Stereochemical and Isotopic Labeling Studies of 4-Oxalocrotonate Decarboxylase and Vinylpyruvate Hydratase: Analysis and Mechanistic Implications

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Abstract: Stereochemical and isotopic labeling studies of 4-oxalocrotonate decarboxylase (EC 4.1.1.-; 4-OD) and vinylpyruvate hydratase (EC 4.2.1.-; VPH) from *Pseudomonas putida mt-2* have been completed. The two enzymes, reportedly a complex, catalyze successive reactions in the catechol meta-fission pathway and convert 2-oxo-3-hexenedioate (**1**) to 2-oxo-4-hydroxypentanoate (**2**) using either manganese or magnesium as a cofactor. 2-Oxo-4-pentenoate (**3**) and 2-hydroxy-2,4-pentadienoate (**4**) have been detected by UV and ^{1}H NMR spectroscopy in the 4-OD-catalyzed decarboxylation of **1**. Incubation of **4** with 4-OD in ^2H_2O resulted in its highly stereoselective ketonization to afford (3*S*)-[3-^{2}H]**3**. A reasonable hypothesis to explain these observations is that 4-OD catalyzes the decarboxylation of **1** to **3** through the intermediacy of the dienol **4**. It was further shown that 4-OD converts (5*S*)-[5-^{2}H]**1** to 4*E*-[5-^{2}H]**4** in ^2H_2O. These stereochemical results coupled with the previously established *S* configuration of [5-^{2}H]**1** indicate that the loss of carbon dioxide and the incorporation of a deuteron occur on the same side of the dienol intermediate. The product of the 4-OD/VPH complex was also isolated and identified unequivocally as (4*S*)-**2**. Finally, it was determined by an ^{18}O labeling experiment that the hydroxyl group at C-4 of **2** is derived from solvent water and that it is unlikely that either 4-OD or VPH utilizes a Schiff base intermediate.

4-Oxalocrotonate decarboxylase (EC 4.1.1.-; 4-OD) and vinylpyruvate hydratase (EC 4.2.1.-; VPH) from *Pseudomonas putida mt-2* convert 2-oxo-3-hexenedioate (**1**) to 2-oxo-4-hydroxypentanoate (**2**; Scheme 1).[1,2] The two enzymes reportedly exist as a complex and utilize either manganese or magnesium as a cofactor.[1,2] These enzymes are 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]

The results of early investigations using partially purified cell extracts led to a general consensus that 2-oxo-4-pentenoate (**3**) is generated from **1** by 4-OD and hydrated by VPH to afford **2** (Scheme 2, route a).[4] Later, Collinsworth et al.[1] purified VPH to homogeneity and reported that the enzyme produces the *S*-isomer of **2** using as substrate a mixture of **3** and 2-hydroxy-2,4-pentadienoate (**4**, Scheme 2).[5] More recently, Harayama and co-workers[2] found thay they were not able to separate 4-OD from VPH during the purification process and suggested that the two enzymes form a complex *in vivo*.[6] Furthermore, they identified **4** as the product of the 4-OD reaction and the species hydrated by VPH (Scheme 2, path b), in contrast to the previous claims.

There are two fundamental problems with these studies that leave the conclusions uncertain. First, the identities of **2** and **3** were established primarily on the basis of chemical degradation studies using relatively harsh conditions.[4] Consequently, the product of the VPH reaction, **2**, has never been directly observed nor isolated and has only been characterized as its lactone. In addition, it is not possible to assign the position of the double bond in **3** using the methods reported. A second problem concerns the mixture of isomers commonly used as the substrate for VPH in these experiments. The mixture results from the amino acid oxidase-catalyzed deamination of 2-amino-4-pentenoate and is assumed to consist solely of **3** and **4**. However, Marcotte and Walsh found that several species are generated in this reaction including **3**, **4**, and 2-oxo-3-pentenoate (**5**, Scheme 2), depending on the incubation time and buffer.[7] Therefore, it is not clear whether **3**, **4**, or **5** (Scheme 2, path c) is the substrate for VPH.

In order to resolve the questions raised above and to narrow the range of mechanistic possibilities, we undertook a rigorous examination of the reactions catalyzed by the 4-OD/VPH complex. The results of this investigation demonstrate that **3** and **4** are generated in the course of the 4-OD-catalyzed decarboxylation of **1** and show definitively that (4*S*)-**2** is the exclusive product of the series of reactions catalyzed by these two enzymes (Scheme 3). Stereochemical experiments suggest that 4-OD catalyzes the decarboxylation of **1** to **3** through the intermediacy of the dienol **4**. Finally, isotopic labeling studies establish the origin of the hydroxyl group at C-4 of **2** and argue

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(1) Collinsworth, W. L.; Chapman, P. J.; Dagley, S. *J. Bacteriol.* **1973**, *113*, 922–931.

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

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

(4) (a) Dagley, S.; Gibson, D. T. *Biochem. J.* **1965**, *95*, 466–474. (b) Bayly, R. C.; Dagley, S. *Biochem. J.* **1969**, *111*, 303–307.

(5) It was concluded by Harayama et al.[2] that the previous group[1] had also copurified the two enzymes so that their preparation of VPH was not, in fact, a single enzyme. Apparently, the sample of "purified" VPH was not examined for decarboxylase activity even though SDS-PAGE using β-mercaptoethanol showed the presence of two polypeptides.[1]

(6) It was not possible to separate the two enzymes by using either the procedure described by Harayama et al.[2] or other HPLC columns. Hence, the two enzymes will be referred to as the 4-OD/VPH complex in order to simplify the discussion.

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

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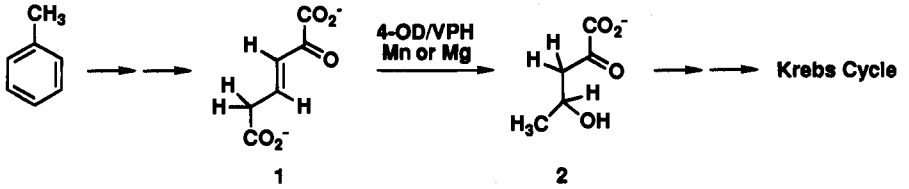
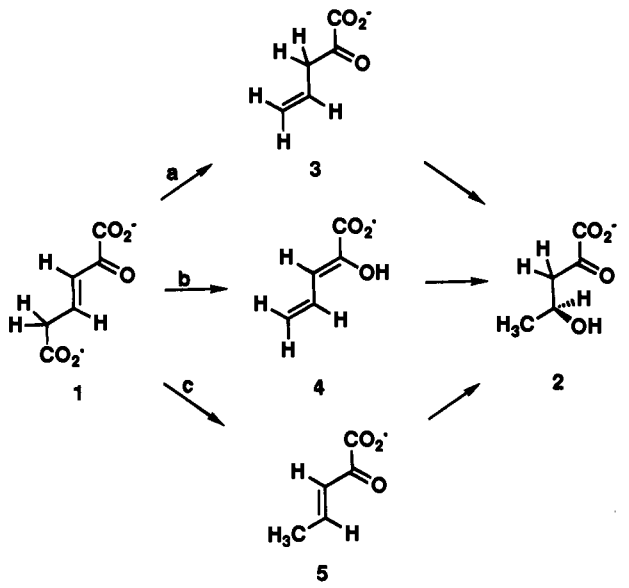
Scheme 1



Scheme 2



against the involvement of a Schiff base intermediate in either the 4-OD or VPH reaction. On the basis of these results, plausible mechanisms for 4-OD and VPH can be formulated.

Results

Spectroscopic Identification and Isolation of 2. The substrate for 4-OD, **1**, was generated by the action of 4-oxalocrotonate tautomerase (4-OT) on 2-hydroxymuconate (**6**, Scheme 4) in H_2O. Addition of the 4-OD/VPH complex and a small quantity of $MnCl_2$ results in the formation of a single product consistent with the structure of **2** as determined by ^1H NMR analysis. The ^1H NMR spectrum of **2** (not shown) presents a set of three peaks at δ 1.20 (d, 3H), 2.90 (d, 2H), and 4.27 (m, 1H). The signals are readily assigned to the methyl protons on C-5, the methylene protons on C-3, and the single proton on C-4, respectively. Subsequently, the product was isolated by anion exchange chromatography and characterized by ^1H and ^{13}C NMR spectroscopy. The ^{13}C NMR spectrum of the isolated product confirmed the presence of a ketone carbonyl carbon (C-2) and a carboxylate group (C-1). Overall, the chemical shifts of the signals in the spectrum are consistent with the structure of **2**.[8] These results clearly show that **2** is the product resulting from the action of the 4-OD/VPH complex on **1**.

