Inactivation of 4-Oxalocrotonate Tautomerase by 2-Oxo-3-pentynoate†

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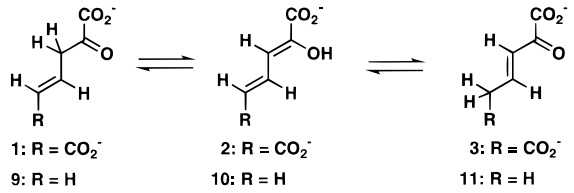
ABSTRACT: The compound, 2-oxo-3-pentynoate, has been synthesized and tested as an inhibitor of the enzyme 4-oxalocrotonate tautomerase. The enzyme is rapidly and irreversibly inactivated by the acetylenic product analogue in a time-dependent fashion. The enzyme displays saturation kinetics and is protected from inactivation by the presence of substrate. These observations are consistent with inactivation taking place at the active site. Partial reactivation (∼18%) occurs by incubating the inactivated enzyme with 10 mM hydroxylamine (pH 7.3). The partition ratio, determined to be ∼0.4, suggests that the inactivation of 4-OT by 2-oxo-3-pentynoate shows half-of-the-sites stoichiometry. The same phenomenon is observed in the inactivation of 4-OT by 3-bromopyruvate and can be explained by examination of the crystal structure. Mass spectral analysis shows that a single residue is modified on the enzyme which has been localized to the nine residue amino-terminal fragment Pro-1 to Glu-9. It can be reasonably concluded that Pro-1 is the site of covalent attachment. Inactivation of 4-OT can occur by either a Michael addition of 4-OT to C-4 of 2-oxo-3-pentynoate or by the enzyme-catalyzed rearrangement of 2-oxo-3-pentynoate to an allene derivative which alkylates Pro-1. These results provide the foundation for the use of 2-oxo-3-pentynoate in future mechanistic studies and as a ligand in an inactivated 4-OT complex that can be studied by X-ray crystallography. Finally, 2-oxo-3-pentynoate is an acetylene analogue of a variety of 2-oxo acids and as such may have general utility as an inhibitor of reactions that bind and process these compounds.

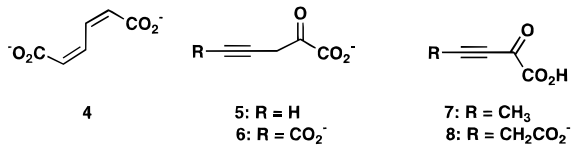
4-Oxalocrotonate tautomerase (4-OT)[1] from *Pseudomonas putida mt-2* catalyzes the isomerization of α-keto acids such as 2-oxo-4-*trans*-hexenedioate (**1**) to 2-oxo-3-*trans*-hexenedioate (**3**) through the intermediate 2-hydroxy-2,4-hexadienedioate (**2**) (Scheme 1) (*1*). The enzyme is expressed as part of a set of inducible enzymes that oxidatively catabolizes toluene, *m*- and *p*-xylene, 3-ethyltoluene, and 1,2,4-trimethylbenzene to intermediates in the Krebs cycle. The entire pathway is encoded by the TOL plasmid pWW0 and enables strains of soil bacteria carrying this plasmid to utilize these simple aromatic hydrocarbons as their sole sources of carbon and energy (*2*).

Stereochemical studies indicate that 4-OT catalyzes a suprafacial allylic rearrangement of **1** → **3** consistent with a one-base mechanism (*3*). On the basis of a crystal structure of an isozyme of 4-OT (73% identical) from *Pseudomonas sp.* CF600 (*4*, *5*), the amino-terminal proline was implicated as the catalytic base and two arginines (Arg-39 and Arg-11) were proposed to bind the two carboxylate groups of **1** (C-1 and C-6, respectively) (*6*). It was further postulated that Arg-39 functions as a general acid catalyst (*6*). Subsequently, the role of Pro-1 in the reaction was confirmed by mechanistic studies showing that 3-bromopyruvate (3-BP) acts as an active-site-directed irreversible inhibitor which modifies the amino-terminal proline with a stoichiometry of one site per monomer (*7*) and kinetic and NMR studies demonstrating that Pro-1 has the correct pK_a (∼6.4) to act as the general base under physiological conditions (*8*). In addition, it was inferred from an NMR study of the enzyme complexed with a competitive inhibitor, *cis*,*cis*-muconate (**4**, Scheme 2), that Arg-39 and Arg-11 interact, respectively, with the 1- and 6-carboxylate groups of **1** (*9*, *10*).

Scheme 1



Scheme 2



On the basis of these studies, a picture for the catalytic mechanism of 4-OT has emerged (*6*, *8*). However, the picture is far from complete and major issues remain unresolved. One issue concerns whether Arg-39 can bind the C-1 carboxylate group and act as the general acid catalyst. Another issue concerns whether substrate binding induces a conformational change that moves other residues into the

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[1] Abbreviations: 3-BP, 3-bromopyruvate; 2-OP, 2-oxo-3-pentynoate; 4-OT, 4-oxalocrotonate tautomerase; BCA, bicinchoninic acid; Bu^t, *tert*-butyl; $CDCl_3$, deuteriated chloroform; DMSO, dimethyl sulfoxide; ESI-MS, electrospray ionization mass spectrometry. HPLC, high pressure liquid chromatography; IR, infrared; NMR, nuclear magnetic resonance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; THF, tetrahydrofuran; TFA, trifluoroacetic acid; TLC, thin layer chromatography; UV, ultraviolet.

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active site. The crystal structure of 4-OT from *Pseudomonas sp.* CF600 does not address these questions because it was solved in the absence of a bound ligand (*6*). Attempts to obtain a crystal structure in the presence of either 3-BP, substrate, or a competitive inhibitor have thus far not been successful (*6*).

One approach that provides insight into how residues at the active site interact with the substrate is to obtain a crystal structure of the enzyme complexed with a mechanism-based inhibitor. In order to achieve this goal, the synthesis of a series of β,γ- and α,β-unsaturated acetylenic ketones (**5**−**8**, Scheme 2) is being pursued. The design of the β,γ-unsaturated acetylenic ketones (2-oxo-4-pentynoate, **5**, and 2-oxo-4-hexyn-1,6-dioate, **6**) is based on two observations. First, several isomerases are inactivated by acetylene-containing substrate analogues because they undergo an enzyme-catalyzed rearrangement to a very reactive allene species which alkylates the enzyme (*11*, *12*). Second, 4-OT catalyzes the isomerization of monocarboxylated compounds such as 2-oxo-4-pentenoate (**9**, Scheme 1), converting it to 2-oxo-3-pentenoate (**11**) through the intermediate 2-hydroxy-2,4-pentadienoate (**10**) (*3*). Hence, monocarboxylated acetylenic compounds such as **5** (Scheme 2) may also be inhibitors of 4-OT. The design of the α,β-unsaturated acetylenic ketones (2-oxo-3-pentynoate, **7**, and 2-oxo-3-hexyn-1,6-dioate, **8**, Scheme 2) is based on the additional observation that the 4-OT reaction is reversible (*1*) so that acetylene-containing product analogues can undergo γ-deprotonation (at C-5) to generate the inactivating allene species.

