Ketonization of 2-Hydroxy-2,4-pentadienoate by 4-Oxalocrotonate Tautomerase: Implications for the Stereochemical Course and the Mechanism

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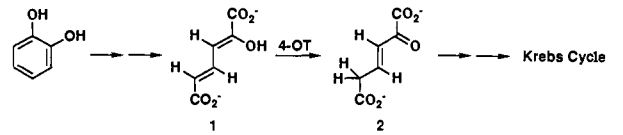
Abstract: 4-Oxalocrotonate tautomerase (EC 5.3.2-; 4-OT), an enzyme involved in the bacterial degradation of catechol to intermediates in the Krebs cycle, catalyzes the ketonization of 2-hydroxymuconate (**1**) to the α,β-unsaturated ketone (*E*)-2-oxo-3-hexenedioate (**2**). Kinetic studies on 4-OT suggest that the enzyme is an isomerase and catalyzes the transformation of (*E*)-2-oxo-4-hexenedioate (**3**) to **2** through the intermediacy of **1**. Isomerases can proceed by either a "one-base" or a "two-base" mechanism. The overall stereochemical course of an isomerase reaction can be used to distinguish between these two mechanisms. The stereochemical analysis of the 4-OT reaction presents a challenge because the proposed substrate, **3**, cannot be synthesized or isolated. This complication is circumvented by utilizing strategies based on the expected stereospecific partitioning of **1** and related dienols in ^2H_2O. It was previously determined that 4-OT ketonizes **1** to (5*S*)-[5-^{2}H]**2**. Because it was not possible to obtain sufficient quantities of [3-^{2}H]**3** for stereochemical analysis, an alternate substrate for 4-OT, 2-hydroxy-2,4-pentadienoate (**4**), was used in order to determine the stereochemistry of deuterium incorporation at the 3-position. The dienol **4** is ketonized rapidly by 4-OT to the β,γ-unsaturated ketone 2-oxo-4-pentenoate (**5**) before a much slower conversion to its α,β-isomer, 2-oxo-3-pentenoate (**6**). This behavior allows for the accumulation of **5** in solution. In order to assign the stereochemistry, the 4-OT-catalyzed ketonization of **4** was performed in ^2H_2O. The product, [3-^{2}H]**5**, was trapped with $NaBH_4$, processed to [3-^{2}H]malate by chemical and enzymatic degradative procedures, and analyzed by ^{1}H NMR spectroscopy. It was concluded that 4-OT ketonizes **4** stereoselectively to (3*R*)-2-oxo-[3-^{2}H]-4-pentenoate. This result and the previous stereochemical finding indicate that the isomerization of **3** to **2** is predominantly a suprafacial process suggesting that 4-OT proceeds by a one-base mechanism.

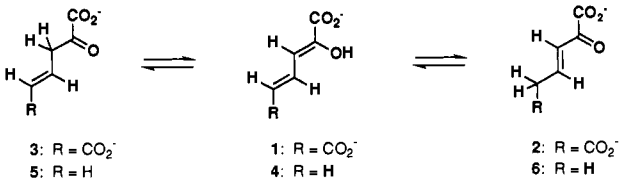
4-Oxalocrotonate tautomerase (EC 5.3.2-; 4-OT) catalyzes the ketonization of 2-hydroxymuconate (**1**) to yield the α,β-unsaturated ketone (*E*)-2-oxo-3-hexenedioate (**2**; Scheme I).[1,2] The enzyme is expressed as part of a set of inducible enzymes that oxidatively catabolizes toluene, *m*- and *p*-xylene, 3-ethyltoluene and 1,2,4-trimethylbenzene to intermediates in the Krebs cycle. The entire pathway is encoded by the TOL plasmid pWW0 and enables strains of soil bacteria carrying this plasmid to utilize these simple aromatic hydrocarbons as their sole sources of carbon and energy.[3,4]

Kinetic studies on 4-OT suggest that the enzyme is an isomerase catalyzing an allylic rearrangement of (*E*)-2-oxo-4-hexenedioate (**3**) to **2** through the intermediacy of **1** (Scheme II).[1] An enzyme-catalyzed allylic rearrangement can proceed by either a "one-base" or a "two-base" mechanism.[5,6] A considerable body of evidence demonstrates that the overall stereochemical course of the reaction (i.e., **3** to **2**) can be used to distinguish between the two possible mechanisms.[5,6] A one-base mechanism is characterized by a suprafacial steric course while a two-base mechanism is characterized by an antarafacial steric course. Because of this relationship between mechanism and active-site structure, the elucidation of the stereochemical course is an essential step in the characterization of an enzyme reaction mechanism.

Scheme I



Scheme II



A straightforward approach to address the stereochemical question for 4-OT is complicated by the fact that the substrate, **3**, has not been synthesized nor isolated—it can only be detected in a rapid equilibrium with **1**.[1] This difficulty can be circumvented by utilizing the observation that **1** is an intermediate in the overall reaction and should be partitioned stereospecifically by the enzyme to [5-^{2}H]**2** and [3-^{2}H]**3** when the reaction is carried out in ^2H_2O. Using this strategy, it was recently demonstrated that 4-OT ketonizes **1** stereospecifically to (5*S*)-[5-^{2}H]**2**.[7] However, the stereochemical analysis of [3-^{2}H]**3** was precluded by the failure to obtain sufficient quantities of the monodeuteriated **3** from the partitioning of **1**. The small quantities of [3-^{2}H]**3** presumably result because the equilibrium constant greatly favors the formation of **2** and any enzymatically generated [3-^{2}H]**3** un-

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

(2) Bayly, R. C.; Barbour, M. G. In *Microbiological Degradation of Organic Compounds*; Gibson, D. T., Ed.; Marcel Dekker: New York, 1984; pp 253–294.

(3) Harayama, S.; Lehrbach, P. R.; Timmis, K. *J. Bacteriol.* **1984**, *160*, 251–255.

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

(5) Hanson, K. R.; Rose, I. A. *Acc. Chem. Res.* **1975**, *8*, 1–10.

(6) Schwab, J. M.; Henderson, B. S. *Chem. Rev.* **1990**, *90*, 1203–1245.

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

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dergoes a facile chemical enolization to [3-^{2}H]**1**.[1] Subsequent enzymatic ketonization of [3-^{2}H]**1** in ^2H_2O yields an achiral molecule.