Assignment of the Stereochemistry of 2 Generated by the 4-OD/VPH Complex. The stereochemistry at C-4 of **2** was assigned by the chemical degradation of **2** to methyl 3-hydroxybutyrate, derivatization with (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetic acid chloride (MTPA-Cl), and ^1H NMR analysis of the resulting diastereomeric Mosher's ester (Scheme 4).[9] The reactions used for this transformation do not disturb the chiral carbon. A quantity of **1** was generated by the 4-OT-catalyzed ketonization of **6**.[10] Addition of the 4-OD/VPH complex and a small quantity of $MnCl_2$ yielded **2** as determined by the absence of absorbance in the UV region between 200 and 350 nm. The degradation of **2** to 3-hydroxybutyrate (**7**) was achieved by treating the reaction mixture with hydrogen peroxide as described elsewhere.[1] It was determined by ^1H NMR spectroscopy that **7** was the major product isolated by anion exchange chromatography of the reaction mixture. Esterification of **7** using diazomethane afforded **8** which was purified by flash chromatography. Reaction with (*R*)-(−)-MTPA-Cl generated Mosher's ester **9**.[9] Similarly, authentic samples of (3*R*)- and (3*S*)-**8**, were converted by reaction with (*R*)-(−)-MTPA-Cl to their Mosher's esters, which were used as reference compounds.[9]

The purified enzyme-derived and authentic samples of **9** were analyzed by ^1H NMR spectroscopy (Figure 1A−D). The methyl group at C-4 of the Mosher ester **9** derived from (3*R*)-**8** gives rise to a doublet at 1.43 ppm (Figure 1A) whereas the methyl group at C-4 of the Mosher ester derived from (3*S*)-**8** gives rise to a doublet at 1.34 ppm (Figure 1B). The methyl group at C-4 of the Mosher ester **9** derived from **2** generated by the 4-OD/VPH complex gives rise to a doublet at 1.34 ppm (Figure 1C). A mixture of the authentic Mosher's ester derived from (3*R*)-**8** and Mosher's ester **9** derived from **2** generated by the 4-OD/VPH complex affords a ^1H NMR spectrum (Figure 1D) presenting a doublet at 1.34 ppm and a second doublet at 1.43 ppm. We conclude, therefore, that the 3-hydroxybutyrate derived from the chemical degradation of **2** is the *S*-isomer and that the product of the reaction catalyzed by the 4-OD/VPH complex was (4*S*)-**2**. The stereochemistry of **2** as determined above is in accord with the previously reported stereochemistry.[1]

Incorporation of ^{18}O into 2 by the 4-OD/VPH Complex. In order to pursue future mechanistic studies of VPH, it is necessary to establish the origin of the hydroxyl group at C-4 of **2**. There are two possibilities for its origin: the hydroxyl group arises either from solvent water by direct addition at C-4 of **3**, **4**, or **5** or from the carboxylate group at C-1 of the substrate. The latter mechanism involves the hydrolysis of a hypothetical lactone intermediate, 4-methyl-2-oxo-butyrolactone (**10**, Scheme 5).[11] In order to address this question, the 4-OD/VPH reaction was conducted in ^{18}O-labeled water and the product analyzed by ^{13}C NMR spectroscopy to see whether there was an isotope shift on the ^{13}C signal for C-1 or C-4 of the isolated product, **2**.[12] Accordingly, a mixture of **6**, 4-OT, 4-OD, VPH, and $MgCl_2$ was incubated in 10% $H_2{}^{18}O$. The product

(8) It has previously been reported that **2** undergoes facile lactonization to 4-methyl-2-oxobutyrolactone (**10**) in the presence of dilute acid.[4a] In order to exclude the possibility that lactonization had occurred during the isolation process, the isolated product was subjected to treatment with HCl which resulted in the formation of **10** as determined by a comparison of its ^1H and ^{13}C NMR spectra to those of the authentically synthesized lactone: Rossi, A.; Schinz, H. *Helv. Chim. Acta* **1948**, *31*, 473−492.

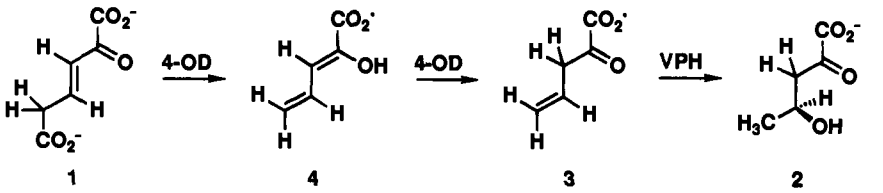
(9) (a) Dale, J. A.; Dull, D. L.; Mosher, H. S. *J. Org. Chem.* **1969**, *34*, 2543−2549. (b) Yamaguchi, S. In *Asymmetric Synthesis*; Morrison, J. D., Ed.; Academic Press: New York; 1983; Vol. 1, pp 125−152.

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

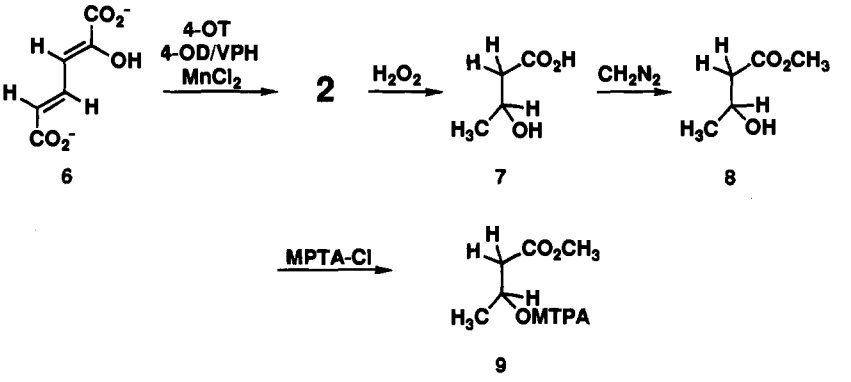
(11) The latter mechanism was suggested by the ability of **2** to undergo acid-catalyzed lactonization. The authentically synthesized **10**[8] was tested as a substrate for the enzyme, but was not processed.

(12) (a) Risley, J. M.; Van Etten, R. L. *J. Am. Chem. Soc.* **1979**, *101*, 252−253. (b) Vederas, J. C. *J. Am. Chem. Soc.* **1980**, *102*, 374−376.

Scheme 3



Scheme 4



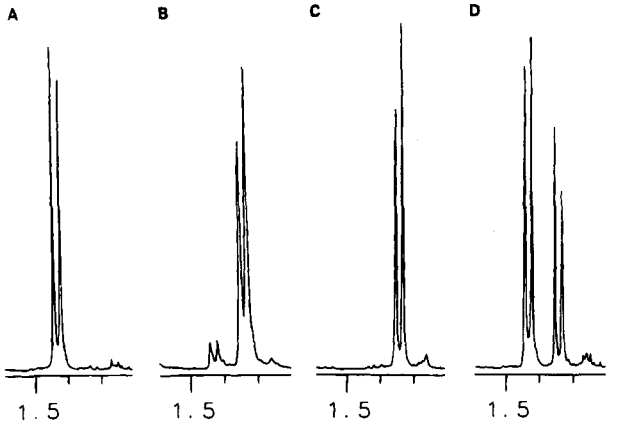


Figure 1. Partial ^1H NMR (300 MHz, $CDCl_3$) spectra showing the signals for the protons on the C-4 methyl group of the Mosher esters (**9**) derived from (A) authentic (3*R*)-**8**, (B) authentic (3*S*)-**8**, (C) **8** that was obtained by the chemical degradation of the 4-OD/VPH product (**2**), and (D) authentic (3*R*)-**8** plus material described in (C).

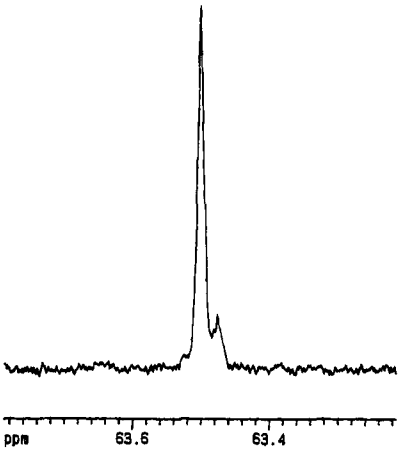
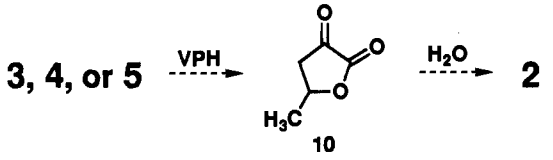


Figure 2. Partial ^{13}C NMR (500 MHz, H_2O) spectrum of **2** generated from **1** by the 4-OD/VPH complex in $H_2{}^{16}O/H_2{}^{18}O$ (90:10). The signal corresponds to C-4 of **2**.

