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The Contribution of the Substrate's Carboxylate Group to the Mechanism of 4-Oxalocrotonate Tautomerase[1]

Huiling Lian, Robert M. Czerwinski, Thanuja M. Stanley, William H. Johnson, Jr., Robert J. Watson, and Christian P. Whitman[2]

Medicinal Chemistry Division, College of Pharmacy, The University of Texas, Austin, Texas 78712-1071

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4-Oxalocrotonate tautomerase (4-OT) converts 2-oxo-4*E*-hexenedioate (**1**) to 2-oxo-3*E*-hexenedioate (**3**) through the dienol intermediate, 2-hydroxy-2,4-hexadiene-1,6-dioate (**2**). Previous studies established that the isomerization of **1** to **3** is primarily a suprafacial process. It was also suggested that the 6-carboxylate group of the substrate maintains the regio- and stereochemical fidelity of the reaction by anchoring the substrate at the active site. A subsequent study suggested an additional role for the 6-carboxylate group in the mechanism: the enzyme may utilize the binding energy of the carboxylate group to facilitate catalysis. In order to explore the role of the carboxylate group in the mechanism further, the nonenzymatic rate constants for mono- and dicarboxylated substrates were measured and compared to the rates obtained for the corresponding enzymatic reactions. The results show that the missing carboxylate group has a profound effect on enzymatic catalysis as evidenced by the significant decreases (a 10^4- and a 10^5-fold reduction) in the values of k_{cat}/K_m observed for the two monocarboxylated substrates. A comparison of the nonenzymatic rate constants indicates that the reduced k_{cat}/K_m values cannot be explained on the basis of the chemical reactivities. The stereochemical course of the 4-OT-catalyzed reaction was also determined using 2-hydroxy-2,4*Z*-heptadiene-1,7-dioate. The stereochemical analysis reveals that the presence of the carboxylate group improves the stereoselectivity of the enzyme-catalyzed ketonization of 2-hydroxy-2,4*Z*-heptadiene-1,7-dioate to 2-oxo-[3-^{2}H]-4*Z*-heptene-1,7-dioate in ^2H_2O—a result that is consistent with its previously assigned role. These findings provide further evidence that the substrate's carboxylate group contributes to the mechanism of the enzyme in two ways: it anchors the substrate at the active site and it facilitates catalysis by destabilizing the substrate or by stabilizing the transition state.

4-Oxalocrotonate tautomerase (4-OT, EC 5.3.2) catalyzes the isomerization of unconjugated α-keto acids such as 2-oxo-4*E*-hexenedioate (**1**) to its conjugated isomer, 2-oxo-3*E*-hexenedioate (**3**) through the dienol intermediate 2-hydroxy-2,4-hexadiene-1,6-dioate (**2**) (Scheme 1) (*1–3*). The enzyme is elaborated by the soil bacterium *Pseudomonas putida mt-2* as part of a degradative pathway that converts various aromatic hydrocarbons to intermediates in the Krebs cycle (*4*). The entire pathway is encoded by the TOL plasmid and enables bacterial strains harboring this plasmid to use aromatic compounds as their sole sources of carbon and energy (*4*).

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[2] To whom correspondence should be addressed. Fax: (512) 232-2606. E-mail: cwhitman@uts.cc.utexas.edu.

