; pp 114–132.
(26) The previously reported[6] values of k_{cat} and k_{cat}/K_M for 4-OT are not correct. These values are high by a factor of 1000.
(27) Chen, L. H.; Kenyon, G. L.; Curtin, F.; Harayama, S.; Bembenek, M. E.; Hajipour, G.; Whitman, C. P. *J. Biol. Chem.* **1992**, *267*, 17716–17721.
(28) Cooper, R. A.; Skinner, M. A. *J. Bacteriol.* **1980**, *143*, 302–306.
(29) (a) Bayly, R. C.; Barbour, M. G. In *Microbiological Degradation of Organic Compounds*; Gibson, D. T., Ed.; Marcel Dekker: New York; 1984; pp 253–294. (b) Sala-Trepat J. M.; Evans, W. C. *Eur. J. Biochem.* **1971**, *20*, 400–413.

Experimental Section

Materials. All chemicals and solvents were purchased from Aldrich Chemical Co. with the following exceptions. Biochemicals and buffers were obtained from Sigma Chemical Co. Centricon (10 000 MW cutoff) centrifugal microconcentrators and ultrafiltration membranes were purchased from Amicon. Dowex 1-X8 resin and electrophoresis chemicals were obtained from Bio Rad Laboratories. The purification of CHMI is described elsewhere.[5,10]

Methods. NMR spectra were obtained on a Bruker AM 250-MHz spectrometer, a Bruker AM 500-MHz spectrometer, or a General Electric QE 300-MHz spectrometer, as indicated. Chemical shifts for spectra recorded in ^2H_2O were standardized to the $^1H^2HO$ resonance at 4.70 ppm. Other spectra were standardized to tetramethylsilane. Kinetic data were obtained on a Perkin-Elmer fast scan UV/vis spectrophotometer Model 553. HPLC was performed on a Waters system using a Waters Protein Pak DEAE 5PW anion-exchange column (10-μm particle size), a Bio Rad Bio-Gel phenyl 5-PW hydrophobic column, and a Pharmacia Superose 12 (HR 10/30) gel filtration column. Protein concentrations were determined using the commercially available bicinchoninic (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL). Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) under denaturing conditions was performed on 15% gels as described elsewhere.[30]

Triethyl 5-Hydroxy-2,4-pentadiene-1,2,5-tricarboxylate (9) (Triethyl 5-(Carboxymethyl)-2-hydroxymuconate). A mixture containing diethyl formylsuccinate[11] (2.7 g, 13.3 mmol), ethyl triphenylphoshoranylidenepyruvate[12] (5.0, 13.3 mmol), and benzoic acid (1.0 g, 8.2 mmol) was stirred in anhydrous DMF (5 mL) under argon at 80–85 °C. After 8 h, the reaction was poured into ethyl acetate (100 mL) and washed with water (3 × 50 mL). The ethyl acetate layer was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness to yield crude product. The compound is readily visible by TLC as a pale yellow spot. The oil was purified by flash chromatography (80% hexanes, 20% acetone) to generate a pale yellow solid in 58% yield (2.3 g). An analytical sample was prepared by crystallization from acetone/pentane, mp 70–73 °C. **9**: ^1H NMR ($CDCl_3$, 250 MHz) δ 1.30 (9 H, m), 3.45 (2 H, s), 4.21 (6 H, m), 6.30 (1 H, d, J = 12.5 Hz), 6.57 (1 H, brd s), 7.77 (1 H, d, J = 12.5 Hz); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 14.1, 14.2 (CH_3 of 1,2,5 $CO_2CH_2CH_3$), 32.9 (C-1); 60.98, 61.05, 63.01 (CH_2 of 1,2,5 $CO_2CH_2CH_3$), 105.2 (C-4), 126.5 (C-2), 132.9 (C-3), 144.2 (C-5), 164.7 (5-CO_2), 166.8 (2-CO_2), 170.5 (1-CO_2). Anal. Calcd for $C_{14}H_{20}O_7$: C, 55.99; H, 6.71. Found: C, 55.86; H. 6.76.