Scheme 5



was isolated and analyzed by ^{13}C NMR spectroscopy. The resulting signal for C-4 appears as two resonances (Figure 2). The smaller resonance is shifted 0.025 ppm upfield from the larger resonance, consistent with the incorporation of ^{18}O from $H_2{}^{18}O$.[13] In contrast, the signals for C-1 (169.6 ppm) and C-2 (204.5 ppm) appear as single resonances, indicating that no ^{18}O was incorporated at these positions from $H_2{}^{18}O$. Finally, the signals for C-1, C-2, and C-4 of [^{16}O]**2**, isolated from a similar mixture conducted in $H_2{}^{16}O$, all present single resonances. These results show that the hydroxyl group at C-4 of **2** arises from solvent water.

This experiment provides an additional piece of mechanistic information. The signal for C-2 (204.5 ppm) appears as a single resonance, indicating no incorporation of ^{18}O from $H_2{}^{18}O$. The failure to observe a ^{18}O isotope shift on the position of this signal argues against the formation of an imine intermediate (i.e., Schiff base) between a carbonyl group on the substrate and an amino group on the enzyme in either the decarboxylation step or the hydration step. Hydrolysis of a Schiff base in ^{18}O-labeled water generally results in the incorporation of the ^{18}O label at the carbonyl position.[14]

Spectroscopic Detection of 3 and 4 in the Reaction of 1 Catalyzed by the 4-OD/VPH Complex. It was previously reported by Harayama and co-workers that **4** is the product of the 4-OD-catalyzed decarboxylation of **1**.[2] This conclusion was based on UV experiments that followed the behavior of a compound (i.e., **4**) with a λ_{max} at 265 nm. Recently, we generated **4** and showed that it undergoes facile decay in aqueous buffer to **3**, which does not have significant UV absorbance above 200 nm.[15] Hence, if **3** were also a product of the 4-OD-catalyzed decarboxylation of **1**, it could have easily

(13) Risley, J. M.; Van Etten, R. L. *J. Am. Chem. Soc.* **1980**, *102*, 4609–4614.

(14) Walsh, C. *Enzymatic Reaction Mechanisms*; W. H. Freeman and Co.: San Francisco, CA, 1977; pp 669–676.

(15) Lian, H.; Whitman, C. P. *J. Am. Chem. Soc.* **1993**, *115*, 7978–7984.

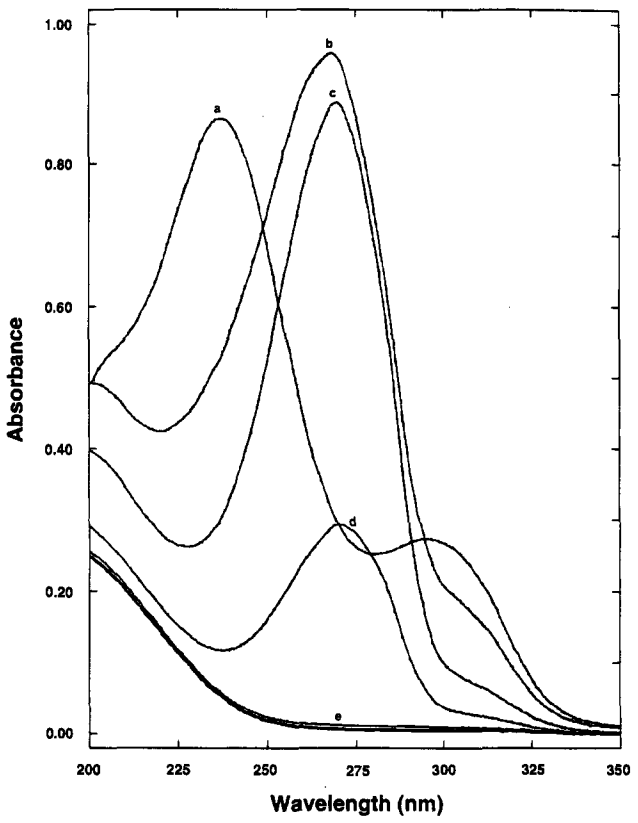


Figure 3. UV spectra (1-cm pathlength) indicating the generation and the transformation of 2-hydroxy-2,4-pentadienoate (**4**; $\lambda_{max} = 265$ nm). Spectrum (a) shows that **1** ($\lambda_{max} = 235$ nm) is the predominant species produced from a mixture of **6** and 4-OT.[10] Addition of 4-OD/VPH (1 μg) and $MgCl_2$ results in the generation of **4** (spectrum b). The enzyme-catalyzed decay of **4** (spectra c and d) results in a new product without significant absorbance above 200 nm (2-min intervals).

escaped detection by UV spectroscopy. In light of this fact, we reinvestigated the 4-OD reaction.

This study was facilitated by our early observations about the behavior of the 4-OD/VPH complex. We observed that when magnesium is substituted for manganese, **2** is generated more slowly from **1**, and two intermediates (**3** and **4**, *vide infra*) accumulate. In the absence of either metal ion, these intermediates become more prominent.[16] There are also dramatic increases in the intensities of the UV spectrum and the corresponding ^1H NMR signals for **4** when it is generated from **1** in ^2H_2O in comparison to when it is generated in H_2O.[16] It appears that by changing these experimental parameters, we can observe the 4-OD-catalyzed reaction separate from the VPH-catalyzed reaction.

Bearing these observations in mind, the substrate for 4-OD is generated by the 4-OT-catalyzed ketonization of **6** in ^2H_2O. After 3 min, UV analysis reveals that **1** ($\lambda_{max} = 235$ nm) is the major species present (Figure 3, spectrum a).[10] Addition of a quantity of the 4-OD/VPH complex and $MgCl_2$ generates **4** as indicated by the prominent λ_{max} at 265 nm (Figure 3, spectrum b). Subsequently, **4** undergoes rapid conversion to the stable product **2** (Figure 3, spectra c–e), previously identified by ^1H NMR spectroscopy.

^1H NMR analysis of a similar reaction mixture (also in ^2H_2O) verified the presence of **4** and revealed the presence of **3** (Figure 4). The key feaures of the spectrum are the two upfield doublets centered at 5.18 ppm and 5.34 ppm which are readily assigned

(16) Although the production of **2** is slowed under these conditions, it is ultimately formed. The accumulation of **3** and **4** in ^2H_2O may reflect the presence of isotopically sensitive steps that occur after decarboxylation.

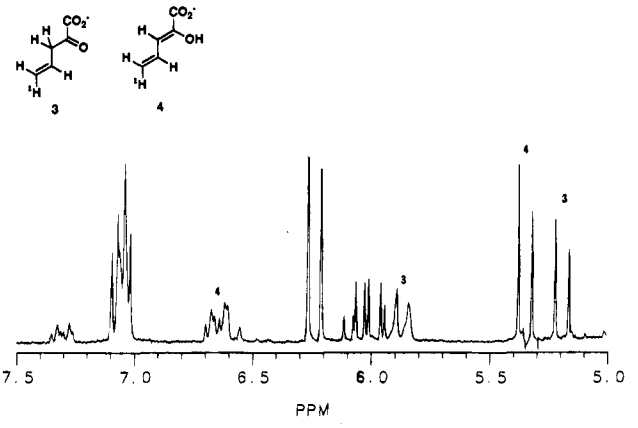


Figure 4. Partial ^1H NMR spectrum (300 MHz, ^2H_2O) indicating the generation of [3,5-$^2H_2$]-2-oxo-4-pentenoate (**3**) and [5-$^2H_1$]-2-hydroxy-2,4-pentadienoate (**4**) from the action of 4-OD on [5-^2H]**1**. The other signals in the spectrum correspond to [5-^2H]**1** and residual 2-hydroxy-muconate (**6**).[10]

to the protons on C-5 of **3** and **4**, respectively, by a comparison to their previously reported ^1H NMR spectra.[15] The observed coupling constant for each C-5 proton ($J = 17$ Hz) indicates a trans relationship to a C-4 proton. The remaining signals in the spectrum are fully consistent with the structures of **3** and **4**. The proton on C-4 of **3** gives rise to a broadened doublet centered at 5.86 ppm. The broadening of the signal presumably results from the incorporation of solvent deuterons at C-3 and C-5.[17] The remaining proton on C-3 of **3** is observed as a broadened doublet at 3.47 ppm (not shown) due to geminal ^1H–^2H coupling. The proton on C-3 of **4** appears as a doublet at 6.04 ppm while the C-4 proton of **4** is observed as a poorly defined multiplet (6.64 ppm). Again, the broadening of the signal is presumably due to the incorporation of a solvent deuteron at C-5 of **4** by the initial ketonization of **6** by 4-OT. Finally, there was no spectral evidence (UV and ^1H NMR) for the presence of **5** in the reaction mixture.[18]