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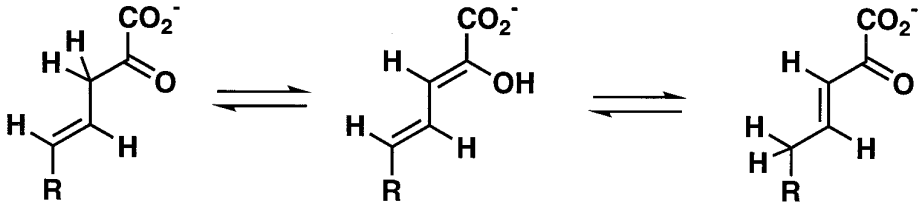
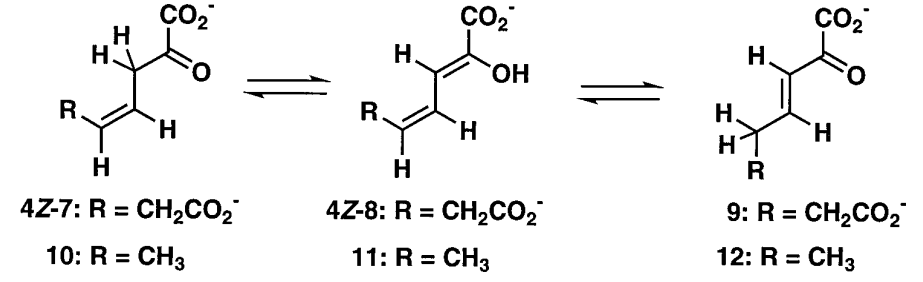


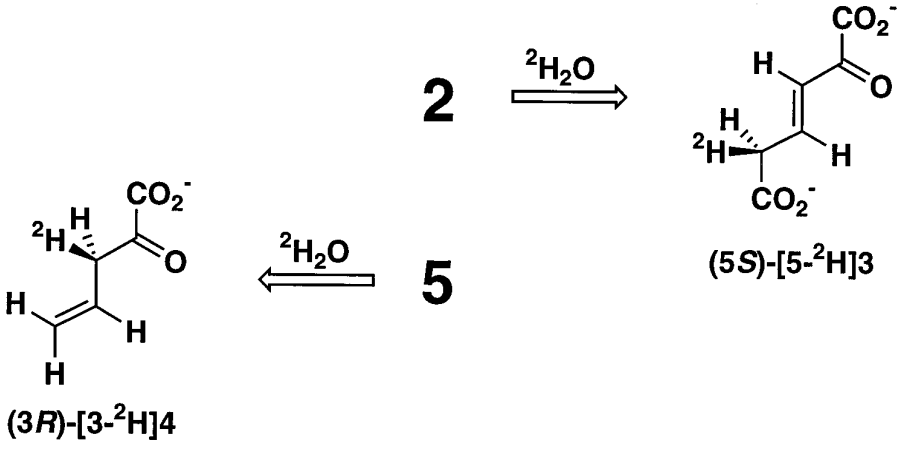
SCHEME 1

The substrate for the enzyme, **1,** cannot be synthesized or isolated—it exists in rapid equilibrium with **2** (*1*). Hence, kinetic, stereochemical, and mechanistic experiments utilize the observation that **2** and the related dienols [2-hydroxy-2,4-pentadienoate, (**5**), 2-hydroxy-2,4*Z*-heptadiene-1,7-dioate (**8**), and 2-hydroxy-2,4-hexadienoate (**11**); Schemes 1 and 2] are partitioned by the enzyme to their respective β, γ- and α,β-unsaturated ketones. Using **2** and **5,** it was demonstrated that 4-OT converts **2** to (5*S*)-[5-^{2}H]**3** and **5** to (3*R*)-2-oxo-[3-^{2}H]-4-pentenoate (**4**), in ^2H_2O (Scheme 3) (*5*, *6*). The stereochemical analysis of 4-OT required the use of two different dienols because only small quantities of [3-^{2}H]**1** were obtained in the enzymatic partitioning of **2** (*5*). Insufficient quantities of [3-^{2}H]**1** presumably result because the equilibrium constant greatly favors the formation of **3** and the facile chemical enolization of [3-^{2}H]**1** results in the formation of [3-^{2}H]**2**. Subsequent ketonization of [3-^{2}H]**2** yields an achiral molecule. On the basis of the stereochemical results, it was concluded that 4-OT catalyzes a suprafacial 1,3-allylic rearrangement consistent with a so-called ''one-base'' mechanism (Scheme 3) (*5*). The single base has been identified as Pro-1 on the basis of affinity labeling and crystallographic studies (*2*, *7*).