During our study of related dienols as possible substrates for 4-OT, we found that one dienol, 2-hydroxy-2,4-pentadienoate (**4**), is processed preferentially by the enzyme to 2-oxo-4-pentenoate (**5**) before a much slower conversion to 2-oxo-3-pentenoate (**6**; Scheme II). Moreover, the nonenzymatic enolization of **5** to **4** is sufficiently slow so that **5** accumulates in solution. This behavior makes it possible to do a stereochemical analysis of [3-^{2}H]**5** which is generated when the enzymatic reaction is carried out in ^2H_2O. The analysis was based on the chemical and enzymatic conversion of [3-^{2}H]**5** to [3-^{2}H]malate. The configuration of [3-^{2}H]malate was established by ^{1}H NMR spectroscopy. It was determined that 4-OT ketonizes **4** stereoselectively to (3*R*)-[3-^{2}H]**5**. The observation of stereoselective ketonization strongly suggests that the conversion of **1** to **3** is also catalyzed by 4-OT and supports the isomerase hypothesis. In addition, this stereochemical finding and the previously reported analysis of [5-^{2}H]**2** indicate that the isomerization of **3** to **2** is predominantly a suprafacial process. On the basis of the stereochemical course, it can be reasonably inferred that 4-OT proceeds by a one-base mechanism.[8]

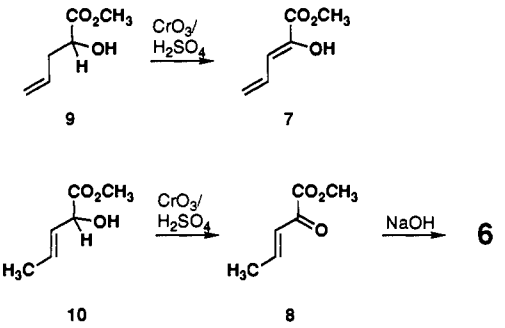
Results

Generation of 4 by the Thermal Decarboxylation of 1. A synthesis for **4** has not been reported. Collinsworth *et al.* first reported that the amino acid oxidase-catalyzed deamination of 2-amino-4-pentenoate produces a mixture of **4** and **5** on the basis of the observation of a λ_{max} at 265 nm.[9] Later, Marcotte and Walsh found that several species are generated by this deamination reaction, including **4**, **5**, and **6**.[10] The deamination reaction requires a complex set of incubation conditions and generally results in poor yields of **4**. In order to address the stereochemical question as well as future mechanistic questions, it is essential to have a more reliable source of appreciable quantities of **4**. The necessary quantities of **4** can be obtained by the thermal decarboxylation of **1**. Incubation of a solution of **1** in dimethyl sulfoxide at 120 °C for 3 min, under argon, resulted in the thermal decarboxylation of **1** and generated a mixture of **4** and **6** in about equal amounts. No other compounds were present in this mixture, as determined by ^{1}H and ^{13}C NMR spectroscopy.

Synthesis of 6 and Methyl 2-Hydroxy-2,4-pentadienoate (7). The identity of **4** was established by the synthesis of its methyl ester (**7**) while the identity of **6** was confirmed by its authentic synthesis. Jones oxidation of methyl 2-hydroxy-4-pentenoate (**9**) and methyl 2-oxo-3-pentenoate (**10**) generated **7** and **8**, respectively (Scheme III).[11] The synthesis of **9** and **10** has been described elsewhere.[10] Alkaline hydrolysis of **8** yielded **6**. Several attempts to generate **4** from **7** by a variety of methods resulted only in uncharacterized material.

Identification of the Products of Chemical and 4-OT-Catalyzed Decay of 4. In aqueous phosphate buffer (20 mM Na_2HPO_4, pH 7.28), UV analysis revealed (Figure 1A) that **4** (λ_{max} = 268 nm) undergoes facile chemical decay to leave a mixture that initially consists of **4** and a product without significant UV absorbance above 200 nm. As time progressed, the mixture was slowly converted to a stable product with a λ_{max} at 230 nm.[4] Addition of 4-OT (11 units) to an identical mixture afforded the same spectral changes (Figure 1B) although at a faster rate.

Scheme III



The initial product of decay is most reasonably identified as 2-oxo-4-pentenoate (**5**) while the final product of decay, responsible for the absorbance at 230 nm, is most reasonably identified as 2-oxo-3-pentenoate (**6**). The identity of **5** was confirmed by ^{1}H NMR spectroscopy and by its conversion to 2-hydroxy-4-pentenoate (**11**; Scheme IV) with $NaBH_4$ and subsequent identification as discussed below. The identity of **6** was confirmed by its λ_{max} and by comparison of its ^{1}H NMR spectrum to that of the authentic compound. These conclusions are entirely consistent with those reported by Marcotte and Walsh on the amino acid oxidase-catalyzed deamination of 2-amino-4-pentenoate[10] as well as with the behavior of similar dienols.[1,12]

It is apparent from the UV tracings that both processes (the formation of **5** and the formation of **6**) are catalyzed by 4-OT and that the rate of formation of **5** is faster than the rate of formation of **6**. The difference in rates results in the accumulation of **5**. In addition, the observation that 4-OT catalyzes the conversion of **4** to **5** provides a reasonable argument that the enzyme also catalyzes the ketonization of **1** to **3**.

^{1}H NMR Identification of 5 in the 4-OT-Catalyzed Ketonization of 4. The action of 4-OT on **4** was monitored by ^{1}H NMR spectroscopy in order to provide additional evidence for the mechanism shown in Scheme II (R = H). The ^{1}H NMR spectra (Figure 2A,B) recording the fate of **4** were consistent with an initial partitioning of **4** to **5**. Phosphate buffer made up in H_2O was added to a mixture of **4** and **6** generated by the thermal decarboxylation of **1**. The pH of the resulting solution was adjusted from 2.5 to 6.4 with 1 N $NaHCO_3$. The complex ^{1}H NMR spectrum shown in Figure 2A resulted. The signals in the downfield region corresponding to **6** (6.21 and 7.08 ppm) were readily assigned by a comparison to the ^{1}H NMR spectrum of the authentic compound. The methyl protons of **6** appeared upfield as a doublet (not shown) at 1.99 ppm. Likewise, the signals corresponding to the four protons of **4** (5.19, 5.38, 6.07, and 6.67 ppm) were easily assigned by a comparison to the ^{1}H NMR spectrum of the authentic methyl ester. The remaining signals (5.22, 5.26, and 5.96 ppm) in the downfield region most reasonably corresponded to **5** and resulted from the nonenzymatic ketonization of **4**. The methylene protons of **5** appeared upfield (not shown) as a doublet at 3.43 ppm.[13]

Addition of 4-OT to the mixture produced the ^{1}H NMR spectrum shown in Figure 2B after 14 min. The presence of the enzyme caused the intensity of the signals in the downfield region corresponding to **4** to decrease while those signals in the downfield region assigned to **5** increased in intensity. Likewise, the doublet assigned to the methylene protons of **5** at 3.43 ppm also increased in intensity. The intensity of the two signals in the downfield region assigned to **6** remained for the most part unchanged as did the doublet assigned to the methyl protons of **6** at 1.99 ppm. The

(8) The one-base/two-base analysis ignores, for the moment, the probable involvement of an electrophilic catalyst at the active site to assist in substrate deprotonation: Gerlt, J. A.; Kozarich, J. W.; Kenyon, G. L.; Gassman, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 9667–9669.

(9) Collinsworth, W. L.; Chapman, P. J.; Dagley, S. *J. Bacteriol.* **1973**, *113*, 922–931. The major contributer to the λ_{max} at 265 nm is presumably **4**.

(10) Marcotte, P.; Walsh, C. *Biochemistry* **1978**, *17*, 5620–5626.

(11) Pike, J. E.; Lincoln, F. H.; Schneider, W. P. *J. Org. Chem.* **1969**, *34*, 3552–3555.