Stereospecific Ketonization of 4 to [3-^2H]3 in ^2H_2O and Conversion of [3-^2H]3 to [3-^2H]Malate. The observation that both **3** and **4** are present as a result of the action of 4-OD and VPH on **1** raises the question of which isomer is the product of the 4-OD reaction. Because the substrate for 4-OD, **1**, is a vinylogous β-keto acid, it is reasonable to propose that 4-OD catalyzes the decarboxylation of **1** to **3** through the intermediacy of **4**.[19] One consequence of this mechanism would be the stereospecific ketonization of **4** to [3-^2H]**3** in ^2H_2O.[20] In order to investigate the stereochemical question, **4** was converted by 4-OD in ^2H_2O to [3-^2H]**3**, which was processed to a mono-deuteriated malate (Scheme 6). Compound **4** was generated

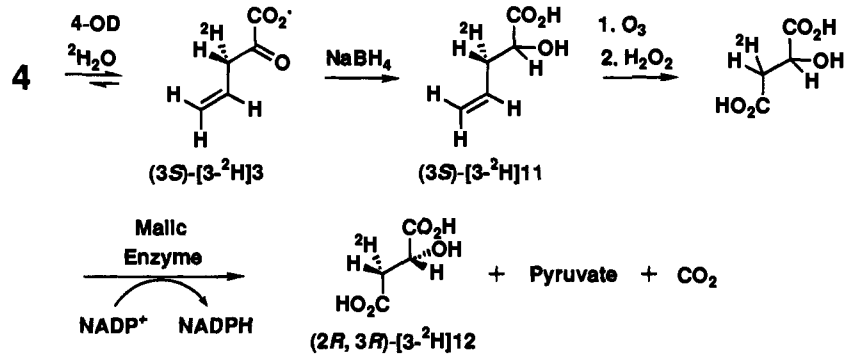
(17) The deuteron at C-5 results from the action of 4-OT on **6**: Whitman, C. P.; Hajipour, G.; Watson, R. J.; Johnson, W. H.; Bembenek, M. E.; Stolowich, N. J. *J. Am. Chem. Soc.* **1992**, *114*, 10104–10110. The deuteron at C-3 results from the action of 4-OD on **4** as discussed in the text.

(18) The remaining signals in the ^1H NMR spectrum correspond to [5-^2H]**1** and residual **6**.

(19) (a) Pollack, R. M. In *Transition States of Biochemical Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum Press: New York, 1978; pp 467–492. (b) Rozell, J. D.; Benner, S. A. *J. Am. Chem. Soc.* **1984**, *106*, 4937–4941. (c) Grissom, C. B.; Cleland, W. W. *J. Am Chem. Soc.* **1986**, *108*, 5582–5583. (d) Piccirilli, J. A.; Rozell, J. D.; Benner, S. A. *J. Am. Chem. Soc.* **1987**, *109*, 8084–8085. (e) Peliska, J. A.; O'Leary, M. H. *J. Am. Chem. Soc.* **1991**, *113*, 1841–1842. (f) O'Leary, M. H. *The Enzymes*, 3rd Ed.; Academic Press, Inc.: San Diego, 1992; Vol. 20, pp 235–269. In this discussion, β refers to the position of the departing CO_2 at C-6 of **1**. If the double bond of **1** were removed, then the departing CO_2 would be β to the carbonyl group at C-2.

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

Scheme 6



by the thermal decarboxylation of **6** as described elsewhere.[15] In ^2H_2O, in the presence of 4-OD and VPH, ketonization of **4** afforded [3−^{2}H]**3**. In order to minimize the formation of product **2**, divalent metal ion (Mn or Mg) was not added to the mixture.[16] Reduction of [3-^{2}H]**3** by $NaBH_4$ made C-3 non-epimerizable. The reduced compound, **11**, was the major product isolated by anion exchange chromatography, as determined by ^{1}H NMR spectroscopy. Subsequently, **11** was subjected to ozonolysis, and the resulting ozonide was treated with H_2O_2.[21] These reactions generated the 2*R* and 2*S* isomers of **12** because the initial $NaBH_4$ reduction of **3** is stereorandom. Incubation of the mixture with malic enzyme and purification of the product by anion exchange chromatography afforded the 2*R* isomer, monodeuteriated malate **12**.[22]

^{1}H NMR Analysis of (2*R*)-[3-^{2}H]Malate. Each diastereotopic proton at C-3 of unlabeled malate appears as a doublet of doublets at 2.33 or 2.63 ppm.[23] Stereospecific incorporation of a deuteron at C-3 results in the loss of one signal and the collapse of the remaining signal into a broadened doublet. The resonances for (2*R*)-[3-^{2}H]malate have been assigned by the reaction of maleic acid with maleate hydratase.[24]

The ^{1}H NMR spectrum of the purified (2*R*)-[3−^{2}H]malate derived from the 4−OD reaction is shown in Figure 5A. The ^{1}H NMR spectra of (2*R*)-[3−^{2}H]malate, obtained from the non-enzymatic ketonization of **4** to **3** in ^2H_2O and processed by the series of reactions described above, is shown in Figure 5B. The two major signals present, both doublets of triplets, are centered at 2.33 and 2.63 ppm and correspond to (2*R*,3*S*)- and (2*R*,3*R*)-[3-^{2}H]malate, respectively.[24,25] In Figure 5A, the height of the integral for the signal assigned to the remaining C-3 hydrogen of the (2*R*,3*R*) isomer is about 5.7 times greater than the corrresponding integral for the (2*R*,3*S*) isomer. In Figure 5B, the height of the integral for the signal assigned to the (2*R*,3*R*) isomer is about equal to the corrresponding integral for the (2*R*,3*S*) isomer. This analysis clearly shows that (2*R*,3*R*)-[3-^{2}H]malate is the major isomer recovered. The stereochemisty at C-3 of malate indicates that the stereochemistry at C-3 of [3-^{2}H]**11** is *S*, because the priority numbering changes upon unsaturation at C-4. This, in turn, indicates that in ^2H_2O, 4-OD ketonizes **4** to (3*S*)-[3-^{2}H]**3** (Scheme 6).[26]

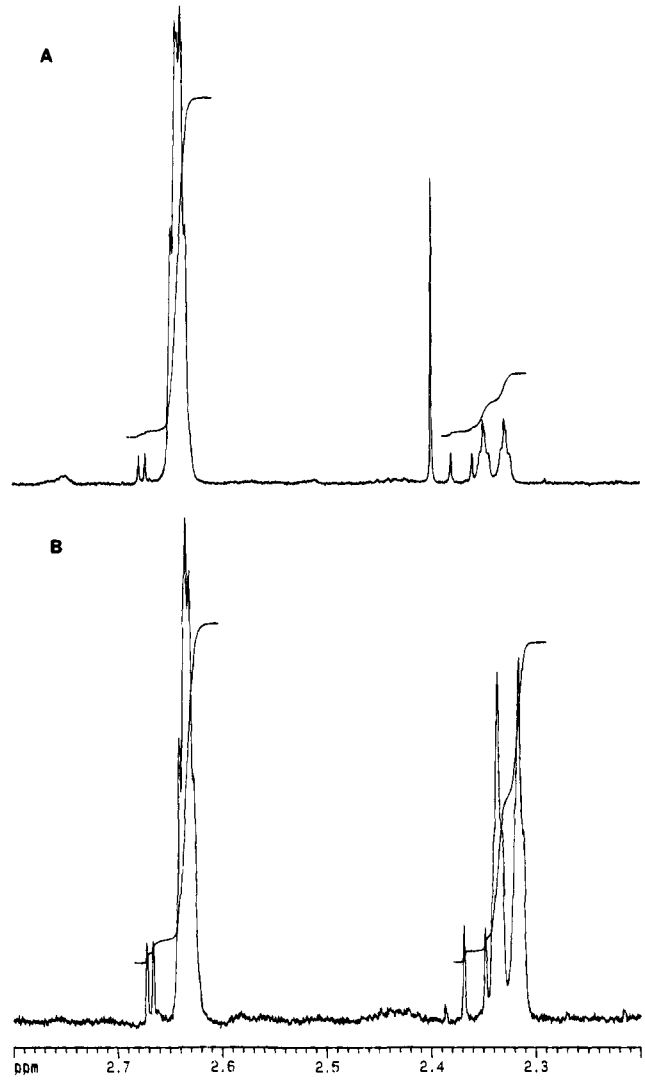


Figure 5. ^{1}H NMR (500 MHz, ^2H_2O) spectra of (2*R*)-[3-^{2}H]malate obtained from the chemical and enzymatic conversion of 2-oxo-[3-^{2}H]-4-pentenoate (**3**) generated by the (A) 4-OD-catalyzed ketonization of 2-hydroxy-2,4-pentadienoate (**4**) in ^2H_2O and the (B) non-enzymatic ketonization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

Determination of the Configuration of [5-^{2}H]4 Generated from (5*S*)-[5-^{2}H]1 by 4-OD/VPH. It has previously been demonstrated that 4-OT ketonizes **6** to (5*S*)-[5^2-H]**1** in ^2H_2O.[17] It has been shown above that 4-OD ketonizes **4** to (3*S*)-[3−^{2}H]**3**. In order to assign the overall stereochemical course of the decarboxylation reaction, it is necessary to establish whether 4-OD generates the 4*E* or the 4*Z* isomer of [5-^{2}H]**4** from (5*S*)-

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

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

(23) (a) Alberty, R. A.; Bender, P. *J. Am. Chem. Soc.* **1959**, *82*, 542−546. (b) Gawron, O.; Glaid, A. J.; Fondy, T. P. *J. Am. Chem. Soc.* **1961**, *83*, 3634−3640.