In course of these stereochemical experiments, two intriguing observations were made regarding the reaction of **2** and **5** with 4-OT (*5*). First, the enzyme-catalyzed



SCHEME 2



SCHEME 3

partitioning of **5** results in the accumulation of the β,γ-unsaturated isomer, **4,** whereas the enzyme-catalyzed partitioning of **2** results in the accumulation of the α,β-unsaturated isomer, **3.** Although 2-oxo-3-pentenoate (**6**) is the thermodynamically favored product of the reaction of 4-OT and **5,** its rate of formation is substantially slower than the rate of formation of **4** from **5.** Second, the enzyme-catalyzed ketonization of **5** to (3*R*)-[3-^{2}H]**4** is stereoselective, whereas the 4-OT-catalyzed ketonization of **2** to (5*S*)-[5-^{2}H]**3** is stereospecific. These observations suggest that the C-6 carboxylate group of **2** maintains the regio- and stereochemical fidelity of the reaction. In the reaction of **2** and 4-OT, the two carboxylate groups and the hydroxy group presumably anchor the substrate in a fixed position at the active site, thus allowing for facile and stereospecific protonation at C-5 (and presumably C-3). In the reaction of **5** and 4-OT, the substrate may be able to bind in two different modes because of the missing ''anchoring'' carboxylate group at C-6 (the third point of attachment). This allows for the production of both stereoisomers, (3*R*)-[3-^{2}H]**4** and (3*S*)-[3-^{2}H]**4,** in a ratio of 3 : 2. Moreover, it may be easier for the single base to protonate the relatively fixed C-3 position rather than the more mobile C-5 position.

A comparison of the kinetic properties obtained for the reaction of 4-OT with **2** to those obtained for the reaction of 4-OT with **5** provided additional insight into the role of the substrate's carboxylate group. The k_{cat}/K_m value for the conversion of **2** to **3** was determined to be 10^4-fold greater than that observed for the conversion of **5** to **6.** Because the affinity of 4-OT for **2** and **5** differs at most by a factor of 120, it was concluded that the higher value of k_{cat}/K_m was due primarily to a decrease in the overall kinetic barrier for the formation of **3** (*3*). This conclusion suggests that the enzyme utilizes the binding energy of the 6-carboxylate group to facilitate catalysis (*3*).

In order to define further the contribution of this carboxylate group to the mechanism of 4-OT, the nonenzymatic rates for the ketonization of **2, 5, 8,** and **11**

(Schemes 1 and 2) to their respective α,β-unsaturated ketones (**3, 6, 9,** and **12**) were determined and compared to the rates of the corresponding enzymatic reactions. In addition, a stereochemical analysis was performed on the reaction using **8.** The results implicate a role for the carboxylate group in both binding and catalysis. The stereochemical findings provide further evidence that the carboxylate group anchors the substrate while a comparison of the nonenzymatic and enzymatic rates of ketonization suggests that enzymatic catalysis is facilitated by the utilization of the binding energy of the carboxylate group.

EXPERIMENTAL PROCEDURES

Materials. All reagents, enzymes, buffers, and solvents were obtained from either Aldrich Chemical Co. or Sigma Chemical Co. unless noted otherwise. The Dowex anion-exchange resin was purchased from Bio-Rad Laboratories (Hercules, CA). Tryptone and yeast extract were obtained from Difco (Detroit, MI). The YM-3 ultrafiltration membranes and Centricon (10,000 MW cutoff) centrifugal microconcentrators were obtained from Amicon. The syntheses of 2-hydroxy-2,4-hexadiene-1,6-dioate (**2**), 2-hydroxy-2,4-pentadienoate (**5**), and 2-hydroxy-2,4*Z*-heptadiene-1,7-dioate (**8**) have been described elsewhere (*1*, *5*, *8*). 4-OT was purified according to published procedures (*11*).