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

(13) Upon the initial addition of **4** and **6** to buffer, the pH of the solution was lowered to about 2.5. At this pH (2.5), the signals assigned to the protons of **5** were not observed. When the pH was adjusted to 6.4, however, the signals were observed.

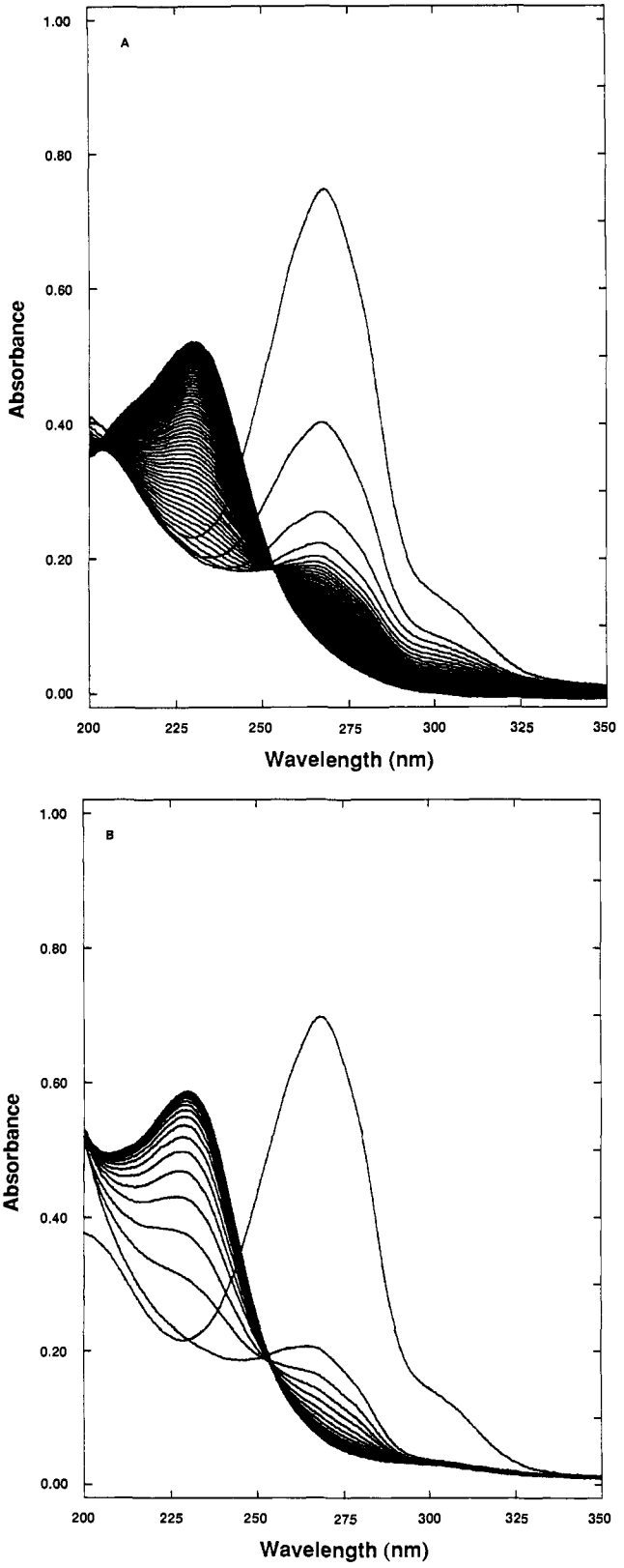
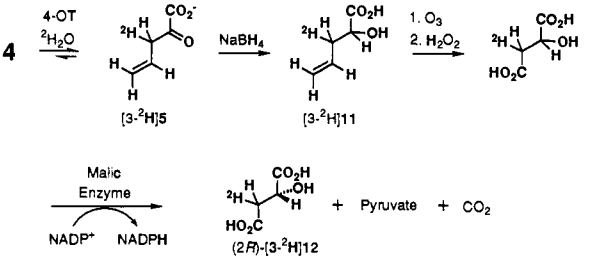


Figure 1. UV spectra (1-cm path length) of the chemical and enzymatic reactions of 2-hydroxy-2,4-pentadienoate (**4**; λ_{max} = 268 nm) in 20 mM Na_2HPO_4 (pH 7.28): (A) buffer-catalyzed decay (10-min intervals); (B) reaction catalyzed by 4-oxalocrotonate tautomerase (11 units; 5-min intervals).

nonenzymatic decay of a similar sample of **4** and **6** in phosphate buffer produced the same spectral changes although at a much slower rate.

Stereoselective Ketonization of 4 to [3-^{2}H]5. The stereochemical analysis of 4-OT was based on the enzymatic and chemical

Scheme IV



conversion of **4** in ^2H_2O to a stereoselectively monodeuteriated malate (Scheme IV). In ^2H_2O, in the presence of 4-OT, ketonization of **4** afforded [3-^{2}H]**5**. Reduction of [3-^{2}H]**5** by $NaBH_4$ made C-3 of the resulting [3-^{2}H]**11** nonracemizable. The reduced product **11** was the major product isolated by anion-exchange chromatography, as determined by ^{1}H NMR spectroscopy.[14] The ketonization of **4** to **5** required a sufficient quantity of enzyme in order to minimize the facile nonenzymatic decay of **4** to **5** and the resulting stereorandom incorporation of deuterium at C-3. However, as the concentration of enzyme increased, there was an increase in the concentration of **6**. The amount of enzyme necessary for the predominant conversion of **4** to **5** was determined by monitoring a small-scale reaction with ^{1}H NMR spectroscopy.

Our strategy for the stereochemical analysis of **11** was based on its further conversion to the monodeuteriated malate **12**. Hence, **11** was subjected to ozonolysis.[15] Treatment of the resulting ozonide with H_2O_2 generated the 2*R* and 2*S* isomers of **12** because the initial $NaBH_4$ reduction of **5** was stereorandom. Subsequent incubation of the mixture with malic enzyme and purification by anion-exchange chromatography afforded the 2*R* isomer of the monodeuteriated malate **12**.[16]

^{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.[17] 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. The resonances for (2*R*)-[3-^{2}H]malate have been assigned by the reaction of maleic acid with maleate hydratase. When the hydration is performed in ^2H_2O, (2*R*,3*R*)-[3-^{2}H]malate is obtained.[18] The resulting ^{1}H NMR spectrum shows the loss of an upfield signal and the presence of a downfield doublet.

The ^{1}H NMR spectrum of the purified (2*R*)-[3-^{2}H]malate derived from the 4-OT reaction is shown in Figure 3A. The ^{1}H NMR spectrum of (2*R*)-[3-^{2}H]malate, obtained from the nonenzymatic ketonization of **4** to **5** in ^2H_2O and processed by the series of reactions described above, is shown in Figure 3B. The two major signals present, both doublets of triplets, are centered at 2.35 and 2.63 ppm and correspond to (2*R*,3*S*)- and (2*R*,3*R*)-[3-^{2}H]malate, respectively.[19] In Figure 3A, the height of the integral for the signal assigned to the (2*R*,3*S*) isomer is ~54% greater than that of the corresponding integral for the (2*R*,3*R*) isomer. In Figure 3B, the height of the integral for the signal assigned to the (2*R*,3*S*) isomer is within experimental error of that of the corresponding integral for the (2*R*,3*R*) isomer (~8–

(14) The other product present was 2-hydroxy-3-pentenoate, which resulted from the $NaBH_4$ reduction of **6**.[10] The presence of this compound did not interfere with the stereochemical analysis.