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

(25) The other signals present in the spectra shown in Figure 6 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. Finally, the singlet located at 2.40 ppm corresponds to the presence of a contaminant.

(26) The small amount of (2*R*,3*S*)-[3-^{2}H]malate present presumably results from the non-enzymatic conversion of **4** to **3** in ^2H_2O.

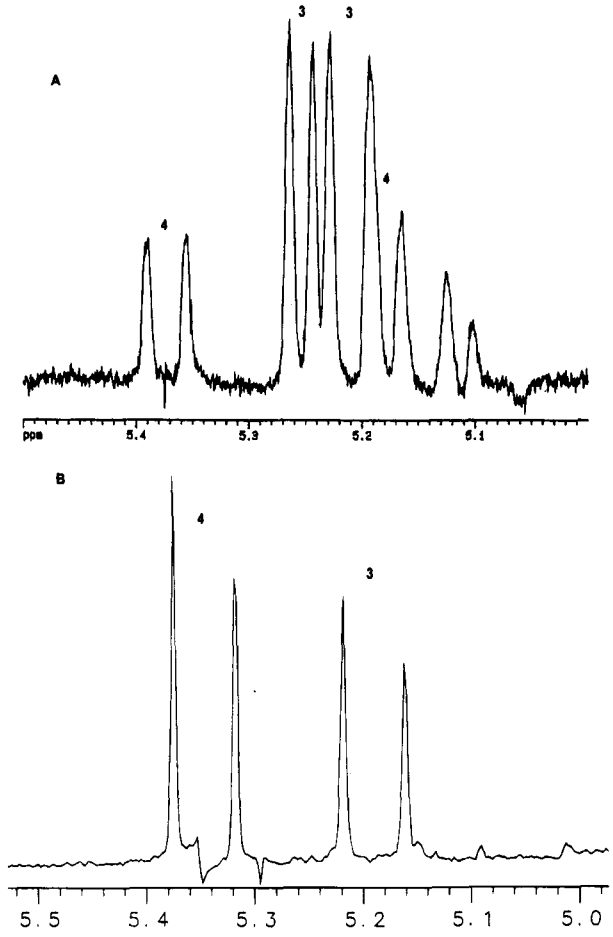


Figure 6. Partial ^{1}H NMR spectra indicating the generation of (A) 2-oxo-4-pentenoate (**3**) and 2-hydroxy-2,4-pentadienoate (**4**) by the action of the 4-OD/VPH complex on **1** in H_2O (500 MHz) and (B) [3,5-$^2H_2$]**3** and [5-$^2H_1$]**4** by the action of the 4-OD/VPH complex on **1** in ^2H_2O (300 MHz). The broadened doublet (~5.12 ppm) corresponds to an impurity.

[5-^{2}H]**1**. Accordingly, **1** was generated from **6** by 4-OT in H_2O and (5*S*)-[5-^{2}H]**1** was generated similarly in ^2H_2O. Subsequently, the 4-OD/VPH complex was added and the separate reactions were followed by ^{1}H NMR spectroscopy. In H_2O, the protons on C-5 of **3** and **4** produce a complex pattern (Figure 6A) due to geminal and vicinal proton coupling. One proton on C-5 of **4** is observed as a downfield doublet (5.37 ppm) with a characteristic trans coupling constant (J = 17.5 Hz) while the other proton on C-5 of **4** produces an upfield doublet (5.17 ppm) with a characteristic cis coupling constant (J = 13.5 Hz). Likewise, one proton on C-5 of **3** appears as a doublet at 5.25 ppm and has a cis coupling constant (J = 10.5 Hz) and the other proton on C-5 of **3** generates a doublet immediately upfield at 5.21 ppm which has a trans coupling constant (J = 17 Hz). In ^2H_2O, a solvent deuteron is stereospecifically incorporated at C-5 of **1** by 4-OT.[17] Subsequent decarboxylation results in a simplified spectrum (Figure 6B) that clearly shows the loss of two doublets: one doublet corresponds to the proton on C-5 of **4** (5.17 ppm) and the other doublet corresponds to the proton on C-5 of **3** (5.25 ppm). In both compounds, the deuteron at C-5 is cis to the proton at C-4. It can be concluded, therefore, that 4*E*-[5-^{2}H]**4** and, in turn, 4*E*-[5-^{2}H]**3** were the products generated from **1** by the action of 4-OT and 4-OD. The combination of the stereochemical results indicates that decarboxylation and the incorporation of a deuteron occur on the same side of the dienol intermediate (Scheme 7).

Discussion

These results shed considerable light on the sequence of steps involved in the 4-OD/VPH-catalyzed conversion of 2-oxo-3-hexenedioate (**1**) to 2-oxo-4−hydroxypentanoate (**2**). The highly stereoselective 4-OD-catalyzed ketonization of **4** to [3-^{2}H]**3** suggests that the decarboxylation of **1** produces **3** through the dienol intermediate, **4**. Moreover, the enzymatic decarboxylation of [5-^{2}H]**1** to [3-^{2}H]**3** is established as a syn process. The mechanism and the stereochemical course are fully consistent with literature precedent.[19e,f, 27]

The substrate for the 4-OD/VPH complex, **1**, is a vinylogous β-keto acid. As such, the mechanism for its decarboxylation is presumably analogous to the mechanisms described for the variety of β-decarboxylases.[19] The generally accepted mechanism for β-decarboxylases involves either the formation of a metal-stabilized enol intermediate which ketonizes to product or the formation of a Schiff base intermediate which undergoes hydrolysis to release product.[19f] In the latter mechanism, a metal ion is not required. The results of the ^{18}O-labeling experiment indicate that the origin of the C-4 hydroxyl group in **2** is solvent water and strongly suggest that a Schiff base intermediate is not involved in either the decarboxylation or the hydration reaction.

Although dienols have been extensively studied as intermediates in enzyme-catalyzed 1,3-allylic rearrangements,[28,29] there are few studies concerning their role in enzyme-catalyzed decarboxylation of vinylogous β-keto acids. 5-(Carboxymethyl)-2-oxo-3-hexene-1,6-dioate decarboxylase (COHED)[30] and 4-carboxymuconolactone decarboxylase (CMD)[27] are the two examples described in the literature that belong to this class of enzymes. In the COHED reaction, the dienol, 2-hydroxy-2,4-heptadiene-1,7-dioate, has been isolated, characterized, and shown to be kinetically competent in the overall reaction.[30] The stereochemical course of the reaction has not yet been determined. In the CMD reaction, a dienol has not been isolated or observed, although it is a plausible intermediate. The stereochemical course of the CMD reaction has been reported to be a syn process.[27] Hence, the stereochemical outcome of the 4-OD reaction as well as the observation of a dienol intermediate are in agreement with the observations reported for other decarboxylases that act on vinylogous β-keto acids.[31]

The product of the 4-OD-catalyzed turnover of **1** is presumably the substrate for VPH, suggesting that the enzyme catalyzes the addition of water to an unactivated double bond. While

(27) Chari, R. V. J.; Whitman, C. P.; Kozarich, J. W.; Ngai, K.-L.; Ornston, L. N. *J. Am. Chem. Soc.* **1987**, *109*, 5520−5521. Like 4-carboxymuconolactone decarboxylase, the allylic rearrangement **1** to **3** is coupled to the loss of carbon dioxide.

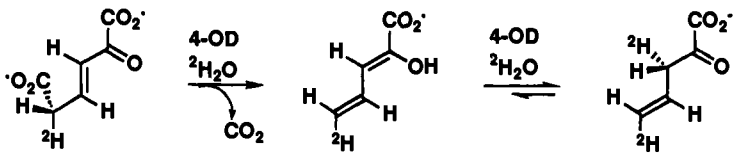
(28) (a) Pollack, R. M.; Bounds, P. L.; Bevins, C. L. In *The Chemistry of Enones*; Patai, S.; Rappoport, Z., Eds.; John Wiley & Sons Ltd: New York, 1989; pp 559−597. (b) Kuliopulos, A.; Mildvan, A. S.; Shortle, D.; Talalay, P. *Biochemistry* **1989**, *28*, 149−159. (c) Eames, T. C. M.; Hawkinson, D. C.; Pollack, R. M. *J. Am. Chem. Soc.* **1990**, *112*, 1996−1998.

