



## Bioavailable affinity label for collagen prolyl 4-hydroxylase



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### ABSTRACT

Collagen is the most abundant protein in animals. Its prevalent 4-hydroxyproline residues contribute greatly to its conformational stability. The hydroxyl groups arise from a post-translational modification catalyzed by the nonheme iron-dependent enzyme, collagen prolyl 4-hydroxylase (P4H). Here, we report that 4-oxo-5,6-epoxyhexanoate, a mimic of the  $\alpha$ -ketoglutarate co-substrate, inactivates human P4H. The inactivation installs a ketone functionality in P4H, providing a handle for proteomic experiments. *Caenorhabditis elegans* exposed to the esterified epoxy ketone displays the phenotype of a worm lacking P4H. Thus, this affinity label can be used to mediate collagen stability in an animal, as is desirable in the treatment of a variety of fibrotic diseases.

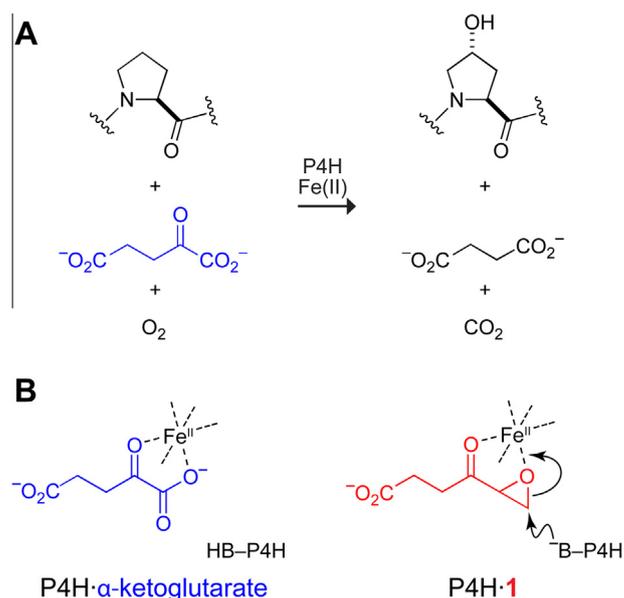
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### 1. Introduction

Collagen is the most abundant protein in animals, and maintains the structural integrity of various connective tissues.<sup>1</sup> The overproduction of collagen is associated with fibrotic diseases, including myocardial infarction, stroke, peripheral vascular disease, diabetes, and severe anemias.<sup>2</sup> The stability of collagen relies on catalysis by collagen prolyl 4-hydroxylase (P4H).<sup>3</sup> This enzyme is located in the lumen of the endoplasmic reticulum, where it catalyzes a remarkable post-translational modification—the conversion of (2S)-proline residues (Pro) to (2S,4R)-4-hydroxyproline residues (Hyp) in procollagen strands. The human enzyme consists of an  $\alpha_2\beta_2$  tetramer in which each  $\alpha$  subunit contains an active site. P4H is essential for animals,<sup>4–6</sup> as the conformational stability of mature collagen relies on its hydroxylation.<sup>7–9</sup> Moreover, P4H is a validated target for the treatment of fibrotic diseases.<sup>2</sup>

P4H is a nonheme iron(II) dioxygenase that uses O<sub>2</sub> and  $\alpha$ -ketoglutarate as co-substrates (Fig. 1A). The three-dimensional structure of mammalian P4H is unknown. We reasoned that an electrophilic analog of  $\alpha$ -ketoglutarate could serve as an irreversible inhibitor of the enzyme and, hence, a useful probe for active-site residues. Inspired by natural products such as trapoxin and epolactaene as well as artificial affinity labels,<sup>10</sup> we designed 4-oxo-5,6-epoxyhexanoate (**1**) for this purpose. Our thought was that the epoxide oxygen would chelate to the active-site iron and thereby be activated for nucleophilic attack (Fig. 1B). We also noted that  $\alpha$ -ketoglutarate and epoxy ketone **1** have the same number and a similar arrangement of nonhydrogen atoms. This attribute is important because of the inability of larger  $\alpha$ -ketoglutarate

analogues to inhibit catalysis by human P4H.<sup>11</sup> Epoxy ketone **1**, which also resembles 5-aminolevulinic acid, is a known competitive inhibitor of 5-aminolevulinic acid dehydratase but does not alkylate that enzyme.<sup>13</sup>