The synthesis and evaluation of the monocarboxylated α,β-unsaturated acetylenic ketone, 2-oxo-3-pentynoate (**7**), as an inhibitor of 4-OT is described in this article. The data show that **7** is a potent active-site-directed irreversible inhibitor of 4-OT and suggest that Pro-1 is the sole target of modification by this compound. Inactivation of 4-OT can result by either a Michael reaction between **7** and Pro-1 or by the enzyme-catalyzed rearrangement of **7** to the allene derivative. The potency of **7** makes it a promising candidate for further use in mechanistic studies and as a ligand in an inactivated 4-OT complex that can be studied by X-ray crystallography. In addition, the compound, as an acetylene analogue of pyruvate, may have general utility as an inhibitor of a variety of enzyme-catalyzed reactions that bind and process pyruvate or other 2-oxo acids.

MATERIALS AND METHODS

Materials. All chemicals and solvents (including the anhydrous solvents) were purchased from Aldrich Chemical Co. with the following exceptions. The synthesis of **2** has been described (*1*). Biochemicals and buffers were obtained from Sigma Chemical Co. Centricon (10 000 MW cutoff) centrifugal microconcentrators and ultrafiltration membranes were purchased from Amicon. 4-Oxalocrotonate tautomerase was purified to homogeneity as assessed by SDS−PAGE according to published procedures (*1*, *7*).

General Methods. For the purification of 4-OT, HPLC was performed on a Waters system using either a Bio-Gel Phenyl 5-PW hydrophobic column or a Pharmacia Superose 12 (HR 10/30) gel filtration column. The remaining HPLC work was performed on either the Waters system or a Rainin dual-pump high-pressure mixing system with 214-nm UV detection as noted below. Protein concentrations were determined using either the commercially available bicinchoninic (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL) or the method of Waddell (*13*). Kinetic data were obtained on a Hewlett Packard 8452A Diode Array spectrophotometer. Enzyme activity was monitored by following the formation of **3** at 236 nm (*1*). The kinetic data were fitted by nonlinear regression data analysis using the Grafit program (Erithacus Software Ltd., Staines, U.K.) obtained from Sigma Chemical Co. IR spectra were recorded on a Nicolet Magna-IR 550 spectrometer. The mass spectral data for **7** were provided by the mass spectrometry facility at the University of Texas (Department of Chemistry). Mass spectra of the modified enzyme and peptides were obtained on a Sciex API-III quadrupole electrospray mass spectrometer. NMR spectra were obtained on a Bruker AM-250 spectrometer unless noted otherwise. Chemical shifts were referenced as noted below.

Chemical Syntheses

Caution. The toxicity of **7** has not been studied. It is an acetylene analogue of pyruvate and could potentially interfere with several enzymes that metabolize pyruvate or pyruvyl-like compounds. The compound should be inactivated by treatment with 1 M NaOH before disposal.

*Preparation of tert-Butyl Glyoxylate (**12**).* The synthesis of **12** was carried out under argon as described elsewhere (*14*) with the following modifications. After all of the fumaryl chloride had been added, the mixture was allowed to stir overnight at ambient temperature. The ether layer was filtered through Celite, concentrated, and subjected to flash chromatography (90% hexanes, 10% ethyl acetate) to generate di-*tert*-butyl fumarate as a solid. The fumarate ester was processed to **12** as described (14). A ^{1}H NMR spectrum corresponded to the previously reported spectrum (*14*).

*Preparation of tert-Butyl 2-Hydroxy-3-pentynoate (**13**).* To a stirring solution of **12** (5.2 g, 40 mmol) in 100 mL of anhydrous THF at −78 °C was added 1-propynylmagnesium bromide (80 mL, 0.5 M in THF) dropwise over a 30 min period. The solution was stirred under argon and allowed to warm to ambient temperature overnight. Subsequently, it was concentrated under reduced pressure to remove most of the THF. The temperature was maintained below 25 °C during this process. An aqueous solution of 10% NaH_2PO_4 (200 mL) was added to the residual THF, and the resulting solution was extracted with a mixture of hexanes and ether (3:1, 3 × 200 mL). The organic layers were pooled, dried over anhydrous $MgSO_4$, filtered through a small plug of silica, and concentrated to dryness to yield a viscous liquid (5.3 g). The crude product was purified further by flash chromatography (8:1 hexanes, ethyl acetate) to give **13** as a yellow liquid (3.0 g, 45% yield). Although a ^{1}H NMR spectrum revealed the presence of minor impurities, the liquid was not purified further. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 1.47 (9 H, s), 1.81 (3 H, s, C-5), 3.82 (1 H, s, C-2). ^{13}C NMR ($CDCl_3$, 250 MHz): δ 3.6 (C-5), 27.9 (CH_3 of Bu^t), 72.8 (C-3), 74.8 (C-2), 82.5 (C-4), 84.2 (C of Bu^t), 169.1 (C-1).

*Preparation of tert-Butyl 2-Oxo-3-pentynoate (**14**).* To a solution of **13** (3.0 g, 17.6 mmol) in 100 mL of anhydrous methylene chloride was added lead tetraacetate (7.8 g, 17.6 mmol) in small portions (*15*). The resulting solution was allowed to sit at ambient temperature under argon. After 3 h, the starting material was no longer present as determined by TLC (50% hexanes, 50% methylene chloride). Hexanes

(100 mL) was added, and the solution was filtered to remove the solids. The solution was concentrated to near dryness, and the residue subjected to flash chromatography (50% hexanes, 50% methylene chloride). Fractions were collected until the UV-active fractions were isolated. Fractions were pooled and concentrated to dryness to afford a pale-yellow liquid (1.3 g, 44% yield). The liquid formed crystals in the freezer at −20 °C, but they melt upon warming to ambient temperature. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 1.51 (9 H, s), 2.10 (3 H, s, C-5). ^{13}C NMR ($CDCl_3$, 250 MHz): δ 4.48 (C-5), 27.5 (CH_3 of Bu^t), 78.8 (C-4), 84.6 (C of Bu^t), 97.3 (C-3), 158.4 (C-1), 170.7 (C-2).