(15) Stork, G.; Meisels, A.; Davies, J. E. *J. Am. Chem. Soc.* **1963**, *85*, 3419–3425.

(16) Spina, J.; Bright, H. J.; Rosenbloom, J. *Biochemistry* **1970**, *9*, 3794–3800.

(17) (a) Alberty, R. A.; Bender, P. *J. Am. Chem. Soc.* **1959**, *81*, 542–546. (b) Gawron, O.; Glaid, A. J.; Fondy, T. P. *J. Am. Chem. Soc.* **1961**, *83*, 3634–3640.

(18) Englard, S.; Britten, J. S.; Listowsky, I. *J. Biol. Chem.* **1967**, *242*, 2255–2259.

(19) The other signals present in the spectra shown in Figure 3 correspond to the fully protio malate and result from the presence of H_2O in the reaction mixture. Incorporation of deuterium at C-3 accounts for the triplet multiplicity of the signals due to geminal ^{1}H–^{2}H coupling.

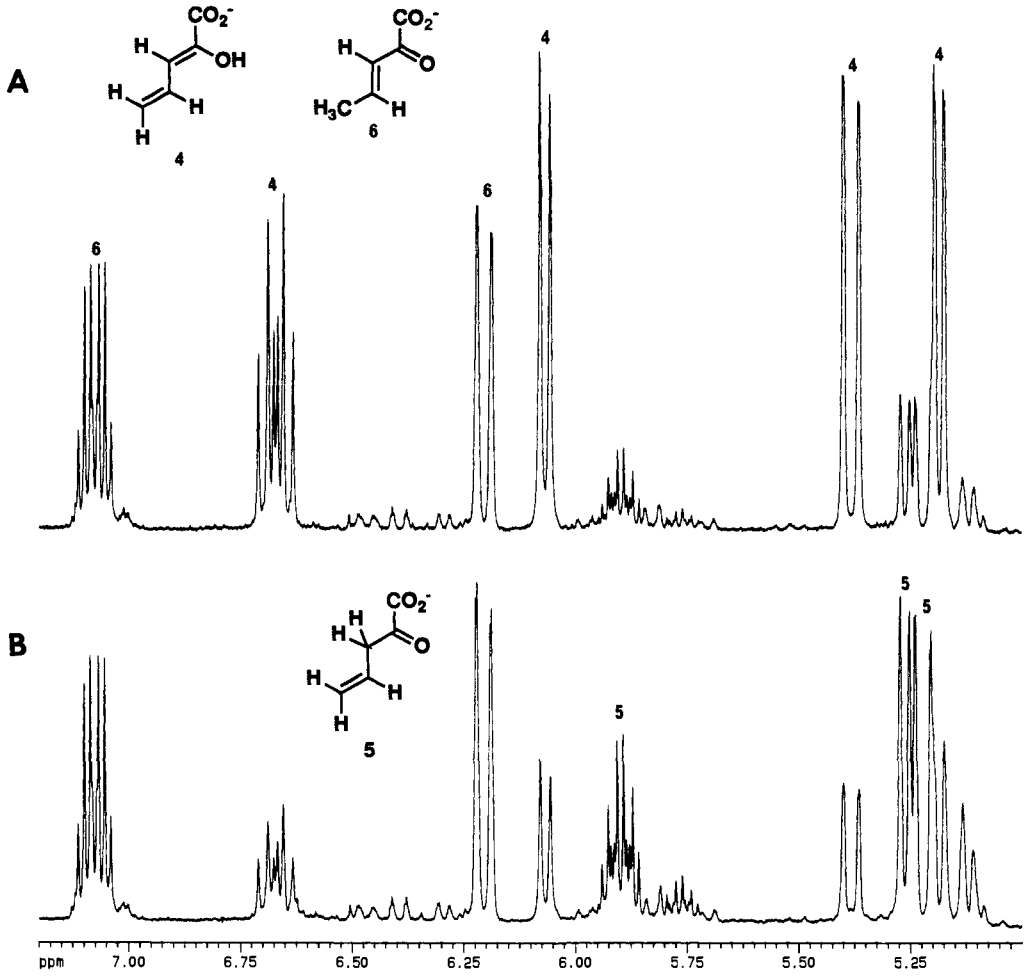


Figure 2. Partial ^{1}H NMR (500 MHz, H_2O) spectra indicating the generation of 2-oxo-4-pentenoate (**5**) by buffer-catalyzed and the 4-oxalocrotonate tautomerase-catalyzed ketonization of 2-hydroxy-2,4-pentadienoate (**4**) in 20 mM Na_2HPO_4 (final pH 6.4): (A) buffer-catalyzed decay of **4** after ~4.5 min in buffer; (B) enzyme-catalyzed decay of **4** after ~9.5 min. The other signals present in both spectra correspond to the protons of 2-oxo-3-pentenoate (**6**), which is produced along with **4** in the thermal decarboxylation of 2-hydroxymuconate (**1**).

9%). This analysis clearly shows that the enzymatic reaction is stereoselective and that (2*R*,3*R*)-[3-^{2}H]malate is the predominant isomer recovered.

Assignment of the Stereochemical Course of 4-OT. The stereochemistry at C-3 of malate indicates that the stereochemistry at C-3 of [3-^{2}H]**11** is *R* because the priority numbering changes upon unsaturation at C-4. This, in turn, indicates that 4-OT ketonizes **4** to (3*R*)-[3-^{2}H]**5** (Scheme V). We have previously demonstrated that 4-OT ketonizes **1** to (5*S*)-[5-^{2}H]**2**.[7] Although the steric courses of the two 4-OT-catalyzed tautomerizations (to the β,γ- and α,β-unsaturated ketones) were probed using different substrates, it can be reasonably concluded from the two stereochemical findings that the isomerization of **3** to **2** is predominantly a suprafacial process (Scheme VI).

Discussion

Our analysis shows that 4-OT ketonizes **4** to (3*R*)-[3-^{2}H]**5** by the stereoselective incorporation of a solvent deuteron. It can be reasonably inferred from this result that the conversion of **1** to **3** is also catalyzed by 4-OT and that the steric course at C-3 is identical. This conclusion and the previously described finding (i.e., 4-OT ketonizes **1** to (5*S*)-[5-^{2}H]**2**)[7] indicate that the isomerization of **3** to **2** is predominantly a suprafacial process. The stereochemical outcome implicates a single-base mechanism.[5,6] These findings are fully consistent with the earlier findings on the mechanism of 4-OT[1,7] and with the stereochemical outcomes of a large class of enzyme-catalyzed allylic rearrangements.[5,6] Finally, the preferential partitioning and the relaxed stereospecificity observed in the reaction of 4-OT suggest a hypothesis for the role of the carboxylate groups in the binding of **1** to 4-OT.