**Figure 1.** (A) Reaction catalyzed by prolyl 4-hydroxylase. (B) Notional image of  $\alpha$ -ketoglutarate (left) or epoxy ketone **1** (right) bound in the active site of P4H and interacting with an enzymic base. B. Alkylation is more likely to occur by nucleophilic attack at C6 than C5.<sup>12</sup>

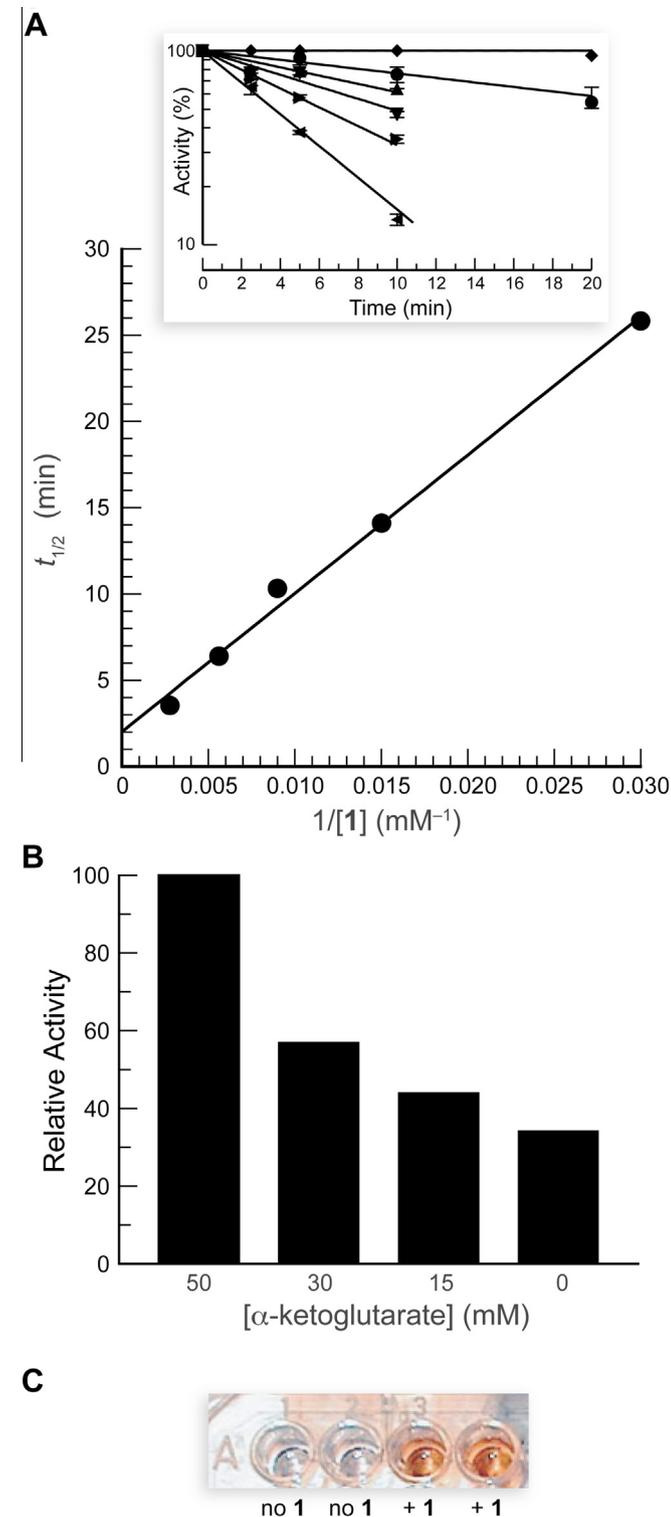
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## 2. Results and discussion

### 2.1. Effect of epoxy ketone 1 on catalysis by P4H

We produced human P4H by heterologous expression in *Escherichia coli*,<sup>14–18</sup> and we synthesized racemic **1**. We assayed the



**Figure 2.** In vitro assays of epoxy ketone **1**. (A) Kitz–Wilson plot<sup>19</sup> showing the effect of different concentrations of epoxy ketone **1** on the catalytic activity of P4H.  $k_{\text{inact}}/K_i = 0.09 \text{ M}^{-1} \text{ s}^{-1}$ . Inset: raw data. (B) Effect of  $\alpha$ -ketoglutarate concentration on the inactivation of P4H by epoxy ketone **1**. (C) Immobilization of P4H after its reaction with epoxy ketone **1**. Wells 1 and 2,  $A_{490} = 0.14$ ; wells 3 and 4,  $A_{490} = 0.98$ .