Preparation of 2-Oxo-3-pentynoic Acid (7). To a solution of **14** dissolved in 100 mL of anhydrous methylene chloride was added anhydrous trifluoroacetic acid (5 mL). After stirring at ambient temperature under argon overnight, the solution was evaporated to dryness *in vacuo*, and hexanes were added to the residue. Removal of the hexanes by evaporation *in vacuo* resulted in a pale-yellow solid. The solid was dissolved in anhydrous methylene chloride and transferred to a preweighed flask, and the solvent was removed. The residual solvent was removed by mechanical vacuum (∼1 mm) for 1 h to leave 0.59 g (69% yield). ^{1}H NMR ($CDCl_3$, 250 MHz): δ 2.15 (3 H, s, C-5), 10.0 (1 H, s). ^{13}C NMR ($CDCl_3$, 250 MHz): δ 4.93 (C-5), 77.9 (C-4), 102.5 (C-3), 159.4 (C-1), 169.8 (C-2). UV (20 mM NaH_2PO_4, pH 6.75): λ_{max} 234 nm (ϵ = 6490 M^{-1} cm^{-1}). IR υ_{max} (neat): 2223 (acetylene), 1741 (carboxylic acid carbonyl group), 1680 (carbonyl group) cm^{-1}. HRMS *m/z* calculated for $C_5H_5O_3$ (MH^+) 113.0239, found 113.0238.

Nonenzymatic Decomposition of 7. The nonenzymatic decomposition of **7** was followed by UV and ^{1}H NMR spectroscopy at different pH values. In the UV spectroscopic experiments, a solution **7** (25 μM) in 1 mL of 20 mM NaH_2PO_4 buffer (pH 6.75) was incubated with 10 μL of a 5 M NaOH (50 mM) solution. The final pH of the solution was 12.4. The λ_{max} corresponding to **7** (234 nm) decreased as two new peaks slowly appeared: a narrow peak with a λ_{max} = 216 nm and a broader peak with a λ_{max} = 300 nm. Similar observations were made in solutions at pH 10 and pH 8.5, although the rates (for the disappearance of **7** and the appearance of the two new products) were slower. In the ^{1}H NMR spectroscopic experiments, a solution of **7** (4 mg, 36 μmol) in DMSO-d_6 (30 μL) was added to 100 mM Na_2HPO_4 buffer (0.6 mL, pH 9.1) and placed in a NMR tube. The reaction was initiated by the addition of 25 μL of a 5 M NaOH (125 mM) solution. The final pH of the solution was 12.5, and the final concentration of **7** was 55 mM. The species responsible for the λ_{max} at 216 nm was identified as 2-butynoate by comparison of its ^{1}H NMR spectrum to that of the authentic compound [δ 1.62 (3 H, s, C-4)]. It was not possible to identify the species responsible for the λ_{max} at 300 nm by NMR spectroscopy. A ^{1}H NMR spectrum showed several resonances between 1.0 and 2.6 ppm. Spectra are recorded in 100% H_2O using selective presaturation of the water signal with a 2-s presaturation interval. The lock signal is DMSO-d_6. Spectra were acquired on a Varian Unity INOVA-500 spectrometer.

Enzymological Methods

Kinetics of Irreversible Inhibition. The inhibition of 4-OT with **7** was carried out as previously described (*7*) with the following modifications. Incubation mixtures (total volume = 1.0 mL) containing varying amounts of inhibitor (1−100 μM) and enzyme (15 μM) in 20 mM sodium phosphate buffer (pH 6.75) at 23 °C were made up in 1.5 mL eppendorf micro test tubes. Aliquots (5 μL) were removed at various time intervals, diluted into 1 mL of 20 mM sodium phosphate buffer (pH 7.3) and assayed for residual activity. The assay was initiated by the addition of **2** to give a final concentration of 300 μM. Stock solutions of **7** (1.12 mg/1 mL) were made up fresh daily in 20 mM sodium phosphate buffer (pH 6.75). Stock solutions of **2** were made fresh daily in ethanol.

The observed rate constant for inactivation (k_{obsd}) was determined from a k_{obsd} least-squares fit of the data obtained from the initial linear portion of the decrease in activity to the equation for a first-order decay. At all concentrations of **7** used, the decrease in activity was linear in enzymatic activity for at least three half-lives. The rate constants (K_I and k_{inact}) were determined by fitting the data as previously described (*7*).

Protection from Inhibition of 4-OT by 7. The protection against inactivation of 4-OT by an equilibrium mixture of **1**−**3** was carried out as described elsewhere (*7*) with the following modifications. 4-OT (15 μM) was incubated with varying concentrations of **2** (0−4.5 mM) in 20 mM sodium phosphate buffer (pH 6.8) at 23 °C. After a 5 min interval in which **2** is converted into an equilibrium mixture of **1**−**3** (*1*), a fixed concentration of **7** (20 μM) was added to the mixture, and aliquots (5 μL) were removed at various time intervals over a 3 min period and assayed for residual activity (see above). The data were plotted and analyzed as described above and elsewhere (*7*).

Irreversibility of the Inactivation. The irreversibility was established as described elsewhere (*7*) with the following modifications. 4-OT (0.12 μmol) was incubated with an excess of **7** (0.4 μmol) in 8 mL of 20 mM sodium phosphate buffer (pH 6.75) for 20 min at 4 °C. In a separate control, the same quantity of enzyme was incubated without **7** under identical conditions. Both samples were dialyzed against 20 mM sodium phosphate buffer (pH 6.75) for 5 days. Aliquots (5 μL) were removed and assayed in sodium phosphate buffer (1 mL) using 300 μM of **2**. After 5 days, the control sample of enzyme lost 54% of its original activity. The inactivated enzyme regained ∼8% activity when compared to the activity of the control sample after 5 days.

Hydroxylamine as a Nucleophilic Scavenger. 4-OT (0.1 mg, 15 μM) was incubated with varying concentrations of NH_2OH (0−50 mM) in 1 mL of 20 mM sodium phosphate buffer (pH 6.75) for 15 s at 23 °C. The NH_2OH was made up as a 1 M solution in 20 mM sodium phosphate buffer, and the pH adjusted to 6.75. Subsequently, the reaction mixture was made 40 μM in **7** and allowed to incubate for 60 s. An aliquot was removed (5 μL) and assayed for activity as described above. At all concentrations of NH_2OH, there was no detectable enzymatic activity. In a control experiment, 4-OT (0.1 mg, 15 μM) was incubated with 50 mM NH_2OH in 1 mL of 20 mM sodium phosphate buffer (pH 6.8) for 60 s at 23 °C. Under these conditions, the enzyme retains ∼84% of its original activity. In a second control experiment, **7** (40 μM) was incubated with 50 mM NH_2OH in 1 mL of 20 mM sodium phosphate buffer (pH 6.8) for 15 s at room temperature. Subsequently, 4-OT (0.1 mg, 15 μM) was added and the reaction mixture was allowed to incubate for 60 s. An aliquot was removed (5 μL) and

assayed for activity as described above. There was no detectable activity.