General methods. Protein concentrations were determined using the commercially available bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL) or the method of Waddell (*12*). 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 12.5% gels as described elsewhere (*11*). Kinetic data were obtained on either a Hewlett Packard 8452A Diode Array spectrophotometer or a Perkin–Elmer Model 553 fast scan UV/vis spectrophotometer. Ozone was generated by the passage of oxygen through a Welsbach ozonator. NMR spectra were obtained on a Bruker AM-250 spectrometer or a Varian Unity INOVA-500 spectrometer as indicated. Chemical shifts were referenced as noted below.

Preparation of 2-hydroxy-2,4E-heptadiene-1,7-dioate (*4E-**8***). A mixture containing approximately equivalent amounts of the 4*Z* and 4*E* isomers of **8** was prepared as described previously using 200 mg of 5-(carboxymethyl)-2-hydroxymuconate in dimethyl sulfoxide (1 mL) (*8*). After being heated at 120°C for 5 min, a solution of I_2 (3.6 mg) dissolved in ethyl acetate (1 mL) was added. Subsequently, the reaction mixture was diluted to 5 mL with ethyl acetate and allowed to stir at room temperature overnight under a fluorescent light. The reaction mixture was diluted to 50 mL with ethyl acetate and extracted (5×) with an equivalent amount of a 0.1 M solution of HCl. The organic layer was collected, dried over anhydrous Na_2SO_4, and evaporated to dryness. The residue was suspended in $CHCl_3$ (25 mL) and stirred for 10 min. The solution was filtered and the filtrate washed with excess

$CHCl_3$ until the purple color was no longer present. A ^1H NMR spectrum of the product showed that it was predominantly 4*E*-**8** (~90% 4*E*-**8** and 10% 4*Z*-**8**) (*8*).

Preparation of 5-(methyl)-2-hydroxy-2,4-hexadiene-1,6-dioate. Sodium ethoxide was generated by the addition of sodium metal (5.4 g, 0.2 mol) to a stirring mixture of toluene (500 mL) and ethanol (200 mL). The reaction was carried out in a 2-L round-bottom flask under an argon atmosphere following a literature procedure (*13*). Subsequently, the dried sodium ethoxide was chilled in an ice bath and diethyl oxalate (28.5 g, 26.5 mL, 0.19 mol) and ethyl tiglate (25 g, 27 mL, 0.19 mol) were added in succession. The reaction mixture was allowed to warm to room temperature by removal of the ice bath. After being stirred at room temperature for 48 h, the mixture was filtered and the precipitate washed with ether until the filtrate was clear. The precipitate was air-dried to yield 29 g (59%) of the crude diethyl ester of 5-(methyl)-2-hydroxy-2,4-hexadiene-1,6-dioate: ^1H NMR ($CDCl_3$, 250 MHz) δ 1.30, 1.35 (6H, overlapping triplets, CH_3 of $-OCH_2CH_3$), 1.95 (3H, s, CH_3 at C-5), 4.20 (3H, q, CH_2 of $-OCH_2CH_3$), 4.35 (3H, q, CH_2 of $-OCH_2CH_3$), 6.40 (1H, d, J = 8.3 Hz, H3), 7.60 (1H, d, J = 8.3 Hz, H4); ^{13}C NMR ($CDCl_3$, 250 MHz) δ 13.0 (CH_3), 14.1, 14.3 (CH_3 of $-OCH_2CH_3$), 60.7, 62.8 (CH_2 of $-OCH_2CH_3$), 106.3 (C-3), 129.9 (C-5), 130.2 (C-4), 142.9 (C-2), 164.9 (C-1), 167.9 (C-6).