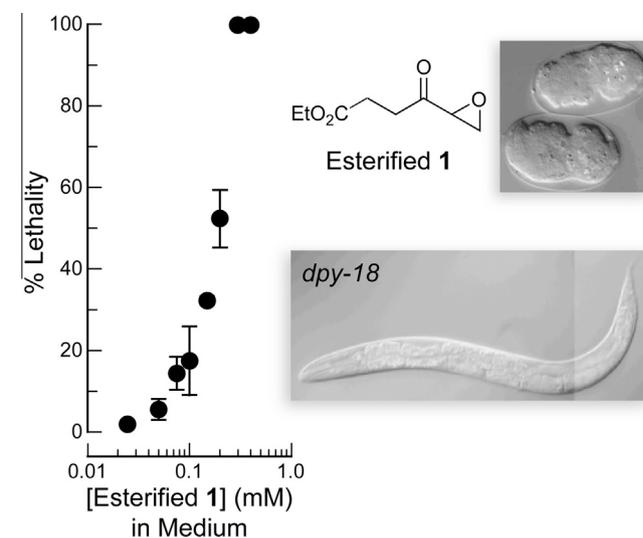
effect of this putative affinity label on the ability of P4H to catalyze the hydroxylation of a fluorescent peptidic substrate, dansyl-Gly-Phe-Pro-Gly-OEt.<sup>14–17</sup> The data revealed that epoxy ketone **1** elicits irreversible inactivation of P4H, and does so in a concentration-dependent manner (Fig. 2A).  $\alpha$ -Ketoglutarate protected P4H against inactivation (Fig. 2B), suggesting that epoxy ketone **1** competes with this co-substrate for binding to the active site.

### 2.2. Utility of epoxy ketone 1 as a proteomic probe

Noting that a ketone has chemical reactivity orthogonal to that in natural amino acids,<sup>20</sup> we examined the utility of epoxy ketone **1** in a model proteomic experiment. Epoxy ketone **1** was incubated with P4H in the presence of iron(II). The mixture was treated with biocytin hydrazide and then  $\text{NaBH}_4$  to reduce the incipient *N*-acyl hydrazone (which is unstable<sup>21</sup>). Small molecules were removed by gel-filtration chromatography. The P4H was added to wells of a microplate containing immobilized streptavidin. The wells were washed and exposed to a monoclonal antibody directed against P4H. Visualization with a secondary antibody conjugated to horseradish peroxidase revealed that P4H is associated with streptavidin only in the presence of epoxy ketone **1** (Fig. 2C). The conjugation was confirmed by SDS–PAGE (data not shown). These data indicate that epoxy ketone **1** could serve as a chemo-selective probe for P4H. Thus, epoxy ketone **1** complements a (2*S*,4*S*)-4-fluoroproline substrate that spawns a ketone upon P4H catalysis.<sup>16</sup>

### 2.3. Effect of epoxy ketone 1 on an animal

Finally, we determined the effect of epoxy ketone **1** on a live animal. Like all animals (including dinosaurs<sup>22</sup>), *Caenorhabditis elegans* has an abundance of collagen. In wild-type worms, P4H exists largely as a tetramer of two  $\alpha$  subunits (DPY-18 and PHY-2, which each contain an active site) and two  $\beta$  subunits.<sup>23</sup> The *dpy-18* gene is essential for body morphology, whereas the function of the *phy-2* gene is not apparent.<sup>45</sup> The deletion of both genes results in embryos that grow to the twofold stage, but are unable to maintain their shape and consequently explode.<sup>5</sup> These two forms of the  $\alpha$  subunit have 56.5% amino-acid sequence identity with each other and ~50% identity with their human homologues.  $\alpha$ -Ketoglutarate



**Figure 3.** In vivo assay of epoxy ketone **1**. *Dpy-18 C. elegans* worms (four in stage L4) were exposed to known concentrations of epoxy ketone **1** in the growth medium. Lethality refers to eggs that do not hatch. Insets: Image of lethality observed in the embryos of either a *dpy-18/phy-2* animal or a *dpy-18* animal exposed to epoxy ketone **1** (top); image of an adult *dpy-18* animal not exposed to epoxy ketone **1** (bottom).

analogues can eliminate the remaining P4H activity in *dpy-18 C. elegans* at concentrations that do not affect the wild-type worm.<sup>5</sup> This synthetic lethality provided the basis for our assay.