On the basis of kinetic studies, it was previously proposed that 4-OT catalyzes an allylic rearrangement of **3** to **2** through **1**.[1] These studies were not entirely conclusive for two reasons: the kinetic parameters for **3** were not determined directly, and the parameters determined for **1** showed that it was not kinetically competent to be an intermediate in the overall reaction.[1] A more convincing argument for the isomerase mechanism can be made by the demonstration that the intermediate dienol is partitioned with some degree of stereoselectivity by 4-OT to the β,γ-unsaturated ketone and the α,β-unsaturated ketone.[20] The observation of stereoselectivity is compelling evidence that a reaction is enzyme-catalyzed.[21] To this end, it was shown that 4-OT converts the dienol to the α,β-unsaturated ketone with a high degree of stereoselectivity.[7] The present result, the stereoselective ketonization of **4** to [3-^{2}H]**5**, provides evidence for the enzyme-catalyzed conversion of the dienol to the β,γ-unsaturated ketone. The combination of these results leads to the conclusion that 4-OT catalyzes an allylic isomerization. The observation that **1** is not kinetically competent in the overall reaction can be explained by two possibilities. The dienol present in solution may either have a different double-bond configuration or a

(20) Hawkinson, D. C.; Eames, T. C. M.; Pollack, R. M. *Biochemistry* **1991**, *30*, 6956–6964.

(21) Retey, J.; Robinson, J. A. In *Stereospecificity in Organic Chemistry and Enzymology*; Ebel, H. F., Ed.; Verlag Chemie: Weinheim, Germany, 1982; Vol. 13, pp 38–51.

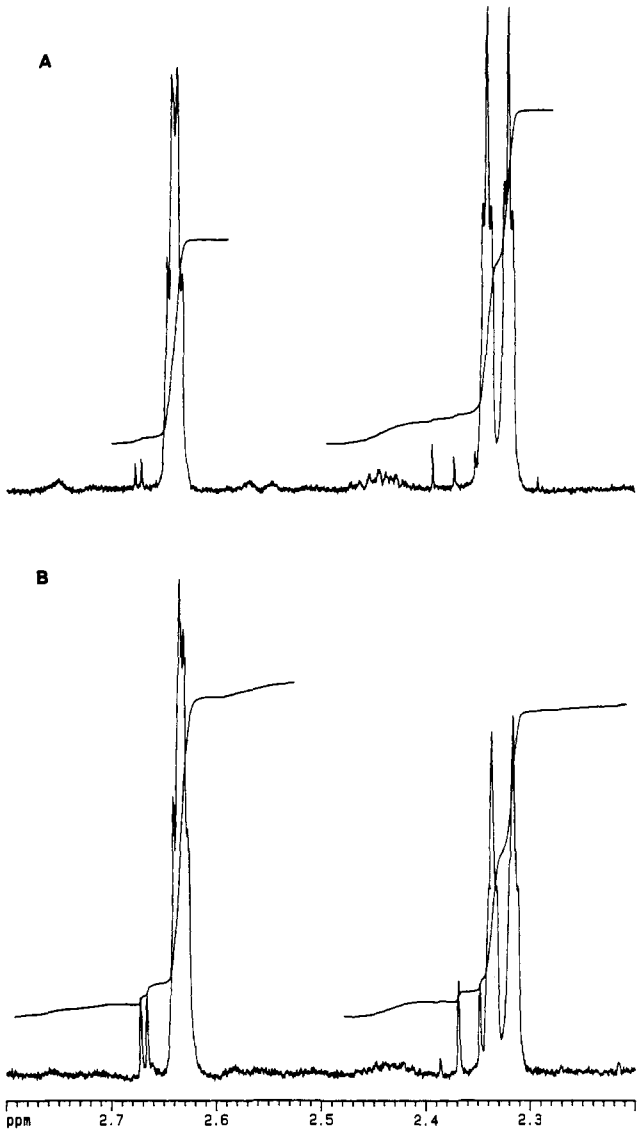
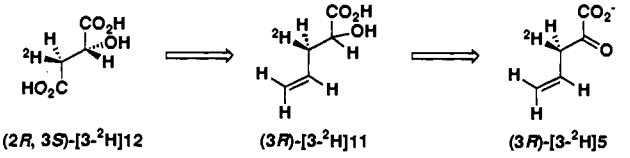
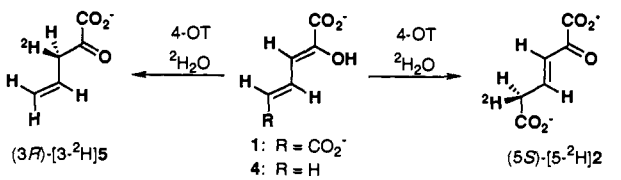


Figure 3. ^1H NMR (500 MHz, ^2H_2O) spectra of (2*R*)-[3-^{2}H]malate obtained from chemical and enzymatic conversion of 2-oxo-[3-^{2}H]-4-pentenoate (**5**) generated by the (A) 4-oxalocrotonate tautomerase-catalyzed ketonization of 2-hydroxy-2,4-pentadienoate (**4**) in ^2H_2O and the (B) nonenzymatic ketonization of **4** in ^2H_2O.

Scheme V



Scheme VI



different state of protonation than the dienol generated at the active site of the enzyme. Alternatively, the catalytic groups on the enzyme may not be in the appropriate protonation state for the turnover of **1**.[1,7]

As an isomerase, 4-OT belongs to the class of isomerases that catalyzes a 1,3 allylic rearrangement.[6] Isomerases employ either a one-base or a two-base mechanism to effect this rearrangement.[5,6] 3-Ketosteroid isomerase (KSI) is the prototypical one-base isomerase and catalyzes the suprafacial transfer of a proton by a single active site residue.[22] Isopentenyl pyrophosphate isomerase is the classic two-base isomerase.[23] The mechanism involves an antarafacial hydrogen shift and requires two active site groups. 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.[24] The substrates for those reactions which proceed suprafacially undergo facile deprotonation due to the presence of an adjacent ketone or thiol ester carbonyl group.[24] Deprotonation of the α-proton affords a readily stabilized intermediate. On the other hand, allylic rearrangements which proceed antarafacially do so because proton removal from the allylic site is particularly difficult.[24] On the basis of the structural characteristics of **3**, it can be predicted that the 4-OT-catalyzed isomerization of **3** to **2** is a suprafacial process. Hence, the observed stereochemical outcome of the 4-OT isomerase reaction is entirely expected and consistent with the trend.