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

(30) Johnson, W. H., Jr.; Hajipour, G.; Whitman, C. P. *J. Am. Chem. Soc.* **1992**, *114*, 11001−11003.

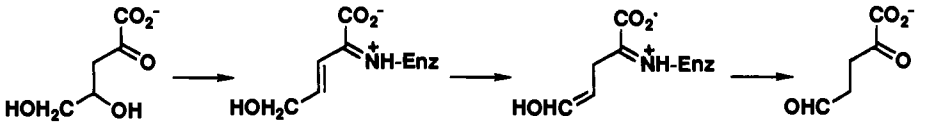
(31) The results of the UV and ^{1}H NMR experiments following the enzymatic decarboxylation of **1** in ^2H_2O show a pronounced accumulation of 4 in solution (e.g., Figure 3). At first glance, this observation could suggest that 4-OD catalyzes an unusual two-stage process in which 4 is generated from **1**, released from the enzyme into solution, bound again by the enzyme, and then ketonized to **3**. It seems more likely that the accumulation of **4** in ^2H_2O reflects the presence of isotopically sensitive steps occurring after decarboxylation because a similar observation is not made when the reaction is carried out in H_2O. In H_2O, the accumulation of **4** is considerably less pronounced and comparable to that expected from the kinetic equilibrium between **3** and **4**.[7] Presumably, the presence of **4** under these conditions results from the non-enzymatic enolization of **3** after **3** has been released from the enzyme. Experiments are underway to resolve this issue.

Scheme 7



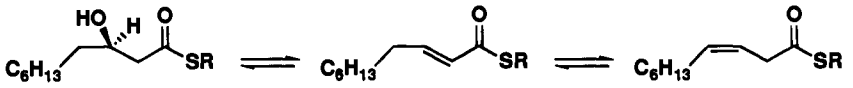
(5*S*)-[5-^{2}H]1 (4*E*)-[5-^{2}H]4 (3*S*)-[3-^{2}H]3

Scheme 8



13 14

Scheme 9



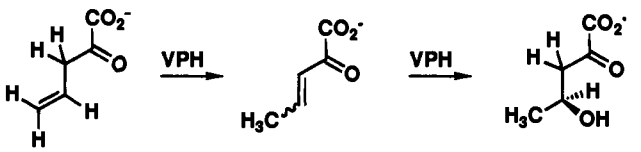
15 16 17

such a reaction may initially appear to be an unusual one in the absence of a cofactor such as iron or the B_{12} coenzyme (which are not required), a reasonably straightforward mechanism can be envisioned based on two examples described in the literature. The two examples are the reactions catalyzed by arabonate dehydrase and β-hydroxydecanoyl thiol ester dehydrase.[29,32] These enzymes catalyze a dehydration and subsequent 1,3-allylic isomerization of their respective substrates.[33]

In the reaction catalyzed by the arabonate dehydrase, Abeles et al. proposed a dehydration/isomerization sequence (Scheme 8) to account for the enzyme-catalyzed elimination of water from 2-keto-3-deoxy-L-arabonate (**13**) to generate α-ketoglutarate semialdehyde (**14**).[32] At first glance, the dehydratase appears to catalyze the loss of water β,γ to the α-keto acid resulting in an enol which ketonizes to α-ketoglutarate semialdehyde. However, it was determined that the loss of water is actually α,β to the carbonyl group. Subsequently, the same enzyme catalyzes an allylic isomerization to result in a β,γ-unsaturated α-keto acid which ketonizes to product **14**. These transformations are facilitated by the formation of a Schiff base between the substrate and the enzyme. In the reaction catalyzed by β-hydroxydecanoyl thiol ester dehydrase (Scheme 9), several studies have shown that the enzyme catalyzes the interconversion of the thiol esters of (*R*)-3-hydroxydecanoic acid (**15**) with those of (*E*)-2-decenoic acid (**16**) and (*Z*)-3-decenoic acid (**17**).[29] The enzyme has no reported coenzymes or metal ion requirements.[29]

On the basis of these two examples, we propose that VPH carries out a metal-assisted isomerization/hydration sequence to convert **3** to **2** (Scheme 10).[34] In this mechanism, the enzyme first catalyzes the isomerization of **3** to **5** and then follows with the Michael addition of water to **5**. While the ^{18}O labeling studies argue against the involvement of a Schiff base in this mechanism, a metal ion (e.g., Mn^{2+} or Mg^{2+}) could presumably serve as the "electron sink". Although there is no experimental evidence to support our working hypothesis, the appropriate experiments are in progress.[35]

Scheme 10



3 5 2

Finally, these results confirm the presence of **2** and **3** as metabolites in the catechol meta-fission pathway. These results also lend support to an earlier suggestion about the metabolic significance of the 4-OD/VPH complex.[2] A complex ensures that **3**, the product of 4-OD, is efficiently transformed by VPH to **2**.[2,36] Presumably, efficient transformation of the chemically labile **3** prevents a potential metabolic loss through an unwanted non-enzymatic side reaction. The nature of the 4-OD/VPH complex and its ability to process labile intermediates are under investigation.

Experimental Section

Materials. All chemicals and solvents were obtained from Aldrich Chemical Co. with the following exceptions. (*R*)-(−)-α-Methoxy-α-trifluoromethylphenylacetic acid chloride was obtained from Fluka Chemical Corp. 2-Hydroxymuconate was synthesized according to the method of Lapworth.[37] Buffers, biochemicals, and catalase (from bovine liver) were purchased from Sigma Chemical Co. Diazomethane was generated from Diazald according to directions. Ion exchange resins were obtained from Bio Rad Laboratories. Centricon (10000 MW cutoff) centrifugal microconcentrators were obtained from Amicon. 4-Oxalocrotonate tautomerase, 4-oxalocrotonate decarboxylase, and vinylpyruvate hydratase were purified according to published procedures.[2,38]

(32) Portsmouth, D.; Stoolmiller, A. C.; Abeles, R. H. *J. Biol. Chem.* **1967**, *242*, 2751−2759.

(33) Both reactions are reversible, further strengthening the analogy.[29,32]

(34) A mechanism involving the generation of an allylic or secondary carbocation (from **4** or **3**, respectively) cannot be ruled out. However, this mechanism seems less likely in the absence of literature precedent and in view of the fact that the molecule is "set up" for electrophilic catalysis: Gerlt, J. A.; Gassman, P. G. *J. Am. Chem. Soc.* **1992**, *114*, 5928−5934.

(35) The authentically synthesized (3*E*)-**5**[15] was tested as a substrate for the enzyme, but was not processed. The (3*Z*) isomer has not yet been tested. It should be noted that there are examples in the literature where a chemically reasonable intermediate for an enzymatic reaction was tested as a substrate, but was not processed: (a) Cleland, W. W. *Biochemistry* **1990**, *29*, 3194−3197. (b) Anderson, K. S.; Johnson, K. A. *Chem. Rev.* **1990**, *90*, 1131−1149.

(36) The fact that the product of 4-OD was mistakenly identified as **4** in the previous work does not invalidate the suggestion about the metabolic significance of the 4-OD/VPH complex.[2]

(37) Lapworth, A. J. *J. Chem. Soc.* **1901**, *79*, 1265−1284.

Methods. Protein concentrations were determined using the commercially available bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL). HPLC was performed on a Waters system using a Waters Protein Pak DEAE 5PW anion-exchange column (10-μm particle size), a Bio-Gel Phenyl 5-PW hydrophobic interaction 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.[39] UV data were obtained on a Perkin-Elmer fast scan UV/vis spectrophotometer Model 553. Nuclear magnetic resonanace spectra were obtained on either a General Electric QE-300 spectrometer or a Bruker AM 500 spectrometer, as indicated.

Spectroscopic Identification and Isolation of 2. The disodium salt of 2-hydroxymuconate (**6**) was prepared by the addition of solid $NaHCO_3$ (~2 equiv) to **6** (76 mg, 0.5 mmol) dissolved in DMSO (1 mL). The subsequent addition of acetone (10 mL) to the solution generated a white precipitate, the disodium salt of **6**, which was collected by filtration. The disodium salt of **6** (20 mg, 0.1 mmol) was dissolved in 20 mM Na_2HPO_4 (0.54 mL, pH 7.1) and ^2H_2O (0.06 mL), and the solution was transferred to a NMR tube. Subsequently, 4-OT (30 μg in 0.05 mL) was added to the solution. A ^1H NMR spectrum indicated that **1** was the predominant species present.[10] A solution of 4-OD/VPH (150 μg in 50 μL) was added to the NMR tube (final pH 8.2), and ^1H NMR spectra were acquired at 10-min intervals. After 30 min, only the set of signals corresponding to **2** was present. The ^1H NMR spectra were recorded on a Bruker AM 500 spectrometer using a composite pulse for selective presaturation of the water signal with a 2-s presaturation interval. **1**: ^1H NMR (90% H_2O/10% ^2H_2O, 500 MHz) δ 3.25 (2H, d, $J_{4,5}$ = 7 Hz, H-5), 6.26 (1H, d, $J_{3,4}$ = 16.5 Hz, H-3), 7.08 (1H, dt, $J_{3,4}$ = 16.5 Hz, $J_{4,5}$ = 7 Hz, H-4).