The free acid was prepared by alkaline hydrolysis of the diethyl ester and subsequent acidification as follows. The diethyl ester (29 g, 0.13 mol) was suspended in a solution of 0.5 M NaOH (500 mL) and stirred at ambient temperature for 16 h. The reaction mixture was filtered and the filtrate was adjusted to pH 1 by the addition of concentrated HCl. The precipitate was collected by filtration and suspended in ethyl acetate (~50 mL). After being stirred overnight, the solution was filtered to yield 5.4 g (25%) of the free acid: ^1H NMR (CD_3OD, 250 MHz) δ 1.90 (3H, s, CH_3), 6.40 (1H, d, J = 11.4 Hz, H3), 7.65 (1H, d, J = 11.4 Hz, H4); ^{13}C NMR (CD_3OD, 250 MHz) δ 12.8 (CH_3), 107.1 (C-3), 128.9 (C-5), 132.9 (C-4), 146.9 (C-2), 166.9 (C-1), 171.7 (C-6).

Preparation of 2-hydroxy-2,4-hexadienoate (***11***). 2-Hydroxy-2,4-hexadienoate was generated by the thermal decarboxylation of 5-(methyl)-2-hydroxy-2,4-hexadiene-1,6-dioate following a similar procedure (*5*). A solution of 5-(methyl)-2-hydroxy-2,4-hexadiene-1,6-dioate (104 mg, 0.6 mmol) dissolved in anhydrous dimethyl sulfoxide (0.5 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 15 min. The reaction mixture was chilled in powdered dry ice, diluted with ethyl acetate (4 mL), and filtered. Subsequently, the filtrate was diluted with ethyl acetate (200 mL) and washed with a solution of 0.1 N HCl (3 × 33 mL). The organic layer was collected, dried over anhydrous Na_2SO_4, and filtered, and the filtrate was evaporated to dryness to give a solid (34 mg, 43%) which decomposes upon prolonged standing (>24 h). The solid was identified as a mixture of 4*Z*-**11** (~55%), 4*E*-**11** (~31%), and 4*E*-**12** (~14%) by ^1H and ^{13}C NMR spectroscopy. 4*Z*-**11**: ^1H NMR (DMSO-d_6, 500 MHz) δ 1.71 (3H, dd, H6), 5.58 (1H, dq, J = 9.3 Hz, H5), 6.07 (1H, d, H3), 6.33 (1H, q, J = 9.5 Hz, H4); 4 *E*-**11**: ^1H NMR (DMSO-d_6, 500 MHz) δ 1.75 (3H, dd, H6), 5.83 (1H, dq, J = 13.5 Hz, H5), 6.26 (1H, d, H3), 6.35 (1H, brd m, H4). 4*Z*-**11** and 4*E*-**11**: ^{13}C NMR (CD_3OD, 250 MHz) δ 13.6, 18.7 (C-6), 108.3, 113.9 (C-3), 123.7, 126.2 (C-4), 129.1, 132.5 (C-5), 140.1, 141.7 (C-2), 167.9 (C-1).

In order to obtain the spectral data for 4*E*-**12,** it was made the predominant product in the mixture described above (4*Z*-**11,** 4*E*-**11,** and 4*E*-**12**) using 4-OT. Accordingly, a solution of 5-(methyl)-2-hydroxy-2,4-hexadiene-1,6-dioate (68 mg, 0.4 mmol) dissolved in anhydrous dimethyl sulfoxide (0.5 mL) was heated at 120°C for 20 min. A portion of the resulting mixture (30 μL) was added to 100 mM NaH_2PO_4 buffer (0.6 mL, pH 8.8) and transferred to an NMR tube. The addition of the mixture to the buffer adjusted the pH to 7.4. The reaction was initiated by the addition of 4-OT (0.2 mg in 20 μL of 20 mM NaH_2PO_4 buffer, pH 7.1). After 18 h, 4*E*-**12** was the predominant product present in solution as determined by the corresponding NMR signals. Spectra were recorded in 100% H_2O using selective presaturation of the water signal with a 2-s presaturation interval. The lock signal is (methyl sulfoxide)-d_6. Chemical shifts were referenced to (methyl sulfoxide)-d_6. 4*E*-**12**: ^{1}H NMR (100 mM NaH_2PO_4 buffer, 500 MHz) δ 0.88 (3H, t, H6), 2.16 (2H, quintet, H5), 5.99 (1H, d, J = 17.5 Hz, H3), 6.94 (1H, dq, H4); ^{13}C NMR (100 mM NaH_2PO_4 buffer, 500 MHz) δ 12.7 (C-6), 27.5 (C-5), 126.9 (C-3), 161.3 (C-4), 174.0 (C-1), 199.9 (C-2).