*Dpy-18* worms were placed onto solid medium containing known concentrations of esterified epoxy ketone **1**. The ester group, which can be hydrolyzed by cellular esterases, served to mask the anionic carboxylate and thus enhance uptake.<sup>5,24</sup> Concentration-dependent embryonic lethality of *dpy-18 C. elegans* was observed with LD<sub>50</sub> = 0.11 mM (Fig. 3). These data are consistent with lethality being due to P4H inactivation.

### 3. Conclusions

The need for small-molecule probes and inhibitors of P4H is evident from the lack of structural information about this essential enzyme as well as the list of fibrotic diseases associated with collagen overproduction.<sup>2</sup> The newly discovered role of P4Hs in hypoxia<sup>25–27</sup> and cancer,<sup>28</sup> and the cardioprotection conferred by P4H inhibitors<sup>29–31</sup> increase the imperative.<sup>32</sup> Our identification of 4-oxo-5,6-epoxyhexanoate as an affinity label for P4H that is bioavailable upon esterification provides a means to address this need. On-going efforts in our laboratory are directed at identifying the alkylated enzymic residue, improving the potency of the epoxy ketone as an affinity label, and evaluating its selectivity towards other P4Hs.

### 4. Experimental procedures

#### 4.1. General

BakerDry™ solvents in cyclotainers™ (DMF, <20 ppm water; CH<sub>2</sub>Cl<sub>2</sub>, <30 ppm water; THF, <10 ppm water) were from Baker (Phillipsburg, NJ). Unless noted otherwise, all other chemicals were from Aldrich Chemical (Milwaukee, WI), Fisher Scientific (Pittsburgh, PA), or NovaBiochem (San Diego, CA), and were used without further purification.

Human collagen prolyl 4-hydroxylase (P4H) was produced in *E. coli* and purified as described previously.<sup>14–18</sup> Dansyl-Gly-Phe-Pro-Gly-OEt, a fluorescent P4H substrate, was synthesized as described previously.<sup>14–17</sup>

In the synthetic procedures, the term ‘concentrated under reduced pressure’ refers to the removal of solvents and other volatile materials using a rotary evaporator at water aspirator pressure (<20 torr) while maintaining the water-bath temperature below 50 °C.

#### 4.2. Instrumentation

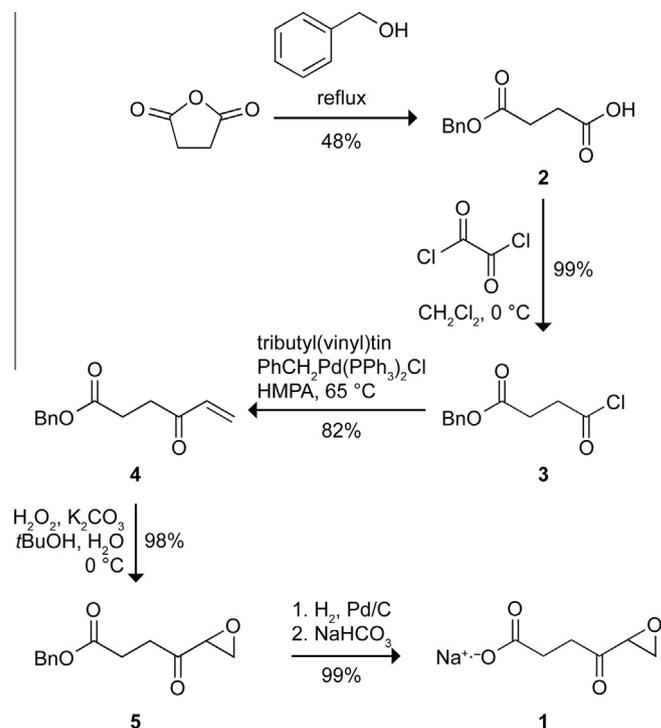
NMR spectra were obtained with a Bruker AC+ 300 MHz, Bruker DMX 400 WB, or Bruker Avance III 500i spectrometer. Mass spectra were obtained with a Micromass LCT® electrospray ionization, time-of-flight analyzer from Waters (Milford, MA). HPLC analyses were performed with an instrument from Waters controlled with the Millennium (version 3.20) software package and equipped with two 515 pumps, a 717 plus autosampler, and a 996 photodiode array detector.