In a separate reaction, **6** (6 mg, 0.04 mmol) was dissolved in 20 mM Na_2HPO_4 (6 mL, pH 7.40) containing $MnCl_2$ (30 μL of 60 mM solution) and 4-OD/VPH complex (320 μg in 0.1 mL). The hydration reaction was monitored spectrophotometrically and was complete in about 2 h as indicated by the absence of any significant absorbance between 200 and 350 nm. The solution was subjected to chromatography on a Dowex-1 (chloride) column (0.8 × 15 cm), eluted with a sodium nitrate gradient (0−1 M $NaNO_3$, 60 mL total volume). Compound **2** eluted at about 0.45 M $NaNO_3$. Appropriate fractions were collected and evaporated to dryness under mechanical vacuum. **2**: ^1H NMR (H_2O, 500 MHz) δ 1.20 (3H, d, $J_{4,5}$ = 6.5 Hz, H-5), 2.90 (2H, d, $J_{3,4}$ = 6.5 Hz, H-3), 4.27 (1H, tq, $J_{2,3}$ = 6.5 Hz, $J_{3,4}$ = 6.5 Hz, H-4). ^{13}C NMR (H_2O, 500 MHz) 22.1 (C-5), 47.8 (C-3), 63.5 (C-4), 169.6 (C-1), 204.5 (C-2).

Conversion of 2 to 4-Methyl-2-oxobutyrolactone (10). The product of the VPH reaction, **2**, was generated by the action of 4-OT, 4-OD, and VPH on **6** (27 mg, 0.17 mmol) as described above. The solution containing **2** was then subjected to chromatography on a Dowex-1 (chloride) column (0.8 × 15 cm), eluted with a hydrochloric acid gradient (0−4 M HCl, 60 mL total volume). The lactone elutes at about 2 M HCl. The ^1H NMR spectrum indicates that both the keto and enol form of **10** are present. **10** (keto): ^1H NMR ($CDCl_3$, 300 MHz) δ 1.62 (3H, d, $J_{4,5}$ = 6.0 Hz, H5), 2.54 (1H, dd, J_{gem} = 19.8 Hz, $J_{3,4}$ = 6.0 Hz, H3), 3.13 (1H, dd, J_{gem} = 19.8 Hz, $J_{3,4}$ = 7.2 Hz, H3), 5.06 (1H, m, H4). **10** (enol): ^1H NMR ($CDCl_3$, 300 MHz) δ 1.45 (3H, d, $J_{4,5}$ = 6.6 Hz, H5), 5.06 (1H, m, H4), 6.22 (1H, d, $J_{3,4}$ = 2.1 Hz, H3); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 19.9 (C-5), 76.0 (C-4), 120.0 (C-3), 141.8 (C-2), 170.2 (C-1).

Assignment of the Absolute Configuration of 2 Generated by the 4-OD/VPH Complex. 2-Hydroxymuconate (**6**, 158 mg, 1.0 mmol) in ethanol (10 mL) was added in portions (10−1.0-mL portions) at 10 min intervals to a stirred solution containing 4-OT (58 μg/mL) in 50 mM Na_2HPO_4 (150 mL, pH 7.23). After the reaction mixture had been stirred slowly at room temperature for 2 h, a solution of 4-OD/VPH (100 μL, 2.9 mg/mL) and a solution of $MnCl_2$ (50 μL, 60 mM) were added to the reaction mixture. The hydration reaction was monitored spectrophotometrically and was complete in about 1.5 h, as indicated by the absence of any significant absorbance between 200 and 350 nm. The reaction mixture was then placed in a water bath at 30 °C and treated with H_2O_2 (40 mL of a 3% solution in Na_2HPO_4 buffer, pH 7.23) added slowly over a 10-min period.[1] After the mixture had been stirred for an additional 30 min, catalase (~5 mg, 13000−17000 units) was added to the reaction mixture. When the reaction mixture no longer generated gas bubbles (~10 min), it was concentrated in vacuo. The resulting solution (~5 mL) was diluted with H_2O (100 mL), and the pH was adjusted to 8.0 with small amounts of a NaOH solution. The resulting solution was subjected to chromatography on a Dowex-1 (formate) column (2 × 16 cm), eluted with a formic acid gradient (0−2 M formic acid, 600 mL total volume). 3-Hydroxybutyric acid (**7**) eluted at approximately 0.6 M formic acid. Appropriate fractions were pooled and evaporated to dryness to give **7** as a colorless oil (84 mg, 80%). ^1H NMR (^2H_2O, 300 MHz) δ 1.06 (3H, d, J = 6.3 Hz, H-4), 2.25 (2H, ddd, $J_{2,3}$ =2.4, 6.6 Hz, J_{gem} = 1.2 Hz, H-2), 4.05 (1H, m, H-3).

Methyl (3*S*)-Hydroxybutyrate (8). To a solution of (3*S*)-**7** (84 mg, 0.8 mmol) in ether (100 mL) was added an ethereal solution of freshly generated CH_2N_2 until the yellow color persisted. After the mixture had been stirred overnight, it was washed with 10% $NaHCO_3$ (2 × 50 mL), and the ether layer was dried over anhydrous $MgSO_4$ and filtered, and the filtrate was evaporated to yield **8** as a colorless oil (11 mg, 12%). ^1H NMR ($CDCl_3$, 300 MHz) δ 1.21 (3H, d, J = 6.3 Hz, H-4), 2.45 (2H, dd, H-2), 3.69 (3H, s, OCH_3), 4.18 (1H, m, H-3).

Preparation of Mosher's Ester Using (3*S*)-8 Derived from the 4-OD/VPH Reaction. Methyl (3*S*)-3-[(*S*)-(−)-α-Methoxy-α-(trifluoromethyl)phenylacetyl]butyrate (9). To a solution of (3*S*)-**8** (11 mg. 0.1 mmol) in dry CCl_4 (~25 drops) were added dry pyridine (~25 drops) and (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetic acid chloride (240 mg, 1.0 mmol).[9] After the reaction mixture had been stirred at room temperature for 12 h, H_2O (2 mL) was added. Subsequently, the reaction mixture was added to ether (20 mL). The ether layer was removed, washed with dilute HCl (10 mL), 10% $NaHCO_3$ (10 mL), and H_2O (10 mL), dried over $MgSO_4$, and filtered. The filtrate was evaporated to yield a colorless oil. The product was further purified by flash chromatography (12.5% ethyl acetate, 87.5% hexanes) to afford (3*S*)-**9** (28 mg, 86%). ^1H NMR ($CDCl_3$, 300 MHz) δ 1.34 (3H, d, J = 6.3 Hz, H-4), 2.57 (1H, dd, J =16.2, 4.5 Hz, H-2), 2.73 (1H, dd, J =16.2, 8.7 Hz, H-2), 3.53 (3H, s), 3.67 (3H, s), 5.54 (1H, m), 7.40 (3H, m), 7.51 (2H, m).

Preparation of Authentic Mosher's Ester (9). Methyl (3*R*)- and (3*S*)-3-[(*S*)-(−)-α-Methoxy-α-(trifluoromethyl)phenylacetyl]-butyrate (9). To a solution of (3*R*)-methyl 3-hydroxybutyrate (20 mg, 0.17 mmol) in dry CCl_4 (5 drops) and dry pyridine (5 drops) was added (*R*)-(−)-MTPA-Cl (43.4 mg, 0.17 mmol).[9] The reaction mixture was processed by the procedure described above to generate (3*R*)-**8**. The yield was quantitative. ^1H NMR ($CDCl_3$, 300 MHz) δ 1.43 (3H, d, J = 4.2 Hz, H-4), 2.54 (1H, dd, J = 16.2, 5.1 Hz, H-2), 2.69 (1H, dd, J = 15.9, 8.4 Hz, H-2), 3.54 (3H, s), 3.58 (3H, s), 5.55 (1H, m, H-3), 7.41 (3H, m), 7.54 (2H, m).

(3*S*)-**9** was prepared from (3*S*)-methyl 3-hydroxybutyrate by the above procedure to yield (3*S*)-**9** in a quantitative yield. ^1H NMR ($CDCl_3$, 300 MHz) δ 1.34 (3H, d, J = 6.3 Hz, H-4), 2.57 (1H, dd, J = 16.2, 4.5 Hz, H-2), 2.73 (1H, dd, J =16.2, 8.7 Hz, H-2), 3.53 (3H, s), 3.67 (3H, s), 5.54 (1H, m), 7.40 (3H, m), 7.51 (2H, m).