Nonenzymatic ketonization of ***2, 5,*** *4E-**8**, 4Z-**8**, and* ***11.*** The nonenzymatic rate for the conversion of each dienol to its β,γ-unsaturated ketone was determined by following the decrease in absorbance at 295 nm (**2**), 264 nm (**5**), 276 nm (4*E*-**8** and 4*Z*-**8**), or 268 nm (**11**). Absorbance readings for the decomposition of each dienol were measured as follows: for **2** every 30 s for 30 min, for **5** every 20 s for 20 min, for 4*Z*-**8** every 10 s for 10 min, for 4*E*-**8** every 20 s for 20 min, and for **11** every 90 s for 60 min. The nonenzymatic rate for the formation of each α,β-unsaturated ketone was determined by following the increase in absorbance at 236 nm (**2** $\rightarrow$ **3,** ε = 6580 M^{-1} cm^{-1}) or at 232 nm (**5** $\rightarrow$ **6,** ε = 5900 M^{-1} cm^{-1}; 4*E*- and 4*Z*-**8** $\rightarrow$ **9,** ε = 8250 M^{-1} cm^{-1}; **11** $\rightarrow$ **12,** ε = 4300 M^{-1} cm^{-1}) (*1*, *5*, *8*). Absorbance readings were collected for each α,β-unsaturated ketone at the indicated wavelength as follows: for **3** every 2.5 min for 3 h, for **6** every 30 min for 16 h, for **9** (from 4*Z*-**8**) every 1 h for 53 h, for **9** (from 4*E*-**8**) every 1 h for 50 h, and for **12** every 1 h for 63 h. All reactions were carried out in 20 mM NaH_2PO_4 buffer (pH 7.3) at 23°C. Kinetic runs were initiated by the addition of 5–17.5 μL of a stock solution (1.8–3.5 mg/mL) made up in ethanol.

For the formation of the β,γ-unsaturated ketones, the readings were fitted to the equation for a single exponential decay by nonlinear regression analysis. For the formation of the α,β-unsaturated ketones, the readings were fitted to a first-order equation and the rates were determined from the initial portion. Both sets of data were fitted using the grafit program (Erithacus Software Ltd., Staines, UK) obtained from Sigma Chemical Co.

Kinetic parameters of 4-OT using ***2, 5,*** *4E-**8**, 4Z-**8**, and* ***11.*** Enzyme activity was monitored by following the formation of the α,β-unsaturated ketone as indicated by an increase in absorbance at 236 nm (**2** $\rightarrow$ **3,** ε = 6580 M^{-1} cm^{-1}) or by an increase in absorbance at 232 nm (**5** $\rightarrow$ **6,** ε = 5900 M^{-1} cm^{-1}; 4*E*- and 4*Z*-**8** $\rightarrow$ **9,** ε = 8250 M^{-1} cm^{-1}; **11** $\rightarrow$ **12,** ε = 4300 M^{-1} cm^{-1}) (*1*, *5*, *8*). Because the thermal generation of **11** results in a mixture of isomers including the nonreactive **12** (~14%), the substrate concentrations in the kinetic experiments using **11** were corrected accordingly. The cuvettes were mixed by a stir/add

cuvette mixer. The kinetic data were fitted by nonlinear regression data analysis using the Grafit program.