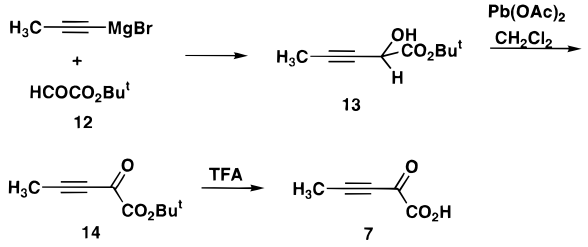
Partial Reactivation of 7-Inactivated 4-OT Using Hydroxylamine. 4-OT (0.015 μmol) was incubated with **7** (0.025 μmol) in 1 mL of 20 mM sodium phosphate buffer (pH 6.75) for 10 min at room temperature. The final pH of the solution was 6.6. An aliquot (50 μL) of the inactivated enzyme was removed and added to different samples of 10 mL of 20 mM sodium phosphate buffer containing 0, 1, 5, and 10 mM NH_2OH. The final pH of these samples was 7.3. At various time intervals, aliquots (1 mL) were removed and assayed for activity using 300 μM of **2**. After 48 h, the enzyme regained 5% (1 mM NH_2OH), 14% (5 mM NH_2OH), and 18% (10 mM NH_2OH) of its original activity.

Determination of the Partition Ratio for 7. 4-OT (15 μM in monomer) was incubated with varying amounts of **7** (0−147 μM) at 23 °C for 2 min in 1 mL of 20 mM sodium phosphate buffer (pH 6.8). Aliquots (5 μL) were removed and assayed for residual activity as described above. The partition ratio was determined by plotting the percentage of residual activity versus the ratio of inhibitor to enzyme concentration. The percentage of residual activity was determined by dividing the residual activity of the modified sample by the activity of the unmodified sample and multiplying by 100% (*16*, *17*).

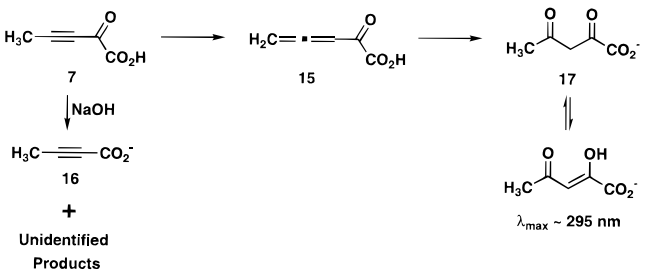
HPLC Analysis of the Inactivated 4-OT. A quantity of **7** (2.3 mg in 0.25 mL 100 mM Na_2HPO_4, pH 9.2, 20 μmol) was added to a small volume of 4-OT (1.1 mg, 0.16 μmol), and the reaction mixture was diluted to a final volume of 0.5 mL with water. The addition of **7** to the Na_2HPO_4 buffer adjusted the pH to 6.5. The final pH of the incubation mixture was ~6.75. The incubation mixture was allowed to stand at 23 °C for 1 h. There was no residual enzyme activity. In a separate control, the same quantity of enzyme was incubated without **7** under identical conditions. The samples were then reduced with a solution of $NaBH_4$ (22.8 mg/200 μL H_2O) and allowed to stand at room temperature overnight. The final pH of the two solutions was ~10.6. The modified 4-OT and unmodified 4-OT were then purified on a Waters system using an Econosil C_{18} reverse-phase HPLC column (250 mm × 22 mm, 10 μ) in 250 μL portions washing first with H_2O/0.05% trifluoroacetic acid for 5 min and eluting with a linear gradient (0 to 100% acetonitrile/0.05% TFA) over the next 45 min at a flow rate of 5 mL/min. The effluent was monitored at 214 nm, and fractions were collected at 1 min intervals. The modified and unmodified 4-OT monomers eluted as one broad peak centered at ~40 min after the time of injection. The fractions were concentrated to dryness *in vacuo* and analyzed by ESI mass spectrometry.

Peptide Mapping. A quantity of 4-OT (2.2 mg, 0.3 μmol) was incubated with **7** (3.4 mg in 0.4 mL 100 mM Na_2HPO_4, pH 9.2, 30 μmol) for 12 h, reduced with $NaBH_4$ (25.6 mg/200 μL H_2O), and purified by reverse-phase HPLC as described above. Another sample of 4-OT was incubated without **7** and subjected to the same treatment to be used as a control sample. The samples were concentrated to dryness *in vacuo*, dissolved in 900 μL of 40 mM ammonium acetate (pH 4), combined with a solution containing 50 μg of protease V-8 from *Staphylococcus aureus* in 100 μL of the same buffer, and incubated for 18 h at 37 °C (*18*). Subsequently, the peptide fragments in the two incubation mixtures were separated on the Rainin system using a Vydac C-18 analytical column (5 μm, 0.46 × 15 cm) by a linear gradient of 0 to 60% acetonitrile containing 0.1% TFA. The major peaks were collected and analyzed by ESI mass spectrometry.

Scheme 3



Scheme 4



Mass Spectrometry. The mass of the purified, modified, and reduced 4-OT-monomer was determined using a Sciex API-III quadrupole electrospray mass spectrometer. Samples were analyzed in a solution of 30% acetonitrile in water and 0.1% TFA at concentrations of approximately 5 μM.

RESULTS

Synthesis of 2-Oxo-3-pentynoic Acid. The chemical synthesis of **7** is outlined in Scheme 3. Condensation of the commercially available 1-propynylmagnesium bromide with the freshly distilled *tert*-butyl ester of glyoxylate (**12**), synthesized by a modification of a literature procedure (*14*), affords **13** as the major product. A ^{1}H NMR spectrum reveals the presence of another *tert*-butyl species which is removed in the subsequent oxidation step. The *tert*-butyl ester was chosen because it can be removed readily under anhydrous conditions. Our experience with related compounds shows that the presence of any water under either acidic or basic conditions results in rapid decomposition of the acetylene. Oxidation of **13** by lead tetraacetate generates **14** (*15*), which is easily purified by flash chromatography. Two other reagents (manganese dioxide and pyridinium dichromate) were tried but not used because the rate of oxidation was too slow. The *tert*-butyl group is removed by anhydrous trifluoroacetic acid to result in the formation of **7** as the free acid.

Nonenzymatic Decomposition of 7. One possible mechanism for the inactivation of 4-OT by **7** involves the enzyme-catalyzed rearrangement of the acetylene to the allene which is the actual inactivating species (**7** → **15**, Scheme 4). Attempts to isolate or to observe the allene by the base-catalyzed decomposition of **7** were not successful. The major products resulting from the nonenzymatic decomposition of **7** are 2-butynoate (**16**, Scheme 4) and a compound with a λ_{max} at 300 nm which has not been identified. The mechanism resulting in the transformation of **7** to **16** is not known, but Chiu and Jordan report a similar reaction (*19*).