#### 4.3. Synthesis of 4-oxo-5,6-epoxyhexanoate (1)

Epoxy ketone **1** was synthesized in five steps with an overall yield of 38% by the route shown in Scheme 1 and described below.

##### 4.3.1. Benzyl succinic acid (2)

Benzyl succinic acid was synthesized by following a known procedure.<sup>33</sup> Here, succinic anhydride (30 g, 300 mmol) was dissolved



Scheme 1. Route for the synthesis of epoxy ketone **1**.

in benzyl alcohol (31.6 mL, 33 g, 300 mmol), and the resulting solution was heated at reflux for 4 h. The reaction mixture was dissolved in ether (100 mL), and the insoluble succinic acid was removed by filtration. The filtrate was extracted with saturated aqueous Na<sub>2</sub>CO<sub>3</sub> (3 × 100 mL), and the combined aqueous extracts were acidified with 2 M HCl (2.0 L). The precipitate was collected by filtration and dried under vacuum to give **2** (30 g, 48%). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ 7.35 (m, 5H), 5.15 (s, 2H), 2.70 (m, 4H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz) δ 177.7, 172.1, 135.7, 128.8, 128.5, 128.4, 66.9, 29.1, 29.0.

##### 4.3.2. Benzyl 4-chlorooxobutanoate (3)

Benzyl 4-chlorooxobutanoate was synthesized by following a known procedure.<sup>34</sup> Here, benzyl succinic acid (4.14 g, 20 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (150 mL), and the resulting solution was cooled to 0 °C. Oxalyl chloride (13.8 g, 109 mmol) was added slowly, and the reaction mixture was stirred at 0 °C for 5 min. The reaction mixture was allowed to warm to 15 °C and stirred for an additional 30 min. The reaction mixture was then concentrated under reduced pressure, and the residue was dissolved in benzene. The residue was concentrated under reduced pressure to give **3** (4.5 g, 99%) as a clear viscous liquid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ 7.34 (m, 5H), 5.13 (s, 2H), 3.19 (t, *J* = 7 Hz, 2H), 2.69 (t, *J* = 7 Hz, 2H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz) δ 173.2, 170.9, 135.6, 128.8, 128.6, 128.4, 67.1, 29.5, 28.5.

##### 4.3.3. Benzyl 4-oxo-5-hexenoate (4)

Benzyl 4-oxo-5-hexenoate (**4**) was synthesized in 82% yield from benzyl 4-chlorooxobutanoate in the same manner as was ethyl 4-oxo-5-hexenoate (**6**) from ethyl 4-chlorooxobutanoate, *vide infra*. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ 7.35 (m, 5H), 6.38 (dd, *J* = 10 and 18 Hz, 1H), 6.26 (dd, *J* = 1 and 18 Hz, 1H), 5.86 (dd, *J* = 1 and 10 Hz, 1H), 5.13 (s, 2H), 2.94 (t, *J* = 7 Hz, 2H), 2.70 (t, *J* = 7 Hz, 2H).

##### 4.3.4. Benzyl 4-oxo-5,6-epoxyhexanoate (5)

Benzyl 4-oxo-5,6-epoxyhexanoate (**5**) was synthesized in 98% yield from benzyl 4-oxo-5-hexenoate in the same manner as was

ethyl 4-oxo-5,6-epoxyhexanoate (**6**) from ethyl 4-oxo-5-hexenoate (**7**), *vide infra*.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$  7.34 (m, 5H), 5.10 (s, 2H), 3.46 (dd,  $J=2$  and 5 Hz, 1H), 2.99 (dd,  $J=5$  and 6 Hz, 1H) 2.94 (dd,  $J=2$  and 6 Hz, 1H), 2.64 (m, 4H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75 MHz)  $\delta$  206.1, 172.4, 135.9, 128.7, 128.5, 128.4, 66.6, 53.7, 46.3, 31.3, 27.5.