Incorporation of ^{18}O into 2 by the 4-OD/VPH Complex. To 100 mM Na_2HPO_4 in 10% $H_2{}^{18}O$ (5 mL) containing 4-OT (6.2 μg), 4-OD/VPH (145 μg), and $MgCl_2$ (10 μL of a 60 mM solution) was added a mixture of **6** (30 mg, 0.2 mmol) and $NaHCO_3$ (8.1 mg, 0.1 mmol). The reaction mixture was stirred at room temperature for 2 h, after which time there was no significant absorbance above 200 nm. The solution was filtered through a Centricon-10 microconcentrator, and the filtrate was collected and evaporated to dryness to yield **2** as a yellow solid. The sample was dissolved in H_2O and filtered through a Chelex 100 column (0.5 × 5 cm). The ^1H NMR spectrum was identical to that reported above for **2**. ^{13}C NMR (H_2O, 500 MHz) δ 22.1 (C-5), 47.8 (C-3), 63.501 (C-4, ^{18}O), 63.476 (C-4, ^{16}O), 169.6 (C-1), 204.5 (C-2).

Spectroscopic Detection of 3 and 4 in the Reaction of 1 Catalyzed by the 4-OD/VPH Complex. Spectral scans were performed from 350 to 200 nm at 30 °C. The enzyme solutions had been previously exchanged by repeated dilution and concentration in ^2H_2O in a

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Centricon-10 microconcentrator and stored overnight in ^2H_2O. The UV spectra monitoring the 4-OD-catalyzed decarboxylation of **1** were generated in the following manner. A quantity of **1** was produced by the addition of **6** (5 μL) from a stock solution (16.5 mM) in methanol and 4-OT (2 μL, 20 μg/mL in ^2H_2O) to 20 mM $Na_2{}^2HPO_4$ (1.0 mL; pD 7.1). The final methanol concentration was 0.5%. After the reaction mixture had incubated at room temperature for 3 min, a single UV spectrum was recorded. Subsequently, the 4-OD/VPH complex (2 μL, 0.5 mg/mL in ^2H_2O) and $MgCl_2$ (5 μL of a 60 mM solution) were added to the reaction mixture in the cuvette. After 1 min, a second spectrum was recorded. The remaining spectra were recorded at 2 min intervals.

The ^{1}H NMR spectra monitoring the enzymatic decarboxylation of **1** were generated as follows. A solution of **6** (9.0 mg, 0.06 mmol) in dimethyl sulfoxide-d_6 (50 μL) was added to 20 mM $Na_2{}^2HPO_4$ in ^2H_2O (0.7 mL, pD 8.6) containing $MgCl_2$ (2 μL of a 60 mM solution) and 4-OT (2 μL, 7.1 mg/mL in ^2H_2O). The pD of the solution was adjusted to 7.4 with NaO^2H in ^2H_2O. After the reaction mixture had incubated at room temperature for 2 min, it was transferred to an NMR tube. A ^{1}H NMR spectrum indicated that **1** was the predominant product, in accord with previous work.[10] Subsequently, a quantity of 4-OD and VPH (40 μL, 3.3 mg/mL in ^2H_2O) was added, and additional ^{1}H NMR spectra were recorded at 7, 16, and 23 min. The reported ^{1}H NMR spectrum (Figure 5) was recorded at 7 min. **3**: ^{1}H NMR spectrum (^2H_2O, 300 MHz) δ 3.47 (1H, br s, H3), 5.18 (1H, d, $J_{4,5}$ = 17.1 Hz, H5), 5.86 (1H, br d, J = 14.4 Hz, H4). **4**: ^{1}H NMR spectrum (^2H_2O, 300 MHz) δ 5.34 (1H, d, $J_{4,5}$ = 17.1 Hz), 6.04 (1H, d, $J_{3,4}$ = 11.4 Hz, H3), 6.64 (1H, br m, H4).

Stereospecific Ketonization of 4 to [3-$^2H_1$]3 in ^2H_2O and Conversion of [3-$^2H_1$]3 to [3-$^2H_1$]11. 2-Hydroxymuconate (15 mg, 0.9 mmol) was heated in dimethyl sulfoxide (0.4 mL) as described elsewhere to generate **4**[15]. Buffer (10 mL, 20 mM $Na_2{}^2HPO_4$, pD 8.6) containing 4-OD/VPH (40 μL, 3.3 mg/mL) was added to the residual oil. The final pD was 8.15. 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 $NaBH_4$ (about 10 equiv) after 12 s and 1, 2, and 5 min. After the reaction mixture had been stirred for an additional 30 min, it was subjected to chromatography on a Dowex-1 (formate) column (0.8 × 15 cm) eluted with a formic acid gradient (0−2 M formic acid, 60 mL total volume). The product, [3-^{2}H]**3**, eluted at 1.2 M formic acid. Appropriate fractions were pooled and evaporated to dryness under mechanical vacuum. The ^{1}H NMR spectrum (^2H_2O, 300 MHz) corresponded to the previously reported spectrum.[15]

Conversion of [3-$^2H_1$]11 to [3-$^2H_1$]Malate. A solution of [3-$^2H_1$]**11** (10 mg, 0.09 mmol) in dioxane (6 mL) and methanol (0.02 mL) at 5 °C was subjected to a stream of O_3 (1 L/min) for 20 min.[21] After the solvent was evaporated to dryness, H_2O_2 (0.3 mL, 30%) and glacial acetic acid (2.0 mL) were added to the residual oil, and the mixture was stirred at room temperature overnight. The solution was evaporated to dryness, and 5% $NaHCO_3$ (2 mL) was added. The [3-$^2H_1$]malates were subjected to chromatography on a Dowex-1 (formate) column (0.8 × 15 cm), eluted with a formic acid gradient (0−4 M formic acid, 60 mL total volume). Malate eluted at 2.3 M formic acid. The ^{1}H NMR spectrum (^2H_2O, 300 MHz) corresponded to the previously published spectrum.[15]

The purified [3-$^2H_1$]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.[22] After the reaction mixture had been stirred at room temperature overnight, (2*R*)-[3-$^2H_1$]-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-$^2H_1$]**12**: ^{1}H NMR (^2H_2O, 500 MHz) δ 2.34 (~0.14H, dt, $J_{2,3}$ = 10.0 Hz, H3), 4.28 (~0.14H, d, $J_{2,3}$ = 9.5 Hz, H2). (2*R*,3*R*)-[3-$^2H_1$]**12**: ^{1}H NMR (^2H_2O, 500 MHz) δ 2.64 (~0.86H, dt, $J_{2,3}$ = 2.5 Hz, J_{gem} = 2.0 Hz, H3), 4.28 (~0.86H, d, $J_{2,3}$ = 3.0 Hz, H2).

Chemical Ketonization of 4 to [3-^{2}H]3 in ^2H_2O and Conversion of [3-^{2}H]3 to [3-^{2}H]Malate. The non-enzymatically-derived malate was produced by a previously described procedure.[15] (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, br d, $J_{2,3}$ = 2.5 Hz, H2).

Determination of the Configuration of [5-^{2}H]4 Generated from 1 by 4-OD/VPH in ^2H_2O. The procedure for the ^{1}H NMR analysis of the 4-OD reaction in ^2H_2O is described above. The reaction was also monitored in H_2O using the following procedure. To an NMR tube was added the disodium salt of **6** (27 mg, 0.13 mmol), prepared by the procedure described above, dissolved in 20 mM Na_2HPO_4 (0.54 mL, pH 7.1) and ^2H_2O (0.06 mL). The final pH of the solution was 8.0. After 30 min, a ^{1}H NMR spectrum indicated that **1** was the predominant species present.[10] A solution of 4-OD/VPH (20 μL, 2.9 mg/mL) was then added to the NMR tube, and ^{1}H NMR spectra were acquired at timed intervals. The ^{1}H NMR spectra were recorded on a Bruker AM 500 spectrometer using a composite pulse for selective presaturation of the water signal with a 2-s presaturation interval. After 20 min, ^{1}H NMR analysis indicated the presence of **1**, **2**, **3**, **4**, and **6**. **3**: ^{1}H NMR spectrum (90% H_2O/10% ^2H_2O, 500 MHz) δ 3.49 (2H, d, $J_{3,4}$ = 6.5 Hz, H3), 5.20 (1H, d, $J_{4,5E}$ = 17.0 Hz, H5), 5.24 (1H, d, $J_{4,5Z}$ = 10.5 Hz, H5), 5.87 (1H, m, H4). **4**: ^{1}H NMR spectrum (90% H_2O/10% ^2H_2O, 500 MHz) δ 5.17 (1H, d, $J_{4,5Z}$ = 13.5 Hz, H5) 5.36 (1H, d, $J_{4,5E}$ = 17.5 Hz), 6.05 (1H, d, $J_{3,4}$ = 12 Hz, H3), 6.64 (1H, m, H4).

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