5-Hydroxy-2,4-pentadiene-1,2,5-tricarboxylic Acid (1) (5-(Carboxymethyl)-2-hydroxymuconic Acid). A solution of **9** (1.4 g, 4.7 mmol) and NaOH (10 mL, 30% w/v) was stirred at ambient temperature for 18 h. The pH of the solution was then adjusted to pH 1 by the dropwise addition of concentrated HCl and extracted with ethyl acetate (3 × 50 mL). The ethyl acetate extracts were combined, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness under reduced pressure to yield a pale yellow solid. The crude product was crystallized from acetone/methylene chloride to afford 0.33 g (33%) of pale yellow product (**1**): mp 172 °C dec; ^1H NMR (CD_3OD, 250 MHz) δ 3.22 (2 H, s), 6.14 (1 H, d, J = 12.5 Hz, H-3), 7.62 (1 H, d, J = 12.5 Hz); ^{13}C NMR (CD_3OD, 250 MHz) δ 33.16 (C-1), 106.20 (C-4), 126.31 (C-2), 135.37 (C-3), 148.24 (C-5), 166.61 (5-CO_2), 170.63 (2-CO_2), 174.61 (1-CO_2). Anal. Calcd for $C_8H_8O_7$: C, 44.46; H, 3.73. Found: C, 44.32; H, 3.99.

Kinetics of Nonenzymic Ketonization of 1. Buffers are made up by the addition of calculated amounts of 100 mM stock solutions of NaH_2PO_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 is calculated to be 0.2 M. If necessary, small amounts of HCl or NaOH are added to adjust the pH of the buffer solution before use.

Decay of **1** is followed at 300 nm in a Perkin-Elmer 553 spectrophotometer equilibrated at 30 °C. Decay of **3** is followed at 295 nm under the same conditions. Kinetic runs are initiated by the addition of 5 μL of a stock solution of **1** or **3** in ethanol to 1.0 mL of buffer (20 mM Na_2HPO_4/NaH_2PO_4), which is equilibrated to 30 °C prior to use. An identical solution without **1** or **3** is used in the reference cell. The final concentration of ethanol is 0.5%. The final concentration of **1** is 84 μM and the final concentration of **3** is 43 μM. Buffer and **1** or **3** are mixed by inversion. It generally takes 10 s to mix buffer with **1** or **3** and to start the run. The rate constants obtained are reproducible in multiple runs. Stock solutions of **1** and **3** are freshly made after 24 h. The reactions are monitored for 2 h.

(30) Laemmli, U. K. *Nature* **1970**, *227*, 680–685.

Absorbance readings for the decomposition of **1** and **3** are measured from the curve every 3 s for the first 3 min and every minute thereafter. The readings are subsequently fit to the kinetic model (Scheme II) by nonlinear regression analysis. The model does not take into consideration the decarboxylation of **2**. The program assumes a 10-s delay before the first reading. The model fits are excellent (correlation constant >0.9999) for all the runs at this pH in this buffer.

^1H NMR Proof for the Formation of 6 during Chemical Ketonization of 1. To a NMR tube containing 100 mM $Na_2{}^2HPO_4$ in ^2H_2O (0.6 mL, pD 9.32) is added a quantity of **1** (4.5 mg/0.01 mL, 1.7% v/v dimethyl sulfoxide) from a stock solution made in dimethyl sulfoxide-d_6. The final pD of the solution is 7.0. The concentration of **1** in the NMR tube is 34.7 mM. After the addition of **1**, spectra are recorded at 2, 9, 14, 20, and 80 min. Both the E and Z isomers of **6** are present.[17] **6**: ^1H NMR (^2H_2O, 250 MHz) δ 3.07 (E, 2 H, s, C-6), 3.10 (Z, 2 H, s, C-6), 6.36 (Z, 1 H, s, C-4), 6.50 (E, 1 H, s, C-4). The protons on C-3 of **6** appear transiently after 2 min at 3.53 ppm as a broadened and poorly defined multiplet. The broadening and loss of this signal are consistent with the exchange of these methylene protons with solvent deuterons by the interconversion of **1** and **6**.