#### 4.3.5. 4-Oxo-5,6-epoxyhexanoic acid (**1**, free acid)

Benzyl 4-oxo-5,6-epoxyhexanoate (340 mg, 1.45 mmol) was dissolved in EtOAc (20 mL). Pd/C (10% w/w, 40 mg) was added, and the reaction mixture was stirred under  $\text{H}_2(\text{g})$  for 4 h. The reaction mixture was filtered through Celite<sup>®</sup>, and concentrated under reduced pressure to give the free acid of epoxy ketone **1** (183 mg, 99%). ESI MS EMM  $m/z$ :  $[\text{M}-\text{H}]^-$  143.0340, calcd 143.0344.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$  10.74 (br s, 1H), 3.48 (dd,  $J=2$  and 5 Hz, 1H), 3.03 (dd,  $J=5$  and 5 Hz, 1H), 2.96 (dd,  $J=2$  and 5 Hz, 1H), 2.64 (m, 4H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75 MHz)  $\delta$  206.1, 178.3, 53.7, 46.4, 31.1, 27.2.

#### 4.3.6. Sodium 4-oxo-5,6-epoxyhexanoate (**1**, sodium salt)

4-Oxo-5,6-epoxyhexanoic acid (206 mg, 1.4 mmol) was dissolved in aqueous  $\text{NaHCO}_3$  (3.6 mL, 400 mM), and the resulting solution was stirred for 1 h. The reaction mixture was concentrated to dryness by lyophilization to give the water-soluble sodium salt of epoxy ketone **1** (237 mg, quant), which was used for assays *in vitro*.  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 500 MHz)  $\delta$  3.69 (dd,  $J=3$  and 5 Hz, 1H), 3.00 (t,  $J=5$  and 6 Hz, 1H), 2.80 (dd,  $J=3$  and 6 Hz, 1H), 2.70 (m, 2H), 2.34 (t,  $J=7$  Hz, 2H).  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ , 125 MHz)  $\delta$  211.7, 182.1, 54.2, 48.7, 36.1, 31.5.

### 4.4. Synthesis of ethyl 4-oxo-5,6-epoxy-hexanoate (**6**, esterified **1**)

Esterified **1** was synthesized in two steps with an overall yield of 70% by the route shown in Scheme 2 and described below.

#### 4.4.1. Ethyl 4-oxo-5-hexenoate (**7**)

Ethyl 4-oxo-5-hexenoate was synthesized by following a known procedure.<sup>35</sup> Here, ethyl 4-chlorooxobutylate (2.5 g, 15.2 mmol) was dissolved in HMPA (10 mL). Tributylvinyltin (5.0 g, 15.2 mmol) and benzylchlorotriphenylphosphine palladium(II) (52 mg) were added, and the reaction mixture was heated open to air to 65 °C for 5 min. At that time, the reaction mixture turned black. The reaction mixture was allowed to cool to room temperature.  $\text{H}_2\text{O}$  (50 mL) and NaCl (1.0 g) were added, and the resulting solution was stirred for 5 min. The solution was extracted with diethyl ether (3 × 60 mL). The combined organic extracts were washed with  $\text{H}_2\text{O}$  (4 × 60 mL), and the organic layer was

dried over  $\text{MgSO}_4(\text{s})$  and concentrated under reduced pressure to ~20 mL. A half-saturated solution of KF in MeOH (25 mL) was added to precipitate the  $\text{SnBu}_3\text{F}$ , which was removed by filtration through Celite<sup>®</sup>. The filtrate was concentrated under reduced pressure and purified by chromatography on silica gel, eluting with ethyl acetate (10% v/v) in  $\text{CH}_2\text{Cl}_2$  to give **7** (1.944 g, 82%). ESI MS  $m/z$ :  $[\text{M}+\text{Na}]^+$  179.0, calcd 179.1.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  6.35 (dd,  $J=10$  and 18 Hz, 1H), 6.23 (dd,  $J=1$  and 18 Hz, 1H), 5.84 (dd,  $J=1$  and 10 Hz, 1H), 4.10 (q,  $J=7$  Hz, 2H), 2.89 (t,  $J=7$  Hz, 2H), 2.59 (t,  $J=7$  Hz, 2H), 1.21 (t,  $J=7$ , 3H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)  $\delta$  198.5, 172.7, 136.2, 128.4, 60.6, 34.0, 27.9, 14.1.