On the basis of the stereochemical course, it can be concluded that 4-OT utilizes a single-base mechanism.[25] In order to be chemically reasonable and to account for the rate of the enzymatic reaction, there are two constraints on this mechanism.[8,26] First, the abstraction of the α-proton from the carbon acid **3** by a single, general base catalyst must be concerted with the protonation of the carbonyl group by a general acid catalyst.[8,26] Protonation of the carbonyl group reduces the pK_a value of the α-proton to one that is comparable to that of a potential active site base.[8,26] While the identities of the two catalysts remain unknown for the 4-OT reaction, a general base catalyst and a general acid catalyst have been clearly identified in the KSI reaction[27] and tentatively identified in the allylic rearrangement catalyzed by β-hydroxydecanoyl thiol ester dehydrase.[28] Enolization also generates an intermediate which is consistent with the fact that a concerted, suprafacial, 1,3-proton transfer is symmetry forbidden.[29] A second constraint on the mechanism is that the pK_a values of the α-proton of the carbonyl-protonated **3** and the γ-proton of the carbonyl-protonated **2** must be comparable. Comparable pK_a values enable a single base to abstract either proton. A comparison of the estimated pK_a value for **2** ($\sim$5.5) to that of **3** ($\sim$4.5) indicates that this constraint is met.[30]

Finally, **4** exhibits two surprising properties in its reaction with 4-OT. First, the dienol ketonizes preferentially to the β,γ-unsaturated ketone **5**, in contrast to **1**, which ketonizes rapidly to the α,β-unsaturated ketone **2**. Second, the ketonization of **4** to [3-^{2}H]**5** is stereoselective whereas the ketonization of **1** to

(22) Hawkinson, D. C.; Eames, T. C. M.; Pollack, R. M. *Biochemistry* **1991**, *30*, 10849–10858.

(23) (a) Muehlbacher, M.; Poulter, C. D. *Biochemistry* **1988**, *27*, 7315–7328. (b) Reardon, J. E.; Abeles, R. H. *Biochemistry* **1986**, *25*, 5609–5616.

(24) Schwab, J. M.; Klasse, J. B. *J. Am. Chem. Soc.* **1984**, *106*, 7217–7227.

(25) The demonstration of intramolecular hydrogen transfer is additional proof of a one-base mechanism.[24] Although intramolecular hydrogen transfer has not been shown for 4-OT, a one-base mechanism is the most reasonable explanation of the data. The alternative mechanism is to have two bases acting on the same side of the substrate. A mechanism involving two bases acting in such close proximity to one another is unnecessarily complex and would presumably result in an unproductive transfer of hydrogen between the two bases.[26]

(26) Gerlt, J. A.; Gassman, P. G. *J. Am. Chem. Soc.* **1992**, *114*, 5928–5934. Protonation of a carbonyl group by the general acid catalyst does not involve the complete transfer of a proton from the general acid catalyst to the carbonyl oxygen. Partial protonation of a carbonyl group via a hydrogen-bonded species is sufficient to explain the observed rates of enzyme-catalyzed reactions: Gerlt, J. A.; Gassman, P. G. *J. Am. Chem. Soc.*, submitted for publication.

(27) (a) Kuliopulos, A.; Mildvan, A. S.; Shortle, D.; Talalay, R. *Biochemistry* **1989**, *28*, 149–159. (b) Zeng, B.; Bounds, P. L.; Steiner, R. F.; Pollack, R. M. *Biochemistry* **1992**, *31*, 1521–1528.

(28) Annand, R. R.; Kozlowski, J. F.; Davisson, V. J.; Schwab, J. M. *J. Am. Chem. Soc.* **1993**, *115*, 1088–1094.

(29) Woodward, R. B.; Hoffmann, R. *The Conservation of Orbital Symmetry*; Verlag Chemie: Weinheim, Germany, 1970; pp 114–132.

[5-^{2}H]**2** is stereospecific.[7] The absence of the carboxylate group at C-5 of **4** may reflect a fundamental role for the carboxylate group at C-6 of **1** in maintaining the regio- and stereochemical fidelity of the reaction. In the reaction of **1** and 4-OT, the two carboxylate groups of **1** presumably bind to specific groups on 4-OT and hold the substrate in a fixed position at the active site. This allows for protonation at either C-3 or C-5 of **1** by a proximal residue. Moreover, protonation should be stereospecific at C-3 and C-5 because the two ends of **1** are held firmly in place. In the reaction of **4** and 4-OT, one end of the molecule is held in place by the C-1 terminus while the remaining part of the molecule is not. Because the C-1 terminus is "anchored" at the active site, it may be easier for the single base (as its conjugate acid) to protonate the relatively fixed C-3 position rather than the more flexible C-5 position. However, there still must be a considerable amount of flexibility about the entire molecule to result in the lack of stereospecificity at C-3. While there is no additional data in support of this hypothesis presently available, it is under investigation.

Experimental Section

Materials. All chemicals and solvents were purchased from Aldrich Chemical Co. with the following exceptions. 2-Hydroxymuconate was prepared according to Lapworth.[31] Biochemicals, buffers, malic enzyme, and β-nicotinamide adenine dinucleotide phosphate (β-NADP) were obtained from Sigma Chemical Co. Centricon (10 000 MW cutoff) centrifugal microconcentrators and ultrafiltration membranes were purchased from Amicon. 4-Oxalocrotonate tautomerase was purified according to published procedures.[32]

Methods. Protein concentrations were determined using the commercially available bicinchoninic (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 as described elsewhere.[33] Kinetic data were obtained on a Perkin-Elmer fast-scan UV/vis spectrophotometer Model 553. Ozone was generated by the passage of oxygen through a Welsbach ozonator. NMR spectra were obtained on a Bruker AM-250 spectrometer, a Bruker AM-500 spectrometer, or a General Electric QE-300 spectrometer, as indicated. Chemical shifts were referenced as noted below.

Generation of 4 by the Thermal Decarboxylation of 1. A solution of **1** (40 mg, 0.25 mmol) dissolved in anhydrous dimethyl sulfoxide (1 mL) was placed in a test tube, sealed with a rubber septum, and purged with argon. The stirring solution was heated at 120 °C for 3 min. After the mixture was chilled in an ice bath, it was added to ethyl acetate (50 mL) and washed with H_2O (2 × 25 mL). The organic layer was dried over $MgSO_4$ and filtered, and the filtrate was evaporated to dryness to give a yellow oil. The oil was identified as a mixture of 2-hydroxy-2,4-pentadienoate (**4**) and 2-oxo-3-pentenoate (**6**) by ^{1}H and ^{13}C NMR. **4**: ^{1}H NMR (DMSO-d_6, 300 MHz) δ 5.13 (1H, d, $J_{4,5Z}$ = 10.8 Hz, H-5), 5.34 (1H, d, $J_{4,5E}$ = 16.8 Hz, H-5), 6.09 (1H, d, $J_{3,4}$ = 10.8 Hz, H-3), 6.66 (1H, ddd, $J_{3,4}$ = 10.8 Hz, $J_{4,5Z}$ = 10.2 Hz, $J_{4,5E}$ = 16.8 Hz, H-4); ^{13}C NMR δ 112.3 (C-3), 119.0 (C-4), 131.0 (C-5), 142.4 (C-2), 166.4 (C-1). **6**: ^{1}H NMR (DMSO-d_6, 300 MHz) δ 1.95 (3H, d, $J_{4,5}$ = 6.9 Hz, H-5), 6.48 (1H, d, $J_{3,4}$ = 16.2 Hz, H-3), 7.04 (1H, dq, $J_{3,4}$ = 16.2 Hz, $J_{4,5}$ = 6.6 Hz, H-4); ^{13}C NMR δ 19.2 (C-5), 127.7 (C-3), 151.2 (C-4), 165.4 (C-1), 187.1 (C-2).