Conversion of 6 to 2-Hydroxy-5-(carboxymethyl)-4-hexenedioate (10). A solution of **1** (23.7 mg; 0.11 mmol) dissolved in CH_3OH (1.0 mL) is added in portions (0.1 mL) to a solution of 20 mM Na_2HPO_4/NaH_2PO_4 aqueous buffer (30 mL, pH 7.23). The solution is treated with small quantities of $NaBH_4$ (approx. 2 equiv) at 5-min intervals for 50 min. An aliquot is removed from the solution and analyzed by UV spectroscopy. There is no appreciable UV absorbance in the 200–300-nm region. The final pH of the solution is 9.38. After the pH is adjusted to 8.0 with HCl, the solution is subjected to chromatography on a Dowex-1 (formate) column (0.8 × 9.5 cm) eluting with a formic acid gradient (0–5 M formic acid, 60 mL total volume). **10** elutes at approximately 4–5 M formic acid. Appropriate fractions are pooled and evaporated to dryness under mechanical vaccum to yield 22 mg (94%) of **10**. **10**: UV λ_{max} (20 mM NaH_2PO_4, pH 7.23) 214 (ϵ 6590); 236 (shd ϵ 2421); ^1H NMR (^2H_2O, 500 MHz) δ 2.48 (2, H, ddd, H-3), 3.19 (2 H, s, H-6), 4.22 (1 H, dd, $J_{2,3}$ = 5 Hz, $J_{2,3'}$ = 7 Hz, H-2), 6.81 (1 H, t, $J_{3,4}$ = 7 Hz, H-4); ^{13}C NMR (^2H_2O, 500 MHz) δ 33.21 (C-3), 34.19 (C-6), 70.06 (C-2), 129.17 (C-5) 142.64 (C-4), 171.31 (C-6), 176.62 (C-1), 177.68 (C-7).

Identification of the Product of the CHMI-Catalyzed and Chemical Decay of 1. Wavelength scans are performed from 350–200 nm with a Perkin-Elmer 553 spectrophotometer at 30 °C. The buffer concentration used in both the chemical and enzyme-catalyzed ketonization of **1** is 20 mM NaH_2PO_4/Na_2HPO_4 (pH 7.23). Chemical ketonization of **1** is performed by adding a quantity of **1** (18 μL) from a stock solution (11.0 mM) in ethanol to 1.0 mL of buffer (final CH_3CH_2OH concentration is 1.8%). It is mixed by inversion, and spectra are recorded every 6 min for 3 h. Enzyme-catalyzed ketonization of **1** is done by making up an identical solution of **1** in buffer and recording a single spectra. Subsequently, a quantity of CHMI from *E. coli C* (0.5 μL, 0.32 μg; specific activity 1400 units/mg) is added and spectra are recorded every 3 min for 3 h.