#### 4.4.2. Ethyl 4-oxo-5,6-epoxy-hexanoate

Ethyl 4-oxo-5,6-epoxyhexanoate was synthesized by following a known procedure.<sup>13</sup> Here, ethyl 4-oxo-5-hexenoate (1.107 g, 7.09 mmol) and  $\text{H}_2\text{O}_2$  (30% v/v, 1.75 mL) were dissolved in *t*BuOH (11.81 mL) and  $\text{H}_2\text{O}$  (5.9 mL) at 0 °C. Saturated aqueous  $\text{K}_2\text{CO}_3$  (606  $\mu\text{L}$ ) at 0 °C was added slowly. The reaction mixture was stirred for 1 h at 0 °C, allowed to warm to room temperature, and then stirred for an additional 3 h. The reaction mixture was diluted with  $\text{H}_2\text{O}$  (100 mL) and extracted with diethyl ether (3 × 30 mL). The combined organic extracts were dried over  $\text{MgSO}_4(\text{s})$  and concentrated under reduced pressure. The product was purified by chromatography on silica gel, eluting with ethyl acetate (10% v/v) in  $\text{CH}_2\text{Cl}_2$  to give **6** (1.04 g, 85%). ESI MS  $m/z$ :  $[\text{M}+\text{Na}]^+$  195.0, calcd 195.1.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$  4.11 (q,  $J=7$  Hz, 2H), 3.48 (dd,  $J=3$  and 4 Hz, 1H), 3.03 (dd,  $J=4$  and 6 Hz, 1H), 3.01 (dd,  $J=3$  and 6 Hz, 1H), 2.66 (m, 4H), 1.25 (t,  $J=7$  Hz, 3H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75 MHz)  $\delta$  206.3, 172.6, 60.9, 53.7, 46.3, 31.3, 27.5, 14.3.

### 4.5. In vitro assay of P4H activity

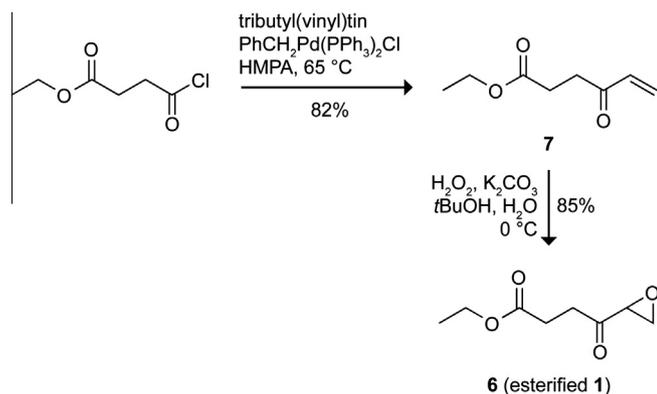
The enzymatic activity of P4H was assayed as described previously.<sup>14</sup> Briefly, substrate (dansyl-Gly-Phe-Pro-Gly-OEt) was added to 1 mL of 50 mM Tris-HCl buffer, pH 7.8, containing P4H (0.43  $\mu\text{M}$ ),  $\text{FeSO}_4$  (0.1 mM), BSA (1 mg/mL), catalase (0.1 mg/mL), ascorbate (2 mM), DTT (0.1 mM), and  $\alpha$ -ketoglutarate (0.5 mM). Aliquots (30  $\mu\text{L}$ ) were removed at 60, 180, and 300 s, quenched by boiling for 30 s, and injected onto a Microsorb-MV C18 reversed-phase HPLC column (4.6 × 250 mm, 100 Å pore size) from Varian (Palo Alto, CA). The column was eluted at 1 mL/min with aqueous acetonitrile (50% v/v), and the absorbance of the eluent was monitored at 337.5 nm. All assays were performed in triplicate.

### 4.6. In vitro assay for irreversible inhibition of P4H

P4H (43  $\mu\text{M}$ ) was incubated for 2.5, 5, 10, or 15 min with  $\text{FeSO}_4$  (2 mM) and epoxy ketone **1** in 50 mM Tris-HCl buffer, pH 7.8. BSA (1 mg/mL), catalase (0.1 mg/mL), ascorbate (2 mM), DTT (0.1 mM), and  $\alpha$ -ketoglutarate (0.5 mM) were added to this reaction mixture. Substrate (dansyl-Gly-Phe-Pro-Gly-OEt) was added to initiate the reaction, and assays for P4H activity were performed as described above. All assays were performed in triplicate. Data are reported as activity relative to control reaction mixtures that lacked epoxy ketone **1**. Half-times for inactivation at various inhibitor concentrations are determined from linear least-squares regression analysis of the log of the relative rates. Half-times for inactivation were plotted against the reciprocal of inhibitor concentration in a Kitz-Wilson plot<sup>19</sup> to determine the value of  $k_{\text{inact}}/K_i$ .