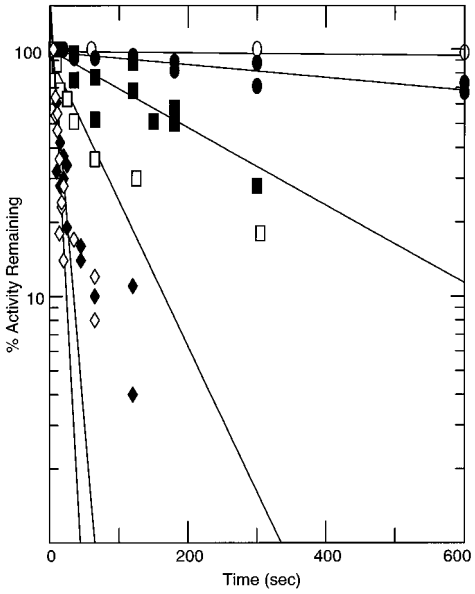


FIGURE 1: Time-dependent irreversible inactivation of 4-OT by **7**. A logarithmic plot showing the percent of 4-OT activity remaining as a function of the incubation time with varying amounts of **7** (open circles, 1.0 μM; filled circles, 2.5 μM; filled rectangles, 5.0 μM; open rectangles, 7.5 μM; filled diamonds, 10 μM; open diamonds, 15 μM).

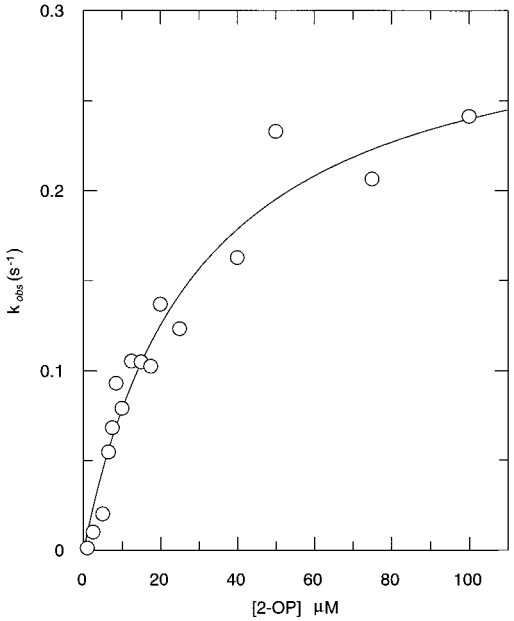


FIGURE 2: Determination of the k_{inact} and K_I values for **7**. A plot of the k_{obsd} values for inactivation measured in 16 experiments as a function of varying amounts of **7** (1−100 μM). The values of k_{inact} and K_I values obtained from this plot are 0.31 ± 0.03 s^{-1} and 29.5 ± 5.4 μM, respectively.

Alkaline treatment of ethyl 2-oxo-4-phenyl-3-butynoate generates phenylpropynoate in addition to other products. The allene, if generated, is not likely to be stable in aqueous solution as it will be quickly converted to acetopyruvate (**17**) by the addition of water to C-4 of **15**. The presence of **17** is readily detected because it has a λ_{max} at 295 nm at alkaline pH due to the presence of the enol form (20).[2] There is no evidence indicating that any significant amount of **17** is generated in these reactions mixtures. Moreover, the unidentified compound with a λ_{max} at 300 nm generated by base-catalyzed decomposition of **7** is not **17**. Significant quantities of either **16** or **17** are not observed in the time course used for the inactivation studies of 4-OT by **7**.

Kinetics of Irreversible Inhibition. Incubation of 4-OT with **7** in phosphate buffer results in the rapid time-dependent, irreversible inhibition of the enzyme. The initial decrease in activity is linear for approximately three half-lives (Figure 1). Subsequently, the decrease occurs more slowly. The k_{obsd} values measured in 16 experiments were plotted versus the inhibitor concentration and fit to a rectangular hyperbola (Figure 2). The values of k_{inact} and K_I values obtained from this plot are 0.31 ± 0.03 s^{-1} and 29.5 ± 5.4 μM, respectively. The hyperbolic inactivation is consistent with the formation of a dissociable complex between enzyme and **7** before covalent attachment and inactivation. Binding at the active site is further indicated by the observation that an equilibrium mixture of **1**−**3** protects the enzyme against inactivation (Figure 3). Exhaustive dialysis does not result in significant reactivation of the enzyme, suggesting that a covalent bond has formed between 4-OT and **7**.

Hydroxylamine as a Nucleophilic Scavenger. In order to determine whether the inactivation of 4-OT results from the release of a reactive species, 4-OT and **7** were incubated together in the presence of increasing concentrations of hydroxylamine. This has no observable effect on the rate of inactivation. This suggests that the inactivation of 4-OT does not result from covalent modification by a reactive species released from the active site into solution. In separate control experiments, it was found that NH_2OH does not significantly inhibit 4-OT nor react with **7** to result in its nonenzymatic decomposition.

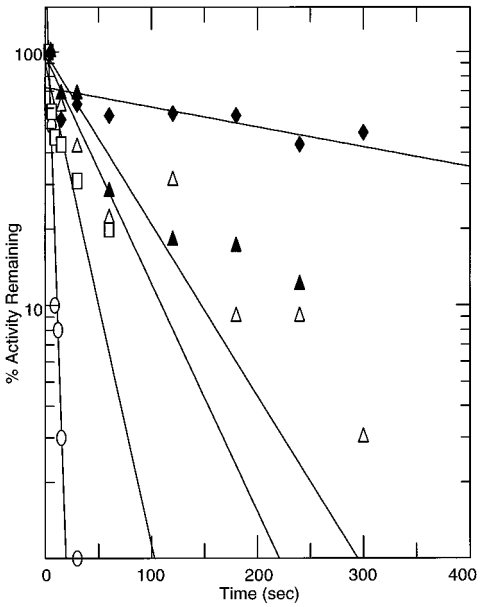


FIGURE 3: Protection against inactivation by **7** using an equilibrium mixture of **1**−**3**. 4-OT was incubated with varying amounts of **2** (open circles, 0.0 mM; open rectangles, 0.09 mM; open triangles, 1.0 mM; filled triangles, 3.0 mM; filled diamonds, 4.5 mM) for 5 min before the addition of **7** (20 μM).

Reactivation of 7-Inactivated 4-OT Using NH_2OH. A modest gain in activity is obtained by incubating the inactivated enzyme with increasing amounts of hydroxy-

[2] Another compound, 2-keto-4-hydroxy-2,4-pentadienoate γ-lactone, can form by the intramolecular addition of the carboxylate anion to C-4 of the allene. In aqueous base, however, the lactone is readily hydrolyzed to **17** (*20*).