Conversion of 2 to (*E*)-2-Hydroxy-5-(carboxymethyl)-3-hexenedioate (11). 5-(Carboxymethyl)-2-hydroxymuconate (**1**; 57.4 m; 0.26 mmol) in dimethyl sulfoxide (0.3 mL) is added in portions (0.015 mL) at 1-min intervals to a solution containing CHMI from *E. coli C* (9 mg) in 20 mM NaH_2PO_4 (30 mL; pH 7.20). Small amounts of a solution of NaOH (1 M) are added periodically to maintain the pH at about 7.10. The ketonization process is monitored spectrophotometrically (λ_{max} = 300 nm) and is complete in about 15 min. The mixture is treated with a large excess of $NaBH_4$ (~12 equiv). After 10 min, the mixture is passed through an Amicon filtration cell in order to remove the protein. The effluent is collected, concentrated to dryness, and dissolved in a small amount of water (1–2 mL). The solution is subjected to chromatography on a Dowex-1 (formate) column (1 × 44 cm) eluting with a formic acid gradient (0–5 M formic acid, 300 mL total volume) in 4-mL fractions. At the end of the gradient, the column is washed with 50 mL of 5 M formic acid to yield 57.4 mg (99%) of **10** and **11**. Typically, **10** elutes at the end of the gradient and **11** elutes during the 50-mL wash. A comparison of the integrals corresponding to the proton on C-2 in **10** and **11** indicates that **10** makes up 38% of the mixture while **11** constitutes the other 62%. **11**: ^1H NMR (CD_3OD, 250 MHz) δ 2.00 (1 H, dd, J = 5 Hz, H-6), 2.35 (1 H, dd, J = 10 Hz, H-6), 2.95 (1 H, dt, H-5), 4.10 (1 H, dd, $J_{2,3}$ = 5 Hz, H-2), 5.27 (1 H, dd, $J_{3,4}$ = 15.9 Hz, $J_{2,3}$ = 5 Hz, H-3), 5.42 (1 H, dd, $J_{3,4}$ = 15.9 Hz, $J_{4,5}$ = 7.5 Hz, H-4).

^1H NMR Detection of 2. To a NMR tube containing 100 mM Na_2HPO_4 in H_2O (0.6 mL, pH 8.86) is added a quantity of **1** (4.5 mg/10

μL, 1.7% v/v dimethyl sulfoxide) made up in dimethyl sulfoxide-d_6. The concentration of **1** in the NMR tube is 34.7 mM. The final pH of the solution is 6.54. The reaction is initiated by the addition of CHMI (10 μL, 6.3 μg, 9 units). The first NMR spectrum is recorded 15 min after the addition of CHMI and every 10 min thereafter. No significant changes occur in the spectra recorded after 55 min except the signals associated with the products of decarboxylation become increasingly apparent.[19]

Spectra are recorded in 100% H_2O using a composite pulse selective presaturation of the water signal with a 2-s presaturation interval. The lock signal is DMSO-d_6. Chemical shifts are standardized to the residual H_2O resonance of 4.7 ppm. **1**: ^{1}H NMR (H_2O, 500 MHz) δ 3.27 (2 H, s, H-6), 6.14 (1 H, d, J = 13 Hz, H-3), 7.30 (1 H, d, J = 13 Hz, H-4); **2**: ^{1}H NMR (H_2O, 500 MHz) δ 2.36 (1 H, dd, J = 8.7 and 17 Hz, H-6), 2.60 (1 H, dd, J = 6.5 and 17 Hz, H-6), 3.47 (1 H, dd, H-5), 6.22 (1 H, d, J = 17.4 Hz, H-3), 6.96 (1 H, dd, J = 8.7 and 17.4 Hz, H-4); **6**: ^{1}H NMR (H_2O, 500 MHz) δ 3.12 (2 H, s, H-6), 3.15 (2 H, s, H-6), 3.58 (2 H, d, J = 8.7 Hz, H-3), 6.42 (1 H, t, H-4), 6.56 (1 H, t, H-4).