*Stereoselective ketonization of 4Z-**8** to [3-^{2}H]**7** by 4-OT and conversion to [3-^{2}H]**13.*** The ketonization of 4*Z*-**8** to [3-^{2}H]**7** in ^2H_2O by 4-OT was carried out by a modification of a literature procedure (*5*). Accordingly, to a stirring mixture of 4-OT (12 μL, 7.1 mg/mL) and $NaBH_4$ (~5 eq) in buffer (10 mL, 20 mM $Na_2[^2H]PO_4$, p[^{2}H] 8.6) was added a solution of 4*Z*-**8** (8 mg, 0.05 mmol) dissolved in (methyl sulfoxide)-d_6 (0.3 mL). 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 stirred for 1 min and a second aliquot of $NaBH_4$ (~5 eq) was added. After being stirred for 30 min, the solution 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). The product elutes at ~3 M formic acid. Appropriate fractions were pooled and evaporated to dryness. The reaction was repeated three times and the products combined. The ^{1}H NMR spectrum of [3-^{2}H]**13** corresponded to a previously published spectrum (*14*).

*Conversion of [3-^{2}H]**13** to [3-^{2}H]malate (**14**).* The chemical and enzymatic degradation of [3-^{2}H]**13** to [3-^{2}H]malate (**14**) was carried out by a modification of a literature procedure (*5*). Accordingly, a solution of [3-^{2}H]**13** (23.6 mg, 0.13 mmol) in a mixture of 1,4 dioxane (7 mL) and methanol (50 μL) 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 CH_3CO_2H (2.5 mL) were added to the residual oil, and the reaction mixture was stirred overnight. The solution was evaporated to dryness and the pH was adjusted to ~8 by the addition of a solution of 5% $NaHCO_3$ (2 mL). The diastereomeric [3-^{2}H]malates were purified by anion-exchange chromatography as described above, eluting at ~2.3 M formic acid. The ^{1}H NMR spectrum of [3-^{2}H]malates corresponded to a previously published spectrum (*5*, *8*).

The purified [3-^{2}H]malates were treated with malic enzyme and the resulting (2*R*)-isomers were recovered by anion-exchange chromatography as described elsewhere (*5*, *8*). (2*R*, 3*S*)-[3-^{2}H]**14**: ^{1}H NMR (^2H_2O, 500 MHz) δ 2.34 (~0.7 H, dt, $J_{2,3}$ = 11.5 Hz, H3), 4.28 (~0.7 H, dt, $J_{2,3}$ = 11.5 Hz, H2). (2*R*, 3*R*)-[3-^{2}H]**14**: ^{1}H NMR (^2H_2O, 500 MHz) δ 2.64 (~0.35 H, dt, $J_{2,3}$ = 3.8 Hz, H3), 4.28 (~0.35 H, brd d, H2).

*Stereoselective ketonization of 4Z-**8** to [5-^{2}H]**9** by 4-OT and conversion to [5-^{2}H]**15.*** A portion (~10 μL) of a solution of 4*Z*-**8** (28.3 mg, 0.16 mmol) dissolved in (methyl sulfoxide)-d_6 (60 μL) was added to seven separate reactions mixtures containing 100 mM $Na_2[^2H]PO_4$ buffer (0.6 mL, p[^{2}H] = 9.2). The addition of 4*Z*-**8** to the buffer adjusted the p[^{2}H] to 7.1. To the mixtures being stirred was added 4-OT (10 μL, 7.1 mg/mL). After 5 min, an aliquot of $NaBH_4$ (~5 eq) was added to each reaction mixture and they were allowed to stand overnight. The final p[^{2}H] of the solution was 9.6. The p[^{2}H] of the solution was adjusted to 8.4 and it was subjected to chromatography on a Dowex-1 (formate) column (0.8 × 14 cm), eluting with a formic acid gradient (0–2 M formic acid, 60 mL total volume). The product, 5-[^{2}H]**15,** elutes as a broad peak from 1.3–2 M formic acid and is recovered in 69%

(20 mg) yield. The ^{1}H NMR spectrum of 5-[^{2}H]**15** corresponded to a previously published spectrum (*15*).