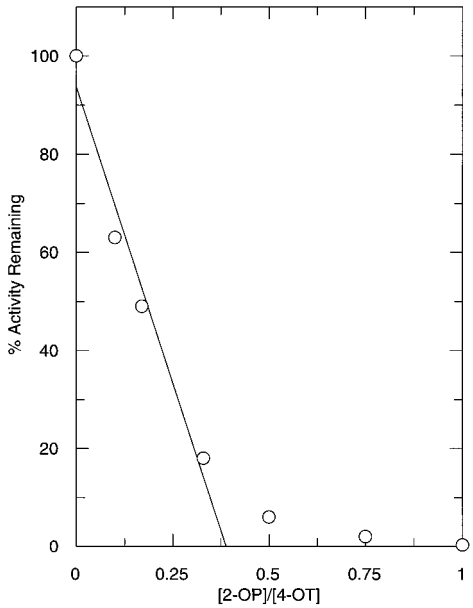


FIGURE 4: Determination of the partition ratio for **7**. A plot showing the percent of residual 4-OT activity as a function of the ratio of the moles of **7** to the moles of 4-OT. The value of the partition ratio determined from this plot is ∼0.4.

lamine. The partial reactivation of the enzyme by hydroxylamine is consistent with the transamination of an eneamine linkage between the enzyme and adduct (*21*, *22*). Transamination results in the transfer of the adduct to the hydroxylamine and the concomitant generation of unmodified enzyme. An eneamine can form between the allene (the putative species generated from **7**) and either the primary ϵ-amino group of a lysine (Lys-16, Lys-47, or Lys-59), one of the imidazole nitrogens of a histidine (His-6 or His-49), or the secondary amino group of the amino-terminal proline (Pro-1).

Partition Ratio Determination. The partition ratio is a measure of the number of molecules of inhibitor processed by the enzyme to number of molecules that result in the inactivation of the enzyme. A plot of the percent of activity remaining vs the ratio of moles of **7** to the moles of 4-OT (Figure 4) yields a partition ratio of ∼0.4. The small partition ratio has two implications. First, if an allene species is generated from the enzyme-catalyzed isomerization of **7**, it has a marked preference for alkylation of the enzyme as opposed to dissociation from the active site. The allene does not accumulate in solution to result in nonspecific covalent modification or decomposition products (i.e., acetopyruvate). Second, the value suggests that the inactivation of 4-OT by **7** shows half-of-the-sites stoichiometry in that three molecules of **7** effectively inactivate all six active sites of 4-OT. The same phenomena was observed in the inactivation of 4-OT by the affinity label, 3-bromopyruvate (*7*).

Isolation of the 7-modified 4-OT and Mass Spectrometry. In order to determine further whether a species derived from **7** was covalently attached to 4-OT, the enzyme was incubated with **7** for 1 h and reduced with $NaBH_4$, and the modified 4-OT was purified by reverse-phase HPLC. The modified sample, a sample of 4-OT treated identically although not incubated with **7**, and an untreated sample of 4-OT were analyzed by electrospray ionization mass spectrometry (ESI-MS) (Figure 5). ESI-MS analysis of the untreated 4-OT used in these experiments is shown in Figure 5A. The reconstruct of the electrospray mass spectrum shows that the enzyme is highly pure and that the observed molecular mass of the monomer (6810.0 Da) is in good agreement with the expected molecular mass, 6810.7 Da (average isotopic composition) (*23*). The reconstruct of the electrospray mass spectrum of the sample of 4-OT incubated for 1 h without inhibitor, reduced with $NaBH_4$, and purified by reverse-phase HPLC reveals that it consists of two components: the unmodified 4-OT (6809.3 Da) and the unmodified 4-OT without the three C-terminal residues (6397.7 Da) (Figure 5B). It appears that the large quantity of $NaBH_4$ combined with the overnight incubation at alkaline pH resulted in cleavage at the carboxyl side of Lys-59 to release the tripeptide, Val-Arg-Arg. The reconstruct of the electrospray mass spectrum of the modified sample of 4-OT reveals that it consists of four major components (Figure 5C). One set of signals corresponds to the expected molecular mass of modified 4-OT (6925.4 Da) and unmodified 4-OT (6809.4 Da). The difference between these masses is 116 mass units. Hence, the mass of the bound species is 117 mass units.[3] A second set of signals, also separated by 116 mass units, corresponds to the molecular mass of the modified 4-OT without the three C-terminal residues (6513.1 Da) and the mass of the unmodified 4-OT without the three C-terminal residues (6397.9 Da). In both sets, the signal for the unmodified enzyme is slightly more intense than that for the modified enzyme.

A comparison of the signal intensities suggests that a little less than half the enzyme is modified by **7**.[4] This observation is consistent with the partition ratio of 0.4 suggesting half-of-the-sites stoichiometry. If the enzyme is incubated with **7** for longer periods of time (12 h), there is no significant additional incorporation of adduct.

Identification of the Modified Peptide by Mass Spectrometry. The result above shows that incubation of 4-OT with **7** leads to the covalent attachment of a species with a mass of 117 to the enzyme. In order to identify the site of modification, 4-OT was inactivated, reduced, and purified as described above. The modified 4-OT was digested with endoproteinase glu-C (protease V-8), and the resulting peptide mixture was separated by reverse-phase HPLC. Subsequently, the fragments were analyzed by mass spectrometry. At pH 4.0, in 40 mM ammonium acetate buffer, protease V-8 cleaves at the carboxyl side of glutamate (*18*). There are seven glutamate residues in 4-OT so that complete digestion by protease V-8 should result in eight fragments.

Chromatographic separation of the mixture and mass spectral analysis of the fragments reveals incomplete digestion of the modified protein with proteolytic cleavage occurring predominately at Glu-9, Glu-22, and Glu-44. The HPLC chromatogram shows three well-defined peaks with retention times of approximately 14.5, 19.5, and 24.5 min as well as a region of poorly defined peaks from 15−17 min (Table 1). Analysis of these peaks by ESI-MS shows a single modification by a residue having a mass of 117 on three

[3] In the modified 4-OT, the adduct replaces the proton on the nitrogen of the amino-terminal proline. Hence, the mass of the adduct is the difference between the mass of the modified 4-OT and the mass of 4-OT without a proton on the nitrogen of the amino-terminal proline.

[4] The relative ionization efficiency of different analytes can affect their quantitation by ESI mass spectrometry. However, for two nearly identical compounds, the intensities of the signals in an ESI mass spectrum generally provide a good indication of the relative amounts.