### 4.7. Protection of P4H by $\alpha$ -ketoglutarate

Epoxy ketone **1** (356 mM), P4H (43  $\mu\text{M}$ ), and  $\text{FeSO}_4$  (2 mM) were incubated for 5 min with  $\alpha$ -ketoglutarate (0, 15, 30, or 50 mM) in 50 mM Tris-HCl buffer, pH 7.8. BSA (1 mg/mL), catalase



Scheme 2. Route for the synthesis of **6** (esterified **1**).

(0.1 mg/mL), ascorbate (2 mM), DTT (0.1 mM), and substrate (dansyl-Gly-Phe-Pro-Gly-OEt) (2.5 mM) were added to initiate the reaction. Aliquots were removed at 15, 60, 120, 180, 240, and 300 s, quenched by boiling for 30 s, and injected onto the C18 reversed-phase HPLC column described above. The column was eluted at 1 mL/min with aqueous acetonitrile (50% v/v), and the absorbance of the eluent was monitored at 337.5 nm. Reaction rates were compared to the reactions without inhibitor.

#### 4.8. Immobilization of P4H

P4H (50 µg) was incubated for 30 min at 37 °C with FeSO<sub>4</sub> (0.1 mM), ascorbate (5 mM), and epoxy ketone **1** (13 mM) in 1 mL of PBS. Biocytin hydrazide was added (to 11 mM), and the reaction mixture was incubated for an additional 30 min at 37 °C. NaBH<sub>4</sub> (30 mg/mL in 10 mM NaOH) was added to the reaction mixture to reduce the acylhydrazide. The P4H complex was desalted using a NICK™ column from Amersham Pharmacia (Uppsala, Sweden) and incubated on Reacti-Bind™ streptavidin coated microtiter plates from Pierce Chemical (Rockford, IL) for 1 h at 37 °C. The wells were washed (4×) with blocking buffer (0.1% w/v BSA and 0.05% v/v Tween 20® in PBS). A mouse monoclonal antibody specific to the α-subunit of human P4H (ICN Biomedicals, Costa Mesa, CA) was added to the wells, and incubated for 1 h at 37 °C. The wells were washed again (4×) with blocking buffer. Anti-mouse IgG–peroxidase conjugate from Sigma Chemical (St. Louis, MO) was added to the wells, and the plate was incubated for 1 h at 37 °C. The wells were washed again (4×) with blocking buffer. O-Phenylenediamine solution (0.05 M citric acid/0.05 M sodium phosphate buffer, pH 5.0, containing 1 mg/mL O-phenylenediamine and 0.003% v/v H<sub>2</sub>O<sub>2</sub>) was added to the wells, and catalysis of O-phenylenediamine oxidation by peroxidase was monitored at 490 nm after quenching the reaction with H<sub>2</sub>SO<sub>4</sub> (2.5 M).

#### 4.9. In vivo assay of epoxy ketone **1**

The effect of epoxy ketone **1** on *dpy-18 C. elegans* was assayed by modification of a known procedure.<sup>5</sup> Here, **6** (esterified **1**) in DMSO was added to a plate containing solid growth medium (4 mL), which consisted of agar (17 g/L), Bacto-Peptone (2.5 g/L), NaCl (50 mM), cholesterol (5 mg/L), CaCl<sub>2</sub> (1 mM), and MgSO<sub>4</sub> (1 mM) in 1 mM potassium phosphate buffer, pH 6.<sup>36</sup> The inhibitor was allowed to diffuse throughout the agar for 24 h. Four L4 worms were then added to each plate. After sufficient time for the L4 worms to grow to adult stage and lay eggs (24–48 h), the adult worms were removed to simplify counting. After sufficient time for all live eggs to hatch into worms (24 h), live worms and dead embryos (typically, 40–100) were counted by using a light microscope.

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