Conversion of [5-^{2}*H*]***15*** *to* [2-^{2}*H*]*succinate* (***16***). A solution of 5-[^{2}H]**15** (20 mg, 0.1 mmol) in a mixture of methanol and 1,4-dioxane was treated with O_3 as described above for 30 min. After the solvent was evaporated to dryness, the residual oil was treated with H_2O_2 and glacial CH_3CO_2H as described above. The [2-^{2}H]succinate (**16**) was purified using anion (formate)-exchange column chromatography. It elutes as a broad peak from 1–1.7 M formic acid and is recovered in 70% yield (9.5 mg): $[\theta]_{210} = 180°$. The ^{1}H NMR spectrum of 2-[^{2}H]**16** corresponded to a previously published spectrum (*16*).

RESULTS

Nonenzymatic ketonization of ***2, 5,*** *4E-****8,*** *4Z-****8,*** *and* ***11.*** The ketonization of each dienol (**2, 5,** 4*E*-**8,** 4*Z*-**8,** and **11**) was monitored in aqueous phosphate buffer by observing the decay in UV absorbance at 295, 264, 276, and 268 nm, respectively. In all cases, a rapid drop in absorbance followed by a much slower decrease was observed. For **5, 8** (both isomers), and **11,** the slower decrease was noticeable only after an appreciable time interval. The rapid drop in absorbance corresponds to the ketonization of each dienol to its β,γ-unsaturated ketone (**1, 4, 7, 10**) as has been established in previous studies (*1*, *5*, *8*). The slower decrease in absorbance corresponds to the formation of the α,β-unsaturated ketone (**3, 6, 9, 12**) as previously shown (*1*, *5*, *8*). At equilibrium, **6, 9,** and **12** are the predominant products (>95%) while **3** constitutes ~80% of the equilibrium mixture (*1*, *5*, *8*).

The absorbance data collected during the initial decay were fitted to the equation for a single exponential decay by nonlinear least-squares regression analysis in order to estimate the rate constant (defined as k_1) for the formation of each β,γ-unsaturated ketone from its dienol. The slow rate of formation of **6, 9,** and **12** precluded an accurate measurement of the first-order rate constant. Hence, the initial rate of formation (defined as k_2) for each α,β-unsaturated ketone was measured and compared to that measured for **3.** The rates are summarized in Table 1. Three features are apparent from these rate constants. First, protonation of these dienols occurs more rapidly at the α-carbon (to form the β,γ-unsaturated ketone) rather than the γ-carbon (to form the α,β-unsaturated ketone) under these conditions. Second, the rates for the formation of the β,γ-unsaturated ketones from their respective dienols do not differ significantly. The largest difference is that between the rates of formation for **7** (from *4Z*-**8** and *4E*-**8**) (35-fold). Finally, the initial rates for the formation of the α,β-unsaturated ketones differ somewhat more significantly, with the largest difference in rates being the nearly 320-fold difference observed between **3** and **12** and the 390-fold difference observed between **3** and **9** (generated from 4*E*-**8**).

Kinetic parameters of 4-OT using ***2, 5,*** *4E-****8,*** *4Z-****8,*** *and* ***11.*** The kinetic properties for the 4-OT-catalyzed ketonization of **2, 5,** 4*E*-**8,** 4*Z*-**8,** and **11** to their respective α,β-unsaturated ketones were measured. The observed values for K_m, k_{cat}, and k_{cat}/K_m are summarized in Table 2. A comparison of the parameters measured for