Methyl 2-hydroxy-2,4-pentadienoate (7) was prepared by the Jones oxidation of **9**,[11] which was synthesized by a method reported elsewhere.[10] To a stirring solution of **9** (0.5 g, 3.85 mmol) dissolved in acetone (150 mL) at 0 °C was added dropwise Jones reagent (2.8 mL; 2.0 g of CrO_3 in 7.5 mL of 6 M H_2SO_4) over a 2-min period.[11] The reaction was stirred vigorously for 1 additional min. The reaction was stopped by the addition of an ice-cold saturated NaCl solution (10 mL). The resulting mixture was extracted with ether (3 × 100 mL). The ethereal extracts were combined, washed with 10% $NaHCO_3$ (2 × 50 mL), dried over $MgSO_4$, and filtered, and the filtrate was evaporated to yield an oily residue. The residue was purified further by flash chromatography (15% ethyl acetate, 85% hexanes) to give **7** (0.1 g, 20%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.86 (3H, s), 5.25 (1H, d, $J_{4,5Z}$ = 10.5 Hz, H-5), 5.38 (1H, d, $J_{4,5E}$ = 16.5 Hz, H-5), 6.0 (1H, s, O*H*), 6.21 (1H, d, $J_{3,4}$ = 11.1 Hz, H-3), 6.78 (1H, ddd, $J_{4,5E}$ = 17.4 Hz, $J_{4,5Z}$ = 10.6 Hz, $J_{3,4}$ = 11.5 Hz, H-4). ^{13}C NMR ($CDCl_3$, 200 MHz) δ 53.0 (O*C*H$_3$), 112.6 (C-3), 120.3 (C-4), 129.7 (C-5), 139.2 (C-2), 166.1 (C-1).

Methyl 2-oxo-3*E*-pentenoate (8) was prepared by the Jones oxidation of **10**,[11] which was synthesized by a method reported elsewhere.[10] To a stirring solution of **10** (1.41 g, 10.85 mmol) dissolved in acetone (30 mL) at 0 °C was added dropwise Jones reagent (8 mL; 2.0 g of CrO_3 in 7.5 mL of 6 M H_2SO_4) over a 2-min period.[11] The reaction was stirred vigorously for exactly 1 additional min. The reaction was stopped by the addition of an ice-cold saturated NaCl solution (20 mL). The resulting mixture was extracted with ether (3 × 100 mL). The ethereal extracts were combined, washed with 10% $NaHCO_3$ (2 × 50 mL), dried over $MgSO_4$, and filtered, and the filtrate was evaporated to yield an oily residue. The residue was purified further by flash chromatography (10% ethyl acetate, 90% hexanes) to give **8** (0.6 g, 42%) as a pale-yellow oil. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.02 (3H, d, $J_{4,5}$ = 7.2, H-5), 3.89 (3H, s, OC*H*$_3$), 6.70 (1H, d, J = 16.2 Hz, H-3), 7.21 (1H, dq, $J_{4,5}$ = 7.2 Hz, $J_{3,4}$ = 15.6 Hz, H-4); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 18.9 (C-5), 52.8 (O*C*H$_3$), 127.0 (C-3), 150.6 (C-4), 162.9 (C-1), 183.2 (C-2).

Sodium 2-oxo-3*E*-pentenoate (6). To a solution of **8** (0.26 g, 2.0 mmol) in ethanol (25 mL) was added NaOH (0.52 mL of a 4 M solution). The reaction was stirred at room temperature for 20 min and then was stored in a refrigerator overnight for precipitation. The precipitate was collected by filtration to give **6** as a yellow solid (85 mg, 30%). ^{1}H NMR (^2H_2O, 300 MHz) δ 2.15 (3H, d, $J_{4,5}$ = 6.9 Hz, H-5), 6.23 (1H, d, $J_{3,4}$ = 16.2 Hz, H-3), 7.09 (1H, dq, $J_{4,5}$ = 6.9 Hz, $J_{3,4}$ = 15.8 Hz, H-4); ^{13}C NMR (^2H_2O, 300 MHz) δ 21.4 (C-5), 130.7 (C-3), 157.1 (C-4), 175.4 (C-1), 201.0 (C-2).

Identification of the Products of Chemical and 4-OT-Catalyzed Decay of 4. Wavelength scans were performed from 350 to 200 nm with a Perkin-Elmer 553 spectrophotometer at 30 °C. The buffer composition in both the chemical and the enzyme-catalyzed ketonization of **4** was 20 mM Na_2HPO_4 (pH 7.28). Chemical ketonization of **4** was performed by adding a quantity of **4** (5 μL) from a stock solution made up in methanol to 1.0 mL of buffer (final methanol concentration was 0.5%). It was mixed by inversion, and spectra were recorded every 10 min for 10 h. The enzyme-catalyzed ketonization of **4** was done by making up an identical solution of **4** in buffer. A single spectrum was recorded. Subsequently, a quantity of 4-OT (11 units) was added and 20 spectra were recorded at 5-min intervals.

^{1}H NMR Detection of 5 Generated by the 4-OT-Catalyzed Ketonization of 4. A mixture of **4** and **6**, generated by the thermal decarboxylation of **1** (15 mg, 0.9 mmol) in DMSO (1.0 mL), was treated as described above. The resulting oily residue was dissolved in 20 mM Na_2HPO_4 (0.63 mL; 0.07 mL of ^2H_2O; pH 8.8) and transferred to an NMR tube. After mixing, the pH of the solution was ~2.5. Only **4** and **6** were present, as determined by ^{1}H NMR spectroscopy. The pH of the solution was adjusted to 6.4 using 1 M $NaHCO_3$ (0.04 mL). After 4.5 min, a ^{1}H NMR spectrum (Figure 2A) indicated that **4**, **5**, and **6** were present. 4-OT (~11 units) was added to the NMR tube, and ^{1}H NMR spectra were acquired after 14 (Figure 2B), 24, 29, and 35 min, where the interval refers to the time elapsed after the pH of the reaction was adjusted to 6.4. Spectra were acquired in 90% H_2O solution using a selective composite pulse presaturation of the water signal with a 2-s duration. Chemical shifts were referenced to the residual H_2O resonance at 4.75 ppm. **5**: ^{1}H NMR (H_2O, 500 MHz) δ 3.43 (2H, d, $J_{3,4}$ = 7.0 Hz, H-3),

(30) The values were estimated using the following analysis and assumptions. The pK_a of the α-proton of carbonyl-protonated carbon acid is the difference between the pK_E of the carbon acid and the pK_a of the carbonyl-bound proton of the carbonyl-protonated carbon acid.[26] The pK_E values for **2** (~1) and **3** (~0) were calculated from the ratio of the previously measured rate constants for the equilibrium among **1**, **2**, and **3**.[1,8,26] The pK_a of the carbonyl-bound proton of the carbonyl-protonated **2** and the carbonyl-protonated **3** was estimated to be ~−3 on the basis of the pK_a of the carbonyl-protonated acetone.[8] The resulting pK_a values (~−2 for **2** and ~−3 for **3**) correspond to the γ-proton of the completely protonated carbonyl group of **2** and the α-proton of the completely protonated carbonyl group of **3**. These values are 15 pK_a units lower than the estimated pK_a value of the γ-proton of **2** (~13) and the α-proton of **3** (~12). These latter values represent the difference between the pK_E values for **2** and **3** and the estimated pK_a value for the OH proton of **1** (~12).[8,26] Assuming protonation of the carbonyl group is about 0.5,[26] then the pK_a values of the γ-proton of **2** and of the α-proton of **3** are reduced by ~7.5 pK_a units.