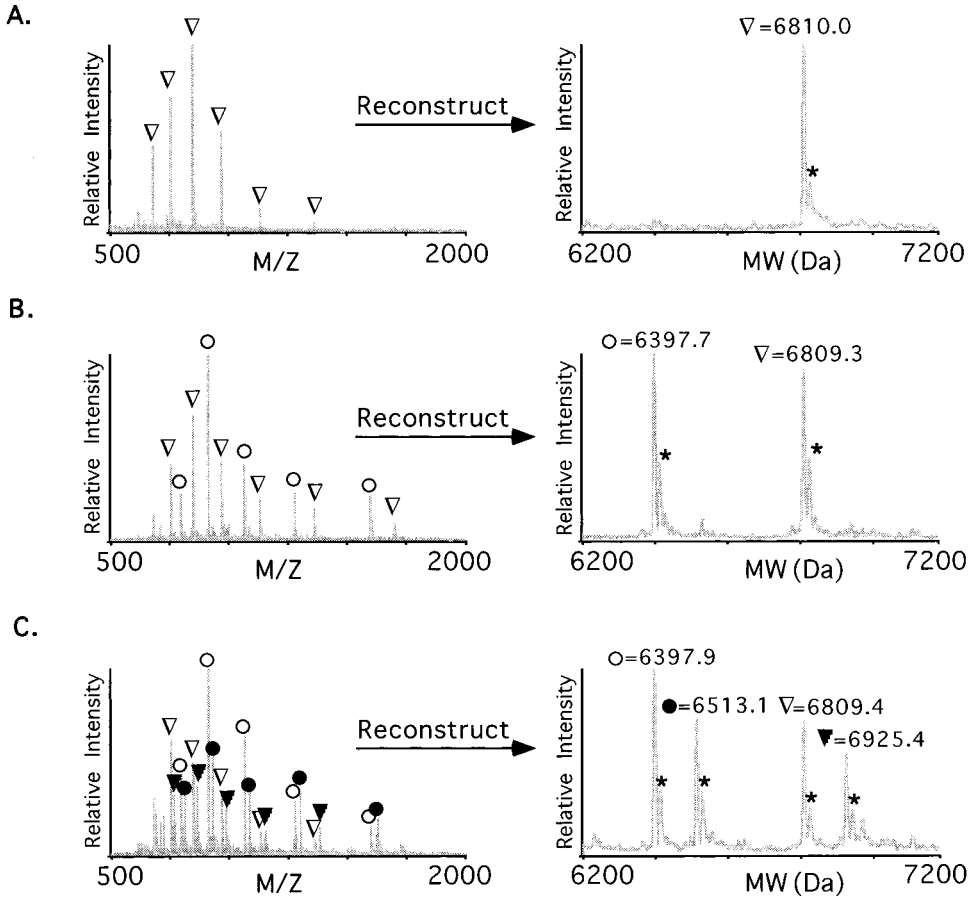


FIGURE 5: Electrospray mass spectra of (A) purified 4-OT, (B) 4-OT incubated without **7**, reduced with $NaBH_4$, and purified by reverse-phase HPLC, and (C) 4-OT incubated with **7** for 1 h, reduced with $NaBH_4$, and purified by reverse-phase HPLC. The mass spectra (left column) show multiple charge states arising from the distribution of protonated residues. The reconstruction of the spectrum (right column) displays a single charge state. The signals designated by the asterisk correspond to the presence of oxidized monomer resulting form the conversion of Met-45 to methionine sulfoxide (*23*).

Table 1: Identification of the Modified Peptides Isolated after Protease V8 Digest of C5H9O3-4-OT by Electrospray Ionization Mass Spectrometry

retention time (min)	calculated mass[a]	observed mass	fragment
14.5	1502.7	1501.8	met-45 to Lys-59
15−17	2575.9	2575.5[b]	Pro-1 to Glu-22
	2691.9	2690.4	$C_5H_9O_3$-Pro-1 to Glu 22
	1033.2	1032.7	PIAQIHILE
	1149.2	1148.2	$C_5H_9O_3$-PIAQIHILE
19.5	2356.7	2355.7	Val-23 to Glu-44
24.5	4914.6	4913.6	Pro-1 to Glu-44
	5030.6	5029.8	$C_5H_9O_3$-Pro-1 to Glu-44

[a] These values are calculated using the average molecular mass (Da). [b] The other masses observed in this region do not correspond to a modified fragment.

fragments: Pro-1 to Glu-44; Pro-1 to Glu-22; and Pro-1 to Glu-9. Analysis of the remaining peaks shows no modification of other fragments. These data indicate that a single site on the enzyme has been modified and that the site of modification is localized to the nine residue amino-terminal fragment Pro-1 to Glu-9 (PIAQIHILE). The most likely targets for alkylation are Pro-1 and His-6. In view of the fact that the inactivation of 4-OT by **7** is active-site-directed and that His-6 is not in the active site, it can be reasonably concluded that the amino-terminal group of Pro-1 is the site of modification.[5]

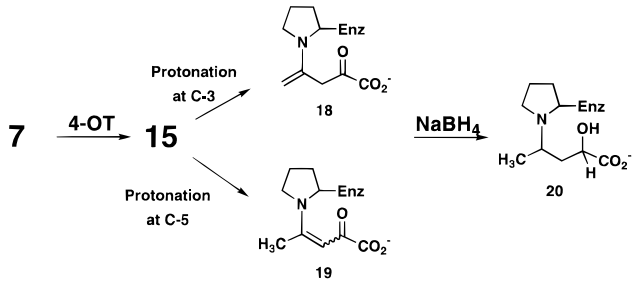
DISCUSSION

The results clearly show that 2-oxo-3-pentynoate is a potent active-site-directed irreversible inhibitor of 4-OT. A single site on the enzyme is modified. The most likely site of this modification is the amino-terminal proline, previously implicated as the catalytic base in the reaction (*6*, *7*). At saturation, the compound is 5-fold more potent than 3-BP. The compound is also a highly efficient inactivator in that modification of three of the six active sites completely abolishes enzymatic activity.

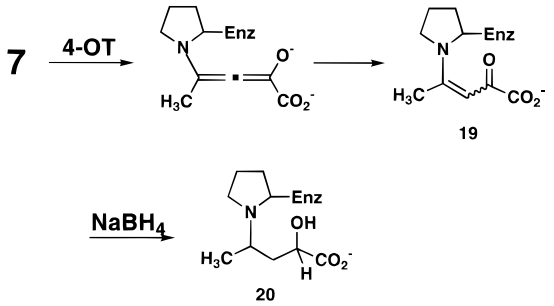
Several acetylenic compounds have been studied as mechanism-based inhibitors of isomerases (*11*, *12*). On the basis of these studies, one mechanism to explain the inactivation of 4-OT by **7** is summarized in Scheme 5. In this mechanism, 4-OT catalyzes an allylic rearrangement of **7** to the allene, **15**, by the abstraction of a proton at C-5. The subsequent transfer of the proton to C-3 is analogous to the normal mechanism (although in the reverse physiological direction). The amino-terminal proline can then react with the electrophilic allene at C-4 resulting in the inactivation of the enzyme by the formation of a covalent bond. The position of the double bond will be determined by whether protonation occurs at either C-3 or C-5. Protonation at C-3 results in the formation of species **18** with the double bond

[5] A crystal structure of the enzyme modified by a species derived from **7** confirms that the amino-terminal proline is the sole site of attachment [A. B. Taylor, and M. L. Hackert, (1997) unpublished results].