Substrate Specificity of CHMI for 1 and 6. CHMI activity is assayed spectrophotometrically at 30 °C by following either the rate of disappearance of substrate (**1**) at 300 nm ($\epsilon = 18.8 \times 10^4$ M^{-1} cm^{-1} in 20 mM Na_2HPO_4 pH 7.53, $\epsilon = 22.9 \times 10^4$ M^{-1} cm^{-1} in ethanol) or the rate of appearance of product (**2**) at 236 nm ($\epsilon = 6.7 \times 10^3$ M^{-1} cm^{-1}). All initial velocities reported in these kinetic experiments are determined by the latter assay. In the experiments utilizing **1**, the assay mixture contains 20 mM Na_2HPO_4 buffer (1.0 mL, pH 7.49) and an aliquot (5 μL; 0.16 μg) of sufficiently dilute enzyme to obtain a linear rate. The assay is initiated by the addition of a quantity (1–19 μL) of **1** from a 18.5 mM stock solution made up in ethanol. In the experiments utilizing a mixture of **1** and **6**, the assay mixture contains 20 mM Na_2HPO_4 buffer (1.0 mL, pH 7.49) and a quantity (1–19 μL) of substrate from the same stock solution. After 45 sec or 2 min, an aliquot (5 μL; ~0.16 μg) of the same dilute enzyme is added to the mixture. The cuvettes are mixed by inversion. A cuvette containing only buffer is used as a blank in both experiments. At each substrate concentration, a velocity generated from the enzymatic processing of **1** is obtained immediately following the determination of a velocity generated from the enzymatic processing of a mixture of **1** and **6**. No lag time in the production of **2** is observed in either experiment. The initial velocities are measured from the linear portion of the first 5–10 sec of the tracing. Stock solutions of **1** are made up just prior to the start of the experiment. Detectable decomposition of the solution is not observed during the lifetime of these experiments. Purified CHMI is relatively unstable in the dilutions necessary to obtain accurate kinetic studies. Dilution of the pure enzyme in phosphate buffer results in complete loss of activity after 3 h. The inclusion of various concentrations of glycerol did not stabilize activity. However, CHMI is active for several hours after dilution in 10 mM ethylenediamine buffer (pH 7.3) made 0.15 M in NaCl and 0.02% NaN_3. Significant inhibition of CHMI by ethanol is not observed at the concentrations of ethanol used in these experiments. All results are reproducible in multiple runs.

Because **2** has not been synthesized nor isolated, the molar absorptivity coefficient (ε) was determined by an indirect method. A large quantity of CHMI was used to generate **2** from **1**. The absorbance contributed from **1** and **6** at 236 nm was subtracted from the absorbance generated by CHMI at 236 nm. The absorbance due to **6** was estimated from the reduced compound **10** while the absorbance due to **1** was measured directly. The amounts of **2** and **6** in this enzyme generated equilibrium were estimated from peak areas in a ^{1}H NMR spectrum and kinetic data reported in Table I. This method produced the ε value reported above for **2** at 236 nm.

Analysis of Enzyme Kinetic Data. The data from the kinetic experiments are fitted by a commercially available nonlinear regression data analysis program known as Enzfitter (Elsevier Science Publishers, Amsterdam).

For a mechanism in which two substrates A and B compete for the active site of the enzyme, the rate of product formation is described by eq 1, where [A] is the concentration of **1** at 45 sec or 2 min, [B] is the

$$v = (V/K)_A[A] + (V/K)_B[B]/1 + [A]/K_A + [B]/K_B \quad (1)$$

concentration of **6** at 45 sec or 2 min, K_A and K_B are the Michaelis constants for **1** and **6**, and V is the maximal velocity measured when the enzyme is fully saturated with substrate. The kinetic parameters K_B and $(V/K)_B$ in eq 1 are obtained from eqs 2 and 3, where K_{A+B} and $(V/K)_{A+B}$

$$K_B = \%B/1/(K_{A+B}) - \%A/K_A \quad (2)$$

$$(V/K)_B = (V/K)_{A+B} - \%A(V/K)_A/\%B \quad (3)$$

are the kinetic constants observed in the equilibrium experiment, %A is the percentage of A present at 45 s or 2 min, and %B is the percentage of B present at 45 s or 2 min. Because the CHMI reaction is not significantly inhibited by the product, **2**, it is not necessary to include a product inhibition term in this analysis.

Acknowledgment. We gratefully acknowledge the Donors of the Petroleum Research Fund, administered by the American Chemical Society (ACS-PRF# 23993-AC4), and the National Institutes of Health (GM 41239) for support of this research. In addition, we thank Professor A. I. Scott (Texas A & M University) for the use of the Bruker AM 500-MHz NMR spectrometer and Steve D. Sorey (Department of Chemistry, The University of Texas) for his expert assistance in the acquisition of the ^{1}H NMR spectra in H_2O.