(31) Lapworth, A. J. *J. Chem. Soc.* **1901**, *79*, 1265–1284.

(32) 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. The specific activity is ~3500 units/mg.

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

5.22 (1H, d, $J_{4,5E}$ = 17.0 Hz, H-5), 5.26 (1H, d, $J_{4,5Z}$ = 10.0 Hz, H-5), 5.96 (2H, ddt, $J_{3,4}$ = 7.0 Hz, $J_{4,5E}$ = 17.2 Hz, $J_{4,5Z}$ = 10.0 Hz, H-4).

Stereoselective Ketonization of 4 to [3-^{2}H]5 by 4-OT and Conversion to [3-^{2}H]11. 2-Hydroxymuconate (**1**; 15 mg, 0.9 mmol) was heated in 0.4 mL of DMSO as described above to generate a mixture of **4** and **5**. Subsequently, the chilled mixture of **4** and **5** in DMSO (0.4 mL) was added to buffer (10 mL; 20 mM $Na_2{}^2HPO_4$; pD = 8.6). The final pD was 7.39. A solution of 4-OT (60 μg, 210 units) was added immediately to the stirring mixture. The enzyme solution had been previously exchanged by repeated dilution and concentration in ^2H_2O in a Centricon-10 microconcentrator and stored overnight in ^2H_2O. The reaction mixture was treated with $NaBH_4$ (10 equiv) at 1.5-, 3-, and 5-min intervals. After being stirred for an additional 30 min, the reaction mixture was subjected to chromatography on a Dowex-1 (formate) column (0.8 × 15 cm), eluting with a formic acid gradient (0–2 M formic acid, 60 mL total volume). The product elutes at ~1.2 M formic acid as the major peak. Appropriate fractions were pooled and evaporated to dryness under mechanical vacuum. **11**: ^{1}H NMR (^2H_2O, 300 MHz) δ 2.35 (1H, m, H3), 2.48 (1H, m, H3), 4.24 (1H, dd, $J_{2,3}$ = 6.6, 6.9 Hz, H2), 5.03 (1H, d, $J_{4,5Z}$ = 10.2 Hz, H5), 5.06 (1H, d, $J_{4,5E}$ = 17.1 Hz, $J_{3,5}$ = 1.5 Hz, H5), 5.71 (1H, ddt, $J_{4,5E}$ = 17.1 Hz, $J_{4,5Z}$ = 10.2 Hz, H4). [3-^{2}H]**11**: ^{1}H NMR (^2H_2O, 300 MHz) δ 2.43 (0.5H, dd, $J_{2,3}$ = 6.9 Hz, $J_{3,4}$ = 7.2 Hz, H3), 2.54 (0.5H, dd, J = 5.1 Hz, H3), 4.34 (0.5H, d, $J_{2,3}$ = 6.6 Hz, H2), 4.34 (0.5H, d, $J_{2,3}$ = 4.8 Hz, H2), 5.14 (1H, d, $J_{4,5Z}$ = 10.2 Hz, H5), 5.16 (1H, d, $J_{4,5E}$ = 17.1 Hz, H5), 5.81 (1H, ddd, $J_{3,4}$ = 7.2 Hz, $J_{4,5Z}$ = 10.0 Hz, $J_{4,5E}$ = 17.1 Hz, H-4).

Conversion of [3-^{2}H]11 to [3-^{2}H]Malate. A solution of [3-^{2}H]**11** (20.3 mg, 0.17 mmol) in dioxane (7 mL) and methanol (0.05 mL) at 5 °C was subjected to a stream of O_3 (1 L/min) for 25 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.[15] 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 (0–4 M formic acid, 60 mL total volume). Malate elutes at ~2.3 M formic acid. (2*R*,3*R*)- and (2*S*,3*S*)-[3-^{2}H]**12**: ^{1}H NMR (^2H_2O, 300 MHz) δ 2.61 (1H, brd s, H3), 4.25 (1H, brd d, $J_{2,3}$ = 2.4 Hz, H2). (2*S*,3*R*)- and (2*R*,3*S*)-[3-^{2}H]**12**: ^{1}H NMR (^2H_2O, 300 MHz) δ 2.30 (1H, d, $J_{2,3}$ = 10.2 Hz, H3), 4.25 (1H, d, $J_{2,3}$ = 9.9 Hz, H2).

The purified [3-^{2}H]malates were dissolved in Na_2HPO_4 buffer (20 mM; 3 mL; pH 7.5) containing $MgCl_2$ (1 mM), β-NADP (12 mg), and malic enzyme (3.5 units) from chicken liver.[16] After the reaction mixture was stirred at room temperature overnight, (2*R*)-[3-^{2}H]malate was recovered by chromatography on a Dowex-1 (formate) column (0.8 × 15 cm), eluting with a formic acid gradient (0–4 M formic acid, 60 mL total volume). (2*R*,3*S*)-[3-^{2}H]**12**: ^{1}H NMR (^2H_2O, 500 MHz) δ 2.34 (~0.6H, dt, $J_{2,3}$ = 10.0 Hz, J_{gem} = 2.0 Hz, H3), 4.28 (~0.6H, d, $J_{2,3}$ = 10.0 Hz, H2). (2*R*,3*R*)-[3-^{2}H]**12**: ^{1}H NMR (^2H_2O, 500 MHz) δ 2.64 (~0.4H, dt, $J_{2,3}$ = 3.0 Hz, J_{gem} = 2.0 Hz, H3), 4.28 (~0.4H, brd d, H2).

Chemical Ketonization of 4 to 5 in ^2H_2O and Conversion to [3-^{2}H]-Malate. A mixture of **4** and **5** was generated by the thermal decarboxylation of **1** (15 mg, 0.9 mmol) using the procedure described above. The chilled mixture in DMSO (0.4 mL) was added to buffer (25 mL; 20 mM $Na_2{}^2HPO_4$; pD = 8.6) and treated with $NaBH_4$ (10 equiv) at 2-, 5-, and 10-min intervals. After the reaction mixture was stirred for 2 h, [3-^{2}H]-**11** was recovered by anion-exchange chromatography. Subsequently, **11** (5.9 mg, 0.05 mmol) was converted to (2*R*)-[3-^{2}H]malate by the procedures described above. (2*R*,3*S*)-[3-^{2}H]**12**: ^{1}H NMR (^2H_2O, 500 MHz) δ 2.33 (~0.5H, dt, $J_{2,3}$ = 10.0 Hz, J_{gem} = 2.0 Hz, H3), 4.28 (~0.5H, d, $J_{2,3}$ = 10.0 Hz, H2). (2*R*,3*R*)-[3-^{2}H]**12**: ^{1}H NMR (^2H_2O, 500 MHz) δ 2.63 (~0.5H, dt, $J_{2,3}$ = 2.0 Hz, J_{gem} = 2.5 Hz, H3), 4.28 (~0.5H, brd d, $J_{2,3}$ = 2.5 Hz, H2).

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