Scheme 5



Scheme 6



β,γ to the carbonyl group while protonation at C-5 generates species **19** with the double bond α,β to the carbonyl group. Species **18** is the kinetically controlled product of the reaction which presumably undergoes isomerization to **19**, the thermodynamically controlled product (*12*, *24*, *25*).

Alternately, the mechanism shown in Scheme 6 can account for the inactivation of 4-OT by **7**. In this mechanism, Pro-1 attacks the C-4 position of **7** in a Michael reaction. This produces the enol (or enolate) which tautomerizes to generate **19**. It is well-known that α,β-unsaturated acetylenic ketones are susceptible to nucleophilic attack (*26*, *27*). The low pK_a for Pro-1 ($\sim$6.4) suggests that it can act as a nucleophile and react with **7**. While it has been shown that 2-oxo-3-butynoate readily reacts with cysteine at neutral pH, model chemistry with amino groups was precluded by that fact that 2-oxo-3-butynoate decomposes under the alkaline conditions required for the presence of a nucleophilic amine (*28*).

The observation that the covalent adduct on Pro-1 has a molecular mass of 117 Da is consistent the formation of a bond between 4-OT and C-4 of **7** or a species derived from **7**. The most reasonable series of events leading to a species with a molecular mass of 117 Da begins with the reaction of 4-OT and **7** to generate either species **18** or species **19** (Scheme 5). Rearrangement of either enamine species results in the corresponding iminium ion which is readily reduced by $NaBH_4$ (*29*). Reduction of the iminium ion species and the carbonyl group accounts for the molecular mass of 117 Da. Such a sequence of events is consistent with either mechanism.

The detection of the allene (**15**) or acetopyruvate (**17**) which results from the addition of water to C-4 of **15** would provide a key piece of evidence to support the mechanistic scenario presented in Scheme 5. If the allene is generated by the enzyme, it does not dissociate from the active site but results in rapid alkylation as there is no evidence for its accumulation in solution. It is interesting to note that if the allene is generated, it is quite reactive, but it does not result in the modification of a group other than the proline which is known to be involved in the mechanism. This is in contrast to an allene generated from a steroidal acetylene compound, designed to be a mechanism-based inhibitor of 3-oxo-Δ^5-steroid isomerase (*22*). The residue modified was identified as Asn-57, which does not play a role in the mechanism (*30*).

Attempts to generate the allene chemically by the alkaline treatment of **7** were not successful. Several products result including a species with a λ_{max} at 300 nm. This observation was initially encouraging because a species with a λ_{max} at 300 nm has been generated upon exhaustive oxidation of propargylglycine (2-amino-4-pentynoate) by amino acid oxidase (*20*). The major species contributing to this λ_{max} was identified as 2-amino-4-hydroxy-2,4-pentadienoate γ-lactone which forms by the intramolecular addition of the carboxylate anion to C-4 of the allene generated from 2-amino-4-pentynoate.[2] The minor product was identified as acetopyruvate (**17**). However, we were unable to identify significant quantities of either of these two compounds. While small amounts of the allene may be generated by alkaline treatment, it appears to be forming a polymeric material which may be responsible for the λ_{max} at 300 nm.

Because the allene has thus far remained elusive, a better understanding of the inactivation of 4-OT by **7** is being pursued by other experiments. One such experiment involves the synthesis and evaluation of 2-oxo-3-butynoate as an inhibitor of 4-OT. Because there is no methyl group at C-5, the allene cannot be generated and inactivation of 4-OT can only occur by a Michael reaction. In preliminary studies, it has been found that 2-oxo-3-butynoate is a more potent inhibitor than **7**.[6] This observation is consistent with the mechanism shown in Scheme 6 in which a Michael reaction results in the inactivation of 4-OT.

The inactivation of 4-OT by **7** contrasts to the inactivation of 4-OT by 3-bromopyruvate, which modifies all six active sites in a hexamer, although three sites are modified rapidly and three sites are modified more slowly (*7*). This phenomenon, exhibited previously by 3-bromopyruvate and now **7**, is known as half-of-the-sites stoichiometry and is explained by the crystal structure (*7*). The enzyme is a trimer of dimers where each dimer consists of two interacting monomers (*6*, *7*). The two active sites of one dimer consist of residues from each monomer so that covalent modification of one active site disrupts the second active site. In the case of **7**, only three of the six prolines are covalently modified. The are two possible explanations for this observation depending on whether **7** is a mechanism-based inhibitor or an affinity label. If **7** behaves as a mechanism-based inhibitor, then the covalent modification of one active site may disrupt the second active site such that it can no longer generate the allene from **7** which precludes modification of the other monomer within a dimer. 3-Bromopyruvate, on the other hand, is already a potent alkylating agent not requiring activation by the enzyme. Hence, the disruption of the one active site will result in slower alkylation at the second site but not preclude it. If **7** is also found to be an affinity label of the enzyme, then its different behavior may be due to a greater disruption of the second active site caused by the binding of the linear acetylene compound followed by its transformation to the more flexible covalent adduct.

[6] W. H. Johnson, Jr., R. M. Czerwinski, and C. P. Whitman, (1997) unpublished results.

Pyruvate and other 2-oxo acids play a central role in metabolism (31). Hence, analogs of these compounds have been used extensively as covalent inhibitors and alternate substrates to explore the mechanisms of the many enzymes involved in the metabolism of 2-oxo acids (*19*). This compound may be a useful addition to the reagents available for these studies.

CONCLUSIONS

An acetylene analog of the product resulting from the 4-OT-catalyzed reaction was synthesized and tested as a potential inhibitor. It was found to be a potent inhibitor resulting most likely in the labeling of the amino-terminal proline. Inactivation results either by Michael addition of the enzyme to the α,β-unsaturated acetylenic ketone or by alkylation of the enzyme by an allenic species generated from the enzyme-catalyzed rearrangement of the α,β-unsaturated acetylenic ketone. This compound, as an acetylene analog of pyruvate, may also have general utility as an inhibitor of a variety of enzyme-catalyzed reactions that process pyruvate or pyruvyl-like compounds.

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